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Proteins in food processing

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1

Introduction

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Through their provision of amino acids, proteins are essential to human growth, but they also have a range of structural and functional properties which have a profound impact on food quality. *Proteins in food processing* reviews the growing body of research on understanding protein structure and developing proteins as multi-functional ingredients for the food industry.

Chapter 2 describes what we know about the common chemical and physical properties of proteins and the range of factors that influence how these properties are expressed in particular food systems. It provides a context for Part I which discusses the diverse sources of proteins, whether from milk, meat or plants. Individual chapters review the structure and properties of these groups of proteins and ways of improving their functionality as food ingredients.

Part II builds on Part I by summarising the range of recent research on analysing and modifying proteins. A first group of chapters reviews ways of testing and modelling protein behaviour, understanding enzyme activity and detecting allergenic proteins. They are followed by chapters reviewing the range of techniques for extracting, purifying and modifying proteins. The book concludes by analysing the many applications of proteins as ingredients, from their use as edible films to their role in modifying textural properties and improving the nutritional quality of food.

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2

Properties of proteins in food systems: an introduction

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2.1 Introduction

The word 'protein' is defined as

> any of a group of complex organic compounds, consisting essentially of combinations of amino acids in peptide linkages, that contain carbon, hydrogen, oxygen, nitrogen, and usually, sulfur. Widely distributed in plants and animals, proteins are the principal constituent of the protoplasm of all cells and are essential to life. ('Protein' is derived from a Greek word meaning 'first' or 'primary,' because of the fundamental role of proteins in sustaining life.) (Morris, 1992)

Proteins play a fundamental role not only in sustaining life, but also in foods derived from plants and animals. Foods vary in their protein content (Table 2.1), and even more so in the properties of those proteins. In addition to their contribution to the nutritional properties of foods through provision of amino acids that are essential to human growth and maintenance, proteins impart the structural basis for various functional properties of foods.

The objective of this chapter is to provide an introduction to the chemical and physical properties of food proteins that form the basis for their structural and functional properties. However, food scientists wishing to study proteins in food systems must be cognizant of the complexity of such systems in terms of composition and spatial organization. Food systems are usually heterogeneous with respect to (a) protein composition (foods usually do not contain a single protein entity, but multiple proteins); (b) other constituents (most foods contain not only water and other proteins, but also lipids, carbohydrates as major components, and various other minor components such as salt, sugars,

Table 2.1 Total protein contents of the edible portion of some foods and beverages[a]

Food	Total protein (%)
Almonds	21.1
Apples (raw, eating)	0.4
Bananas	1.2
Beans (canned, baked)	5.2
Beer (bitter)	0.3
Beef (lean, raw)	20.3
Beansprouts (raw)	2.9
Bread (white)	8.4
Cabbage (raw)	1.7
Cheese (Cheddar)	25.5
Cheese (Parmesan)	39.4
Chicken (lean, raw)	20.5
Chocolate (milk chocolate)	8.4
Chocolate (plain chocolate)	4.7
Cod fillet (raw)	17.4
Cornflakes	7.9
Egg (whole)	12.5
Ice cream	3.6
Lentils (dried)	24.3
Milk (cow's whole)	3.2
Milk (human)	1.3
Pasta	3.6
Potatoes (new)	1.7
Rice	2.6
Sweetcorn (canned)	2.9
Soya milk	2.9
Tofu (steamed)	8.1
Tuna (canned)	27.5
Yogurt (plain)	5.7

[a] Adapted from Table 5.1 of Coultate (2002).

micronutrients, minerals, phenolic compounds, flavour compounds, etc.); and (c) structural or spatial organization (proteins exist in foods as tissue systems, gels, coagula, films, emulsions, foams, etc., and not usually as the dilute solutions or crystalline forms that are typically investigated in model systems). Furthermore, significant changes in the properties of the proteins are induced by environmental factors and processing conditions that are typical of food systems.

Lluch *et al*. (2001) have written an excellent chapter describing the complexity of food protein structures. The diversity of the structural role of proteins in various food raw materials is illustrated by comparing protein structures in the muscle tissues of meat, fish and squid, the protein bodies of plant tissues such as cereals, legumes, oilseeds and shell (nut) fruits, and the casein micelle structure of bovine milk. Interactions of proteins with other components are exemplified in protein-starch interactions observed during dough processing and baking, protein-hydrocolloid interactions in dairy

products, protein-fat interactions in comminuted meat emulsions, mayonnaise and cheese, protein-water as well as protein-protein matrix interactions in fish surimi gels, yogurt and cheese (Lluch *et al*., 2001).

With this complexity in mind, in addition to describing the basic chemical and physical properties of proteins and their amino acid building blocks, this chapter provides an overview of the factors that can influence the properties of proteins in food systems, and suggests approaches that may be useful to elucidate the structure–function relationships of food proteins.

2.2 Chemical and physical properties of food proteins

2.2.1 Amino acids commonly found in proteins

It is commonly recognized that 20 amino acids form the building blocks of most proteins, being linked by peptide (amide) bonds formed between α-amino and α-carboxylic acid groups of neighbouring amino acids in the polypeptide sequence. Nineteen of these 20 amino acids have the general structure of H_2N-$C_\alpha H$ (R)-CO_2H, differing only in R, which is referred to as the side chain, while the 20th amino acid is in fact an 'imino' acid, in which the side chain is bonded to the nitrogen atom. With the exception of the amino acid glycine, in which the side chain is a hydrogen atom, the α-carbon atom exhibits chirality. Typically, only the L-form of the amino acids is found in proteins, being incorporated through the transcription and translation machinery of the cell. The D-enantiomers of amino acids are present in some peptides.

Table 2.2 shows the three-letter and single letter abbreviations as well as some key properties of the 20 amino acids. The reader is referred to Creighton (1993) and Branden and Tooze (1999) for illustrations depicting the structure of the side chains of the 20 amino acids. Similar information can also be viewed at numerous internet sites, such as those maintained by the Institut für Molekulare Biotechnologie (2003a), and the Birbeck College (University of London) School of Crystallography (1996). As shown in Table 2.2, the 20 amino acids can be classified according to their side chain type: acidic (Asp, Glu), basic (Arg, His, Lys), aliphatic (Ala, Ile, Leu, Val), aromatic (Phe, Tyr, Trp), polar (Ser, Thr), thiol-containing (Cys, Met), amide (Asn, Gln). In addition, as noted above, two amino acids are unique in being achiral (Gly) or an imino rather than amino acid (Pro).

It is interesting to note that the two amino acid residues occurring at greatest frequency in proteins possess aliphatic side chains (9.0 and 8.3% for Leu and Ala, respectively), while Gly is the third most frequently occurring amino acid at 7.2% (Creighton, 1993). With the exception of His, more than 80 or 90% of the basic and acidic amino acid residues in proteins usually locate such that they are primarily exposed to the solvent (Institut für Molekulare Biotechnologie, 2003a; Bordo and Argos, 1991). Similarly, amino acid residues with polar side chains (Ser, Thr, Asn, Gln) as well as Pro are also primarily accessible to the solvent. Conversely, with the exception of Tyr, which contains an aromatic phenolic

Table 2.2 Some properties of the 20 amino acid residues commonly found in proteins

Amino acid			Mass[a]	Side chain type	pK_a [b]	Residue nonpolar surface area [c] ($Å^2$)	Estimated hydrophobic effect, side chain burial (kcal/mol)	Percentage with solvent exposed area[c] >30$Å^2$	<10$Å^2$	Frequency in proteins[b] (%)
Alanine	Ala	A	71.09	aliphatic hydrocarbon		86	1.0	48	35	8.3
Arginine	Arg	R	156.19	basic δ-guanidyl	12.0	89	1.1	84	5	5.7
Aspartic acid	Asp	D	114.11	acidic β-carboxyl	3.9–4.0	45	−0.1	81	9	5.3
Asparagine	Asn	N	115.09	acid amide		42	−0.1	82	10	4.4
Cysteine	Cys	C	103.15	thiol	9.0–9.5	48	0.0	32	54	1.7
Glutamic acid	Glu	E	129.12	acidic γ-carboxyl	4.3–4.5	69	0.5	93	4	6.2
Glutamine	Gln	Q	128.14	acid amide		66	0.5	81	10	4.0
Glycine	Gly	G	57.05	hydrogen		47	0.0	51	36	7.2
Histidine	His	H	137.14	basic imidazole	6.0–7.0	43+86	1.3	66	19	2.2
Isoleucine	Ile	I	113.16	aliphatic hydrocarbon		155	2.7	39	47	5.2
Leucine	Leu	L	113.16	aliphatic hydrocarbon		164	2.9	41	49	9.0
Lysine	Lys	K	128.17	basic ϵ-amino	10.4–11.1	122	1.9	93	2	5.7
Methionine	Met	M	131.19	thio-ether		137	2.3	44	20	2.4
Phenylalanine	Phe	F	147.18	aromatic phenyl		39+155	2.3	42	42	3.9
Proline	Pro	P	97.12	heterocyclic imino		124	1.9	78	13	5.1
Serine	Ser	S	87.08	polar hydroxyl		56	0.2	70	20	6.9
Threonine	Thr	T	101.11	polar hydroxyl		90	1.1	71	16	5.8
Tryptophan	Trp	W	186.12	aromatic indole		37+199	2.9	49	44	1.3
Tyrosine	Tyr	Y	163.18	aromatic phenol	9.7	38+116	1.6	67	20	3.2
Valine	Val	V	99.14	aliphatic hydrocarbon		135	2.2	40	50	6.6

[a] Mass of the amino acid (from NIST Chemistry WebBook, 2001) minus the mass (18.00) of a water molecule.
[b] From Creighton (1993).
[c] From Institut für Molekulare Biotechnologie (2003a) and Karplus (1997); aliphatic and aromatic surface areas are reported separately for aromatic amino acids; percentages of each residue with solvent exposed area >30$Å^2$ or <10$Å^2$ were calculated based on 55 proteins in the Brookhaven database using solvent accessibility data of Bordo and Argos (1991).

group, less than 50% of the aliphatic and aromatic groups have solvent exposed areas greater than 30Å. Nevertheless, only 40–50% of aliphatic and aromatic residues would be considered to be 'buried', with solvent exposed areas of less than 10Å. These observations indicate that while charged residues are almost always located near the surface or solvent-accessible regions of protein molecules, the converse cannot be assumed for nonpolar aliphatic or aromatic residues, probably due to insufficient capacity in the interior of the molecule. Thus, both charged and hydrophobic groups reside at the surface or solvent-accessible regions of protein molecules, whereas charged groups are found much less frequently in the buried interior of protein molecules. In fact, it has been reported that approximately 58% of the average solvent accessible surface or 'exterior' of monomeric proteins is nonpolar or hydrophobic, while 29% and 13% of the surface may be considered polar and charged, respectively (Lesk, 2001).

Table 2.2 shows that 54% of Cys residues are 'buried' with solvent-exposed area <10Å, although the estimated hydrophobic effect of Cys side chain burial is 0.0 kcal/mol. The highly reactive thiol groups of Cys residues may interact with other thiol-containing residues to undergo sulfhydryl-disulfide interchange reactions or oxidation to disulfide groups. Internal disulfide bonds frequently play an important role in the stability of the three-dimensional structure of globular proteins, while disulfide bonds between Cys residues on the surface of molecules may be responsible for the association of subunits or the formation of aggregates from denatured molecules.

Similarly, as mentioned previously, the percentage of buried His residues is higher than that observed for the other basic amino acid residues. The pK_a of His residues lies near neutrality, and the ionization state of imidazoyl groups has been implicated in important biological or catalytic functions of His residues, particularly those located in the interior of protein molecules, which may be related to the unusual ionization properties that can result from the influence of environment in the folded protein molecule.

2.2.2 Other naturally occurring amino acids

While most of this chapter will be focused on food proteins composed of the 20 amino acids listed in Table 2.2, it is important to acknowledge the presence of other naturally occurring amino acids, as these can confer distinctive and interesting properties to some food systems. Over 300 naturally occurring amino acids have been reported, and the reader is encouraged to consult Mooz (1989) and the references cited therein for a listing of these amino acids and their properties. Some of these amino acids exist as free amino acids, while others have been found in peptides or proteins.

Some examples of the unusual amino acids that have been reported from food sources include O-phosphoserine in casein, 4-hydroxyproline in gelatin, 4-hydroxy-4-methyl-proline, 4-methylproline and pipecolic acid in apples, citrulline in watermelon, 1-aminocyclopropane-1-carboxylic acid in pears and

apples, 2-alanyl-3-isoxazolin-5-one in pea seedlings, S-methylcysteine-sulfoxide in cabbage, δ,ϵ-dihydroxynorleucine in bovine tendon, ϵ-N-methyl-lysine in calf thymus histone, S-(2-carboxypropyl)-cysteine, S-allylcysteine and other sulfur derivatives in onions, S-methylmethionine in asparagus, S-methylcysteine in Phaseolus vulgaris and hercynin (histidine betaine) in mushrooms.

Other amino acids may be found as a result of processing, such as furosine (ϵ-N-(2-furoyl-methyl)-lysine) and pyridosine (ϵ-(1,4-dihydro-y-methyl-3-hydroxy-4-oxo-1-pyridyl)-lysine) in heated milk, or N-ϵ-(2-amino-2-carboxyethyl)-lysine in alkali-treated protein. In addition, α, β-unsaturated amino acids stabilized by peptide bond formation are present in natural products. Examples include dehydroalanine and β-methyldehydroalanine in the peptides nisin and subtilin (Fasman, 1989).

Incorporation of amino acids that are not coded by mRNA into peptides or peptidomimetic compounds, has generated much interest due to the increased diversity in physicochemical properties with potential pharmacological interest, as well as to the possibility for reduced sensitivity of such peptides to biodegradation by peptidases (Sandberg *et al*., 1998). Recent research reports have also appeared on methods for genetic encoding of additional amino acids, beyond the 20 amino acids commonly occurring in living organisms. For example, Mehl *et al*. (2003) reported the generation of a completely autonomous bacterium *Escherichia coli* with a 21 amino acid genetic code. The bacterium demonstrated the capacity to synthesize the additional amino acid p-aminophenylalanine from simple carbon sources and to incorporate it into proteins with fidelity rivaling the common 20 amino acids. The authors concluded that their pioneering research could open the door to allow investigations into the evolutionary consequences of adding new amino acids to the genetic repertoire, and to generate proteins with novel or enhanced biological functions.

2.2.3 Levels of structural organization

Four levels of hierarchical organization are used to describe protein structure or architecture. The primary structure of a protein refers to its peptide bond linked sequence of amino acids, described from the N-terminus to the C-terminus. The primary structure also includes other covalently bonded structures, such as the location of disulfide bridges and the sites of posttranslational modifications of side chains (e.g. methylation, glycosylation, phosphorylation). The enormous potential for diversity of proteins arises from the fact that theoretically, each site in the primary sequence could be occupied by one of the 20 amino acids. Thus, for example, excluding posttranslationally modified residues and unusual amino acids, there would be 20^{100} unique sequences of proteins containing 100 amino acids. In fact, only a small percentage of the potential sequences have actually been found to exist in nature. As described later, the native structure of most proteins possess only marginal stability conferred by specific intramolecular

interactions in the folded state. Furthermore, the planar nature of the atoms around the peptide bond and the bulky side chains of some of the amino acid residues impose restrictions on the flexibility of the polypeptide chains, and thus the primary structure dictates the final three-dimensional structure of a protein molecule.

The secondary structure describes the regular local conformations of the polypeptide backbone, which are determined by the planarity of the peptide bond, hydrogen bonding between the C=O acceptor and N-H donor groups of peptide bonds, and the possible rotation around N-C_α and C_α-C bonds. Periodic structures, such as the α-helix or β-sheet structures, are characterized by recurring values of the dihedral phi (Φ) and psi (Ψ) angles, generating a uniformity of backbone conformation (Ludescher, 1996; Lesk, 2001). Images of some of these periodic secondary structures can be viewed at the IMB Jena Image Library (Institut für Molekulare Biotechnologie, 2003b). In contrast, aperiodic structures such as reverse (β) turns or loops involve regular backbone conformations, but without a repeating sequence of dihedral angles. Many variants of β-turns have been described, including β-hairpins that link the strands of an antiparallel β-sheet. Reverse turns are commonly found on the surface of proteins, providing purely structural roles in some cases, and functional residues accessible to the solvent in other cases.

In the most commonly found helical structure, the right handed α-helix, with 3.6 residues per turn, the characteristic Φ and Ψ angles are approximately -60^o and -50^o, respectively. Intrachain hydrogen bonding occurs between the C=O group at position i with the NH group at position i+4, resulting in a dipole moment along the helical axis, with a positive pole at the N-terminus and negative pole at the C-terminus. The side chains of the residues point away from the surface of the helix, and many α-helices possess hydrophilic and hydrophobic faces (Lesk, 2001). The Φ and Ψ angles are approximately -70^o and -20^o or less, respectively, for the more tightly packed 3_{10} helix with an i+3 hydrogen bonding pattern (Ludescher, 1996; Institut für Molekular Biotechnologie, 2003b). The polyproline II conformation found in collagen and gelatin is also an example of a periodic secondary structure, but is an extended, left-handed helical structure with 3.3 residues per turn, and Φ and Ψ angles of -80^o and $+150^o$, respectively. Unlike the other helical structures, the polyproline II structure is not stabilized by intra chain hydrogen bonds, but by specific conformational restraints resulting from the many proline and hydroxyproline residues that are characteristic of the collagen molecule (Ludescher, 1996).

The individual β-strands of a β-pleated sheet have a helical structure arising from the recurring Φ and Ψ angles of 120^o and $+140^o$, respectively, while the fully extended polypeptide chain has both Φ and Ψ angles at 180^o. Inter-chain hydrogen bonding occurs between two or more β-strands or extended chains pointing in the same direction (parallel β-sheet) or in opposite directions (anti-parallel β-sheet), and the side chains of residues point alternately above and below the plane of the pleated sheet (Ludescher, 1996).

The regular geometry of the periodic secondary structures favours interactions between secondary structural elements, which can also lead to the formation of supersecondary structures, including coiled-coils, the triple helix, helix-loop-helix, beta-alpha-beta motif, DNA-binding motif and calcium-binding motif (Branden and Tooze, 1991). The triple helix of collagen molecules and the coiled-coil structure of myosin molecules are well-known examples of supersecondary structures in food proteins.

The description of the overall three-dimensional folding of the protein is referred to as its tertiary structure. It involves the folding pattern of the polypeptide backbone including the secondary structures, arrangements of motifs into domains, and conformations of the side chains. In essence, the tertiary structure provides information on the complete folding pattern of the primary and secondary structures of the protein molecule, which dictate its final dimensions of size and shape. In addition, the locations of each of the amino acid residues in three-dimensional space are described, thus providing information on the accessibility or degree of exposure and the potential noncovalent interactions of the side chains.

Finally, the quaternary structure refers to the fourth-dimensional level of structure of protein complexes that may arise from the association of identical or heterogeneous polypeptide chains. The molecular weight of monomeric globular proteins usually falls in the range of 10,000 to 100,000 daltons, yielding diameters in the range of 3–6 $\times$ 10^{-9} m (Ludescher, 1996). Proteins with a quaternary level of structure are usually larger than monomeric proteins. For example, the 11 S globulins of various monocotyledonous as well as dicotyledonous plants such as soybean, are composed of acidic and basic subunits assembled in hexameric structures, with molecular weights of approximately 300,000–370,000 daltons (Marcone *et al*., 1998). Other examples of protein complexes in food systems include the glutenins from wheat, which have a molecular weight of >1,000,000 daltons (Cheftel *et al*., 1985). Some food systems involving association of multiple proteins into biologically functional units may be viewed as having a fifth level of protein structure. Thus, for example, κ-casein B, αS_1-casein B and β-casein B with monomeric molecular weights of 19,000, 23,500 and 24,000 daltons, respectively (Cheftel *et al*., 1985) associate to form casein micelle structures ranging in size from 20 to 600 nm (Schmidt, 1982). Other food systems are composed of fibrous proteins that are asymmetric in their shape, and usually much longer than globular proteins. For example, the myosin filament is about 1.5 $\times$ 10^{-6} m in length, being composed of longitudinally aligned myosin molecules, each with two heavy chains and four light chains and a total molecular weight of 475,000 daltons (Cheftel *et al*., 1985).

The molecular forces that play key roles in stabilizing the secondary and tertiary structures of the protein molecule, as well as in determining the surface properties and intermolecular interactions leading to quaternary structures, are described in the following section.

2.2.4 Molecular forces affecting physicochemical and functional properties

The three-dimensional as well as quaternary structure of a protein in its native state is dependent not only on the covalent bonds that link the amino acid residues in sequence or that create disulfide bonds between cysteinyl residues, but also on the numerous non-covalent interactions that occur within the protein molecule, or between the protein molecule with solvent molecules (usually water in the case of food systems), or between proteins and other molecules in the food system. These molecular forces are instrumental in stabilization of the protein's folded structure in a specific native conformation. They influence molecular flexibility, size and shape of the protein molecule. Furthermore, these forces may be involved in intermolecular interactions including aggregation. Therefore molecular forces play an important role in determining the physicochemical and functional properties of the protein.

Table 2.3 compares typical ranges of energy and interaction distance found in covalent bonds and noncovalent interactions. Folding of proteins into a stable conformation is enthalpy driven; however the increases in enthalpy achieved by interactions between functional groups of proteins are accompanied by decreases in entropy. The entropic cost of folding has been estimated as 20 kJ/mole of residue (Ludescher, 1996). Based on this estimate, the energy cost of folding even a small protein such as hen egg white lysozyme with 129 residues would be approximately 2580 kJ per mole of protein. Thus, formation of the stable folded state would be energetically favourable only if there are sufficient specific intramolecular interactions to yield an increase in enthalpy that at least would compensate for the loss in entropy. In fact, most native proteins are only marginally stable at 25°C, typically with only 20–100 kJ per mole of protein additional energy beyond that required for folding into a stable well-defined structure, and this marginal stability provides a physical basis for their properties (Ludescher, 1996). Greater stability is observed when the folded structure is

Table 2.3 Molecular forces involved in protein interactions[a]

Type	Energy (kilojoules/mole)	Interaction distance (Å)	Functional groups
Covalent bond	330–380	1–2	disulfide
Hydrogen bond	8–40	2–3	amide, hydroxyl, phenolic groups
Hydrophobic interactions	4–12	3–5	aliphatic and aromatic side chains
Electrostatic interactions	42–84	2–3	carboxylic acid and amino groups
van der Waals	1–9	2–3[b]	permanent, induced and instantaneous dipoles

[a] Adapted from Table 5 of Cheftel *et al.* (1985).
[b] Optimal van der Waals interactions generally occur at a distance of 1.2Å greater than the covalent bond length (Creighton, 1993).

stabilized by covalent bonds such as intramolecular disulfide bonds. Using again the example of lysozyme, the presence of four intramolecular disulfide bonds contributes to its resistance to denaturation by heat and to unfolding under shearing conditions such as those used for emulsification or foaming (Li-Chan and Nakai, 1991a and 1991b).

The predominant, noncovalent molecular forces involved in protein properties can be classified as electrostatic interactions and hydrophobic interactions (Creighton, 1993; Ludescher, 1996), which are strongly dependent on the water molecules which form the solvating medium in most foods. Approximately 10–20 molecules of water, referred to as 'structural water' are tightly bound by hydrogen bonding to specific groups and assist in stabilization of the protein structure (Damodaran, 1996). Monolayer water refers to highly structured water molecules that are tightly bound to the protein surface via hydrogen bonding and ion-dipole interactions to polar and charged groups, and hydrophobic hydration of nonpolar groups (Damodaran, 1996).

Electrostatic interactions can occur between point charges (e.g., positively charged amino groups and negatively charged carboxyl groups), between point charges and permanent dipoles (charged amino group and water), between fluctuating transient dipoles (van der Waals or London dispersion forces between nonpolar hydrocarbon chains), and between hydrogen bond donors and acceptors (OH of water to O of water; carbonyl and amino groups of the polypeptide backbone or amino acid residues). 'Salt bridges' that form between closely located, oppositely charged groups in a protein, arise from electrostatic interactions as well as usually some degree of hydrogen bonding (Creighton, 1993). The π electrons in the aromatic rings of tryptophan, tyrosine and phenylalanine residues, are localized above and below the face of the rings, leading to small net negative charges to the face of the rings, and corresponding positive net charges at the edge due to the hydrogen atoms. Thus, aromatic residues interact with each other not by stacking their rings one above the other, but through the negative face of one ring interacting with the positive edge of another ring. For the same reason, positively charged amino groups interact with aromatic rings at the negatively charged face, while electronegative oxygen or sulfur groups tend to interact with the positively charged edges of aromatic rings.

Both enthalpic and entropic considerations are involved in the influence of hydrogen bonding of water molecules as a driving force leading to hydrophobic interactions between nonpolar side chains of amino acid residues of proteins in water (Ludescher, 1996; Nakai and Li-Chan, 1988). A decrease in enthalpy results from the disruption of hydrogen bonds between water molecules in the presence of nonpolar molecules or groups, such as the side chains of aliphatic amino acid residues, which lack the ability to form hydrogen bonds with the solvent water molecules. The enthalpy of hydrogen bond formation is partially recovered by re-orientation of water molecules around the nonpolar molecules into highly ordered, hydrogen bonded ice-like clathrate structures, but this is accompanied by a loss of entropy. The hydrophobic effect, which is manifested

as the hydrophobic interactions arising through association of nonpolar groups with each other, may thus be viewed as being energetically favourable in terms of both enthalpy considerations (generating attractive interactions between nonpolar groups) and entropy considerations (releasing the clathrate ordered water molecules). Since water is ubiquitous in food systems, the hydrophobic effect plays a dominant role in both the intramolecular interactions that stabilize the three-dimensional structure of proteins, as well as in the intermolecular interactions of proteins with other molecules in food systems.

Furthermore, the absence of water molecules in the nonpolar regions resulting from hydrophobic interactions of nonpolar side chains provides a favourable environment for hydrogen bonding between polar side chains or peptide bond groups located in these nonpolar regions. The greater stability arising from cooperativity of hydrogen bond formation between intramolecular groups may explain why their formation is favoured over that of hydrogen bonds between the solvent and unfolded protein (Darby and Creighton, 1993).

In addition to molecular forces stabilizing the protein structure, the physicochemical and functional properties of proteins are influenced by stereochemical constraints related to planarity of the peptide bond as well as size and shape of bulky side chains. These steric effects may dictate molecular flexibility of the polypeptide chain. The role of molecular flexibility in food protein functionality was introduced by Townsend and Nakai (1983). Although molecular flexibility is not a readily quantifiable parameter, it is manifested in dynamic fluctations in volume, which can be directly assessed from compressibility of globular proteins (Damodaran and Razumovsky, 1998). Compressibility values have been suggested to be a useful guide or descriptor of protein stability (Apenten *et al*., 2000) and surface activity (Damodaran and Razumovsky, 1998).

2.3 Factors affecting properties of proteins in food systems

Intrinsic factors, extrinsic or environmental factors, and processing treatments or other intentional modifications can all contribute to influence the chemical and functional properties of proteins in food systems, as depicted in Table 2.4. Intrinsic factors include the basic chemical and physical properties of the amino acids comprising a particular protein, as described previously in Section 2.2. As mentioned previously, about half of the surface or solvent accessible areas of most globular protein molecules are nonpolar. The distribution of nonpolar and polar or charged side chains on the surface protein plays a key role in the chemical properties and ultimately the functions of the proteins. These surface properties can be altered by de-stabilization of the tertiary structure leading to unfolding or exposure of previously buried residues, or by formation of quaternary structures (complexes or aggregates) leading to shielding of previously solvent-accessible sites. Given the marginal stability of the folded structure, conformational changes as well as subsequent intermolecular

Table 2.4 Examples of structural or chemical properties and functional properties of food proteins[a]

Structural or chemical properties			Functional properties		
Intrinsic factors	Extrinsic factors	Processing	Surface or interface	Hydrodynamic	Bioactivity
amino acid composition 1^o, 2^o, 3^o, 4^o structures conjugates subunits	pH redox status temperature salt, other ions solvent other major or minor constituents	heating cooling, freezing drying concentrating storage shear force pressure chemical or enzymatic modification	solubility wettability dispersibility foaming emulsification fat binding flavour binding	viscosity thickening gelation coagulation film formation	enzyme hormone antimicrobial antihypertensive immunomodulatory antioxidant opioid

[a] Adapted from Damodaran (1996).

interactions may be readily induced by other food constituents or by environmental factors and processing conditions typical of food systems, as described in the following sections.

2.3.1 Protein interactions with other food constituents

The chemical properties and functions of proteins are shaped by their interactions both with major food constituents such as water, other proteins, lipids and carbohydrates, as well as with any number of minor constituents such as salts, metal ions, acidulants, flavour components and phenolic compounds. The published literature describes many studies of the interactions of proteins with other food constituents, and only a few examples are included here to illustrate how these interactions may affect the properties of proteins in food systems.

Water

Water molecules that are hydrogen bonded to protein molecules may be critically important for the structural stability of the protein (Damodaran, 1996). The balance between protein-water interactions versus protein-protein interactions is important in functional properties such as swelling, water-binding capacity and solubility of protein ingredients, as well as their ability to form network structures such as gels or films including those surrounding foam bubbles.

Salts

Salts may promote either solubilization (salting-in) or precipitation (salting-out) of proteins, depending on the concentration and nature of the salt involved (Regenstein and Regenstein, 1984; Li-Chan, 1996). The increased β-sheet content that has been observed in the salt-induced aggregated state may be attributed to the relatively large surface area and opportunities for hydrogen bonding provided by the β-sheet structure (Przybycien and Bailey, 1991). Furthermore, the weaker strength of water hydration to β-sheet than to α-helix structures, due to different geometry of the water-carbonyl group interactions in these secondary structure conformations, may influence the changes in water-protein and protein-protein interactions that favour aggregate or network formation (Li-Chan and Qin, 1998).

Acidulants

Acidulants alter the net charge and isoelectric point of the protein molecule and may also affect the local distribution of positive or negative charges on the protein surface, again with possible alterations of the protein-solvent and protein-protein balance and associated properties. The specific binding of metal ions can also affect stability of proteins. The binding of calcium ions to α-lactalbumin, and of iron ions to lactoferrin or ovotransferrin, are examples of food proteins whose properties are dependent on their interactions with specific metal ions.

Carbohydrates

Carbohydrates, with their multiple hydroxyl groups, may contribute to the structural stability of proteins, either by exclusion of the carbohydrate from the protein surface resulting in preferential hydration of the protein, or by interaction of the carbohydrate with hydroxyl or ionic functional groups of the protein molecule (Arakawa and Timasheff, 1982; MacDonald *et al*., 2000). These carbohydrate-protein interactions affect stability of food proteins to processes such as thermal treatment, dehydration or frozen storage, and are the basis for addition of ingredients such as sucrose or sorbitol to stabilize fish muscle proteins during frozen storage or drying (MacDonald *et al*., 2000). Attractive (complexation) or repulsive (segregation) interactions may occur between proteins and anionic polysaccharides such as carrageenan or pectin, leading to precipitate or gel formation, depending on conditions such as biopolymer concentration, pH and ionic strength (Dickinson, 1998).

Nonpolar residues

Nonpolar residues of proteins are primarily responsible for hydrophobic interactions with lipid molecules at oil-water interfaces such as emulsions (Howell *et al*., 2001) although electrostatic, covalent, hydrogen and hydrophobic forces may all contribute to protein-lipid interactions (Alzagtat and Alli, 2002). Protein-lipid interactions may also be implicated in protein-protein interactions; for example, protein-lipid complexes in wheat gluten have been associated with the lipid-mediated aggregation of high and low molecular weight polypeptides in the gliadin fraction (Carcea and Schofield, 1996).

Flavour components

Since many flavour components (particularly aroma compounds) are nonpolar, the binding of flavour compounds has been correlated with the hydrophobic residues of proteins (Kim and Min, 1989; Kinsella, 1989; O'Neill, 1996). Tannins also can interact through hydrophobic interactions and hydrogen bonding with nonpolar and polar residues of proteins. The tannins from a wide variety of plant foods such as beach pea, canola hulls, evening primrose and faba bean have been demonstrated to form complexes with proteins, often leading to precipitation (Naczk *et al*., 2001). The role of tannin-protein complexes in haze formation and quality of wine, beer and juices is well recognized (e.g., Beveridge, 1999; Waiblinger, 2002; Yokotsuka and Singleton, 1996), and the benefits of tannins, including those complexed with proteins, as potential antioxidants or radical scavengers have also received attention recently (e.g., Carbonaro *et al*., 1996; Riedl and Hagerman, 2001).

2.3.2 Changes induced by food processing

Protein structures can be readily destabilized by relatively minor changes in pH, temperature, the addition of oxidizing or reducing agents or different salts, or under the stress of pressure or shear, as well as numerous combinations of these

conditions. The properties of proteins can therefore be expected to be altered as a result of food processing operations such as thermal or high pressure processing, freezing and frozen storage, dehydration, concentration, mixing, homogenization, extrusion, membrane processes such as ultrafiltration, etc. Such changes are not necessarily undesirable, and processing may be controlled to modify intentionally the structure and functionality of food proteins.

The term 'denaturation' is usually used to refer to changes from the original native structure, without alteration of the amino acid sequence. Denaturation has also been defined with respect to its effect on protein functionality, in terms of changes in chemical, physical or biological properties (Li-Chan, 1998). Both of these definitions are important in the context of understanding the properties of food proteins. An excellent description of the chemistry of protein denaturation as well as detailed examples of denaturation of food proteins may be found in Kilara and Harwalkar (1996).

For many single-domain proteins, a simple two-state transition (native → denatured) is used to describe the transition from folded, native structure to the unfolded, denatured structure, and implies a high degree of cooperativity in the interactions that stabilize the native structure. In other words, disruption of a limited number of interactions will lead to destabilization of the native structure (Darby and Creighton, 1993). However, it is now widely recognized that the two-state transition model does not always adequately describe the process of denaturation. Instead, a stable, partially folded state referred to as the 'molten globule' has been characterized for certain proteins under particular conditions. The molten globule state frequently has similar secondary structural composition and degree of compactness as the native protein structure, but may possess little or no tertiary structure and may exhibit greater exposure of nonpolar residues. An important role of the molten globule state in functional properties of foods has been described in many studies (e.g., Hirose, 1993; Smith *et al*., 2000; Cornec *et al*., 2001; Farrell *et al*., 2002).

2.3.3 Other intentional modifications

The application of chemical and enzymatic methods intentionally to modify chemical and functional properties of food proteins has a long history of usage, as illustrated by the enzymatic modification of milk proteins to produce yogurt and cheese (Howell, 1999).

Chemical methods of derivatization which have been reported in the literature include (a) acylation (acetylation, succinylation) or alkylation of amino groups, (b) esterification or amidation of carboxyl groups, (c) acylation or electrophilic substitution of phenolic groups, (d) oxidation or alkylation of sulfhydryl, thioether, imidazole or indole groups, (e) oxidation or reduction of disulfide groups, (f) glycosylation or phosphorylation through hydroxyl (O-linked) or amino (N-linked) groups (Howell, 1999). By selecting the appropriate derivatizing reagent, the charge, polarity, hydrophobicity and, indirectly, even the molecular size or shape of protein molecules may be modified to yield

desirable functional properties. However, most of these chemical modifications are not approved or acceptable for modification of proteins that are intended for human consumption. Notable exceptions are oxidizing and reducing agents such as sulfite, hydrogen peroxide, ascorbic acid and cysteine, which are commonly used for food protein modification, for example in controlling dough properties in the baking industry. In addition, acid or alkali treatments are currently being used for the purpose of deamidation or peptide bond cleavage to produce hydrolysates.

In contrast to chemical modification, enzymatic modification is generally considered to be milder, more specific, and less prone to yielding undesired side reactions. Furthermore, enzymes can usually be readily inactivated after the desired functional property has been achieved, leading to better control of the final product quality.

Proteolysis, or hydrolysis of the peptide bond, is probably the most common application of enzymatic modification of proteins. A variety of enzymes from microbial, animal and plant sources are commercially available for modification of food proteins, with different specificities and sites of action. The optimum degree of hydrolysis depends on the desired product, with limited proteolysis being used to enhance functional properties such as emulsification, foaming or curd formation, while extensive proteolysis yielding amino acids and peptides is being used to produce flavour hydrolysates or pre-digested nutritional products for enteral feeding or for individuals who have protein hypersenstitivity, gastro-intestinal disorders or other special medical concerns (Mahmoud and Cordle, 2000).

Well-known examples of enzymatic modification of protein foods include the coagulation of milk through the action of bovine rennet, recombinant chymosin or microbial rennets, the development of cheese texture and flavour through proteolytic and lipolytic action of microbial enzymes, and the tenderization of meat by application of plant proteinases such as papain, ficin and bromelain. In addition to the hydrolytic action of proteinases, the crosslinking action of microbial transglutaminase has also found commercial application in the production of restructured meat and surimi seafood products. In recent years, there has also been an increasing demand for enzymatically produced protein hydrolysates that contain peptides with specific biological properties, which can be marketed as functional food ingredients. The properties reported for these bioactive peptides include antimicrobial, antioxidant, antihypertensive, immunostimulatory or immunomodulatory, and opioid activities (Korhonen and Pihlanto-Leppala, 2001; Meisel, 1998).

2.4 Structure and function of proteins: classification and relationships

2.4.1 Classification of proteins – by structure or function

Food proteins can be classified based on either structural or functional attributes. Structural attributes that may be used as the basis for distinguishing between

groups of proteins include the amino acid composition (e.g. hydrophobic or hydrophilic balance, acidic or basic proteins, sulfur-containing proteins, proline-rich proteins), shape (e.g. globular or fibrous), secondary structure propensity (e.g. proteins with predominantly β-sheet or predominantly α-helical structures, $\alpha + \beta$, α/β, α/β linear, α/β barrel structures, or those with little ordered structure), and quaternary structure (i.e. monomeric, dimeric, etc.). Hierarchical classifications of families of protein structures are found on the world wide web, including SCOP (Structural Classification of Proteins at *http://scop.mrc-lmb.cam.ac.uk/scop/*) and CATH (Class, Architecture, Topology, Homology at *http://www.biochem.ucl.ac.uk/bsm/cath/*). Proteins can also be categorized as simple or conjugated proteins, and in the latter case, by the particular conjugating group, for example as phosphoproteins, metalloproteins, lipoproteins, glycoproteins, etc. (Regenstein and Regenstein, 1984).

Functional attributes used for classification may be based on biological functions, for example, as enzymes, hormones, transport proteins, structural proteins, contractile proteins, storage or nutrient proteins, regulatory proteins, defense proteins, etc. Alternatively, proteins can be viewed in terms of their functional role in food systems. Food proteins differ in their solubility, viscosity, water-binding, gelation, cohesion, adhesion, elasticity, emulsification, foaming, and fat or flavour binding properties (Damodaran, 1996).

The Osborne classification of proteins such as albumins, globulins, glutelins and prolamins, is an example of applying a functional attribute (solubility) to distinguish food proteins (Regenstein and Regenstein, 1984; Li-Chan, 1996). Albumins are defined as those proteins that are readily soluble in water, while globulins require salt solutions for solubilization. Glutelins are soluble in dilute acid or base, and prolamins require alcoholic media as solvents. Examples of food protein fractions prepared and identified based on these solubility classes include the gluten and non-gluten fractions of wheat, the globulins from various oilseeds, and the sarcoplasmic, myofibrillar and stroma proteins from muscle foods (Li-Chan, 1996).

Unfortunately, to date, although there are anecdotal bases for defining protein ingredients as 'good' or 'poor' with respect to particular functional properties, there has not been any systematic classification of food proteins based on functional properties other than solubility. Furthermore, our understanding of the specific structural characteristics that form the basis for discrimination between different classes of functional properties remains limited.

2.4.2 Quantitative structure-activity relationships

The function of amino acids as building blocks of proteins is based on charge, hydrophobicity and structure-forming capacity including covalent bonds and noncovalent bonds (Nakai *et al*., 1994). As reviewed by Nakai and Li-Chan (1993), quantitative structure-function or structure-activity relationships (QSAR) analysis has been successfully used to predict various properties of peptides, such as bitterness, opioid activity, hormonal function or interfacial

properties. Amino acid 'z scores', obtained by principal component analysis of parameter descriptors representing hydrophilicity (or polarity), side chain bulk (molecular size) and electronic properties, have been successfully applied to model a number of biological effects of small peptides as a function of the z-score values of the constituent amino acids in each position in a peptide (Hellberg *et al*., 1987; Sandberg *et al*., 1998; Siebert, 2001). To recognize the potential contribution of the position of particular amino acid residues within a sequence on the function of that peptide, homology pattern similarities were investigated for selected segments within sequences of the antimicrobial peptide lactoferricin and its derivatives (Nakai *et al*., 2003a).

Liebman (1998) described a virtual database, composed of a variety of existing databases. Integration of the data and information from these different databases involves developing the concepts of inheritable characteristics that a protein exhibits. To define the 'protein object concept', a set of descriptors is required that provides information on structural parameters, physico-chemical properties, and both *in vitro* and *in vivo* function (Liebman, 1998). Higher order concepts obtained through database mining using neural networks or other methodologies would be used to unify the underlying data defined by these descriptors.

A number of databases containing nucleic acid sequences, amino acid sequences, and secondary or tertiary structural information, are accessible to the public, and can be used to provide or derive information for the structural parameters and some physico-chemical properties. However, proprietary (private company) databases are the likely sources of information for the function descriptors (Liebman, 1998). Similarly, to apply this virtual distributed database concept to understand structure-function relationships of food proteins, databases must be developed to provide information for structural, functional and physicochemical descriptors of proteins in food systems. Database mining of this information to develop quantitative structure-function relationships could then be used to achieve a better understanding of the chemical and physical properties of proteins, and how these properties are affected by intrinsic parameters, interactions with other constituents, changes induced by processing, or by intentional modifications such as chemical or enzymatic treatments. These changes in the basic structural properties would in turn affect the functional properties of the food proteins.

In order to apply QSAR to understand the function of proteins, it is necessary to take into account not only the composition and characteristics of the amino acid building blocks, but also the distribution of the amino acid residues in three-dimensional space. Unfortunately, the absence of sufficiently detailed and accurate structural information for most food proteins, as well as the complexity of food systems, are major obstacles encountered in applying QSAR techniques to food proteins. Nonetheless, the development of powerful data mining techniques in recent years has led to great strides towards successful QSAR of proteins (Nakai *et al*., 2003b). In the end, selection of the appropriate technique for analysis of food protein data in the quest for QSAR will depend on an

evaluation of the benefits of increasingly complicated computer-aided techniques, balanced with the desire for simple techniques applicable to food processing (Nakai *et al*., 1994).

2.5 Future trends

The past decade has seen great advances in the understanding of structure and function of proteins in model systems. The strategy for elucidating the relationship between protein structure and function has matured from the application of chemical or enzymatic modification, as reported in earlier studies to investigate the role of particular types of amino acid functional groups, to the present-day application of molecular biology tools such as site-directed mutagenesis and protein engineering tools to pinpoint the effects of modifying specific residues in the protein sequence. These trends are likely to increase exponentially in the future, with the ever-expanding tools and database of information gathered in the '-omics' era of genomics, transcriptomics, proteomics, and metabolomics, all of which are dependent on effective data analysis via bioinformatics.

Traditionally, the spectrum of techniques for studying protein structure has been classified into those tools providing 'global' information on various functional groups or overall secondary structural composition, and those providing domain or residue-specific information. Ultraviolet absorbance, fluorescence, circular dichroism and vibrational spectroscopy are examples of techniques that can provide global information on protein structure, while high-resolution nuclear magnetic resonance spectroscopy and X-ray crystallography are capable of conveying sequence-specific information (Li-Chan, 1998). While the latter tools are obviously desirable to provide the detailed information on three-dimensional structure necessary to elucidate QSAR, the large molecular size and multi-domain characteristic of many food proteins, as well as the complexity of space and composition of food systems have been major obstacles in their practical application for studying food protein systems. Therefore, significant advances in the understanding of food proteins will require a new paradigm of the approach for analysis of their properties.

Firstly, the characteristics of the proteins must be analyzed in food systems *in situ*, or at least under conditions that are of relevance. The utilization of confocal Raman microspectroscopy to probe spatial distribution of protein and phenolic constituents in wheat grain (Piot *et al*., 2000), the monitoring of protein structure and interaction changes during cheese ripening by infrared and fluorescence spectroscopy (Mazerolles *et al*., 2001), the investigation of food protein structural networks using Fourier transform infrared spectroscopy or Raman spectroscopy (Li-Chan *et al*., 2002; Li-Chan and Qin, 1998), and the determination of secondary and tertiary structures of protein adsorbed at the interface of refractive index matched emulsions by circular dichroism and infrared spectroscopy (Husband *et al*., 2001), are examples of recent approaches in this context.

Furthermore, performing site- or domain-specific modification prior to structural or functional analysis should be considered, so that more detailed information can be obtained even using global methods of analysis. For example, recent studies have demonstrated the merit of utilizing isotope effects (such as $^{13}C=^{18}O$ labeling) to enhance the information that can be obtained from spectroscopic analysis of larger proteins or protein complexes (e.g. Faruskov Nielsen *et al*., 1996, Li *et al*., 2000; Torres *et al*., Dong *et al*., 2001). At the same time, advances in protein fusion molecular biology techniques provide the knowledge base to prepare segmental isotope-edited proteins (e.g. Otomo *et al*., 1999). New methods are being developed for ligation of segments using the protein or peptide splicing elements referred to as inteins, which are in-frame intervening sequences that disrupt a host gene and its gene product, the exteins (Perler, 2002). Application of such techniques with global methods such as vibrational spectroscopic methods would be particularly useful to study proteins under conditions that are typical of food systems, such as solids, films, emulsions, gels.

The establishment in the past decade of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometry for investigation of proteins, has also provided powerful new tools to obtain structural information even for large and complex proteins, again especially when used in conjunction with enzymatic and chemical modification or genetic engineering (Andersen *et al*., 1996).

Finally, various multivariate regression techniques and artificial neural networks have been used in recent years for correlation, classification, and optimization of structural or chemical and functional properties of food proteins (Nakai *et al*., 1994), and the trend to search for ever-more powerful algorithms will continue (Nakai *et al*., 2003a and 2003b). However, computer-aided analysis for data mining and elucidation of structure-function relationships can be effectively applied only if accurate databases of information are available. Thus, coordinated efforts of the worldwide research community are required, to promote easy access to existing and future knowledge of the diverse proteins that are present in foods. Although many databases currently exist, food scientists may need to consider what additional parameters or even what new databases or classification schemes might be considered, that would be specifically relevant for food systems. For instance, chemical and functional properties such as reactivity to Maillard browning, or affinity to an oil-water interface, or antioxidant activity, might be parameters that are of particular interest to producers and users of food proteins. ‘Standardized’ methods or protocols for systematically evaluating these properties should be established (Hall, 1996). By depositing such information into databases that are accessible through the internet so that scientists may both contribute new data and take advantage of the abundance of prior knowledge of either related or diverse proteins, the virtual database system suggested by Liebman (1998) may be used to facilitate elucidation of the distinct characteristics of proteins in foods.

2.6 Sources of further information and advice

There are a number of excellent books that the reader should consult for further information on properties of proteins in general (e.g., Branden and Tooze, 1999; Creighton, 1993; Darby and Creighton, 1993; Lesk, 2001) and in the specific context of food proteins (e.g., Hettiarachchy and Ziegler, 1994; Nakai and Modler, 1998; Sikorski, 2001). The worldwide web also contains many valuable sources of information about proteins. Table 2.5 lists key starting points for a web-based search of information on protein structure and function, and the reader may wish to consult Appendix 2 of Lesk (2001) for other 'useful web sites'. However, the reader is cautioned about the gap and the discrepancies that may exist when comparing the properties of proteins analyzed in model systems versus 'real' systems. A true understanding of the properties of food proteins will be possible only if their structure, chemical and functional properties have been investigated under conditions relevant to the actual, complex food system. It is hoped that databases cataloguing the relevant characteristics of food proteins will become valuable sources of further information in the not too distant future.

Table 2.5 Internet resources – a few useful starting points for information on chemical and structural properties of proteins

Internet resource	URL
Protein Data Bank	www.rcsb.org
National Center for Biotechnology Information, including Entrez (sequence retrieval)	www.ncbi.nlm.nih.gov www.ncbi.nlm.nih.gov/Entrez
National Biomedical Research Foundation Protein Identification Resource (PIR)	http://www-nbrf.georgetown.edu/pirwww/search/searchdb.html
Swiss Institute of Bioinformatics, including the Expert Protein Analysis System or ExPASy Molecular Biology Server	http://www.isb-sib.ch/ www.expasy.ch

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Part I

Sources of proteins

3

The caseins

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3.1 Introduction: the caseins

There are two main types of proteins in milk, which can be separated based on their solubility at pH 4.6 at 20ºC. Under these conditions, some of the proteins precipitate; these are called caseins. The proteins that remain soluble at pH 4.6 are known as serum or whey proteins. Approximately 80% of the total nitrogen in bovine, ovine, caprine and buffalo milk is casein; however, casein represents only ~40% of the protein in human milk. About 3% of the total nitrogen in bovine milk is soluble in 12% trichloroacetic acid (TCA) and is referred to as non-protein N (NPN); its principal constituent is urea. The fat globule membrane contains several specific proteins, including many enzymes, at trace levels; these represent ~1% of the total protein in milk.

Probably because of their ready availability, the milk proteins have been studied since the very beginning of protein chemistry. The first research paper on milk proteins (curd) appears to have been published by J. Berzelius in 1814. The term 'casein' appears to have been used first in 1830 by H. Broconnet, i.e., before the term 'protein' was introduced in 1838 by G. J. Mulder, whose studies included work on milk proteins. Early researchers were very confused as to the nature of proteins; they believed that there were three types of protein: albumin (e.g., egg white), fibrin (muscle) and casein (milk curd), each of which occurred in both animals and plants (Johnson, 1868). The caseins were considered to be those plant or animal proteins that could be precipitated by acid or by calcium or magnesium salts.

The preparation of casein from milk by isoelectric precipitation was improved and standardised by Hammarsten (1883); milk was diluted 1:5 with water and made to 0.1% acetic acid (which gave a pH of ~4.6); isoelectric casein

is still often referred to as casein *nach* Hammarsten. The preparation of isoelectric casein was further refined by van Slyke and Barker (1918). Isoelectric casein was considered initially to be homogeneous; the first evidence that it is heterogeneous was published by Osborne and Wakeman (1918). Further evidence of heterogeneity, based on fractionation with ethanol-HCl mixtures, was presented by Linderstrøm-Lang and Kodama (1925) and Linderstrøm-Lang (1925, 1929). However, heterogeneity was not generally accepted until the application of free boundary electrophoresis to the study of milk proteins by Mellander (1939), who showed that isoelectric casein consists of three proteins, α-, β- and γ-caseins, representing 75, 22 and 3% of total casein, respectively. Heterogeneity was also demonstrated by analytical ultracentrifugation (Svedberg *et al*., 1930; Pedersen, 1936) but protein-protein association is now known to be mainly responsible for the heterogeneity observed on ultracentrifugation.

The α-casein resolved by free boundary electrophoresis is, in fact, a mixture of three proteins: α_{s1}-, α_{s2}- and κ-caseins. Waugh and von Hippel (1956) resolved α-casein into calcium-sensitive (α_s-) and calcium-insensitive (κ-) fractions. The α_s-casein fraction was resolved further into two distinct proteins, now known as α_{s1}- and α_{s2}-caseins, by Annan and Manson (1969).

The very extensive literature on various aspects of milk proteins has been reviewed at regular intervals, including textbooks by McKenzie (1970, 1971), Fox (1982, 1989, 1992), Walstra and Jenness (1984), Wong (1988), Barth and Schlimme (1988), Cayot and Lorient (1998) and Fox and McSweeney (1998, 2003). All the principal milk proteins have been isolated and characterised thoroughly at the molecular and physico-chemical (functional) levels. However, the milk proteins are still an active and fertile subject for research: knowledge of the structure of the caseins is being refined, new biological functions are being identified and the genetic control of milk protein synthesis is being elucidated, creating the possibility of altering the protein profile of milk and exploiting the mammary gland to synthesise exogenous, possibly pharmaceutically-important, proteins.

In this chapter, the heterogeneity, molecular and functional properties of the caseins, the structure and properties of the casein micelle, the role of caseins as food ingredients and bioactive peptides derived from the caseins will be discussed.

3.2 Heterogeneity of the caseins

The four proteins in bovine casein, α_{s1}-, α_{s2}-, β- and κ-, represent approximately 38, 10, 36 and 12%, respectively, of whole casein. Each of the caseins exhibits microheterogeneity, for one or more reasons:

- variation in the degree of phosphorylation
- variation in the degree of glycosylation in the case of κ-casein

- genetically controlled amino acid substitutions, leading to genetic polymorphism
- formation of disulphide-linked polymers in the case of α_{s2}- and κ-caseins
- proteolysis by indigenous proteinases.

3.2.1 Phosphorylation of the caseins

All of the caseins are phosphorylated: most molecules of α_{s1}-casein contain 8 PO_4 residues but some contain 9; β-casein usually contains 5 PO_4 residues but some molecules contain 4; α_{s2}-casein contains 10, 11, 12 or 13 PO_4 residues; most molecules of κ-casein contain only 1 PO_4 residue but some contain 2 or perhaps 3. The phosphate groups of the caseins are esterified as monoesters of serine or, to a very minor extent, of threonine. The phosphate group for phosphorylation is provided by ATP and transfer is catalysed by casein kinases. A specific sequence, Ser.X.A (where X is any amino acid and A is an anionic residue, i.e., Glu, Asp or SerP), is required for phosphorylation. As a result of this requirement, not all Ser residues are phosphorylated; furthermore, although a few Ser residues in the sequence cited above are not phosphorylated, probably for steric reasons, no Ser residue without an adjacent anionic residue is phosphorylated. Most of the phosphoserine residues in the caseins occur in clusters.

The phosphate groups *per se* are very important from a nutritional viewpoint but they also bind polyvalent cations strongly. In milk, the principal cation bound is calcium, with smaller amounts of other cations, including Zn; these cations are very important nutritionally. Binding of cations causes charge neutralisation and precipitation of α_{s1}-, α_{s2}- and β-caseins. κ-Casein, which usually contains only 1 PO_4 residue, binds cations weakly and is not precipitated by them; furthermore, it can stabilise up to ten times its weight of calcium-sensitive caseins through the formation of micelles, the significance of which will be discussed below.

3.2.2 Glycosylation of the caseins

κ-Casein is the only glycosylated casein; it contains galactose, galactosamine and *N*-acetylneuraminic (sialic) acid, which occur either as trisaccharides or tetrasaccharides attached to theronine residues in the C-terminal region. κ-Casein may contain 0 to 4 tri- or tetra-saccharides and there are at least nine variants differing in carbohydrate content and type. The presence of oligosaccharides in the C-terminal region of κ-casein increases its hydrophilicity.

3.2.3 Genetic polymorphism of the caseins

All the caseins exhibit genetic polymorphism, which involves the substitution of 1 or 2 amino acids or, very rarely, the deletion of a sequence of amino acid residues, e.g., α_{s1}-CN A and α_{s2}-casein D (Ng-Kwai-Hang and Grosclaude,

2003). Polymorphism is determined by simple Mendelian genetics. To date, 32 genetic variants of the bovine caseins have been identified. However, since genetic polymorphism is normally detected by electrophoresis, only substitutions that cause a change in charge are detected. It is almost certain that there are numerous undetected (silent) substitutions involving uncharged residues; such variants can be detected by mass spectrometry. The presence of certain genetic variants in milk has a significant effect on some of its properties, e.g., protein content and profile, cheesemaking properties and heat stability. Goats may possess so-called null alleles, as a result of which a particular protein is absent from the milk; obviously, such an event has a major impact on the properties of milk. To date, null variants have not been detected in cattle.

3.2.4 Disulphide linking of caseins

α_{s2}- and κ-caseins contain two cysteine residues, which normally exist as intermolecular disulphide bonds; α_{s2}-casein usually exists as disulphide-linked dimers but up to at least ten molecules of κ-casein may be polymerised by intermolecular disulphide bonds. The absence of cysteine or cystine from α_{s1}- and β-caseins increases their flexibility.

3.2.5 Impact of indigenous proteinases

Milk contains a number of indigenous proteinases (Kelly and McSweeney, 2003), the most important of which is plasmin, to which the caseins show different susceptibilities. Plasmin is a serine proteinase which is highly specific for peptide bonds, the carbonyl group of which is supplied by lysine or, to a lesser extent, arginine. β-Casein is the most susceptible of the caseins and in milk is cleaved rapidly at three sites, Lys_{28}-Lys_{29}, Lys_{105}-His_{106} and Lys_{107}-Glu_{108}, yielding peptides known as the γ-caseins and the proteose peptones (PP) PP 5, PP8 slow and PP8 fast. The casein-derived peptides are now named after the parent casein, e.g., γ_1-, γ_2- and γ_3-caseins are now designated β-CN f29–209, f106–209 and f108–209, respectively. Similarly, the proteose peptones PP5, 8 fast and 8 slow are β-CN f1–105/107, f29–105/7 and f1–28, respectively.

Isolated α_{s2}-casein is rapidly hydrolysed by plasmin (Le Bars and Gripon, 1989) but α_{s2}-CN-derived peptides have not been identified in milk. Isolated α_{s1}-casein is also readily hydrolysed by plasmin (Le Bars and Gripon, 1993; McSweeney *et al*., 1993) and a group of minor peptides, known as the λ-caseins, are produced from this protein in milk (Aimutis and Eigel, 1982; O'Flaherty, 1997). κ-Casein is very resistant to hydrolysis by plasmin, whether in buffer or in milk. Milk contains several other indigenous proteinases, including cathepsins B and D; it is probable that these proteinases produce some unidentified peptides in milk. Proteolytic enzymes of microbial origin may also be present in poor-quality milk.

Clearly, the casein system is very heterogeneous and a logical nomenclature system is necessary. Rapid progress in the fractionation of the caseins was made

in the 1950s and 1960s and this led the American Dairy Science Association to establish a Nomenclature Committee with a brief to develop nomenclature guidelines. The original report (Jenness *et al*., 1956) has been revised five times, most recently by Eigel *et al*. (1984); a sixth revision is in preparation, under the chairmanship of Dr R. Jiménez-Flores.

In addition to establishing and updating the nomenclature of milk proteins, these reports contain very useful summaries of the heterogeneity and properties of milk proteins. The presently accepted nomenclature is as follows:

- The casein family is indicated by a Greek letter with a subscript, if necessary, i.e., α_{s1}-, α_{s2}-, β-, κ-.
- This is followed by CN (for casein).
- The genetic variant is indicated by a Latin letter, A, B, C, etc., with a superscript, if necessary, e.g., α_{s1}-CN B, β-CN A^1.
- Finally, the number of phosphate residues is indicated, e.g., α_{s1}-CN B-8P, β-CN A^1-5P.

3.3 Molecular properties of the caseins

The caseins are very well characterised proteins; their more important properties are summarised in Table 3.1. Some of their important molecular properties were discussed in the preceding section; additional properties are discussed below.

The caseins are quite small proteins, with a molecular mass of 20–25 kDa, which probably contributes significantly to their remarkably high stability. The caseins, especially β-casein, contain a high level of proline; in β-casein, 35 of the 209 amino acid residues are proline; these are uniformly distributed throughout the molecule. The presence of a high level of proline prevents the formation of α-helices, β-sheets and β-turns.

The caseins are relatively hydrophobic and, in particular, have a high surface hydrophobicity owing to their open structures. Experimental studies indicate that the caseins have low levels of secondary and tertiary structures (they have little α-helical structure, no denaturation temperatures and high hydrodynamic volumes), although theoretical calculations indicate that they do possess some higher structures. It has been suggested that, rather than lacking secondary structures, the caseins have very flexible, unstable structures and therefore they have been described as ‘rheomorphic’ (i.e., the casein molecules, in solution, are sufficiently flexible to adopt structures that are dictated by their environment; Holt and Sawyer, 1993). The lack of stable secondary and tertiary structures renders the caseins stable to denaturing agents, e.g., heat or urea, and contributes to their high surface activity. This latter property also confers good foaming and emulsifying properties, and renders the caseins readily susceptible to proteolysis, which is important for digestibility, in cheese ripening and for the production of protein hydrolyzates for dietary applications.

Table 3.1 Properties of the principal caseins in cows' milk

Protein	Molecular mass	Amino acids	Proline residues	Cysteine residues	PO_4 groups	Concentration (g/L)	Glyco-protein	Genetic variants
α_{s1}-casein	23164	199	17	0	8	10	No	A, B, C, D, E, F, G, H
α_{s2}-casein	25388	207	10	2	10–13	2.6	No	A, B, C, D
β-casein	23983	209	35	0	5	9.3	No	A^1, A^2, A^3, B, C, D, E, F, G
κ-casein	19038	169	20	2	1	10.3	Yes	A, B, C, E, F^S, F^I, G^S, G^S

The primary structures of the four bovine caseins, and of the principal genetic variants, are known (Swaisgood, 2003). Hydrophobic, polar and charged residues on the caseins are not uniformly distributed throughout the sequences but occur as hydrophobic or hydrophilic patches, giving the caseins strongly amphipathic structures which make them highly surface active. For example, the N-terminal 2/3 of κ-casein is hydrophobic while the C-terminal 1/3 is strongly hydrophilic; this structural feature is highly significant for the properties and stability of casein micelles. The hydrophobicity of the caseins explains why their hydrolyzates have a high propensity to bitterness, which causes sensory defects in many cheese varieties.

3.3.1 Non-bovine caseins

The milk of all mammalian species that have been studied contain casein but the concentration, types and molecular properties of the individual caseins vary considerably. An interspecies comparison of the caseins was prepared by Martin *et al*. (2003). The principal, perhaps the only, function of the caseins is nutritional, and therefore it is not surprising that there is a good correlation between the growth rate of the neonate and the casein content of its mother's milk (Bernhart, 1961). However, since a low level of amino acid substitution due to genetic mutation, particularly of non-essential amino acids, will not significantly affect the nutritional value of casein, the caseins have evolved rapidly and are, in evolutionary terms, the most divergent family of mammalian proteins (Holt and Sawyer, 1993; Martin *et al*., 2003).

In terms of the individual caseins, β-casein is the most homologous casein, although it shows only a low level of homology; the only homologous sequences of the β-caseins of the cow, sheep, goat, camel, pig, rabbit and human are the signal peptide, the two N-terminal residues of the mature protein and the sequence Ser-Ser-Ser-Glu-Glu (residues 17–21 of the mature protein, which is the principal phosphorylation site; in mouse and rat β-casein, Ser_{18} is missing). The sequence of the phosphorylation sites is also conserved in α_{s1}- and α_{s2}-caseins. In κ-casein, not even the chymosin cleavage site is conserved; in bovine, buffalo, ovine and caprine κ-caseins the rennet-sensitive bond is Phe_{105}-Met_{106}, but in camel, porcine, human, rat and mouse κ-caseins it is Phe-Ile, Phe-Ile, Phe-Ile, Phe-Leu and Phe-Leu, respectively.

It has been proposed (Gustchina *et al*., 1996) that chymosin exists in active and inactive forms and that the latter is activated by the sequence His-Pro-His-Pro-His, residues 98–102 of κ-casein. This sequence occurs in bovine, buffalo, ovine and caprine κ-caseins, but in porcine, camel and human κ-caseins, the corresponding sequences are Arg-Pro-Arg-Pro-His, Arg-Pro-Arg-Pro-Arg and Arg-Pro-Asp-Leu-His, respectively. If the proposal of Gustchina *et al*. (1996) is correct, calf chymosin would not be activated by, and should be unable to hydrolyse, the κ-casein of these latter species; in fact, they are hydrolysed, although data on the kinetics of hydrolysis are not available.

3.4 The caseins as food constituents and ingredients

3.4.1 Overview of role of caseins in dairy products

In western countries, milk and dairy products supply about 30% each of dietary energy, lipids and protein, as well as a substantial portion of many vitamins, especially riboflavin and vitamin B12, and minerals, especially calcium, about 80% of which is derived from dairy products. Some raw (unprocessed) milk is consumed, but essentially all milk is processed to a greater or lesser extent. Although precise data are lacking, relatively little milk is consumed directly as a beverage, most being used as an ingredient in, or as a raw material for, other food products.

With the exception of butter, anhydrous milk fat, high-fat creams and lactose, most dairy products can be regarded as protein-rich, and their key properties depend on certain properties/characteristics of milk proteins, especially of the caseins. The key attributes/functions of proteins in the principal dairy products are:

- Pasteurised liquid milk: appearance (colour), heat stability, mouth-feel, (off)-flavour (especially bitterness and sunlight oxidised flavour).
- In-container and UHT-sterilised products: appearance, colour (Maillard browning), flavour (cooked, caramel, Maillard products), heat stability, age gelation, viscosity (body).
- Fermented milks: gel formation, rheological properties of the gel, mouth-feel, syneresis of the gel, (off)-flavours.
- Butter and anhydrous milk fat: no significant effects.
- Creams: emulsion stability, rheology (body), whippability.
- Ice-creams: emulsion stability, rheological properties (body), whippability (over-run).
- Milk powders: wettability, dispersibility, solubility, flavour, colour; other important properties depend on the end-use of the powder.
- Cheese: texture, body and other rheological properties; functional properties such as meltability, stretchability, sliceability, adhesiveness, water-binding properties; (off-)flavours.

The nutritional properties of the proteins are of primary importance, but most western diets provide more protein than is necessary.

It is not possible to review all of the technologically-important properties of caseins here. As well as the books on milk proteins cited earlier, there are one or more text-books on each of the major families of dairy products, e.g., general dairy science and technology (Hui, 1993; Walstra *et al*., 1999), cheese (Fox, 1993; Robinson and Wilbey, 1998; Law, 1999; Eck and Gilles, 2000; Fox *et al*., 2000, 2003), liquid milk (Henderson, 1971; Lewis and Heppell, 2000), milk powder (Caric, 1994; Pisecky, 1997), ice-cream (Marshall and Arbuckle, 1996), yoghurt (Tamime and Robinson, 1999) and functional milk proteins (Fox, 1989). In the following section, we will discuss developments in functional milk proteins.

3.4.2 Casein products

Functional milk proteins are of major significance to the dairy and general food industries (see Fox, 2001). Owing to the ease with which casein can be produced by isoelectric precipitation or rennet-induced coagulation, it has been produced commercially since the early 20th century. Initially, casein was used only for industrial applications, e.g., glues, plastics and paper glazing, and was essentially a by-product of minor economic importance. Pioneering work in New Zealand and Australia in the 1960s up-graded casein for use as a food ingredient; consequently, it became a much more valuable product and is now one of the principal functional food proteins. The production of functional food-grade casein in the 1960s coincided with the development of processed food products that require functional proteins. The most important applications of caseins are in cheese analogues, especially pizza cheese, as an emulsifier in coffee whiteners, synthetic whipping creams and cream liqueurs, and in fabricated meats, some cereal products and various dietetic foods.

Technology for the production of acid and rennet caseins and caseinates is well established and has been improved regularly (Mulvihill, 1992; Mulvihill and Fox, 1994; Mulvihill and Ennis, 2003). A number of new methods, e.g., cryoprecipitation and ethanol precipitation, for the preparation of casein products with interesting properties have been developed but have not been applied industrially. Casein co-precipitates, which include denatured or, less commonly, native whey protein, have also been developed as food ingredients.

The development of large-pore membranes, with a cut-off in the range 0.01–10 μm, makes it possible to separate the casein micelles from the whey proteins by microfiltration (MF). Relatively tight MF membranes (0.1 μm) in cross-flow conformation are used to produce native micellar casein. The composition of this product is similar to that of calcium caseinate; it exhibits good rennet coagulation properties and is particularly well suited for increasing the protein content of cheesemilk, thereby improving the quality of cheese and increasing the capacity of a cheese plant (Kelly *et al*., 2000). Garem *et al*. (2000) described the manufacture of a milk powder with improved cheesemaking properties; the process involves removal of whey proteins by a combination of microfiltration and ultrafiltration. The production of micellar casein powders with significant potential applications for cheese manufacture was reviewed by Saboya and Maubois (2000).

3.4.3 Fractionation of casein

Since the 1940s, it has been possible to fractionate whole casein into its component proteins on a laboratory scale by exploiting differences in the solubility of the individual caseins in solutions of urea at ~pH 4.6, or in $CaCl_2$ or by various forms of chromatography. However, these methods are not suitable for the industrial-scale production of individual caseins, for which there are believed to be potential opportunities because:

- β-Casein has very high surface activity and may have applications as a high-quality emulsifier or foaming agent.
- Human milk contains mainly β- and κ-caseins but very little or no α-caseins; hence, β-casein should be an attractive ingredient for the manufacture of bovine milk-based infant formulae.
- κ-Casein, which is responsible for the stability of casein micelles, might be a useful additive for certain milk products.
- It has been reported that fortification of milk with β-casein improves its cheesemaking properties.
- All the milk proteins contain sequences which have biological activity when released by proteolysis; the production of biologically-active peptides requires purified protein substrates.

As mentioned earlier, a number of methods for the large-scale preparation of β-casein have been described, leaving a fraction enriched in α_s- and κ-caseins. These methods are based on the dissociation of β-casein at a low temperature, due to its high hydrophobicity. Some of these methods use UF or MF to separate monomeric β-casein from the more aggregated α_{s1}-, α_{s2}- and κ-caseins in milk or sodium caseinate (see Mulvihill and Fox, 1994), others exploit the dissociation of β-casein from renneted Ca-caseinate (Ward and Bastian, 1996) or from renneted milk (Fig. 3.1). The latter method is capable of giving a very high yield of β-casein, with γ-caseins as the principal contaminants, and can be included readily in the industrial process for the production of rennet casein. κ-Casein-rich protein dissociates from the micelles when milk or a serum protein-free dispersion of casein micelles is heated to at least 90 ºC at pH $\geq$6.7; in milk, the dissociated κ-casein is complexed with whey proteins (Singh *et al*., 1993).

3.4.4 Bioactive peptides in milk protein hydrolysates

The bovine caseins contain peptide sequences which have specific biological activities when released by enzymatic hydrolysis, including the following:

- phosphopeptides
- caseinomacropeptide (CMP)
- caseinomorphines
- immunomodulating peptides
- blood platelet-modifying (antithrombic) peptides (e.g., casoplatelin)
- anginotensin-converting enzyme (ACE) inhibitors, sometimes referred to as casokinins
- bacteriocidal peptides.

Casein-derived bioactive peptides have been the subject of considerable research for several years and the very extensive literature has been reviewed by Fox and Flynn (1992), Mulvihill and Fox (1994), Pihlanto-Lappälä (2002), Gobbetti *et al*. (2002) and FitzGerald and Meisel (2003).

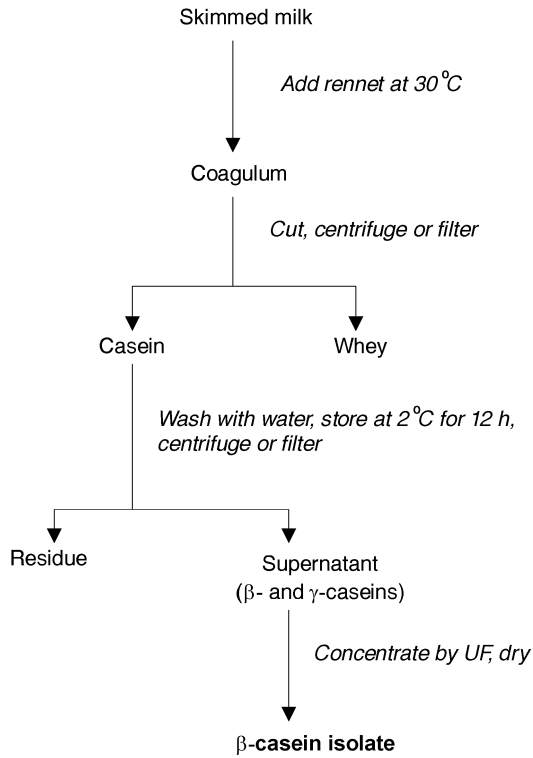

Fig. 3.1 Procedure for the isolation of β-casein from rennet casein curd (P. F. Fox, Shakeel-Ur-Rehman and T. Considine, unpublished).

β-Casomorphins, which are derived from β-casein residues 60–70 may inhibit gastrointestinal motility and the emptying rate of the stomach by direct interaction with opioid receptors. ACE is a dipeptidyl carboxypeptidase that catalyses the production of the vasoconstrictor, angiotensin II, and plays an important role in the regulation of blood pressure and hypertension.

CMP (κ-CN f106–169) results from hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein on renneting; it diffuses into the whey, while the remaining portion of κ-casein (*para*-κ-casein, κ-CN f1–105) remains with the curd. Relatively high levels of CMP are present in whey (~4% of total casein, 15–20% of protein in cheese whey; 180×10^3 tonnes *per annum* are available globally from whey), and can be quite easily recovered therefrom. CMP has several interesting biological properties, as it:

- has no aromatic amino acids and hence is suitable for individuals suffering from phenylketonuria (although it lacks several essential amino acids)
- inhibits viral and bacterial adhesion
- promotes the growth of bifidobacteria
- suppresses gastric secretions

- modulates immune system responses
- inhibits the binding of bacterial toxins (e.g., cholera and *E.coli* toxins).

Peptides derived from CMP by proteolysis may have antithrombotic properties or may act as growth promoters for *Lc. lactis* subsp. *lactis*.

Currently, few milk-derived biologically active peptides are produced commercially although many of them have been purified partially by UF and hence are amenable to large-scale production. Also, in general, the biological activity of most or all of these peptides *in vivo* remains to be demonstrated. Perhaps the peptides most likely to be commercially viable in the short-term are the caseinophosphopeptides, which contain clusters of serine phosphate residues. It is claimed that these peptides promote the absorption of metals (Ca, Fe, Zn), through chelation and acting as passive transport carriers for the metals across the distal small intestine, although evidence for this is equivocal. Caseinophosphopeptides are currently used in some dietary and pharmaceutical supplements, for example in treatment of dental caries.

3.5 The casein micelle: introduction

3.5.1 What is a micelle?

A colloid or colloidal dispersion (sol) consists of solid particles in the range 1 to ~1000 nm dispersed in a liquid. The dispersed particles may be:

- very finely divided solids, having the same internal structure as the bulk solid
- large molecules (e.g., proteins, polysaccharides, nucleic acids, synthetic polymers) with at least one dimension > 1 nm
- aggregates formed by the association of small molecules; these are referred to as *association colloids* or *micelles*.

According to Sørensen (1930), the word 'micelle' or 'micella' (diminutive of the Latin, *mica*, meaning crumb) was introduced by the botanist C. von Nägeli in 1879 to describe the crystalline particles or molecular groups that he considered to be constituents of high molecular weight natural substances. Classically, micelles are aggregates of amphipathic molecules (e.g., surfactants, detergents, soaps) which are in dynamic equilibrium with surfactant monomers (Hunter, 1987; Dickinson, 1992). These classical micelles were studied extensively during the early decades of the 20th century (McBain and Salmon, 1920).

The caseins in milk exist as colloidal particles (50–500 nm), which are now almost always referred to as 'casein micelles'. However, this is a misnomer (Dickinson, 1992); casein micelles are irreversibly aggregated over normal time-scales, although a solution of pure β-casein does form reversible aggregates at concentrations above the critical micelle concentration in a way which has some resemblance to classical surfactant micelles. Dickinson (1992) urges that, to avoid confusion, the term 'casein micelle' should never be shortened to 'micelle'.

3.5.2 Historical development of the concept of the casein micelle

It has been known since the work of Schubler in 1818 that casein is merely 'suspended' in milk (Palmer and Richardson, 1925) and, by the end of the 19th century, it was recognised that the casein in milk exists as large particles which are retained by Pasteur-Chamberland porcelain filters (Blyth, 1896; Richmond, 1899). The individual casein molecules are non-dialysable and can scatter light and therefore are classified as colloids. Since the term 'casein micelles' is a misnomer it seemed of interest to attempt to trace the usage of this term.

During the early years of the 20th century, a number of studies were reported on the 'colloidal chemistry of milk' (Wiegner, 1914) in which the term 'casein particles' or 'large casein particles' was used to describe the form of casein. The stability of these particles, especially how they are destabilised during the rennet-induced coagulation of milk, attracted much attention. It was suggested by Alexander (1910) that the particles are stabilised by a protective colloid, which he proposed is 'lactalbumin'. He suggested that the poor renneting properties of human and ass's milk are due to the low level of casein and the high level of lactalbumin in those milks. Early work on the rennet coagulation of milk was reviewed by Palmer and Richardson (1925), who dismissed the protective colloid idea and proposed that the rennet coagulation of milk is due to the 'precipitation of micellae by cations in the presence of a suspensoid which is peptidised by the precipitating ion'. This is the only mention of 'micellae' in that paper and no explanation of the term is given.

The term 'casein micelle' was not used by Clayton (1918) and Palmer and Scott (1919) in papers on the colloidal and physicochemical properties of milk proteins. As far as we can ascertain, the first author to use the term 'casein micelle' was Beau (1921), who introduced the term 'lacteine', for complexes, which he called 'micelles', comprised of caseins and whey proteins. Perhaps this concept is based on the proposal of a 'protective colloid' in milk. The term 'micelle' was used extensively by Porcher (1923), in his review on the chemistry of milk constituents. Unfortunately, Beau (1921) and Porcher (1923) did not explain why they used the term and the articles are not referenced. Marui (1926), who discussed the rennet coagulation of milk, also used the term 'casein micelle' without explanation and, again unfortunately, the paper is sparsely referenced. Marui (1926) also used the term 'Schutzkolloid' (protective colloid) in relation to micelle stability and rennet-induced coagulation. Linderstrøm-Lang (1929) also used the term 'Schutzcolloid' but did not use the term 'casein micelle'. Richardson and Palmer (1929) freely used the terms 'micellae', 'caseinate micellae', 'calcium caseinate micellae' and even 'rennin micellae'.

In a long series of articles on the rennet coagulation of milk from the colloidal viewpoint, published in *Le Lait*, volumes 9, 10 and 11 (1929, 1930, 1931), Porcher used the term 'micelle' freely; the best description of the micelles is given in Porcher (1929). The term 'micelle', in relation to the colloidal state of casein, was also used by Brigando (1933). Interestingly, most of the early papers in which casein micelles were mentioned involved attempts

to explain the rennet coagulation of milk. In the textbook *Fundamentals of Dairy Science* (Associates of Rogers, 1928, 1935), the term was not used in the chapters on Milk Proteins or Physical Equilibria of Milk but was used to a very limited extent by L. S. Palmer in the section on the Rennet Coagulation of Milk.

Sørensen (1930) compiled an extensive review of micelles formed by the association of soluble proteins, including casein. He considered casein to be particularly interesting because, as discussed earlier, isoelectric casein had just been shown to be heterogeneous. He proposed that casein dissolved in acid or alkali existed as micelles but, surprisingly, he did not discuss the natural casein colloidal particles and did not reference any of the earlier studies in which the colloidal aspects of casein had been discussed. The term 'casein micelles' was not used in the Dairy Chemistry textbooks of the period (Davies, 1936, 1939; Ling, 1946) or in the comprehensive review by McMeekin and Polis (1949).

Without offering an explanation for doing so, Eilers (1947) described the casein particles in milk as 'casein micellae' and listed several of their properties, as determined by him and others. However, the terms 'casein particles', 'calcium caseinate particles', 'calcium caseinate-calcium phosphate' or 'colloidal particles' were used by others during this period (e.g., Nichols *et al*., 1931; Ford and Ramsdell, 1949; Hostettler and Imhof, 1953; Ford *et al*., 1955). Jenness and Patton (1959) acknowledged the term 'micelle' but decided to use the term 'calcium caseinate-phosphate complex'. In his review on the chemistry of casein, Pyne (1955) usually used the term 'caseinate-phosphate complex' but used the term 'caseinate micelle' occasionally. Without any explanation for doing so, von Hippel and Waugh (1955) used the term 'casein micelle' extensively and it has since come into general use.

The proposals of the involvement of a protective colloid (Alexander, 1910; Marui, 1926; Linderstrøm-Lang, 1929) in the stability of casein micelles could be regarded as attempts to describe their structure. There were also proposals that casein acts as a protective colloid for calcium phosphate (now referred to as colloidal calcium phosphate). The relationship between the colloidal casein particles and calcium phosphate was investigated by van Slyke and Bosworth (1915); using differential centrifugation, they showed that the ratio of casein (organic) phosphate to inorganic phosphate depended on the length of centrifugation and concluded that calcium caseinate and inorganic calcium phosphate were not chemically linked. Perhaps rather surprisingly, the faster-sedimenting larger particles were reported to contain less inorganic phosphate than the slower-sedimenting, presumably smaller, particles; however, McGann *et al*. (1979) showed that the ratio of colloidal phosphate to casein is higher in large micelles than in smaller ones, while Dalgleish *et al*. (1989) found no relationship between casein micelle size and the concentration of colloidal calcium phosphate.

The development of a meaningful model of casein micelle structure became possible only after the isolation of κ-casein and the description of its key properties (Waugh and von Hippel, 1956). The first, very simplistic, model of the casein micelle was published by Waugh (1958). During the next decade or

so, several papers on the formation and properties of artificial casein micelles were published by Waugh and his colleagues. These studies were reviewed by Waugh (1971), who included a brief explanation for his choice of the term 'casein micelle' and elaborated on a model for the structure of casein sub-micelles.

Since the pioneering work of Waugh (1958), there has been continuous work on the properties and structure of the casein micelle, and structural models have been refined progressively. The extensive literature has been reviewed at regular intervals (Rose, 1969; Waugh, 1971; Garnier, 1973; Farrell, 1973; Slattery and Evard, 1973; Farrell and Thompson, 1974; Slattery, 1976; Schmidt, 1980, 1982; Payens, 1979, 1982; Walstra and Jenness, 1984; McMahon and Brown, 1984; Ruettimann and Ladisch, 1987; Rollema, 1992; Visser, 1992; Holt, 1992, 1994; Walstra, 1990, 1999; Holt and Horne, 1996; Horne, 1998, 2002; de Kruif, 1999, Walstra *et al*., 1999; de Kruif and Holt, 2003). Before discussing the more significant of these models, the general properties of the casein micelles will be described.

3.6 Properties and stabilisation mechanisms of casein micelles

As discussed above, it has been known since the beginning of the 19th century that casein in milk exists as large colloidal particles. It is now clear that the caseins have a strong tendency to form large macromolecular aggregates. Even in solution, individual caseins display a strong tendency to self-associate; β-casein, which is an amphiphatic molecule with a hydrophilic head (residues 1–60) and a hydrophobic tail, forms classical detergent-like micelles, with a critical micelle concentration in the range 0.3 to 0.7 mg/ml, depending on pH, ionic strength and temperature (Rollema, 1992). α_{s1}-Casein forms long polymers of individual molecules, while κ-casein polymerises via interactions between the hydrophobic C-terminii of individual molecules, and by intermolecular disulphide bonds.

Casein micelles may be studied either in milk or after separation therefrom; they can be readily recovered from milk by ultracentrifugation or ultrafiltration. Preparative ultracentrifugation is most widely used; ~95% of the micelles are sedimented by ultracentrifugation at 100,000 g for 1 h. Under such conditions, only very small κ-casein-rich micelles and some β-casein, which dissociates from the micelles at low temperatures, are lost in the supernatant. The pellet is readily dispersible (using a mortar and pestle or Potter homogeniser) in milk dialysate or a synthetic milk salts buffer (Jenness and Koops, 1962), preferably with continuous stirring overnight at 4 ºC using a magnetic stirrer or other suitable device. The properties (size, hydration, heat stability, rennet coagulability) of the dispersed micelles are similar to those of the native micelles. The micelles can be fractionated according to size by differential ultracentrifugation at progressively increasing gravitational force. Ultrafiltration (UF) using porcelain filters was used in the 1880s to recover casein particles but

these filters became blocked easily and were difficult to use. UF can now be applied readily on a laboratory or industrial scale.

Electron microscopy shows that casein micelles are generally spherical in shape with a diameter ranging from 50–500 nm (average ~ 120 nm) and a mass ranging from 10^6 to 3×10^9 Da (average ~ 10^8 Da). There are very many small micelles, but these represent only a small proportion of the mass. There are 10^{14}–10^{16} micelles mL^{-1} milk, and they are roughly two micelle diameters apart, i.e., they are quite tightly packed. Since the micelles have colloidal dimensions, they are capable of scattering light and the white colour of milk is due largely to light scattering by the casein micelles, with a contribution from fat globules; the white colour is lost if the micelle structure is disrupted. As the temperature is lowered, caseins, especially β-casein, dissociate from the micelles; the amount of β-casein that dissociates varies from 10 to 50%, depending on the method of measurement.

The micelles are highly hydrated, binding ~ 2.0 g H_2O g^{-1} protein; the dry material in the micelles consists of ~94% protein and ~6% other material, mainly calcium and phosphate, with small amounts of magnesium and citrate and trace amounts of other metals, e.g., zinc. This non-protein material, generally referred to as colloidal calcium phosphate (CCP), has a major influence on the properties and behaviour of the micelles. α_{s1}-, α_{s2}- and β-caseins, which together represent ~ 85% of whole casein, are precipitated by Ca at a concentration > 6 mM. Since bovine milk contains ~ 30 mM Ca, it might be expected that these caseins would precipitate in milk. However, as stated earlier, κ-casein is insensitive to Ca^{2+} and can stabilise the other caseins against precipitation by Ca^{2+} by forming a type of quaternary structure, i.e., the casein micelle.

The Ca-binding properties of the caseins and their organisation into micelles enables a high concentration of calcium phosphate to be maintained in a 'soluble' form in milk; without this property, $Ca_3(PO_4)_2$ would precipitate in, and block, the ducts of the mammary gland, resulting in the death of the gland and perhaps of the animal. The ability to 'solubilize' calcium phosphate is considered to be a biological function which has influenced the evolution of the caseins. Without the presence of the caseins, milk would be unable to sustain the high levels of calcium and phosphorus it contains, and hence would be a far less nutritionally-valuable product (Holt, 1994). The principal properties of the casein micelle are summarised in Table 3.2.

3.6.1 Mechanism of stabilisation of casein micelles

It is universally accepted that κ-casein is the principal stabilising factor in the casein micelle and represents the stabilising colloid proposed, e.g., by Linderstrøm-Lang (1929). There have been occasional proposals, e.g., Parry and Carroll (1969) and Garnier and Ribadeau-Dumas (1970), that κ-casein is located within the micelle, possibly as nodes in a molecular network, but there is widespread, probably now unanimous, support for the view that this protein is

Table 3.2 Average characteristics of casein micelles (from Fox and McSweeney, 1998)

Characteristic	Value
Diameter	50–500 nm (mean 120)
Surface area	8×10^{-16} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g/cm^3
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 cm^3/g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^8 Da
Number of peptide chains	10^4
Number of particles/mL milk	10^{14}–10^{16}
Surface of micelles/mL milk	2×10^4 cm^3
Mean free distance	240 nm

located predominantly on the surface of the micelle (Holt, 1992, 1994; Holt and Horne, 1996; Horne, 1998, 2002; de Kruif and Holt, 2003).

Evidence for a surface location includes the observations that:

- κ-casein can stabilise 8–10 times its weight of Ca-sensitive caseins (~ 85% of total casein) and a surface location would facilitate this, analogous to the stabilisation of lipid emulsions by a thin surface layer of amphipathic molecules (emulsifiers),
- small casein micelles have a higher content of κ-casein than large ones, reflecting the larger surface area-to-volume ratio of the latter (see, e.g., McGann *et al*., 1980; Davies and Law, 1983; Dalgleish *et al*., 1989),
- κ-casein is readily available for hydrolysis by chymosin and other acid proteinases. Chymosin has a molecular mass of ~36 kDa, suggesting that the micelle is either very porous, allowing chymosin to move freely through it, or that κ-casein is located at the micelle surface. Since the active site of chymosin and other acid proteinases is located in a deep cleft, which is very small in comparison with the casein micelle (MW ~ 10^8 Da), the sequence of κ-casein entering this cleft must be very exposed, also favouring a surface location,
- following thermal denaturation, β-lactoglobulin (dimeric MW ~ 36 kDa) interacts readily with micellar κ-casein via sulphydryl-disulphide interchange interactions. A surface location of κ-casein or a very porous micellar structure would be necessary to facilitate such a reaction.

How does κ-casein function as a micelle stabiliser? κ-Casein has an amphiphatic structure, with a rather hydrophobic N-terminal region (the *para*-κ-casein formed on hydrolysis by chymosin; κ-CN f1-105) and a hydrophilic C-terminal, the caseinomacropeptide, κ-CN f106-169, which contains the oligosaccharide moieties, has no aromatic residues and has quite a strong net

negative charge (12 anionic residues but only 3 cationic residues). Thus, κ-casein is a major contributor to the micellar zeta-potential of -20 mV. As will be discussed later, the C-terminal region protrudes from the micelle surface, creating a steric stabilising layer, the idea of which probably originated with Hill and Wake (1969), who considered the amphipathic structure of κ-casein to be an important feature of its micelle-stabilising properties. This protruding layer, which has been estimated to be ~7 nm thick, gives micelles a hairy appearance; this layer has been described as a salted polyelectrolyte brush (Tuinier and de Kruif, 2002).

It has been suggested that the surface of the casein micelle is only partly covered by κ-casein, and that the κ-casein is heterogeneously distributed over the surface (Dalgleish, 1998). This surface coverage is sufficient to provide steric stabilisation against the approach of large particles, such as other micelles, but the small-scale heterogeneities and the gaps between κ-casein molecules provide relatively easy access for molecules with dimensions of individual proteins or smaller.

The extension, and hence the protective effect, of the κ-casein brush layer is affected directly by brush density (it can be reduced, for example, by the action of proteinases during rennet coagulation of milk), charge density along the chain (pH-dependent), concentration of divalent salt ions (e.g., calcium) and polarisability of the solvent (e.g., ethanol content). Such changes destroy the colloidal stability of the micelles, and they coagulate or precipitate (Holt and Horne, 1996). The effects of such factors on the casein micelle will be discussed later in this chapter.

3.7 Structure models of the casein micelle

The structure of the casein micelle has attracted the attention of scientists for many years. Knowledge of the structure of the micelle is important because reactions undergone by the micelles are central to many dairy products and processes (e.g., cheese manufacture; stability of sterilised, sweetened-condensed, and reconstituted milks; and frozen products). Without knowledge of the structure and properties of the casein micelle, attempts to solve many technological problems faced by the dairy industry can only be empirical and not generally applicable; many processing operations are the result of trial and error. From an academic viewpoint, the casein micelle presents an interesting and complex problem in protein quaternary structure.

The micelles cannot be seen by light microscopy but can be visualised by ultramicroscopy (e.g., Alexander, 1910; Van Slyke and Bosworth, 1915) which gives no information on the structure of the micelles. Electron microscopy (EM) has been used widely in the study of casein micelles; the first EM study was probably that of Shimmin and Hill (1964) and various methods of sample preparation and staining techniques have been applied since. EM provides valuable information on the size and shape of the micelles but individual casein molecules cannot be seen or differentiated. Therefore,

attempts to describe the structure of the casein micelle have relied strongly on the effects of various treatments and reagents on their behaviour and the elaboration of models.

The models proposed to describe the structure of the casein micelle fall into four general categories, although there is some overlap: core-coat, internal structure, submicelles (in many of these models, it is proposed that the submicelles have a core-coat structure) and gel-like aggregates. The evidence supporting the first three of these alternative structures was discussed by Ruettimann and Ladisch (1987). The core-coat and internal structure models, e.g. those proposed by Payens (1966), Parry and Carroll (1969), Rose (1969) and Garnier and Ribadeau-Dumas (1970), have been largely abandoned and will not be discussed further.

For a long time, there has been strong support for the view that the casein micelle is composed of submicelles, 10–15 nm in diameter and of mass ~10^6 Da. In this type of model, first proposed by Morr (1967), the sub-micelles are considered to be held together by nano-crystals of calcium phosphate (colloidal calcium phosphate, CCP) and by hydrophobic and hydrogen bonds. The seminal idea for the model was the appearance of the micelles on electron microscopy: the micelles appear variegated, with regions of high and low electron density. This was referred to as a 'raspberry-like' structure, and was assumed to be due to a sub-micelle structure (Shimmin and Hill, 1964).

On removal of CCP (e.g., by acidification/dialysis, EDTA, citrate or oxalate) the micelle disintegrates and particles in CCP-free milk, with a mass of ~500 kDa, are considered to be the sub-micelles, although there is no direct evidence for this. The properties of CCP-free milk are very different from those of normal milk (e.g., it is sensitive to, and precipitated by, relatively low levels of Ca^{2+}, it is more stable to heat coagulation and it is not coagulable by rennets). Many of these properties can be restored, at least partially, by increased concentrations of calcium. However, CCP is not the only integrating factor, as indicated by the dissociating effect of urea, SDS, ethanol, or alkaline pH (as will be discussed later).

Waugh (1971) proposed a rosette-type structure, very similar to that of a classical soap micelle, for the casein sub-micelles; it was proposed that the polar regions of α_{s1}-, β- and κ-caseins are orientated towards the outside of the submicelle to reduce electrostatic repulsion between neighbouring charged groups and that each submicelle is surrounded by a layer of κ-casein, which also provides a κ-casein coat for the entire micelle. The role of CCP was not considered in the development of this model, which appears to be a major weakness. It may also be difficult to explain by this model why some β-casein dissociates from the casein micelle on cooling.

The submicelle model of Morr (1967) has been modified by several authors. Slattery and Evard (1973) and Slattery (1976) proposed that the submicelles are not covered completely by a layer of κ-casein and that there are κ-casein-rich, hydrophilic and κ-casein-deficient, hydrophobic regions on the surface of each submicelle. The submicelles aggregate *via* their hydrophobic patches such that

the entire micelle assumes a κ-casein-rich surface layer; some of the other caseins are also exposed on the surface.

Ono and Obata (1989) proposed two types of subunits: one consisting of α_s- (α_{s1}- and α_{s2}-) and β-caseins, which are present in the core of the micelle, and the other of α_s- (α_{s1}- and α_{s2}) and κ-caseins, which form a surface layer. Kimura *et al*. (1979) proposed that the casein molecules are folded within the submicelles such that the hydrophilic portions are at the surface, with the hydrophobic sections in the interior, but without preferential distribution of the casein types.

Schmidt (1980, 1982) suggested that the κ-casein content of submicelles varies and that the κ-casein-deficient submicelles are located in the interior of the micelle, with the κ-casein-rich submicelles concentrated at the surface, thereby giving the overall micelle a κ-casein-rich surface layer. However, super-polymerised carboxypeptidase A, which is unable to penetrate the micelles, can release the C-terminal residue from α_{s1}- and β-caseins, suggesting that some of these caseins are also exposed on the surface (Cheryan *et al*., 1975). That all the casein components are present at the surface of the micelle is in accord with the results of Heth and Swaisgood (1982), who covalently coupled casein micelles to glass beads, removed unbound proteins, and analysed the bound protein, which was found to consist of amounts of all the caseins is the same proportions as in milk.

Walstra and Jenness (1984) proposed that the hydrophilic C-terminal region of κ-casein protrudes from the surface, forming a layer 5–10 nm thick and giving the micelles a hairy appearance. This hairy layer is responsible for micelle stability through major contributions to zeta potential (-20 mV) and steric stabilisation. It was proposed that the submicelles are linked by CCP (Fig. 3.2). This model was modified by Walstra (1999) and Walstra *et al*. (1999) who proposed that, instead of linking the submicelles as proposed by Walstra and Jenness (1984), the CCP is located within the submicelles, which are held

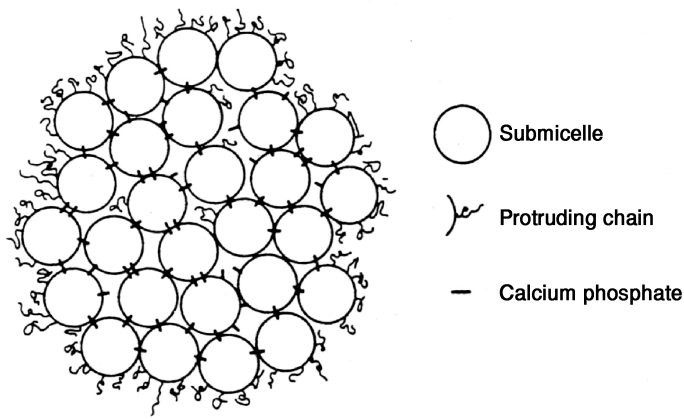

Fig. 3.2 Model of casein micelle (Walstra and Jenness, 1984).

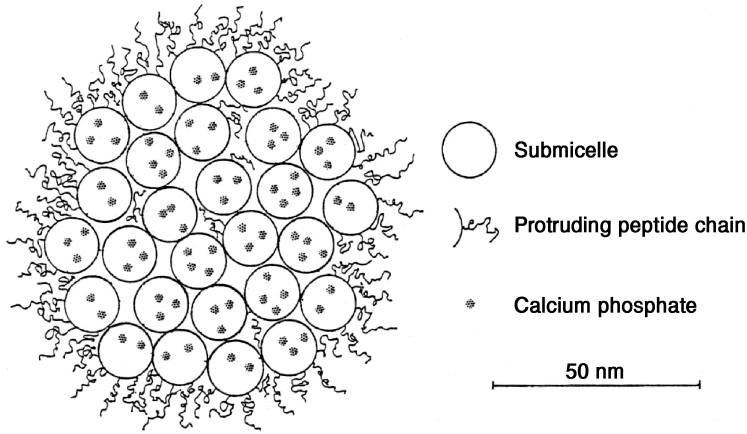

Fig. 3.3 Model of a casein micelle (reproduced from Walstra *et al*., 1999).

together by secondary forces (Fig. 3.3). The rationale for this apparent *volte face* is not clearly explained. Walstra (1999) suggested that when submicelles are formed in the mammary gland, they contain little or no CCP at first; subsequently, CCP is deposited in the sub-micelles, reducing their negative charge and their size and leading to their self-assembly into micelles. This mechanism is supported by electron microscopic studies of casein micelle formation within lactating cells, which indicate a succession of fractal clusters of particles in various stages of aggregation.

Although the submicelle model adequately explains many of the principal features of, and physicochemical reactions undergone by, the casein micelles and has been supported widely, it has never enjoyed unanimous support. For instance, Holt (1998) concluded that none of the submicelle models of casein micelle structure explained the results of gel permeation chromatography of micelles dissociated by removal of CCP or by urea. A number of alternative models for the structure of the micelle have thus been proposed. Visser (1992) suggested that the micelles are spherical conglomerates of casein molecules randomly aggregated and held together partly by salt bridges in the form of amorphous calcium phosphate and partly by other forces (e.g., hydrophobic bonds), with a surface layer of κ-casein. Holt (1992, 1994) depicted the casein micelle as a tangled web of flexible casein molecules forming a gel-like structure in which microgranules of CCP are an integral feature and from the surface of which the C-terminal region of κ-casein extends, thereby forming a hairy layer (Fig. 3.4). de Kruif (1998) supported the structure of the casein micelle as depicted by Holt (1992, 1994) and described the behaviour and properties of the micelles in terms of adhesive hard spheres.

In the latest model (the dual binding model, Fig. 3.5; Horne, 1998, 2002), it is proposed that α_{s1}-casein has two hydrophobic and one hydrophilic region, which includes the phosphoserine cluster, and that it can link to two neighbouring α_{s1}-, α_{s2}- or β-casein molecules via the hydrophobic sequences and to a third

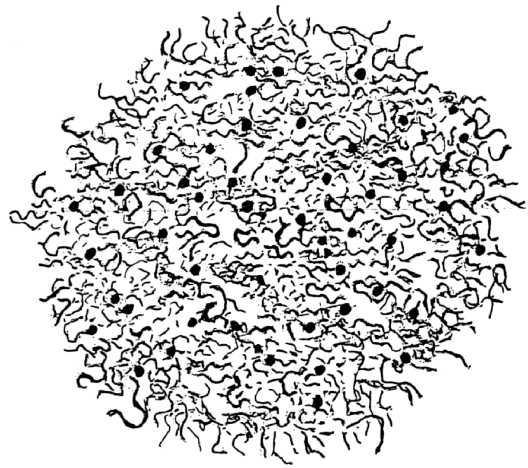

Fig. 3.4 Model of a casein micelle (according to Holt, 1994, as modified by Fox and McSweeney, 1998).

molecule (α_{s1}-, α_{s2}- or β-casein) via nanocrystals of CCP attached to seryl residues. α_{s2}-Casein has two hydrophobic and two phosphoseryl clusters and can bind to α_{s1}-casein via either type of group and thus extend the network. β-Casein, with only one hydrophobic and one phosphoseryl group, can link to α_{s1}- or α_{s2}-casein, either hydrophobically or via CCP. κ-Casein has a hydrophobic N-terminal region but no phosphoseryl cluster; therefore it interacts hydrophobically with one of the other caseins but, having done so, cannot extend the network and micelle growth ceases. These three models (i.e. those of Visser (1992), Holt (1992, 1994) and Horne (1998, 2002) retain two of the key features of the submicellar model (i.e., the cementing role of CCP and the predominantly

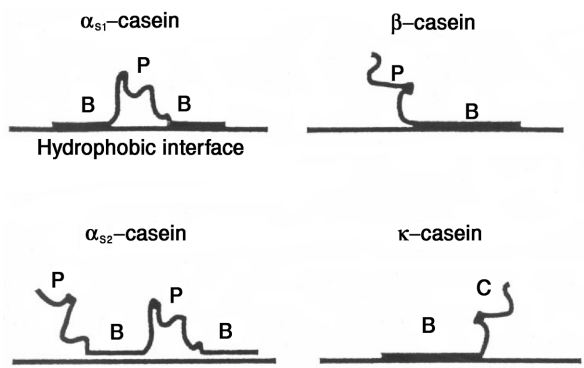

Fig. 3.5 Schematic diagram of part of the polymer network that can be produced by the caseins according to the dual-binding model of micelle structure. Phosphoserine clusters (P), hydrophobic regions (B), and the C-terminal of κ-casein (C) are indicated (reproduced from Horne, 2002).

surface location and micelle-stabilising role of κ-casein) and differ from it mainly with respect to the internal structure of the micelle.

As stated earlier, much of the evidence for a submicellar structure came from electron microscopy images. However, artefacts may arise in electron microscopy owing to fixation, exchanging ethanol for water, air-drying, or metal coating. Using a new cryo-preparation stereo-imaging microscopy technique, McMahon and McManus (1998) found no evidence to support the submicellar model; they concluded that, if the micelles do consist of submicelles, these must be smaller than 2 nm or less densely packed than previously presumed. The TEM micrographs appear very similar to the model proposed by Holt (1994).

While the current views on the structure of the casein micelle will probably be refined based on the results of further research, they are adequate to explain the technologically important properties of the micelles, including rennet coagulation, which results from the specific hydrolysis of the micelle-stabilising κ-casein, and resultant loss of the stabilising surface layer.

3.8 Stability of the casein micelles

The micelles are stable to:

- compaction – the pellet recovered by ultracentrifugation (e.g., at 100,000 *g* for 1 h) can be redispersed readily in milk diffusate or suitable buffer and the properties of the dispersion are essentially similar to those of the original micelles
- homogenisation at normal or high pressure
- high Ca^{2+} concentrations, up to at least 200 mM, at temperatures up to 50 ºC.

However, a number of processes can destabilise, disperse or otherwise affect the properties of the casein micelles, and hence of milk. Most of the principal dairy products are produced either by destabilising the casein micelles, e.g., cheeses or fermented milks, or preventing their destabilisation, e.g., heat-treated, dried or frozen products. The principal changes are discussed below.

3.8.1 Effects of acidification on casein micelles

Reducing the pH of milk has several significant implications for the physicochemical properties of the casein micelles, and hence the properties of milk; it has been known for millennia that acidification of milk, e.g., due to bacterial fermentation, yields a gelled milk product, i.e., yoghurt.

The changes in casein micelles on reducing the pH are mediated in part by changes in the colloidal calcium phosphate (CCP) content of the micelles. CCP may be dissolved and removed from the micelles by acidification in the cold, followed by dialysis against a large excess of bulk milk. The same result can be achieved by addition to milk of a calcium sequestrant (e.g., citrate, oxalate, polyphosphate). CCP is fully dissolved at pH 4.9 and any level of CCP between

0 and 100% of the normal value can be achieved by acidification to the appropriate value between pH 6.7 and 4.9. Conversely, the CCP content of casein micelles can be increased by raising the pH of milk, followed by dialysis against bulk milk. If acidified cold milk is readjusted to pH 6.7 and dialysed against bulk milk, the micelles reform but their properties are altered compared to the native micelles (Lucey *et al*., 1996).

Reducing the pH of milk also results in temperature-dependent dissociation of individual caseins (especially β-casein) from the micelles into the serum; >25 ºC, little dissociation apparently occurs (Dalgleish and Law, 1989). Dissociation of caseins decreases between the natural pH of milk (6.7) and pH 6.2, but thereafter increases to a maximum at pH 5.3–5.6. In the pH range 6.7–5.2, micelle solvation and porosity also increase as the pH decreases.

As a result of solubilisation of CCP and dissociation of individual caseins, reducing the pH of milk results in gradual disintegration of the casein micelles. Eventually, due to the reduction of the net negative charge on the casein micelles (and hence reduced intermicellar repulsive forces), reduced casein hydration (in the range pH 4.6–5.4) and increasing ionic strength of the serum, due to the increased concentration of calcium and phosphate ions, aggregation forces begin to dominate over the tendency to dissociate, and gelation occurs, typically commencing around pH 5.2, with a three-dimensional network of loose porous strands of linked casein particles being present at the isoelectric point of casein, pH 4.6.

Thus, aggregation or gelation of the caseins occurs when the pH is reduced to the isoelectric point (4.6), which in practice is achieved either by acidification with a mineral acid, an acidogen, usually gluconic acid-δ-lactone, or by *in situ* fermentation of lactose to lactic acid using a suitable culture of lactic acid bacteria. The latter is the principle involved in the manufacture of fermented milk products and acid-coagulated cheeses. Precipitation of casein in the region of the isoelectric point is temperature-dependent (i.e., does not occur at a temperature < 5–8 ºC and occurs over a wide pH range (perhaps 3.0–5.5) at higher temperatures); micelles identical to those found in milk probably do not exist at < pH 5 owing to the dissolution of CCP and perhaps other factors. However, electron microscopy studies of yoghurt show a network of structured protein, indicating that some micelle-like structure, or aggregates of dissociated caseins, are present at pH values around 4.6 (Bottazzi, 2002).

Whereas, at neutral pH, the casein micelles behave as hard spheres, it has been suggested (de Kruif, 1998) that destabilisation by renneting or acidification changes their behaviour to that of adhesive or 'sticky' spheres. This theoretical approach allows good prediction of the gelation of casein micelles under various conditions during milk processing.

3.8.2 Hydrolysis of κ-casein by proteolytic enzymes (rennet coagulation of milk)

It has been known for a long time that treatment of milk with NaCl extracts of the stomachs of neonatal mammals (known as rennets) result in its coagulation;

on gentle agitation, the coagulum separates into curds and whey. The principal active agent in calf rennet is the proteoytic enzyme, chymosin, with lesser amounts of pepsin. Calf rennet has, traditionally, been the principal milk coagulant. However, the proteinase from the thistle, *Cynara cardunculus,* has been used to coagulate milk for certain cheeses in Portugal and Spain and, from the mid-20th century, the use of pepsins and acid proteinases from the fungi, *Rhizomucor meihei, R. pucillus* and *Cryphonectria parasitica*, have been used widely as milk coagulants, due to a shortage of calf rennet. About 1990, the natural or synthesised calf chymosin gene was cloned in selected yeasts (*Kluyveromyces lactis, Saccharomyces cerevisiae*), bacteria (*E. coli*) and moulds (*A. nidulans, A. niger*) and so-called 'fermentation-produced chymosin' is now widely used as a coagulant in many countries.

Chymosin, and many other proteinases, coagulates milk in a two-stage process. In the first (primary) stage, the hydrophilic C-terminal segment of κ-casein (κ-CN f106–169, referred to as the caseino(glyco)macropeptide, CMP) is separated by hydrolysis of the Phe_{105}-Met_{106} peptide bond from the hydrophobic N-terminal region (κ-CN f1–105, referred to as *para*-κ-casein) and diffuses into the serum. The loss of the CMP from the surface of the micelles destabilises them through a decrease in the micellar zeta-potential to about -10 mV and a loss of steric stabilisation. *Para*-κ-casein is unable to stabilise the Ca-sensitive caseins and the rennet-altered micelles coagulate in the presence of Ca^{2+}, at a temperature of greater than ~18 ºC.

Once a critical level of κ-casein (~80%) has been hydrolysed, the secondary stage of coagulation begins; this stage occurs only at a temperature >18 ºC and in the presence of a critical concentration of calcium. It involves the aggregation of CMP-depleted micelles, at first into chains or clumps and eventually, under quiescent conditions, into a cross-linked three-dimensional gel network, at which stage the milk has visibly coagulated into a viscoelastic gel. The rennet-induced coagulation of milk is exploited in the manufacture of rennet-coagulated cheese varieties (~75% of all cheese) and rennet casein. Chymosin is very specific for the Phe_{105}-Met_{106} bond of κ-casein, with very low activity on other bonds in the casein system. This high specificity minimises the losses of peptides in the whey and maximises cheese yield.

The effects of several compositional and environmental factors on the secondary, non-enzymatic, phase of rennet coagulation is markedly different from those on the primary enzymatic phase.

- The temperature coefficient, $Q_{10\,°C}$, of the primary stage is ~2, i.e., typical for an enzyme reaction, but the $Q_{10\,°C}$ for the secondary reaction >20 ºC is ~16 and it is essentially instantaneous at 40 ºC. Interestingly, porcine milk coagulates readily on renneting at 4 ºC (Hoynes and Fox, 1975). This marked difference in the temperature-dependence of the primary and secondary stages of rennet coagulation is exploited in the cold renneting technique for continuous rennet coagulation of milk; this technique works well in the laboratory but has not been adopted commercially.

- Coagulation of rennet-altered casein micelles is absolutely dependent on the concentrations of Ca^{2+} and CCP. Presumably, Ca^{2+} functions by reacting with anionic groups on the caseins ($-COO^-$ and $-PO_4^{3-}$), thereby reducing micellar charge. Palmer and Richardson (1925) reported that *para*-casein binds more Ca than casein, but did not propose a mechanism for this. It has been suggested that Ca cross-links rennet-altered micelles via PO_4 groups (Hsu *et al*., 1958) but why this occurs only in rennet-altered micelles is not obvious.
- The mechanism by which CCP affects rennet coagulation is not obvious. As discussed earlier, CCP is one of the factors responsible for the integrity of the micelles, in which it is linked to the organic PO_4 groups of casein via Ca; hence, its removal should increase net negative charge on the micelles, thereby stabilising them. However, although CCP-free milk is very sensitive to Ca^{2+}, it is not coagulated by rennet.
- Rennet coagulation is promoted by decreasing pH, with an optimum of pH 6.0; the reaction is particularly sensitive at pH >6.4, and chymosin is inactive >pH 7.0. The effect of pH is primarily on the primary enzymatic phase. However, the aggregation phase is also affected by pH, with clotting starting at lower degrees of hydrolysis of κ-casein at lower pH values.
- The enzymatic phase and, especially, the secondary phase are adversely affected by pre-heating the milk; milk heated to 90 ºC for 10 min does not coagulate unless the pH is reduced and/or the $[Ca^{2+}]$ is increased. On heating to >70 ºC, whey proteins are denatured, and the sulphydryl group of β-lactoglobulin becomes active and interacts with micellar κ-casein *via* sulphydryl-disulphide exchange reactions. Electron microscopy shows the β-lg attached to the micelles as appendages, which presumably prevent the gelation of renneted micelles.

A rennet-induced milk gel is stable under quiescent conditions but if cut or broken or subjected to an external pressure, it synereses (contracts) with the expulsion of whey. Syneresis is promoted by reducing the size of the curd pieces, increasing the temperature, reducing the pH and subjecting the curd to pressure (i.e., stirring and/or pressing). Manipulation of one or more of these parameters is exploited by cheesemakers to vary the moisture content of cheese, and hence the texture and the rate and pattern of ripening and the quality of cheese. Syneresis is also affected by increasing the calcium concentration and, up to a certain level, by the concentration of casein; at very high casein concentrations (e.g., UF retentates), the gel is very rigid, with little tendency to synerese.

The strength of the renneted milk gel at cutting is very important with respect to cheese yield; if the gel is too soft, extensive shattering will occur, with high losses of fat and protein in the whey. Conversely, if the gel is too firm, it is not possible to cut it cleanly, or at all, using normal cheesemaking equipment and special apparatus is required; shattering of the curd may also occur, with high losses of curd fines in the whey.

Owing to its significance as a phenomenon in colloidal chemistry and the commercial importance of cheese as a dairy product (~35% of all milk is

converted to cheese), all aspects of the rennet coagulation of milk have been studied intensively since the work of O. Hammarsten in the 1870s. Numerous reviews of the very extensive literature are available, e.g., Fox (1984), Dalgleish (1992), Walstra (1992), Fox and McSweeney (1998) and Hyslop (2003).

3.8.3 Changes in micelles on heating milk

In general, the structure and properties of casein micelles are quite resistant to mild or even moderately severe heating but very severe heating (e.g., at 140 ºC) causes simultaneous dissociation and aggregation, and eventual coagulation, of the micelles. The ability of milk to withstand high-temperature processing without undergoing major sensory or physico-chemical changes is one of its principal technological attributes. Except under very rare circumstances, unconcentrated milk readily survives the thermal processes to which it is exposed, e.g., HTST pasteurisation (72–74 ºC × 15 s), UHT (140 ºC × 5 s) or in-container (115 ºC × 10–15 min.) sterilisation.

The heat stability of milk at high temperatures (> 120 ºC) has been of interest since the development of sterilised evaporated milk in 1884 and has been studied systematically since the first paper on the subject was published by Sommer and Hart (1919). The very extensive literature on this subject has been reviewed at regular intervals, most recently by O'Connell and Fox (2003).

Heating at a temperature >75 ºC has several important effects on the casein micelles. Firstly, denaturation of whey proteins occurs: of particular significance is the unfolding of β-lg, which results in the formation of disulphide-linked κ-casein/whey protein complexes through sulphydryl-disulphide interchange reactions, either at the micelle surface, or in the milk serum, depending on temperature and pH. This has several profound implications for the properties of casein micelles. For example, the rennet coagulation properties of milk heated under conditions sufficient to result in the attachment of denatured whey proteins are severely impaired (see previous section). Heat treatment also causes the precipitation of soluble calcium phosphate, probably as $Ca_3(PO_4)_2$, with the concomitant release of H^+ and a decrease in pH.

The casein micelles are relatively stable at higher temperatures, coagulating at 140 ºC after 15–20 min at the normal pH of milk. Such coagulation is not due to protein denaturation *stricto sensu*, but to changes that occur in the milk system as a result of such high-heat treatment, i.e., a decrease in pH due to the pyrolysis of lactose, mainly to formic acid, dephosphorylation of the casein, cleavage of κ-casein, denaturation of the whey proteins and their interaction with and precipitation onto the casein micelles, and precipitation of soluble calcium phosphate on the micelles as $Ca_3(PO_4)_2$.

One of the most striking features of the heat stability of milk is its very strong dependence on pH; the HCT-pH profile of most milk samples shows a maximum at ~pH 6.7 and a minimum at ~pH 6.9 (known as a Type A heat stability profile). A small proportion of individual-cow milk samples exhibit continuously increasing heat stability with increasing pH (Type B heat stability

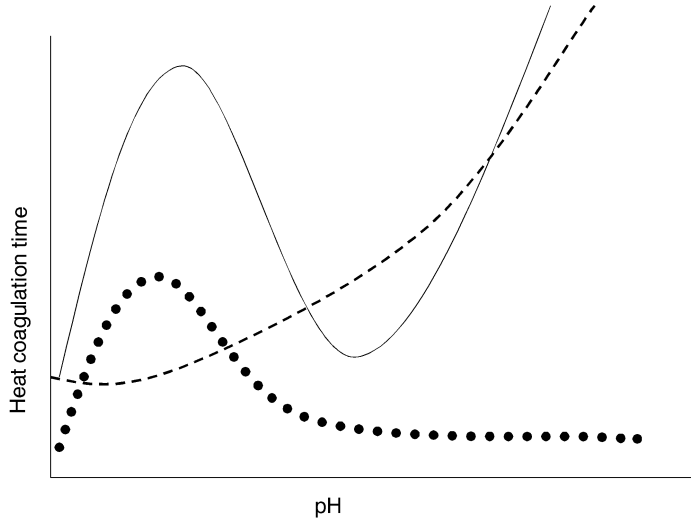

Fig. 3.6 Heat coagulation time (HCT)-pH profiles of typical type A bovine milk (whole line) and type B or serum protein-free milk (dashed line), as determined at 140 °C, and concentrated milk (dotted line), as determined at 120 °C.

profile), which is as expected in view of the increasing charge on the casein micelles. The occurrence of a maximum/minimum in the HCT-pH profile is affected by several factors, especially on the ratio of β-Lg to κ-casein; the HCT of a serum protein-free casein micelle dispersion increases continuously with increasing pH (Fig. 3.6). In the presence of β-Lg, stability increases in the pH range 6.4–6.7 but decreases in the range 6.7–7.0. It is believed that the maximum/minimum in the HCT-pH profile is due to the dissociation of κ-casein from the surface of the casein micelles on heating, the extent of which depends on pH and on the presence of β-Lg. In the pH range 6.4–6.7, β-Lg reduces the dissociation of micellar κ-casein but at pH >6.7 it promotes it; the reason for the pH-dependent effect of β-Lg is not known. The κ-casein-depleted micelles are unstable and undergo Ca-dependent precipitation, i.e., in the region of the minimum HCT. Dissociation of κ-casein is even more marked at >pH 7, but stability is high due to the high net negative charge.

The heat stability of concentrated (2–2.5 fold) milk is greatly reduced, especially for milk concentrated by thermal evaporation (less so by UF) and it may not withstand sterilisation unless certain process modifications are made. The HCT of concentrated milk is also markedly pH-dependent; it shows a maximum at pH ~ 6.5 but, in contrast to unconcentrated milk, stability does not increase at pH values >7.0. Interestingly, the shape of the HCT-pH profile of unconcentrated caprine or ovine milk is similar to that of concentrated bovine milk, with a maximum at pH ~ 6.7, but without a minimum. The heat stability of skim milk is not affected by homogenisation, but this process destabilises fat-containing liquid dairy products to an extent directly dependent on fat content and homogenisation pressure.

Various techniques have been developed over the past 140 years to enable sterilised concentrated milk to be produced successfully; the most significant, and almost universally used, of these are forewarming (preheating) milk at 90 ºC for 10 min. or 120 ºC for 2 min before concentration and the addition of orthophosphates, which shift the pH and chelate Ca^{2+}. Other methods by which heat stability can be increased include:

- the addition of diacetyl, e.g., as a culture distillate (Shalabi and Fox, 1982)
- hydrolysis of lactose using β-galactosidase (Tan-Kintia and Fox, 1996)
- addition of certain polyphenols (O'Connell and Fox, 2001)
- pre-treatment of the milk with transglutaminase (O'Sullivan *et al*., 2001, 2002a).

The first three of these increase the propensity of milk to Maillard browning and therefore care is required to balance the positive and negative effects but TGase treatment seems to be without undesirable side-effects.

3.8.4 Effects of dissociating agents or alcohols

Agents such as urea (>6 M), sodium dodecyl sulphate (SDS; >1%), ethanol (>35% and >70 ºC) or other alcohols, or exposure to a high pH value (>9.0) dissociate the casein micelles. Since CCP is not dissolved by any of these agents, their effectiveness clearly indicates that, in addition to CCP, hydrophobic and hydrogen bonds and electrostatic interactions contribute to micelle integrity.

It has been known for about 100 years that milk is coagulated by the addition of ethanol to ~ 35% and that the ethanol stability of milk is related to, although not highly correlated with, its heat stability. The alcohol stability (AS) of milk, usually measured by mixing equal volumes of milk and aqueous ethanol (e.g., 80%), was, and still is in some countries, widely used as a selection test for milk for processing. The AS of milk is strongly affected by pH and [Ca^{2+}] but today, the quality of milk is rarely determined by pH alone; therefore, the AS of milk is not a good predictor of its quality and has not been used commercially in developed dairying countries for about 40 years. However, the AS of milk products, especially sodium caseinate, assumed new importance with the development of cream liqueurs.

The influence of various compositional and processing factors on the AS of milk was investigated thoroughly in the 1970s and 1980s, principally by Dr David Horne, who has reviewed the subject a number of times, most recently in Horne (2003). AS decreases with decreasing pH: a plot of AS as a function of pH shows a sigmoidal relationship, with a sharp increase in the range 6.3–7.0 and an inflection point at ~ pH 6.6. The AS-pH curve is shifted to more alkaline values at high Ca^{2+} concentrations and to more acidic values when Ca^{2+} is sequestered by, e.g., phosphates or citrate. At any particular pH, AS decreases when [Ca^{2+}] is increased and *vice versa*.

Considering that ethanol at a concentration >35% precipitates the casein in milk at room temperature, it has the rather unexpected effect of dissociating the casein

micelles if the mixture is heated > 70 ºC, an effect first observed by Zadow (1993). When the system is recooled, or the ethanol evaporated off at 70 ºC, the white appearance of milk is restored. Zadow (1993) suggested that the alcohol/temperature-induced dissociation of the micelles might be exploited to modify the composition, structure and properties of the casein micelles, e.g., to incorporate additional β- or κ-casein. Ethanol-induced, temperature-dependent dissociation of casein micelles was investigated in more detail by O'Connell *et al*. (2001a,b, 2003). These studies showed that ethanol does indeed dissociate the micelles on heating and that the effect depends on the concentration of ethanol and temperature. The dissociation is reversible, but with a hysteresis effect; in fact, if milk containing ethanol/heat-dissociated micelles is cooled to 0–4 ºC, a gel is formed, which dissolves reversibly on warming. It is possible that a new range of gelled dairy products could be developed based on this effect.

If the ethanol is removed by evaporation, very large aggregates (average diameter, ~ 3,000 nm) are formed that have very different properties from those of natural micelles; these have been referred to as 'reformed micelles'. The dissociating effect of ethanol is promoted by increasing the pH (35% ethanol causes dissociation at 20 ºC at pH 7.3) or adding NaCl. Methanol and acetone have a dissociating effect similar to ethanol, but propanol causes dissociation at ~25 ºC. The mechanism by which ethanol and similar compounds cause the dissociation of casein micelles is not fully established, but it is not due to the solubilisation of CCP, which is unaffected.

Milk containing reformed micelles has a type-B HCT-pH profile and its rennet coagulation time is reduced. Casein-whey protein interactions, albeit different from those observed on heating, appear to play a significant role in the observed phenomena. The implications of these recent studies, both for elucidating the stability of the milk protein system and for dairy product technology, remain to be evaluated fully.

Casein micelles are also dissociated by urea > ~4 M; this dissociation does not involve dissolution of CCP, and is presumably due to rupture of the hydrogen bonds, suggesting that these contribute to casein micelle structure. On removal of urea by dialysis against a large excess of bulk milk, micelle-like particles with properties generally similar to the native micelles are formed (McGann and Fox, 1974).

Sodium dodecyl sulphate (SDS) is a widely used protein dissociating agent which functions by its hydrophobic hydrocarbon chain interacting with hydrophobic segments of proteins, thus rupturing hydrophobic bonds; its $—SO^{3-}$ group increases the negative charge on the proteins, thereby creating intermolecular electrostatic repulsion. SDS has rather interesting and specific effects on the structure and properties of the casein micelle. Possibly the first study of the effects of SDS on the caseins was that of Noelken (1966), who produced artificial micelles by interacting caseins with detergents. He showed that κ-casein bound 0.4 g SDS per 1 g and formed soluble complexes. α_{s1}-Caseinate could be dispersed by polyoxyethylene lauryl ether, which functions like κ-casein.

In a thorough study, Cheeseman (1968), Cheeseman and Jeffcoat (1970) and Cheeseman and Knight (1970) investigated various aspects of the dissociation of individual caseins by SDS; it was found that κ-, α_{s1} and β-caseins bound a total of 73, 107 and 244 mol of SDS per mol of protein, respectively, and that tyrosine and tryptophan are probably involved in the binding. At concentrations up to 0.5%, SDS progressively increases the heat stability of milk and shifts the HCT-pH profile to more acidic values; at concentrations >0.5%, SDS dissociates the casein micelles (Fox and Hearn, 1978). At 0.15 or 0.25%, SDS markedly increases the HCT of a serum protein-free casein dispersion in synthetic milk ultrafiltrate in the pH range 6.0–6.5 but reduces it at pH 6.5–7.0, thereby introducing a pronounced maximum–minimum into the HCT-pH profile, similar to the effect of β-Lg. As far as we are aware, the effect of SDS on the heat stability of milk has not been investigated further. As shown by Noelken (1966), SDS forms micelles with κ-casein and preferentially dissociates this protein from the micelles (at 0.4%, Lefebvre-Cases *et al*., 1998b, 2001a), i.e., an effect similar to that of β-Lg.

SDS increases the rennet coagulation time of milk (Pearce, 1976; Marshall and Green, 1980), perhaps because it binds Ca^{2+} but it also inhibits the hydrolysis of κ-casein, to which it binds (Marshall and Green, 1980). The ability of SDS to increase the HCT of milk could also be due to its ability to bind Ca^{2+} but Ca-binding would be expected to eliminate the minimum in the HCT-pH profile, which is not the case.

The effect of SDS on various properties of casein micelles has been reported by Lefebvre-Cases *et al*. (1998a,b, 2001a,b). Working with low-heat skim milk powder reconstituted to 12%, w/v, in water containing SDS at a concentration in the range 0–28 mM (0 – ~0.8%, w/v), they reported that:

- turbidity increased with SDS concentration up to 21 mM (0.6%) but decreased at 28 mM (0.8%)
- at concentrations of SDS > 28 mM (0.8%), the micelles were dispersed
- a gel was formed on treatment with 21 mM SDS (0.6%), as shown by rheological measurements and electron microscopy
- κ-casein was completely dissociated from the micelles by 14 mM (0.4%) SDS but 28 mM (0.8%) was required to completely dissociate α_s- and β-caseins
- the diameter of the micelles was unchanged by 7 mM (0.2%) SDS but was increased by 14 mM (0.4%) SDS
- zeta-potential was increased by 7 mM (0.2%) SDS (from −22 to −32 mV) but it decreased at higher concentrations
- micelle solvation decreased to a minimum at 7 mM (0.2%) SDS
- the formation of an acid-induced milk gel was reduced by 7 mM (0.2%) SDS but reduced at 14 mM (0.4%).

Partial dissociation of micelles occurs at 0.8% SDS and dissociation is complete at 2.0% SDS (P. F. Fox and N. P. O'Sullivan, unpublished). In contrast to the effects of ethanol at 70 ºC or urea, the dissociating effect of SDS was not

reversed by dialysis against bulk milk; presumably, the SDS is not removed by dialysis. Interestingly, dialysis of SDS-dissociated milk against bulk milk caused non-selective precipitation of some casein.

3.8.5 High-pressure treatment of milk

High-pressure (HP) treatment is a novel processing technology of commercial interest due to its ability to increase the safety and shelf-life of food products, generally without undesirable loss of sensory or nutritional quality (Balci and Wilbey, 1999). While covalent bonds are generally not affected by HP (exceptions include effects of HP on disulphide bonds of β-lg), changes in secondary and higher-order structures of proteins are known to occur, chiefly through disruption of ionic and hydrophobic interactions. HP can thus induce structural changes or denaturation of proteins, and inactivation of enzymes. The effects of HP on milk have been examined in a number of studies over the last 10 years (for review see Huppertz *et al*., 2002); in general, besides proven anti-microbial efficacy, HP has been shown to induce some interesting and unusual changes in milk proteins, and hence in the properties of milk.

The effects of HP on casein micelles in milk are probably partly due to the above influences of pressure on protein structure; in particular, it is known that whey proteins are denatured under HP and probably interact with casein micelles, with α-la being significantly more baroresistant than β-lg (Gaucheron *et al*., 1997; Schrader and Buchheim, 1998; Huppertz *et al*., 2004). HP treatment also causes considerable dissociation of caseins from the micelles into the serum phase of milk (Lopez-Fandino *et al*., 1998). In addition, considerable dissolution of CCP from the micelles occurs under pressure (Schrader and Buchheim, 1998). Interestingly, the net result of the influence of each of the HP-induced changes in the casein micelles depends on the pressure applied and is probably related to the relative contribution of forces which tend towards aggregation reactions and those which promote micelle dissociation.

Generally, the lowest pressure at which effects have been noted is ~250 MPa; treatment of milk at this pressure increases the size of the casein micelles (by around 20%), possibly due to aggregation (Needs *et al*., 2000b; Huppertz *et al*., 2004). However, treatment at a higher pressure (e.g., >300 MPa) significantly reduces casein micelle size, typically by around 50%, probably due to solubilisation of CCP and disruption of electrostatic and hydrophobic interactions; these effects are strongly dependent on the duration of treatment, milk pH and temperature (Cheftel and Dumay, 1996; Huppertz *et al*., 2002, 2004).

Such effects of HP on casein micelles, perhaps not surprisingly, have significant effects on the properties of milk. For example, the whiteness of HP-treated milk is reduced significantly (Johnston *et al*., 1992; Gaucheron *et al*., 1997). The rennet coagulation time of milk is reduced markedly at around 200 MPa (Lopez-Fandino *et al*., 1997; Needs *et al*., 2000b), but effects on gel firmness and gel structure are highly dependent on treatment pressure and

duration (Huppertz *et al*., 2002). At pressures above 300 MPa, denatured whey proteins may interfere with the process of gel structure formation, and while cheese yield is increased following such treatments, the increase in yield is due largely to a higher level of moisture in the cheese (Drake *et al*., 1997). The firmness and structure of yoghurt may also be enhanced by the HP treatment of milk (Needs *et al*., 2000a).

3.8.6 Cooling and freezing of milk

At refrigeration temperatures, a significant quantity of β-casein (up to 50% of the total) and a lower amount of κ-casein dissociate reversibly from the micelles in a thermoreversible process. The selective dissociation of β-casein reflects its high hydrophobicity (Sullivan *et al*., 1955; Payens and van Markwijk, 1963; Downey and Murphy, 1970; Davies and Law 1983). Since the effect is reversible, temperature-induced dissociation of micellar casein is probably of little practical significance. The selective dissociation of β-casein forms the basis of methods for its isolation on a potentially industrial scale by UF (Murphy and Fox, 1991), MF (Famelart and Surel, 1994) or from renneted Ca-caseinate (Ward and Bastian, 1996) or rennet-coagulated milk (Fig. 3.1).

Casein micelles are destabilised by freezing (cryodestabilisation) due to a decrease in pH and an increase in the concentration of ionic calcium in the unfrozen phase of milk; concentration of milk increases its susceptibility to cryodestabilisation. Cryodestabilised casein can be dispersed in water to give particles with micelle-like properties (Lonergan, 1983). Cryodestabilisation of the casein micelles limits the commercial usefulness of frozen storage of milk; however, it could form the principle of a novel method for the industrial-scale production of casein but, as far as we are aware, the procedure is not used commercially.

3.8.7 Treatment of milk with transglutaminase

Transglutaminase (TGase) modifies the properties of many proteins through the formation of new cross-links, incorporation of an amine or deamidation of glutamine residues. To create protein cross-links, TGase catalyses an acyl-group transfer reaction between the γ-carboxyamide group of a peptide-bound glutamine residue and the primary amino group of a variety of amine compounds, including lysine residues in proteins.

The caseins are good substrates for TGase, due to their open structure (Han and Damodaran, 1996; Lorenzen and Schlimme, 1998). TGase treatment of casein micelles stabilises them to the action of dissociating agents, such as calcium chelators or urea (O'Sullivan *et al*., 2002b). The heat stability of milk and concentrated milk is very markedly increased by treatment with TGase, presumably by preventing the dissociation of κ-casein from the micelles (O'Sullivan *et al*., 2001, 2002a). Milk which has been extensively cross-linked by TGase is not coagulable by rennet (O'Sullivan *et al*., 2002b), presumably

because the micelles become very rigid and the segment of κ-casein containing the Phe_{105}-Met_{106} bond is unable to fit into the active site of coagulant acid proteinases, such as chymosin.

3.9 Future trends

The proteins of milk have been the subject of research for >100 years, especially since 1950. It is not surprising, then, that the milk proteins are the best characterised food protein system. All the principal proteins and many of the minor ones have been well characterised at the molecular level. Progress on the chemistry of milk proteins will depend on developments in protein chemistry generally. Developments which are likely to have a significant impact on the technological aspects of milk proteins are:

- improved fractionation techniques
- application of molecular biology techniques to modify proteins through point mutations
- modification of proteins by chemical (which was not discussed here), physical or enzymatic methods
- more extensive inter-species comparison of various aspects of milk proteins – the milk proteins of very few of the ~ 4500 mammalian species have been studied to date. It is highly probable that very interesting new milk proteins remain to be discovered.

It is very likely that useful studies on the chemistry and technology of milk proteins will continue for many years to come.

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4

Whey proteins

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4.1 Introduction: whey proteins as food ingredients

4.1.1 Introduction

Whey is the fluid by-product resulting from the precipitation of proteins in milk. The precipitation can be facilitated by the growth of microorganisms (e.g. cheese whey), addition of acid (acid casein manufacture) or by the addition of enzymes (rennet casein manufacture). Thus whey is classified as either sweet whey or acid whey. Acid whey results from Cottage cheese and acid casein manufacturing processes. All other whey is termed sweet whey. A majority of the whey produced globally is sweet whey. Approximately 94% of the whey produced in the United States is sweet whey and the remaining 6% is acid whey (Anon., 2002).

Whey is a dilute nutrient stream. Its composition is approximately 94% water (6% total solids), 4.5% lactose, 0.8% protein, and 0.7% minerals (Morr, 1989b). This dilute stream can be dried to provide a product called sweet whey powder or whey powder. The lactose content of the whey can be reduced and then the resulting product is dried to yield a product called reduced lactose whey powder. Similarly, processes, such as electrodialysis, can reduce the mineral content of the whey, to obtain demineralized whey that in its dried form is called demineralized whey powder. The next stage of whey processing is to concentrate the proteins by removing minerals and lactose. When the protein content of the powder is at least 25% or more the product is termed whey protein concentrate. The most common protein levels in whey protein concentrates are 35%, 55% and 80%. The highest form of purification yields whey protein isolates with >90% protein on a dry weight basis.

The by-products of protein concentration are lactose and calcium-rich milk minerals. These by-products are becoming increasingly useful in pharmaceutical

Table 4.1 Composition of various dry whey products (Anon., 2001)

Product	Protein	Lactose	Fat %	Ash	Moisture
Whey powder	11–14.5	63–75	1–1.5	8.2–8.8	3.5–5.0
Reduced lactose whey powder	18–24	52–58	1–4	11–22	3–4
Demineralized whey powder	11–15	70–80	0.5–1.8	1–7	3–4
Whey protein concentrate-34	34–36	48–52	3–4.5	6.5–8.0	3–4.5
Whey protein concentrate-50	50–52	33–37	5–6	7.5–8.5	3.5–4.5
Whey protein concentrate-80	80–82	4–8	4–8	3–4	3.5–4.5
Whey protein isolate	90–92	0.5–1.0	0.5–1.0	2–3	4.5

and dietary supplement markets. Lactose is mainly used as an excipient in the tableting process and the milk minerals are used for the fortification of various foods and beverages with calcium.

The compositions of whey products are summarized (Table 4.1). The protein make up of whey is different from that of skim milk. The main difference is the virtual absence of caseins in whey. The major proteins in cheese whey are known (Table 4.2). The unique characteristics of whey proteins are their solubility over a wide range of pH, favourable nutritional profile with respect to essential amino acids, diverse functionality and relatively low cost. In the next section the use of whey proteins as ingredients in foods is discussed. The other aspects discussed in this chapter are the analytical methods used for whey protein analysis, the structure of the major whey proteins, improving functionality of whey proteins and sources for further information on this subject.

4.1.2 Use of whey proteins as ingredients

The composition of whey products depends upon the methods employed to reduce lactose and ash contents. Morr and Foegeding (1990) and deWit *et al.* (1986, 1988) studied samples of whey protein concentrates and isolates from various countries and observed differences in the proportions of individual whey proteins and also individual minerals in the ash. There can be several reasons for

Table 4.2 Typical proteins in cheese whey and some of their characteristics (Anon., 2001)

Protein	Abundance (%)	Molecular weight	Isoelectric point
β-lactoglobulin	48	18,400–36.900	5.2
α-lactalbumin	19	14,200	5.1
Proteose-Peptone	20	4,000–80,000	5.1–6.0
Serum albumin	6	69,000	4.8
Immunoglobulins	8	160,000	5.5–6.8

the observed differences and these include seasonal changes and lipid composition (Kilara, 1994).

Physicochemical attributes that make a protein useful in foods are called functional properties. Composition of whey protein products varies and a number of different types of whey protein products are available in the marketplace. Morr (1979) observes that for any food protein ingredient to be useful, it must be free from toxic and anti-nutritional factors, free of off flavours and off colours, compatible with other processes and ingredients in the formulation. Functional properties can be evaluated in model systems, model food systems and in real foods. The complexity of evaluation increases from model systems to real foods. Further, functionality testing in model systems has not been standardized. Lack of standardization presents challenges in evaluating and comparing results within the same laboratory and between laboratories. Many empirical methods exist for functionality testing.

Water-protein interactions

Macromolecules are not soluble in the same manner as small molecules are. However, the amino acid side chains in the proteins can interact with water and proteins can be suspended in water. This property is often used as an indicator of whey protein denaturation. Protein solubility is a function of temperature, pH, presence of other ions, and the values obtained for solubility are highly dependent on the methods used to achieve the solubility (Kilara, 1984). Proteins are least soluble at their isoelectric point but whey proteins are soluble over a wide range of pH values. This property of whey proteins makes it desirable for use in beverages.

Increase in temperature generally results in increased solubility of low molecular weight solutes. For proteins, however, increasing temperatures can lead to denaturation and, in turn, a decrease in solubility. There is a positive correlation between solubility and enthalpy for denaturation of whey proteins (Kilara and Mangino, 1991). During ultrafiltration of whey the resulting retentates can be spray dried directly or a pasteurization treatment can be provided prior to spray drying. Pasteurization of retentates decreases the β-lactoglobulin content of the subsequent whey protein concentrate manufactured (Mangino *et al*., 1987).

Another related property is the interaction of proteins with water. This property leads to thickening or an increase in apparent viscosity. Hydrogen bonding, ion dipole and dipole-dipole interactions are all important mechanisms for water-protein interactions. Physical forces such as adsorption are also important in increasing viscosity. Insoluble proteins bind a lot of water. Heat denatured whey protein (lactalbumin) absorbs more water than undenatured whey protein (Morr 1989a).

Viscosity which results from water-protein interactions have been discussed extensively by deWit (1989). He observed that viscosity of a whey protein solution increases above 65 ºC and even greater increase occurs at temperatures greater than 85 ºC. Between 65 and 85 ºC whey proteins denature and above 85 ºC denatured proteins aggregate leading to further viscosity increases.

Practical uses of whey protein concentrates in which water-protein interactions are utilized include yogurt drinks, hard pack ice cream, low fat ice cream, non-fat ice cream, soft serve ice cream, yogurt, sour cream and coffee whiteners. In cheese sauces, low fat cream soups, creamy salad dressings, refrigerated pasta and orange marmalade, viscosity and the ability of whey proteins to bind water are useful. Nutritional beverages, meal replacement beverages, sports beverages and protein fortified-citrus beverages also rely on the solubility of whey proteins for successful formulation.

Foaming

Foams are the result of the behaviour of proteins at air-water interfaces. Rapid diffusion of molecules to the interface followed by molecular rearrangement allows these films to entrap air. With whey proteins heating is a prerequisite for foaming (Devilbiss *et al*., 1975). This is suggestive of a partial denaturation of whey proteins resulting in molecular rearrangements conducive to rigid high viscosity surface films. This confirmed the work of Reichert *et al*. (1974) who reported that heating whey protein concentrates to a temperature 55–60 ºC led to improvements in foaming properties of whey protein concentrates. Cooling whey protein solutions to below 4 ºC reduces foaming. It has been speculated that this temperature-dependent foaming may be due to the effects of heat on β-lactoglobulin (Haggett, 1976).

Hydrophobicity and sulfhydryl content are predictors of foaming in whey protein concentrates derived from acid whey (Liao and Mangino, 1987). These observations are similar to those made with commercial whey protein concentrates (Peltonen-Shalaby and Mangino, 1986). It has also been reported that native confirmation of β-lactoglobulin strongly affected foaming performance (Kim *et al*., 1987). Pasteurization of retentates reduces foaming properties of the subsequently dried whey protein concentrates (Mangino *et al*., 1987). When 11 commercial protein concentrates were tested for their foaming properties, Morr and Foegeding (1990) found considerable variability. In some samples even though the foam volume was adequate the foams were not stable. Foaming properties of whey proteins play an important role in baked goods and confectionery creams.

Emulsification

This pertains to the behaviour of the protein at oil-water interfaces. Emulsions are formed when energy is applied to disperse one phase into another of two normally immiscible phases. If the dispersed phase is oil and the continuous phase is water an oil in water emulsion results. When the continuous phase is oil and the dispersed phase is water, a water in oil emulsion results. Emulsions can be liquid, semi-solid or solid. In addition to the work performed for dispersing the two phases an energy barrier is necessary to prevent coalescence of the dispersed phase. This energy barrier is provided by surfactants (emulsifiers) and proteins are macromolecular surfactants. Emulsions are metastable systems and four types of instability, namely creaming, flocculation, coalescence and phase

inversion, may be observed. Proteins retard gravitational separation of phases (creaming) and are not as efficient as small molecular weight surfactants at stabilizing emulsions against coalescence.

Whey proteins are not extensively used as emulsifiers. In one study by Pearce and Kinsella (1978) the oil phase volume was maintained at a constant 25% and whey protein concentrations were increased from 0.5 to 5% in emulsions. It was observed that the oil droplet size decreased as the protein concentration increased (Pearce and Kinsella, 1978). It has been demonstrated that whey proteins adsorb at the interfaces at a slower rate than other proteins like β-casein (Tornberg and Hermannson, 1977). Factors affecting whey protein emulsions include pH and ionic strength. Around their isoelectric point (pI) whey proteins form poor unstable emulsions (deWit, 1989). If the milk used for cheesemaking is pasteurized or if the whey resulting from the cheesemaking is pasteurized the emulsification properties of the whey proteins are not adversely affected (Mangino *et al*., 1987). Pasteurization of the retentate greatly diminished the emulsion capacity of the proteins. Mangino *et al*. (1987) therefore demonstrated that the effects of heat treatment on whey proteins in milk, whey and retentates have differing effects on emulsification functionality.

Whey proteins do help in emulsification in infant formula, meal replacement beverages, soups and gravies and coffee whiteners. They are used in conjunction with low molecular weight emulsifiers.

Gelation

Under the right circumstances the balance between polymer-polymer and polymer-water interactions results in the formation of networks or structures known as gels. Gels are capable of holding large amounts of water and other nutrients within the network. Coagula are not gels and are incapable of holding large amounts of water. In the two-step process of gel formation, the first step involves the denaturation of the protein and the second step is a rearrangement of the denatured molecules leading to aggregation and network formation (Ferry, 1948). Foegeding and Haman (1992) have suggested a more detailed description of the thermal gelation of proteins.

Ions such as calcium, sodium and magnesium affect gelation of whey proteins (Varunsatian *et al*., 1983). At pH > 8 the chloride salts of anions increased the rate of aggregation and calcium was the most effective cation. Divalent cations lowered the denaturation temperature of the proteins. Sodium chloride increased the denaturation temperature. Heat sensitivity of β-lactoglobulin was enhanced by the presence of calcium ions. Even though this study did not study gelation, some insights into one aspect of gelation can be gained by the results presented.

Alkane binding by proteins is a measure of the hydrophobicity of the protein. Calcium content and hydrophobicity are predictors of gel strength (Kohnhorst and Mangino, 1985). Mangino *et al*. (1987) demonstrated that pasteurizing milk used for cheesemaking affected the ability of whey protein to gel at pH 6.5 but not at 8.0. Heating retentates significantly reduced gel strength but

pasteurization of whey did not significantly alter gel strength. It has been reported that whey protein isolates heated at 90 ºC for 15 min. at pH 6.5–8.5 form reversible gels at protein concentrations of 9–10% (Rector *et al*., 1989). The melting temperature of gels at pH 8 ranged from 24.5 to 57.8 ºC. The maximum enthalpy of formation was −858 calories per mole of crosslinks and a maximum storage modulus of 240 dynes/cm^2 was obtained after holding for 7h at 8 ºC.

Rinn *et al*. (1990) reported that whey protein concentrates prepared by microfiltration through 0.6 μm pores exhibited superior gels at 4 and 5% protein concentration. In comparison, conventionally prepared whey protein concentrates required 9% protein concentration to form non-pourable gels. When 11 commercial whey protein concentrates were tested for their abilities to gel, some did not form gels at all, some required high protein concentrations in order to gel. Addition of sodium chloride to solutions led to a decrease in the minimum concentration required for gelling (Morr and Foegeding, 1990).

Gelation is an important functionality that is useful in baked goods, processed meats, surimi, desserts, and sour cream applications. In many food products multiple functionalities are at play and it is difficult to specify the degree of importance of each property relative to its successful application in formulations.

4.2 Analytical methods for determining protein content

Determination of total protein can be accomplished by a number of different techniques. Some of these include gravimetric, nitrogen determination, amino acid analysis, colorimetric methods, spectrophotometric and fluorometric methods (Darbre, 1987). When dealing with mixtures of proteins like whey protein concentrates and isolates a mere determination of protein content is not as meaningful as knowing the proportion of individual components in the mixture. Thus, such an exercise requires the separation of components in a mixture followed by a determination of the concentration of the separated entities. Separation may be achieved by utilizing the ionic nature of proteins where ion exchange chromatography or electrophoresis is the technique of choice. Other techniques may rely on the separation of proteins based on their size or on their shape as in gel permeation or size exclusion chromatography. Proteins can also be separated on the basis of their polarity as in high-performance liquid chromatography (HPLC) (Holme and Peck, 1993). In these techniques, the identity of the separated components is determined by a combination of prior knowledge of the nature of the mixture or by indirect comparison to known standards. For example, components separated via electrophoresis under dissociative conditions can with the help of standards reveal the molecular weight of the molecules but it cannot tell the analyst that the band is a certain protein.

4.2.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of whey proteins was first performed to quantify the individual components (Darling and Butcher, 1965). The separation and staining procedures were standardized and during each electrophoresis run a standard protein solution of whey proteins was also separated and stained under the same conditions as the test materials. In this way the standard solutions were subjected to the same treatment as the test solutions. Densitometric scanning of the stained protein containing gels followed by peak area determinations was carried out. By comparison with standard peak areas individual protein concentrations of the test samples were determined.

4.2.2 Liquid chromatography

Pearce (1984) reported that a high-performance liquid chromatography method could be used for the separation and quantitation of whey proteins. The separation was achieved using an alkyl C6 reverse phase column with an acidic saline/acetonitrile gradient. The major whey proteins were resolved completely in 30 min. In addition, genetic variants A and B of β-lactoglobulin were separated to better than 70% in the same analyses. Reproducibility of peak retention times and peak areas were 1 and 3%, respectively. Analyses of purified whey proteins revealed impurities not detected by electrophoresis. Analysis was applicable to whey from a number of different sources of casein and cheese manufacture.

Whey protein isolates recovered by ion exchange and whey protein concentrates obtained by ultrafiltration were compared in terms of gross composition and in terms of more detailed protein content using both size exclusion and reverse phase high performance liquid chromatography (Barry *et al*., 1988). The size exclusion analysis was performed on a TSK G 300 column with eluting buffer of 0.05 M sodium phosphate, pH 7.4 containing 0.15 M sodium sulfate at a flow rate of 0.3 ml/min. Eluted proteins were detected by their absorbance at 280 nm. The high-performance liquid chromatographic separation was performed with a reverse phase Ultrasphere RPSC column containing 5 μm particle size C3 propyl bonded phase (Rector *et al*., 1989, 1991). Solvent A was 0.15 M sodium chloride/HCl pH 2.1 and solvent B was acetonitrile. The gradient program was held at 0% B for 4 min, 0–30% B in 3 min, 30–42% B in 24 min, 42–0% B in 4 min.

4.2.3 Immunoturbidimetric methods

Immunoturbidimetric methods have also been used to measure the whey protein content of milk- and buttermilk-powders (Greiner *et al*., 1985). Antibodies to whole bovine whey were developed for rapid screening of whey protein in non-fat dry milk and buttermilk. Milk samples are heat treated prior to analysis to denature the whey proteins for a more uniform response to antibodies. Of the whey proteins tested the assay is most sensitive to bovine serum albumin and least sensitive to β-lactoglobulin. Precision of the method is about 4%

coefficient of variation with a minimum level of detection of 3% whey protein concentrate added to non-fat dry milk.

Kim *et al*. (1987) determined the β-lactoglobulin, α-lactalbumin and bovine serum albumin contents of eight whey protein concentrate samples using reverse phase high-performance liquid chromatography and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The chromatographic column used was a C4 bonded reverse phase column with 300 A pore size. A non-linear gradient was used consisting of 30–45% acetonitrile containing 0.1% trifluoroacetic acid. The absorbance was measured at four different wavelengths of 210, 237, 250 and 280 nm. The coefficient of variation was 3.87% for bovine serum albumin, 4.39% for α-lactalbumin and 6.29% for β-lactoglobulin.

High-performance liquid chromatography has also been used to determine the denaturation of whey proteins (Parris and Baginski, 1991). Denatured whey protein and casein were isolated from undenatured whey protein by isoelectric precipitation at pH 4.6. Whey protein denaturation was determined by comparing reverse phase high-performance liquid chromatography protein profiles of isolates of heat-treated and unheated non-fat dry milk. In general, protein profiles for heat treated skim milk indicated whey protein denaturation began at 40 ºC, became more rapid at 70 ºC and was 95% complete at 85 ºC. Undenatured whey protein was also quantified as whey protein nitrogen based on their absorbance and nitrogen content compared to known whey protein standards or by augmenting the same samples with a known amount of lysozyme. Whey protein nitrogen values were obtained by a modified Kjeldahl nitrogen procedure. A C4 reverse phase bonded column of 10 mm particle size was used with solvent A being 0.1% trifluoroacetic acid in water and solvent B being acetonitrile. Absorbance at 280 nm was monitored to detect protein elution.

Alpha-lactalbumin, β-lactoglobulin and bovine serum albumin in raw and ultra high temperature pasteurized milks were determined using capillary electrophoresis (Cifuentes *et al*., 1993). The separation buffer contained 40 mM Tris boric acid, 0.1% sodium dodecyl sulfate and 10% polyethylene glycol 8000. Detection of the separated components was performed by monitoring the absorbance at 214 nm. The migrations times were reproducible and results agreed well with high-performance liquid chromatographic separations.

Uncoated capillaries were used in the quantitation of whey proteins by capillary electrophoresis (Recio *et al*., 1995). Separations were performed using 100 mM borate buffer, pH 8.2 containing 30 mM sodium sulfate. The use of high pH and high ionic strength buffer reduced adsorption of proteins on the capillary walls making their separation possible. Reproducibility of migration times and peak areas are improved by optimising the capillary equilibration procedure and by an internal standard. Relative standard deviations ranging between 0.74 and 1.03% for migration times and 2.14 to 5.23% for areas of major components are obtained. Detection limit of equal to or less than 0.5 mg/100 ml was achieved. Linear relationships of peak area to concentration have been used to quantitate bovine serum albumin, α-lactalbumin, β-lactoglobulin A and β-lactoglobulin B in cow's milk subjected to various thermal treatments.

Capillary zone electrophoresis has also been successfully applied to the quantitation of whey proteins in heat-treated milk (Recio and Olleman, 1996). The amount of denatured whey protein in heat-treated skim milk could be estimated by analyzing the casein fraction obtained by isoelectric precipitation at pH 4.6. A hydrophilically coated capillary was used in combination with 6 M urea in citrate buffer at pH 3. Optimization of the sample and running buffer minimized the adsorption of serum proteins, especially that of bovine serum albumin. This afforded a detection limit down to about 5–65 μg/ml for the three main components in milk serum. The detector response at 214 nm was linear in the range of 0.05–0.35 and 0.05–0.85 mg/ml for α-lactalbumin and β-lactoglobulin, respectively. Bovine serum albumin showed a slightly less linear behaviour due to residual adsorption to capillary walls. The recovery of serum proteins was in the range of 89–107%.

A capillary electrophoresis method for the determination of casein and whey protein has also been reported (Miralles *et al*., 2001). The effects of several parameters such as pH, ionic strength, concentration of urea and applied voltage on time and separation efficiency were studied. Using a hydrophilically coated capillary in combination with an electrophoresis buffer of 0.48 M citric acid-0.13 mM citrate containing 4.8 M urea at pH 2.3 and a separation voltage of 25 kV allowed complete separation of β-lactoglobulin and para-κ-casein permitting the quantitation of both compounds.

What the preceding examples show is that there are a number of methods available for the detection and quantitation of whey proteins. Is there a preferred method that is recommended? A study was conducted to compare the three common analytical methods of polyacrylamide gel electrophoresis, high-performance liquid chromatography (HPLC) and capillary electrophoresis (Norris *et al*., 1998). The electrophoretic procedures included native polyacrylamide gel electrophoresis (native PAGE) and polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). The capillary electrophoresis procedures used were capillary electrophoresis (CE), capillary gradient electrophoresis (CGE), capillary zone electrophoresis (CZE) and the HPLC procedures included size exclusion, ion exchange, reverse phase and IgG affinity HPLC.

The best method depends upon which component is critical in the analysis. For α-lactalbumin the preferred methods in order of preference were reverse phase HPLC, size exclusion HPLC, reduced PAGE and non-reduced PAGE. For β-lactoglobulin reverse phase HPLC and reduced SDS-PAGE were found to be the most suitable. For bovine serum albumin size exclusion and native PAGE were deemed the best and for IgG reduced SDS-PAGE and Affinity Protein G HPLC were optimal.

In another comparative study, CE, SDS-CE and UV 4th derivative spectra were compared for their sensitivities and efficiencies of quantitation of whey proteins (Miralles *et al*., 2000). Samples tested in this study were raw milk and heat-treated milk. All methods effectively measured the whey protein to total protein ratios independently of the heat treatment applied to the samples. Mean

values obtained by CE, SDS-CE and 4th derivative UV spectroscopy were respectively 17.1, 18.5, 17.3 for raw milk samples, 16.6, 17.7 and 18.8% for pasteurized milks and 16.8, 17.0 and 17.2 for ultra high temperature treated milks. The composition or states of the proteins in whey were not determined in this study.

4.3 Structure of whey proteins

4.3.1 β-lactoglobulin

The most prevalent protein in whey is β-lactoglobulin. It comprises 10% of the total milk protein or about 58% of the whey protein. It contains 162 amino acids with a molecular weight of about 18,300. There are two genetic variants, A and B that differ in the substitution of a glycine in Variant B for an aspartic acid in Variant A. The molecule contains two disulfide and 1 free sulfhydryl groups and no phosphorus (Swaisgood, 1982).

The primary sequence of β-lactoglobulin (Fig. 4.1) shows one of the disulfide groups between Cys 66 and 160. The other seems to be a dynamic one that involves 106 and is sometimes found with Cys 121 and sometimes with Cys 119. Thus, 1/2 of the Cys 119 and 1/2 of the Cys 121 exist as free sulfhydryl groups (Kinsella, 1984).

Below pH 3.0 and above pH 8.0, β-lactoglobulin exists as a monomer. Between pH 3.1 and 5.1 at low temperatures and high protein contents, it associates to form an octamer. This polymerization seems to be mediated through the action of carboxyl groups and thus the A Variant forms better

1 **11**
Leu Ile Val Thr Gln Thr Met Lys Gly Leu Asp Ile Gln Lys Val Ala Gly Thr Thr Trp
21 **31**
Ser Leu Ala Met Ala Ala Ser Asp Ile Ser Leu Leu Asp Ala Gln Ser Ala Pro Leu Arg
41 **Gln in Variant D** **51** **Variant C His**
Val Tyr Val Glu Glu Leu Lys Pro Thr Pro Glu Gly Asp Leu Glu Ile Leu Leu **Gln** Lys
61 **Gly in Variants B, C** **71**
Asp Glu Asn **Asp** Glu Cys Ala Gln Lys Lys Ile Ile Ala Glu Lys Thr Lys Ile Pro Ala
81 **91**
Val Phe Lys Ile Asp Ala Leu Asn Glu Asn Lys Val Leu Val Leu Asp Thr Asp Tyr Lys
101 **111** **Variants B, C Ala**
Lys Thr Leu Leu Phe Cys Met Glu Asn Ser Ala Glu Pro Glu Gln Ser Leu **Val** Cys Gln
121 **131**
Cys Leu Val Arg Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys Phe Asp Lys Ala Leu
141 **151**
Lys Ala Leu Pro Met His Ile Agr Leu Ser Phe Asn Pro Thr Gln Leu Glu Glu Gln Cys
161 **162**
His Ile OH

Fig. 4.1 Primary structure of bovine β-lactoglobulin A. The locations of the amino acid substitutions in the genetic variants are indicated. There is a disulfide bond between cys 66 and cys 160. Another disulfide bond is formed between cys 119 and cys 121. There is a 50:50 distribution of the bond between positions 119 and 121. Cys 121 is always involved in the bond.

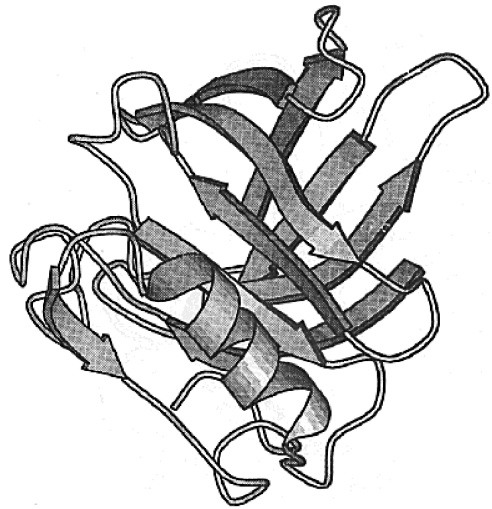

Fig. 4.2 Structure of bovine β-lactoglobulin.

octamers than does the B Variant. At other pH values, including the pH of milk, β-lactoglobulin tends to be found as a dimer. These dimers are spherical with diameters of about 18 A. The complex association-dissociation behaviour of β-lactoglobulin has been the subject of extensive study (Whitney, 1977).

Beta-lactoglobulin is manufactured specifically in the mammary gland for inclusion in milk where its role is unknown. All ruminant milk contains β-lactoglobulin while the milk of almost all non-ruminants does not. While biological functions have been speculated to exist for β-lactoglobulin, to date none has been fully accepted. The molecule has a very hydrophobic area that is quite effective in binding retinol. Some speculate that the binding of Vitamin A may have a regulatory role in the mammary gland. Because of its prevalence in bovine milk, to a large extent the properties of whey protein concentrates are in effect, the properties of β-lactoglobulin.

The secondary structure of β-lactoglobulin is homologous to that of retinol-binding proteins. It contains nine strands of β structure, eight of them arranged to form a β barrel. The lone α helix is located on the surface of the molecule. The centre of the barrel is hydrophobic and can be involved in the binding of hydrophobic molecules. The three-dimensional structure of β-lactoglobulin is (Fig. 4.2) similar to plasma retinol binding protein (Papiz *et al*., 1986).

4.3.2 α-lactalbumin

The second most prevalent protein in whey is α-lactalbumin that comprises about 2% of the total milk protein that is about 13% of the total whey protein. The molecule consists of 123 amino acids and has a molecular weight of 14,146.

1 **Arg in Variant B**

Glu Gln Leu Thr Lys Csy Glu Val Phe **Gln** Glu Leu Lys Asp Leu Lys Gly Tyr Gly Gly

21 **31**

Val Ser Leu Pro Glu Trp Val Cys Thr Thr Phe His Thr Ser Gly Tyr Asp Thr Glu Ala

41 **51**

Ile Val Glu Asn Asn Gln Ser Thr Asp Tyr Gly Leu Phe Gln Ile Asn Asn Lys Ile Trp

61 **71**

Cys Lys Asn Asp Gln Asp Pro His Ser Ser Asn Ile Cys Asn Ile Ser Cys Asp Lys Thr

81 **91**

Leu Asn Asn Asp Leu Thr Asn Asn Ile Met Cys Val Lys Lys Ile Leu Asp Lys Val Gly

101 **111**

Ile Asn Tyr Trp Leu Ala His Lys Ala Leu Cys Ser Glu Lys Leu Asp Gln Trp Leu Cys

121 **123**

Glu Lys Leu OH

Fig. 4.3 Primary structure of bovine α-lactalbumin B. The position of the amino acid substitution that occurs in genetic variant A is indicated. Disulfide bonds are formed between the following pairs of cys residues: 6 and 120, 28 and 111, 61 and 77 and 73 and 91.

The molecule contains four disulfide linkages and no phosphate groups. Its primary structure (Fig. 4.3) has been elucidated (Brew *et al*., 1970). The three-dimensional structure for α-lactalbumin from baboon is also shown (Fig. 4.4).

Alpha-lactalbumin has been shown to modify the activity of the enzyme galactosyl transferase. In the absence of α-lactalbumin, this enzyme adds UDP-galactose to N-acetyl glucosamine groups that are attached to proteins. It can transfer the UDP galactose to glucose, but the K_m for glucose is 1400 mM and

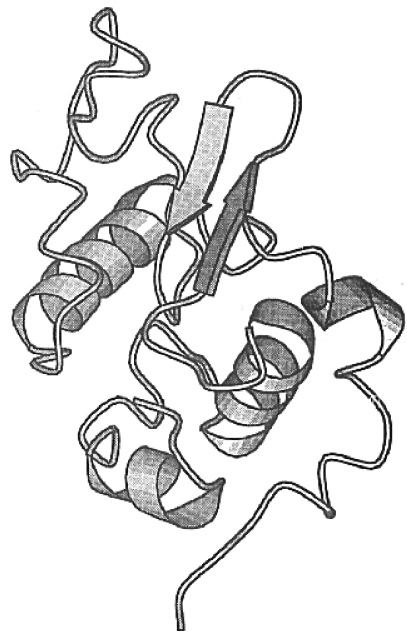

Fig. 4.4 Three-dimensional structure of baboon α-lactalbumin (Bernstein *et al*. 1977).

thus, the reaction proceeds slowly, if at all. Alpha-lactalbumin serves to lower the K_m for glucose to 5 mM and the enzyme complex now will add UDP-galactose to glucose to produce lactose and UDP. Thus, the milk of all mammals that contain lactose also contains α-lactalbumin. The α-lactalbumin of any species isolated so far will serve to modify bovine galactosyl transferase activity (Brew and Grobler, 1990).

When the sequences of α-lactalbumin and lysozyme are compared, about 40% of the residues are found to be the same, including all the cysteine residues. Another 20% of the residues have similar structures. This information coupled with the fact that α-lactalbumin helps to synthesize the same linkage that lysozyme cleaves, suggests that the molecules are closely related. In fact, knowledge of the three-dimensional structure of lysozyme has been utilized to predict the three-dimensional structure of α-lactalbumin (Browne *et al*., 1969).

Despite their similarity, they do not work on the same substrates and are not related antigenically. The site of synthesis of α-lactalbumin like β-lactoglobulin is the mammary gland. Alpha-lactalbumin is unusual in that the molecule is more stable to heat in the presence rather than the absence of calcium. Most proteins show increased heat sensitivity in the presence of calcium. This is probably due to the ability of calcium to promote the formation of ionic intermolecular cross-links with most proteins. These crosslinks hold the molecules in proximity and increase the likelihood of aggregation upon heating. Alpha-lactalbumin, on the other hand, uses calcium to form intramolecular ionic bonds that tend to make the molecule resistant to thermal unfolding. Under favourable conditions of calcium and pH, α-lactalbumin can remain soluble after exposure to 100 ºC. The structure of α-lactalbumin is presented in Fig. 4.3 (Swaisgood, 1996).

4.3.3 Bovine Serum Albumin

The Bovine Serum Albumin (BSA), isolated from milk, is identical to the blood serum molecule. Thus, BSA is not synthesized in the mammary gland, but rather into the milk through passive leakage from the bloodstreams. The protein has a molecular weight of 69,000. It contains no phosphorus, 17 disulfides and one free sulfhydryl group. In blood plasma albumin is a carrier of free fatty acids. The molecule has specific binding sites for hydrophobic molecules and may bind them in milk as well (Brown, 1977).

4.3.4 Immunoglobulins

The immunoglobulins comprise at least 2% of the total milk protein. There are four classes of immunoglobulins found in milk: IgG1, lgG2, lgA and lgM. All of these molecules have a similar basic structure being composed of two light chains with molecular weights of 20,000–25,000 and two heavy chains, having molecular weights of 50,000–70,000 (Swaisgood, 1982).

These molecules are not synthesized in the mammary gland and thus must first enter into the gland and then be transported through it to be able to enter the milk. In the case of at least one class of antibodies, lgG1, a specific receptor site has been located on the membrane of the cells of the mammary gland that facilitates the entry of this protein into the gland. The immunoglobulins supply passive immunity to the calf when supplied in the colostrum. This protection lasts until the animal is old enough to begin synthesis of its own antibodies (Whitney, 1977).

4.3.5 Proteose peptones

This fraction of milk has been defined as those proteins that remain in solution after milk has been heated at 95 ºC for 20 minutes and then acidified to pH 4.7 with 12% trichloroacetic acid (Swaisgood, 1982). This fraction can be divided into four major components while other minor components are recognized. Proteose peptone component 3 is found only in whey and is not associated with casein. This protein contains over 17% carbohydrate and has a molecular weight of 20,000. Antibody to proteose peptone component 3 will cross react with fat globule membrane and it has been suggested that this component is of membrane origin (Girardet and Linden, 1996).

Proteose peptone component 5 has a molecular weight of 13,000 and is associated with both the whey and casein fractions of milk. The molecule contains phosphorus and has been shown to consist of the N-terminal 107 amino acids of β-casein that arise from the proteolytic cleavage that yields the γ-caseins (Swaisgood, 1982).

In a like manner, proteose peptone component 8 fast, with a molecular weight of 3,900, represents the N terminal 28 amino acids released from the cleavage of β-casein. The other major proteose peptone component, 8 slow, has not yet been shown to be derived by the proteolysis of any milk proteins. In time, however, this will probably occur. The protein has a molecular weight of 9,900. As a group, the proteose peptones are by definition resistant to heating. They are also very surface-active due in part to their low molecular weights and also to the carbohydrate associated with component 3. About 1.1% of the total milk protein consists of proteose peptone. As some of these molecules are derived from the proteolysis of β-caseins, their concentration in any given milk can be expected to increase with time (Swaisgood, 1982).

4.4 Improving functionality of whey proteins in foods: physical processes and enzymatic modification

Two main methods of improving whey protein functionality are modification of proteins through physical treatments and enzymatic modification of proteins. Chemical modifications of proteins are also possible but due to the necessity to prove the safety of such modifications to humans they are not generally practised.

4.4.1 Physical processes to enhance functionality

The observed difference in functionality of commercial whey protein concentrates is attributable to differences in the proteins or in their non-protein components (Morr, 1992). These non-protein constituents are lactose, lipids and minerals. Therefore, Morr *et al*. (1967) and Brandenburg *et al*. (1991) attempted to remove several of the low molecular weight components. The treatments used by these researchers removed 85–90% of the lactose but only 5–35% of the minerals and none of the lipids associated with the protein products. These treatments were detrimental to the gelation functionality of the protein products.

Another approach attempted by Morr (1992) was the addition of ions to improve gelation. Centrifugally treated whey proteins were fortified with 0.1 and 0.2 M NaCl, KCl, $CaCl_2$ and Na_2HPO_4. The pH of the whey protein solutions was either adjusted to 3, 4, 5, 6, or 7.5 and protein concentrations from 2–12%. Proteins with lowest lipid content were effective for forming stable gels at the lowest protein concentration. Gels with highest shear strain and stress were obtained at pH 6 and 7.5 irrespective of the type of ions added. Ion additions generally resulted in firmer whey protein concentrate gels.

Removal of residual lipids during the manufacture of whey protein concentrates has been reported (Melachouris, 1984; Maubois *et al*., 1987; Kim *et al*., 1988; Rinn *et al*., 1990). The industry has not adopted the methods researchers have developed to any major extent. Microfiltration has also been attempted to remove residual phospholipoproteins from whey (Morr, 1992). Microfiltration increased the clarity of the whey and greatly improved flux rates during the subsequent concentration by ultrafiltration.

On a pilot scale, whey protein concentrates were prepared by treating whey with calcium chloride and heat (Vaghela and Kilara, 1996b). These and commercial whey protein concentrates were subjected to proximate analyses, and analyses of lipid classes, phospholipid classes, free fatty acids and monoacylglycerol composition. The pre-treatment of whey with calcium chloride and heat followed by centrifugal clarification, resulted in whey protein concentrate that had significantly lower total lipids and lower lipid to protein ratio. Higher removal of lipids through higher calcium addition also resulted in higher protein losses. The commercial whey protein concentrate had ratios of lipid to protein that were significantly higher than all experimental whey protein concentrates. Triacylglycerols were the highest lipid class followed by phospholipids, diacylglycerols, free fatty acids, cholesterol esters, cholesterol and monoacylglycerols. The pre-treatment significantly increased the proportion of phospholipid and monoacylglycerol and decreased the proportion of triacylglycerol. Three major phospholipids in whey protein concentrates were sphingomyelin, phosphotidylcholine, and phosphotidylethanolamine followed by phosphotidylinositol, phosphotidylserine and cerebrosides. The pre-treatment significantly reduced the proportion of phosphotidylethanolamine but had no effect on free fatty acid composition of the whey protein concentrate.

Laboratory manufactured whey protein concentrates of 35 and 75% protein content were prepared by treating whey with calcium chloride and heat (Vaghela

and Kilara, 1996a). Solubility, thermal, foaming and emulsification properties were compared with commercial samples of whey protein concentrates. Pre-treatment increased calcium and phosphorus contents and decreased the contents of all other minerals. The pre-treatment had no effect on solubility, denaturation enthalpy and onset temperature of denaturation for the whey protein samples. These values were comparable with commercial whey protein samples. Foaming capacity and emulsion stability were unaffected but foam stability increased and emulsifying capacity decreased due to the pre-treatment. Overall total lipids and lipid class contents of whey protein concentrates were too low to affect the surface properties of the whey protein concentrates.

Industry's reluctance to use techniques proven in the laboratory is due to increased costs and processing steps. Other pre-treatments that have been attempted are shifting pH of whey to acidic levels, adding citrate at pH 6.1, adding a sequestrant (EDTA) at pH 4.1, adding calcium chloride at pH 4.0 and adding sodium polyphosphate at pH 5.1 (Melachouris, 1984). Changes in the functional performance of whey proteins can be achieved by thermal treatment, biopolymer complexing or texturization. Heat treatments around 70 ºC are critical to physical properties of whey proteins (Kester and Richardson, 1984). At or near this temperature, protein solubility, foaming activity and emulsifying activity begin to decline, but water binding and viscosity begin to increase (de Wit and de Boer, 1975). Partial denaturation of whey proteins as an intentional way of modifying functionality has been suggested (Ryan, 1977).

Thermal treatment to induce partial denaturation of whey proteins and to enhance surface properties of the resulting whey protein concentrate has been reported in literature (de Wit, 1975; McDonough *et al*., 1974; Richert *et al*., 1974). Heating whey protein concentrate at 60–65 ºC for 30 min. increased foam capacity and reduced whipping time (Reichert *et al*., 1974).

The ability of whey proteins to form gels at room temperature or at incubation temperature of enzymes has been reported (Barbut and Foegeding, 1993; Nakamura *et al*., 1995; Sato *et al*., 1995). This phenomenon was called cold-set gelation (McClements and Keogh, 1995). Cold-set gelation requires an initial pre-heating step to denature the whey proteins followed by incubation with such additives as salts, acidulants or proteases.

After whey protein or β-lactoglobulin solution was heat denatured, soluble aggregates were detected with size exclusion chromatography (Ju *et al*., 1997; Matsudomi *et al*., 1992), SDS-PAGE (Laligant *et al*., 1991) and electron microscopy (Nakamura *et al*., 1995). The percentage of native whey protein converted to soluble aggregates depended on the amount of heat. Salt and acid-induced gelation of denatured whey protein isolate solution resulted from changes in the surface charges of the aggregates (Sato *et al*., 1995). Such cold-set gels from the denatured whey protein solution had fine stranded structure (Barbut and Foegeding, 1993; Kawamura *et al*., 1993; Nakamura *et al*., 1995; Ju *et al*., 1997) even at high ionic strength or pH close to the isoelectric point. Heat-set gels under similar conditions had particulate microstructures (Mangino, 1992; Langton and Hermansson, 1992). Both covalent and non-

covalent bonds were suggested to be involved in cold-set gelation (Sato *et al*., 1995).

Cold set gelation has potential application in the food industry. A commercial whey protein product has been manufactured (Thomsen, 1995). This ingredient can be used in various foods such as surimi, pressed ham, dressings, spreads, and bakery products. A few patents have also been awarded for cold-set gelation (Tamaki *et al*., 1991; Kawachi *et al*., 1993).

A 9% whey protein isolate solution at pH 7 was heat denatured at 80 ºC for 30 min. (Ju and Kilara, 1998a). Size exclusion chromatography showed that native whey protein formed soluble aggregates after heat denaturation. Addition of $CaCl_2$ (10–40 mM), NaCl (50–400 mM) or glucono–delta-lactone (0.4–2%) or hydrolysis by a protease from *Bacillus licheniformis* caused gelation of the denatured protein at 45 ºC. Textural parameters, hardness, adhesiveness and cohesiveness of the gels so formed changed markedly with concentration of added salts or pH by added glucono-delta-lactone. Maximum gel hardness occurred at 200 mM NaCl or pH 4.7. Increasing $CaCl_2$ concentration continuously increased gel hardness. Generally, glucono-delta-lactone induced gels were harder than salt induced gels and much harder than the protease induced gels.

Ju and Kilara (1998b) denatured various concentrations of whey protein isolates in solution by heating them to 80 ºC for 30 min. Size exclusion chromatography and dynamic light scattering revealed the formation of soluble aggregates in the denatured whey protein solutions. Size and content of the aggregates increased with increases in preheated whey protein concentration. The 4–9% denatured whey protein solutions were diluted to 3% protein concentrations with distilled water. Upon addition of 20 mM $CaCl_2$ or 0.6% glucono-delta-lactone all denatured whey protein solutions formed gels at 37 ºC. Hardness of the 3% whey protein gels remarkably increased with whey protein concentrations during heating or with aggregate size and content. The high-performance liquid chromatography profiles showed that prolonging the heating time at 80 ºC for 8% whey protein solutions also gradually increased aggregate size and concentration which then led to an increase in the hardness of the cold-set gels.

The thermal properties of the whey protein aggregates were also investigated by Ju *et al*. (1999). Aggregation of 10% whey protein solution was induced by addition of calcium salt, acidification, or proteolysis at 45 ºC. Effects of pre-aggregation on thermal properties of whey proteins were examined by differential scanning calorimetry. The different types of aggregation had three effects (a) one endothermic peak, representing the denaturation of the whey protein aggregates, instead of two endothermic peaks representing the denaturation of β-lactoglobulin and α-lactalbumin in the control; (b) a narrower range of denaturation temperature than the control and (c) significantly greater enthalpy values than the control. Denaturation temperatures of the aggregates were also different from those of α-lactalbumin (67 ºC) and β-lactoglobulin (76 ºC) of the control. Aggregates induced by calcium salts (~ 74 ºC) and

protease (~73 ºC) had intermediate denaturation temperatures. The pH induced aggregates had high denaturation temperatures (80–91 ºC) at low pH (3.5–7.5). An exothermic peak was detected during calcium- salt- or protease-induced aggregation of whey proteins at 45 ºC. Thus the pre-aggregation changed the thermal properties of whey proteins.

Aggregation of whey proteins induced by $CaCl_2$ was investigated by measuring turbidity and aggregate size (Ju and Kilara, 1998c). The effect of this aggregation on thermal gelation was determined by measuring gel hardness. Standard conditions were set at 10% whey protein solution, 20 mM $CaCl_2$ and 45 ºC in order to observe the influence of various variables on the aggregation. The addition of 20 mM $CaCl_2$ to a 10% whey protein solution resulted in a continuous increase in turbidity during 5 h incubation at room temperature (21 ºC). As expected an increase in temperature (21–45 ºC) or $CaCl_2$ concentration (5–20 mM) increased the rate and extent of aggregation. Stable colloidal aggregates were obtained with 10–30 mM $CaCl_2$. An increase in protein concentration from 5 to 20% remarkably reduced the rate and extent of aggregation during a 5 h incubation. The aggregation was dependent on the weight ratio of $CaCl_2$ to whey proteins. Aggregation induced by $CaCl_2$ before heat-induced gelation greatly affected the hardness of the gels that were formed by heat treatment (80 ºC for 30 min.). The formation of the hardest gels required the optimal extent of aggregation and aggregate size.

Effects of pH (3.0–7.0) on aggregation of 18% whey protein solutions have also been investigated (Ju and Kilara, 1998d) by examination of turbidity, aggregate size and microstructure. As expected, maximum turbidity and aggregate sizes occurred at the isoelectric point (5.2) of whey proteins. Lower or higher pH than the isoelectric point resulted in a steady decrease in the turbidity and aggregate size. Microstructure analysis revealed that the whey protein solution at pH 5.7 contained loose and irregular aggregates with 200–400 nm size. From the pH-aggregated whey heating, hydrolysing with a protease from *Bacillus licheniformis*, increasing ionic strength with $CaCl_2$ and quiescently acidifying with glucono–delta-lactone, respectively, induced protein solution gelation. The hardness, colour and microstructure of the gels so formed were determined. Micrographs of protease and $CaCl_2$ induced gels showed aggregates similar in size and shape to the parent aggregates (500 nm and 1–2 μm). Separating the process of formation of aggregates and gels may provide a means of manipulating protein gel structures.

Gelation that was induced by heat protease, calcium salt or acidulant from a solution of Ca^{2+} aggregated whey protein was investigated by analyses of the rheological, textural and microstructural properties of the gel (Ju and Kilara, 1998e). The addition of 40 mM $CaCl_2$ to 18% whey protein solution resulted in aggregation during 4 h of incubation at 45 ºC. The occurrence of aggregation was determined by turbidity and in mean aggregate size. Hydrolysis by a protease from *Bacillus licheniformis* (1% enzyme to protein ratio), a decline in pH by glucono-delta-lactone (1.5% wt/vol), an increase in ionic strength by $CaCl_2$ (60 mM) or heat treatment (80 ºC, 30 min.) all led to gelation of the

aggregated whey protein within 40 min. The gels formed differed widely in texture and rheological properties. The heat-induced gel was over 20 times stronger than gels induced by enzyme, acid and salt. The heat-induced gel showed considerably higher adhesive properties than other gels. The gels induced by acid and salt showed considerably more cohesiveness than gels induced by heat or enzyme.

The structure of particulate gels resulting from salt-induced cold-set gelation of heat denatured whey proteins was studied (Marangoni *et al*., 2000). Near ultraviolet spectroscopy, circular dichroism and fluorescence spectroscopy reveal that whey proteins lose their tertiary structure to form soluble aggregates during heat denaturation. These aggregates can be induced to form gels when ionic strength is manipulated by the addition of NaCl or $CaCl_2$. Charge dispersal and calcium bridging are thought to be operative mechanisms facilitating the gelation of aggregates. Light microscopy revealed that these gels were composed of flocs of aggregated primary particles. Flocs were termed microstructures while primary particles were termed microstructural elements. The size of the microstructures ranged from 10 to 20 μm while the size of the microstructural elements ranged from 0.5 to 1.0 μm. These gels were structured and behaved rheologically as stochastic mass fractals where the elastic constant of the gels was related to the volume fraction of protein in a power law fashion. The gel network was found to be in a weak link regime. These researchers described the other characteristics of the fractal nature of these gels.

4.4.2 Enzymatic modification to enhance functionality

Another method available to modify the molecular properties of proteins is to subject the proteins to enzymatic hydrolysis. Endopeptidases cleave peptide linkages in the primary sequence of a protein yielding two or more peptides. Enzymatic hydrolysis of proteins is affected by enzyme specificity, extent of denaturation of the protein substrate, enzyme concentration, pH, temperature, ionic strength and presence or absence of inhibitors. Specificity of an enzyme is a key factor affecting the number and location of peptide linkages hydrolyzed (Panyam and Kilara, 1996). Enzymatic hydrolysis of proteins leads to a decrease in the molecular weight of the protein, an increase in the ionisable groups and the exposure of hydrophobic groups hitherto concealed. The change in functional properties is a direct result of these changes.

One obvious consequence of hydrolysis is the increase in solubility of the protein. Whey proteins, in general, have good solubility over a wide range of pH values. Denaturation at low pH leads to aggregation and insolubility. A product called lactalbumin (not to be confused with α-lactalbumin) is manufactured by heat denaturing whey proteins. Heat denatured whey protein isolate was prepared by heating a solution of protein isolate to 85 ºC for 30 min. at pH 4.6 (Mutilangi *et al*., 1995). This preparation of lactalbumin was hydrolysed with trypsin, chymotrypsin, Alcalase or Neutrase to 2.8, 4.3, 6.0 and 8.0% degree of hydrolysis. The hydrolysates were subjected to ultrafiltration (Mutilangi *et al*.,

1996). Ultrafiltration increased solubility and permeates and retentates had higher solubilities than the hydrolysates.

Solubility is not the only functionality that has been demonstrated to be altered with enzymatic hydrolysis. Water holding or water binding capacities impact the rheological properties of proteins. β-Lactoglobulin binds 6.7 grams of water per 100 g. Mietsch *et al*. (1989) report that partially hydrolyzed milk proteins have lower water binding capacities than unhydrolyzed substrates.

Gelation is thought to be hindered by hydrolysis, presumably due to the reduced hydrophobicity of the hydrolysates (Mahmoud, 1994). The increased net charge on proteins results in increased repulsion between peptides. Interestingly, limited proteolysis using various enzymes can also be used to control the gelling ability as well as gel strength. Tryptic hydrolysis of whey proteins to between 2.3 and 6.7% degree of hydrolysis prevented gelation at pH 3.0 and 7.0, whereas hydrolysis to 2.3% degree of hydrolysis with a *Bacillus subtilis* protease dramatically increased the gelling ability and gel strength at neutral pH (Ju *et al*., 1995).

Whey protein concentrate was hydrolyzed by trypsin to obtain 2.5, 3.9 and 5.3% degree of hydrolysis (Chobert *et al*., 1988). In all instances the hydrolyzed whey proteins had better emulsion capacities than the unhydrolyzed control. These researchers also suggested that in order to maintain good emulsifying properties the apparent molecular weights of the peptides should not be less than 5,000. In addition, the important role played by enzyme specificity in generating desirable peptides with amphiphilic properties was also reinforced.

Gauthier *et al*. (1993) hydrolyzed commercial 35% whey protein concentrate and heat-treated 35% whey protein concentrate with trypsin and chymotrypsin. The hydrolyzates were separated with a 1,000 molecular weight cut off membrane. The interfacial properties of these peptides revealed that tryptic hydrolyzates had higher rates of adsorption at the oil water interface than intact proteins. Enzyme specificity was considered important because the tryptic hydrolyzates were more surface active than the chymotryptic hydrolyzates. Turgeon *et al*. (1991, 1992b) also earlier reported similar observations.

The predominant component in whey protein is β-lactoglobulin. Turgeon *et al*. (1992a, b) demonstrated that an oligopeptide from β-lactoglobulin (f 41–60) with a size of 2300 Daltons possessed improved surface activity over the native β-lactoglobulin. A number of reports have shown that major fragments of the β-barrel domain can be produced by limited trypsinolysis of β-lactoglobulin (Chen *et al*., 1993; Huang *et al.*, 1994, 1996).

Fragments of the β-barrel domain of β-lactoglobulin were obtained by membrane fractionation of a limited proteolysate prepared with an immobilized trypsin reactor (Huang *et al*., 1996). Analysis of the fraction by a size exclusion chromatography under physiological conditions indicated that the fraction contained a predominant peptide with a size of 8,400 Daltons and several other peptides with sizes ranging from 2,000 to 30,700 Daltons. When the fraction was analyzed by gel electrophoresis under reducing conditions a major peptide with a molecular weight of 6,400 Daltons was observed. The authors suggest the

presence of a smaller peptide linked to this 6,400 Dalton peptide via a disulfide linkage. The isolated peptide had a reduced surface hydrophobicity and a slightly higher surface and interfacial tension than native β-lactoglobulin. The emulsifying activity index for the domain peptide was twice the size of the intact protein. Droplet size measurement revealed that domain peptides formed smaller emulsion droplets than the intact protein. Their results suggested that the emulsifying properties of the f 41–100 + f 149–162 were greater than those of β-lactoglobulin f 41–60.

Foaming properties can also be improved by enzymatic hydrolysis. Kuehler and Stine (1974) treated casein, laboratory prepared whey protein concentrate and commercial protein concentrate with pepsin, Pronase and Prolase. Pepsin and Pronase hydrolyzates produced foams that were inferior to the untreated control. Hydrolysis of whey protein by Prolase resulted in improving the foaming capacity and foam stability of the hydrolyzates over the control.

Whey protein isolate was modified to enhance foaming characteristics by controlled hydrolysis using Alcalase, acid fungal protease, chymotrypsin, pepsin and trypsin to achieve 2.5–3.0% degree of hydrolysis (Althouse *et al*., 1995). Hydrolyzates were separated by ultrafiltration (10,000 molecular weight cutoff) to obtain a permeate and a retentate fraction for each enzyme treatment. Foam capacity, foam stability and surface tension at the air–water interface were measured. Permeate from Alcalase treatment exhibited the best foaming characteristics, comparable to that of egg white.

Innocente *et al*. (1998) report that proteose peptone 3, a component that results from the milk fat globule membrane protein, has good foaming properties. At concentrations of 0.05, 0.10 and 0.20 g/100ml of proteose peptone 3 the equilibrium surface tension at the air-water interface were reported to be 44.75, 36.14 and 32.11 mN/m, respectively. The compression isotherms for the total proteose peptone films were different from those of the proteose peptone 3 films. The maximum surface elasticity of the proteose peptone 3 films was at surface values of 0.3 and 0.6 m^2. mg^{-1}. This property was considered important for foaming capacity and foam stability parameters.

Another developing aspect of properties of proteins is their potential nutraceutical functions. Pihlanto-Leppala *et al*. (2000) have reported that peptides from whey show angiotensin -1 converting enzyme (ACE) inhibition. The bovine whey proteins α-lactalbumin and β-lactoglobulin were hydrolyzed with pepsin, trypsin, chymotrypsin, pancreatin, elastase or carboxypeptidase alone and in combination. The total hydrolyzate was fractionated in a two-step ultrafiltration process, first with a 30,000 molecular weight cut-off (MWCO) membrane and then with a 1,000 MWCO membrane. Inhibition of ACE was monitored spectrophotometrically. The peptides were isolated by chromatography and analyzed by mass spectrometry for sequencing. Inhibition of ACE was observed after hydrolysis with trypsin alone and with an enzyme combination containing pepsin, trypsin and chymotrypsin. Whey protein digests showed a 50% inhibition of ACE activity at concentration ranges of 345–1733 μg/ml. Values for 1–30,000 fractions ranged from 485–1134 μg/ml and for the

< 1,000 fraction from 109–837 μg/ml. Potencies of the purified peptide fractions indicated that concentrations required to achieve 50% inhibition of ACE ranged from 77–1062 μM. The ACE inhibitory peptides arising from α-lactalbumin hydrolysis were f 50–52, f 99–108, and f 104–108 while those from β-lactoglobulin were f 22–25, f 32–40, f 81–83, f 94–100, f 106–111 and f 142–146 (Pihlanto-Leppala *et al*., 2000).

4.5 Sources of further information and advice

This chapter attempts to provide a digest of the proteins present in whey and their commercial importance. There are a number of books and articles on this topic and some of these have been cited as references in this chapter. There are many more that have not been cited here due to limitations of space.

Commercially available whey protein products may have similar gross composition but their functionality and their ease of use can vary widely. When food manufacturers are developing specifications for ingredients, these functional attributes should become a part of the process. In order to achieve meaningful descriptions of desirable functionalities, food manufacturers and ingredient suppliers will have to agree upon test methodologies that will be used to assess the desired functionalities. Such methodologies may have to be validated so that the factors that affect the test method are well understood.

Manufacturing variables lead to differences in functionalities. These variables include source of whey, pre-handling conditions of whey, pre-treatment, ultrafiltration and microfiltration membrane performance and spray drier conditions and characteristics.

Analytical methods currently available make it easier to analyze for specific protein fractions in an ingredient. Therefore, if gelation is the functionality of interest, methods can precisely determine the amount of β-lactoglobulin in the sample in a relatively short period of time. Such methodologies provide a means of controlling and understanding the composition of protein products during the manufacturing process.

As a greater understanding is developed about the peptide sequences necessary for functionalities and nutraceutical properties, the possibility of using biotechnology to improve protein composition exists. Research from Iowa State University demonstrated that cattle producing the BB form of β-lactoglobulin also secrete more casein and less β-lactoglobulin than do cattle that produce the AA form of β-lactoglobulin. Cows that produce β-lactoglobulin BB secreted more fat than their AB and AA counterparts, and BB cows made more of the fatty acids that are synthesized directly in the mammary gland. It was concluded that, if increased cheese yield is desired, then the use of bulls with β-lactoglobulin BB genotype could be increased.

It has also been reported from New Zealand (Hill and Patterson, 1994) that bulk milk collected from groups of β-lactoglobulin AA phenotype cows has a different composition to bulk milk collected from groups of β-lactoglobulin BB

phenotype cows. Differences in composition were similar with the β-lactoglobulin AA phenotype cow group producing milk that contained less casein, fat and total solids than the β-lactoglobulin BB phenotype cow group. No significant differences were observed in the total protein, lactose or ash content in the milk of the two phenotypic groups. In comparison with the β-lactoglobulin AA and BB phenotype bulk milks, large variations in composition were observed in the milk from individual cows. As all cows were fed on identical pasture and subjected to identical farm management practices these results indicate that in addition to β-lactoglobulin phenotype other genetic factors can have a significant influence on the composition of milk.

Since whey is a by-product of cheese manufacture and cheese yields are of paramount economic importance, biotechnology may lead to milk production practices that favour improvement in cheese yields. Such changes in the genetics of milch animals may lead to an altered composition of whey and consequently altered functionalities in whey protein products. Preliminary reports indicate that New Zealand is developing herds of cattle that produce only β-lactoglobulin BB.

The future for whey protein products is bright and as the world's demand for protein grows, whey protein will become increasingly important in providing nutrition and functionality to humans.

4.6 References

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5

Muscle proteins

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5.1 Introduction

Proteins are the major constituents in muscle, comprising approximately 20% of the total muscle weight or 80% of dry mass in the lean tissue. The roles of proteins in muscle foods are twofold. On one hand, they contain all the essential amino acids with a composition profile closely resembling that of the human body, and hence, are highly nutritious. On the other hand, through imparting specific functionalities (gelation, emulsification, water-binding, etc.), muscle proteins greatly contribute to the overall properties of meat and meat products (red meat, poultry, and fish), including texture, appearance, mouthfeel, and juiciness, as well as physical stability during storage. The latter role is of particular importance because essentially all the functional properties exhibited by muscle proteins cannot be reproduced by any other food proteins or nonprotein functional ingredients. An example is meat analogs made from vegetable proteins that, even with a sophisticated extrusion technology and creative formulations, have not been able to closely replicate the type of texture and mouthfeel of meat hamburgers, letting alone the savory meaty flavor. Consequently, preparation of functional proteins from muscle sources is a necessity in the development of high-quality muscle foods that the consumers are so accustomed to. Table 5.1 lists the major muscle proteins and muscle-derived protein ingredients for use in a variety of foods.

The sensitivity of muscle protein functionality to various processing factors, including temperature, heating rates, pH, salts, ionic strength, oxidizing agents, and nonmeat ingredients, makes the proteins a vital component in producing the type of product with a desirable physical characteristic. However, if these environmental parameters are not carefully controlled, poor protein

Table 5.1 Muscle proteins as indigenous components or as functional ingredients in food products

Protein	Source	Function/application
In situ protein		
Myofibrillar protein	Lean tissue	Meat binding; water holding; fat emulsification; texture of meat products
Sarcoplasmic protein	Lean tissue	Water binding; fat emulsification; color
Collagen	Connective tissue	Texture of meat products
Ingredient protein		
Myofibrillar protein	Meat trimming; animal by-products	Meat binding; water holding; fat emulsification
Collagen	Pig, turkey, chicken and fish skins	Water holding; juiciness; product yield
Gelatin	Hydrolyzed collagen from animal skins, hides, and cartilage; cooked pig and poultry skins	Water holding; emulsion stabilization; meat binding
Surimi	Fish; mechanically separated and washed meat; meat by-products	Seafood analogs; meat binding; water holding; fat emulsification
Plasma protein	Beef plasma	Meat binding; water holding; protease inhibitors for surimi

performance, and hence, low-quality meat products, could result. Thus, a clear understanding of the structure-functionality relationship as well as how the various meat processing conditions affect protein's structure and interactions is essential to a successful utilization of muscle-derived proteins in food processing as well as in new muscle food product development.

5.2 Structure of muscle proteins and endogeneous proteases

5.2.1 Structure of muscle proteins

Based on their solubility characteristics, muscle proteins are divided into three groups – sarcoplasmic, myofibrillar and stromal proteins. They make up approximately 35, 60 and 15%, respectively, of the estimated 1,000 or so total proteins present in the muscle tissue as revealed by proteomic analysis

(Lametsch and Bendixen, 2001). The sarcoplasmic protein fraction consists of at least 500 individual proteins, including many metabolic enzymes, and they are soluble in water and dilute salt solution (ionic strength $\Gamma = 0–0.2$) (Pearson and Young, 1989). Most sarcoplasmic proteins are of a globular structure with a high density of exposed polar and charged side chains. Myoglobin, a 17,000-dalton protein, is probably the best known member in this superfamily because of its prominent role as the chief pigmentation protein in fresh meat.

Myofibrillar proteins, the most abundant protein fraction that makes up the myofibril, are comprised of fewer proteins, but the total number may well be in the neighborhood of one hundred, estimated from two-dimensional gel electrophoretic analysis (Lametsch and Bendixen, 2001). This group of proteins is soluble in salt solutions (>2% or $\Gamma > 0.5$), and is responsible for much of the functional characteristics of fresh and processed muscle foods. Myosin and actin, the two most preponderant constituents in the myofibril, account for more than 70% of total myofibrillar protein (Pearson and Young, 1989). Myosin is comprised of approximately 4,500 amino acids with a molecular weight of 500,000, and has a fibrous structure made up of six subunits (two heavy chains; four light chains) that are associated into a hydrophilic helical rod (tail) and a hydrophobic globular head (Fig. 5.1). On the other hand, actin exists as globulins that are linked to one another to form an elongated chain (Murray and Weber, 1974). In postrigor meat, myosin and actin are cross-linked in the form of actomyosin, which is the dominant functional protein complex in low-salt processed meats. There are a number of other proteins present in the myofibrils, including titin and nebulin (scaffold proteins), tropomyosin, troponins, α-actinin, desmin, C-, M-, and X-proteins, and many other minor polypeptides (Asghar *et al*., 1985; Xiong, 1997). The roles for most of these secondary proteins in muscle foods are not fully understood. Ultrastructurally, proteins in the myofibril are located within four structural units – the thick filaments (e.g., myosin, C-protein, and M-protein), the thin filaments (e.g., actin, tropomyosin, and the troponin complex), the cytoskeletal filaments (i.e., titin and nebulin), and the Z-disks (e.g., α-actitin, zeugmatin, and desmin) (Fig. 5.2).

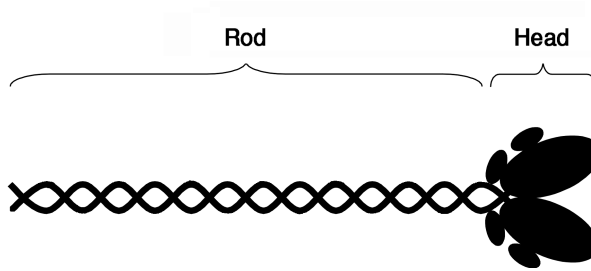

Fig. 5.1 Schematic representation of a myosin molecule that consists of a hydrophobic head and a hydrophilic tail (rod). Each heavy chain has a globular head (filled large oval) and a helical tail. The four light chains are shown as filled small ovals.

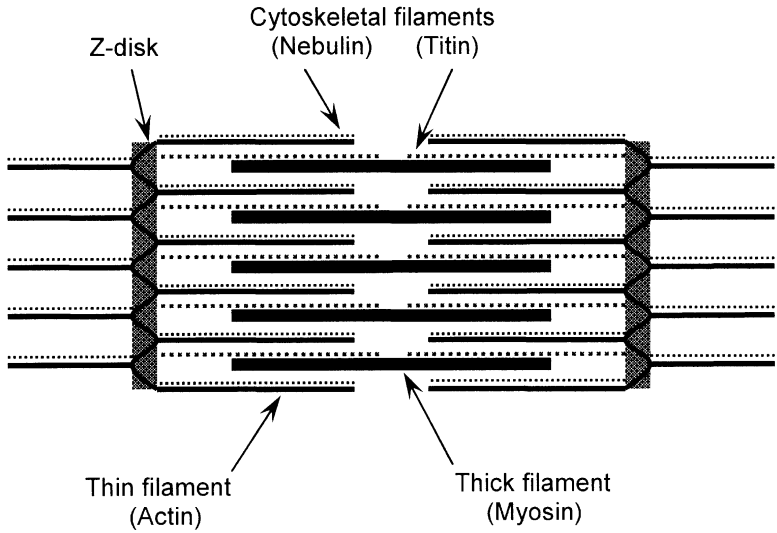

Fig. 5.2 Schematic representation of a basic unit (sarcomere) of a myofibril.

The third group of muscle proteins are stromal proteins. They are soluble only in acid or alkaline solutions but can be solubilized also with slow, moist cooking in the absence of acid or alkali compounds. They are mostly located in the interstitial space of muscle cells and are generally referred to as connective tissue proteins for their presence therein. Three of the connective tissue proteins – collagen, reticulin, and elastin – have been extensively studied (Pearson and Young, 1989). These extracellular proteins are composed of fine collagenous and reticular fibrils that are the major constituents in endomysium, perimysium, and epimysium. The connective tissues encasing the individual muscle fibers (endomysium) and the muscle fiber bundles (perimysium) contribute to toughness of meat. Other water- and salt-insoluble proteins, including membrane proteins of intracellular organelles, are also grouped into the stromal protein fraction. Because of their minute quantity, none of the membrane proteins has a practical significance in the quality and palatability of meat and meat products.

The basic unit of a collagen molecule is a triple-helical structure composed of three polypeptide chains that are stabilized via hydrogen bonds (Gross, 1961). Individual tropocollagen monomers, in turn, are cross-linked to form fibrils via hydrogen and covalent bonds. The amount and type of covalent linkages determine collagen's solubility and digestibility. Collagen from mature animals contains more stable, nonreducible cross-links derived from lysine residues (e.g., hydroxylysyl pyridinoline). Hence, it is more difficult to hydrate and solubilize when compared with that from young animals. At least ten genetic types of collagen have been identified in meat animals; types I, III, and IV have a major presence in the three layers of connective tissues and therefore, are major toughness factors (Bailey *et al*., 1979).

5.2.2 Endogenous proteases

Enzymes are bioactive proteins, and proteases are a special group of enzymes that are involved in protein turnover during growth and development of animals. Some of the endogenous proteolytic enzymes cease their functions shortly after death, while others remain active throughout the entire postmortem ageing process and contribute either to flavor (exopeptidases) or to tenderness (endopeptidases) of meat. Two well-studied enzyme systems that are implicated in meat tenderization are calpain and cathepsins.

Calpain is a calcium-dependent protease located around the myofibrils. It exists in two forms, i.e., μ-calpain and m-calpain, so designated due to their micromolar (μM) and millimolar (mM) range calcium concentration requirements for maximal activity (Edmunds *et al*., 1991). The μ-calpain and m-calpain are isomers with a high degree of sequence homology; both are composed of two polypeptide subunits with molecular weights of 80,000 and 28,000. The active site is located at the larger subunit. Since the calcium concentration in cytosol is in the micromolar range, only μ-calpain would be active and play a significant role in the degradation of the specific mofibrillar proteins, and hence, meat tenderization. Biochemical studies have shown that μ-calpain is able to degrade titin, nebulin, desmin, tropomyosin, troponin-T, and C-proteins, but interestingly, it does not affect myosin and actin (Goll *et al*., 1983; Huff-Lonergan and Lonergan, 1999). Cleavage of these proteins leads to the disruption of the Z-disks as well as the peripheral structure of the myofibrils. Although the optimal pH for calpain is 7.0–7.2, the enzyme still retains a significant amount of activity at postrigor muscle pH (5.5–5.6) (Koohmaraie *et al*., 1986). Because μ-calpain can reproduce all the proteolytic changes in meat under normal ageing conditions, it is believed that the calpain protease system plays a dominant role in meat tenderization. To accelerate meat ageing and to reduce tenderness variability between beef cuts, calcium infusion/injection has been proposed (Koohmaraie *et al*., 1989). Injection of a 2.2% calcium chloride solution up to 5% of the meat weight has been found to activate both μ-calpain and m-calpain, thus, greatly enhancing tenderness of otherwise tough cuts. Meat that is subjected to a normal postmortem ageing process rarely becomes mushy. This is because calpain is susceptible to autolysis and is regulated by its endogenous inhibitor calpastatin. The low pH condition (pH 5.5–5.6) in postrigor muscle tissue would also limit the activity of the enzymes.

Cathepsins are a group of acidic proteases located in the lysosomes. Like the calpain protease system, cathepsins are believed to be involved in the postmortem degradation of selective myofibrillar components. Furthermore, they are active against collagen. At least five lysosomal endopeptidases are implicated in meat tenderness, i.e., cathepsins B, D, E, H, and L (Ouali *et al*., 1987). These proteases are capable of degrading most of the same substrates affected by calpain (e.g., tropomyosin, troponins, and titin) within the pH 3.5–4.5 range. Moreover, they are effective against myosin and actin (Asghar and Bhatti, 1987). However, the role of cathepsins in meat postmortem ageing is subject to debate, and a popular school of thought is that cathepsins may not

have practical significance in meat tenderization (Koohmaraie, 1992; Huff-Lonergan and Lonergan, 1999). The premise is developed on the basis of three factors. First, cathepsins in intact muscle tissue are confined within the lysosomal membrane, i.e., they are not in direct contact with myofibrils. Second, these proteases have a very low pH requirement for optimal activity (1 to 2 pH units lower than postrigor meat pH). Third, electrophoresis of aged meat does not show any appreciable change in myosin nor in actin, both of which are favored substrates by cathepsins as shown in model systems. This last evidence is perhaps the strongest indication of minimal involvement of this group of enzymes. Nevertheless, it is assumed that the pH drop (from neutrality, due to glycolysis) and high temperature condition in the early stage of postmortem storage, especially with carcass electric stimulation, could cause the lysosomal membrane to rupture, and the released cathepsins would then diffuse to the intermyofilamental space to initiate protein degradation (Moeller *et al*., 1977). A disruption of the lysosome compartment appears to be a prerequisite for cathepsin activity, and this is supported by the findings that in surimi, a crude protein concentrate prepared by washing macerated fish muscle tissue, cathepsins B, L, and an L-like protease are highly active, causing rapid degradation of myosin, actin, and other myofibrillar proteins, thereby weakening of surimi gels (Jiang and Chen, 1999).

5.3 Muscle protein functionality

Protein functionality can be defined as the physical and chemical performances of proteins during processing and storage that affect the texture-related properties of the final meat product. The term ‘functionality’ referred to hereof should be distinguished from a protein’s physiological function which is concerned with the health and wellbeing of the human body. The principal protein functionalities in processed meats are gelation and related properties (e.g., meat particle binding and adhesion), emulsification, and water-holding (Table 5.2). Depending on the product formulation and processing protocols, muscle proteins undergo specific structural changes and interactions, producing various product physical characteristics. Most processed muscle foods are a manifestation of a combination of several protein functionalities, but the resultant product palatability could vary greatly. For example, the firm texture and juiciness of a frankfurter are attributed to a composite protein network system made possible through protein-protein interaction (gelation), protein-fat interaction or fat encapsulation (emulsification), and protein-water interaction (water-binding). Often, a slight variation in the product ingredient composition or processing condition is sufficient to initiate a significant shift in the physical characteristics of a cooked meat product from being desirable to undesirable or vice versa.

Table 5.2 Functional properties of muscle proteins in meat and meat products

Category	Property	Mode of action	Food example
Hydration	Water binding, holding, and absorption	Protein-water interaction via hydrogen bonds; water entrapment in myofibril lattices	Fresh meat; pumped/injected meat; marinated and other processed (salted) meats
	Solubility	Protein-water interaction via hydrogen bonds; protein charge repulsion by the presence of Na^+ and Cl^- ions and phosphates	Salted meats; tumbled/massaged meats
	Swelling	Water penetration into myofibril lattices	Marinated meats; pumped/injected meats
Structure/texture	Gelation	Matrix formation by extracted myofibrillar proteins; collagen protein-protein interaction	Restructured meat rolls and loaves; luncheon meats; gelatin gel foods
	Cohesion/adhesion/binding	Gels of salt-soluble proteins serve as a binding agent; surimi protein-protein interaction; gelation of collagen protein	Restructured meat rolls and loaves; boneless ham; kamoboko; shellfish analogs; gelatin-bound luncheon meats
Surface	Emulsification	Protein adsorption on fat particles to reduce surface tension; formation of rigid protein membrane in fat emulsion	Sausage; frankfurters; bologna

5.3.1 Gelation

A protein gel generally refers to a viscoelastic entity comprised of strands or chains cross-linked into a continuous network structure capable of immobilizing a large amount of water. The process of forming a gel, i.e., gelation, occurs in muscle foods as a result of unfolding and subsequent association of extracted proteins, usually in the presence of salt and sometimes also phosphates. The rate of structural change, i.e., denaturation, is critically important. A slow unfolding process, which typically occurs with a mild heating condition, allows polypeptides to align in an ordered manner into a cohesive structured network capable of holding both indigenous and extraneous water (Ziegler and Foegeding, 1990). Examples of gel-type products are bolognas and various luncheon meats made from comminuted muscle. Because of its adhesion ability, the gel formed at the

junction of meat chunks in restructured products (e.g., boneless ham and turkey rolls) is largely responsible for the product integrity and sliceability.

Theoretically, all the three muscle protein fractions are capable of forming a gel. Practically, however, sarcoplasmic and connective tissue (stromal) proteins play only a small role in the overall gelation phenomenon in muscle foods. In processed meats, where 2–3% salt is typically added, most of the sarcoplasmic proteins are readily coagulated when the meat is cooked to 40–60 ºC and they do not form a gel (Macfarlane *et al*., 1977). In fact, when the sarcoplasmic protein extract is mixed into salted myofibril suspensions, it has a tendency to interfere with myofibril gelation. Partially hydrolyzed collagen (gelatin) is the best known gelling protein and its gelation is relatively insensitive to ionic strength. Gelatin forms reversible, cold-set gels, which are stabilized by hydrogen bonds. However, dissociation and degradation of collagen into soluble gelatin (the gelling component) requires moist, prolonged heating, a condition that is not employed in the production of most meat products. Sometimes, connective tissues from animal skins are frozen, powdered, and then incorporated into meat to modify the product texture and increase its protein content. Overall, both sarcoplasmic and stromal proteins contribute insignificantly to gelation and meat binding in muscle foods.

On the other hand, myofibrillar proteins, a superior gelling material as a whole, have a vital role in producing desirable textural characteristics in processed muscle foods. In particular, myosin (prerigor) or actomyosin (postrigor) accounts for most of the gel-forming capacity of the myofibril protein system (Asghar *et al*., 1985). In order to form a gel, myofibrillar proteins must first be extracted, and this is usually initiated by mixing with salt (NaCl or KCl) and phosphates. The gelling properties of myofibrillar proteins are influenced by the proteins' structure and size, their concentration, the source or type of meat, and the various processing conditions such as pH, ionic strength, and heating rate. Thus, myosin, which has a large length-to-diameter ratio (approximately 100 nm in length and 1.5–2 nm in diameter), can form a highly viscoelastic gel, whereas actin, which is a globular protein of about one-tenth of the myosin size, is a poor candidate for gelation (Samejima *et al*., 1969). Nevertheless, extracted actin may reinforce myosin gels, depending on the specific soluble myosin-to-actin ratio (Asghar *et al*., 1985).

The ionic strength has a profound influence on myosin gelation. Under a typical meat processing condition (with 2–3% or 0.5–0.6 M added NaCl, and up to 0.5% polyphosphate), myosin molecules in the thick filaments depolymerize into monomers. When the ionic strength is lowered to 0.1–0.3 M NaCl through dilution or dialysis, myosin molecules re-associate forming filaments. Therefore, myosin gels prepared at low salt concentrations are more rigid and translucent than those prepared at high salt concentrations (Egelandsdal *et al*., 1986; Hermansson *et al*., 1986). Because of the high salt concentration effect as well as the ATP-like function of pyrophosphate and tripolyphosphate, it is believed that myosin monomers, instead of the actomyosin complex or myosin filaments, are prevalent in the brine (protein extract) of salted raw meat.

Myosin exists in various isoforms that are specific to muscle or fiber types, i.e., fast-twitch oxidative glycolytic white (type IIA), fast-twitch glycolytic white (type IIB), slow-twitch oxidative red (type I), and a number of 'intermediate' types. These fiber-type-dependent myosin variants have different morphologies and produce gels of different rigidities (Xiong, 1994). For example, chicken pectoralis major consists exclusively of white fibers, and its myosin or mixed myofibrillar proteins can form a gel several-fold stronger than that of chicken gastrocnemius (preponderantly red fibers) at pH around 6.0 (Xiong and Blanchard, 1994; Liu and Foegeding, 1996). Similar findings have been made in mammalian species, such as pork (Robe and Xiong, 1993) and beef (Vega-Warner *et al*., 1999). A microscopic examination shows that white fiber myosin has a tendency to cross-link, forming long filaments, while red fiber myosin produces principally short aggregates (Morita *et al*., 1987). As the consumer demands for 'lighter' meat are high, the meat animal industry is breeding animals with increased white fibers. A good understanding of the gelation properties of myosin from different fiber types would help the development of specific processing strategies for different muscles or types.

The gelling system in many processed meats, especially finely comminuted products, is quite complex. For example, the protein extract alone is a heterogeneous unit that encompasses various soluble proteins (myosin, actin, actomyosin, etc.), myofibrils, and collagen fragments that are suspended in the viscous tacky solution. Therefore, upon cooking, the protein mixture will form a composite gel matrix. Moreover, if a meat batter is emulsified as in the case of frankfurters, fat globules (which can be viewed as 'hydrophilic' particles due to the polar protein membrane coating) will be readily imbedded into the gel, thereby producing a complex gel product.

As is for myosin gelation, the formation of an adhesive gel by mixed myofibrillar proteins in meat processing is induced by heat. Characteristics of the gels depend on the protein source (species and fiber types) and are affected by a range of processing factors. In general, gel strength of myofibrillar proteins increases with protein concentration and temperature of heating up to 65–70 ºC. Maximum gel strengths are typically obtained at pH around 6.0 although the specific pH requirement may differ between muscle fiber types and animal species (Xiong, 1997). The superior gelling capability of mixed myofibrillar proteins extracted from white fibers over those obtained from red fibers, especially at relatively low protein concentrations (<3%), has been demonstrated. Figure 5.3 shows typical dynamic rheograms of salt-extracted white and red myofibrillar proteins when subjected to a thermal process. The protein extracts from chicken white fiber muscles (pectoralis major and minor) form more elastic and rigid gels than those from predominantly red fiber muscles (drumstick and thigh). The transition in the storage modulus (G′) for both muscle fiber types at about 52 ºC results from major structural changes in myosin globular head and is indicative of rearrangements of protein networks that may involve actin detachment from myosin and, hence, a shift in elasticity of the gel (Xiong, 1994). The rapid ascending after 55 ºC probably reflects the

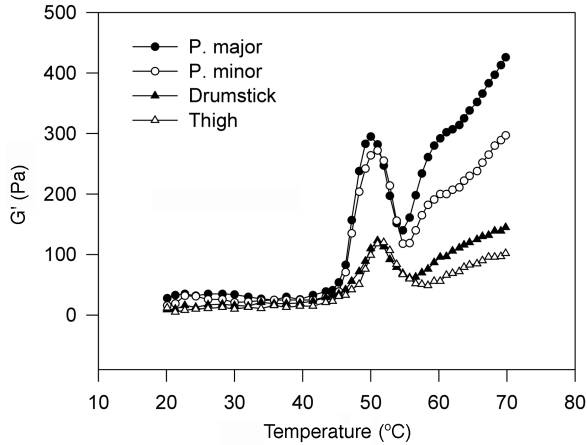

Fig. 5.3 Rheograms of salt-soluble myofibrillar protein mixtures extracted from chicken white (P. major and minor) and red (drumstick and thigh) muscles. Gelation condition: 20 mg/mL protein, 0.6 M NaCl, pH 6.0, 1 ºC/min heating rate.

formation of permanent cross-links and aggregates involving both the hydrophobic head and the hydrophilic tail of myosin.

It has also been observed that rigidity of myofibril gels gradually increases during storage of the salt extract (in 0.6 M NaCl) at 2–5 ºC, probably due to an increased extraction of myosin (Xiong and Brekke, 1989). This 'cold-setting' phenomenon may explain the beneficial effect of 'pre-blending' of meat with salt before it is further processed, an operation that is often employed for improved meat and water binding. The effect of polyphosphates (pyrophosphate, tripolyphosphate, etc.) on myofibrillar protein gelation is controversial. While they can enhance protein extraction, they have the tendency to interfere with protein matrix formation, which is required for developing a cohesive and elastic gel for meat binding (O'Neill *et al*., 1993; Robe and Xiong, 1993; Torley and Young, 1995). Microbial transglutaminase, an enzyme that catalyzes acyl transfer reactions thereby cross-linking proteins through the glutamine-lysine bridge, has a remarkable effect on myofibrillar protein gelation. Incorporation of this enzyme into the gelling solution results in as much as ten fold increases in myofibril gel strength, and therefore appears to be an excellent food ingredient for use in meat and surimi processing where meat binding is of main importance (Kuraishi *et al*., 1997; Lee *et al*., 1997; Ramirez-Suarez and Xiong, 2003a).

5.3.2 Emulsification

Proteins are amphoteric molecules containing both hydrophobic and hydrophilic groups. With sufficient energy input, i.e., a mechanical process such as shear, large fat particles are chopped into fine granules. This process also allows protein molecules to adsorb at the fat-water interface where they align in such a

manner that the nonpolar groups are imbedded in fat (hydrophobic) while the polar groups extend into the aqueous phase, resulting in an overall reduction of free energy and an improved stability of the meat emulsion system. The relative emulsifying activity of muscle proteins follow the order of myosin > actomyosin > sarcoplasmic proteins > actin (Hegarty *et al*., 1963; Galluzzo and Regenstein, 1978). The excellent emulsifying capability of myosin is attributed to its unique structure, namely, its high length-to-diameter ratio and perhaps more importantly, its bipolar structural arrangement with a hydrophobic head interacting with fat and a hydrophilic tail interacting with water.

An electron microscopic examination shows that the fat globules in comminuted meats are dispersed in the continuous aqueous phase comprised of salt-soluble proteins, segments of fibers, myofibrils, connective tissue fibers, collagen fragments, and various ingredients suspended in water (Jones, 1984). It has been suggested that stability of meat emulsions is attained by two mechanisms: formation of protein coatings on fat particles to reduce the interfacial tension, and immobilization of the fat particles in protein matrices largely through physical entrapment (Gordon and Barbut, 1992). The physicochemical and rheological properties of the fat globule membrane and the continuous protein matrices are the determinants of the emulsion stability, and they are influenced by many factors, such as pH, viscosity of the aqueous phase, time and temperature of chopping, and the lean meat-to-fat ratio.

Meat processors use different food ingredients to modify the composition and properties of both the fat globule membrane and the continuous aqueous phase to obtain a high emulsion stability against coalescence at high cooking temperatures. For example, addition of preheated and hydrolyzed soy proteins may help stabilize the emulsion system by reinforcing the interfacial membrane and the protein matrix (Feng *et al*., 2003). Mixed myofibril-whey or myofibril-soy proteins exhibit good emulsifying ability but the emulsion is not stable under normal meat cooking conditions (70–75 ºC). However, stability of the cooked product is rather high when microbial transglutaminase (0.1%) is incorporated into the emulsion prior to cooking, because it enables the amorphous emulsion to transform into a stable gel matrix system in which the fat globules are imbedded (Ramirez-Suarez and Xiong, 2003b). It appears that the enzyme effects by means of promoting the interaction and cross-linking of membranes of fat particles as well as the interaction of the fat globules with the continuous protein matrix.

5.3.3 Water-holding

The ability to bind, immobilize and retain water in cooked meat and meat products is another key functionality of muscle proteins. The amount of moisture present in cooked meats determines the product juiciness and influences its tenderness. Water accounts for about 75% of the fresh meat weight, and in injected or pumped ('enhanced') meat, the water content can be as high as 85%. Water in meat is either bound or in a free state. Bound water is

tightly associated with proteins through hydrogen bonds, which is influenced by the surface charge and polarity of the proteins. Free water, on the other hand, is held via capillary forces in different compartments of the muscle tissue, e.g., in the spaces between myofilaments, between myofibrils, and outside the fibers. This form of water makes up the bulk of the water in meat (70–90%). In comminuted meats, a large portion of water is also retained via entrapment in the matrix of myofibrillar protein gels. Denaturing conditions, such as frozen storage, oxidation, and fast acid accumulation (glycolysis) while the muscle temperature remains high, lead to reduced water-binding in meat. A common case of poor water-binding meat relevant to acid-induced protein denaturation is pale, soft, exudative (PSE) pork, turkey, and chicken.

Because most of the water in muscle is confined within the myofibrils in the spaces between the thick (myosin) and thin (actin) filaments, any chemical, physical, or enzymatic means that increases the interfilamental spaces can bring about an enhanced water-binding potential in intact meat and entrapment of extraneous water in processed meat. Thus, high concentrations of monovalent salts (NaCl or KCl), usually in the form of a brine, is commonly incorporated into meat by marination through blending or injection. Hydration and retention of added water are made possible through the NaCl-induced myofibril expansion due to increased electrostatic repulsion, thus, transverse swelling (Offer and Trinick, 1983; Hamm, 1986). A variety of phosphate compounds, such as sodium pyrophosphate, sodium tripolyphosphate, and sodium hexametaphosphate, are used in conjunction with salt to further improve the moisture-retention capability of meat. Injected loins and tenderloins, which have emerged as novelties in recent years in response to consumers' demand for convenient, flavor-enhanced foods in the U.S., usually contain both salt (0.5–2.0%) and phosphate (0.25–0.40%). When an alkaline phosphate is used, it confers an additional benefit by raising the meat pH from around 5.5–5.6 (which is close to the isoelectric point of actomyosin, pH ~ 5.0) to 5.8–6.0 where myosin and most other muscle proteins will bind water more strongly due to increased net charges. This would also allow the interfilamental spaces to further expand via electrostatic repulsions for additional water to be immobilized.

The mechanism for the NaCl and phosphate effects on meat hydration and water-holding has been studied. In addition to effecting electrostatic repulsions between adjacent myofilaments, high concentrations of NaCl (usually greater than 2.5% or 0.6 M), which impart high ionic strengths, are able to dissociate myosin filaments, thereby creating a bulky polypeptide matrix for moisture retention. Furthermore, NaCl at elevated concentrations (>2%) shifts the isoelectric point of myosin (pH ~ 5.0) to lower pH through a screening effect of Cl^- for positive charges in proteins (Hamm, 1986). As a result, myosin or actomyosin will carry more surface charges within the normal range of meat pH, thereby increasing inter-peptide repulsive forces to enable a stronger protein-water interaction and a greater water-retaining capacity of meat (Fig. 5.4). An augmented electric double layer surrounding the protein also allows for a more extensive myofibril hydration.

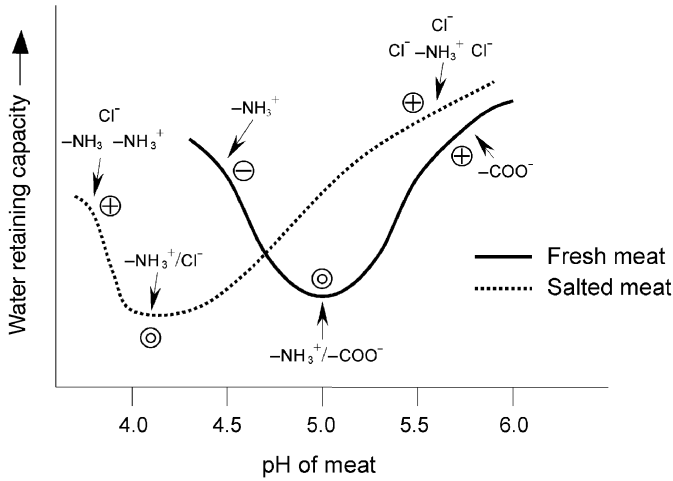

Fig. 5.4 Schematic representation of the relationship between pH and water immobilization in fresh and salted meat. The signs inside the open circles indicate protein net charges.

On the other hand, low concentrations of pyrophosphate and tripolyphosphate (<0.5% or 5–15 mM) are capable of dissociating actomyosin. In the presence of magnesium, the dissociation effect of pyrophosphate is very similar to that exerted by adenosine tripolyphosphate (ATP) (Granicher and Portzehl, 1964). These biochemical processes result in weakening of the myofibril structure, thereby allowing water molecules to more readily diffuse into the interfilamental spaces. Significant muscle fiber swelling occurs as the NaCl concentration is raised from 0.1 M to about 0.6 M in the absence of phosphate, or to 0.4 M in the presence of phosphate (Xiong *et al*., 1999). The extent of swelling and hydration continues until 1.0 M NaCl (approximately 4.0% of muscle weight) is incorporated where the swollen fiber will start to shrink due a salt-out effect (Parsons and Knight, 1990).

The NaCl- and phosphate-mediated hydration and moisture retention in meat are accompanied by partial extraction of myofibrillar proteins. Selective extraction of proteins from the myofibril backbone may be necessary in order to create a sufficient space for water to diffuse into the protein matrix. Indeed, phase contrast microscopy reveals a simultaneous myofibril 'swelling' and removal of proteins from the thick filaments (myosin) in the 0.6–1.0 M NaCl solution (Offer and Trinick, 1983; Xiong *et al*., 2000). The addition of 10 mM pyrophosphate or tripolyphosphate greatly facilitates the hydration process, and causes myosin to be extracted from the ends of the A-band where the amount of myosin-actin cross-linking is maximal. Moreover, certain transverse structural polypeptides that play a role in maintaining the myofibril integrity, e.g., M-protein, X-protein, and C-protein, may serve as a structural constraint to myosin extraction. The removal of the structural components seems to

correlate with the ultrastructural changes in irrigated myofibrils (Xiong *et al*., 2000).

5.4 Prepared muscle proteins as functional ingredients

5.4.1 Surimi protein

Surimi is a crude myofibrillar protein concentrate prepared by washing minced, mechanically deboned fish muscle to remove sarcoplasmic constituents and fat, followed by mixing with cryoprotectants (usually polyols) to prevent protein denaturation during frozen storage (Fig. 5.5). Surimi is considered to be an 'intermediate' product because it is usually further processed to make various kamaboko products and seafood analogs, such as imitation crab meat and lobster. For economic reasons, surimi is mostly prepared from under-utilized marine fish, such as Alaska pollock, Pacific whiting, mackerel, threadfin bream, croaker, bigeye snapper, and sardine (Morrissey and Tan, 2000). A main quality concern with some of the fish species is that they contain endogenous proteases that are detrimental to the texture of surimi-based products. For example,

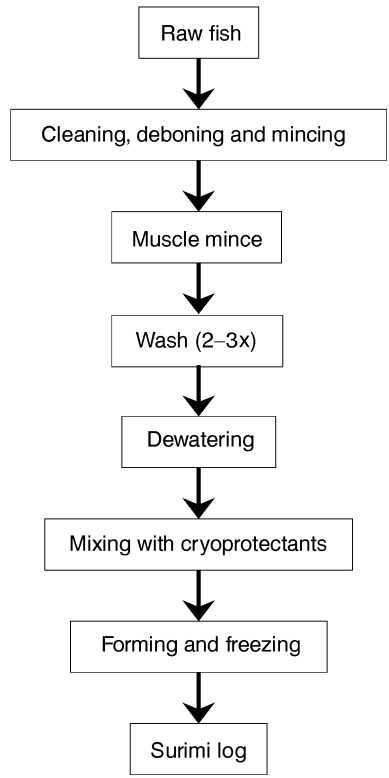

Fig. 5.5 Flow diagram for traditional surimi processing.

cathepsins B, L and an L-like protease are difficult to remove completely by the washing process, and their presence causes degradation of actomyosin, thereby impairing the gelation of surimi protein during the manufacture of seafood analogs (An *et al*., 1994; Jiang and Chen, 1999). This gel-weakening phenomenon, referred to as 'Modori', occurs in the 45–55 ºC temperature range where the enzymes have a high activity. To overcome this problem, different protease inhibitors, e.g., beef plasma protein, egg white, and potato extract, are commonly incorporated into the surimi paste before the heating process is initiated. Because the majority of the catheptic enzyme superfamily, including cathepsins B, H, and L, are cysteine proteases, a specific cathepsin inhibitor, cyctatin, has been produced using recombinant technology. This protease inhibitor has been found highly effective in preventing surimi gel weakening during cooking (Chen *et al*., 2001; Jiang *et al*., 2002).

The rapid decline, due to over-catching, in the fish species traditionally used for surimi making has prompted a search for alternative species as muscle sources for surimi. For example, paddlefish, a freshwater species, seems to be an excellent species to meet the need. Surimi prepared from paddlefish meat forms gels with high elasticity and rigidity, and the gels are cohesive and therefore can be utilized as a binding material in other foods (Lou *et al*., 2000a,b). Surimi-like myofibrillar protein concentrates have also been prepared from land animals, such as mechanically separated chicken meat (Yang and Froning, 1992) and animal by-products (Wan *et al*., 1993; Parkington *et al*., 2000). The surimi-like protein produced from beef heart muscle is of high quality; it forms moderately firm gels and has a good water-binding ability especially in the presence of a small quantity of transglutaminase (Ramirez-Suarez and Xiong, 2003a). When formulated into restructured pork as a functional ingredient, beef heart surimi exerts a strong cohesive force to bind meat pieces together (Xiong *et al*., 1993).

Recently, an alternative approach to the traditional surimi preparation method has been developed. Unlike the traditional surimi process, which employs repeated washing of the minced fish muscle tissue to concentrate myofibrillar protein, the new method involves either acid (pH 2.5–3.5) or alkaline (pH 9–10) solubilization of both myofibrillar and sarcoplasmic proteins from homogenized muscle tissue and then recovery of the soluble proteins through isoelectric precipitation (pH 5.0–5.5) (Hultin and Kelleher, 2000). This technique has the main advantage of high product yields (protein recovery >90% compared to 55–65% from the traditional washing method). The acid solubilization-isoelectric precipitation method is particularly suitable for dark-muscle and fatty fish; it may also be of potential application for the preparation of functional ingredients from under-utilized terrestrial animal species or meats, especially animal by-product meats.

5.4.2 Collagen protein

Collagen can be obtained from animal skins, hides, bone extracts, offal meats, and skeletal muscle. Native collagen tissue from the animal carcasses is of little

usefulness in food. However, by means of denaturation followed by partial hydrolysis, collagen can be converted into a highly functional protein ingredient known as gelatin. Gelatin derived from young collagen, such as the skin of the pig, chicken, and some fish, is termed 'Type A gelatin' and is suitable for a broad range of food applications. A mild acid treatment is generally sufficient to extract gelatin from the collagen material. In contrast, gelatin extracted from mature (highly cross-linked) collagen sources, such as cattle hides, bones and cartilage, is called 'Type B gelatin', and it is of limited use in food but more in nonfood applications. This type of gelatin requires more vigorous treatments for solubility, e.g., pretreated in an alkali-lime solution for a few days. Type A gelatin has a high isoelectric point (pH 6–9) and thus carries a net positive charge in most food uses, whereas Type B gelatin has an isoelectric point close to pH 5.0 and thus carries a net negative or positive charge depending on the acidity of the food (Stainsby, 1987). Chemically, skin contains a mixture of types I and III collagen, while bone and cartilage have a preponderance in type I and III collagen, respectively (Weiss, 1984).

The relative independence of pH (4–9) for gelation makes gelatin particularly useful for a broad range of applications. Yet, the single largest food application of gelatin is cold desserts because of the unique cold-set gelling and melt-in-mouth property of the protein. A typical gelation process will involve heating the gelatin solution to pass its denaturation point and then cooling rapidly to allow renaturation and interaction of gelatin molecules to form a protein gel matrix. Because the hydrogen bond is the predominant force that stabilizes the gel and no inter-peptide covalent linkages are present, the gelatin gel is thermo-reversible and readily melts at body temperature. Rapid gel setting is essential to immobilization and an even distribution of fruit bits and other particles in the gel matrix. Gelatin is also used in frozen dairy products, such as frozen desserts, to inhibit ice crystal growth and recrystallization (graininess) as well as lactose recrystallization (sandiness) during frozen storage (Jones, 1977). In confections, gelatin is used in soft candies, such as Gummy Bears, to stabilize sucrose, corn syrup, and other ingredients through its gelling property.

In meat products, collagen is used to make edible casing and as an ingredient in sausage products to increase the product protein content. For skeletal muscle collagen, it is concentrated by mechanical desinewing or by partial defatting through a low-temperature rendering process. Collagen added to emulsified meats, such as turkey and beef frankfurters, improves the raw meat emulsion stability. However, upon cooking, significant product shrinkage due to loss of water and collapse of emulsified fat droplets can occur, resulting in an increased meat toughness (Gillett, 1987). This is because collagen denaturation and ensuing abrupt shrinkage occur at around the final cooking temperature (65–75 ºC) (Davey and Gilbert, 1974), which disrupts the fat globule membrane (where collagen serves as a constituent) as well as the gel matrix made of myosin and suspended collagen fibrils.

To improve the functional performance of collagen in muscle foods, native collagen can be subjected to pretreatment to increase its solubility and eliminate

cook-induced shrinkage. Heat-predenatured collagen, when added at a level of no more than 10% of the product weight, was found to improve bind strength and juiciness in restructured low-fat, low-salt beef (Kenney *et al*., 1992). Recent industrial developments include low-temperature processing to make gelatin-rich protein. Extracted crude collagen from pork trimmings and poultry (chicken and turkey) skins can form a firm, elastic cold-set gel when formulated into emulsified muscle foods. The gel serves as a matrix for fat entrapment and water immobilization as well as for adhesion of meat particles, and is used in frankfurters, bologna, chicken nuggets, and boneless hams (Prabhu, 2003). With a careful control of time and temperature of moist heating and limited enzyme hydrolysis, it is now possible to make gelatin that remains liquid at room temperature, which can be pumped into restructured meat to bind water and to form a cohesive gel when chilled to the refrigerator temperature (Prabhu, Pers. Comm.).

5.4.3 Plasma protein

Plasma constitutes about two-thirds of the weight of blood and can be separated from red cells by centrifugation. The liquid plasma is then filtered and spray dried. Beef plasma, which contains about 70% protein, is widely used in the meat industry for its high solubility and excellent gelling property. The latter is attributed to the presence of fibrinogen (~5%), which is a superior gelling protein (Foegeding *et al*., 1986), and albumin (~65%). Plasma protein can be dissolved in brine and injected into meat. It will form an elastic, irreversible gel when cooked to above 65 ºC, and therefore is suitable for a variety of sectioned, formed, and restructured meats that require a strong bind at the junction of meat chunks and particles. Another important application of beef plasma, as aforementioned, is in surimi products. Beef plasma exhibits a remarkable capability to inhibit Modori, i.e., gel weakening, during cooking of surimi prepared from some fish species or animal by-product meats. Electrophoretic analysis has shown that myosin degradation, which occurs in control surimi samples heated to above 45 ºC, is prevented when as little as 0.5% beef plasma powder is added (Lou *et al*., 2000b; Wang *et al*., 2001). This strongly suggests that beef plasma contains protease inhibitors, although the nature of such inhibitor(s) has not been elucidated.

5.5 Future trends

Muscle proteins play a vital role in the quality of fresh and processed muscle foods. Solubility (protein extraction), water-binding ability, emulsifying capacity, and gelation are the most important functional properties exerted by muscle proteins during meat processing, and they are responsible for the texture and water-holding capacity of cooked products. A quick survey indicates that muscle foods – even the same type processed under similar conditions – often

vary in textural characteristics intentionally or unintentionally. Thus, future research and product development will be likely to continue focusing on the manipulation and control of processing factors (nonmeat ingredients, pH, temperature, etc.) to produce products that meet all the specifications. Because low-fat, convenience meat products are greatly desired by consumers, isolated meat proteins as water- and meat-binding ingredients (e.g., pre-treated collagen and protein from low-value meats) will be likely to have an expanding market. Furthermore, the meat consumption trend in the past decade shows a steadily increased demand for poultry and light meat and further processed meat products. Functional protein ingredients derived from poultry species may therefore be of particular importance in all-poultry products in the near future.

5.6 Sources of further information and advice

The following selected books contain excellent treatments of the subject matter. By no means are they a complete resource including all the detailed aspects discussed in this chapter. For the latest information, the readers are encouraged to consult other scientific literature available at the time of publication of this book. Additional sources of information about meat proteins and their food applications can be found in the 'References' section following. Of particular usefulness, perhaps, are several comprehensive review articles that address various intrinsic and extrinsic (processing) factors that can be manipulated to enhance the extraction and performance of myofibrillar proteins as functional protein ingredients *in situ*, for example, Hamm (1986), Asghar *et al.* (1985), and Xiong (1994, 1997).

- *Muscle and Meat Biochemistry*, edited by Pearson and Young, 1989, Academic Press, Inc., San Diego, CA, p. 457. This book presents an in-depth coverage of basic meat science and muscle protein biochemistry. It contains a comprehensive discussion of all the major and numerous minor myofibrillar, sarcoplasmic and collagen proteins. Hence, it serves as an excellent information source in this subject area.
- *Meat Science*, 6th edition, edited by R.A. Lawrie, 1998, Woodhead Publishing, Ltd., Cambridge, UK, p. 336. This is perhaps the best-known meat science source book because of its long period of availability (first edition was printed in 1966). This latest revision by Professor Lawrie reviews a broad range of processing factors that affect meat quality and it discusses the role of muscle proteins and enzymes involved in meat quality and palatability. A unique aspect of the book is the inclusion of meat science and protein research that has evolved over half a century.
- *Advances in Meat Research, Vol 4, Collagen as a Food*, edited by A.M. Pearson, T.R. Dutson, and A.J. Bailey, 1985, Van Nostrand Reinhold Co, New York, p. 396. This volume delivers a comprehensive and thorough discussion of collagen as an important food protein. The topics range from

the synthesis of collagen, its basic structure and genetic types, the molecular mechanism of collagen cross-linking and how this influences meat texture and tenderness, thermal and chemical properties of collagen, and applications of collagen proteins. Although the book was published almost two decades ago, the basic science and fundamental principle of collagen presented in the book remain current.

- *Surimi and Surimi Seafood*, edited by J.W. Park, 2000, Marcel Dekker, Inc., New York, p. 500. This is a very useful book for researchers and industry personnel who work with surimi and surimi products. Its coverage includes the latest surimi processing technologies, the chemistry and enzymology of surimi proteins, and interactions between surimi proteins and nonprotein ingredients.
- *Quality Attributes of Muscle Foods*, edited by Y.L. Xiong, C.T. Ho, and F. Shahidi, 1999, Kluwer Academic/Plenum Publishers, New York, p. 433. This volume, based on a research symposium, presents a comprehensive, contemporary review of muscle food quality and stability by a group of more than 30 of the world's leading meat scientists. Particular emphasis is placed on the physicochemical and biochemical characteristics and reactions of muscle food constituents, including the relationship between muscle protein biochemistry and fresh meat quality, and that of muscle protein functionality and processed meat quality.

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6

Soy proteins

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6.1 Introduction

For more than 2,000 years people throughout East Asia have consumed soybeans in the form of traditional soy foods, such as nimame (cooked whole soy), edamame (green fresh soy) (Fukushima, 2000a), soy milk (Fukushima, 1994), tofu (Fukushima, 1981), kori-tofu (freeze-denatured and dry tofu) (Fukushima, 1980 and 1994), abura-age (deep-fat-fried tofu) (Fukushima, 1981), sufu or tofu-yo (fermented tofu) (Fukushima, 1981 and 1985), soy sauce (Fukushima, 1985 and 1989), miso (Fukushima, 1985), natto (Fukushima, 1985), tempeh (Fukushima and Hashimoto, 1980), etc. In Western countries, soybeans had attracted people's attention in the 1960s as an economical and high-quality vegetable protein source for humans. In the United States, new soy protein products were developed, such as soy flour, soy protein concentrates, soy protein isolates, and their texturized products. These soy products were introduced into Japan at the end of the 1960s, but their consumption remains only 40,000 metric tons as products. The major methods of consumption of soybeans in Japan are traditional, for which about one million metric tons of soybeans and soybean meal are used, as shown in Table 6.1. The manufacturing techniques and equipment for these traditional soy foods had made great progress through the technical innovations following World War II and the modernization of the manufacturing process had almost been achieved, by the end of 1980.

In Western countries, the history of soybeans for human consumption covers only several decades, where the non-traditional protein products described above are mainly used as ingredients in formulated foods for their functional properties, such as water and fat absorption, emulsification, foaming, gelation,

Table 6.1 Consumption of traditional soy food products in Japan

	Soybeans[(1)]	Soybean meal[(2)]	Total
Tofu and its derivatives	496,000	0	496,000
Kori-tofu	28,000	0	28,000
Natto	128,000	0	128,000
Miso	162,000	0	162,000
Soy sauce	26,300	157,600	183,900
Soy milk	4,200	0	4,200
Major traditional products (Total above)	844,500	157,600	1,002,100
Non-traditional products (Soy proteins)		4,000 (as product)	4,000 (as product)
Food use total	1,032,000	401,000[(3)]	1,433,000[(3)]

[(1)] Shokuhin Sangyou Shinbunsha. [(2)] Ministry of Agriculture, Forestry, and Fisheries. [(3)] Including non-food meal other than feeds.

binding, etc. These soy foods have penetrated steadily into Western countries as healthy foods, but the growth is not so high as they expected, perhaps owing to the strong off-flavors associated with the products. However, the consumption of soy foods in the United States has begun to increase abruptly with 1997 as a turning point (Liu, 2000). It is obvious that this increase is due to the realization of the physiological properties which soybeans possess. Numerous investigations during the 1990s put soybeans in the spotlight, where soybean storage proteins and soybean minor components traditionally considered to be antinutritional factors have been recognized to have exciting roles in the prevention of chronic disease. Furthermore, the FDA confirmed the 'Soy Protein Health Claim' on 26 October, 1999, that 25 grams of soy protein a day may reduce the risk of heart disease. The market is very much responsive to this Health Claim. Therefore, taking this opportunity, soy foods will penetrate rapidly into Western cultures and diets.

In the processing and utilization of soybeans, the following four points are very important. One is the nutritional and physiological aspects, second is the functional properties working physicochemically in food systems, third is the unfavorable substances such as off-flavors, allergens, etc., and fourth is the creation of the beneficial cultivars. This chapter deals with the molecular structures and physicochemical functions of soybean storage proteins, the reevaluation of the nutritive value of soy proteins, the physiological effects of soy proteins and their fragments, the allergenic proteins in soybeans and the genetic improvements of soybean storage proteins, etc.

6.2 Soybean storage proteins: structure-function relationship of β-conglycinin and glycinin

Approximately 90% of the proteins in soybeans exist as storage proteins, which mostly consist of β-conglycinin and glycinin. β-Conglycinin (Koshiyama, 1965; Catsimpoolas and Ekenstam, 1969; and Koshiyama and Fukushima, 1976a) has the sedimentation coefficients (SC) of 7S, whereas glycinin (Mitsuda *et al*., 1965) has 11S. There are two kinds of globulins having the SC of 7S other than β-conglycinin, namely γ-conglycinin (Catsimpoolas and Ekenstam, 1969; Koshiyama and Fukushima, 1976b) and basic 7S globulin (Yamauchi *et al*., 1984). However, these two 7S globulins are minor components which account for less than a few percent. The major storage proteins, namely, β-conglycinin and glycinin, possess a variety of functional properties physicochemically for food applications as shown in the introduction. These functional properties are ascribed to the intrinsic physicochemical characteristics which are based on the molecular structures. Therefore, this section focuses on recent developments in the structure-function relationship of β-conglycinin and glycinin.

6.2.1 Basic structures of β-conglycinin and glycinin

β-Conglycinin is a glycoprotein and a trimer which consists of three subunits with a molecular mass of 150–200 kDa. Major subunits are α', α, and β and their molecular weights are 72, 68, and 52 kDa, respectively (Thanh and Shibasaki, 1977). Besides these, there is a minor subunit called γ in β-conglycinin (Thanh and Shibasaki, 1977). The amino acid sequences of these subunits are similar each other (Hirano *et al*., 1987). Each of α' and α subunits possesses one cysteine residue (-SH) near the N-termini, whereas β subunit does not possess any cysteine residue (Utsumi *et al*., 1997). No cystine residues (-SS-) exist in these subunits. β-Conglycinin exhibits molecular heterogeneity, where six molecular species are identified as $\alpha'\beta_2$, $\alpha\beta_2$, $\alpha\alpha'\beta$, $\alpha_2\beta$, $\alpha_2\alpha'$, and α_3 (Thanh and Shibasaki, 1978; Yamauchi *et al*., 1981). In addition, Yamauchi *et al.* (1981) found another species of β_3. β-Conglycinin trimers cause association or dissociation depending upon the pH and ionic strength of the solution (Thanh and Shibasaki, 1979).

Glycinin is a hexamer with a molecular mass of 300–380 kDa. Each subunit is composed of acidic (~35 kDa) and basic (~20 kDa) polypeptides, which are linked together by a disulfide bond (Staswick *et al*., 1984). In glycinin, five subunits are identified as $A_{1a}B_{1b}$ (53.6 kDa), A_2B_{1a} (52.4 kDa), $A_{1b}B_2$ (52.2 kDa), $A_5A_4B_3$ (61.2 kDa) and A_3B_4 (55.4 kDa), which are classified into group I ($A_{1a}B_{1b}$, A_2B_{1a} , $A_{1b}B_2$) and group II ($A_5A_4B_3$, A_3B_4) by the extent of the homology (Nielsen, 1985; Nielsen *et al*., 1989). Each subunit in group I has two cysteine and three cystine residues, whereas each subunit in group II has two cysteine and two cystine residues (Utsumi *et al*., 1997). Glycinin subunits exhibit polymorphism, that is, there are some amino acid replacements in the same kind of subunit among soybean cultivars (Mori *et al*., 1981; Utsumi *et al.*,

1987). Moreover, glycinin exhibits molecular heterogeneity, because the molecule is a hexamer with a different subunit composition (Utsumi *et al*., 1981). Glycinin hexamers dissociate to their constituent polypeptides, subunits, and half-molecules, depending upon pH, ionic strength, and heating temperature (Wolf and Briggs, 1958; Mori *et al*., 1982).

6.2.2 The physicochemical properties of β-conglycinin, glycinin, and their subunits

In Table 6.2 are shown the properties of β-conglycinin, glycinin, and their subunits on the gel formation, thermal stability, and emulsification, (Utsumi *et al*., 1997). The mechanisms on the gel formation of β-conglycinin (Nakamura *et al*., 1986) and glycinin (Mori *et al*., 1982; Nakamura *et al*., 1984) are studied in detail. Glycinin forms a turbid, hard, and not inelastic gel, whereas β-conglycinin forms a transparent, soft, but rather elastic gel, in 100 ºC heating (Utsumi *et al*., 1997). The A_2 polypeptide of glycinin A_2B_{1a} subunit closely relates to gel turbidity, whereas the A_3 polypeptide of the A_3B_4 relates to the gel hardness. The hardness of glycinin gel increases in proportion to the content of A_3 polypeptide. The $A_5A_4B_3$ subunit relates to the easiness of gel formation, because of the easy cleavage of the hydrophobic bonds between the A_5 and A_4 acidic chains during heating. Further, the existence of A_4 acidic polypeptide raises the elasticity of the gel, and at the same time it makes the gel softer and more fragile (Lee *et al*., 2002). β-Conglycinin is more unstable thermally than glycinin, but the emulsifying and emulsion-stabilizing abilities of β-conglycinin are much stronger than those of glycinin.

Table 6.2 Functional properties of soybean storage proteins and their subunits working physicochemically in food systems[(1)]

Functionality	Proteins or subunits	Property or its difference
Gel formation	β-conglycinin	Transparent, soft, but rather elastic gel
	Glycinin	Turbid, hard, and not so fragile gel
	A_2B_{1a} subunit	A_2 polypeptide relates to gel hardness
	A_3B_4 subunit	A_3 polypeptide relates to gel hardness
	$A_5A_4B_3$ subunit	$A_5A_4B_3$ subunit relates to the easiness of gel formation
Thermal stability	Soybean storage proteins	β-conglycinin $<$ Glycinin
	β-conglycinin subunits	$\alpha < \alpha' < \beta$
Emulsification	Soybean storage proteins	β-conglycinin $>$ Glycinin
	β-conglycinin subunits	$\alpha \geq \alpha' \gg \beta$

[(1)]Utsumi *et al*., 1997.

Table 6.3 Number of cysteine and cystine in each subunit of β-conglycinin and glycinin[1]

	Subunit	Cysteine (-SH)	Cystine (-SS-)
	α'	1	0
β-conglycinin	α	1	0
	β	0	0
	$A_{1a}B_{1b}$	2	3
	A_2B_{1a}	2	3
Glycinin	$A_{1b}B_2$	2	3
	A_3B_4	2	2
	$A_5A_4B_3$	2	2

[1]Utsumi *et al*., 1997.

The physicochemical functions of proteins depend upon their three-dimensional structures substantially. The polypeptide chains of the protein molecules are unfolded through the heat treatment of soybeans and as a result the amino acid side residues buried inside a molecule are exposed on the surface. The exposed -SH or hydrophobic residues combine the protein molecules through -SH, -SS- interchange reaction or hydrophobic bonding, respectively. In this case, it is very important that these active residues are present at an accessible location of the molecules. Table 6.3 shows the numbers of -SH and -SS- groups in each subunit. The larger numbers of SH groups and their topology in glycinin make glycinin gel much harder and more turbid in comparison with β-conglycinin gel, whereas the higher hydrophobicity and more easily unfolded structure in β-conglycinin make its emulsifying ability much stronger than that of glycinin (Utsumi *et al*., 1997).

6.2.3 The three-dimensional structures of β-conglycinin and glycinin

In order to improve these functional properties, it is necessary to know the theoretical relations between the functional properties and the three-dimensional structures of the molecules. The research on the three-dimensional structures of soybean storage proteins started 35 years ago. We investigated the three-dimensional structures of β-conglycinin and glycinin molecules through optical rotatory dispersion (ORD), circular dichroism (CD), infra-red absorption spectra, ultraviolet difference spectra, deutration studies, and so on (Fukushima, 1965, 1967, and 1968). However, the results obtained by these methods are indirect ones. For a direct and complete analysis of three-dimensional structures, soybean proteins must be crystallized, followed by X-ray analysis.

The complete amino acid sequence of molecular subunits of soybean storage proteins was determined in the early 1980s through the sequence analysis of full-length cDNA and a genomic clone (see the review of Fukushima, 1988, 1991a, and 1991b). For a long time, however, X-ray analysis of soybean proteins has not been

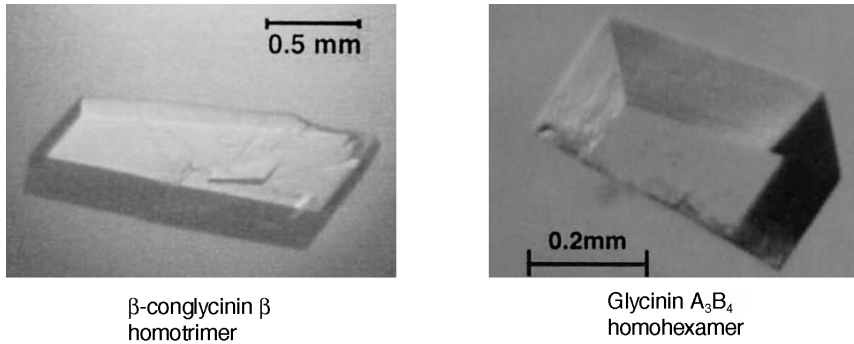

Fig. 6.1 The crystals of (a) β-conglycinin β homotrimer and (b) glycinin A_3B_4 homohexamer (Courtesy of Dr S. Utsumi).

carried out, because the molecular heterogeneities in both β-conglycinin and glycinin obstructed their crystallization. Utsumi's group has overcome these difficulties by using a special soybean variety, in which β-conglycinin molecules or glycinin molecules are composed of the same kinds of subunits. In the crystallization of β-conglycinin they used the soybean variety, of which β-conglycinin is composed only of β homotrimer, that is, 3β, whereas in the crystallization of glycinin, they used the variety of which glycinin is composed only of A_3B_4 homohexamer, that is $6A_3B_4$. Thus, they have succeeded in the crystallization and subsequent complete analysis of the three-dimensional molecular structures of both β-conglycinin and glycinin, as shown in Figs. 6.1, 6.2, and 6.3 (Maruyama *et al*., 1999; Adachi *et al*., 1999; Fukushima, 2000b and 2001; Adachi *et al.*, 2003).

This success should be recognized as epoch-making in the basic research of soybean proteins, because most of the properties of proteins are ascribed to the conformation of the molecular surface in the three-dimensional structures of the molecules. Furthermore, the elucidation of the detailed three-dimensional

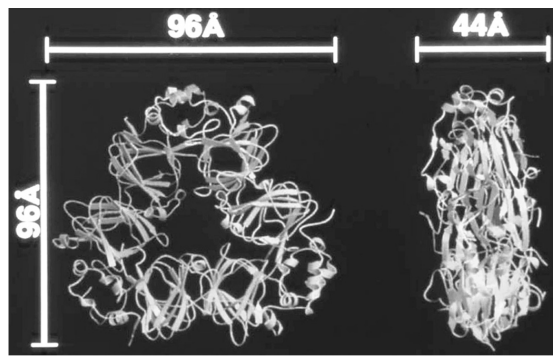

Fig. 6.2 Three-dimensional molecular structures of β-conglycinin β homotrimer (Courtesy of Dr S. Utsumi).

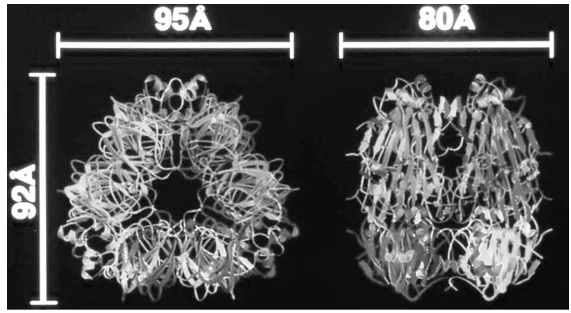

Fig. 6.3 Three-dimensional molecular structures of glycinin A_3B_4 homohexamer (Courtesy of Dr S. Utsumi).

structures enables us to understand the theoretical modifications of the molecules, leading into the improvement of soybean protein properties in genetic level. Table 6.4 shows the comparison between the X-ray data of Utsumi's group (Fukushima, 2000b and 2001) and our ORC and CD data (Fukushima, 1965, 1967, and 1968; Koshiyama and Fukushima, 1973) on the per cent of the secondary structures. It is very interesting that the results of X-ray analysis are in good accordance with the results of our indirect CD method around 30 years ago.

6.3 Soy protein as a food ingredient: physicochemical properties and physiological functions

6.3.1 Physicochemical properties of soy protein

It is generally known that soy protein ingredients have appropriate functional properties for food applications and consumer acceptability. These functional properties are intrinsic physicochemical characteristics of soy protein, which affect the behavior of protein in food systems during preparation, processing, storage, and consumption. These properties are not only important in

Table 6.4 Contents of secondary structures contained in soybean storage protein molecules

	β-conglycinen		Glycinin	
	X-ray	CD[(1)]	X-ray	CD[(1)]
α-helix	10	5	8	5
β-structure	33	35	36	35
Disordered structure	57	60	56	60

[(1)]Circular dichroism.

determining the quality of the final product, but also in facilitating processing, for instance, improved machinability of cookie dough or slicing of processed meats. Physicochemical functions performed by soy protein preparations in actual food systems are solubility, water absorption and binding, viscosity, gelation, cohesion-adhesion, elasticity, emulsification, fat adsorption, flavor-binding, foaming, color control, etc.

As examples, gelation is important in comminuted meats, while emulsification and foaming are important in coffee creamers and dessert toppings, respectively. However, it should be noted that the physicochemical properties of the whole proteins contained in soybeans considerably differ depending upon soybean cultivars. As already described in section 6.2.2, the physicochemical properties of each component of soybean proteins are quite different not only between β-conglycinin and glycinin, but also among the subunits of β-conglycinin or glycinin molecule. Further, both the ratio of β-conglycinin to glycinin and the subunit compositions of β-conglycinin or glycinin molecules are fairly different among soybean cultivars. This is the reason why the physicochemical properties differ among soybean cultivars.

Lee *et al*. (2002) compared the physicochemical properties of the heat-induced gels among the glycinin preparations produced from seven different soybean cultivars. In Table 6.5 is shown the relationship between the contents of A_1, A_2, A_3, A_4, and A_5, the acidic polypeptides of glycinin subunits, and the physicochemical properties of the gels. There were many differences in the gel textures among soybean cultivars. The glycinin gels were divided into two groups. One is the group of Shirotsurunoko, Hill, and York, which contains A_4 polypeptide and the other is Raiden, Suzuyutaka, Matsuura, and Yamabe, which lacks A_4 polypeptide. The former showed lower compressibility (CM), higher cohesive property (LC), and two to three times greater resiliency (RS) than the latter, indicating that A_4 polypeptide raises the elasticity of the gels and makes them softer and more fracturable. Among the gels of these seven cultivars, the most fracturable gel was of Shirotsurunoko and the most unfracturable gel was of Yamabbe-A3. On the elasticity, the gel from Hill was the highest and that from Matsuura was the lowest. This indicates that the selection of the cultivars is important for the application of soybean proteins to food systems.

6.3.2 Reevaluation of nutritive value of soy protein

The quality of soybean proteins has actually been undervalued until recently, because the protein efficiency ratio was based upon the growth of laboratory rats. Growing rats not only possess a much higher requirement for proteins than infants, but also a much higher need for certain amino acids than humans (Steinke, 1979). Particularly, the rat requirement for methionine is about 50% higher (Sarwar *et al*., 1985). According to the Report of a Joint FAO/WHO/UNU Expert Consultation in 1985, the amino acid requirements are different depending upon human age and methionine is not a limiting amino acid for

Table 6.5 Relationships between the acidic polypeptide compositions and physicochemical properties of glycinin gels of seven soybean cultivars[1]

Cultivars	Acidic polypeptides				Mechanical parameters at rupture			
	A_1	A_2	A_3	A_4	F[2]	RS[3]	CM[4]	LC[5]
Shirotsurunoko	40.6	29.7	15.6	14.0	195.7	11.66	53.33	0.96
Hill	40.8	31.6	13.6	14.0	324.5	8.46	67.14	0.84
York	37.4	30.4	17.8	15.5	453.3	8.07	72.30	0.87
Raiden	42.7	32.0	25.3	Negl.[6]	410.3	3.74	83.33	0.67
Suzuyutaka	41.1	39.8	19.1	Negl.[6]	455.0	4.68	81.41	0.73
Matsuura	44.3	30.2	25.5	Negl.[6]	557.0	4.04	84.20	0.72
Yamabe-A3	40.0	34.4	25.7	Negl.[6]	685.3	3.85	88.10	0.63

[1]Lee *et al*. (2002). [2]Force. [3]Resiliency. [4]Compressibility. [5]Linearity of the compression process. [6]Negligible.

Table 6.6 Patterns of amino acid requirements and soybean amino acid composition[1]

Amino acid (mg/g protein)	Pattern of requirement				Amino a. comp. of soybeans
	3–4 Mo	2–5 Yr.	10–12 Yr.	Adult	
His	26	19	19	16	27
Ile	46	28	28	13	48
Leu	93	66	44	19	78
Lys	66	58	44	16	61
Met + Cys	42	25	22	17	26
Phe +Tyr	72	63	22	19	90
Thr	43	34	28	9	35
Trp	17	11	9	5	13
Val	55	35	25	13	48
Total (including His)	460	339	241	127	426
Total (minus His)	434	320	222	111	399

[1]Joint FAO/WHO/UNU Expert Consultation (1985).

soybean proteins, except in infants (see Table 6.6) (Fukushima, 1991a). Both the World Health Organization (WHO) and the United States Food and Drug Administration (FDA) adopted the protein digestibility corrected amino acid score (PDCAAS) method as the official assay for evaluating protein quality. Soybean proteins have a PDCAAS of 1.0, indicating that it is able to meet the protein needs of children and adults when consumed as the sole source of protein at the recommended level protein intake of 0.6g/kg body wt. (Young, 1991). It is now concluded that the quality of soybean proteins is comparable to that of animal protein sources such as milk and beef.

6.3.3 Physiological functions of soy protein

Formerly, soybean proteins had been considered to play a role only as traditional nutrients. In the latter half of the 1970s, however, it was found that soybean proteins have a hypocholesterolemic effect. As shown in Fig. 6.4 (Descovich *et al*., 1980), the serum cholesterol is lowered markedly when the animal proteins in the diet are exchanged with soybean proteins. Since then, numerous investigations on the hypocholesterolemic effect of soybean proteins have been carried out. According to a meta-analysis of 38 separate studies involving 743 subjects, the consumption of soy protein resulted in significant reduction in total cholesterol (9.3%), LDL cholesterol (12.9%), and triglycerides (10.5%), with a small but insignificant increase (2.4%) in HDL cholesterol (Anderson *et al*., 1995). In linear regression analysis, the threshold level of soy intake, at which the effects on blood lipids became significant, was 25g. Thus, soy protein represents a safe, viable, and practical nonpharmacologic approach to lowering cholesterol.

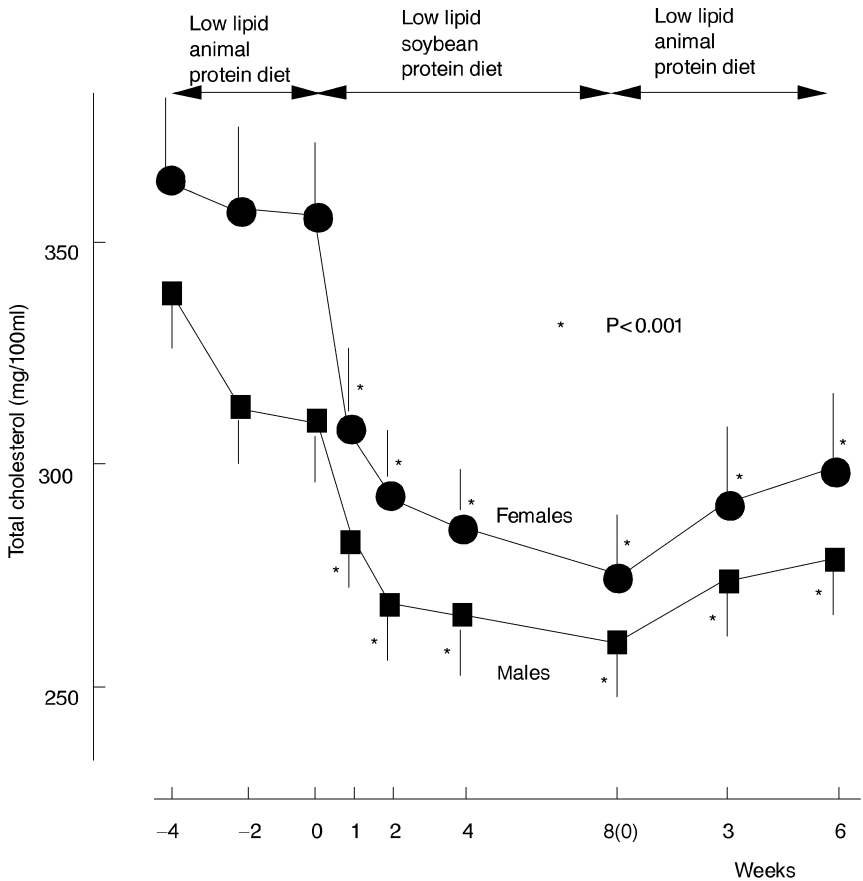

Fig. 6.4 Total cholesterol levels in type II patients treated with soy protein diets. Mark (*) indicates highly significant difference from mean plasma lipid levels during the term before soy protein diets. Reprinted with permission from Elsevier Science (*The Lancet*, 1980, No. 8197, 709–712 by Descovich *et al.*).

It is clear that soybean storage proteins possess the hypocholesterolemic effect in themselves, because the plasma total cholesterol of the rats fed casein-cholesterol diets was reduced by 35 and 34% by the administration of purely isolated β-conglycinin and glycinin, respectively (Lovati *et al.*, 1992). The exact mechanism of the cholesterol reduction has not been established fully. Some suggest that cholesterol absorption and/or bile acid reabsorption is impaired when soybean proteins are fed, while others propose that changes in endocrine status, such as alteration in insulin to glucagon ratio and thyroid hormone concentrations, are responsible (Potter, 1995).

In addition to the cholesterol-lowering effects described above, soybean proteins suppress the lipogenic enzyme gene expression in the livers of genetically fatty rats (Wistar fatty rats), indicating that dietary soybean proteins are useful for the reduction of body fats (Iritani *et al.*, 1996).

6.3.4 Physiologically active fragments derived from soybean storage protein molecules

In Table 6.7 are put together the physiological active peptide fragments derived from soybean proteins, which have hypocholesterolemic, anticarcinogenic, hypotensive, immunostimulating and/or antioxidant effects.

Iwami *et al*. (1986) found that the hydrophobic peptide fragments which appeared through the proteinase digestion of soybean proteins are responsible for their plasma cholesterol-lowering action. Since the hydrophobic peptides bound well to bile acids, the fecal excretion of bile acids is increased. Consequently, the bile acid synthesis in the liver is stimulated, resulting in the reduction of serum cholesterol. Soybean protein digests have the highest hydrophobicity among commonly used protein sources and give the lowest cholesterol level. Major hydrophobic peptides to bind to bile acids are A_{1a} and A_2, which are the acidic peptides of glycinin subunits, $A_{1a}B_{1b}$ and A_2B_{1a}, respectively (Minami *et al*., 1990). The region comprising residues 114–161 (48 amino acid residues) represents the most hydrophobic area of the A_{1a} subunit. This hydrophobic region is also highly conserved in the A_2 subunit.

Sugano *et al*. (1988) made the hydrophobic and high molecular weight fraction (HMF) resistant to microbial proteases, which exerts an extraordinarily strong hypocholesterolemic effect in rats through the increase of the fecal excretion of cholesterol and/or bile acids, compared to the parent soybean proteins. Most recently, however, Yoshikawa *et al*. (2000) found that Leu-Pro-

Table 6.7 Physiologically active peptide fragments from soybean proteins

Peptide fragments	Physiological activity	Protein source
High Molecular Weight Fraction[(1)]	Hypocholesterolemic and anticarcinogenic	Soybean proteins
LPYPR[(2)]	Hypocholesterolemic	Soybean glycinin
MLPSYSPY[(3)]	Anticarcinogenic	Soybean proteins
Peptide Fraction[(4)]	Hypotensive through ACE-inhibition	Soybean proteins
MITLAIPVNKPGR[(2)]	Phagocytosis-stimulating	β-conglycinin α'-subunit
MITLAIPVN[(2)]	Phagocytosis-stimulating	β-conglycinin α'-subunit
MITL[(2)]	Phagocytosis-stimulating and protection from hair loss	β-conglycinin α'-subunit
HCQRPR[(5)]	Phagocytosis-stimulating	Glycinin A_{1a}-subunit
QRPR[(5)]	Phagocytosis-stimulating	Glycinin A_{1a}-subunit
VNPHDHQN[(6)]	Antioxidant	β-conglycinin
LVNPHDHQN[(6)]	Antioxidant	β-conglycinin
LLPHH[(6)]	Antioxidant	β-conglycinin
LLPHHADADY[(6)]	Antioxidant	β-conglycinin
VIPAGYP[(6)]	Antioxidant	β-conglycinin
LQSGDALRVPSGTTYY[(6)]	Antioxidant	β-conglycinin

[(1)]Sugano *et al*., 1988. [(2)]Yoshikawa *et al*., 2000. [(3)]Kim *et al*., 2000. [(4)]Wu and Ding, 2000. [(5)]Yoshikawa *et al*., 1993. [(6)]Chen *et al*., 1995.

Tyr-Pro-Arg, the low molecular weight peptide fragment derived from soybean glycinin, also reduced serum cholesterol in mice after oral administration. This may be a different mechanism from that of HMF, because there was no increase in the excretion of the fecal cholesterol and bile acids.

It is known that bile acid is an intrinsic promoter of colon cancer. Azuma *et al*. (1999, 2000a, and 2000b) and Kanamoto *et al*. (2001) found that HMF described above suppresses the tumorigenesis in the liver and colon in rats through the inhibitory effect on the reabsorption of bile acids in the intestine. Kim *et al*. (2000) discovered that Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr, a low molecular weight peptide fragment derived from soybean proteins, has anticarcinogenic properties.

Using an *in vitro* assay exposes a variety of food-derived peptides which inhibit angiotensin-converting enzyme (ACE). *In vivo,* however, most of them did not show any antihypertensive effect. The peptides which showed a real antihypertensive effect *in vivo* are only Ile-Lys-Pro from bonito muscle (Yokoyama *et al*., 1992), Ile-Pro-Pro and Val-Pro-Pro from fermented milk (Nakamura *et al*., 1995), and Ile-Lys-Trp from chicken muscle (Fujita *et al*., 2000). Many of the reported peptides were mere substrates of ACE and they showed apparent inhibitory activity by competing with a synthetic substrate which was used for the enzyme assay. Using a strong acid cationic exchange resin, Wu and Ding (2001) separated the peptide fraction from the enzyme hydrolyzate of soybean proteins, which is composed of three peaks. The molecular weights and lengths of the peptides in this fraction were below 954 and between 2 and 8, respectively. This peptide fraction inhibited ACE and showed the hypotensive effect *in vivo*, when it was orally administrated on spontaneously hypertensive rats.

Immunostimulating peptides are expected to improve senile immuno-deficiency. Yoshikawa *et al*. (2000) isolated a peptide stimulating phagocytosis by human polymorphonuclear leukocytes from soybean proteins. It is Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg which was derived from the α' subunit of β-conglycinin and named soymetide. Soymetide-4, the tetrapeptide at the amino terminus, that is, Met-Ile- Thr-Leu, is the shortest peptide stimulating phagocytosis. Soymetide-9 (Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn) is the most active in stimulating phagocytosis *in vitro*. Besides these, soymetide-4 prevents hair loss induced by a cancer chemotherapy agent. The peptides derived from soybean glycinin A_{1a} subunit, Gln-Arg-Pro-Arg and His-Cys-Gln-Arg-Pro-Arg, also stimulated phagocytotic activity of human polymorphonuclear leukocytes, but their activities are weaker than those of soymetide described above (Yoshikawa *et al*., 1993).

Chen *et al*. (1995) isolated six antioxidative peptides against peroxidation of linoleic acid from the protease hydrolysates of soybean β-conglycinin. They are

1. Val-Asn-Pro-His-Asp-His-Gln-Asn
2. Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn
3. Leu-Leu-Pro-His-His
4. Leu-Leu-Pro-His-His-Ala-Asp-Ala-Asp-Tyr

5. Val-Ile-Pro-Ala-Gly-Tyr-Pro
6. Leu-Gln-Ser-Gly-Asp-Ala-Leu-Arg-Val-Pro-Ser-Gly-Thr-Thr-Tyr-Tyr.

These peptides are characterized by the hydrophobic amino acids such as valine or leucine at the N-terminal positions and proline, histidine, or tyrosine in the sequences. Yokomizo *et al*. (2002) also isolated four antioxidative peptides from the protease hydrolysates of the water-insoluble residues of soybeans. The amino acid sequences were

1. Ala-Tyr
2. Ser-Asp-Phe
3. Ala-Asp-Phe
4. Gly-Tyr-Tyr.

These peptides possess aromatic amino acid at the C-terminal end. Gly-Tyr-Tyr has the strongest antioxidative activity among these four peptides, which is nearly equal to that of carnosine. It should be noted that the molecular weights of these four peptides are much lower than those of the other antioxidative peptides previously isolated from soybean proteins (Chen *et al*., 1995), from Alaska Pollack skin (Kim *et al*., 2001), and from fermented milk (Kudoh *et al*., 2001).

6.3.5 Physiological functions of soybean minor components

Another important factor in the physiological action of soybeans is that soybean minor components have exciting roles in the prevention of chronic disease (see Table 6.8). Although most of these minor components are not proteins, they coexist more or less in soy protein products as a food ingredient (Anderson and Wolf, 1995). Hitherto, these minor components, such as isoflavones, saponins, trypsin inhibitors, phytic acid, lectin, etc., were thought to be antinutritional factors, but now they are recognized to have preventative effects on cancer (Messina and Barnes, 1991). Among these, isoflavones (mainly genistein and daidzein) are particularly noteworthy, because soybeans are the only significant dietary source of these compounds. Isoflavones have not only anticarcinogenic activities, but also preventative effects on osteoporosis (Anderson and Garner, 1997) and the alleviation of menopausal symptoms (Albertazzi *et al*., 1998).

6.3.6 Off-flavors and allergenic proteins contained in soy protein

The most difficult problem limiting the expanded use of soy protein products in Western countries is the strong off-flavors associated with these products. There are two types of off-flavors. One is grassy and beany flavors and the other is bitter, astringent, and chalky flavors. The grassy-beany flavors are developed through the action of the three kinds of lipoxygenases 1, 2, and 3 present in soybeans. The bitter, astringent, and chalky flavors are caused by saponins and isoflavones (Okubo *et al*., 1992). The off-flavors of isoflavones are enhanced by the hydrolysis of their aglycones through the action of three kinds of β-

Table 6.8 Physiological functions of minor components contained in soybeans

Isoflavones	Anticarcinogenic activities[1], prevention of cardiovascular diseases[2], prevention of osteoporosis[3], antioxidant activities[4], and alleviation of menopausal symptoms.
Saponins	Anticarcinogenic activities[1, 6, 7], hypocholesterolemic effects[6], Inhibition of platelet aggregation, HIV preventing effects (group B saponin)[8], and antioxidant activities (DDMP saponin) [9].
Phytosterol	Anticarcinogenic activities[1].
Phytic acid	Anticarcinogenic activities[1, 6].
Lectin (Hemagglutinin)	Activation of lymphocytes (T cell)[8] and aggregating action of tumor cells[8].
Nicotianamine	Inhibitor of angiotensin-converting enzymes[10, 11].
Protease inhibitors	Anticarcinogenic activities[1, 6].

[1]Hawrylewicz *et al*., 1995; [2]Setchell and Cassidy, 1999; [3]Anderson and Garner, 1997; [4]Yoshiki and Okubo, 1997; [5]Albertazzi *et al*., 1998; [6]Messina and Barnes, 1991; [7]Rao and Sung, 1995; [8]Harada, 1999; [9]Yoshiki and Okubo, 1995; [10]Kinoshita *et al*., 1993; and [11]Kinoshita *et al*., 1994.

glucosidases A, B, and C in soybeans (Matsuura and Obata, 1993). Thus, both lipoxigenases and β-glucosidases contained in soybeans play an important role in the production of off-flavors. Moreover, the lipid hydroperoxides produced by lipoxygenases oxidize the free -SH groups of soybean proteins, resulting in a decrease of their gel-forming ability (Fukushima, 1994). For a long time, a number of attempts have been made to remove or mask these off-flavors during processing. However, it was impossible to remove or mask the off-flavors to a satisfactory extent by these methods.

In addition to off-flavors, another unwanted characteristic in soybeans is allergenic proteins. The major allergenic proteins in soybeans are shown in Table 6.9 (Ogawa *et al*., 1991). It is noticeable that two of the three subunits of β-conglycinin have allergenic proteins. It is impossible to remove all of these major allergens through the usual treatments.

6.4 Improving soy protein functionality

6.4.1 Improvement through conventional breeding

In the last two decades, the various soybean mutant genes which control the production of enzymes, allergenic proteins, storage proteins, etc., have been identified in the world soybean germplasm. Using these mutants, commercially available soybean cultivars have been bred without having undesirable substances but with the beneficially modified composition of storage proteins. For instance, the cultivar 'Kunitz' (Illinois Agricultural Experiment Station)

Table 6.9 Major allergenic proteins in soybeans[(1)]

Protein assignment	Molecular wt. (k Da)	Frequency[(2)] (%)
Gly m Bd 30 k	30	65.2
Gly m Bd28 k	28	23.2
α subunit of β-conglycinin	68	23.2
β subunit of β-conglycinin	45	10.1

[(1)]Ogawa *et al*., 1991. [(2)] Detection frequency among 69 soybean-sensitive patients with atopic dermatitis.

lacking Kunitz's soybean trypsin inhibitor (Bernard *et al*., 1991) and the cultivar 'Ichihime' (Kyushu Agricultural Experiment Station) lacking all of the lipoxygenases 1, 2, and 3 (Nishiba *et al*., 1995) can be taken as examples. The development of a lipoxygenase-free cultivar will be beneficial for the production of non-traditional soy products, since Western people are very sensitive to beany flavors. However, the soybean cultivars lacking the β-glucosidases have not been developed yet, which are the enzymes enhancing the off-flavors by changing the isoflavones into their aglycones.

There was some progress recently on the removal of allergenic proteins. The cultivar with a high ratio of glycinin to β-conglycinin was developed by the group of Tohoku National Agricultural Experiment Station, named Tohoku 124. This cultivar lacks the two major allergenic proteins of 28K and α subunit, while it still possesses allergenic proteins of 30K andβ-subunit (Samoto *et al*., 1996). Fortunately, the 30K protein can be removed easily by centrifugation, which is bound to IgE antibodies most strongly and frequently.

Another group of Kyushu National AES found the wild soybean line, named QT_2, which lacks all of β-conglycinin (Hajika *et al*., 1998). This line grows normally and produces successive generations, indicating the possibility of breeding the soybean varieties where storage proteins are mainly composed of glycinin without containing any β-conglycinin. Using this QT_2 line, they obtained the line lacking all the subunits of β-conglycinin by back-crossing with Fukuyutaka. This line contains only glycinin as storage proteins and it lacks the three major allergenic proteins of 28K, α, and β subunits (Takahashi *et al*., 2000). This performed as well in the field as Fukuyutaka in the on-campus experiment and was named Kyu-kei 305. Kyu-kei 305 should be mentioned as being the variety with the fewest allergens so far.

Besides these, the eight isogenic breeding lines with a different ratio of glycinin to β-conglycinin have been obtained by back-crossing, using Enrei as a recurrent parent in Nagano Chushin Experiment Station (Yagasaki *et al*., 1999). In each of these lines, not only the ratio of glycinin to β-conglycinin, but also the subunit composition of glycinin is varied systematically (see Table 6.10). The breaking stress of tofu gels made from the soybeans of these lines increases markedly with the increase of the contents of glycinin and its A_3B_4 subunit. The

Table 6.10 Hardness of tofu gel made from soybeans with different ratio of 11S and 7S proteins and different composition of glycinin subunits[1]

Breeding line	Glycinin subunit			11S/7S in soy milk	Breaking stress of tofu gel
	Group I	$A_5A_4B_3$	A_3B_4		
Enrei (control)	+	−	+	58/42	9,891
EnB_2-111	+	+	+	66/34	9,989
EnB_2-110	+	+	−	62/38	8,955
EnB_2-101	+	−	+	57/43	10,171
EnB_2-100	+	−	−	45/55	7,162
EnB_2-011	−	+	−	52/48	6,791
EnB_2-010	−	+	−	33/67	4,835
EnB_2-001	−	−	+	25/75	5,381
EnB_2-000	−	−	−	12/88	3,002

[1]Yagasaki *et al*., 1999. Crops: Enrei (control), 380 kg; and others, 384–441kg/10 a. Protein content of seeds: Enrei (control), 42%; and others, 39.3–40.7%.

yields and protein contents of the soybean seeds in these lines are substantially the same as the parent Enrei, indicating the possibility of the breeding of practical cultivars, from which we will be able to produce a variety of soybean protein products with different physicochemical properties.

6.4.2 Improvement through modern genetic engineering

Now that the three-dimensional structures have been elucidated completely in the molecules of β-conglycinin and glycinin, it is possible to improve the qualities of soybean storage proteins both physiologically and physicochemically through genetic modification. For instance, Kim *et al*. (1990) made a modified $A_{1a}B_{1b}$ gene which has four additional methionyl residues near the C-termini. This modified $A_{1a}B_{1b}$ is excellent in both gelling and emulsifying properties. If this gene is introduced into the soybean lines lacking β-conglycinin such as Kyu-kei 305, the resultant transgenic soybeans are expected to have beneficial functional properties in food systems, together with a high content of methionine. We can also improve the properties of the storage proteins by introducing various kinds of physiologically active peptides into the molecules on the basis of the three-dimensional structures. In addition, transgenic rice (Momma *et al*., 1999) and potato (Utsumi *et al*., 1994) with glycinins have already been bred.

6.5 Conclusion

For a long time, the removal of off-flavors has been a primary concern in research and technology on the utilization of soy protein as a food ingredient. At present, however, the concern is changing to the physiologically active

substances as well as the physicochemical functions of soy protein in food systems. For instance, isoflavones were considered simply as undesirable substances having strong off-flavors, but now they are recognized as being useful substances with an excellent preventative or alleviating effect on cancer, osteoporosis, and menopausal symptoms, etc. However, it should be remembered that the isoflavones are substances having both favorable and unfavorable properties for the development of new soy products.

The creation of new soybean cultivars is one of the most effective methods to obtain new soy protein products with beneficial characteristics, because the desirable or undesirable components of soybeans can be controlled essentially at a DNA level, in either conventional breeding or modern genetic engineering. The preventative effects of soy protein on chronic diseases have been proved scientifically and at the same time the relationship between the physicochemical properties and molecular conformations has been elucidated. Most recently, the three-dimensional molecular structures of both β-conglycinin and glycinin were cleared up completely therefore the improvement of soybean cultivars through modern biotechnology will be continued extensively. The day is not far distant when ideal soybean cultivars for obtaining soy protein products as a food ingredient will appear.

6.6 References

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7

Proteins from oil-producing plants

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7.1 Introduction

With a few exceptions, oil-producing plants have not generally been regarded as prime sources of protein for human consumption. Yet many of the oil-producing plants contain an appreciable level of protein, which has great potential for use in the human diet. Included in this group of oil-producing plants are soybean, canola (rapeseed), sunflower, safflower, peanut, corn, cottonseed, sesame, flax and even hemp. The best known protein source from an oil-producing plant is soybean and this topic has been extensively covered in Chapter 6 and will, therefore, not be a topic for discussion in this chapter. In this chapter, a range of proteins from oil-producing plants will be introduced. This will be followed by an examination of antinutritional components that limit their use for human food and methods used to isolate the proteins which will minimize or inactivate these components. A discussion of the functional properties of these protein isolates and ways to improve these properties will come next. The final issues that will be addressed are the possible uses for these proteins as well as future direction for increasing their value. It should be noted that the proteins will be evaluated only in relation to human use (i.e. not as animal feed) and that the canola and sunflower proteins will be used for the majority of the examples addressed in this chapter.

7.2 Oilseed protein characteristics

There are many oil-producing plants which have the potential to serve as protein sources for human consumption as shown in Table 7.1. The proteins found in

Table 7.1 Oil-producing crops and the major storage proteins suitable for human consumption

Botanical name	Oilseed – common name	Major storage protein
Brassica species	Canola/rapeseed	Cruciferin or 12S protein
Zea mays L.	Corn	Zein
Gossypium species	Cottonseed	11S protein
Linum usitatissimum L.	Flax	12S protein
Cannabis sativa L.	Hemp	12S protein
Arachis hypogaea L.	Peanut	Arachin
Carthamus tinctorius L.	Safflower	Carmin
Sesamum indicum	Sesame	α-globulin
Glycine max	Soybean	Glycinin
Helianthus annuus	Sunflower	Helianthin

seeds such as these are classified as biologically active (e.g. enzymes), structural or storage (Marcone, 1999). The storage proteins are the prominent proteins in seeds and are therefore of prime interest when using these protein sources for human consumption. The physical and chemical properties of many of these oil-producing proteins have been extensively studied by Prakash and Narasinga Rao (1986). The levels of protein in these seeds range from 13–17% for safflower (Prakash and Narasinga Rao, 1986; Paredes-López 1991) to as high as 37% for soybean (Lampart-Szczapa, 2001). Despite these differences in protein levels, there are many similarities between these oilseed proteins.

All crops contain a mixture of albumins (water soluble), globulins (salt soluble) and glutelins (alkali soluble) proteins. Almost all of these proteins contain the same four protein fractions with sedimentation coefficients of 2S, 7S, 11S (12S) and 15S (Prakash and Narasinga Rao, 1986). The exceptions are the *Brassica* species, which lack the 15S fraction. The proportions of these fractions, however, are highly variable. The two major protein fractions are the 11S and the 2S, with the 7S thought to represent the product resulting from dissociation of the 11S fraction. The similarity of the 11S proteins in oilseeds as well as legumes has been the subject of several investigations (Prakash and Narasinga Rao, 1986; Grinberg *et al*., 1989; Marcone, 1999; Lampart-Szczapa, 2001). These proteins have similar molecular weights, subunits, amino acid profiles and secondary structure (Marcone, 1999). This has resulted in similar hydrophobicity values and similar association–dissociation behaviour in response to pH. These oilseed proteins, however, exhibit distinct differences in terms of tertiary structure and surface properties (Marcone, 1999).

7.2.1 Sunflower

While the types of proteins and some of the characteristics of the main storage proteins are the same, there are some unique characteristics associated with the various oilseed proteins. For sunflower, the low molecular weight 2S fraction is

of importance due to the fact that chlorogenic acid preferentially associates with this fraction (Venktesh and Prakash, 1993a), without significantly changing its molecular weight. This results in unfavourable colour production and is undesirable in foods destined for human consumption. While this binding can be reduced by washing with acidic butanol, the physicochemical properties are also altered in the process (Venktesh and Prakash, 1999a). This low molecular weight fraction has been shown to consist of eight polypeptide chains, with molecular weights of 10 000 to 18 000 (Kortt and Caldwell, 1990). Two of these chains have higher than normal levels of methionine (16%) and cystine (8%) (Kortt and Caldwell, 1990). As these proteins often become a component in protein isolates, the high level of these sulphur containing amino acids in this component may be important to the nutritional and functional value of the resulting isolates.

Oleosin proteins have also been found in sunflower (Li *et al*., 1993; Alexander *et al*., 2002). These proteins, with molecular weights of 15 000 to 20 000 are associated with the oil bodies in the seed and represent 14% of these oil bodies. The proteins are characterized as having a hydrophobic core of 70–80 residues, flanked by polar C and N terminal domains. The N terminal domain is made up of 10% α-helix, 20–30% β-strands, 8% β-turns and 60% random coil (Li *et al*., 1993), while the hydrophobic core is primarily an α-helix hairpin structure in which two helices are separated by a turn region (Alexander *et al*., 2002). As these proteins are present in small amounts and are associated with the oil bodies, these are not usually considered when looking at protein isolation from sunflowers.

Even for a given species, there are number of factors that can influence the levels and types of proteins found in the seed and meal produced by oil extraction. The choice of sunflower cultivar may influence the proteins present. In a study by Raymond *et al*. (1991), there were only minor differences in protein level (30–36%) or protein composition for nine sunflower cultivars. In work done by Durante *et al*. (1989), some lines of sunflowers contained a 190 000 protein rather than the typical 300 000 (12S) protein. This difference was due to the absence of a 39 200 component that was present in the 300 000 protein, along with the 32 500, 25 600 and 23 200 proteins that were common to both.

Handling practices also affect the proteins present. Attempts to sift the seeds into different sizes had only limited success in producing a size with increased protein content in that it was effective for only one of the cultivars tested (Nel *et al*., 1999). During storage, change in the relative proportions of the protein fractions (albumins, globulins and glutelins) have been reported (Minakova *et al*., 1996), although the amino acid content was unaffected. Germination of the seeds also affected the proteins, resulting in increases in lysine, tryptophan, free amino acid and non-protein nitrogen (Balasaraswathi and Sadasivam, 1997). While the albumins and globulins decreased and the glutelins increased during germination, the overall effect of germination was a reported increase in protein and nutritional value. The heat associated with the oil extraction process has also been shown to have a negative impact on protein in that the protein structure was

altered, interactions between proteins and polyphenolics were promoted and physicochemical properties of the protein were changed (Venktesh and Prakash, 1993b).

7.2.2 Canola

Canola proteins also have some unique characteristics. Both the terms canola and rapeseed have been used in the literature. The distinction between these is related to the requirement for reduced levels of erucic acid and glucosinolates in canola. The terms rapeseed, canola and *Brassica* species will all be used in this chapter and will reflect the various terms used in the literature. Of note for these *Brassica* proteins is the high level of water-soluble proteins or albumins, which make up 45–50% of the total protein (Appleqvist, 1972). The globulins, which are the most common protein in most other oil-producing plants (Prakash and Narasinga Rao, 1986), represent about 25% of the protein (Appleqvist, 1972). This may be related to the fact that only 87% of the nitrogen is present in the form of protein, with the rest present as water-soluble peptides and free amino acids (Niewiadomski, 1990).

Another unique characteristic of canola protein is the isoelectric point, which is around pH 7 (Schwenke *et al*., 1981) compared to pH 4.5 to pH 5 as is the case for other oilseed proteins (Prakash and Narasinga Rao, 1986). The effect of this difference can be seen in the protein solubility where minimal solubility has been reported at both pH 4.0 and pH 8.0 compared to a single minimum around pH 4.5 for other oilseed proteins.

In a comparison of different varieties of canola/rapeseed (all *Brassica napus* L.), the main differences were seen in the metabolic proteins rather than in the storage proteins. In terms of storage proteins, differences were seen in the 2S fraction but not the 12S fraction (Raab *et al*., 1992). In a comparison of yellow genotypes from *Brassica napus*, *Brassica rapa*, *Brassica juncea* and *Brassica carinate* with the more traditional brown canola, higher levels of protein were found in the yellow-seeded varieties, presumably due to a lower level of fibre in these seeds (Simbaya *et al*., 1995).

The *Brassica* species also contain proteins which are not storage proteins, but which may have some impact on protein isolation. For example, myrosinase-binding proteins, having molecular weights of 50 000 to 52 000, have been well characterized (Falk *et al*., 1995). These proteins, in conjunction with the myrosinase-binding protein-related proteins, myrosinase and glucosinolates provide a defence mechanism for the seed. Since myrosinase and glucosinolates can influence the properties and value of isolated storage proteins, these proteins should not be overlooked.

7.2.3 Other oilseeds

Other oilseed proteins also have some unique characteristics that will not be addressed here. However, there are reviews for many of these proteins, including

peanut (Bhushan *et al*., 1986), corn (Lawton, 2002), cottonseed (Alford *et al*., 1994; 1996), flax (Oomah and Mazza, 1993) and safflower (Paredes-López, 1991). Safflower proteins are unique in that the alkaline soluble proteins are the most prevalent at 39% (Salazar Zazueta and Price, 1989).

7.3 Factors limiting protein utilization

As is the case with other plants, oilseed products have a range of chemical compounds which aid their growth and serve as a defence to factors such as insects and microorganisms that might limit their ability to grow. These compounds generally end up in the meal component that is left after oil extraction and have presented problems in making use of these proteins for human consumption. In addition to compounds such as fibre, protease inhibitors, phytic acid and phenolics which are common to all oil-producing plants, there are specific compounds associated with certain crops that must not be overlooked. These include gossypol in cottonseed, chlorogenic acid in sunflower and glucosinolates, which are most often associated with canola/rapeseed. These compounds will be examined in terms of their occurrence, why their presence represents a problem, and what can be done to minimize the problem.

7.3.1 Fibre

The high fibre content of most oilseed meals produced during oil extraction limits the availability of nutrients in the meal and creates processing problems (Prakash and Narasinga Rao, 1986). Since the fibre is largely associated with the hull of the seed, a dehulling step can be an effective option for decreasing the fibre content of most oilseeds. Dehulling sunflowers, for example, can be achieved quite effectively because of the seed size, but canola seeds, for which the hull represents 16% of the weight of the seed, are small and mechanical dehulling does not provide a good separation (Niewiadomski, 1990). Although the hulls of flaxseed, which represent 30–39% of the seed weight, contain a soluble fibre fraction with demonstrated hypoglycaemic activity, protein recovery from flaxseed is improved if the hulls and mucilage associated with the hulls are removed (Wanasundara and Shahidi, 1997). As a result, dehulling (when possible) in conjunction with the preparation of protein concentrates and protein isolates seems to be the most effective way to minimize problems of excess fibre.

7.3.2 Proteinase inhibitors

Proteinase inhibitors are generally water-soluble low molecular weight proteins that protect the plant from insects and microorganisms (Ceciliani *et al*., 1994; Whitaker, 1997; Lampart-Szczapa, 2001). These compounds are a problem when consumed because of their ability to complex with proteinases such as

trypsin and chymotrypsin in the body thus rendering these enzymes inactive. This results in reduced pancreatic activity and growth inhibition (Shahidi, 1997). While there is some evidence that proteinase inhibitors may be able to play a role in preventing cancer and HIV infections (Lampart-Szczapa, 2001), they are still a concern if oilseed proteins are to provide the essential amino acids that are naturally present.

Perhaps the best known of the proteinase inhibitors are the Kunitz and Bowman-Birk inhibitors associated with soybean protein, but a range of proteinase inhibitors have also been isolated from other oil-producing plants. Several cystine proteinase inhibitors, called phytocystatins, have been isolated from sunflower and characterized with respect to their structure and mode of action (Kousuma *et al*., 2001). Serine proteinase inhibitors from *Brassica* species have also been well characterized (Ceciliani *et al*., 1994). Three serine proteinase inhibitors that vary in terms of heat stability and mode of action have been identified in rapeseed. The thermostable inhibitor, referred to as rapeseed trypsin inhibitor (RTI) has been shown to bind to the trypsin differently than other serine proteinases inhibitors, which are more sensitive to heat (Ceciliani *et al*., 1994). As oilseed proteins are usually subjected to heat prior to consumption, most proteinase inhibitors are inactivated prior to consumption. The presence of thermally stable inhibitors means that heating alone may not completely eliminate this antinutritional factor and the viability of proteinase inhibitors should be monitored during the development of oilseed protein products.

7.3.3 Phytic acid

Phytic acid and phytates, the salts of phytic acid, are commonly found in the cotyledons of oil-producing plants (Shahidi, 1997), where they serve as phosphate stores for the plant. The highly charged phosphate groups make the phytic acid molecule very reactive, and if present in foods, they tend to bind divalent cations, such as calcium, iron, zinc and magnesium, rendering them nutritionally unavailable. Despite serious concerns about phytate consumption, studies indicating that phytic acid may play a role in reducing blood glucose and plasma cholesterol and aid in cancer prevention have been reported (Shahidi, 1997). The presence of phytic acid can also create problems in the processing of oilseed proteins as phytate has been shown to interact with proteins, thereby altering protein solubility. Insoluble phytate-protein complexes have been reported in acidic environments (Bulmaga *et al*., 1989).

Two approaches have been used to address the concerns about phytic acid in oilseed protein products. One approach is to remove phytic acid and phytate from the protein source. Preparation of protein isolates can be very effective in this respect as will be seen later in this chapter. An alternate approach is to use the enzyme phytase to breakdown the phytic acid molecule to a point where it is no longer able to bind to minerals in the diet. Canola contains some endogenous phytase, which is present at its highest levels shortly after germination (Houde *et*

al., 1990). Despite the endogenous phytase, phytate levels in canola remain high and protein purification seems to be the preferred route to address this problem.

7.3.4 Oxalic acid

A similar problem of mineral binding has been noted for oxalic acid or the oxalate salts. This problem is primarily associated with sesame seeds (Prakash and Narasinga, 1986). As the oxalic acid is located primarily in the hull, both dehulling and protein isolation are effective methods of controlling oxalic acid levels (Bandyopadhyay and Ghosh, 2002).

7.3.5 Phenolic compounds

Phenolic compounds have received a lot of attention in relation to their impact on the proteins from oil-producing plants. These phenolic compounds are usually present either as phenolic acids or condensed tannins (Naczk and Shahidi, 1997). The phenolic acids, including caffeic, vanillic, syringic and coumaric can be found in free, esterified and bound forms (Sosulski and Krygier, 1983; Naczk and Shahidi, 1997) and are responsible for astringent flavours, dark colours and decreases in mineral availability (Shahidi, 1997).

Canola phenolic acids have become more of an obstacle to the utilization of canola protein for human consumption as the glucosinolates problems have been reduced through breeding efforts. Canola contains about 10 times more phenolic compounds than soybean (Xu and Diosady, 2002) at a level of 1540–1840 mg/100g (Shahidi and Naczk, 1992; Naczk *et al*., 1998). The main phenolic acid (sinapic acid) is unique to canola/rapeseed where it represents 70–85% of the free phenolic acids and 71–97% of the esterified phenolic acids (Shahidi and Naczk, 1992). It should be noted that the majority (~80% in meal) of the phenolic acids exist in the esterified form as the sinapine choline ester. Insoluble bound phenolics represents a relatively low proportion of the phenolic acids (Sosulski and Krygier, 1983; Shahidi and Naczk, 1992), yet the ability of sinapic acid to interact with proteins can lead to colour and taste problems in canola protein products (Xu and Diosady, 2000).

An examination of the interactions between canola phenolic compounds and proteins has shown that the primary mode of interaction is ionic (~30%) with minor roles for hydrophobic interactions, hydrogen bonding and covalent bonding (<10% each) (Xu and Diosady, 2000). Changes in phenolic acids as a function of pH can also have an influence on the quality and characteristics of protein products. The formation of the dimer of sinapic acid, which has been identified as thomasidioic acid, can negatively impact the functional properties of the isolated protein (Rubino *et al*., 1996). Methods to reduce the phenolic compounds have focused on minimizing the interaction between the phenolics and proteins, thus creating a situation where the phenolic compounds can be removed from the protein due to a difference in solubility or size. The use of a methanol-ammonia-water treatment (Naczk and Shahidi, 1989; Shahidi and

Naczk, 1992; Wanasundara and Shahidi, 1994a; 1994b) and treatment with Na_2SO_3 and polyvinyl pyrolidone (PVP) (Xu and Diosady, 2002) have been shown to be effective at significantly reducing the levels of phenolic compounds associated with canola proteins.

Sunflower protein use is also limited by the phenolic acids, in particular, chlorogenic acid (CGA) which represents 70% of the phenolic acids present in the seed (Domínguez *et al*., 1993). The majority of the CGA exists in the free form (75%) and the interaction between CGA and the sunflower protein can lead to discolouration in protein isolates (Vaintraub and Kratch, 1989). The binding of CGA, its hydrolysis products, caffeic and quinic acids, and its oxidation products, o-quinones, also play a role in determining the impact of these phenolic compounds on the properties of the protein. CGA binding was shown to be dependent on pH and CGA concentration with minimum binding at pH 5 (Saeed and Cheryan, 1989). Increases in temperature and salt concentration, as well as the inclusion of 0.01M Na_2SO_3 or 8M urea resulted in lower levels of binding (Shamanthaka Sastry and Narasinga Rao, 1990). While hydrophobic interactions were thought to play a role at pH 5, the binding of CGA at low pH values was due to hydrogen bonding, and at high pH values binding was due to electrostatic interactions (Saeed and Cheryan, 1989). The caffeic acid has been shown to interact with the amino acids tryptophan, tyrosine and lysine while the quinic acid interacts with lysine, arginine and tryptophan, although to a lesser extent than caffeic acid (Suryaprakash *et al*., 2000). The o-quinones bound covalently to free sulphydryl groups or amino groups (Saeed and Cheryan, 1989).

Attempts to reduce the CGA levels have included treating the meal directly or incorporating appropriate steps into protein isolation procedures. Isolation procedures will be examined later in this chapter. Extractions with acidic aqueous acetone and acidic butanol have been effective in reducing sunflower phenolics, but have also resulted in protein denaturation and a decrease in protein solubility (Prasad 1990a; 1990b). Treatment of the sunflower kernels with HCl prior to oil extraction reduced CGA levels without protein denaturation; when CGA levels were further reduced by boiling the meal in water, the protein solubility was also affected (Domínguez *et al*., 1993). A combination of autoclaving and treatment with gamma radiation was also effective in reducing CGA, but resulted in a decrease in protein solubility and available lysine (Farag, 1999).

7.3.6 Tannins

Tannins are complex structures, with molecular weights ranging from 500 to 3000, which can be hydrolyzed to produce phenolic acids (from condensed tannins) or anthocyanins (from polymerized tannins) (Naczk and Shahidi, 1997). The predominant type is the condensed tannins, which have been found to be associated with a range of leaf proteins (Hussein *et al*., 1999) and canola hulls (Naczk and Shahidi, 1997). The structure and effects of the canola condensed

tannins have been reviewed by Naczk and Shahidi (1997). They reported condensed tannin content in canola meal is highly variable (0.1% to 3%) depending on the type of seed analyzed and the method of analysis used (Naczk and Shahidi, 1997), though values around 0.7% seem to be a reasonable estimate. The concern with having tannins in the meal relates to the ability of these compounds to precipitate protein (Naczk *et al*., 1996) and form complexes with minerals and amino acids thereby reducing the nutritional value of the isolated protein (Naczk and Shahidi, 1997). It has been shown that the extent to which protein precipitation is induced increases with the amount and the molecular weight of the tannin as well as the type and concentration of the protein (Naczk *et al*., 2001a; 2001b). Elimination of tannins is often done in conjunction with the removal of phenolic acids and the same approaches apply.

7.3.7 Glucosides and glycosides

Glucosides and glycosides are present in a number of oil-producing plants. In terms of nutritional problems, perhaps the best known are the glucosinolates, found in rapeseed and canola but cyanogenic glycosides in peanuts (Wanasundara and Shahidi, 1994a,b; Shahidi and Wanasundara, 1997) and the phenolic glucosides in safflower (Tasneem and Prakash, 1992), also represent nutritional problems. The problems created by the glucosinolates cannot be overlooked. In fact, one of the factors that distinguishes canola from the early rapeseed varieties is the glucosinolate content. Canola, by definition, must contain less than 30 μmol/g of glucosinolates. Glucosinolates from *Brassica* species have been reviewed by Shahidi *et al*. (1997). There are a large number of glucosinolates that can exist as there are many different R groups that can be attached to the basic molecule. However, the glucosinolates most commonly found in *Brassica* species are limited, and are shown in Table 7.2. The concern with glucosinolates is not the glucosinolates themselves, but the degradation products that result when the glucosinolates degrade to produce compounds such as isothiocyanates, thiocyanates, cyanides, oxazolidinethiones and nitriles (Niewiadomski, 1990; Shahidi *et al*., 1997; Pecháček *et al*., 2000).

These compounds have been associated with thyroid enlargement and growth retardation as well as problems with the kidney and pancreas (Shahidi *et al*., 1997). It is interesting to note that the indolyl glucosinolates may actually be beneficial in the prevention of cancer (Shahidi *et al*., 1997). Despite the fact that glucosinolates levels have been significantly reduced in canola varieties, the presence of glucosinolates continues to be an impediment to the utilization of canola protein for human consumption. As a result, in processing canola, a number of treatments have been considered to further minimize this problem, including inactivation of the myrosinase, extraction of the glucosinolates or breakdown products, chemical (or microbiological) degradation of the glucosinolates and preparation of protein isolates (Niewiadomski, 1990; Shahidi *et al*., 1997). The use of a methanol-ammonia-water treatment has been effective in removing glucosinolates in their original form or as breakdown products

Table 7.2 Structures of glucosinolates from *Brassica* species

Type of glucosinolate	Common name	R group *
Alkyl	Sinigrin	Allyl
	Gluconapin	3-butenyl
	Glucobrassicanapin	4-pentenyl
	Progroitrin	2-hydroxy-3-butenyl
	Gluconapoleiferin	2-hydroxy-4-pentenyl
	Gluconasturtiin	Phenylethyl
	Glucoerucin	4-methyl thiobutyl
	Glucosinalbin	4-hydroxybenzyl
Sulphinyl	Glucoalyssin	5-methylsulphinylpentyl
Indolyl	Glucobrassicin	3-indolymethyl
	4-hydroxyglucobrassicin	4-hydroxy 3-idolymethyl
	Neo glucobrassicin	1-methoxy-3 indolymethyl

R
/
* Basic structure: Glucose-S-C=NSO_4
Adapted from Slominski and Campbell, 1987, Shahidi *et al*., 1997, Linsinger *et al*., 2001

(Shahidi *et al.*, 1990; Pecháček *et al*., 2000). Similar approaches have been used to remove glucosides in other oilseeds. Aqueous ethanol washing has been effective in reducing safflower phenolic glucosides (Tasneem and Prakash, 1992).

7.3.8 Gossypol

Gossypol limits the use of cottonseed; however, there are techniques available to reduce the gossypol to a level below the recommended maximum of 0.06% (Mohamed, 1993). These include treatments with alkaline and hydrogen peroxide, acetic acid or a mixture of acetone/hexane and water (with or without acetic acid). The acetic acid treatment was the most effective (gossypol was $< 0.03\%$), and also improved the available lysine, solubility, and water holding capacity of the protein (Mohamed, 1993).

7.3.9 Allergenic proteins

Largely as a result of the antinutritional factors found in oilseed meals, isolation of the protein is often required to make these proteins suitable for human consumption. If a problem exists with the protein itself, such as the allergenic response some people have to soybean and peanut protein (Lampart-Szczapa, 2001; Frøkliær *et al*., 1997), protein isolation will be of no benefit. In introducing proteins not previously consumed into human food, the potential for allergenic reactions should not be discounted.

7.4 Extraction and isolation of proteins

Protein extraction and isolation can be effective in reducing many of the antinutritional problems, while creating a protein-rich commodity with marketable nutritional and functional properties. This topic is covered in detail in Chapter 14. While protein isolation has been clearly demonstrated for soybean, where many commercial products are available, other oil-producing plants often have unique properties that require modification of the standard soybean protocols. A range of approaches specific to the isolation of oilseed proteins are discussed below, including adaptations required, or preferred, for certain crops.

7.4.1 Dehulling

The removal of the hulls from oil-producing plants is an effective way to reduce fibre as well as other antinutritional factors such as phytic acid and phenolic compounds. For many oil-producing plants, including soybean, cottonseed and sunflower, hulls can be easily removed through milling or mechanical abrasion, but this is not the case for some of the smaller seeds, such as canola, where the hull tends to adhere to the endosperm making separation difficult (Thakor *et al*., 1995). Several pretreatments have been examined to improve canola dehulling including infra-red heating (McCurdy, 1992) and a hydrothermal treatment involving soaking and drying the seeds prior to mechanical dehulling (Thakor *et al*., 1995). These treatments improved dehulling efficiencies, with the hydrothermal treatment reducing the fibre content in the meal to 3.5% (from 9.5%) compared to a level of 7.5% if dehulled without pretreatment (Thakor *et al*., 1995). However, the hull fraction was shown to contain 18% oil and 18% protein, a situation which is not helpful to the overall recovery of these components. While the infra-red pretreatment also produced meals with lower fibre content, meal yields were at unacceptably low levels regardless of the type of mill used for dehulling (McCurdy, 1992).

The removal of hulls, however, continues to be a desirable step in the protein isolation process. In addition to its role in helping to purify the protein, dehulling has been shown to improve flax protein solubility (Oomah and Mazza, 1993). As oil recovery is still the main reason for processing oilseeds, dehulling can be included only if it does not adversely affect oil recovery. As a result, for crops such as canola, a dehulled meal as a starting material for protein isolation is rarely available.

7.4.2 Oil extraction conditions

Most oil is recovered from oil-producing plants by prepressing and extraction with hexane. It has been suggested that hexane may not be the best solvent for subsequent use of the protein. Sunflower seeds (dehulled), extracted with isopropanol, rather than hexane, have lower CGA levels and show significant

improvement in nutritional value, based on growth rate and feed efficiency ratios when fed to rats (Sen and Bhattacharyya, 2000). The use of a methanol-ammonia-hexane treatment has been investigated for use with canola where it has been shown to reduce glucosinolates and polyphenolic compounds (Diosady and Rubin, 1993) with minimal changes to essential amino acids and no change in the predicted protein efficiency ratios (Shahidi *et al*., 1992). A similar approach, using alkaline and ethanol has been used to extract oil and protein from corn (Hojilla-Evangelista *et al*., 1992).

7.4.3 Preliminary meal treatments

Treatment of the meal with methanol following hexane extraction has also been shown to reduce the CGA level in sunflower (González-Pérez *et al*., 2002) prior to further protein isolation. Prewashing the meal in water at a pH close to the isoelectric point of the protein has been used to reduce the antinutritional factors in safflower prior to protein isolation (Ordorica-Falomir *et al*., 1990). A similar approach that has been investigated for defatted sunflower meal that had not been dehulled was to separate the meal into three fractions by dispersing it in water at pH 4.5 (Parrado *et al*., 1991). This produced an insoluble protein-rich fraction, a soluble fraction containing many of the polyphenolic compounds and carbohydrates and a floating layer of lignocellulosic material, mostly from the hulls. The protein-rich fraction was then used for further protein isolation.

7.4.4 Protein solubilization

There have been a number of different solvents used to solubilize the protein in oilseed meals, but the most common solvent seen in the literature is an aqueous alkaline solution and this has been used for corn (Myers *et al*., 1994), cotton seed (Berot *et al*., 1995), safflower (Paredes-López, 1991), sesame seed (Bandyopadhyay and Ghosh, 2002), peanut (Jangchud and Chinnan, 1999a), flaxseed (Oomah and Mazza, 1993; Oomah *et al*., 1994), sunflower (Ordóñez *et al*., 2001; González-Pérez *et al*., 2002) and canola (Wilska-Jeszka and Zajac, 1984; Diosady *et al*., 1989; Deng *et al*., 1990; Tzeng *et al*., 1990a; Chen and Rohani, 1992; Xu and Diosady, 1994; Klockeman *et al*., 1997; Aluko and McIntosh, 2001). The choice of alkali has been popular because of the high degree of solubility that can be obtained. Despite the fact that these are all alkaline extractions, the pH of the solution has varied from 9 to 12.5, with higher protein yields associated with higher pH values (Betschart and Saunders, 1978). High pH values (especially pH 12 and above) are associated with increased lysinoalanine formation (Deng *et al*., 1990) and do not always produce the best quality isolates. For this reason, a number of researchers have preferred to solubilize proteins with lower pH alkaline solutions.

A variety of salt solutions have been evaluated as solubilizing agents, with NaCl being used most frequently. Although oilseed proteins tend to be less soluble in salt solutions compared to alkaline solutions, the combination of

alkali and salt has often been used to improve protein solubility (Lasztity *et al*., 1993; Oomah *et al*., 1994; Klockeman *et al*., 1997). The use of NaCl extraction has been used for a number of proteins including flax (Oomah *et al.*, 1994), safflower (Ordorica-Falomir *et al*., 1990; Paredes-López, 1991), sunflower (Shamanthaka-Sastry and Narasinga Rao, 1990) and canola (Ismond and Welsh, 1992; Léger and Arntfield, 1993; Murray, 1997; 2001a; 2001b). The attraction in using NaCl is that it avoids using pH extremes and the associated changes in protein structure, and is an accepted food ingredient. Phosphate salts, such as NaH_2PO_4 and sodium hexametaphosphate (SHMP), have also been used to enhance protein extraction (Rubin *et al*., 1990; Ismond and Welsh, 1992; Bland and Lax, 2000).

An alternative approach to increase protein solubility that has been used primarily with sunflower proteins is the use of enzymes. Treatment of the dehulled seed with pectinase (with and without cellulase) produced a meal with reduced CGA, which led to the production of a protein-rich fraction following oil extraction and washing with water at pH 4.8–5.0 (Domínguez *et al*., 1995; Sen and Bhattacharyya, 2001). No further purification was done on these protein-rich fractions. Alternatively, proteolytic enzymes, such as fungal proteases, trypsin, papain (Cai *et al*., 1996) and kerase (Parrado *et al*., 1991; 1993) render the protein highly soluble so that it can be extracted in water. Protein fractions containing 78% protein and low levels of CGA and carbohydrates have been obtained with this approach (Parrado *et al*., 1991). A similar approach, using cell-wall degrading enzymes, has been used to produce a dehulled protein-rich rapeseed meal from rapeseed (Jensen *et al*., 1990).

7.4.5 Protein precipitation/recovery

A number of approaches have been investigated for recovering the solubilized protein. For those proteins extracted using alkaline solutions, the most common approach has been to precipitate the protein with pH adjustment. For sesame protein, a pH of 4.9 was used (Bandyopadhyay and Ghosh, 2002), for flaxseed protein pH 4.1 was more effective (Oomah and Mazza, 1993) and for peanut (Jangchud and Chinnan, 1999a) and sunflower (Parrado *et al*., 1991; Cai *et al*., 1996) protein has been precipitated at a pH of 4.5. Canola protein has been more of a challenge in terms of protein recovery. While maximum precipitation has been reported at pH 4.0 (Aluko and McIntosh, 2001), pH 3.7 (Chen and Rohani, 1992) or pH 3.5 (Klockeman *et al*., 1997), only about 53% of the protein precipitates (Chen and Rohani, 1992). The inclusion of SHMP (Chen and Rohani 1992) or $CaCl_2$ (Tzeng *et al*., 1990a), while precipitating at pH 3.5–4, has been used to improve the recovery of canola protein. Alternatively, Xu and Diosady (1994) found pH 6.5 was adequate for protein precipitation, and Aluko and McIntosh (2001) prepared an isolate where the protein was precipitated at a pH of 6.0 with 1M $CaCl_2$.

For salt-extracted canola proteins, the main method of protein precipitation is by reducing the ionic strength either by dialysis or dilution of the extracted

protein (Ismond and Welsh, 1992; Léger and Arntfield, 1993; Murray, 1997; 2001a; 2001b). This dilution technique has also been applied to safflower protein (Paredes-López, 1991; Ordorica-Falomir *et al*., 1990).

7.4.6 Other purification steps

In addition to the basic steps of extraction and precipitation, there can be a number of extra steps included in a protein isolation procedure that aid in protein purification. Washing of the isolate following precipitation with an aqueous pH-adjusted solution helps reduce non-proteinaceous components. When precipitating protein using the dilution technique, a protein concentration step using ultrafiltration is often necessary to get the protein to a concentration that will allow the proteins to interact to form micelles during the precipitation step (Ismond and Welsh, 1992, Murray, 1997; 2001a, 2001b). This ultrafiltration step has an added benefit of removing low molecular weight antinutritional factors including phenolics, glucosinolates and phytic acid (Ismond and Welsh, 1992). A schematic diagram of this entire process can be seen in Fig. 7.1.

Because of the high level of soluble protein in the supernatant following precipitation of canola proteins, efforts have been made to recover this protein, which represents the 2S canola protein. This has been effectively accomplished by ultrafiltration and drying of the supernatant (Tzeng *et al*., 1990a; 1990b; Rubin *et al*., 1990). The overall process which results in the recovery of two distinct protein fractions can be seen in Fig. 7.2.

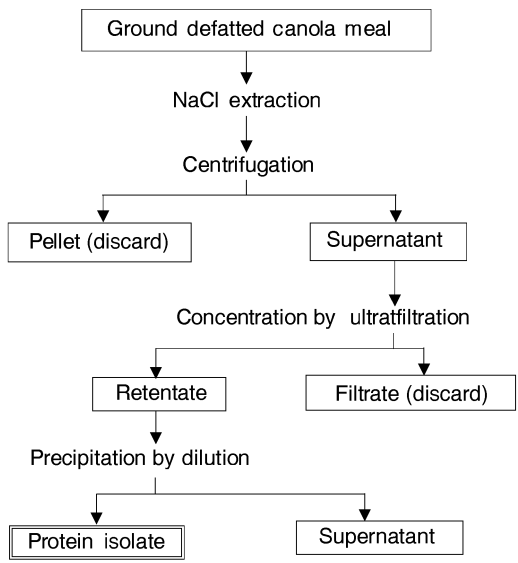

Fig. 7.1 Generic schematic diagram for canola protein isolation using salt solubilization and precipitation by dilution. Based on Ismond and Welsh (1992); Léger and Arntfield (1993).

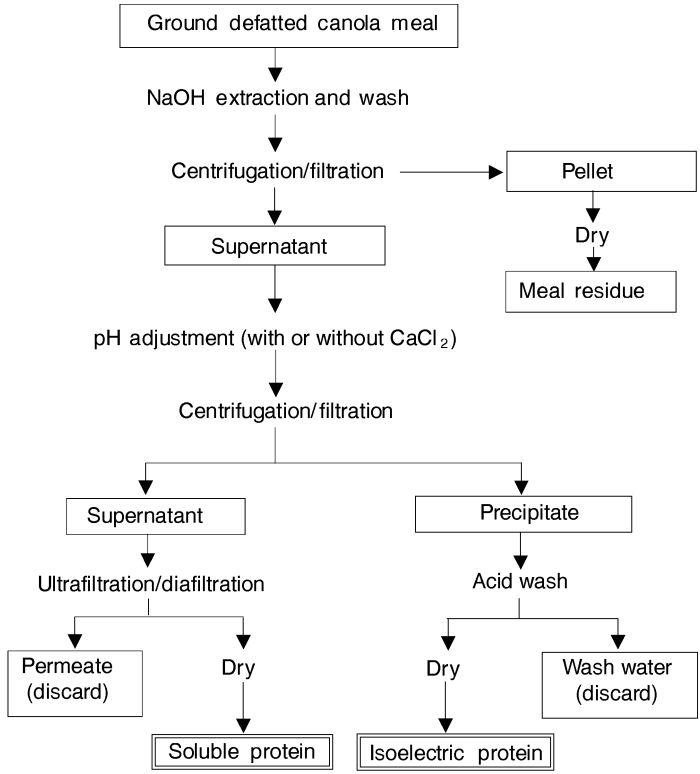

Fig. 7.2 Generic schematic diagram for canola protein isolation using alkaline solubilization and recovery of two protein streams. Adapted from Tzeng *et al*. (1990a).

One other approach that has been used to remove glucosinolates and their degradation products from a canola protein extract is the use a strongly basic anion exchange resin, followed by drying of the extract (Wilska-Jeszka and Zajac, 1984). While this technique effectively removed glucosinolates, its use is limited by expense and the size of anion exchange resins.

7.5 Functional properties of proteins

Protein isolation is only the first step towards incorporating proteins from oil-producing plants into food products. To reap the benefits of these nutritious proteins, it is critical that their incorporation into food products be such that the food remains appealing to the consumer. Functional properties of proteins provide valuable information as to how effectively proteins will be able to do this. The functional properties that seem to receive the most attention when characterizing isolated oilseed proteins are solubility, water absorption capacity (water-holding capacity), fat-binding capacity, emulsification activity and

stability, foaming capacity and stability and gelation. The tendency when evaluating functional properties of proteins from oil-producing plants has been to compare them to soybean proteins or to evaluate changes in these properties as a function of conditions of preparation or handling. Comparison of functional properties of different oil-producing proteins is difficult because of the influence of the protein isolation technique and the variability in techniques used to evaluate functional properties.

7.5.1 Solubility and absorption capacity

Solubility is often used as an indicator of other functional properties. However, solubility data for protein from oil-producing plants is limited as solubility is often influenced by the isolation method used. As many of these proteins are globulins (salt soluble), an evaluation of water solubility is not always beneficial. Nevertheless, in a study by Oomah and Mazza (1993), a low mucilage protein isolate from flax seed was shown to have better water-absorption properties than a soybean protein isolate. The pH used for protein precipitation affected the solubility of safflower protein with higher solubility when the protein was precipitated at pH 6 rather than pH 5 (Betschart *et al*., 1979). Testing protocol can also have an impact on solubility. Proteins in a concentrate prepared from sesame seeds, like most plant proteins, had reduced solubility around the isoelectric point, but if salt was included during the evaluation of solubility, protein solubility increased (Inyang and Iduh, 1996).

7.5.2 Fat-binding and emulsification properties

Fat-binding and emulsification properties can be of value when incorporating proteins in mixed systems. Safflower proteins that had been neutralized to pH 7 following precipitation at pH 5 exhibited fat binding and emulsification properties that were equivalent to soybean (Betschart *et al*., 1979) while the fat absorption and emulsification properties of a low-mucilage flax protein isolate were superior to those from a soybean protein isolate (Oomah and Mazza, 1993). Fat absorption and emulsification properties of canola protein were reported to be good, but were not compared to soybean (Thompson *et al*., 1982). Pawar *et al*. (2001) demonstrated that the protein isolation technique used can influence oil absorption and emulsification properties, which were improved when phytates and polyphenolic compounds were reduced during isolate preparation. In a study by Inyang and Iduh (1996), emulsifying activity of a sesame protein concentrate increased as the pH or salt concentration during evaluation was increased and emulsion stability was also improved by including salt during testing.

7.5.3 Foaming properties

In addition to the functional properties noted above, the foaming properties of the low mucilage protein isolate from flax seed studied by Oomah and Mazza,

(1993) were shown to be better than those for a soybean protein isolate evaluated using a similar protocol. The canola protein concentrate examined by Thompson *et al*. (1982) was also reported to have good foaming properties. The impact of the protein isolation protocol on foaming was seen with both safflower and sunflower proteins. With safflower, proteins precipitated at pH 6 had better foaming properties than proteins precipitated at pH 5 (Betschart *et al*., 1979). Sunflower protein for which phytates and polyphenolic compounds were reduced during isolate preparation had improved foaming capacity compared to isolates with higher levels of phytates and polyphenolics (Pawar *et al*., 2001).

7.5.4 Gelation

Isolated sesame globulins were shown to produce harder gels with less syneresis than gels from soy protein (Yuno-Ohta *et al*., 1994), but gels from canola protein isolates were generally not as good as those from soybean (Thompson *et al*., 1982; Owen *et al*., 1992). To produce gels from canola protein upon heating, at least 5.4% protein was required in the system to get any gel formation at pH 7 (Gill and Tung, 1976), and if the pH during testing was increased stronger gels were created (Léger and Arntfield, 1993). The addition of phytates or phenolic compounds, however, resulted in weaker, less elastic gels even at pH 8.5 (Arntfield, 1996). Although the inclusion of salt was able to improve gel strength in the presence of phenolic compounds, the elasticity of the network remained poor (Arntfield, 1996). A similar influence of the phytates and polyphenols on the gelation of sunflower proteins was reported by Pawar *et al*. (2001). These data emphasize the need for isolation procedures which reduce these compounds.

7.6 Improving functionality of oilseed protein

Efforts have been made to improve the functional properties of the oilseed proteins or to isolate proteins with unique properties. Probably the best documented of these efforts involve chemical and enzymatic modification. The benefits of these treatments, and some less frequently used treatments, may be critical to the incorporation of these proteins in food systems.

7.6.1 Presoaking

In an attempt to improve the properties of the proteins in sesame and rapeseed meals, seeds were soaked for 24 h and then dried prior to milling and oil extraction (Mahajan *et al*., 2002). This process resulted in increased solubility, water-holding capacity and foaming properties. Emulsification properties of the rapeseed proteins were superior to those from sesame, but were unaffected by the soaking process.

7.6.2 Selective isolation of proteins

While purification of individual proteins is not normally thought to be a viable approach to preparing protein isolates for incorporation into human foods, there are exceptions to this. With corn, the major 11S protein, zein, has not been considered a particularly good candidate for protein isolation. Nevertheless, a non-zein protein has been extracted from corn that has good solubility and good emulsification properties although it does not form a gel (Myers *et al*., 1994). The isolation of canola proteins can also lead to distinct protein fractions as efforts are made to increase protein recovery (Fig. 7.2) (Rubin *et al*., 1990). While the functional properties of the soluble protein fraction from canola have not been extensively studied, the higher solubility alone will influence the way the protein can be used. It should be possible to recover a similar protein fraction from the supernatant obtained when canola protein is precipitated by dilution rather than pH adjustment (Fig. 7.1).

7.6.3 Chemical modification

Several techniques have been used to chemically modify proteins. One of these is alkylation. This involves the addition of aldehyes to the protein with the intention of binding it to amino, hydroxy, and thiol groups resulting in a decrease in the positive charge on the protein and longer side chains (Sikorski, 2001). Selective alkylation of the 12S rapeseed protein using formaldehyde and sodium borohydrate produced a protein that was unable to form gels (Gill and Tung, 1978). Since the level of the ϵ-amino group for lysine was unchanged, it was suggested that other functional groups, essential for gelation, had been modified.

The chemical modification that has been most used to improve functional properties of proteins from oil-producing plants is an acylation reaction where both acetic anhydride (neutral) and succinic anhydride (negatively charged) have been used. These anhydrides interact with positively charged sites on the protein with the N terminal amino group and ϵ-amino group of lysine being the most readily available (Sikorski, 2001). This results in a change in the net charge of the protein and, at sufficiently high levels of modification, denaturation of protein.

Succinylation of peanut proteins resulted in improved solubility at neutral pH values, as well as improved water absorption, fat absorption and emulsification properties (Monteiro and Prakash, 1996). The succinylation of the sesame α-globulin (Zaghloul and Prakash, 2002) and helianthinin from sunflower (Venktesh and Prakash, 1994) resulted in the accumulation of a 7S fraction as the 12S fraction dissociated and at higher levels of succinylation the proteins were converted to the 2S form. The succinylated sesame protein was more soluble at acidic pH values, had improved fat-binding, water-binding, and emulsification properties compared to the protein which had not been succinylated. The thermal stability of the protein, however, decreased as the denaturation temperature dropped from 84ºC to 62ºC when 40% of the lysine

residues had been succinylated. When the succinylated sunflower was incorporated into bread at levels of 5 and 10%, the bread had the characteristics of being over-fermented (Yue *et al*., 1991). This was overcome to some extent by reducing the fermentation time. There did not seem to be any advantage in using a succinylated protein in this application.

Succinylation and acetylation have both been used to modify canola proteins with the degree of modification varying; levels up 62% acetylation or succinylation (Gruener and Ismond 1996; 1997), 84% succinylation (Paulson and Tung, 1987; 1989) and 93.5% succinylation (Gueguen *et al*., 1990) have been used. Succinic anhydride and acetic anhydride have also been used for acylation of flax protein isolates (Shahidi and Wanasundara, 1998), where the degree of acylation was greater with acetic anhydride. For canola, low levels of modification led to improved foaming and emulsifying activities due to improved interfacial absorption kinetics (Gueguen *et al*., 1990), but foam stability was adversely affected by acylation and emulsion stability decreased at higher levels of modification (Gruener and Ismond, 1997). With flax, acylation improved emulsification properties but had no impact on foaming properties (Shahidi and Wanasundara, 1998). Heat-induced gel characteristics were also improved with the firmest gels being obtained at intermediate levels of modification (Paulson and Tung, 1989; Gruener and Ismond, 1997).

7.6.4 Enzymatic modification

The use of enzymes to aid in protein purification was discussed earlier. Enzymes have also been used to improve the functional properties of the isolated proteins. One approach has been to continue to purify the protein in the isolate by removal of antinutritional factors that impede functional properties. Treating a sesame protein meal with the enzyme phytase improved emulsification properties, but did not change the foaming capacity or stability (Sung *et al*., 1998a).

The more common use of enzymes has been to promote changes in protein structure. A number of techniques exist including phosphorylation, deamidation (Haard, 2001) and cross-linking using a number of different reactions (Feeney and Whitaker, 1985), but the main technique that has been examined in relation to proteins from oil-producing plants is protein hydrolysis. Treating a sesame protein isolate with a combination of phytase and proteinase from *Aspergillus*, resulted in improved oil and water absorption as well as foaming properties (Sung *et al*., 1998b). The choice of enzymes is obviously important to effective hydrolysis. In a comparison of pepsin, papain and trypsin for sesame protein hydrolysis, all three enzymes improved protein solubility, but trypsin was more effective than papain in improving emulsifying activity (Seon *et al*., 1995). Limited hydrolysis (20%) also improved foaming ability, but this advantage was reversed if the level of hydrolysis was too high (Seon *et al*., 1995).

Enzymatic modification of peanut proteins with α-chymotrypsin has also been effective in improving functional properties (Monteiro and Prakash, 1994;

1996). While the different peanut protein fractions responded a little differently to the enzyme treatment, protein solubility in the neutral pH range was improved for all fractions, as were emulsifying and foaming properties.

Sunflower protein functional properties have also been improved by enzymatic hydrolysis. Certainly, the process of using the neutral microbial enzyme kerase to aid in the isolation of protein from sunflower has resulted in a very soluble protein isolate (Parrado *et al*., 1993). Since solubilization of the protein is an initial step for functional properties such as emulsion, foam and gel formation, this isolate was believed to have improved potential for food uses. The high solubility also makes it possible to further fractionate the protein using ultrafiltration. A low molecular weight protein with a low level of aromatic amino acids has been obtained from sunflower using this approach (Bautista *et al*., 1996). A combination of endopeptidase (Alcalase) and exopeptidase (Flavourzyme), or these enzymes used individually, have been used to prepare protein hydrolysates using sunflower protein isolate as a starting material (Villanueva *et al*., 1999). The main advantage to treating with these enzymes was an increase in solubility without changing the amino acid profile. This is not the case with chemical modification where a reduction in available lysine can result. The enzymatic hydrolysates have been recommended for use in high-energy beverages.

While the potential for using enzymes to improve the functionality of proteins from oil-producing crops is clearly demonstrated in these examples, the possibilities in this area are even greater and it may be possible to use enzymes to tailor the properties of isolate for specific applications.

7.6.5 Proteins in mixed systems

Knowing the functional properties of a protein isolate does not necessarily indicate how the protein will behave in food systems. Interactions with other food components and the influence of processing conditions can affect protein performance in food systems. Under the right conditions, protein-polysaccharide interactions have been shown to significantly increase the strength of protein gels. For example, improved gel characteristics for heat-induced canola protein gels have been obtained when guar or methyl cellulose are included (Arntfield and Cai, 1998).

A number of processing treatments, including roasting, puffing, pressure cooking, microwave cooking, germination and fermentation have been evaluated as means to improve the functional properties of proteins in sesame and rapeseed meals (Mahajan *et al*., 1999) Fat binding was improved with all treatments and water absorption improved with all treatments except puffing. Emulsification properties were adversely affected by these treatments, while foaming was enhanced by germination and microwave treatments only. It is not clear if these effects would be seen if protein isolates rather than meals were subjected to these treatments.

7.7 Future trends

7.7.1 Using isolated proteins

The range of food products that can potentially include proteins from oil-producing plants is extensive. The use of proteins as additives for food products is discussed in Chapter 18 of this book. However, with respect to proteins for oil-producing plants, the term 'potential' is significant in that demonstrated uses are primarily at an experimental level and these proteins have yet to receive that commercial recognition seen by soy protein. Nevertheless, for these proteins to become commercially viable, these experimental applications must be researched. Baked goods comprise one area where protein isolates can be used to improve nutritional quality. Adding 18% sesame protein isolate to bread was possible without significantly altering the sensory properties (El-Adawy, 1995). With succinylated sesame proteins, the maximum level of addition was only 10% and required changes to the bread preparation protocol (Yue *et al*., 1991). Peanut proteins, as components in peanut flour, have also been incorporated into baked goods as well as a variety of other products (Singh and Singh, 1991). Sunflower protein was included in an extruded product where 10–20% protein isolate was included with corn starch (Sotillo *et al*., 1994).

Extended or simulated meat products could also benefit from including plant proteins with the appropriate gelling properties available. The regime used by Paulson and Tung (1989) to investigate the gelling properties of canola proteins was designed to emulate that used for comminuted meat products. They were able to demonstrate that succinylated canola proteins possessed the desired properties. Flax proteins have also been recommended for use as additives in meat emulsions (Oomah and Mazza, 1993).

The use of protein isolates to produce films and coatings is another area with great potential. The proteins from oil-producing plants that have been examined for this application include soybean (Anker, 1996), peanut (Anker, 1996; Jangchud and Chinnan 1999a; 1999b), cottonseed (Marquie, 2001) and to a greater extent corn (Anker 1996; Gennadios and Weller, 1990; Gennadios *et al*., 1993; Herald *et al*., 2002; Kleen *et al*., 2002). Variations in the amount of protein, alone or as part of a protein polyethylene system (Herald *et al*., 2002), and the amount of plasticizing agent both contribute to the required tensile strength and stability needed for film production. Whether these films will be edible will also depend on how they are prepared.

Other possible uses for these proteins that have been reported include incorporation of peanut protein into cheeses (El-Sayed, 1997), use of flax proteins as additives in ice cream and fish sauce (Oomah and Mazza, 1993), use of sunflower protein hydrolyzates in high energy beverages (Villanueva *et al*., 1999) and the use of corn protein that had been treated with citric acid to increase metal-binding capacity use as a scavenger in waste-water treatments (Sessa and Wing, 1999).

7.7.2 Future uses of oil-producing proteins

Despite the nutritional value in proteins from oil-producing plants, their use in food systems may be restricted by antinutritional factors and less than ideal functionality. While the isolates and modified isolates have improved the possibilities for these proteins, there are other techniques for protein modification that have not yet been examined. In addition, there is a need to demonstrate how these proteins will behave in food products and what contributions they will make to these products. There must be some advantage (improved nutrition, quality or economy) to facilitate the acceptance of these proteins as food ingredients. Demonstration of these proteins as high quality ingredients that will contribute to the structure and nutrition of food products is certainly a step in the right direction. The production of films has been effective for some of these oil-producing proteins. One would expect to see similar testing using other proteins to examine the full range of properties that can be attained.

The use of these protein and products associated with these proteins may also find a place in the nutraceutical/functional food arena. Peptides from the hydrolysis of these proteins may be bioactive and have a role in disease prevention. Minor components such as phytic acid and phenolic compounds, that have been considered antinutritional and detrimental to protein functionality, may be recovered for use in this area, as both have been shown to have properties that may have health benefits (Shahidi, 1997).

As the production of oilseed will continue to supply the demand for food grade oil, there will be a great deal of protein available from the oilseeds. While more research is required, there will be a place for these proteins in both food and non-food applications.

7.8 References

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8

Cereal proteins

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8.1 Introduction

Cereals are the world's most important food crop, and cereal products are the most important foods. Some are employed as seed (rice, barley, oats, millet, sorghum, maize) others as flour (wheat, rye, maize), or flakes (barley, oats, maize). The protein content of the seeds or the flour determines its technological importance and is related to the quantity and the quality of the proteins. Different cereal varieties are diffuse throughout the world; their production is related to genetic and environmental factors, in particular the amount of proteins. The protein content of the seeds changes every harvest, instead the baked products require seeds and flours with constant properties, this problem may be resolved with knowledge of the chemical physical properties of the main components of the seed: proteins, polysaccharides and their relationships with minor components like enzymes, minerals and lipids. The cereals in the world form a very large family, some of them are grown for traditional use, the most diffuse are reported in Fig. 8.1. Following the biological classification, different species of wheat are commonly grown. The first *Triticum aestivum* forms the classes hard red winter, hard red spring, soft red winter, hard white and soft white. The second *Triticum durum* dsf. includes the durum and red durum wheat. The third species are *Triticum monococcum*, einkorn, *Triticum dicoccum*, emmer and *Triticum spelta*, spelt. Among the minor cereals there is also triticale, a hybrid of wheat and rye.

8.1.1 Proteins in cereals

The cereal seed is a complex structure formed by many individual components and different anatomical parts. The kernel, or caryopsis, is divided into three

Triticeae	*Triticum*	*aestivum*	**Wheat**
		durum dsf	**Durum wheat**
		spelta	**Spelt**
		monococcum	**Einkorn**
		dicoccum	**Emmer**
	Secale cereale		**Rye**
	Hordeum vulgare		**Barley**
	Triticosecale		**Triticale**
Avenae	*Avena sativa*		**Oats**
Oryzeae	*Oryza sativa*		**Rice**
Paniceae	*Pennisetum*		**Millet**
Maydeae	*Zea mays*		**Maize**
Andropogoneae	*Sorghum*		**Sorghum**

Fig. 8.1 Classification of cereals used in food products.

general anatomical regions: the bran, the embryo and the endosperm. The first two are very different in shape and size among the cereals and require specific technology to remove (i.e. corn and rice). The bran is formed by pericarp, testa, nucellar layer and aleurone layer, it contains fiber (pentosan, hemicelluloses, β-glucans cellulose and glucofructans), ash (mineral), high levels of enzymes, vitamins and globulin storage proteins. In the embryo a large number of lipids, enzymes (lipase and lipoxygenase), vitamins and globulin storage proteins are present (Shewry and Halford, 2002). In small cereals the aleurone and the embryo account for only 10% of the grain dry weight and are usually removed by milling (wheat), polishing (rice), pearling (barley) or decorticating (sorghum) before use. The embryo of maize accounts for 10–11% of the grain and its high content of protein and oil are important for nutrition (Shewry and Halford, 2002). The largest portion of the seed is the endosperm, which provides the nutrients necessary for embryo development during germination. The nutrients are made available by the release of enzymes from the aleurone layer and embryo which hydrolysed the reserves. The reserves in the endosperm are starch

granules dispersed in a matrix of storage proteins. A gradient exists to the centre of the grain; the starch concentrations increase as the distance from the periphery increases, while the proteins decrease from the periphery to the centre (Evers and Miller, 2002).

8.2 Protein function in cereals

The storage proteins synthesized in the rough endoplasmic reticulum (ER) are deposited in organelles located within the ER or in storage vacuoles (Vitale and Galili, 2001).

The major function of the storage proteins is to accumulate high levels of amino acids within the limiting space of the seed (Shimoni and Galili, 1996). The entry of the storage proteins into the ER occurs cotranslationally, specified by an N-terminal signal peptide that is cleaved from the nascent polypeptide chain as it enters in the lumen space. Further processing of storage proteins is facilitated by chaperons and enzymes, the BiP, the Binding Protein (Muench *et al*., 1997) and the Disulfide Isomerase (PDI) for correct protein folding and intramolecolar disulfide bonds formation in wheat gliadins (Bulleid and Freedman, 1988). *In vitro* studies of wheat germ extract and microsomes obtained from bean cotyledons were carried out to investigate the maturation of a LMW glutenin. This research suggest the presence of an early step on the pathway of the glutenin polymer assembly which involves the formation of one intrachain disulfide bond and the folding of a soluble monomer (Orsi *et al*. 2001). In maize and rice prolamin accumulate directly within the lumen of the ER leading to the formation of protein bodies surrounded by a membrane, in other cereal, wheat and barley, protein bodies are sequestered into one or more large central vacuoles that contain numerous protein bodies (Herman and Larkins, 1999). Some prolamins, principally gliadins are transported via the Golgi to the protein storage vacuoles whereas others, principally glutenins, are retained within the ER and subsequently absorbed by protein storage vacuoles in a process similar to autophagy. The result in the mature seed is an endosperm formed of a continuous matrix of proteins surrounding the starch granule (Shewry and Halford, 2002).

8.2.1 Protein role in the seed: energy and functionality

Proteins in the seed have a different role: functional, as part of complex structures like membranes and structural proteins, energetic, the storage proteins, as source of amino acid to supply the building blocks required during the protein biosynthesis in the germination, and metabolic, as enzymes necessary for the control of the reactions. During dormancy the proteins, and other components, e.g., lipids and starch, in the cereal seeds are not mobilized from the protein bodies for different reasons, the presence of a low amount of water, the cellular section separation and the presence of different enzyme

inhibitors (Kato *et al*., 2002). During germination the enzymatic machinery starts to work and the protein composition of the seed changes. There is a decrease of prolamins and a corresponding increase in free amino acids between the two and the six days of germination followed by a synthesis of new macromolecules (Kruger and Marchylo, 1985).

8.2.2 Enzymes

The enzymes present in the cereal kernel are necessary for seed development, but also play a role in the processing or are responsible for reactions correlated with the quality of cereal products. α and β amylases are present in all cereals. In mature kernels the amount of α amylase is lower, while it increases abruptly during sprouting or germination. In wheat and rye amylases have an important role to produce sugars from starch for the yeast, but their presence must be controlled. Unfavourable harvest conditions favour sprouting, the amount of α amylase rises and the quality of the wheat decreases. Higher amounts of this enzyme determine an extensive starch degradation during baking that produces a sticky and poor development of the baked goods. Eighty percent of the β amylases in the wheat flour is associated with glutenins. Both α and β amylases are heat labile (Nagodewithana and Reed, 1993). In triticale higher amylase and often protease activity is reported (Macri *et al*., 1986), these enzymes are partially responsible for the rare employment of this cereal in breadmaking.

In the seed acid proteases and peptidases are present. Most endoproteases are sulfhydryl proteases. The protease activity is distributed throughout the cereal grain; their activity increases about 15 fold during germination, these enzymes are not heat stable, their maximal activity was obtained at pH 4.4 (Nagodewithana and Reed, 1993).

In all cereals lipases and lipoxygenases are present and are located in the germ and in the bran. Compared to other cereal, oats contain significant levels of lipase. They are inactivated by heat treatment, an activation of lipoxygenases in pasta products produces an oxidation of carotenoids and lead to a loss of yellow colour (Beliz and Grosh, 1999). Another important enzyme is phytase, about 70% of phosphorus in wheat is bound to phytin, which is 1% of the kernels, this reaction is nutritionally desirable since phytin inhibits the intestinal absorption of iron and calcium ions. Peroxidase and catalase are widely distributed in cereals, the oxidative cross-linking of pentosans catalyzed by peroxidase play an essential role in the dough, in particular in rye. Glutathione dehydrogenase oxidizes glutathione during dough making. It reacts quickly and promotes disulfide interchange in flour proteins. If HMW-glutenins are depolymerized the viscosity of the dough drops. Polyphenoloxidases are presents in the outer layers of the kernels and can cause browning in whole meal flours (Beliz and Grosh, 1999).

8.3 Classification of proteins

The classification of storage proteins is based on Osborne sequential extraction and different solubility. The cereal proteins have been classified in four classes of protein: the water soluble albumins, the salt soluble globulins, the prolamins soluble in alcohol/water mixtures, and glutelins soluble in dilute acid or alkali. Osborne fractionation was a milestone in the development of cereal chemistry but each of these fractions is a complex mixture of different polypeptides with similar solubility, in particular for the gliadins and LMW-glutenin polypeptides. Other classifications and many purification methods have been worked out (Gianibelli *et al*., 2001). In the sixth International Gluten Workshop (Wrigley *et al*., 1996) adopted for wheat a major division on polymeric glutenins and monomeric gliadins, the difference between these two groups of storage proteins was found by analyzing their functionality. In other classifications, utilized by biochemical and genetic researchers, the proteins are divided in three groups: the sulphur-poor (S-poor), the sulphur-rich (S-rich) and the HMW prolamins (Tatham and Shewry, 1995). These groups do not correspond directly to the polymeric and monomeric fraction in wheat but on structural and evolutionary relationships (Shewry and Halford, 2002). The sequences and domains homology of the cereal proteins allowed the identification of a prolamin superfamily which is one of the most widespread groups of plant proteins. It comprises cereal seed storage proteins, low molecular mass sulphur rich proteins (GSP, Pina/b, Trypsin and α amilase inhibitors, 2S albumins) and some cell wall glycoproteins. This superfamily includes several major types of plant allergens (Shewry *et al*., 2002).

The protein concentration in the seeds was calculated from the Kjeldhal or combustion method of Dumas or often by NIR spectroscopy (Brimmer *et al*., 2002). The quantification of the soluble fraction or isolated proteins have been done by micro-kjeldhal or spectroscopic assays. The colorimetric assays often used globular proteins as standard which allows significant errors with gluten protein which present a particular amino acid composition and reaction with Bradford assay, a correct interpretation of the reference curve allowed for routine use (Eynard *et al*., 1994). Other authors use turbidimetry assays and chromatographic areas (Wieser, 1999, Wieser *et al*., 1998). The amount of albumins, globulins and prolamins (gliadins and glutenins) depends on purification methods and the varieties utilized, so a comparison of proteins of different cereals is not easy. In parallel, SDS-PAGE must be carried out for a correct polypeptides identification to avoid interpretation error. In the reduced condition all the prolamin are alcohol soluble and their molecular mass varies greatly from 10,000 to almost 100,000 Da (Shewry and Halford, 2002). The amino acid composition of the cereals is correlated with the botanical genealogy of the cereals. The amino acid composition is similar for wheat, rye and barley. The prolamin composition of oats is intermediate between *Triticaceae* and other cereals. Oats and rice contain 70–80% of globulins and differ from other cereal grains which contain higher amounts of prolamin (i.e. alcohol soluble and

Table 8.1 Amino acid composition of cereal flours[a]

Amino acid	Wheat	Rye	Barley	Oats	Maize	Millet	Rice
Asx	4.2	6.9	4.9	8.1	5.9	7.7	8.8
Thr	3.2	4.0	3.8	3.9	3.7	4.5	4.1
Ser	6.6	6.4	6.0	6.6	6.4	6.6	6.8
Glx	31.1	23.6	24.8	19.5	17.7	17.1	15.4
Pro	12.6	12.2	14.3	6.2	10.8	7.5	5.2
Gly	6.1	7.0	6.0	8.2	4.9	5.7	7.8
Ala	4.3	6.0	5.1	6.7	11.2	11.2	8.1
Cys	1.8	1.6	1.5	2.6	1.6	1.2	1.6
Val	4.9	5.5	6.1	6.2	5.0	6.7	6.7
Met	1.4	1.3	1.6	1.7	1.8	2.9	2.6
Ile	3.8	3.6	3.7	4.0	3.6	3.9	4.2
Leu	6.8	6.6	6.8	7.6	14.1	9.6	8.1
Tyr	2.3	2.2	2.7	2.8	3.1	2.7	3.8
Phe	3.8	3.9	4.3	4.4	4.0	4.0	4.1
His	1.8	1.9	1.8	2.0	2.2	2.1	2.2
Lys	1.8	3.1	2.6	3.3	1.4	2.5	3.3
Arg	2.8	3.7	3.3	5.4	2.4	3.1	6.4
Trp	0.7	0.5	0.7	0.8	0.2	1.0	0.8
Amide groups	31.0	24.4	26.1	19.2	19.8	22.8	15.7

a: total proteins, mole %.

alcohol insoluble polymers). The amount of glutamic acid, glutamine and proline in the *Triticaceae* is higher than in other cereals (Table 8.1) and this difference should be correlated with the Celiac disease (Wieser, 2001).

Prolamin is more variable in structure and sequence than globulin. It is possible that the major groups of prolamins in the *Triticaceae* (wheat, barley, rye) and maize, sorghum, millet had separate evolutionary origins. Most prolamins have two common structural features, first, the presence of distinct regions, or domains, which adopt different structures and may have different origins, the second is the presence of amino acid sequences of reported blocks of short peptide motifs. These features are responsible for their high proportion of glutamin, proline and other specific amino acids (i.e. histidin, glycin, methionin, phenilalanin) (Shewry and Halford, 2002).

8.3.1 Storage proteins

Albumin, globulin and lipid-binding proteins

The water-soluble albumins and salt-soluble globulins are enzymes involved in metabolic activity but several have unknown functions and are not well characterized. Some belong to a family of trypsin/α-amylase inhibitor and are also implicated in plant defence (Singh *et al*., 2001) and they have been demonstrated to be allergens in baker's asthma (Posch *et al*., 1995). Their apparent molecular weight is lower than prolamins (<30,000 Da), their amino

acid composition differs from gluten proteins, they are rich in lysine and poor in glutamic acid. Also present is an HMW-albumin of ≈60,000 Da which forms disulfide linked oligomers and monomers and presents a β-amylase activity. Storage globulins, the legumin-like 11–12S, are located in the starchy endosperm. They comprise subunits of Mr 55,000 that are post-translationally cleaved to give acid (Mr 33,000 in oats, 28–31,000 in rice, 40,000 in wheat) and basic (Mr 23,000, 20–22,000, 22–23,000 respectively) polypeptides chains linked by a single disulfide bond (Shewry and Halford, 2002).

Among the minor proteins two types of lipid-binding protein have been characterized from wheat flour. One belongs to the family of plant non-specific lipid transfer proteins (LTP) and is localized in the aleurone layer and the others have been named purindoline (Pin) and are localized in the aleurone layer in the endosperm; they are related to the endosperm texture. These proteins are present in soft wheat but are absent in durum wheat. They are related to the hardness of the kernel and are also called friabilin. Two types of Pin have been characterized, Pin-a and Pin-b (Dubreil *et al*., 1998). They are monomeric proteins of 15,000 Da in disulfide bridges. In the endosperm they are associated to the starch and this association is mediated by polar lipids (Greenblatt *et al*., 1995). Studies on different wheat lines are carried out to clarify their role in the seed and their relationship with the endosperm texture (Dubreil *et al*., 1998, Morris and Massa, 2003). The Pin have amphiphilic characteristics and have a greater effect on the stability of lipid monolayer films at air-water interfaces. This is an important issue in breadmaking (Gan *et al*., 1995). The structure and composition of the tryptophan-rich region is of major importance for the interaction with the lipids (Kooijman *et al*., 1997).

8.3.2 Monomeric proteins

Prolamins are a group of heterogeneous monomers soluble in 70% aqueous alcohol or other alcohol/mixtures in the native state. According to the electrophoresis mobility in acid-PAGE the wheat gliadins are divided into four groups α- (the fastest mobility) and β- γ- ω- (the lowest mobility). The structure of the gliadins is shown in Fig. 8.2, all gliadins show three different regions, the N and the C terminal regions and the repetitive region, the amino acid length of each is different as shown in Fig. 8.2. The Mr range between 30,000 and 75,000 Da, with two-dimensional electrophoresis up to 50 polypeptides, can be identified and are widely used for cultivars identification. The ω-gliadins are characterized by high levels of glutamine, proline and phenilalanine which represent 80% of the amino acids and by the absence of cysteine; the γ-gliadins differ from α and β in the amount of aspartic acid, proline, methionine, tyrosine, phenilalanine and tryptophan. On the basis of the N-terminal sequence, three different ω-gliadins have been observed and are related to the C hordeins and ω-secalins (Tatham and Shewry, 1995). The α and β gliadins have six cysteines which form three disulfide bonds intramolecular, the non-repetitive domain is rich in α-helix, the repetitive domain is irregular when compared to the γ-

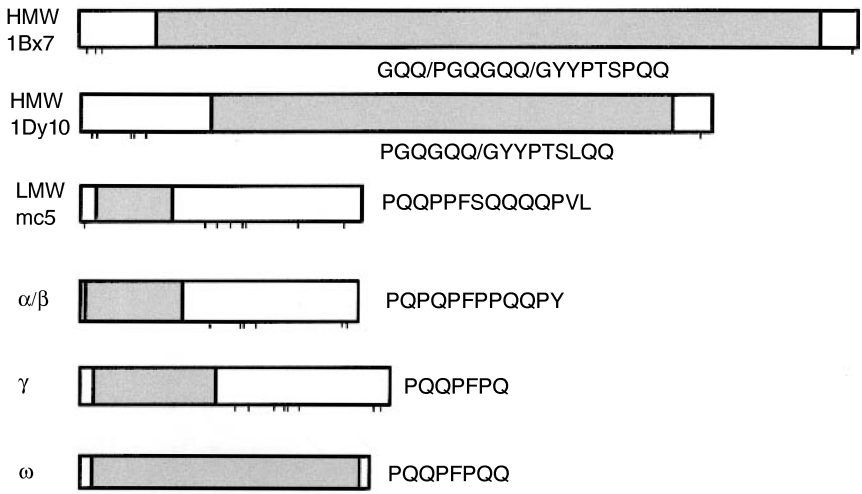

Fig. 8.2 Structure of HMW-GS, LMW-GS and gliadins. The grey areas indicate the repetitive domains; the repetitive sequences are shown. The ticks under the bars indicate the cysteine residues. Data from EMBL, SWISS Prot, Tatham and Shewry, 1995; Gianibelli *et al*., 2001.

gliadins, the last is rich in β reverse turn and forms an extended structure. In the γ-gliadins eight cysteines form intrachain disulfide bonds, these cysteines are located in the C-terminal non-repetitive domains and their position is conserved (Fig. 8.2) (Thompson *et al*., 1994). Prolamins HPLC pattern of other cereals differ greatly from wheat. In rye the hydrophilic ω-secalins are followed by the hydrophobic γ-secalins. In barley a hydrophilic fraction is missing, the C hordeins eluted in the middle area are followed by the hydrophobic B-hordeins. The chromatogram of oats is characterized by two hydrophobic fractions that are close to each other (Belitz and Grosch, 1999).

8.3.3 Polymeric proteins

The molecular weights of glutelin polymers reach over twenty millions of Daltons, in particular glutenins in the wheat fractions are formed by the HMW-glutenins and LMW-glutenins (Stevenson and Preston, 1996). These proteins are a heterogeneous mixture of polymers formed by disulfide-bond polypeptides that can be classified in four groups according to their molecular mobility in SDS-PAGE after reduction (A-B-C-D regions). The A regions (80,000–120,000 Da) correspond to the HMW-glutenins, the B (42,000–51,000 Da) and C regions (30,000–40,000 Da) are LMW-glutenins, γ and α/β gliadins, the D region belongs to the ω gliadins and LMW-glutenins. The genetic and polymorphism of these subunits are review by different authors (Lafiandra *et al*., 2001; MacRitchie and Lafiandra, 2001; Masci *et al*., 2002). The HMW subunit comprises between 630–830 amino acid residues (67,000–88,000 Da) and

consists of three distinct domains. The N-terminal domain (81–104 residues) which contains five cysteines in the y-type and three cysteines in the x-type, and the C-terminal domain where only one cysteine is present. The repetitive domains consist of tandem repeats peptide subunits (Fig. 8.2). Secondary structure prediction on spectroscopic studies of purified HMW and synthetic or recombinant peptides indicated that the repetitive domain adopts an unusual supersecondary structure of loose spirals consisting of regularly repeated β reverse turns. Detailed studies of isolated HMW subunits in the solid and solution states showed the β reverse turn contained glutamine residues that can interact with the solvent (water) to form β turn or form intramolecular hydrogen bonds (β sheet) (Feeney *et al*., 2003). Atomic force microscopy shows that the HMW subunits and the recombinant 58,000 Da peptide are capable of forming self-associating fibrillar structures (Humphris *et al*., 2000).

Studies on the secondary structure of the HMW 1Bx20 and 1Bx7 showed 96% of identity, both proteins formed regularly repeated β reverse turns and their turns prediction were similar in total number and position in the two subunits. The infra-red spectra of the two subunits were almost identical. The conformational stability to urea denaturation measured by CD spectroscopy showed the same pattern and the spectra were indistinguishable in their behaviour. The mixograph analysis with a reduction/re-oxidation procedure has demonstrated a clear difference between the two subunits that is not due to the secondary structure. The only difference which can influence the incorporation of the subunits in the polymer is the substitution of two cysteine residues in the N-terminal domain of subunit 1Bx20 by two tyrosines. This HMW glutenin subunit (HMW-GS) contains only one cysteine in the N-terminal domain instead of three and one cysteine in the C-terminal domain. This difference can influence the incorporation of the polypeptides in the gluten complex and the network formation (Shewry *et al*., 2003).

LMW glutenin subunit (LMW-GS) (B-C-D subunits) represent about 60% of the total glutenins, but they are difficult to identify due to overlapping between LMW-GS and gliadins. Recently, new techniques of purification allow protein characterization (Bean and Lokhard, 2001). Polypeptides with a single cysteine, D-type LMW glutenin subunit can act as terminators during the formation of the gluten polymers and a general decrease of dough strength (Masci *et al*., 1999).

The secondary structures of LMW-GS except for D subunits, have an overall similarity to the structure of the S rich gliadins (Thompson *et al*., 1994). As for the HMW-GS, the length of the repetitive domain defines the variation in size of the LMW-GS. This domain is rich in β turn forming a regular spiral structure, while the non-repetitive domain is rich in α helix and appears to be more compact. These proteins have a cysteine residue in the N-terminal domain (position 5) that is unlikely to form intramolecular disulfide bonds with the cysteine residues of the C-terminal domain because of the rigidity imposed by the repetitive domain and can be involved in polymerization. In addition, LMW-GS have seven cysteines in the C-terminal domain one of which can be involved for intermolecular bonds while the others participate in intramolecular disulfide

bonds (Gianibelli *et al*., 2001). LMW-GS have the ability to form large aggregates that are related to dough strength. Nevertheless, the effect of incorporating LMW-GS was less important for HMW-GS incorporation (Uthayakumaran *et al*., 2000).

8.4 Gluten: formation, properties and modification

8.4.1 Structure

The HMW-GS are minor components in quantity but are a key factor in bread making because they are major determinants of the gluten elasticity (Tatham and Shewry, 1985) and are present in wheat. Several HMW-GS have been associated with bread-making quality. Based on analysis of large numbers of cultivars, a scoring system for HMW-GS has been developed (Payne, 1987) in which individual subunits are graded with numbers based on quality evaluations. The presence of the subunits 5 + 10 was correlated with good quality and the 2 + 12 with poor quality. Such results have been confirmed in laboratories elsewhere, but these results come from complex interactions that define wheat quality including LMW-GS and gliadins. The effect of individual proteins HMW-GS, LMW-GS, gliadins, hordein, can be evaluated studying the mixing properties of a base flour modified by incorporation or addition of specific proteins (Bekes and Gras, 1999), or by specific breeding line (MacRitchie and Lafiandra, 2001). The cysteine residues in the primary structure of the HMW-GS and LMW-GS allow identification of different polymer building subunits: chain extenders (subunits with two or more cysteine residues that can form intermolecular disulfide bonds) and chain terminators (with only one residue of cysteine available for intermolecular disulfide bonds). In the first case we obtain stronger dough, in the last the opposite effect. The chain extender proteins with longer repetitive domain increased the stability and the strength of the gluten, in the durum wheat the higher presence of the LMW-GS, with a short repetitive domain reduced gluten elasticity. Gluten quality is related to the size and structure of the network formed in the dough.

Different models have been proposed over the years to understand the gluten structure. The ‘loop and train’ and the ‘entanglement’ models have a large consensus but are only the first step to resolving the structure-functionality relationships in the wheat flour. In the ‘loop and train’ model the properties of the glutenin are explained with a combination of intermolecular hydrogen bonded and solvated domains. The model predicts that the strength of the elastic restoring force depends on the number and the nature of the intermolecular interactions in the hydrated mass. This depends on the lengths of the repetitive domains and the precise sequence of the repeat motif (Feeney *et al*., 2003; Belton, 1999). The ‘entanglement’ model proposed by MacRitchie (1999) is inspired by the polymer theory. He suggests that the glutenin polymer can be described as an entangled polymer network. In such a network, covalent aggregates become joined through physical entanglements. Their amount is

related to the structure, size and concentration of the polymer. The emphasis on gluten polymer size has prompted research to assess the molecular size distribution of the macropolymer using techniques like multistaking gel electrophoresis, free field flow techniques and light scattering (Hamer and Van Vliet, 2001). The structure of the gluten network is a superimposition of both covalent and non-covalent interactions. Hydrogen bonds, hydrophobic interactions and electrostatic interaction (salt, metal ion bridges) are relatively weak but together give greater strength (Hamer and Van Vliet, 2001).

The protein during mixing forms a continuous viscoelastic network throughout the dough. The surface of the gluten is also apolar and can be measured by ligand binding techniques with hydrophobic probes, ANS (Sironi *et al*., 2001), or chromatographic techniques (Torres *et al*., 2000; Popineau, 1985). The surface hydrophobicity is different among grains and depends also on technological treatment (Guerrieri *et al*., 1996). The stepwise reduction (TCEP as reducing agent in presence of ANS) shows different behaviour in surface hydrophobicity of gluten of bread and durum wheat. Two main steps are present: the reduction start for all gluten with 1mM TCEP, these disulfide bonds are easily accessible in the gluten complex (glutenin and gliadins) but the gluten stability and released polypeptides are different between *Triticum durum* and *aestivum*. A second step was evident at 25mM TCEP for the bread wheat whereas the durum wheat required 50mM TCEP. These data suggest a different organization of the gliadins and glutenins in the gluten that need further investigation (Guerrieri *et al*., 2001a).

Studies with monodisperse polymers showed that for each polymer there exists a narrow temperature or elongation rate window within which maximum drivability occurs (Singh and MacRitchie, 2001). Gliadins generally contribute to the viscosity and extensibility of the gluten. Some authors associate specific gliadins alleles with bread-making and pasta-making quality but they may not have a direct effect on wheat quality (Porceddu *et al*., 1998). They are a marker of quality, their presence in the genes is related to the presence and expression of LMW2 (good quality) or LMW1 type (poor quality) associated with the γ gliadins 45 or 42, and the presence of chain terminator (subunit C) can affect the quality of the gluten as for the cultivar Demetra that shows LMW2 type pattern (Masci *et al*., 2001).

8.4.2 Gluten modification: heat treatments and interactions

The size of glutenin polymers increases strongly for samples heated above about 50 ºC (Lefebvre *et al*. 1994), showing a reorganization of intermolecular covalent bonds between the glutenin subunits and possibly the gliadins. From 10 to 50 ºC the disruption of low energy intermolecular bonds is observed. Above this temperature, the sulphydryl-disulfide interchange imposes a polymer size increase of the glutenin. The increase in flexibility of the mobile label (TEMPO) observed in heated gluten or exposed to urea indicates that the effect of hydrogen bonds is dominant as compared to hydrophobic interactions

(Hargreaves *et al*., 1995). Heating of gliadins and gluten has been reported to decrease their α helix content (Tatham and Shewry, 1985). Compared to globular proteins, prolamins have a high degree of mobility when hydrated. In the first step of the heat denaturation a better solubilization rather than denaturation is reported, probably due to the glutamine residues. One consequence of this behaviour is that wheat gluten appears to have no significant protein-related cooperativity transition on heating. There is no transition from a 'folded' to a 'denatured' state as for globular proteins (Mills *et al*., 2001). The total surface hydrophobicity of the gluten occurring during a process can be measured with a fluorescence hydrophobic probe (ANS) (Guerrieri *et al*., 1996). A first transition was identified in the gluten heated at 45 ºC, the increase in hydrophobicity could be due to conformational changes and to the exposition of previously unavailable hydrophobic areas. The structural modification induced by the temperature (45 ºC) suggests a decrease of the strong interactions between gliadins and glutenins. The glutenins rich in glutamine residues could start to hydrate, instead the more hydrophobic gliadins expose their sites to the probe. These modifications involve also tyrosine residue that becomes more exposed to the solvent. On heating the gluten starts to aggregate, the molecular mass increases and the solubility decreases with interchange of disulfide bridges (Guerrieri *et al*., 1996). A stepwise titration with reducing agent (DTT) indicated that up to 65 ºC only a polypeptide of 55,000 Da and the HMW albumin required increasing amounts of DTT to be released (Lavelli *et al*., 1996). In the flour or in the dough the heat treatments are not the same as in the model system. The starch modifies the heat transfer to the gluten as evidenced by the HTST wheat flour (Guerrieri and Cerletti, 1996). The starch has a 'protection' effect in the gluten, it is more heat resistant, only some minor modifications involve the hydrophobic region of the surface.

The starch-protein interaction plays an important role in the flour and in the dough, the proteins localized on the starch granule surface are responsible for the technological properties of the flour. The rheological properties of model systems (gluten-starch added of fat and oil), were investigated recently by Watanabe *et al*. (2002). During the baking process the damage starch or the dextrin influence the protein-polysaccharide's interaction and the water distribution modifying the bread shelf-life (Martin and Hoseney, 1991) through a less clear mechanism that involves the water migration from the starch to the protein and the starch-dextrin-protein interaction. A model system with starch, amylopectin, amylose and gluten or gliadins was employed to clarify it after a heat treatment an interaction between polysaccharides and proteins can exist. The amyloglucosidase was utilized as a probe to follow the accessibility of the polysaccharide. The gliadins showed a reduction of accessibility to the probe especially with starch and β-dextrin, this behaviour suggests a polysaccharide-protein interaction (Guerrieri *et al*., 1997) that should be further investigated.

8.5 Processing and modification of cereal proteins in cereal products

8.5.1 Bread

The unique properties of the wheat gluten is derived from the structure of the gluten complex and the relationship between glutenins and gliadins. Among other cereals only rye can form a gluten-like structure which is completely different from wheat gluten and it is classified as poor-quality flour because its dough lacks elasticity and gas retention that depends on the different polypeptide structures and assembly (Bushuk, 2001). The presence of pentosans (arabinolxylan polymers) replace the network of the gluten in this cereal. Rye alone or when mixed with wheat flour produced characteristic breads. The baking process is different from wheat bread and the use of sourdough or acid fermentation is fundamental for a good-quality product. The acid fermentation modified positively the protein structure together with the arabinoxylan network. In the triticale, a hybrid of wheat and rye, the presence of the rye glutenins produced a poor quality gluten that could be enhanced only by wheat flour or vital gluten being added to the dough (Macri *et al*., 1986).

Other cereals such as flour, flakes, whole seeds or wheat bran were added to the wheat flour together with vital gluten and improvers to permit correct development of loaf volume in breads. For every mixture technological adjustments must be made to obtain the correct network formation because often these cereals have a detrimental effect on the protein-protein association (Dendy and Dobraszczyk, 2001).

8.5.2 Crackers, cookies and biscuits

In cracker, cookie and biscuit production, the characteristic texture of the product depends primarily on properties of the gluten in the flour used; in Table 8.2 the principal use of cereal is reported. For cookies and biscuits, the texture, flavour and colour of the final product depends also on the raw material used, shortening, sugar, milk, leavening and flavouring agents. Each ingredient has a specific purpose and produces a different final product. Flour in these products often is less than 50% of formula. In soda crackers flour constitutes 88–90% of the formula and the quality of the final product is related to yeast fermentation and gluten development. The addition of bacteria and enzymes improves the modification of the gluten. Proteinases present in flour and yeast split gluten into peptides and amino acids. With the addition of standardized proteinases, gluten breakdown can be optimized to obtain high-quality soda crackers. The two-stage 24-hours fermentation process is generally used for soda crackers to obtain the desired characteristics. Changes in gluten during fermentation include diminished resistance, increased extensibility, formation of smaller components and diminished water-retention capacity (Van Wakeren and Popper, 2002).

Table 8.2 Protein content and food use of cereals

Cereal	Protein[a]	Principal use
Wheat	8.0–17.5	Bread, pasta, crackers, cakes, biscuits, cookies, breakfast cereals, infant foods, feed.
Maize	8.8–11.9	Food, brewing, breakfast cereals, feed.
Barley	7.0–14.6	Food, high-fibre healthy foods, flakes, malting, brewing, feed.
Rice	7.0–10.0	Food, noodles, breakfast cereals, cakes, cookies, bread, baby foods, fermented grain (miso), fermented beverages (sake).
Oats	8.7–16.0	Porridge, breakfast cereals, flakes, biscuits, cookies, infant food, feed.
Rye	7.0–14.0	Bread, distilling, feed.
Triticale	11.7–16.3	Feed.

a: % on dry matter.

8.5.3 Pasta

Pasta products are made from wheat semolina and grist in which the flour extraction grade is less than 70%. The preferred ingredient is durum wheat semolina rather than soft wheat. Pasta products are manufactured continuously by a vacuum extruder that consists of a mixing trough and press segments. The vacuum is used to retard oxidative degradation of carotenoids and to avoid air bubbles in the dough. The pasta quality is related to the granulometry (250 μm is the optimal) and water absorption. The protein network surrounding the granule starch is then stabilized by the drying process. A good-quality gluten is required for a low-temperature process but also a low-quality gluten can be used with a high-temperature drying process. A formation of Maillard components is apparent in these processes and a loss of lysine and other amino acids reduces the nutritional value of these pasta products (Novaro *et al*., 1993). The quality of semolina used for pasta making is defined by final product quality which is influenced by the biochemical composition and physical state of the semolina. The yellow colour of pasta remains a variety characteristic that is not really influenced by milling conditions because the pigment distribution is identical throughout the grain (Abecassis, 2001).

It is possible to produce good pasta quality also with cereals other than wheat, like rice, maize, barley and buckwheat flour but it is necessary to modify the process to promote specific structures. To obtain good-quality pasta from non-traditional raw materials some adjustments may be necessary such as correction of the rheological properties and modification of traditional processing technology. Correction of the rheological and functional properties is possible by adding vital gluten (5–10%) or gluten-like proteins (whey proteins or caroubin) which can be used to form a protein network to hold starch during pasta cooking. Caroubin is a protein isolated from carob germ and can be used to produce pasta for celiac people (Feillet and Roulland, 1998). Some other

ingredients are necessary to promote the network formation or to modify starch swelling during cooking like ascorbic acid, tartrazine, sunset yellow, β-carotene and vitamin E). The traditional process for rice noodles, which involves starch gelatinization followed by retrogradation, can be adapted to improve the resistance of pasta during cooking. Extrusion-cooking technology is highly versatile and permits the use of a wide range of raw materials (Marconi and Carcea, 2001).

8.5.4 Modification of cereal proteins

The traditional modification of the wheat gluten to improve functionality is the limited proteolysis. The peptides obtained are more efficient in stabilizing emulsions and foams. The limited proteolysis modifies the surface properties of the gluten and separation of the hydrophobic fraction improves the foaming and emulsifying capacity (Linarès *et al*., 2000; Popineau *et al*., 2002). Transglutaminase is a relatively new enzyme used in the manufacture of baked goods, it occurs widely in nature in mammalians and in microorganisms and is produced on an industrial scale through biotechnological processes. In a baking application this enzyme is present in preparations containing xylanase and amylase and is used for frozen dough applications to avoid the negative influence of deep freezing on dough quality. The transglutaminase stabilizes the gluten structure through cross-linking among the polypeptides and the network becomes stronger and less sensitive to ice crystal damage (Gerrard *et al*., 2000). It is also used in high-speed mixing pan breads, its effect improves not only the dough process but also the final bake quality in bread with a high proportion of fibres like bran or rye which interferes with the expansion of the loaf. In Asian-type steam bread a good dough stability is essential to obtain an acceptable final product. The use of transglutaminase enhances water absorption of the proteins which are then released to the starch during gelatinization in baking. The same effect is noted in bread baked with rice, corn, barley or rye flour (Poza, 2002; Basman *et al*., 2002a). It is used also to improve the functionality of soft wheat or in bug-damaged wheat (Basman *et al*., 2002b).

8.6 Future trends

Grain cadmium is reported to be higher in durum wheat than in common wheat. New processes involve a screening of the germoplasm for sources of low cadmium and the development of protocols for breeding of low cadmium cultivars (Clarke *et al*., 2002). Breeding research will develop new innovative lines to obtain durum wheat with better characteristics. Some of these new features are shown in Table 8.3.

The development of functional food and functional ingredients has been growing recently. Cereals can be used for non-dairy probiotic products such as fermentable substrates for probiotic microorganisms like lactobacilli and

Table 8.3 Future trends

Products	Features
Cereal based 'functional products'	• Fermentable substrate for probiotic microorganisms. Dietary fibre promoting. • Prebiotics effects: presence of non-digestible carbohydrate. • Encapsulation materials for prebiotic food. • Production of fructooligosaccharides (FOS): soluble dietary fibre use as ingredient (pasta).
Breeding lines	• New bread lines to improve quality. • Durum wheat with low cadmium. • Durum wheat with different hardness.
Novel products	• Pasta made with pseudo-cereals: amaranth, quinoa, buckwheat. • Pasta, biscuits, crackers, bread enriched in barley (β glucans and tocols). • Use of whole seed for cereal breakfast and snack foods. • Texturized wheat gluten added to hamburgers and vegetarian food.
Gluten for non-food use.	• Good film forming and mechanical properties (protective coating, paper coating, adhesives). • High barrier properties for gas (O_2, CO_2) for packaging materials. • Surface active properties (various surfactants).

bifidobacteria. Lactic acid fermentation of cereal is a long-established processing method used in northern Europe, Asia, and Africa for the production of foods in various forms like beverages, gruels, porridge and sourdough production (Charalampopoulos *et al*., 2002). Another growing field in health foods is the nutriceutical foods used as new prebiotic products from germinated barley (Bamba *et al*., 2002).

The supercritical fluid extrusion (SCFX) is a new hybrid extrusion technology which uses supercritical carbon dioxide (SC-CO_2) to expand structure development at lower temperatures to avoid heat-sensitive nutrients and flavour loss (Chen *et al*., 2002). In other processes, the cereal products have finished product textures composed of visible, joined, individually cooked cereal grains. Products with visible-grain textures are achieved by first cooking and infusing in water and milk into individual cereal grains. Finally the mixture is formed into shaped units that are baked or fried and then frozen. Shaped products can be made with whole and modified cereal grains, such as white, brown and wild rice, wheat, soy, millet, corn oats, barely, rye, buckwheat and sorghum. They can be used alone or blended with two or more types of grain. The visible grain technology can be used with three different profiles: breakfast products, rice shell entrées and rice fries, gourmet grain entrées, burgers (Zukerman, 2000).

Texturized wheat gluten is available commercially in several forms differing in size, shape, density, colour, texture and structure coarse powder, flakes, chips,

expanded chips and cylindrical chunks. These products can be used as ingredients for extension in meat foods and in vegetarian foods (Manigat *et al*., 1999).

Wheat gluten can be used in the non-food industry as a very promising biopolymer for coating, adhesives and disposables. Often the gluten for the film forming is modified by esterification, deamidation or other chemical modification which changes the mechanical properties of gluten (De Graaf *et al.*,1999). Wheat gluten films can act as active layers when the film contributes to food preservation. The development of bio-packaging or edible films with selective gas permeability is very promising for controlling respiratory exchange and improving the conservation of fresh or minimally processed fruits and vegetables (Guilbert *et al*., 1999).

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9

Seaweed proteins

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9.1 Introduction: seaweed and protein content of seaweed

9.1.1 Seaweed consumption

Marine plants are mainly exploited for the production of phycolloids or foods. The annual global production is estimated to 2,000,000 t dry weight with 90% coming from China, France, Korea, Japan and Chile (Zemke-White and Ohno, 1999). In total 221 species of algae are used with 145 species as food and 101 species for phycolloid production.

Seaweeds have been used in human or animal diets from early times. Traditionally, they are consumed as sea vegetables in Far East countries especially in Japan (Nisizawa *et al.,* 1987). In this country more than one hundred species of seaweeds are used in food (Fujiwara-Arasaki *et al.,* 1984) and it is reported that Japanese annually consume 1.6 kg (dry weight) per capita of this marine resource.

The main species used as foods belong to the brown, green and red seaweed groups (Table 9.1). Among the more extensively consumed, we find *Laminaria japonica* (kombu*), Undaria pinnatifida* (wakame) and *Porphyra tenera* or *yezoensis* well known under the name of nori. Other species belonging to the green seaweeds are also consumed as sea vegetables, for example species such as *Ulva lactuca* or *Ulva pertusa* which are commercially available under the name of 'sea lettuce'. In contrast, little seaweed is eaten in the Western world and especially in Europe. They are mainly exploited for colloid extraction. However, during the past decade, seaweed consumption as sea vegetables or 'novel foods' has been increasing (Darcy-Vrillon, 1993).

Generally, marine algae are often used in human or animal foods for their mineral content or for the functional properties of their polysaccharides. They

Table 9.1 Examples of seaweed species used in the food industry (Zemke-White and Ohno, 1999)

Species or genus	Common name	Uses	Annual world seaweed production 1995 (tons of dry weight)
Laminaria japonica	Kombu	Sea vegetables, colloids	682,600
Laminaria digitata	Kombu breton	Sea vegetables, colloids	13,400
Undaria pinnatifida	Wakame	Sea vegetables	127,708
Ulva sp (lactuca or *pertusa)*	Sea lettuce, Aosa, Aonori	Sea vegetables	1,500
Chondrus crispus	Irish moss, pearl moss	Sea vegetables, colloids	12,213
Porphyra tenera or *yezoensis*	Amanori laver	Sea vegetables	130,622
Palmaria palmata	Dulse	Sea vegetables	130

are rarely promoted for the nutritional value of their proteins. However, some red seaweeds such *Porphyra tenera* or *Palmaria palmata* can have protein contents higher than those recorded from pulses such as soybean (Fleurence, 1999a). Other algae such as *Ulva*, *Undaria* or *Entormorpha* have protein levels comparable to those reported for common vegetables (Amano and Noda, 1990).

9.1.2 Proteins in seaweeds

The protein content of marine algae differs according to species. Generally, it is low for brown seaweeds (3–15% of dry weight), moderate for green algae (9–26% of dry weight) and high for red seaweeds (maximum 47% of dry weight). Except for species *Undaria pinnatifida* (wakame) which has a protein content of 11 to 24% of dry weight, most of the brown seaweeds used as sea vegetables or for colloid extraction contain less than 15% protein on a dry weight basis (Table 9.2). The situation is different for edible green seaweeds belonging to the genus *Ulva* because their protein concentration can represent 9 to 33% of plant dry mass. *Ulva pertusa* which is frequently consumed under the 'aonori' name by Japanese people shows a high protein level (26% of dry weight) (Table 9.2). Some works gave a value of 32% for protein content of *Ulva lactuca,* but this rate appears to be a seasonal high value.

However, higher protein contents were recorded for certain red seaweeds. For instance, *Porphyra yezoensis* can contain up to 47% of proteins expressed according to dry mass (Fujiwara-Arasaki *et al.,* 1984). This level is higher than those found for high-protein pulses such as soybean. *Palmaria palmata*, another red seaweed can contain 35% of proteins (dry mass) (Morgan *et al.,* 1980). This

Table 9.2 Protein levels of some seaweeds consumed as foods in human nutrition

	Phaeophyta			*Chlorophyta*		*Rhodophyta*		
Seaweed species	*Laminaria digitata* (1)	*Ascophyllum nodosum* (2)	*Undaria pinnatifida* (3,4)	*Ulva lactuca* (5)	*Ulva pertusa* (6)	*Palmaria palmata* (7)	*Porphyra tenera* (3)	*Chondrus crispus* (8)
Protein content (% of dry weight)	8.0–15.0	3.0–15.0	11.0–24.0	8.7–32.7	17.5–26.0	8.0–35.0	33.0–47.0	21.4

(1) Augier and Santimone, 1978; (2) Smith and Young, 1954; (3) Fujiwara-Arasaki *et al*., 1984; (4) Fleurence, 1999a; (5) Abdel-Fattah and Sarry, 1987; (6) Nisizawa *et al*., 1987; (7) Morgan *et al*., 1980; (8) Young and Smith, 1958.

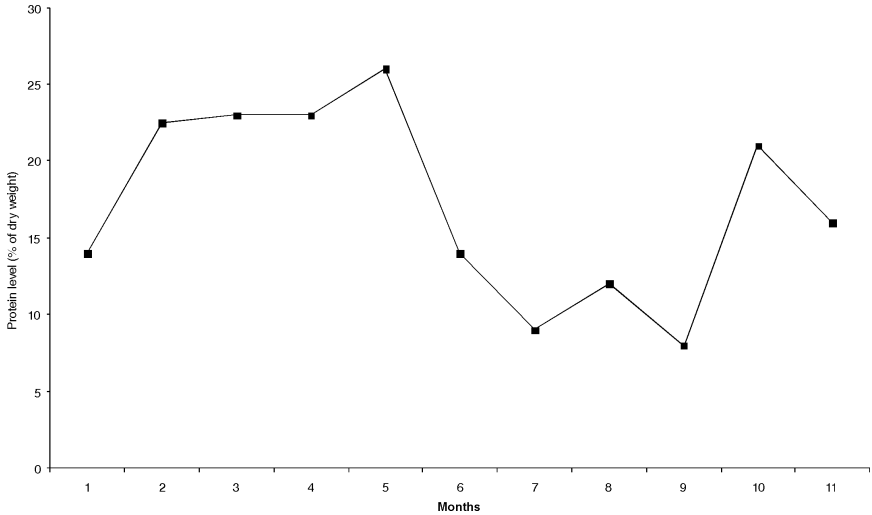

Fig. 9.1 Seasonal variation of protein content of *Palmaria palmata* (dulse) (from Fleurence, 1999a).

species is well known as dulse (Great Britain and North America) or dillisk (Ireland) and is traditionally consumed in northen Europe.

The protein content of seaweeds also varies according to a seasonal cycle. For instance, the protein content of *Palmaria palmata* collected on the French Atlantic coast showed large variations (9 to 25% of proteins) (Fig. 9.1) with the highest values occurring during the winter and spring months (Fleurence, 1999a).

Seasonal variation of the algal protein content has also been reported for other species belonging to green or brown seaweeds. For example, the green seaweed *Ulva lactuca* possesses a protein level that reaches maximum in August (32.7% of dry weight) and a minimum in April (8.7%) (Abdel-Fattah and Sarry, 1987). Variations in protein content according to season were also observed for edible brown seaweeds such *Laminaria digitata* (6.5 to 14.5% of dry weight) (Augier and Santimone, 1978).

9.2 Composition of seaweed proteins

The amino acid composition of algae has been often studied and frequently compared to that of other food proteins such as legumes or eggs. For most seaweeds, a large part of the amino acid fraction is comprised of aspartic and glutamic acids (Table 9.3). It is especially true with proteins from brown seaweeds. For example, in *Fucus* sp. these two amino acids can represent 22 to 44% of the total amino acids or 19 to 41% of the protein fraction (Munda, 1977). For *Laminaria digitata*, species consumed as sea vegetable under the name 'Kombu Breton', the combined glutamic and aspartic acid fractions can reach approximately 18% of the protein fraction (Augier and Santimone, 1978) (Table

Table 9.3 Amino acid composition of some seaweeds and other food proteins (in g amino acid/100 g protein)

	Phaeophyta		*Chlorophyta*		*Rhodophyta*				
Amino acids	*Laminaria digitata* (brown seaweed) (1)	*Undaria pinnatifida* (brown seaweed) (2)	*Ulva armoricana* (green seaweed) (3)	*Ulva pertusa* (green seaweed) (2)	*Palmaria palmata* (red seaweed) (4)	*Porphyra tenera* (red seaweed) (2)	*Chondrus crispus* (red seaweed) (5)	Legumi–nous plants (6)	Ovalbumin (2)
Histidine	1.3	2.7	1.2–2.1	4.0	0.5–1.2	1.4	0.9	3.8–4.0	4.1
Isoleucine	2.7	2.9	2.3–3.6	3.5	3.5–3.7	4.0	1.8	3.6	4.8
Leucine	5.4	5.1	4.6–6.7	6.9	5.9–7.1	8.7	2.9	7.3	6.2
Lysine	3.7	4.3	3.5–4.4	4.5	2.7–5.0	4.5	4.9	6.4–6.5	7.7
Methionine	1.6	2.0	1.4–2.6	1.6	2.7–4.5	1.1	0.5	1.2–1.4	3.1
Phenylalanine	3.2	3.7	5.0–7.1	3.9	4.4–5.3	3.9	1.5	2.4	4.1
Threonine	4.4	2.4	4.5–6.8	3.1	3.6–4.1	4.0	2.2	4.0	3.0
Tryptophan	0.8	0.8	–	0.3	3.0	1.3	–	1.6–1.9	1.0
Valine	4.2	4.1	4.0–5.2	4.9	5.1–6.9	6.4	–	4.5	5.4
Cysteine	1.7	0.5	–	1.2	–	0.3	–	1.1–1.3	1.3
Arginine	0.3	7.5	4.3–8.7	14.9	4.6–5.1	16.4	33.6	13.0–14.0	11.7
Aspartic acid	8.7	5.6	6.0–11.8	6.5	8.5–18.5	7.0	3.8	4.7–5.4	6.2
Glutamic acid	9.4	5.1	11.7–23.4	6.9	6.7–9.9	7.2	4.1	6.4–6.7	9.9
Alanine	14.4	4.8	5.5–7.7	6.1	6.3–6.7	7.4	3.8	–	6.7
Glycine	4.3	4.4	6.3–7.5	5.2	4.9–13.3	7.2	3.5	–	3.4
Proline	3.7	2.8	5.0–10.5	4.0	1.8–4.4	6.4	1.9	–	2.8
Serine	4.0	2.8	5.6–6.1	3.0	4.0–6.2	2.9	2.2	–	6.8
Tyrosine	1.5	1.6	4.4–4.7	1.4	1.3–3.4	2.4	1.0	2.3–2.6	1.8
Alanine	14.4	4.8	5.5–7.7	6.1	6.3–6.7	7.4	3.8	–	6.7

(1) Augier and Santimone, 1978; (2) Fujiwara-Arasaki *et al*., 1984; (3) Fleurence, 1999a; (4) Morgan *et al*., 1980; (5) Young and Smith, 1958; (6) Fowden, 1954.

9.3). A similar result was recorded for the edible brown seaweed *Ascophyllum nodosum* (Smith and Young, 1954).

The species *Undaria pinnatifida* (wakame), a brown seaweed with a high protein level was recorded (to 24% of dry weight), is characterized by a high level of methionine (2 mg/100 g of proteins) in regard to pulse proteins (Table 9.3). For this seaweed, the most abundant essential amino acids are respectively arginine (an essential amino acid for children), leucine, lysine and valine (Table 9.3). Concerning non-essential amino acids, *Undaria pinnatifida* also contains high levels of glutamic and aspartic acids, alanine and glycine (Table 9.3).

The green seaweed proteins are also characterized by a large proportion of glutamic and aspartic acids. The level of these amino acids together can represent up to 26% and 32% of the total amino acids for the species *Ulva rigida* and *Ulva rotundata* (Fleurence *et al.,* 1995b), and can reach up to 35% in the case of *Ulva armoricana* (Table 9.3). *Ulva pertusa* which is frequently consumed in Japan, shows an amino acid profile characterized by high concentrations in essential amino acids such as arginine (14.9 mg/100 g of proteins) or leucine (6.9 mg/100 g of proteins) (Table 9.3). This level of arginine (near 18% of total amino acid fraction) exceeds that of ovalbumin.

Red seaweed proteins seem to possess lower amounts of glutamic and aspartic acids than those recorded from other algae groups. The two *Porphyra tenera* amino acids constitute together only 15% of the total amino acid fraction compared to 13 to 35% for the green seaweed of *Ulva* genus. However, the situation is less clear with *Palmaria palmata* for which a great variation in aspartic acid level is recorded according to season (8.5 to 18.5% of the total amino acid fraction) (Table 9.3). Essential amino acids can constitute almost 46% of the total amino acid fraction of *P. palmata,* an amount quite similar to that recorded for ovalbumin. In addition, proteins from *Palmaria palmata* possess a methionine content similar to ovalbumin and higher than that reported for leguminous pulses (Table 9.3). *P. palmata* proteins also appear to be an interesting food source of threonine and leucine (Table 9.3).

Chondrus crispus, a red seaweed consumed in Ireland as a sea vegetable, is mainly characterized by an abundance of arginine (34% of the total amino acid fraction). Most seaweeds, especially green and red algae, seem to be an interesting and suitable source of proteins for human or animal food. However, algal protein digestibility often appears to be limited by the presence of various anti-nutritional compounds such as polysaccharides or trypsin inhibitors.

9.3 Algal protein digestibility

Generally, the protein nutritional value is determined in terms of two factors, amino acid profiles and protein digestibility. A protein with an excellent amino acid composition would have a fair nutritional value if its digestibility remains low (Wong and Cheung, 2001a). Most studies on algal protein digestibility have been performed *in vitro* from proteins extracted under strong alkaline

Table 9.4 Relative *in vitro* digestibility of protein alkali extracts or protein contained in algal powder (*P. palmata*) (Fujiwara-Arasaki *et al*., 1984; Galland-Irmouli *et al*., 1999)

Seaweed species	Pepsin % digestibility*	Pancreatin % digestibility*	Pronase % digestibility*
Ulva pertusa (green seaweed)	17.0	66.6	94.8
Undaria pinnatifida (brown seaweed)	23.9	48.1	87.2
Porphyra tenera (red seaweed)	56.7	56.1	78.4
Palmaria palmata (red seaweed)		56.0	

* Relative digestibility is expressed as a percentage compared with casein digestibility.

conditions. Digestion of the protein fraction is carried out by means of three enzymes or an enzymatic mixture such as pepsin, pancreatin and pronase for 5 h. The relative digestibility of algal proteins is then referred to the digestibility of casein (100%). Under these conditions, proteins extracted from *Ulva pertusa* are up to 67% digestible in the presence of pancreatin and greater than 90% digestible with pronase (Table 9.4) (Fujiwara-Arasaki *et al.,* 1984). The relative digestibility of proteins from *Porphyra tenera* is 70% in the presence of pronase and 56% with either pepsin or pancreatin. These results suggest a high digestibility of algal proteins when they are submitted to the action of pronase, and more moderate digestibility in presence of other proteolytic systems.

On the other hand, recent studies were performed using water-soluble proteins extracted from *Ulva armoricana*, *Palmaria palmata* and *Sargassum sp.* In the case of *U. armoricana,* the water-soluble proteins are little degraded by trypsin or chymotrypsin. In contrast, they appear more sensitive to the action of enzymatic complexes such as human intestinal juice (Fleurence *et al.,* 1999). Proteins extracted from *Sargassum sp.* show in the multi-enzyme method (trypsin, chymotrypsin, intestinal peptidase), a digestibility up to 75% (Wong and Cheung, 2001a). In another study, it was shown that *in vitro* digestibility of protein concentrates from red seaweed *(Hypnea japonica*) or from green seaweed (*Ulva lactuca*) is near 89 or 85%, respectively (Wong and Cheung, 2001b).

Proteins from raw *Palmaria palmata* show a relative digestibility of 56% after successive action of pepsin and pancreatin (Galland-Irmouli *et al.,* 1999). This digestibility appears moderate and suggests the presence of algal compounds that may limit the proteolysis.

9.3.1 Inhibition of algal protein digestibility

Seaweeds are known to possess high values of soluble fibres, 17–33% on a dry matter basis (Darcy-Vrillon, 1993). Fibres extracted from *Laminaria japonica*

and *Undaria pinnatifida* were described as developing a significant inhibitory effect (55 and 21%) on pepsin activity (Horie *et al.,* 1995). Glycosylation is also a mechanism that protects proteins against proteolysis by digestive enzymes. For the green seaweed *Ulva armoricana,* variations in protein fraction digestibility seem to be related to the seasonal differences in the glycoprotein content (Fleurence *et al.,* 1999), whereas other seaweeds contain high level of lectins that could affect protein digestibility (Benivides *et al.,* 1998). However, the presence of polysaccharides in large quantities remains probably the main cause of fair or moderate digestibility of the algal proteins.

In *Palmaria palmata*, the fibre content is mainly composed of water-soluble xylans (at least 30% of the dry mass) (unpublished data). The interaction between polysaccharides and algal proteins or digestive enzymes has already been reported and suggested as the main cause of weak digestibility of algal proteins (Galland-Irmouli *et al.,* 1999). Therefore, an enzymatic treatment to remove polysaccharides was suggested as a possible way to increase protein accessibility or digestibility (Amano and Noda, 1990; Fleurence, 1999b).

Compounds other than polysaccharides seem also to be involved in decreasing algal protein digestibility. For example, polyphenols or trypsin inhibitor compounds, contained in *Ulva* or *Ascophyllum* inhibit digestive enzymes (Bobin-Dubigeon *et al.,* 1997).

9.3.2 Effects of processes on algal protein digestibility

Physical processes

The effects of the drying methods on algal protein digestibility have received limited study. For the brown seaweeds belonging to genus *Sargassum*, an effect on protein digestibility was reported according to the drying method used (freeze-drying or oven drying) (Wong and Cheung, 2001a). Washing the red seaweed *Palmaria palmata* to remove soluble molecules such as xylans and mineral salts allows improved protein *in vitro* digestibility (unpublished data). However, even if digestibility of proteins from washed samples were increased threefold over unwashed algae, it still would remain remain 50% lower than the digestibility of casein (unpublished data). Therefore, other processes to remove anti-nutritional factors and significantly to improve the nutritional value of proteins were investigated. Two main types of treatment were studied, fermentation and enzymatic maceration.

Fermentation processes

The effects of fermentation treatment on improving protein digestibility were studied. For example, the action of *Rhizopus macroscopus var. chinensis*, *Aspergillus oryzae* and *Trichoderma pseudokoningii* was tested on *Palmaria palmata.* The fermentation processes significantly increased the *in vitro* digestibility of *P. palmata* proteins (Fig. 9.2). The best result was recorded for *Trichoderma pseudokoningii* which should have a relative digestibility rate nearly 73% of that for casein digestibility compared to 50% after treatment by

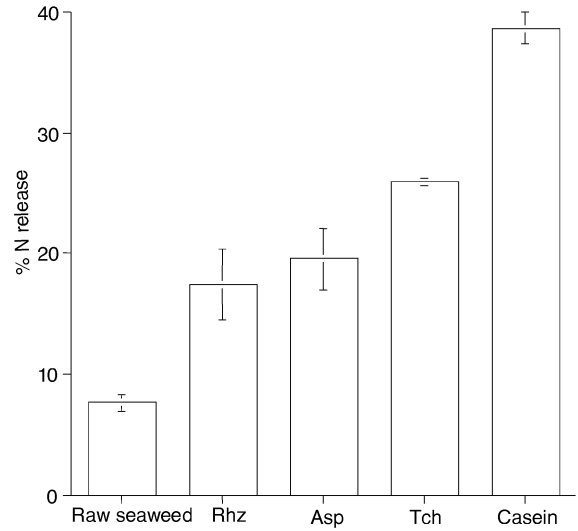

Fig. 9.2 Fermentation effects on *Palmaria palmata* digestibility (pepsin and pancreatin activity). (From Marrion and Villaume, unpublished data.)

Aspergillus oryzae or *Rhizopus macroscopus.* The presence of xylanases in *T. pseudokoningii* probably explains the greater efficiency of this microorganism on *Palmaria palmata*.

Enzymatic processes

Enzymatic maceration is classically used by the food industry for land crop transformation. It is based on the use of cell wall polysaccharide-degrading enzymes to improve yields, modify texture or ease processing operations (McClearly, 1986). The use of enzymatic liquefaction was initially described on *Palmaria palmata* (dulse) (Lahaye and Vigouroux, 1992). Some enzymes or commercial preparations such as Pentasonases, Celluclast, Novozym 188 and purified xylanases from *Aspergillus niger* were tested for their efficiency in liquefying dry or fresh dulse. Liquefaction was higher (> 70%) when enzymes were used either alone or in combination (Lahaye and Vigouroux, 1992).

On basis of these results, the effects of cell wall polysaccharide degrading enzymes were studied on some other red seaweeds. Initially, the action of carrageenase, agarase and xylanase was tested on *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata* respectively (Fleurence *et al*., 1995a). The enzymes were used alone or in combination with cellulases due to the presence of small amounts of cellulose (3% w/w) in their cell walls. The combined action

Table 9.5 Commercial enzyme preparations used to increase the protein digestibility of *Palmaria palmata*

Cellulases	Xylanases	β-glucanases
Celluclast (Novo) Cellulyve (Lyven)	Feedlyve (Lyven) Panlyve (Lyven) Shearzyme (Novo)	Finizym (Novo) Ultraflo (Novo)

of agarase/cellulase or carrageenase/cellulase strongly increased protein extraction from *G. verrucosa* and *C. crispus* (threefold to tenfold) respectively.

The effect of crude and commercial preparation polysaccharide-degrading enzymes on protein digestibility was evaluated from *P. palmata* (Table 9.5). The main enzymes were xylanases from *Aspergillus aculeatus* (Shearzyme from Novo), β-glucanases obtained from *Aspergillus niger* and *Humicola insolens* (Finizym, Ultraflo from Novo). These enzymes were used alone or in combination under operation conditions as described in a patent application (Fleurence *et al.,* 2001).

Protein *in vitro* digestibility of 80% was recorded after seaweed treatment with coupled xylanases (Shearzyme) and cellulases (Celluclast) (Fig. 9.3). This digestibility was higher than that recorded for casein using the same conditions (near 30%). On the other hand, the action of cellulases alone strongly increased the digestibility of *P. palmata* proteins (Fig. 9.3). Therefore, enzymatic maceration of dulse appears to be an efficient process for increasing digestibility of proteins and improving its nutritional value.

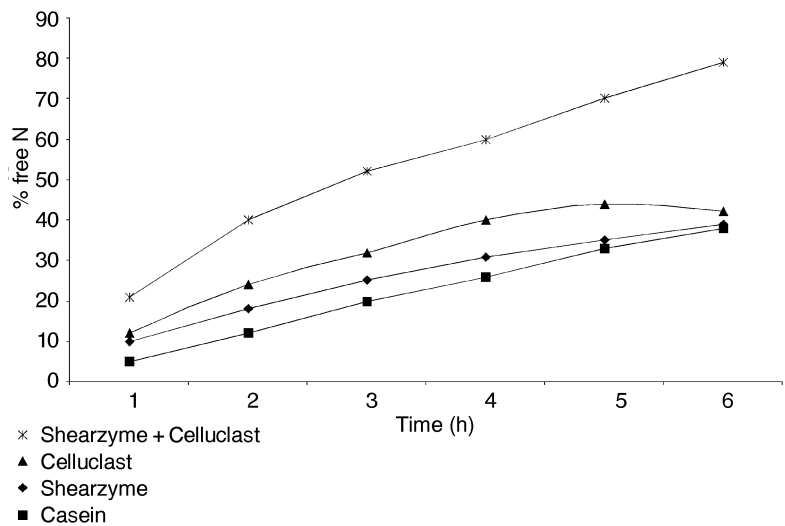

Fig. 9.3 Influence of enzymatic processing on protein digestibility of *Palmaria palmata* (*In vitro* digestibility in presence of porcine pepsin and pancreatin).

9.4 Uses of algal proteins in food

9.4.1 Entire algae

Numerous seaweeds rich in proteins are used in processing and preparing seafoods. The best known are *Porphyra* species which are used in the famous sushi preparations. These seaweeds are also processed into roasted products (yaki-nori) or boiled down in soy sauce (tsukudani-nori) (Nizasawa *et al.,* 1987). Some green seaweeds which also contain a high protein level are also processed for the manufacturing of foods. For example, *Ulva pertusa*, *Enteromorpha sp.* and *Monostroma sp.* which show protein levels of 26, 19 and 20% (dry weight basis) are mixed to create a food product called 'aonori' (green laver) that is very much appreciated by Japanese consumers. In small amounts, green laver are also used as a garnish served with raw fish. The cooked preparation called sashimi is a favourite dish of most Japanese.

In both Europe and Canada, *Palmaria palmata* or dulse is used as a sea vegetable or food ingredient. This seaweed, particularly rich in proteins (up to 35% of dry weight), is processed in dry flakes and mixed with other species (*Ulva*, *Porphyra*) to produce a food product sold in French Brittany under the name of 'Fisherman salad'. This mixture is used as a garnish especially with cooked fishes. Dulse and sea lettuce are also incorporated as food ingredients for making the 'Bread of algae'. This type of product is consumed on the Atlantic coast of France especially during the summer period.

9.4.2 Protein extracts

Algal proteins or purified protein fractions are very little used at present as ingredients in the food industry. However in Japan, a blue chromoprotein belonging to the phycobiliprotein family is used as food colorant. This pigment called phycocyanin is extracted from the *Spirulina* sp. and is commercially available under the name of 'Lina Blue-A'. It is used in chewing gum, soft drinks, dairy products and the green-coloured hot paste served in sushi bars. The same types of pigments (R-phycocyanin, R-phycoerythrin) but with purple or red colours are also present in red algae (Rüdiger, 1994, Roman *et al.,* 2001). These chromoproteins possess unique spectral properties allowing one to envisage their use as food colorants.

9.5 Future trends

9.5.1 Animal feed

Several studies on the food value of algae used for animal feed were performed. Two decades ago an evaluation of the nutritional potential of some green, red and brown seaweeds in feeding herbivorous marine fish was carried out (Montgomery and Gerking, 1980). The fish (damselfish) absorbed at least 20 to 24% of the biomass and 56 to 67% of proteins contained in the algae and the

green seaweeds belonging to the genus *Ulva* seemed to have a nutritional potential higher than the other algae tested (brown or red seaweeds). In a continuation of these studies, the effects of algal biomass in fish diets were studied for several species either herbivorous or carnivorous. Many fish, including carnivores, ingest numerous species of algae.

In this context, the opportunity of using *Ulva pertusa* as a protein source for red sea bream (*Pagrus major*) has been evaluated. The fish feed containing even small quantities of algae meal (5% of w/w of food) showed a significant increase in growth rate and feed efficiency (Mustafa and Nagawaka, 1995). In addition, the algal supplement seemed to foster a better resistance to stress and diseases for the fish submitted to this special diet. The *Ulva* meal diets were also described as affecting the unsaturated fatty acid composition of the fish flesh and thereby modified the final quality of the fish for human consumption (Nakagawa and Kasahara, 1986).

Other seaweed species particularly rich in proteins were also studied as suitable feed for fish aquaculture. For example, *Porphyra spp.* were tested in red sea bream feeding and led to an increase in animal weight gain (+22%) and improved the survival rate of animals (+13%) relative to the control population or in comparison with other algal diets (eg*: Ulva sp.*, *Ascophyllum sp.*) (Mustafa *et al.,* 1995). Because *Porphyra yezoensis* is already consumed daily as a human food, supply or economic costs strongly limit the use of *Porphyra* for fish diets. This situation applies for most algae tested in fish feeding experiments. However in Europe, the use of animal meal has recently been forbidden for animal nutrition thus creating an inducement for the use of plant proteins as an alternative protein source. In this context, the use of proteins from marine algae could become more attractive. Independently of studies on fish nutrition, some research has also been carried out including the use of algae in mollusc diets as with abalone feeding which has a high economic potential in Europe and Japan, also in North America.

Seaweeds with high protein levels such as *Palmaria mollis or P. palmata* were particularly tested as protein sources (Mai *et al.,* 1994; Buchal *et al.,* 1998). According to these studies, use of dulse throughout the abalone life cycle should improve the production process by shortening the growth duration. Using algae in animal feeds also concerns species other than marine animals. For example, in France dulse was eaten by cattle (‘goémon à vache’ in French) and today a new interest in using *Palmaria* or *Ulva* as additives in animal feed has appeared.

9.5.2 Human nutrition

In addition to their use as sea vegetables, algae could be in processed into new dietetic products or novel foods. This is a new market for the seaweeds especially in Europe where algae are not traditionally consumed as sea vegetables. Several red and green seaweeds are rich in proteins (25% of dry weight on average), in carbohydrates (38–74% of dry weight) (Morgan *et al.,* 1980) and low in lipids (less than 2%). In particular, they can be used for amino

Table 9.6 Distribution of taurine (rare amino acid) in various algae (from Arasaki and Arasak, 1983)

Species	*Ulva pertusa*	*Enteromorpha linza*	*Undaria pinnatifida*	*Sargassum confusum*	*Neodilsea yendoana*
Taurine (in mg /100 g of dry weight)	1.8	2.0	11.8	9.7	218

acid supplementation under FAO recommendations. *Palmaria palmata* appears as a suitable source of methionine (2 mg/100 g dry weight) (Schlichting and Purdon, 1969, Fleurence, 1999a). The presence of some free rare amino acids such as taurine in various seaweeds (Table 9.6) (Arasaki and Arasaki, 1983) may be an additional stimulus to develop algal extracts for human consumption.

Algal peptides could also be of nutritional interest and therefore promote the appearance of novel foods. Information is limited about the biochemical composition and biological activities of these peptides. Some peptides rich in glutamic acid derivates were however isolated from brown seaweeds *Pelvetia caniculata* or *Eisenia bicyclis.* Another peptide called carnosine (β-alanyl-L-histidine) was also characterized in red seaweed *Ancanthophora delilei* (Arasaki and Arasaki, 1983). This peptide, generally present in animal muscle, was described as an anti-oxidant and possessing protective functions against free-radical effects. It could be involved as possible modulator of diabetic complications, atherosclerosis and Alzheimer's disease (Hipkiss, 1998). Its involvement as a control factor for muscle activity was also reported during evaluation of dietary protein requirements in athletes (Lemon, 1997). The possible use of seaweeds for the processing of foods for athletes appears therefore to be an alternative for the application of a marine resource in human nutrition.

9.5.3 Food additives

Unlike polysaccharides, knowledge of functional properties of algal proteins is very limited. This situation is a consequence of a lack of study on properties of seaweed proteins and therefore the accessibility of the protein fractions in some species, especially brown algae that are the main resources for the extraction of colloids. However, red seaweeds contain a red coloured protein, phycoerythrin that possesses spectral and colorant properties potentially useful for the food industry. Phycoerythrin belongs to same molecular family as phycocyanin that is already included as a colorant in food preparations.

According to the algal source, two types of phycoerythrin were distinguished. R-phycoerythrin is provided from most red algae (Rhodophyta) and B-phycoerythrin is obtained from Bangiales which are a particular family in the Rhodophyta class. R-phycoerythrin is abundant since this pigment can constitute up to the 12% of dry weight of *Palmaria palmata* in spring (unpublished data). It is

a protein with a relative mass between 240,000 and 260,000 (Hilditch *et al*., 1991; D'Agnolo *et al*., 1993). R-PE consists of an open chain tetrapyrole called bilin that is covalently linked to the apoprotein. Their absorption spectrum shows a maximum of 565 nm with a shoulder at 545 nm and a peak at 497–498 nm. R-PE emits a red fluorescence (λmax = 572–578 nm) and displays an intense fluorescence more than 20 times greater than that reported for other pigments such as fluorescein. The food industry could be interested in the use of a red fluorescent colorant as a complement to the blue fluorescent pigment already available.

Until recently the extraction of phycoerythrin was based on grinding the alga in liquid nitrogen, a procedure that does not allow the recovery of a large quantity of pigment. This process leads to high production costs not compatible with use of R-PE as a food colorant. However, an alternative process using enzymatic hydrolysis of the cell wall for extraction of phycoerythrin was successfully tested on dulse. This new extraction method is based on the action of the commercial enzyme preparations (Xylanases, β Glucanases, Cellulases) on the alga at moderate temperatures (30–40 ºC) and pH7. The process does not require crushing of seaweed, does not denature the pigment and results in the recovery of 4 mg of R-PE/mg of dry alga (tenfold more than the classical process) (Fleurence, 2003). The availability of an easy, less expensive process than those based on pulverizing alga may give new perspectives for the industrial utilization of this pigment. Further, the R-PE thermal and photochemical stabilities recorded previously (Galland-Irmouli *et al*., 2000) suggest a suitable compatibility with the process conditions used in the food industry. The application of phycobiliproteins as food colorants thus appears to have potential and for utilizing algal protein in the future.

9.6 Sources of further information and advice

Lack of knowledge about functional properties of algal proteins is hindering their use as food additives. Therefore, it should be interesting to study different physicochemical characteristics such as water activity or thickening properties of isolated protein fractions. This could be done with green or red seaweeds known to possess high protein levels. The use of protein-rich algae for the development of novel foods seems to be a realistic approach. Effectively, the algae concerned are already consumed as sea vegetables either in Asia or in Europe. Nevertheless, some studies on the allergenic properties of algal proteins seem necessary before the development of novel foods. This recommendation is motivated by the record of a poisoning case due to ingestion of a red alga 'ogo-nori' (*Gracilaria verrucosa*) (Noguchi *et al.,* 1994). Lack of knowledge about food allergens or other toxins present in edible seaweeds could limit the use of this marine resource in the case of the development of new products such as health foods especially in the European or American markets.

In conclusion, seaweeds remain a potential protein source for human or animal nutrition. The algal proteins show original amino acid composition often

complementary to other food proteins, especially leguminous proteins. However, this resource is little exploited and some research work seems necessary to improve our knowledge on the nutritional properties of these proteins and factors limiting their digestibility.

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Part II

Analysing and modifying proteins

10

Testing protein functionality

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10.1 Introduction

10.1.1 Aims

Interest in food proteins predates agriculture. However, the study of protein behavior in foods gathered pace only within the past 30–40 years [1–4]. Protein functionality can be defined as any physical or chemical characteristic of a protein that is relevant for food production, storage, processing, packaging and consumption.[5] Over 120 review papers have been devoted to this topic (Fig. 10.1).

The aim of this chapter is to provide a brief overview of standardized testing methods for protein functionality. Successful food technologists are creative and innovative. Proprietary work in the food industry also encourages competitiveness and novelty. Such considerations may explain why standardized testing received low priority previously.[6–16] Comparing protein test results from different laboratories can be extremely difficult. But there may be reason for optimism. Standardized testing may be occurring spontaneously. This chapter aims to raise awareness of the range of tests currently available. As part of such discussions we shall consider (Section 10.1.2) protein structure and function in food technology. Section 10.2. describes methods for structure determination for commercial food proteins. Section 10.3 deals with the (sometimes opposing) requirements for model foods tests (Type I tests) versus laboratory (Type III) testing. Sections 10.4–10.6 presents the current range of model foods for assessing protein foaming, emulsification and gelation. Representative results are given along with advice on further reading.

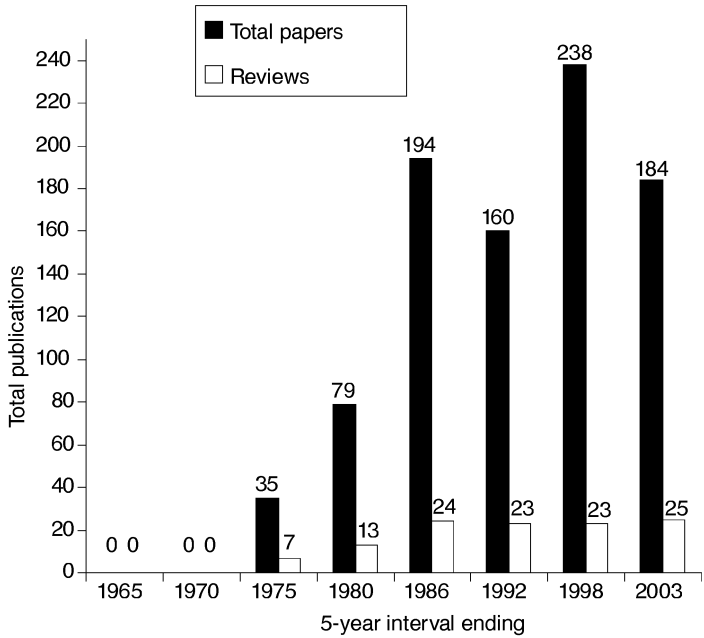

Fig. 10.1 A summary of protein functionality publications from 1965–2003.

10.1.2 Structure-functionality: the paradox

Examples of protein functionality include solubility, emulsification, gelation and foaming (Table 10.1). Early examples of structure-functionality relations for food proteins appeared in the 1970s. Recall that in 1963, the first 3-D structure for a protein (myoglobin) was published by Kendrew and co-workers. C. B. Anfinsen refolded ribonuclease, previously denatured with 8M urea and 2-mecaptoethanol, and established the thermodynamic hypothesis for protein folding.[17] This work revealed that the instructions for folding any protein are held within the primary structure. Furthermore, protein structure (folding) could be studied using chemical thermodynamics principles. From the onset, experts in food proteins embraced the concept of native structure. In contrast, the structure of commercial proteins were poorly studied. Professor Kinsella identified the following paradox twenty years ago[18]

> The food scientist is faced with a situation wherein the protein being used possesses structures and conformations that have … evolved to perform specific biological functions and are not necessarily designed for specific [food] functional applications.

In other words, establishing structure-functionality relations for food systems could be difficult since proteins evolved to function within living systems. Protein structure-functionality relations are concerned, beyond the dictates of evolution and biologic function, with the realm of food technology. The food

Table 10.1 Multiple protein functionality in selected processed foods*

Type of food	Multiple functionality
Beverage	Solubility, grittiness, color
Baked goods	Emulsification, foaming, gelation,
Dairy substitutes	Gelation, foaming, emulsification
Egg substitutes	Foaming, gelation
Meat emulsions	Emulsification, foaming, gelation, adhesion-cohesion
Soups and gravies	Viscosity, emulsification, water adsorption
Topping	Foaming, emulsification
Whipped dessert	Foaming, gelation, emulsification

Notes: *Adapted from refs 15 and 18.

proteins paradox can be resolved by measuring the structure for commercial protein preparations directly and matching this with their performance in a range of real foods. A further requirement for establishing accurate structure-functionality relations was noted by Jim Harper *et al*.[7] from Ohio State University ... '[The] disparity between (laboratory) test results and actual functionality in final food formulations necessitates closer scrutiny by researchers of traditional experimental methods.' The issue on hand is how to predict the behavior of protein ingredients within a host of fabricated foods including beverages, bread, cookies, ice cream, nondairy coffee whitener, whipped topping, comminuted meat products, soups, and sauces.[19]

10.2 Protein structure: sample characteristics and commercial proteins

10.2.1 Sample characteristics

Soybean derived proteins include soy grit, flour, concentrate and soy protein isolate (SPI) with protein contents of 45% to >90%. Commercial protein preparations from cereals include wheat/corn flour or wheat gluten. Fresh milk is processed into a number of high protein ingredients including non-fat dried milk (NDM), milk protein co-precipitates (MPC), acid casein, rennet casein, sodium caseinate, as well as whey protein concentrates (WPC) and isolates. The majority of food protein ingredients are presented to the user as dried powder with increased shelf life, improved microbial stability, lower weight and increased portability.

Techniques for structure determination that work with purified globular proteins may be unsuccessful for commercial proteins which are not wholly soluble and/or contain a range of molecular species. Protein ingredients are also heterogeneous with respect to protein conformation, size, shape, and pI. Furthermore, process-induced changes lead to mixtures of native, non-native, partially denatured and fully denatured proteins.[12] It is necessary to consider such sample characteristics when selecting a method for structure analysis. We

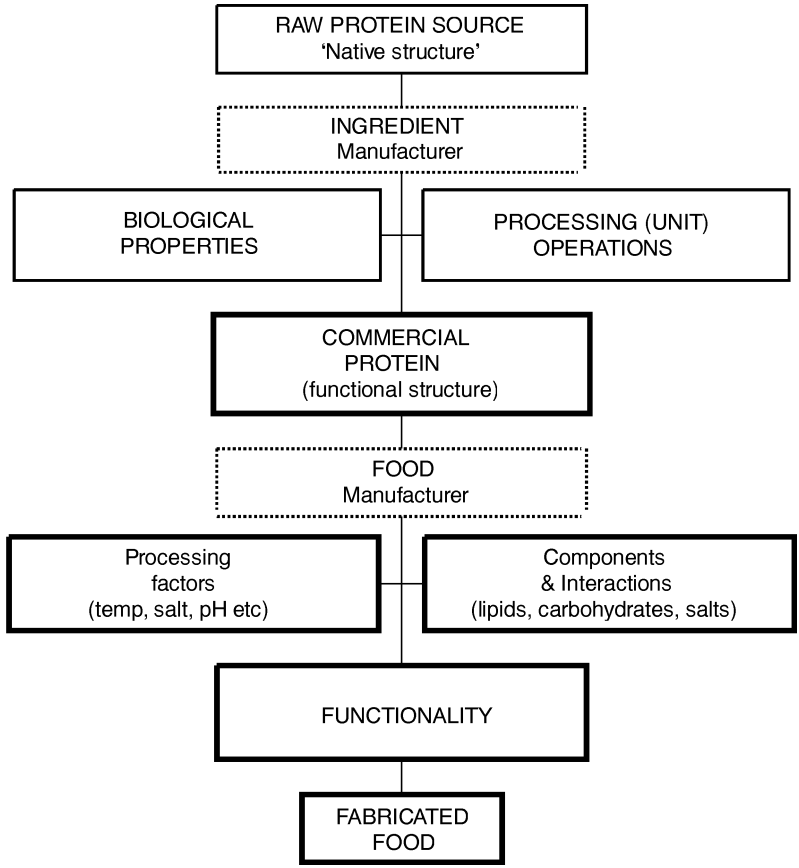

Fig. 10.2 Stages in producing a commercial protein ingredient and its subsequent use.

Table 10.2 Commercial protein ingredients used for food manufacture

Source	Protein ingredients
Soy, wheat, maize and other plants	Grits, flour, concentrates or isolates, fractionated proteins
Milk	Non-fat dried milk, acid casein, rennet casein, caseinate, casein-whey protein co-precipitates, WPC and lactalbumin
Egg	Liquid egg white, dried egg, egg yolk
Meat	Meat hydrolysates, comminuted meat, mechanically recovered protein, gelatin
Fish	Fish protein hydrolysates, collagen, muscle

Table 10.3 The structure of commercial protein preparations

Structure feature	Processing change
Amino acid composition (for hydrolysate)	–
Primary structure post-translational modification (glycosylation, phosphorylation, acetylation, amidation)	+
Hydrophilic-lyophilic balance, charge and hydrophobicity, pI	+
Sulfhydryl disulfide content	++
Size (molecular weight), shape, hydrodynamic properties	+
Secondary structure (intra- and inter-peptide), tertiary fold	++
Quaternary structure (intra- and inter-peptide)	++

Notes: Adapted from ref. 18.

should also take account of instrument cost, cost per analysis, and the degree of personnel training required.

Native proteins consist of a hierarchy of primary, secondary, tertiary and quaternary structures. In contrast, the functionally active structures of commercial food proteins appear after manufacture (Fig. 10.2). These structures may or may not resemble the 'native conformation'. The traditional practice of calling commercial protein preparations (Table 10.2) denatured is over-simplistic.[20–22] As described in relation to the 'food protein paradox', protein ingredients are industrial materials requiring accurate structural analysis.

Food ingredients including proteins are subjected to numerous unit operations during their production (Fig. 10.2). Examples of common processing operations include thermal treatment (pasteurization, sterilization, heat coagulation), particle size reduction, defating (solvent extraction or centrifugation), solubilization, enzymatic treatment, chemical treatment (alkali, acids, oxidizing or reducing agents, chelators), precipitation (by acid, salt, heat, or organic solvents), chromatography (ion exchange or size exclusion), concentration (ultrafiltration, salt or solvent precipitation) and dehydration (freeze drying, roller drying or spray drying). Texturization, aromatization, agglomeration, and extrusion are also used for some protein sources.

As described previously, many of these unit operations will impact on protein structure (Table 10.3). A significant number of commercial proteins also do not have exact biological analogues; gelatin is derived from collagen by extensive processing but the former does not appear in nature. Gluten is another proteinaceous material that has no biological counterpart though formed from wheat storage proteins. Commercial protein products are often composite materials which contain starch, lipids and other components.

10.2.2 Commercial protein structure

Polymer solution chemistry provides the analytic framework for studying food biopolymers.[23] By analogy, material science principles are widely applied to

food solids. The proportion of crystalline and amorphous structure within a material has important consequences on its mechanical behavior. The proportion of crystalline structure within commercial protein preparations is dependent on the amount ordered (2º and super-2º) which persists during processing. Amorphous structure is due to combinations of aperiodic, beta-turns, and other structures. The intermolecular and intramolecular structure of industrial protein has not been determined in most cases. The 2º structure of commercial proteins, without a unique molecular weight, can be quoted per gram of sample rather than per mole.

For materials in solution, the polymer scientist determines the number or weight average molecular weight determined by gel-filtration analysis. Hydrodynamic size can be studied by viscometry, ultracentrifugation, or light scattering. Concentrated solutions, gels or so-called soft solids can be characterized via rheological studies involving destructive testing (Instron Universal testing) or small deformation (non-destructive) testing also called oscillatory rheology. Studies of viscoelastic behavior provide structural information within specific frequency domains related to molecular dynamics, polymer-polymer, and polymer-solvent interactions.[24]

The typical product sheet provided by the ingredient manufacturers includes information on protein content (Kjeldahl), moisture, fat, carbohydrate, ash and microbiological count. From the applications view point, the following structural indices could be useful; (i) Differential scanning calorimetry (DSC). The specific denaturation enthalpy (cal/g) and onset temperature is related to residual ordered structure providing a basis for quality control and/or new structure-functionality relations.[25] (ii) Fourier transform infra-red (FTIR) analysis. This is an affordable method for estimating the degree of crystalline and amorphous structure within biopolymers in solution, gel or solid states.[26] (iii) Multi-angle light scattering (MAL). This technique used in conjunction with SEC, provides a weight average molecular weight distribution and root mean square size of hydrated polymers.[27] (iv) Fluorescence analysis of surface hydrophobicity (So/Se) and (iv) sulfhydryl/disulfide analysis. These last two indices have been shown to be good predictors for certain functionalities.[28, 29] In conclusion, to develop accurate structure-function relations for food proteins, commercial samples need to be analyzed for their structure. Many techniques for materials characterization such as DSC, MAL, FTIR, SEC and fluorescence analysis can be readily adapted for structure determination for commercial protein ingredients.

10.3 Testing functionality

10.3.1 Classes of tests

There are three classes of tests for protein functionality; (i) Type I utility tests employ model foods, (ii) Type II tests analyze physicochemical properties such as surface hydrophobicity and sulfhydryl content and (iii) Type III tests are

idealized laboratory tests using buffered media and well defined (purified) proteins. The ubiquitous phrase 'model system' is frequently used in connection with Type III tests leading to some confusion with model *food* systems, which are always nearly food-like. The relevance of different tests *to the food industry* increases in the order Type I > Type II > Type III because Type I tests employ realistic formulations. By contrast, the order of generality for test results is, Type III $\geq$ Type II >Type I since Type III tests are more abstract.

10.3.2 The rationale for testing

To develop practical methods, one needs instruments and strategies for assessing the performance of protein ingredients within fabricated foods. Testing is concerned with method development. Confounding variables associated with protein samples should be identified, normalized and/or eliminated. This can be achieved by providing standardized samples to all testers. Batch-to-batch variation in the sample should not be an issue during method development and good sampling practices are assumed to be in force.

Model food tests are useful for predicting protein functionality within specific foods. Such tests are also suited for quality control, screening, surveillance or enforcement. Technological developments over the past 30 years led to new ways for manufacturing food proteins. Type I testing is useful for product standardization and for product development.[7, 8, 10–12, 14, 16] Mulvihill and Fox[12] pointed to the need for standardized testing for protein solubility, viscosity, water absorption, gelation, emulsification, whippability/formability and casein meltability. Hall (1994)[14] considered standardized testing for protein solubility, viscosity, gelation, foam formation, emulsification, water and fat holding, and surface hydrophobicity. Zayas[30] identified solubility, water holding, emulsifying properties, oil and fat binding, foaming properties, and gelling as the major forms of protein functionality.

Formal attempts to develop standardized protein tests stalled on many occasions (Dalgleish and Fox; personal communication, May 2003). One possible reason may be the absence of any consensus about what represents legitimate goals for Type I versus Type III testing (see below). The current interest in standardized tests may be due to partnering relationships between ingredient manufacturers and users. Competitive advantage can be gained by designing utility tests for a valued customer. Model food tests can also provide fundamental insights when combined with appropriate experimental design and statistical analysis.

10.3.3 Functionality research

Type III tests are performed under idealized laboratory conditions. Such basic research, though technologically relevant, is a long-term activity aiming to increase our understanding of food proteins.[31] The demands as well as types of samples for Type III testing are different. Highly empirical testing using model

Table 10.4 Type I functionality tests using model foods

First-generation model foods	Further examples
• Angel cake • Bread • Cake • Coffee whitener • Infant formula • Salad dressing • Sausage • Starch pudding • Whipped topping • Yellow cake	• Low fat spread • Beef batters • Beef gravy • Bread • Cream • Beef patties • Food powders • Cheese • Chicken • Glassy foods • Meat • Milk • Processed cheese • Sausages • Wine • Yogurt

foods can be grouped with other methods of protein analysis, such as protein quantitation, determination of nutritional value, detection of adulteration, protein fractionation and characterization. Only a limited number of laboratory tests have undergone collaborative trials.[32–34]

10.3.4 Utility testing

Harper *et al*.[6–8] described the stages for designing model food tests for protein functionality. Start by reviewing many formulations of the type needed and select the lowest 'common denominator' of ingredients found in all recipes. Next, develop a small-scale process for producing the model food. Thereafter, identify the dependent and independent experimental variables and establish simple and reliable procedures for their measurement. Finally, choose an efficient experimental design; the most effective of these includes surface response methods. The number of model foods employed for testing protein functionality has grown greatly in the past 15 years (Table 10.4). In the remainder of this article we present an overview of model foods for testing protein functionality.

10.4 Model foods: foaming

A foam is a collection of air bubbles dispersed within a continuous phase arranged in the form of a thin film (lamellae). For a water-based foam, adjacent air bubbles are separated by an hour-glass shaped film that contains dissolved solutes or particles; sugars, fat droplets, ice crystals, low molecular weight

emulsifiers, non-gelling thickeners, starch and/or protein. Food foams with varying densities can be produced including bakery products (cakes, biscuits, sponges), beverages (beer, stout, soft drinks), desserts (bavaroises, soufflés, mousse, instant puddings), confectionery (nougat, marshmallows, meringues, fondant, fudge), whipped topping and ice cream.[35,36] Protein functionality within simple foams has been reviewed.[12,37,38,39] Foaming capacity (FC) is the volume of gas-phase incorporated. A high FC requires proteins that adsorb rapidly at the gas-water interphase. Foam stability (FS), the extent to which fluid can be retained within the foam, requires a cohesive protein film that entraps air bubbles.

10.4.1 Foaming capacity and stability

A standard test for foaming is described by Philips *et al*.[33] Collaborative testing involved six laboratories using 3.75 g protein sample dispersed in 60 ml of water (6.25% w/v) adjusted to pH 7.0. All participants used an identical brand of instrument (the Sunbeam™ Mixmaster Powerplus 350 Watt mixer) and carefully defined operating conditions (bowl speed ≈78 rpm and beater speeds ≈417 rpm). Spray dried egg white, sodium caseinate and milk protein isolate (MPI) were adopted as standard samples by all laboratories. FC was measured as percent overrun by weighing 100 ml of foam (W_F) and an equal volume of protein solution (W_S).

$$FC(\%) = \left(\frac{W_S - W_F}{W_S}\right) \times 100 \qquad 10.1$$

From Eq. 10.1, FC is also equal to the gas:liquid volume ratio (multiplied by 100). FS was measured by monitoring the rate of liquid drainage using a tarred balance and a timer. The FC for sodium caseinate, MPI or egg white ranged between 750–1100% depending on the whipping time (5–15 minutes). Reproducibility depended on the method of sample dissolution, type of whisking accessory fitted to the mixer, bowl shape, sampling, and the time elapsed before measuring foam drainage.

Peltonen-Shalaby and Mangino[40] found, using eight different WPC samples, that foaming overrun was 770 (±164)% compared to 248 (±19)% for whipping overrun (next section).[41] Some investigators refer to whipping by virtue of the type of mixer used; food foams can be prepared by shaking, whipping or sparging. Paulson *et al*.[42] reported the foaming overrun for canola, SPI and sunflower protein isolates as 423, 150 and 488% respectively. Treatment with SDS produced FC values 885, 725 and 825% respectively; linoleate or trypsin treatment also improved foam volume and drainage. The preceding test was run at pH 7 with no oil added. The FC for sunflower meal was minimum at pH 6 (FC ≅ 500%) and increased to 700–900% at higher or lower pH values.[43] Free-standing foams have been produced under a range of conditions using soybean products (1% w/v suspensions, foaming via shaking, mixing time one min.),[44] succinylated fish protein (1% w/v dispersion in 0.3% NaCl solution, Hobart

mixer, mixing time of ten min.)[45] and sunflower protein (8% w/v in distilled water, Sorval Omni mixer, six min. blending time).[46] So far the standardized foam test has been used mainly in connection with whey protein characterization.[47–50]

10.4.2 Whipped topping

A model whipped topping for functionality testing is described by Harper *et al*.[6–8] (Table 10.5). This product is an analogue for dairy whipped cream.[51,52] They are termed high fat foams, foamed emulsions, or whippable emulsions.[53,54] The total solids content of nearly 40% is greater than the value used for a simple foaming test (10.4.1). Notice also the presence of low molecular weight emulsifiers such as Tween.

Though many investigators refer to whipping characteristics relatively few testers employ high fat conditions. Min and Thomas tested the whipping characteristics of milk proteins using model whipped topping containing 15% milk fat and 5.5% NDM. Additional protein was added at levels of 1.5%. The whipping overrun was 116% (sodium caseinate), 124% (whey protein), 193% (low heat NDM), 181% (skim milk protein colloid) or 184% (CMC-milk protein complex). Addition of Ca^{2+} to sodium caseinate improved whipping overrun and FS suggesting that micellar casein has better whipping characteristics compared to dissociated casein.[55] Milk pasteurization had a positive effect on the FC value for WPC but no significant effect on whipping overrun.[41]

Kolar *et al*.[56] reviewed the use of SPI for manufacturing whipped topping. Milk protein was replaced with SPI (0.4% w/v) leading to products with a overrun of 350–400% and excellent stability. Proteinase treated SPI had superior whipping functionality compared to egg white. The whipping characteristics of soy peptides were unaffected by heating and over whipping.[57] Interestingly, Lah

Table 10.5 The composition of model foam emulsions

Component	Model topping [(a)]	Commercial whipped topping [(b)]		Ice cream mix[(c)]
Oil	30	30	25–26	10–20 [(d)]
Protein	6	2	0.4–1.2	~5 [(e)]
Sucrose	7	7	18	18–20
Corn syrup	–	3–5	–	–
Stabilizer gum	0.2	0.3–0.5	–	0.2–0.5
Emulsifiers [(f)]	0.05	0.35–1	0.6	0.2
Water	qs. to 100	qs. to 100	qs. to 100	qs. to 100

Notes:
(a) From Harper *et al*., cited by Peltonen-Shalaby and Mangino.[40] (b) Commercial formulations using caseinate[51] or soy bean protein isolate.[57] (c) Adapted from Marshall and Arbuckle.[56] (d) Vegetable oil may be used instead of milk fat, non-fat ice cream has <1% added lipid. (e) Added as non-fat dried milk with 37% protein and 55% lactose. (f) Tween 60, 65, 80, etc.

et al.[58] reported inferior whipping using full fat soy protein (60.2% protein, 33.8% fat) which needed preheating at 80 ºC for 10 min. before use. For a 3.5–4.0% solution the whipping overrun was 250–375%. The test system did not contain low molecular molecular weight surfactants or added oil; however, endogenous soy oil and phospholipids may have affected whipping. Chow *et al*.[59] and Abdullah *et al*.[60] evaluated soy beverage and peanut milk for their whipping ability in a system containing corn syrup, fat and low molecular weight emulsifiers. The whipped performance was comparable to commercial brands. Compared to caseinate, whipped topping using SPI had lower overrun but enhanced viscosity and foam stability.

10.4.3 Ice cream

Ice cream is a foamed emulsion with low (50–100%) overrun compared to whipped topping. A model ice cream suitable for protein testing is described by Huse *et al*.[61] and also by Goff and coworkers.[62] The ingredients list (Table 10.6) is similar to the formulary used in commercial products but the batch size (8–10 kg) is smaller. The manufacturing process for ice cream involves: (i) blending ingredients with water and batch pasteurization at 68–70 ºC/30 min., (ii) 2–stage homogenization leading to a protein stabilized water-in-oil emulsion or ice cream mix, (iii) Ageing the mix for 24 hours at 3–5 ºC, (iv) freeze-aeration at 5 ºC followed by (v) hardening at −18 ºC for 3–4 weeks. The final product is a high-fat emulsion foam with ice crystals and partially coalesced fat droplets in a freeze-concentrated lamellae phase. At 5.5 ºC about 50% solvent water is frozen resulting in freeze concentrated mixture with 69% total solids.[62] The fat droplets are covered by milk proteins (casein and whey proteins) and low molecular weight emulsifier.

The function of the non-protein emulsifiers added to ice cream mix is to destabilize oil droplets during the freeze-aeration stage in a process termed

Table 10.6 Model ice cream mix for protein testing

Component	% (w/w)	Ice cream processing steps
Milk fat	10%	1. Blend mixture
Nonfat dried milk*	11%	2. Pasteurize (74 ºC/30 min.)
Sucrose	10%	3. Homogenize
Corn syrup	5%	4. Cool (5 ºC/24 hr)
Polysorbate 80 (Tween 80)	0.08	↓
Water	64	Ice cream mix (p/o/w emulsion)
		↓
		(Freeze-aeration at −5 ºC)
		↓
		Ice cream

Note: * substitute with ~4% (w/w) test protein.

'churning out'. According to Keeney, destabilization leads to dry textured ice cream.[63] The role of the protein present within ice cream mix is (i) emulsion stabilization, (ii) foaming, (iii) fat destabilization due to competitive displacement by low molecular weight emulsifier and (iv) water holding in the lamella aqueous phase.[64–66] Using a model system (Table 10.6) protein emulsification degree can be determined by measuring droplet size and extent of oil phase separated after prolonged storage (creaming). The proportion of protein found in the ice cream mix serum phase can also be determined after centrifugation. A convenient fat destabilization test was devised by Keeney.[63] A small sample of ice cream and ice cream mix are diluted 1/500 with water. The absorbance of the two samples are then measured at 540 nm. The per cent of fat destabilization (FD) is calculated from Equation 10.2:

$$FD(\%) = \left(\frac{A_{mix} - A_{ice\ cream}}{A_{mix}}\right) \times 100 \qquad 10.2$$

During the 1970s ice cream manufacturers began to substitute 20–50% of NDM with cheese whey powder, demineralized whey powder, WPC30 or ultrafiltrated-WPC30. Production costs were reduced by 14–20%.[67] After adding WPC the same functionality (as NDM) could be obtained by altering the formulations slightly. With increasing WPC:NDM ratio the viscosity of ice cream mix increased. Ice cream hardness increased and the color decreased. Sensory attributes were not adversely affected at $\leq$ 50% substitution. Higher levels of substitution caused decreased smoothness, reduced creaminess, reduced flavor and increased iciness. WPC has lower surface activity than caseinate. Adding WPC to ice cream formula allows lower amounts of emulsifier to be used for fat destabilization.[61, 68–72] Acceptable ice cream has been developed using up to 40–80% substitution of NDM by soybean protein[73, 74] peanuts protein isolate,[75, 76] or soluble wheat protein (Amylum SWP®)[77] and a miscellaneous group of plant protein isolates including chick pea and sesame.[78]

10.4.4 Bread

Several AACC approved methods are available for testing baking quality of bread flour and cookie flour (AACC method 10–9, AACC method 10–10, AACC method 10–11). Each test is clearly described with regards to: (i) test definition and purpose, (ii) scope – and list of ingredients, (iii) apparatus – equipment, implements, containers and sizes, and potential suppliers for equipment, and (iv) procedural steps, types of measurements needed (example bread volume) and forms of data treatment.[79–83] Khan *et al.*[84] examined the effect of blood plasma protein isolate (PPI) on bread baking quality. PPI (96% protein −%N × 6.25) was a creamy white free flowing powder with 3–5% water. Substitution of wheat flour at levels of 2, 4 and 6% increased loaf volume especially when formulations did not contain low molecular weight emulsifier. Additional PPI at 8% and 10% (w/w) decreased loaf volume. Crust color, loaf

specific volume as well as crumb color were reduced at all levels of PPI addition to wheat bread.

10.4.5 Angel cake

Angel cake is one of the best known model foods for testing food protein foaming *and* gelation simultaneously. The majority of reports deal with egg white as the benchmark protein for cake formation.[85–94] The angel cake test was also applied to whey protein,[7, 8, 95] blood plasma protein[84, 96] and powdered beef plasma.[97] Egg white (or test protein) is added to flour, sugar, acid salt and water and mixed to form cake batter which is then baked under well-defined conditions.[98–100] The AACC approved angel cake formulation used for testing protein functionality is summarized in Table 10.7.[95]

Angel cake test results correlate with the functionality of individual egg white proteins.[88–90] Cake height, texture, and compressibility appear to be related to four elementary characteristics; viscosity, FC, FS and gelation. Results from idealized laboratory tests showed the relative FC for egg white proteins to be; ovomucin (0%) = ovomucoid, < lysozyme (4%) < conalbumin (11%) < ovalbumin (20%) < whole egg white (100%) < globulins (153%). By comparison, the angel cake test shows relative cake volume increases as: ovomucin (19%) < ovomucoid (20%) = conalbumin (20%) < lysozyme (39%) < whole egg white (100%) < ovalbumin (113%) < globulins (121%). Ovomucin is non-gelling but enhances the viscosity and heat stability of the angel cake foam. Lysozyme had a high gelation onset temperature but formed a relatively stiff gel when heated above 81.5 ºC. Protein-protein interactions between lysozyme and globulin or lysozyme and ovomucin led to reduced foaming capacity, as measured via an idealized laboratory test, and increased angel cake volume.[88–90] Therefore, foaming is not the only functionality required for cake-baking quality.

Heat pasteurization of liquid egg white to inactivate salmonella increased angel cake height, egg white viscosity, and soluble protein content.

Table 10.7 Angel cake formulation for testing protein functionality

Ingredient	Egg angel cake (g)	Angel cake (+WPC)
Flour (commercial, pH 4.3)	86	86
Sucrose	200	200
Flavoring	5	5
Salt	<1	<1
Cream of tartar	5	–
Baking powder	–	5
Egg white (14% total solids)	250	–
WPC	–	50
Water	–	200

Homogenization had no detrimental effects on cake volume.[93] Egg white substitution by 25% plasma proteins isolates produced angle cake with acceptable characteristics. Cakes made by hand mixing had greater variability than machine mixed cakes[84] and therefore the AACC method avoids hand mixing. Proteases (papain, ficin, trypsin −0.34%) treatment increased angel cake volume at zero-time before proteolysis. There were accompanying increases in FC measured by standardized laboratory tests.[87]

During angel cake preparation egg white or WP dispersion (20% w/v) in water is whipped to form a thick foam to which is added sucrose (50% w/w). Flour is then added to produce the final cake batter which may then be baked at 191 ºF for 30 min. Whipping overrun can be determined as described in equation 10.1. However, baking quality is also affected by events subsequent to whipping. The initial protein foam has to support the added flour. Cake batter destabilizes by foam drainage. This can be reduced by the addition of sucrose and other agents which increase the viscosity of the lamellae foam.[95]

10.5 Model foods: emulsification and gelation

An emulsion consists of two immiscible fluids one of which forms a dispersed (droplet) phase within a continuous (outer) phase. Food emulsions are normally prepared from oil and water.[101] One can create an oil-in-water (O/W) or water-in-oil (W/O) emulsion depending on the choice of emulsifier and volume fraction of oil to water. All emulsions are unstable compared to the two phases from which they are prepared. Therefore energy is required to create an emulsion and emulsifier is needed to slow down de-emulsification by creaming, flocculation, droplet coalescence or phase inversion. Food emulsifiers are amphihilic molecules that possess affinity for water and oil according to their hydrophilic-lyophilic balance (HLB) number. The surfactants arrange at the O/W interphase to form a 2–dimensional film that prevents coalescence of droplets. Proteins are widely used as food grade emulsifiers. In addition, low molecular weight emulsifiers can be added including mono-glycerides, diglycerides, phospholipids, Tween and Span.

Interactions between two or more emulsifiers can lead to synergistic or antagonistic effects on emulsion stability.[102] Low molecular weight emulsifiers are more surface active and are rapidly adsorbed compared to proteins. Low molecular weight emulsifiers tend to displace proteins from the oil-water interface in a process called competitive displacement. For reasons discussed by Hill, there are no standardized laboratory test for emulsification though the turbidimetric technique of Pearce, Haque and Kinsella is convenient.[103, 104]

10.5.1 Coffee whitener

A model coffee whitener for protein functionality testing was described by Harper *et al*.[7, 8] Commercial whitener is an analogue for milk cream. Whiteners

include non-dairy creamers which are protein stabilized oil-in-water emulsions with vegetable oil as the dispersed phase. Some specialty coffee whiteners are protein free. A selection of commercial coffee whiteners currently available in North America include Borden cremora™ (Eagle Family food, Gahanna, Ohio), Carnation coffee-mate™ (Nestlé Beverage Corp., Glendale, CA), Farm rich™ (Rich Product Corp., San Francisco), Frontier™ (Frontier Herb, Norway, IA), International delight™, Mocha mix™ (Morningstar Group, Dallas, TX), Westsoy™ (Westbrae Natural Foods/International Foods) and Wegman's non-dairy creamer (Wegman's Food Markets, Inc. Rochester, NY). Apart from Coffee-mate and Borden cremora most non-dairy creamers appear to be casein free. Some whiteners employ SPI or whey protein.[105, 106]

The formulation of commercial coffee whiteners, including liquid and powdered products and the role played by each ingredient has been reviewed (Table 10.8)[107, 108] The patent literature describes whitener formulations using milk protein retentate,[109] phosphate modified milk protein,[110] or reformed casein micelles.[111] To prepare a coffee whitener emulsion non-fat ingredients are first dispersed in water at 60–70 ºC. The oil (normally hydrogenated vegetable oil) and emulsifiers are heated separately to 70–80 ºC and then added to the aqueous phase followed by two-stage homogenization at elevated temperatures. The emulsion is then cooled and stored at refrigeration temperatures or frozen. A liquid whitener can also be spray dried for improved shelf life.

The function of proteins within a whitener is to provide emulsification, reduce oil droplet size, increase whiteness, and increase resistance to oil separation. Flavor improvements arise due to protein binding with the coffee astringent, tannic acid. The quality of non-dairy creamers is tested in terms of (i) powder or coffee whiteness measured on the L*, a* and b* scale, (ii) emulsion stability towards creaming in hot coffee at 80–90 ºC at pH 4.5, and (iii) the extent of feathering or flocculation in the presence of Ca^{2+} and Mg^{2+} ions found with hard tap water.[112, 113] Also important is (iv) powder dispensability, wettability and solubility in hot coffee.[114, 115] A number of vegetable proteins evaluated using a model whitener include SPI,[116] cottonseed protein isolate,[117] MPC, and peanut protein extract.[118]

Table 10.8 Components for a coffee whitener model food system

Component	Liquid whitener (% w/w)	Powder whitener (% w/w)
Protein	0.4–2	4.5–9
Vegetable oil	10–12	35–40
Corn syrup (or malto-dextrin)	2.5	55–60
Emulsifier I	0.2	~1
Emulsifier II	0.3	~1.2
Potassium phosphate	0.2	0.8
Carrageen or xantham gum	0.05–0.25	<2
Water	to 100%	–

Euston and Hirst[119] found that protein functionality measured using a model whitener was similar to results from a laboratory test. Milk protein isolates containing micellar casein (casein, MPC, skim milk powder) formed large oil-in-water emulsion droplets with $d_{3,2} \approx 2–3$ μm and a protein coverage of 2.3–3.6 mg m^2. By contrast, caseinate, total milk proteinate or WPC stabilized emulsions had $d_{3,2}$ values of 0.4–0.5 μm and a protein coverage of 1.2–1.6 mg m^2. Micellar casein was less efficient as an emulsifier. Addition of EDTA, which chelates Ca^{2+}, produced the behavioral characteristics of dissociated casein. Coffee whitener prepared from micellar casein had oil droplets with a $d_{3,2}$ value of 1.7–1.8 μm compared to 0.4 μm for emulsions prepared using dissociated caseinate or milk protein concentrate. Emulsion stability was generally lower for non-dairy creamer compared to simple O/W emulsions probably due to the presence of low molecular weight emulsifiers in the former system.

10.5.2 Meat emulsions

Meat emulsions include products like bologna, frankfurters, sausages, liver sausages, and meat loaf. They are produced from comminuted or finely homogenized meat, mechanically recovered meat, poultry or fish. Sausage can be manufactured on a small scale by homogenizing meat with ice (for temperature control) using a bowl-chopper. Fat is then added followed by further processing in the chopper. Spices are then added followed by rusk or other water binders or fillers. According to the emulsion theory for comminuted meat products – water, protein and fat produce the continuous, emulsifier, and dispersed phase of an oil-in-water emulsion, respectively. The large size of some oil droplets (0.1–50 μm) has led to doubts whether meat emulsions should be considered true emulsions. An alternative model for comminuted meat products is that they are 3–dimensional gel networks with entrapped oil.[120–124] Most reviewers refer to these products as meat emulsions and this practice is adopted here.[125–131]

Standardized tests for protein functionality in meat emulsions have been developed. The emulsification capacity (EC) test of Swift *et al*. measures the volume of oil emulsified per 100 mg of protein at the point of emulsion inversion.[132, 133] Related indices have been proposed including the emulsified volume (volume of oil emulsified per 15 ml of protein solution) or the emulsifying ability (EA), which is the volume of oil emulsified per 25 ml of protein extract. The oil phase volume at the point of inversion is a further index for EC.[134] Applications of Swift's test for a variety of plant proteins were reviewed by McWatters and Cherry.[135] The meat emulsion stability (ES) test of Townsend *et al*.[136] measures the volume of fluid released when an emulsion is cooked to an internal temperature of 68.8 ºC. Standard conditions for assessing EC involves 25 ml initial oil volume, a soluble protein concentration of 11 mg/ml, a mixer speed of 13, 140 rpm and temperature of about <28 ºC.[137] The emulsion breakpoint is more easily visualized by adding 0.3g Oil Red O dye per liter of oil.[138] Tests for EC and ES for comminuted meat products have become

Table 10.9 Variables affecting meat emulsion characteristics

Variable
Chopping – meat extraction temperature
Collagen content
Emulsification – temperature
Emulsification intensity
Fat melting point
Ionic strength
Meat postmortem physiology (rigor, pale exudative meat)
Proportion of fat, protein and water
Salt soluble protein concentration
Type of salt (anion)

widely accepted though they have not undergone formal collaborative testing. A summary of variables affecting protein functionality in meat emulsions is shown in Table 10.9.

The order of increasing EC for isolated muscle proteins was myosin > actomyosin > actin for beef,[139] porcine[140] or chicken muscle.[141] Gillet *et al.*[142] showed, using eight different meat sources, that a plot of soluble protein concentration versus EC or EA produced a linear or inverse curvilinear plot, respectively, by virtue of the algebraic definition of each index. EC was directly proportional to the concentration of salt soluble protein extracted by stirring a 1:4 w/w meat slurry with 7.5% NaCl solution over six minutes. The relation between texture and salt soluble protein levels applies also to Chinese meat balls (Kung-wan).[143] Mechanically deboned poultry meat,[144] and the effect of chopping temperatures[145, 146] on meat emulsions have been assessed using Swift's test.

Large deformation rheological measurements using the Instron universal tester is another routine test for protein functionality in meat emulsions. Substitution of meat protein by vegetable protein leads to a reduction in the texture of cooked emulsions. Gluten, SPI or egg white increased the yield of a cooked meat emulsion. At replacement levels of < 80% egg white and SPI had a positive effect on product texture.[147] Pretreatment with ficin, collagenase, and papain revealed that both salt soluble and connective tissue proteins affected emulsion texture.[148] Corn germ protein at 2% substitution reduced shear force and cooking losses. Adhesiveness and water holding capacity were increased.[149] Canola or SPI were evaluated at 33.3 and 66.7% substitution. Rapture force, first and second bite hardness, and springiness were reduced compared to whole meat emulsions. Adhesiveness and cook stability was improved.[150] The functionality of vegetable proteins within meat emulsions is further discussed by Mittal and Usborne.[151, 152]

Small deformation rheological measurements or thermo-rheological studies provide continuous measurement of the storage modulus (G′) and loss modulus (G″) during thermal treatment.[153–155] Heating meat emulsions produced a fall in

G′ at $T > 20$ ºC probably due to melting of meat fat. There was then a sudden rise in G′ at 60–70 ºC ascribed to myosin denaturation. Adding SPI produced a two-phase transition in G′ at 60–70 ºC and 70–100 ºC. These changes in G′ coincided with protein denaturation temperature measured by DSC. Meat emulsions containing SPI or buttermilk powder had increased rigidity compared with control meat emulsions or those containing modified wheat flour or whey protein.

10.5.3 Filled emulsions or emulsion gels

An emulsion gel is formed when a protein stabilized in oil-in-water emulsions is subjected to heating. As an alternative, the pre-emulsified oil can be added to a protein solution prior to heat gelation. The mechanical properties of these so-called filled gels are modeled by synthetic composite materials in which an amorphous continuous phase surrounds a particulate (filler) phase. The mechanical properties of such reinforced material is superior to the baseline material.[24] Protein filled gels were first made using gelatin or whey protein as the network phase and glass beads or oil droplets as the filler.[156–158] Gel hardness, measured as the relative stress at failure (R), increased with the volume fraction of filler (Φ). Einstein's viscosity equation also applies.

$$R \approx \frac{(1 - \Phi^{0.33})}{(1 - \Phi)^{2.5}} \qquad 10.3a$$

$$G_C/G_0 = 1 + a\Phi + b\Phi^2 \qquad 10.3b$$

where G_C is the modulus of the composite and G_0 is the modulus of the matrix.[156, 159]

The value of G_C increases with decreasing diameter or increasing specific surface area of the filler. Inclusion of hydrophilic (normal) glass beads led to brittle gels. In contrast, protein gels were more elastic than the control if the dispersed phase had hydrophobic surfaces. Equation 10.3 can be modified to take account of interactions between the dispersed phase, adsorbed proteins, and the bulk network phase. Non-interactive fillers do not affect gel network formation other than by their physical presence. In contrast, interactive fillers assist and enhance gel structure.

A wide variety of food grade emulsion gels, heat-set emulsions, or filled (protein) gels appear to confirm predictions from material science.[160–168] Oil-in-water emulsions stabilized using globular proteins behave like interactive fillers within a 3-D protein network. In contrast, oil-in-water emulsions stabilized by low molecular weight emulsifier (Tween 20, Triton X-100, lecithin) behave like non-interactive fillers. Acid induced caseinate gels containing emulsified oil also behave as filled gels.[169, 170] More work is needed to consider the functionality of different protein groups within filled gel systems. Whether filled gels are good models for meat emulsions also needs looking into.

10.6 Conclusions and future trends

This chapter provides an overview of standardized tests for protein functionality. The majority of tests described in this article employ model foods. Owing to their resemblance to real formulations, tests using model foods provide results that are directly relevant to the food technologist. Other notable features of model food tests include their (i) ease of implementation, (ii) non-requirement for specialized instruments (iii) ability to test multiple factors and interactions, (iv) compatibility with product optimization, (v) robustness or resistance to interference, and (vi) increased potential for standardization. However, it is likely that Type I tests using purified systems may continue to predominate in food proteins research. There is increasing tendency to use model food (Type III) tests in conjunction with sophisticated experimental design (such as surface response methodology) for research. Another expectation is the increasing use of utility testing in conjunction with quantitative sensory analysis.

10.7 Sources of further information and advice

Only a small fraction of the general literature on protein functionality could be covered in the space available. In contrast, the majority of work dealing with model food systems and their use to examine protein functionality has been covered. The interested reader is advised to proceed to the original papers and references cited therein for more detailed information. *The Encyclopedia of Food Science and Technology* is perhaps the single most useful source for general background reading on protein functionality.

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11

Modelling protein behaviour

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11.1 Introduction

The modern concept of quantitative structure-activity relationships (QSAR or simply SAR) originated from 'Hansch Analysis', which comprise linear free energy relations of activity in first- or second-order linear models, based on the parameters representing the hydrophobic, steric and electronic properties of compounds.[1] Nakai *et al*.[2] reviewed in 1994 the QSAR study of food proteins. Algorithms discussed in that article were *correlation* (multiple linear regression analysis, principal component regression, partial least square (PLS) regression, and artificial neural networks (ANN)), *classification* (stepwise discriminant analysis, principal component similarity (PCS) analysis, and ANN) and *optimization* (linear programming, constrained simplex optimization, and random-centroid optimization (RCO)).

With the advent of genetic engineering, site-directed mutagenesis became a powerful tool for improving protein functions. As a result, RCO developed for general optimization purposes was modified to apply for protein engineering and thus coined as RCG (RCO for genetics). This optimization successfully improved the thermostability of *B. stearothermophilus* neutral protease[3,4] by mutating one site in the active site with 16 amino acid residues in the amino acid sequence of the enzyme. Later, RCG was extended to mutation of two sites simultaneously, by applying to the entire sequence of recombinant human cystatin C with 120 amino acid residues, in order to enhance its papain inhibitory activity.[5] Recently, we also published another QSAR review to update our previous review[2] and reported progress made in computer-aided technology, especially for use for unsupervised data mining (DM) purposes.[6] The approach employed was to conduct QSAR after PCS for protein sequence analysis

(SPCS). More recently, we have further introduced a new software package by including both homology similarity analysis (HSA)[7] and homology similarity search (HSS),[8] which are more useful for achieving the protein QSAR purposes. In this chapter, the most updated version of our QSAR will be discussed.

In this post-genome era, the structural consequences of genomic data became extremely important from the aspect of proteomics, to achieve the final aim of elucidating the underlying mechanisms of protein functions. Although 3D structure-based functional predictions should be the final, decisive approach to be used, the 3D structure analysis of proteins is an involved process, especially for extracting QSAR information from X-ray crystallographic data of food proteins. Thus, sequence-based functional predictions become simpler, more useful without relying on the information on 3D structures of proteins.

There has been a novel approach reported for protein QSAR using nonlinear signal analysis.[9] According to its authors, this protein QSAR developed from classical QSAR can be characterized by (a) the substitution of the molecular descriptors with self-consistent indices derived from time-series analysis for parameterizing 'hydrophobicity distribution' and (b) the substitution of biological properties with protein structure and/or physiological properties or any other 'global properties' that can be measured for protein folding as a whole. The major algorithm used for the analysis of hydrophobicity sequences or profile was recurrence quantification analysis (RQA), in which the recurrence plot (RP) depicted a single trajectory as a two-dimensional representation of experimental time-series data. Examples of the global properties used in this RQA were thermal stability, protein/peptide interaction and folding behaviour.[9]

The objectives of this chapter are to explain our new QSAR protocol for food proteins by combining SPCS, HSA including HSS, and ANN along with optimization strategy, such as RCG, and to discuss how this approach can be effectively organized for elucidating the underlying mechanism of protein functions on the basis of their molecular structures.

11.2 Computational methodology

For conducting the QSAR study of proteins, correlation analysis is generally performed for predicting functions after classification of the proteins into groups. Although protein functions can be optimized based on the QSAR results thus derived, the optimization of functions can also be directly conducted independently using optimization techniques, e.g., RCG, without relying on the QSAR study.

11.2.1 Classification

There are two groups of classification, 'supervised' and 'unsupervised' depending on whether prior knowledge of which family groups the proteins should belong to is available. Unsupervised classification is usually more useful

than supervised counterparts as there could be a great chance of making an unexpected new discovery with regard to the new class eliciting the underlying mechanism of a newly classified function. Typical examples of popular unsupervised classification are cluster analysis (CA) and principal component analysis (PCA) along with its derivatives, such as our PCS approach that is subsequently discussed in this chapter.

CA

Clustering of data is derived from computation based on the distance between data points. The Euclidian distance (D) has been the most popular form used in many papers:

$$D_{ij} = \left\{ \sum (x_{ik} - x_{jk})^2 \right\}^{1/2} \qquad 11.1$$

Formation of object cluster is achieved in a hierarchical way. However, a disadvantage of the Euclidian distance is the dependency of calculated distances on the units of measures employed, unless normalization of the variables is made.

To account for the correlation among variables, Mahalanobis distance also has been used:

$$D_{ij}^2 = (\bar{x}_i - \bar{x}_j)\, {}^t C^{-1} (\bar{x}_i - \bar{x}_j) \qquad 11.2$$

where $\bar{x}_i$, $\bar{x}_j$ and C (covariance) are matrix variables. Thus, normalization of data is unnecessary; however, this covariance should be accurately computed with a degree of freedom in the order of 100 or more.

PCA

The major function of PCA is to reduce the dimensionality of a data set X, which may consist of a large number of interrelated variables, while retaining the original variability in X as much as possible.[10] This can be achieved by transforming sample data to a new set of variable, principal components T, which are uncorrelated in the order so that the first few principal components retain most of the variation present in the original variables.

$$X = TP^t \qquad 11.3$$

where P is the loading matrix demonstrating relationships of original data with the PC scores derived from PCA. A comprehensive example of PCA computation is neatly shown by Otto.[11]

PCS

The PCS plots slope (s) against coefficient of determination (r^2) obtained from linear regression analysis of deviations of sample PC scores from those of a reference sample, which is shown as a 45° diagonal line (Fig. 11.1). The proportion of variability of each PC score is seen as the distance of the PC score in question from the nearest lower-level score. For instance the portion (%) of total variability

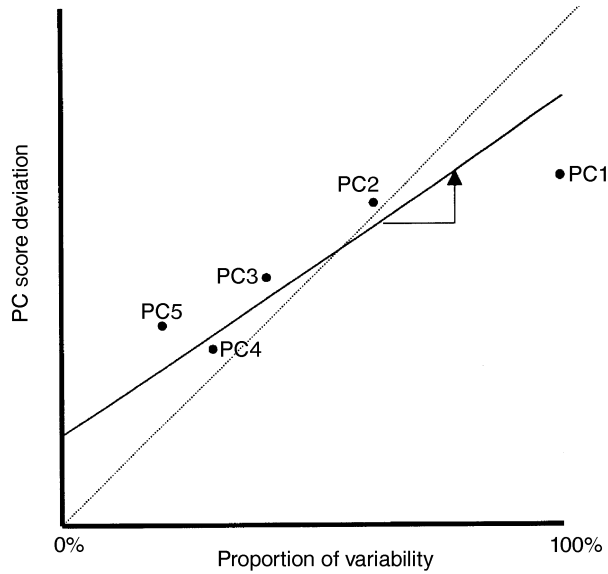


Fig. 11.1 Computation of slope (s) and coefficient of determination (r^2) for drawing a PCS scattergram. PC data points deviated from the 45° diagonal line (thin); regression line (thick).

accounted for by PC1 is represented by the distance between PC1 and PC2 on the abscissa in Fig. 11.1. The original report on the principle of PCS and its application to GC data of mango was made for assaying fruit quality based on cultivars and ripening conditions by Vodovotz *et al.*[12] The main purpose of PCS was to depict more than three PC scores on a 2D scattergram, which was not possible to represent when regular PCA was used for processing the same data. Better and more rational classification enabled by PCS scattergrams than 2D or 3D PCA scattergrams was explicitly demonstrated in our previous publication.[6, 13]

Advantages of the PCS are:

1. Time series changes can be readily and accurately monitored. Examples of application are cheese ripening[13] and bacterial spoilage of meat.[14]
2. By rotating the reference samples, the classification capacity of PCS can be more flexible than that of PCA[6] as well as those based on distance, e.g. CA.[6, 12]
3. Because of the unsupervised nature, there is always a possibility of making an unexpected new discovery by finding a previously unknown factor playing an important role in the underlying mechanisms.[15]
4. Sample grouping observed on PCS scattergrams on the basis of functional strength would assist screening of influential predictors to be used as input variables (descriptors) in the subsequent ANN computation.

Figure 11.2 shows a comparison of classifications made by PCS vs. PCA,[6] which is an example of advantage 2 listed above. Similarity among different

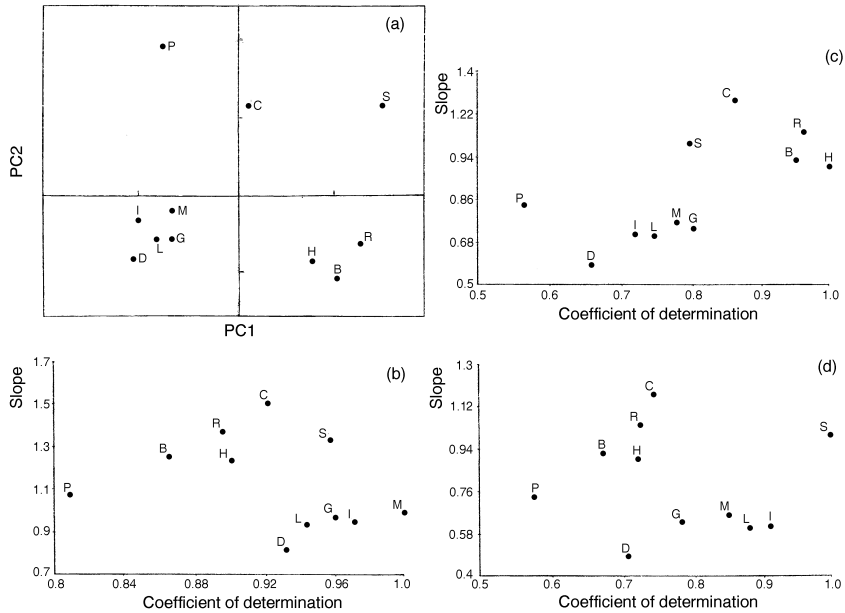


Fig. 11.2 Principal component similarity (PCS) scattergrams of CAP using charge scale for amino acid residues in the sequences. S: salmon protamine, H: human protamine, B: rabit protamine, R: rat protamine, C: chicken protamine, P: PR39 from pig, L; lactoferricin, M: MSI-95 from frog skin, G: BNCP-1 from guinea pig, D: α_1-defensin, I: indolicidin.

antibiotic activities of cationic antimicrobial peptides (CAP) is more clearly observed in PCS (Fig. 11.2b, c and d using different peptides M, H and S as the reference) from PCA (Fig. 11.2a). All of the scattergrams (Fig. 11.2) were drawn by using the charge scale for quantifying amino acid residues in the sequences. This result is indicative of the important property of side-chains of amino acid residues, i.e., charge in this case, for the classification of CAP. With regard to CA, it has been reported that a distance measurement of full dimensionality may easily overlook patterns differing in only a small number of variables.[16]

Classification of CAP is not so straightforward as it is rather difficult to find group difference, for instance, the difference of protamines (H, S, B, R and C) from the helix-type CAP such as indolicidin (I), based on their molecular structure is shown in Fig. 11.2(a) (PCA) vs. Fig. 11.2(b), (c) and (d) (PCS). According to the overview made by Andreu and Rivas,[17] CAPs were classified into peptides with helical conformation, peptides rich in certain amino acids, peptides with single cyclic disulfide and peptides with several internal disulfides. Unfortunately, not all of the CAPs included in Fig. 11.2 have been distinctly grouped in any review article thus far in the literature. For instance, peptides M and G, which could be unique, did not belong to the third group other than the protamine group and the helix-controlled group.[17] Compared to the PCA scattergram (Fig. 11.2(a)), which is rather unilateral, PCS scattergrams

(Fig. 11.2(b), (c) and (d)) show characteristic scattering, even within the same group of protamines, especially by rotating the reference. Difference of fish protamine (S: salmine) from other protamines can be seen in Fig. 11.2(d) by using S as the reference. Similarity of samples with a selected reference peptide can be different depending on the peptide employed as the reference, resulting in different groupings on the scattergrams (advantage 2 listed above). This result suggests that PCS can be a useful tool for family prediction in the functional sequence analysis of peptides.[8]

Figure 11.3(a) and (b) shows PCS scattergrams of human cystatin C mutants[5] with data-points labelled to demonstrate the intensity of papain inhibitory activity (the first two parts of the figure from the original reference[6] are not shown here). There are trends from weak (0) to strong (5) when helix (Fig. 11.3(a)) and strand (Fig. 11.3(b)) propensities were used for the PCS computation. The trend of the plot is that the greater the coefficient of determination as well as the slope, the stronger the antibacterial effects (lower the MIC or the minimum inhibitory concentration), suggesting an important role played by these secondary structures in the peptide function (MIC). Figure 11.3 is an example of the above advantage 4.

Classification ANN

The ANN was originally developed for supervised classification purposes.[18] To make ANN unsupervised, pre-processing of data using the Kohonen net or genetic algorithm is recommended.[19] However, pre-processing of the predictor variables using PCA (such as 3z prediction of peptides[20]) or PCS for homology sequence analysis[6] may be able to achieve the same objectives as discussed subsequently.

11.2.2 Prediction

Most predictions have been made by using multiple linear regression analyses in the past. Among many software packages for regression analysis currently available, ANN was chosen for discussion in this chapter because of its outstanding analytical capacity for nonlinear data, despite several skeptical comments in the past. It must be noted that the ANN software itself has been phenomenally improved during the past decade.

Regression ANN

The ANN uses nonlinear algorithms and is currently most powerful package for prediction purposes. This modern ANN was superior to the prediction made by a microbial modeling approach.[21] The ANN was also reported to be more accurate than PLS (partial least squares regression), especially in DM computation.[22, 23]

Bishop[18] extended the ANN capacity by adopting many important techniques in the modern ANN, for instance Statistica NN (SNN),[19] such as density estimation, error function, parameter optimization algorithms, data-preprocessing and incorporation of Bayes' theorem. As a result, the SNN

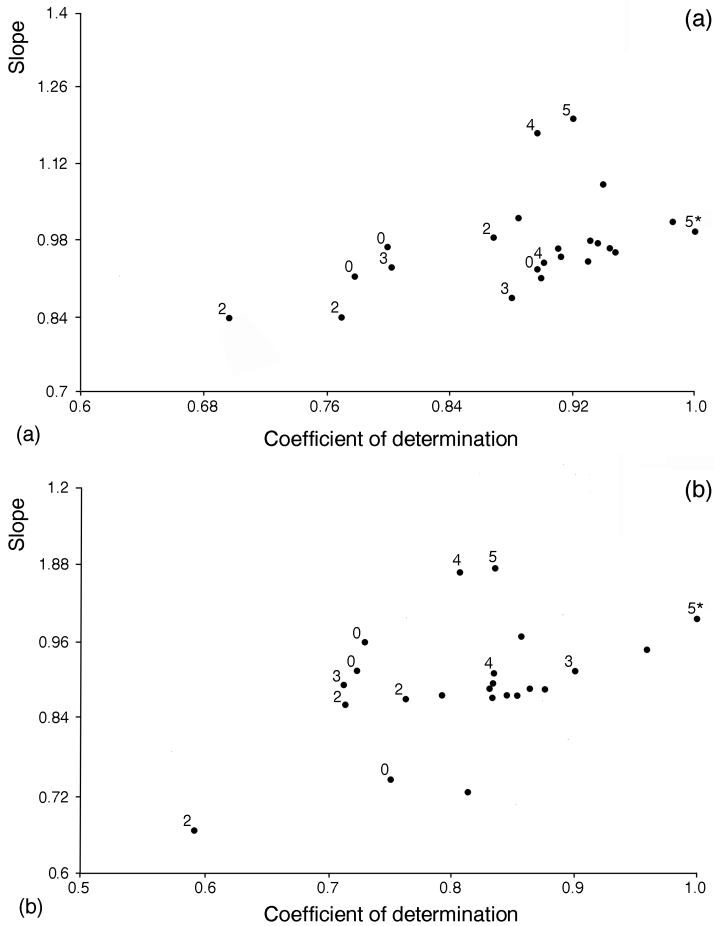


Fig. 11.3 Sequence PCS scattergrams of double mutants of human cystatin using helix propensity (a) and strand propensity (b). Mutant 12W86V was used as the reference. Twelve and 11 PC scores were used in PCS computation to account for 88 and 85% of variability, respectively. Digit labels show multiples of the papain inhibitory activity compared to that of wild type after rounding off. Dots without labels are for single site mutation at helical Zone I. The reference sample* at coefficient of determination 1.0 and slope 1.0 had the highest activity of 5.

includes not only Bayesian NN but also the ability to use PCA to select important input variables for dimensionality reduction, in addition to Kohonen nets and genetic algorithm to train the ANN networks. When the regular, linear PCA fails, the autoassociative network[24] can be used for conducting nonlinear PCA. Furthermore, SNN can compute sensitivity to rank the importance of input variables and draw scattergrams as well as 3D response surfaces without relying on other specific algorithms. Another important feature of the SNN is an easy-to-use wizard for network creation including an automated network designing

system. ANN has been recognized as a useful tool for drug design by using mainly for QSAR purposes.[25]

11.2.3 Optimization

There have been a plethora of computer programs as optimization is the eternal aim of research scientists. This is especially true in the search for the global optimum rather than being stalled at local optima, which has long been the focus of interest in mathematics and computer science. Random-centroid optimization is one of the solutions that we have proposed despite the fact that it is not the perfectly automated technique using computers because human manipulation for reading surface response maps is required during the computing procedure. It may be worth noting that we have successfully automated this visual comparison process by introducing a penalty function.[26] However, in general, complete computerized automation is not always desirable because human judgement should not be ignored in solving problems. The capability of visual comparison to define the regularities in data scattering is unsurpassed by automated computer-aided methods.[16]

RCO

RCO is a sequential search by iterating a search cycle consisting of regulated random design, centroid design, and mapping.[3] The regulated random design for obtaining a near-even distribution of the search design within the preset search spaces is essential to avoid unnecessary searches accidentally chosen, i.e., lopsided local search spaces; this is ineffective in achieving highly efficient optimization. The centroid design also prevents the waste of experimental results obtained during the processing of random search. In addition, mapping is effective in determining the search direction without being trapped in local optima. Mapping aims at defining the search direction towards the global optimum on response surface maps.

In our study, to draw 'trend lines' on a map for approximating the response surface, the entire search space for each factor was divided into three equal subdivisions. Data points qualified to be linked on the map, thereby forming the curvilinear trend lines for the factor, were those that belonged to the same subdivisions for factors other than the factor in question (an example illustration appeared in Nakai *et al*.[3]). However, the trend lines were frequently too few in number to make the correct moves in search of the global optimum, especially during an early search cycle of the entire RCO procedure because of the lack of an adequate number of data points. To circumvent this problem, an intentional factor ignoring process was introduced as shown in Fig. 11.4.[4] The maps drawn by ignoring apparently unimportant factors are shown on the right-hand side of Fig. 11.4 in contrast to its left-hand side. Clearer demonstration of the area of search to be continued is more evident in the former than the latter on the same scattergram without applying the ignoring process. Despite the possible increasing inaccuracy due to inadvertent discarding of potentially informative

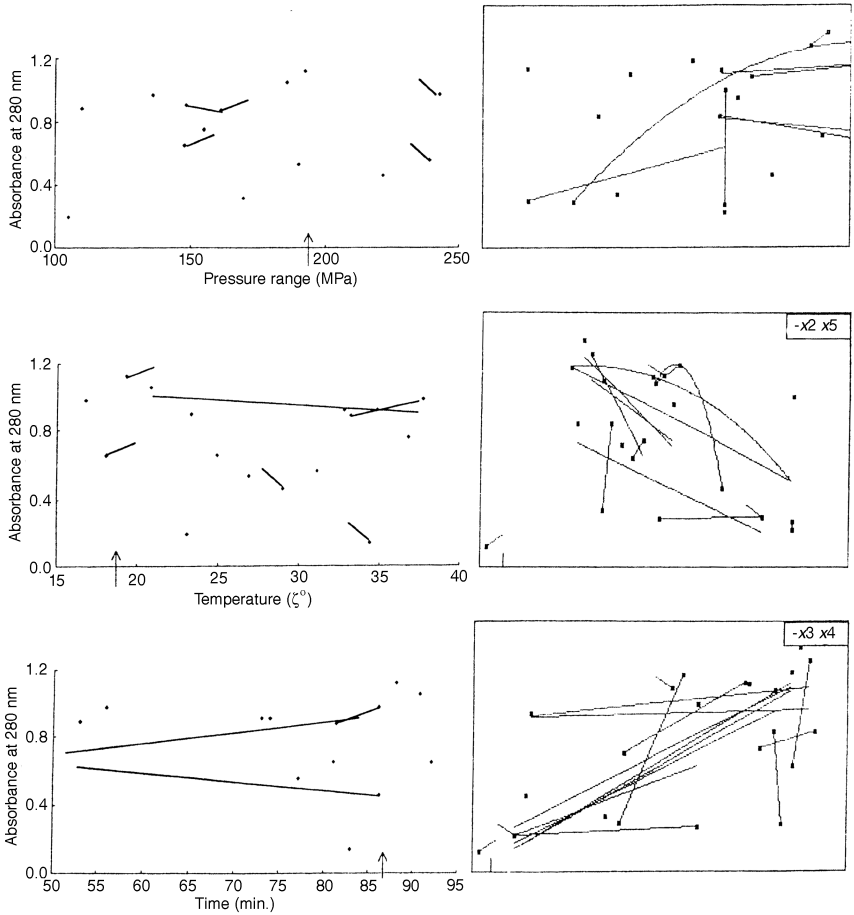


Fig. 11.4 Effect of trend-line drawing by ignoring factors in mapping during RCO optimization of high-pressure treatment of an enzyme. Right-hand side shows the response surfaces improved by ignoring two factors over the left-hand side counterparts without ignoring. For instance, in drawing the response surface map of temperature (X_4), X_2 (NaCl) and X_5 (Time) were ignored. Pressure was X_3.

data, this mapping procedure to approximate the search direction is obviously useful in any global optimization with an extremely small number of the experimental data available, especially at the early stage of optimization processes.

Overall, by combining all those useful tools, RCO proved to outperform the currently available algorithms, such as linear programming, classical surface response methodology and sequential simplex optimization when assessed by using more than ten mathematical models including multimodal models for optimization verification.[2, 3] For global optimization purposes, the currently available and most popular algorithms are simulated annealing and genetic

algorithm, which require thousands of iterations during optimization computations using automated computer-aided procedures without difficulty. Although RCO requires manual manipulation for determining the search direction on maps, the RCO can reach the global optima of the mathematical models as above mostly within 50 iterations.[3] This is crucial, especially in the case of expensive, labour-intensive life science experiments. The great efficacy of RCO owes its success mainly to the centroid design and the mapping as discussed above.

Popular sequential simplex optimization[27] may be, however, still useful for writing a computer program for automated optimization. For wine blending, simplex optimization was easier to use as a subroutine subprogram in the blending optimization software package than the RCO program.[28] Separately conducted GC data of wine volatiles alone could be used without the need for additional blending experimentation. A combination of GC profiles of ingredient wines was optimized to blend varietal and/or stock wines for simulating the bouquet of a target wine in a winery with successful blending results, which were verified by professional taste panel tests.[28]

RCG

The RCO was then modified for optimizing genetic projects, such as site-directed mutagenesis of the active site with 16 amino acid residues within a protease sequence (G139–Y154).[4] The half-life temperature of the optimized mutant V143E was higher by 6.5 ºC than the 68.3 ºC of the wild-type enzyme in addition to a 30% enhanced activity. Unfortunately, this result of the project is still an interim output at the time of writing and research on the entire RCG procedure was not completed due to an expiry of time and funds to continue the project.

The RCG program was subsequently and successfully applied to another optimization project for simultaneous two-site mutagenesis with a larger search range including the whole human cystatin C sequence.[5] The search space in this project covered the entire sequence of 120 residues rather than the short segment of the active site, which consisted of only 16 residues in the case of the previous optimization project.[4] As a result of the former, a fivefold activity increase was achieved by preventing amyloidosis during expression in *Pichia pastoris* and purification of the crude cystatin secreted.[5] To carry out these RCG optimizations, the amino acid residues in the protein sequence were quantified for drawing maps using their property values, i.e. hydrophobicity, charge represented by isoelectric pH of side chains, α-helix propensity, β-strand propensity and bulkiness. The final RCG maps including all mutants prepared (Fig. 11.5) were valuable in elucidating the underlying mechanism of target functions (papain inhibitory activity) depending on effective residue properties for logical explanation of functional changes on maps. The underlying mechanism of amyloidosis, which would immediately destroy the protease inhibitory activity, could be postulated on the basis of the optimization process shown in the maps (Fig. 11.5). Figure 11.5(a) and (b) match the active/binding site 1 at position 12 and another binding site at position 108 as reported in the

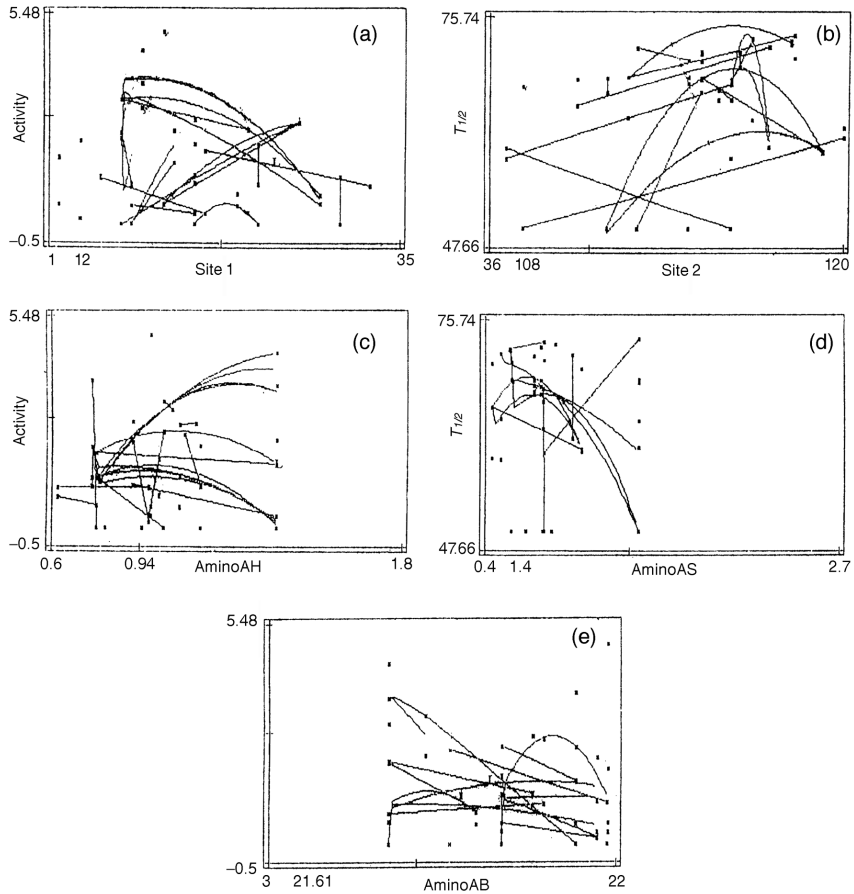


Fig. 11.5 Effect of amino acid scales of papain-inhibitory activity and thermostability of human cystatin C. (a) mutation zone I, (b) mutation zone II, (c) helix in zone I, (d) strand in zone II, (e) bulkiness in zone I. Digit at the bottom between the terminal values of the abscissa is the value of maximal data point of each map.

literature. Figure 11.5(c) and (d) demonstrate lower helix (helix index used here is opposite to the propensity) and lower strand were favourable for activity and thermal stability, respectively. Figure 11.5(e) indicated that lower bulkiness in the first mutation zone (positions 1–35) is favourable in increasing activity. These effects appear as reducing helix and concomitant increase in strand propensity in the strand zone in human cystatin C molecule, which enhanced amyloidosis, thereby resulting in an activity loss. Another advantage of RCG in addition to the mechanism study is that there is no need for prior information on 3D structures because it is a random search. As discussed later, the map-elicited information can be effective in selecting useful predictor variables for potential, successive ANN computation for function prediction.

11.2.4 QSAR and DM

It has been generally agreed that QSAR is not equivalent to DM in concept as there are different approaches used for different DM purposes in the literature. However in chemometrics, QSAR is one of the most logical, powerful as well as popular tools for DM purposes.[16, 29] In general, when the number of input variables (predictors) is exceedingly large, the multi-colinearity problem arises, thereby causing a rapid decline of predictability. Thus, dimensionality reduction becomes essential although the risk of missing truly influential factors may be aggravated. At any rate, it is still a useful practice without any other mathematical replacements, to reduce the number of independent variables (predictors) using PCA of orthogonal nature prior to conducting regression analysis as the final step of QSAR.[20]

Data mining is a technique to extract valuable information from established databases mostly in a supervised mode. The general protocol of DM being used in combinatorial chemistry[30] is to (i) choose a similarity probe; (ii) calculate similarity; (iii) rank all components; (iv) select top compounds; and (v) estimate success rate. Data mining accepts a 'black box' approach, such as ANN and PLS, which can generate valid predictions but is not capable of identifying the interrelations between the input variables.[18] There are a great variety of representations available for DM, including rule bases, decision trees and ANN; and also there are many algorithms to adopt for constructing a DM protocol such as density estimation, classification, regression and clustering. However in conjunction with statistical techniques, the graphical model derived from Bayesian neural networks (BNN) is advantageous for DM study.[31]

Recently, a new mathematical DM that determines local structural information within proteins was developed for protein structure study.[32] However, this DM algorithm cannot indicate the biological implication of the computed outputs. This is probably because the algorithm deals with atomic weight and instability within the least squares routine, thereby evaluating internal chemical symmetry between amino acid residues, by which the ability to match different amino acid residues can be estimated. However, there is no functional consideration being made in this technique. This phylogenetic DM has been so rational that genetic algorithm based on evolutionary conservation can define a family of query DNA arrays or protein sequences; consequently their functionality can be postulated. A problem is that this logic is not always valid mainly because of readily circumstantial alteration of protein structures even if they are originated from the same DNA array due to SNP (single nucleotide polymorphism),[33] thus it is a limitation of this approach. The hypothesis that one gene to one protein eliciting one function is no longer true; multiple protein products are derived from a single gene.

11.3 Computer-aided sequence-based functional prediction

For modern proteomics in the post-genome era, researchers need rapid, easy-to-use, reliable tools for functional characterization of new sequences derived from

yet-unannotated genes. For most of the currently available sequence analyses, the 'Prosite'-type models (Blast and Clustal models are currently most popular) flanking conserved sites (motifs) are used for similarity analysis to align sequences.[34] However, pattern similarity of amino acid side-chains as defined herein *per se* is difficult to use for evaluating functionality, thereby frequently resulting in unreliable functional predictions.

During our QSAR study, we developed a new strategy (protocol) for protein sequence analysis[6] composed of four steps. Step 1: PCS is applied to homology profiles or multiple sequence alignments for classifying sequences into families based on side-chain properties. The resultant PCS scattergrams can identify which side-chain property is playing an important role in the target function. By clustering sample sequences on the PCS scattergrams in terms of intensity of functional activity, influential predictors can be identified, although in crude forms, as shown in Fig. 11.3. Step 2: the HSA can more precisely identify the potential functional motifs within sequences. Step 3: the RCG, which is optional, can be conducted at any time, at any step within this protocol as it does not need structural variables as a prerequisite. From maps drawn on the basis of specific side-chain properties, the influential predictors can be selected as described earlier (Fig. 11.5). Step 4: Using the predictors thus screened as input variables together with output function data, regression ANN can induce functional estimates.

HSA

For the segments selected from a sequence, pattern similarity constants of the query sequence in comparison with the pattern of corresponding segments in the reference sequence with known functional mechanisms as well as the average value of side-chain properties are computed.[6, 7] The properties of amino acids used here were the same as those used in RCG.[4] In other words, the specific side-chain properties in a designated segment within a sample peptide sequence are compared with those of side-chains of the corresponding segment within the sequence of the reference peptide. The most potent sequence within the family is usually selected as the reference. This procedure is repeated for other segments within the same sample sequence. Linear regression analysis is carried out and the resultant coefficient of determination (r^2) is referred to as the homology similarity constant. Average values of the property index values within the segments are also calculated for comparing segments between query sequences and the reference sequence.

Sequence PCS

Equivalent to the sample attributes in the regular PCS computation, side-chain property indices are compared between different peptide sequences.[7] The rest of the PCS procedure is exactly the same as the regular PCS.[12–15] The sequence PCS (SPCS) program thus modified was written accordingly.[7]

11.3.1 Data mining of antimicrobial activity of lactoferricin and its derivatives

A total of 71 sequences of lactoferricin and its derivatives, of which the major sequences used were 15-residue lactoferricin derivatives, were investigated.[7] The SPCS classified them into four groups in addition to one exceptional group, which did not belong to any other of the three classification groups. From the aspect of antimicrobial activity (MIC), 15 residues were divided into three segments at positions 1–3, 4–9 and 10–15. It was concluded from ANN regression analysis that helix propensity of positions 4–9 was the most important in determining MIC against *E. coli*, followed by cationic charge pattern at positions 4–9 and 1–3 in this order. An important discovery was that the pattern similarity of divided segments based on properties of side chains in homology profiles was more important in elucidating the underlying mechanisms of the function than the similar data computed for the entire sequences.[7]

Regression ANN was performed using input variables selected by SPCS and HSA against MIC, yielding r^2 of 0.85–0.97 for [predicted log MIC]/[observed log MIC] correlation (Fig. 11.6). Comparison of these values with those reported by Strøm *et al*.[35] using the similar approach is difficult because our work is for DM study in contrast with the restricted number of 15-residues peptide derivatives used in their study. The DM study usually intends to deduce the most useful information from data collected in large databases, whereas another popular approach is to define the biological function through a small number of

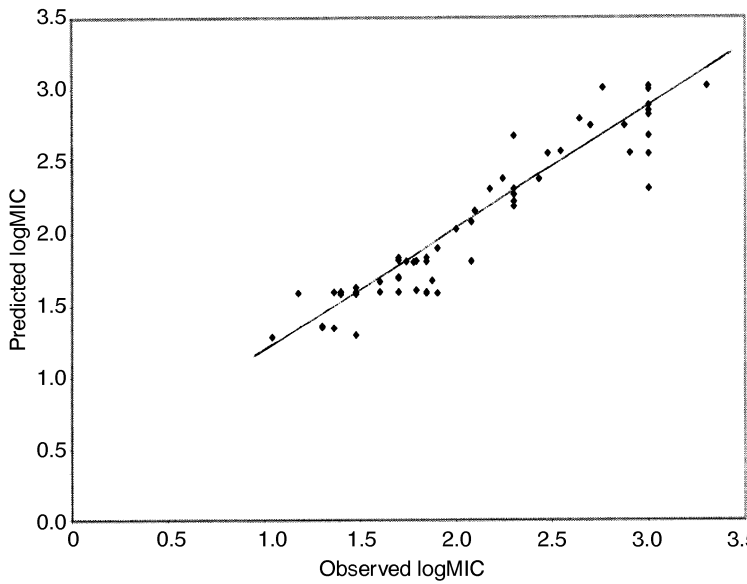


Fig. 11.6 Predicted versus observed MIC values against *E. coli* of 65 different lactoferricin derivatives in logarithmic MIC scale as determined by ANN. The exceptional derivatives group (group III) were not included in ANN computation.

site-directed mutagenesis. In general, the latter is one of the most potent, direct QSAR approach. Furthermore, the PLS work of Strøm *et al*.[35] has used property values of the entire enzyme sequences as input variables in their computation. As a result, we could obtain more detailed elucidation for the MIC mechanism of lactoferricin.

11.3.2 Functional motifs determination in lysozyme sequences

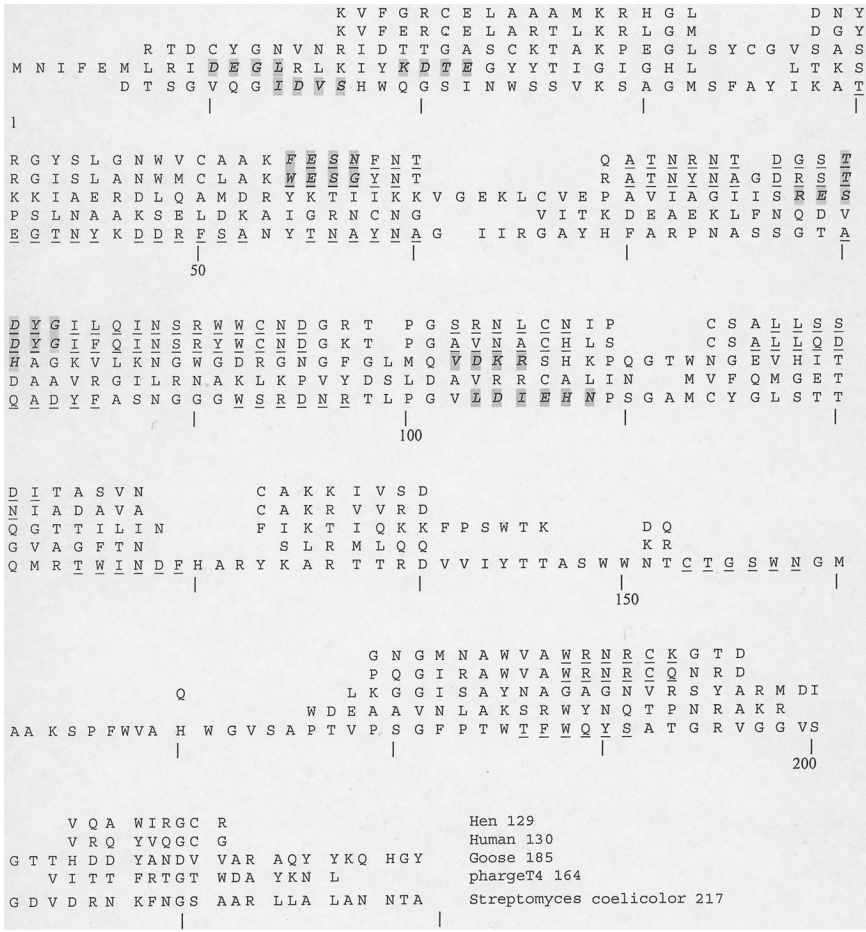
A new program of homology similarity search (HSS) was successful in defining the active sites and the substrate binding sites of lysozyme families. The active site and binding sites of a new CH-type (*Streptomyces coelicolor)* lysozyme, which possesses a unique β/α-barrel fold,[36] thus being quite different in molecular structure from the helix/strand double domain fold of other lysozyme families,[37] were successfully identified.[8]

Table 11.1 shows the multiple sequence alignment of lysozymes of four families: c-type (hen and human), g-type (goose), v-type (T4) and CH-type (*S. coelicolor*). There are considerable differences in not only sequences, but also active sites (shaded) and binding sites (underlined). Figure 11.7 is the PCS scattergram, which shows remote data points for this characteristic CH-type lysozyme from those of other lysozyme families. Figures 11.8(a) and 11.8(b) are HSS patterns of searching for the substrate-binding sites in lysozyme sequences of human and *S. coelicolor*, respectively. Potential sites have to meet a required condition with both high similarity (close to 1.0) and high average property values. For drawing Fig. 11.8(a), hen lysozyme was used as the reference, while for Fig. 11.8(b), *Streptomyces* lysozyme itself was used as the reference because of poor detectability when hen lysozyme was used as the reference. It should be remembered that this reference change was unnecessary in the case of active site detection. The reference change required in the case of binding site detection may be because of a major difference in the enzymatic reaction and mechanism of this lysozyme of unique structure compared to those of other families.[36] Table 11.2 is the result of a binding site search;[8] the active sites detected using HSS were in good agreement with those reported in the literature,[37] whereas the binding sites shown in Table 11.2 are uncertain due to lack of literature data to confirm; this awaits future study. The HSS search used in this study is described below.

HSS

A preliminary study was carried out by changing the size of segment to be searched (mostly consecutive 3–6 residues), flanking the potential functional residue(s), to determine the most appropriate size of segment in differentiating functional segments from nonfunctional segments within the reference sequence. Using the segment thus selected, the search was initiated from the N-terminus of the sample sequences by shifting the search one residue after the other each time towards the C-terminus. For every segment, the pattern similarity constant and the average of property index value were computed.

Table 11.1 Homology patterns of lysozymes belonging to four families



Functional motifs were chosen to be the segments with similarity constant close to 1.0 and average property values closest to the corresponding values of the reference sites, usually the highest value, as exemplified in Fig. 11.8.

11.3.3 DM strategy in post-genome era

An approach that we have recently proposed[6] was to use principal component scores computed after dimensionality reduction through PCA included in the SPCS program as predictor variables in correlation computation using nonlinear ANN algorithm. The advanced multiple sequence alignments, such as BLAST, have frequently enhanced the alignment capacity by using programs such as HMM (hidden Markov model)[38] for identifying functional sites in families of remote

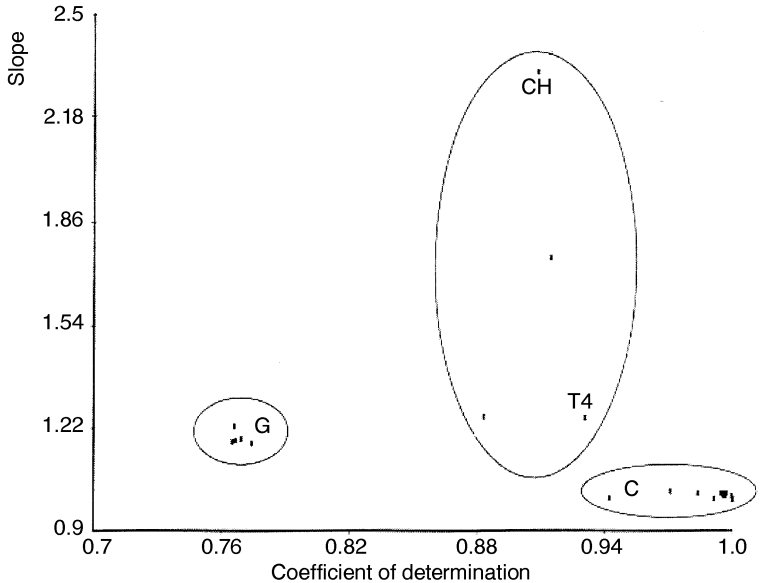


Fig. 11.7 PCS scattergram of lysozyme families when hydrophobicity was used as property index to convert sequences to scale values. Hen lysozyme was used as the reference at coefficient of determination = 1.0 and slope = 1.0. The circles between circles for c-type (C) and g-type (G) families include *Streptomyces coelicolor* lysozyme (CH-type), which is separated from dots for v-type lysozyme including T4 lysozyme.

distances. Instead, we proposed to use pattern similarity of motifs in unannotated sequences vs. those of reference (or lead) sequences by assigning to the motifs with different properties of amino acid side-chains closely relating to target protein functions. This new computer-aided techniques of HSS[8] could fairly accurately predict the locations of active as well as substrate-binding sites in the newly discovered sequences. The new HSA and HSS programs may upgrade the efficiency of not only RCG but also the previously proposed DM plan[6] as follows: BLAST → SPCS → HSA → HSS → (RCG) → ANN where the RCG herein is optional.

This new, upgraded protocol could be more useful when 3D structure is difficult to define, which is not unusual in natural polymers including food proteins. All of the above techniques of sequence analysis *in silico* proposed in this chapter do not require prior information on 3D structure; thus it should be useful for investigating the function of unannotated genes.

All software along with the instructions on how to use the computer programs are open to the public through ftp files (**ftp://ftp.agsci.ubc.ca/foodsci/**) on the Web. In addition, upon request from researchers through e-mail (addressed to shuryo.nakai@ubc.ca) by attaching Excel files of query data, we will provide computation services by returning mails attaching the computation outputs. This service may be helpful to readers, especially when the computations *in silico* require manual adjustments, such as visual comparison of the maps in the RCG procedure.[4, 5]

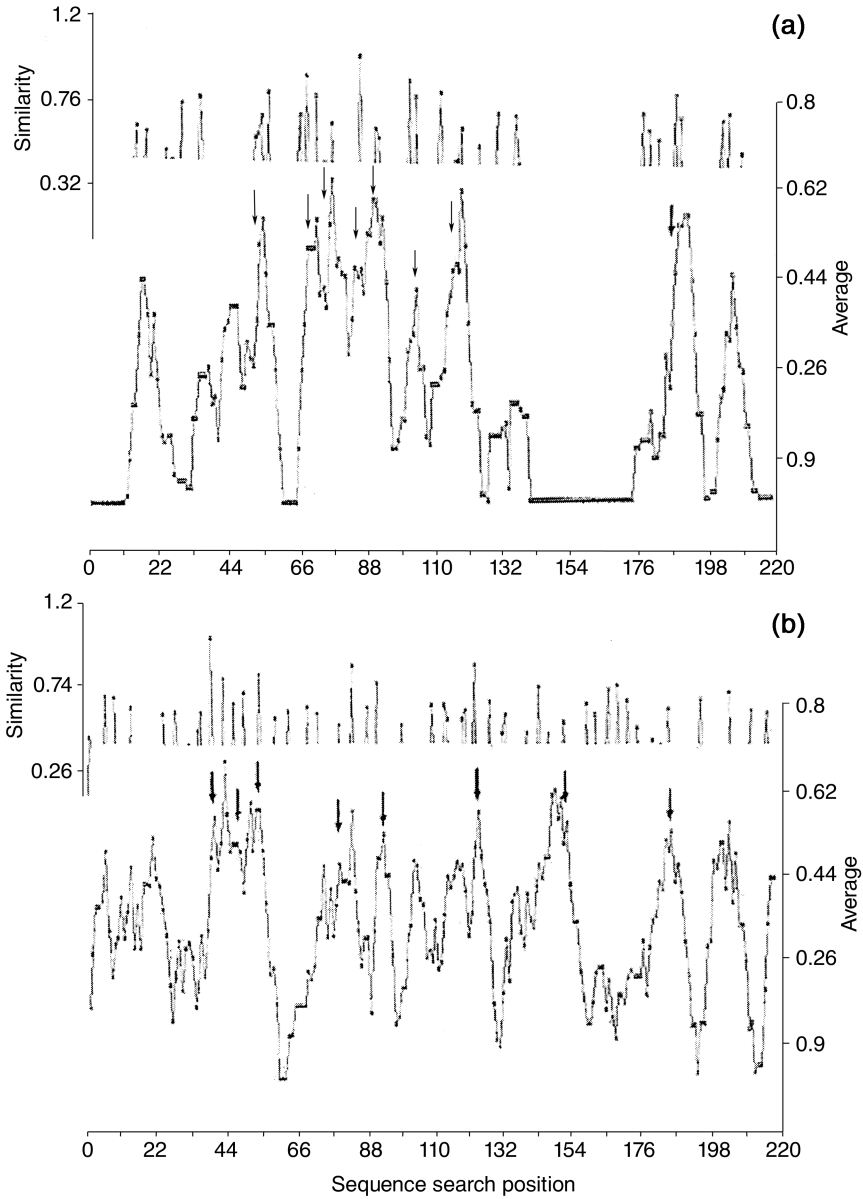


Fig. 11.8 HSS search patterns of substrate-binding sites: (a) human lysozyme against hen lysozyme using the segment at alignment positions 84–89 as the reference. (b) *Streptomyces* lysozyme using the segment at alignment positions 40–45 as the reference (self-search). Arrows show the alignment positions where similarity constant and average hydrogen bonding value are both high, thus most likely to be the substrate binding sites. Abscissa scale shows the alignment positions.

Table 11.2 Determination of substrate binding sites in amino acid sequences of lysozyme families

	Potential sites								
Hen	**55(35)E** **.81/.71**	**70(42)A** **.79/.53**	77(48)D .34/.65	**84(55)I** **1.0/.41**	**90(61)R** **.56/.58**	**102(72)S** **.78/.56**	**117(83)L** **.61/.42**	188(111)W .66/.35	
Human	**54(34)W** **.48/.52**	**70(42)A** **.42/.51**	77(49)D .34/.65	**84(56)I** **.91/.47**	**90(62)R** **.50/.61**	102(73)A .80/.32	**116(83)A** **.61/.46**	**188(112)W** **.89/.45**	
Goose	43(37)I .85/.31	99(93)L .76/.34	**119(113)I** **.67/.46**	**143(133)S** **.81/.55**	181(143)G .82/.36	**201(163)G** **.81/.58**			
T4	**6(6)M** **.74/.34**	**16(16)K** **.99/.38**	53(49)A .78/.30	**66(57)V** **1.0/.42**	**75(66)L** **.52/.54**	94(85)K .67/.38	**123(112)A** **.73/.45**	**186(136)S** **.58/.51**	207(153)F .78/.36
CH	**40(35)T** **1.0/.58**	**47(42)D** **.63/.52**	**55(50)T** **.80/.51**	80(74)A .51/.44	**92(86)W** **.73/.49**	**124(118)T** **.83/.59**	153(147)C .51/.56	186(180)T .56/.54	

The first and second digits are similarity coefficients and average hydrogen bond index values of binding site with segments of six residues beginning with the position shown.
Bold digits show more likely binding sites than medium-face digits.

11.4 Future trends

Our new approach for structure-based functional prediction could be a new method of protein sequence analysis for functional assessment of food proteins. These new data-mining techniques may be more user-friendly when internet software packages are available for conducting QSAR computations on websites, without downloading the software package. As in the case of most of the bioinformatics, internet computation prevails and the historical background of the progress of this technique since 1970 has been reviewed.[39] A recent example of the internet computer software has already been published[40] for calculation of lipophilicity and solubility of chemical compounds. It is obvious that this is the direction to move in the near future, implying that our DM process should be able to be conducted on the computers of individual researchers without downloading the computer programs.

The nonlinear signal analysis methods proposed by Giuliani *et al*.[9] could certainly be a potential approach of protein QSAR. However, their RQA as discussed in the introduction to this chapter has not always been successful in measuring complexity in the protein QSAR;[41] the method is still premature as a protein QSAR routine and the existence of the global properties of proteins cannot be ignored as they have claimed. An important question remains: can hydrophobicity and its distribution alone explain all functionalities of proteins as they have claimed? How can we expect rational explanation of the underlying mechanisms of all different functions on the basis of the molecular folding of a single protein defined by their method? Not only protein structure, but also functions of a single protein may be variable in the human body due to SNP phenomena.[33] The three-dimensional structure itself may be variable due to different pathways of folding *in vivo* under the control of molecular chaperons.[42] Further study is required to ascertain the extent to which biological functions can be elucidated using our approach in comparison with nonlinear signal analysis, such as RQA.

11.5 Further information and advice

In this section a brief commentary on key books to consult is provided. In this regard, a historical perspective of QSAR in toxicology[43] may be recommended. This article would certainly help readers gain deeper knowledge through conceptual discussion of QSAR including its mathematical approaches. However, it should be mentioned that macromolecules including proteins were treated as receptors in the receptor-mediated interactions with small molecular toxicants; there has been no detailed discussion on the protein QSAR in this overview. The QSAR based on protein molecular structure is more complex and it is more difficult to find a good key book. One of the most recent and useful review articles on protein sequence-structure relationships was written by Giuliani *et al*.,[9] in which the importance of hydrophobicity distribution was

strongly emphasized. To conduct protein QSAR study, the following point may be useful.

11.5.1 Similarity vs. dissimilarity

A basic requirement to demonstrate accurately the patterns existing among entities, is that similar entities should be expressed by points that are close together and the fact that the more dissimilar the entities, the more distant should be the points representing them.[44] There is, therefore, a direct connection between the dissimilarity of two entities and the distance between them in the geometrical representation. Because the dissimilarity is so closely linked to the idea of distance, one reasonable way of measuring it is the use of a familiar metric such as Euclidean distance (eqn 11.1). Frequently, a similarity measure is quoted in the literature or calculated in a computer package by using the transformation of [similarity] = 1 − [dissimilarity] (0 < 1). However, it should be carefully remembered that highly correlated variables may distort dissimilarity calculations by giving undue weights to outlying individuals.[44] Mahalanobis distance (eqn 11.2) is recommended for use in this case as already discussed. We have observed that appropriate treatments of outliers are extremely critical in achieving the correct classification of samples.[45]

When the entities to be compared are variables, the correlation coefficient provides a sensible measure of similarity:

$$S(AB) = \sum X_i X_i' / \sqrt{\sum X_i^2 \sum X_i'^2} \qquad 11.4$$

where $A = (X_i, X_2 \ldots X_n)$ and $B = (X_1', X_2' \ldots X_n')$. $S(AB) = 1$ and 0 mean perfect match and absolutely no similarity, respectively. The important fact here is that distance-based dissimilarity and correlation-based similarity are not always so close that the total of these two values is 1.0. In general, people can habitually more readily compare individuals within their own community rather than compare with individuals in other communities. Therefore, correlation-based pattern similarity in PCS by rotating the reference sample does not necessarily yield the same classification as that made by distance-based CA as discussed above.[12,45]

11.5.2 ANN's over-learning

An important limitation of ANN is over-fitting or over-learning. The over-fitting is to fit data-points with extremely high curvatures, which do not represent the true underlying mechanism of a function but sometimes yields an excessively superior fit unexpectedly. Over-learning occurs during training runs usually with a too lengthy procession when the network is extremely complex. It is likely to end up with modeling of the noise in the data rather than the true underlying trend when the functional mechanism is being investigated. This over-fitting herein discussed is exactly the same phenomenon as over-fit during polynomial

curve fitting due to the presence of collinear independent variables in the regression equations.[46]

To secure acceptable accuracy for ANN computation, an adequately large number of cases are needed. The precise number required depends on the number of input variables, which is well known as 'the curse of dimensionality'.[18] Therefore, dimensionality reduction is an essential prerequisite, especially in the case of expensive, labour-intensive classification or regression studies in life science. Reducing numbers of input variables as well as hidden layers and/or hidden units should be tried to make the network as compact as possible. Outliers should be removed by either discarding the causative cases or deliberately converting them into missing values.[16,44] Close verification with similar test errors as obtained during cross-validation is a good sign for avoiding over-fitting. During processing of cross-validation by changing subsets, the training and verification errors should not change much.[7,19]

11.5.3 Segments vs. entire sequences

In *Current Protocols in Molecular Biology*,[47] it is stated that if the protein sequence contains multiple functional domains, it may be useful to perform the search with each of these domains individually. This was exactly the case in our study, thus providing clear evidence to support an advantage of our approach, e.g., inconsistency in explanation for the cause of amyloidosis of cystatin in segment vs. entire sequence.[5] While β-strand decreased in the total cystatin molecules according to a circular dichroism (CD) study, the strand content increased at the expense of decreased helix in the strand zone (Fig. 11.5). It may be worth mentioning that it is extremely difficult to conduct CD measurement of each of helix and strand zones in the cystatin molecule separately. The Protocol[47] also mentioned that historically, local alignment has been very successful at identifying similarity between proteins, however, such rigorous alignment algorithms are often quite slow and impractical for searching current databases on a routine basis. Our HSS approach using stepwise process in an automated fashion may be a potential answer to solve the problem of mathematical complication.

11.5.4 HSA to improve RCG efficiency

During running of the RCG program to optimize a project, the potential of further improving the functionality of mutants designed by RCG exists if the HSA program is used during or prior to the optimization. By running the HSA package including HSS, hypothetical, low function values can be assigned intentionally to potentially negative, abortive mutants designed by RCG on behalf of the better efficacy for continuing the optimization procedure instead of continuing optimization by conducting useless mutation experiments. This manipulation would avoid waste due to tedious genetic experiments, thus accomplishing improved efficacy for the RCG optimization, which is under investigation.

11.6 Conclusion

A protein QSAR protocol is proposed by applying new computer programs of SPCS, HSA and HSS to multiple sequence alignment (BLAST), followed by ANN by entering predictor variables derived from pre-processes used to compute QSAR. The new protocol would endorse efficient, reliable prediction from sequence data of proteins and peptides; however further study is needed to prove its accuracy in order finally to establish the new QSAR protocol.

11.7 Acknowledgement

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12

Factors affecting enzyme activity in foods

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12.1 Introduction

Proteins are essential ingredients in human and other animal foods and feeds. Proteins supply the essential L-amino acids – isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine – required by children and adults to synthesize the unique proteins required for body functions. Histidine is also an essential amino acid for infants.[1] Plants and microorganisms synthesize the specific amino acids required to build their unique proteins. Enzymes, the catalytic group of proteins, are essential for all living organisms.

In all living organisms, there are many different proteins with different biological functions. Table 12.1 lists 13 major essential biological functions of proteins in humans. To be active biologically, these proteins must have unique sequences of amino acids and be folded into secondary and tertiary structures. Some are biologically active only when two or more folded subunits (of proteins) associate to form quaternary structure (hemoglobin for example). Some other proteins are biologically functional when two or more types of proteins, such as the proteins myosin, actin, troponin and tropomyosin associate to form unique structures to permit movement of muscles (for walking, talking, etc.). In vision, the complex formed by the two proteins opsin and alcohol dehydrogenase and non-protein rhodopsin, permit capture of images at a rapid rate.

12.2 Types of enzymes and post-harvest food quality

12.2.1 Enzymes

All the functions, except one, of the proteins listed in Table 12.1 are the result of noncovalent interaction that does not require covalent bond splitting or

Table 12.1 Protein biological functions in humans

1. Catalysis (all enzymes)
2. Regulation
   a. enzyme inhibitors (trypsin inhibitors)
   b. osmotic control (serum albumin)
   c. hormones (insulin)
3. Carriers
   a. hemoglobin and myoglobin (O_2 and CO_2)
   b. serum albumin (ions, lipids, hormones)
4. Protectors
   a. antibodies, skin, hair, nails
   b. blood clotting
   c. chaperones
5. Movement (contractile proteins: myosin, actin, troponin)
6. Connectors
   a. elastin (muscle to bone)
   b. signal transducers (nerve transmission)
7. Recognition
   a. glycoproteins (cell to cell interaction in organs)
   b. blood types (A, B, AB, O)
8. Elicitors (antigens)
9. Collagen (cell coating, cell 'glue'; adhesion)
10. Tongue proteins (taste perception)
11. Opsin, rhodopsin, alcohol dehydrogenase (vision)
12. Protein or DNA? (memory)
13. Functional properties in food (texture, viscosity, foaming, emulsification, gelation)

synthesis. The exception is the catalytic proteins, the enzymes. The enzymes are highly specific in recognition of specific compounds (substrates), binding them and then specific conversion (catalysis) to unique products. An enzyme is defined as 'a protein with catalytic properties due to its power of specific activation.' They make life possible at existing environmental conditions (0º to 40ºC, and even to 100ºC in some microorganisms). Enzymes are positive catalysts in that they increase the rates of reactions by 10^2 to 10^{11} that of non-enzyme-catalyzed reactions (Table 12.2).[2]

12.2.2 Types of enzymes

The latest report (1992) of the Enzyme Nomenclature Committee[3] lists 3196 enzymes that have been purified and investigated in sufficient detail to prove that they differ enzymatically from all other enzymes. Ten years later, the number is probably more than 4000. To date, all enzymes can be listed under six headings based on the types of reactions catalyzed, as identified by the Enzyme Nomenclature Committee (Table 12.3). The six types of reactions catalyzed by enzymes are:

1. oxidoreduction,
2. transfer,

Table 12.2 Effect of catalyst on E_a and on relative rates of some reactions[a]

Substrate	Catalyst	E_a (kcal/mol)	n'/n (25°C)	Relative rates[b] (25°C)
H_2O_2	None	18.0	5.62×10^{-14}	1.00
	I^-	13.5	1.16×10^{-10}	2.07×10^3
	Catalase	6.4	1.95×10^{-5}	3.47×10^8
Sucrose	H^+	25.6	1.44×10^{-19}	1.00
	Invertase	11.0	8.04×10^{-9}	5.58×10^{10}
Carbonic acid	None	20.5	8.32×10^{-16}	1.00
	Carbonic anhydrase	11.7	2.46×10^{-9}	2.96×10^6
Urea	H^+	24.5	9.33×10^{-19}	1.00
	Urease	8.7	3.96×10^{-7}	4.25×10^{11}

[a]From ref. 2, p. 324; courtesy of Marcel Dekker, Inc.
[b]In relation to 1.00 for no catalyst or H^+.

3. hydrolysis,
4. formation of double bonds without hydrolysis,
5. isomerization and
6. ligation.

Thus, the types of enzymes are:

1. oxidoreductases,
2. transferases,
3. hydrolases,
4. lyases,
5. isomerases and
6. ligases.

These six biochemical type reactions, along with the nature of the substrate(s), form the basis of the numerical nomenclature. For example, the enzyme catalyzing the conversion of ethanol to acetaldehyde (12.1)

$$CH_3CH_2OH + NAD^+ \longleftrightarrow CH_3CHO + NADH + H^+$$

Ethanol Acetaldehyde 12.1

is named ethanol:NAD^+ oxidoreductase. Its numerical number is EC 1.1.1.1.

All six types of enzymes are important in living animals, plants and microorganisms. Type 6 enzymes make life possible via biosynthesis of proteins, carbohydrates, lipids, nucleic acids and smaller compounds unique to the organism. Type 3 enzymes are important in the metabolism (hydrolysis) of proteins, carbohydrates, lipids and nucleic acids. Deficiencies of specific enzymes are responsible for more than 600 known diseases in humans. A few examples of these enzyme deficiencies are shown in Table 12.4.[4] In food processing and preservation, the exogenous enzymes used are primarily members of the hydrolases and the oxidoreductases (Table 12.5).

Table 12.3 Key to numbering and classification of enzymes[a]

1. Oxidoreductases
   - 1.1 Acting on the CH-OH group of donors
   - 1.2 Acting on the aldehyde or oxo group of donors
   - 1.3 Acting on the CH-CH group of donors
   - 1.4 Acting on the CH-NH_2 group of donors
   - 1.5 Acting on the CH-NH group of donors
   - 1.6 Acting on NADH or NADPH
   - 1.7 Acting on other nitrogenous compounds as donors
   - 1.8 Acting on a sulfur group of donors
   - 1.9 Acting on a heme group of donors
   - 1.10 Acting on diphenols and related substances as donors
   - 1.11 Acting on hydrogen peroxide as acceptor
   - 1.12 Acting on hydrogen as donor
   - 1.13 Acting on single donors with incorporation of molecular oxygen (oxygenases)
   - 1.14 Acting on paired donors with incorporation of molecular oxygen
   - 1.15 Acting on superoxide radicals as acceptor
   - 1.16 Oxidizing metal ions
   - 1.17 Acting on CH_2 groups
   - 1.18 Acting on reduced ferredoxin as donor
   - 1.19 Acting on reduced flavodoxin as donor
   - 1.97 Other oxidoreductases

2. Transferases
   - 2.1 Transferring one-carbon groups
   - 2.2 Transferring aldehyde or ketone residues
   - 2.3 Acyltransferases
   - 2.4 Glycosyltransferases
   - 2.5 Transferring alkyl or aryl groups, other than methyl groups
   - 2.6 Transferring nitrogenous groups
   - 2.7 Transferring phosphorus-containing groups
   - 2.8 Transferring sulfur-containing groups

3. Hydrolases
   - 3.1 Acting on ester bonds
   - 3.2 Glycosidases
   - 3.3 Acting on ether bonds
   - 3.4 Acting on peptide bonds (peptide hydrolases)
   - 3.5 Acting on carbon-nitrogen bonds, other than peptide bonds
   - 3.6 Acting on acid anhydrides
   - 3.7 Acting on carbon-carbon bonds
   - 3.8 Acting on halide bonds
   - 3.9 Acting on phosphorus-nitrogen bonds
   - 3.10 Acting on sulfur-nitrogen bonds
   - 3.11 Acting on carbon-phosphorus bonds

4. Lyases
   - 4.1 Carbon-carbon lyases
   - 4.2 Carbon-oxygen lyases
   - 4.3 Carbon-nitrogen lyases
   - 4.4 Carbon-sulfur lyases
   - 4.5 Carbon-halide lyases
   - 4.6 Phosphorus-oxygen lyases
   - 4.99 Other lyases

5. Isomerases
   - 5.1 Racemases and epimerases
   - 5.2 *cis-trans*-isomerases
   - 5.3 Intramolecular oxidoreductases
   - 5.4 Intramolecular transferases (mutases)
   - 5.5 Intramolecular lyases
   - 5.99 Other isomerases

6. Ligases
   - 6.1 Forming carbon-oxygen bonds
   - 6.2 Forming carbon-sulfur bonds
   - 6.3 Forming carbon-nitrogen bonds
   - 6.4 Forming carbon-carbon bonds
   - 6.5 Forming phosphoric ester bonds

[a]The third and fourth levels of classification are given in *Enzyme Nomenclature*, 1992 (ref. 3).

Table 12.4 Some human genetic diseases due to established enzymatic defects

Disease	Defective enzyme
Alcaptonuria	Homogentisic acid oxidase
Phenylketonuria	Phenylalanine hydroxylase
Hyperammonemia	Ornithine transcarbamylase
Hemolytic anemia	Erythrocyte glucose 6-phosphate dehydrogenase
Acatalasia	Erythrocyte catalase
Congenital lysine intolerance	L-Lysine-NAD oxidoreductase
Gaucher's disease	Glucocerebrosidase
Refsum's disease	Phytanic acid oxidase
McArdle's syndrome	Muscle phosphorylase
Hypophosphatasia	Alkaline phosphatase
Congenital lactase deficiency	Lactase
Gout	Hypoxanthine-guanine phosphoribosyl transferase
Xanthinuria	Xanthine oxidase
Hereditary fructose intolerance	Fructosel-phosphate aldolase

Source: Adapted from Wacher and Combs (1969) (ref. 4).

Table 12.5 Some important industrial enzymes

α-Amylase – for starch hydrolysis in syrup and dextrin production; bread making and beer production
Glucoamylase – for dextrin hydrolysis to glucose and fructose: 'light' beer production
β-Amylase – for high-maltose syrup production
β-Glucanase – hydrolysis of β-glucans in beer to aid filtration
Cellulase – convert cellulose waste to fermentable feedstock for ethanol or single-cell protein production
Xylose (glucose) isomerase – isomerization of glucose to fructose as sweetener and high fructose corn syrup
Glucose oxidase – conversion of glucose to gluconic acid as acidulate in foods; remove O_2 in food packaging to protect against browning or oxidative deterioration
Lactase – additive to dairy products for individuals lacking lactase; hydrolysis of lactose in whey products for making polyactide
Pectinase – added to fruit pulp to enhance juice yield and clarification; increase filtration rate
Lysozyme – antimicrobial preservative
Acetolactate decarboxylase – reduce maturation time in beer making by converting acetolactate to acetoin for single step process
Papain – meat tenderizer, prevent chill-haze formation in beer
Chymosin – clotting of milk for cheese formation
Microbial proteases – processing of raw plant and animal proteins; production of fish meals, meat extracts, meat extenders and textured proteins
Lipase – enhance flavor development and shorten ripening time of cheeses; production of transesterified lipids with better properties

12.2.3 Food quality

Enzymes are very important in postharvest food quality. Table 12.6 lists some of the enzymes that cause undesirable changes during harvesting and storage of fruits and vegetables. The action of these enzymes can be controlled by inactivation with heat, cold storage or by inhibitors. Polyphenol oxidase causes 'browning' of some fruits and vegetables by oxidation of phenolic compounds to produce black, brown or red discolorations that are unacceptable to consumers. Chlorophyllase affects the green color of some green vegetables, such as peas and beans, by hydrolyzing chlorophyll to pheophytin. The pectic enzymes hydrolyze pectin and pectic acids, causing loss of texture. Amylases and cellulases in some food materials, such as green peas and green beans, also decrease texture.

Figure 12.1 shows the effect of blanching temperature and time on the rates of denaturation of four enzymes of English peas.[5] The four enzymes have different heat stabilities. The increasing order of heat stability is: catalase, lipase, lipoxygenase and peroxidase (most stable). After about 20 minutes at 60ºC, further inactivation of peroxidase and lipoxygenase is very slow, leaving ~55 and ~35% activity left, respectively. At 70ºC all lipoxygenase activity is lost at 20 min., while there is ~40% peroxidase activity left.[5] These types of behavior for peroxidase and lipoxygenase indicate there are two or more isozymes of peroxidase and lipoxygenase with different heat stabilities.

Table 12.6 Some enzymes that cause undesirable changes in foods

Enzyme	Undesirable changes
Lipoxygenase	Destruction of chlorophyll and carotenes Development of oxidative off flavors and aromas Oxidative damage to vitamins and proteins Oxidation of essential fatty acids
Chlorophyllase	Hydrolyzes chlorophyll to phytol and chlorophyllide
Polyphenol oxidase	Browning of fruits and some vegetables; loss of economic value, taste and nutritional quality
Pectic enzymes	Texture loss; invasion by microorganisms
Cellulase	Texture loss; invasion by microorganisms
Pentosanases	Possible texture loss and water-holding ability
Amylases	Starch degradation
Proteases	Texture loss due to protein hydrolysis
Esterases	Decrease in aromas of fruits

12.3 Parameters affecting enzyme activity

Enzymes are catalysts which increase the rate of conversion of compounds (called substrates) to products. Table 12.2 shows the catalysis of four compounds to products by specific enzymes versus no catalysis.[2] The amount of activation energy (k_{cal}/mol) required for the reaction to go is E_a (Table 12.2,

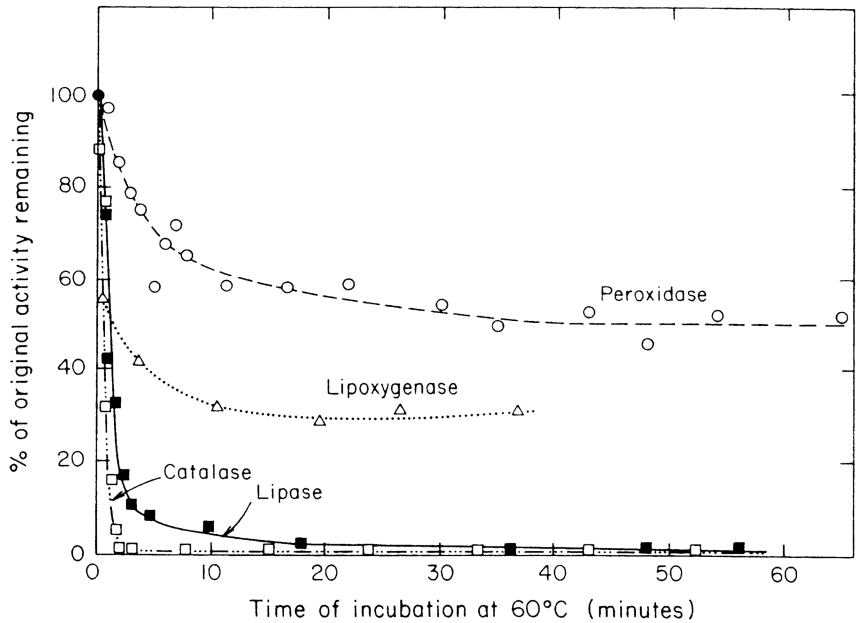

Fig. 12.1 Rate of thermal inactivation of peroxidase, lipoxygenase, catalase, and lipase in English pea homogenates incubated at 60ºC (Williams *et al*. 1986 (ref. 5); courtesy of Institute of Food Technologists).

column 3). The Boltzmann Distribution Equation gives the fraction, n'/n_0 of the molecules of a compound which have energy E_a, thereby giving the relative rates of the reactions under the given conditions. Catalase is 3.47×10^8 more effective in converting hydrogen peroxide to water and oxygen than with no catalyst and it is 1.68×10^5 times more effective than iodide ions. Invertase (sucrase) is 5.6×10^{10} more effective than H^+ as catalyst for sucrose hydrolysis; carbonic anhydrase is 3.0×10^6 more effective in decomposing carbonic acid than no catalyst and urease is 4.25×10^{11} more effective in hydrolysis of urea to ammonia and carbon dioxide than H^+. All concentrations of reactants and products are normalized to one molar at 25ºC.

12.3.1 Specificity and efficiency of enzymes

The question of why and how enzymes are so specific in relation to substrate selection was answered in part by Fischer[6] in 1984 when he determined that enzymes are very selective in the type(s) of compounds converted to products, leading to his 'lock and key' analogy of their action. Henri (1902 and 1903)[7, 8] and Brown (1902)[9] independently suggested that the saturation-type curve obtained when increasing amounts of substrate are added to a fixed amount of enzyme (Fig. 12.2; eqn. 12.2) is the result of an obligate intermediate, the enzyme-substrate complex (E·S), where E is free enzyme, S is free substrate, E·S

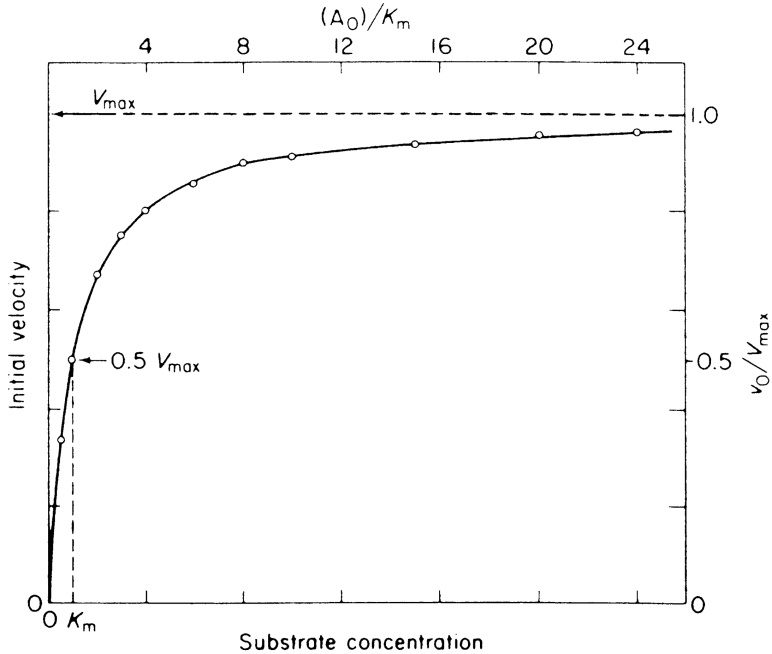

Fig. 12.2 Effect of substrate concentration on rate of an enzyme-catalyzed reaction (Whitaker 1994a, page 168 (ref. 2); courtesy of Marcel Dekker, Inc.).

is the non-covalent complex and P is product. In 1913, Michaelis and Menten[10] derived their now-famous mathematical equation which describes quantitatively the saturation-like behavior (eqn 12.3).

$$E + S \longleftrightarrow E \cdot S \longleftrightarrow E + P \qquad 12.2$$

$$v_0 = V_{max}\ [S]/(K_m + [S]) \qquad 12.3$$

where v_0 is observed initial velocity, V_{max} is the maximum velocity when the enzyme is saturated with substrate, [S] is the substrate concentration, and K_m is the substrate concentration at which $v_0 = 0.5\ V_{max}$.

The question of how enzymes treat all substrates stereospecifically was solved in about 1965 when it was shown that non-asymmetric ethanol (CH_3CH_2OH) can be treated asymmetrically when it binds into the active site of alcohol dehydrogenase. As shown in Fig. 12.3, there are three binding points A, B and C on the enzyme for the substrates.[2] Binding point A binds one of the hydrogens (H) on carbon 1 of ethanol; binding point B binds the -CH_3 group and binding point C binds the -OH group as shown in (a). Other orientations of substrate with respect to the enzyme active site are possible but only one or two contact points can be made. Orientations (b), (c) and (d) are non-productive, since the substrate is not properly positioned with the catalytic locus of the active site (see Fig. 12.3). Experimental evidence for correctness of the above

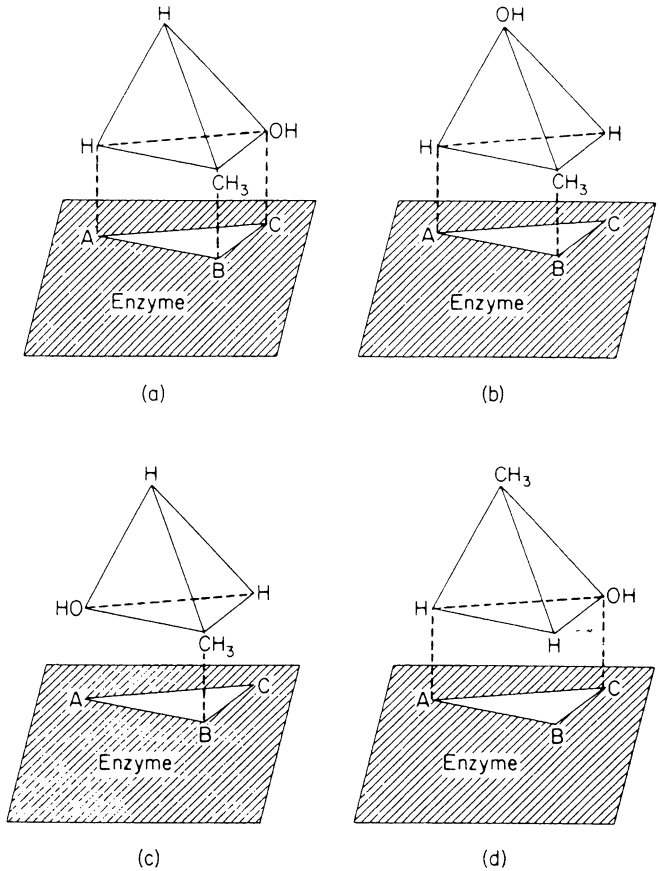

Fig. 12.3 Schematic representation of four possible modes of non-covalent binding of ethanol into the active site of alcohol dehydrogenase (Whitaker 1994a, page 120 (ref. 2); courtesy of Marcel Dekker, Inc.).

orientation came from use of CH_3CHDOH, where D is deuterium. Unlike CH_3CH_2OH, CH_3CHDOH is an asymmetric molecule. Interestly, nature makes both D- and L-alcohol dehydrogenases. With the CH_3CHDOH as substrate, one enzyme removes deuterium from C_2, the other enzyme removes H from C_2.

The second question is why enzymes are much more efficient catalysts than non-enzyme catalysts (H^+, ^-OH, metal ions). Above, the effect of stereospecific binding of the substrate into the active site of alcohol dehydrogenase is described. Figure 12.4 shows both the binding and the catalytic steps.[11] The ethanol (RCH_2OH) is bound into the lipophilic binding site (left side) in proximity to the Zn^{2+} cofactor and the $NADH^+$ cofactor (~2-4 Å). The transition state (high energy state) is shown in the middle diagram, with the bound E·CoF·P state on the right. This electrophilic/nucleophilic reaction has four components (alcohol dehydrogenase, ethanol, Zn^{2+} and $NAD^{+)}$. Rate

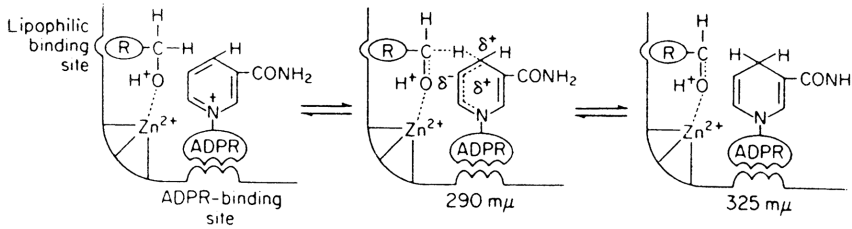

Fig. 12.4 Schematic depiction of NAD^+ and ethanol binding into the active site of yeast alcohol dehydrogenase followed by oxidation of ethanol to acetaldehyde and reduction of NAD^+ to NADH (Theorell and Yonetani 1963, page 552 (ref. 11); courtesy of Springer-Verlag).

enhancement by conversion of a four-component intermolecular system to an intramolecular system is theorically expected to increase the rate by more than 10^{20} over the non-catalyzed rate.

Another catalytic factor in enzyme-catalyzed reactions is the conversion of the active site from a hydrophilic to a hydrophobic environment when all reactants are in the active site. The water molecules are pushed out of the active site. Chymotrypsin, a hydrolytic enzyme, has only one molecule of water, in stereospecifically bound form, at the active site during catalysis.[12] The effect of excluding water from the active site has been demonstrated experimentally for glutathionine synthetase.[13] After the three substrates γ-glutamylcysteine, glycine and ATP bind stereospecifically into the active site, the active site is closed by a 'lid' consisting of a 17–amino-acid 1oop of the enzyme, thereby excluding water during the reaction. The rate constant k_o is 151 sec^{-1} for the overall synthesis. By use of recombinant DNA technology, Kato *et al.*[3] replaced the 17–amino-acid loop with a sequence of three glycine residues, so that the active site could not close. The k_o for the mutant enzyme was 0.163 sec^{-1}, which is 1×10^{-3} that of the wild-type enzyme. Loop replacement did not appear to cause any other change in the physical structure of the enzyme.

12.3.2 Enzyme concentration effect

Rates of enzyme-catalyzed reactions are also controlled by the concentration of enzyme, substrate(s), cofactor, inhibitor(s), pH, temperature and sometimes the dielectric constant and ionic strength of the reaction. In determining rates of enzyme-catalyzed reactions, it is important to use initial velocity (v_0) where no more than 5% of the substrate is converted to product (Fig. 12.5). At longer times the rate may decrease as a result of decreased substrate concentration, instability of enzyme, back reaction due to increased product concentration, inhibition by product and decreased cofactor concentration.

Effect of enzyme concentration on rates of enzyme reactions is shown in Fig. 12.6. In most cases the relationship is linear. However, there are at least five conditions where this is not true.[2] These conditions involve limited solubility of substrate(s), competition of two or more enzymes for cofactors, presence of

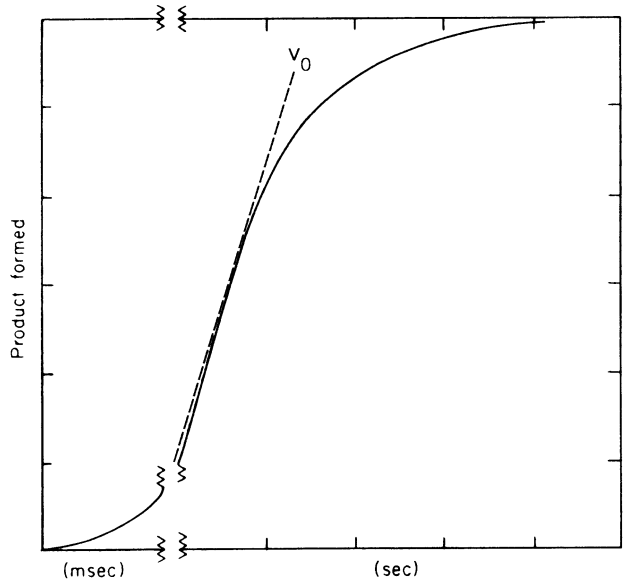

Fig. 12.5 Typical enzyme-catalyzed reaction showing the pre-steady-state (millisecond scale), the constant rate indicated by dashed line (v_o), and the declining rate of progress curve.

irreversible inhibitors (such as Ag^+, Pb^{2+} or Hg^{2+}), competitive inhibitors in the enzyme preparation, and dissociable cofactors in the enzyme preparation.

12.3.3 Substrate concentration effect

The effect of substrate concentration on rates of enzyme-catalyzed reactions is shown in Fig. 12.2. At very low substrate concentration ($\leq 0.1\ K_m$), v_0 increases linearly with substrate concentration increase (first order reaction). At substrate concentration of $> 0.1\ K_m$ to substrate concentration of ~90 K_m the relationship is curvilinear (mixed order reaction). When all the enzyme is bound (saturated) with substrate ([S] >100 K_m), the rate is independent of substrate concentration, a zero-order rate. The behavior shown in Fig. 12.2 is known as Michaelis-Menten behavior. When the data of Fig. 12.2 are plotted as $1/v_0$ vs 1/[S], a linear relation is found (Fig. 12.7). This is the Lineweaver-Burk Method of plotting.[14] As shown in Fig. 12.7, it is easy to determine V_{max} and K_m using substrate concentrations between 0.5 K_m to 5 K_m.

12.3.4 Cofactor effect

Cofactors are required by some enzymes in order to be active. These include compounds derived from some of the vitamins, as shown in Table 12.7. Others are metal cations and a few anions such as Cl^- for salivary α-amylase. Zn^{2+} is a required cofactor of more than 150 enzymes in humans. In most cases, there is no

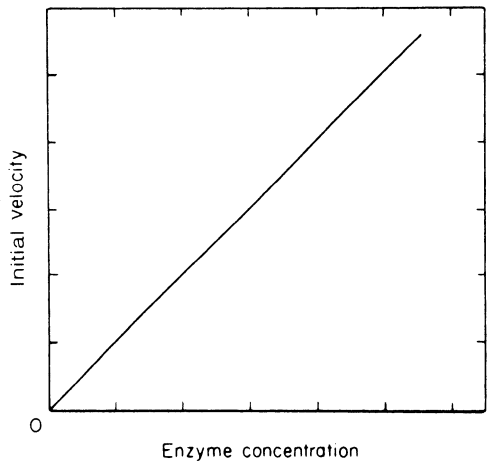

Fig. 12.6 Expected relationship between enzyme concentration and initial velocity of the reaction. Substrate concentration, pH, temperature, cofactor (if any needed) and buffer are kept constant.

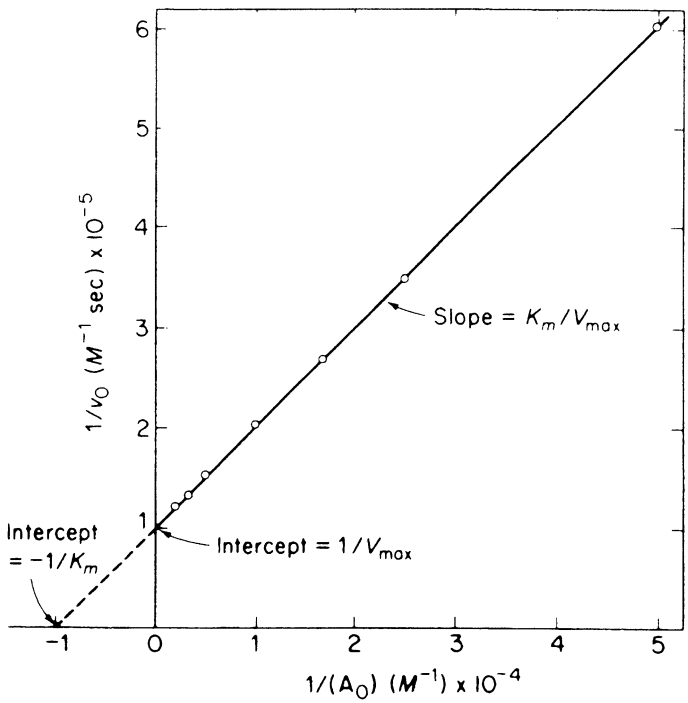

Fig. 12.7 Plot of substrate-velocity data according to Lineweaver-Burk reciprocal method (ref. 14).

Table 12.7 Importance of phosphate, ribose, and purine and pyrimidine bases in cofactors[a]

Cofactor	Vitamin	Phosphate	Ribose	Base
NAD^+	Niacin	+	+	Adenine
$NADP^+$	Niacin	+	+	Adenine
ATP	–	+	+	Adenine
UTP	–	+	+	Uridine
CTP	–	+	+	Cytidine
CoA	Pantothenic acid	+	+	Adenine
Acetyl phosphate	–	+	–	–
Carbamyl phosphate	–	+	–	–
S-Adenosyl methionine	–	–	+	Adenine
Adenosine-3′-phosphate-5′-phosphosulfate	–	+	+	Adenine
FMN	Riboflavin	+	+	–
FAD	Riboflavin	+	+	Adenine
Pyridoxal phosphate	Pyrodoxine	+	–	–
Thiamine pyrophosphate	Thiamine	+	–	–

[a]From ref. 2, page 331; courtesy of Marcel Dekker, Inc.

activity in the absence of a required cofactor. Cofactors are of two types, the coenzymes and the prosthetic groups. The coenzymes (examples NAD^+, $NADP^+$, Table 12.7) are loosely bound to the active site of the enzyme and treated as a second substrate kinetically by the enzyme; therefore they are converted to a product. A plot of v_0 vs cofactor concentration (or by $1/v_0$ vs 1/[CoF]) shows the same behavior as the primary substrate (Figs. 12.2 and 12.7). V_{max} and K_d can be determined by a reciprocal plot of $1/v_0$ vs 1/[CoF]). The cofactors that bind very tightly, even covalently to the enzyme in some cases, are called prosthetic groups. Examples in Table 12.7 are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). There is no change in concentration of the prosthetic group since it is regenerated in the overall reaction.

12.3.5 Inhibitor effect

Enzyme inhibitors are important in inhibiting unwanted reactions in foods (such as polyphenol oxidase-caused browning/blackening of foods), or in controlling or preventing diseases caused by enzymes. Some inhibitors bind tightly to the enzyme so that all the undesirable enzymatic activity is eliminated. These are irreversible inhibitors. Other inhibitors bind less tightly (loose binding) and decrease activity at higher concentrations in relation to the inhibitor·enzyme, enzyme and inhibitor equilibrium constant (eqn 12.4).

$$E + I \underset{}{\overset{Keq}{\longleftrightarrow}} E \cdot I \qquad 12.4$$

Loose-binding inhibitors are of three types – competitive inhibitors (with respect to substrate), non-competitive inhibitors (both substrate and inhibitor bind to

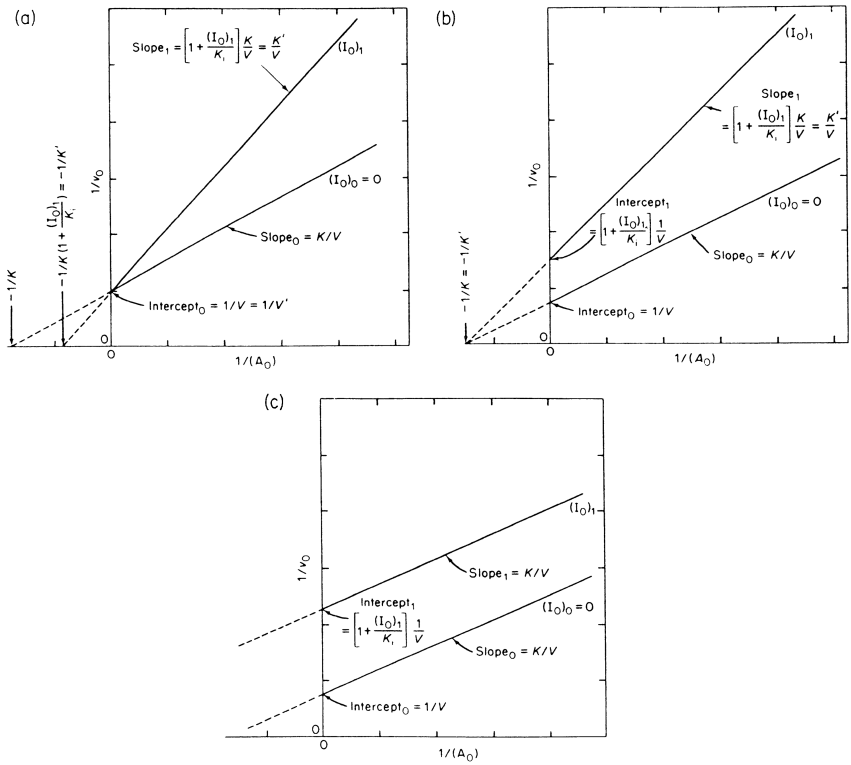

Fig. 12.8 (a) Effect of a competitive inhibitor on $1/v_o$ versus $1/(A)_0$ plot; $(I_0)_1 = K_i$. (b) Effect of a simple linear noncompetitive inhibitor on $1/v_o$ versus $1/(A)_0$ plot; $(I_0)_1 = K_i$. (c) Effect of uncompetitive inhibitor on $1/v_o$ versus $1/(A)_0$ plot; $(I_0)_3 = 2\ K_i$. The control activity (lower line) has no inhibitor.

enzyme without competition, and uncompetitive inhibitors that bind to the enzyme only after substrate has bound. Figure 12.8 demonstrates the kinetic differences among these inhibitors, as well as provide methods to determine K_m, V_{max} and K_i of the enzyme-inhibitor systems. In food preservation that use inhibitors, tight-binding inhibitors are better than loose-binding inhibitors for preventing undesirable changes.

12.3.6 pH effect

Activities of enzymes are generally affected by pH of the medium. Figure 12.9 shows the effect of pH on activity of four enzymes. In order, the pH optima are: pepsin, 2; peroxidase, 6; trypsin, 8; and alkaline phosphatase, 10. All show approximately bell-shaped activity behavior due to two apparent ionizable groups in the active site. Using pepsin as an example, the increasing activity at higher pHs on the left side is due to increasing ionization of a specific aspartate carboxyl group essential for activity (E-COOH $\longleftrightarrow$ E-COO$^-$ + H$^+$) and the decreasing activity is

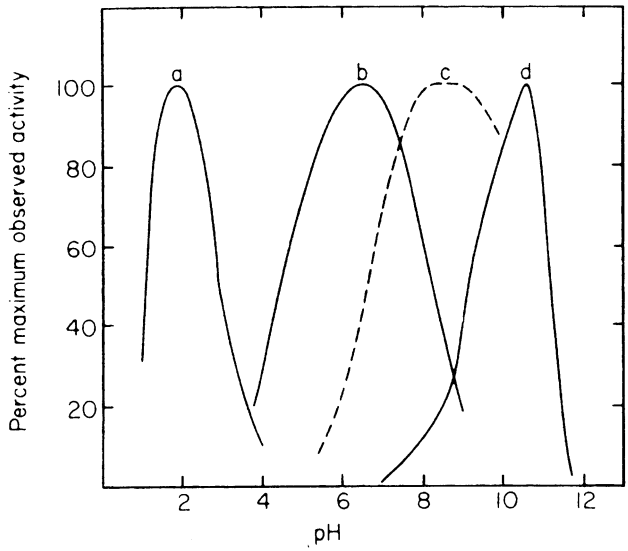

Fig. 12.9 Schematic diagram of the relative effect of pH on enzyme activity: (a) pepsin hydrolysis of N-acetyl-L-phenylalanyl-L-diiodotyrosine; (b) *Ficus glabrata* peroxidase oxidation of guaiacol in presence of H_2O_2; (c) bovine trypsin hydrolysis of casein; and (d) alkaline phosphatase hydrolysis of *p*-nitrophenyl phosphate.

due to a second aspartate carboxyl group which is protonated at the lower pH and ionizes as the pH increases above 2. The enzyme is most active when the low pK_a carboxyl group is ionized and the higher pK_a carboxyl group is protonated.

Active sites of enzymes may contain one or two ionizable groups of the six ionizable groups on the side chains of amino acid residues of the protein. These ionizable groups are: carboxyl side chain groups of aspartic and glutamic acids (pK_a ~2 to 4), imidazole side chain group of histidine (pK_a ~ 6 to 7), sulfhydryl side chain group of cysteine (pK_a ~7.5 to 8.5), ϵ-amino side chain group of lysine (pK_a ~9), hydroxyl side chain group of tyrosine (pK_a ~10), and the guanidino group of arginine (pK_a ~12.5).

As shown in Fig. 12.9, pepsin is less stable below pH 2 while alkaline phosphatase is less stable above pH 10, as shown by the skewed pH/activity curves. Catalase (not shown) has the same maximum activity from pH 4 to 10 indicating that there are no ionizable groups essential for activity. Below 4 and above 10, activity decreases due to instability. Table 12.8 gives the pH optima of several enzymes found/used in foods. pH also affects stability of enzymes.

12.3.7 Temperature effect

Temperature affects activity of enzymes. As shown in Fig. 12.10, the activity is low at 20ºC but increases in a logarithmic fashion ($k = Ae^{-E_a/RT}$) at 30º, 40º and 50ºC (solid line). A is the Arrhenius constant, E_a is the Arrhenius activation energy required for reaction and R is the universal gas constant (Whitaker

Table 12.8 pH-activity optimum of several enzymes[a]

Enzyme	pH optimum
Acid phosphatase (prostate gland)	5
Alkaline phosphatase (milk)	10
α-Amylase (human salivary)	7
β-Amylase (sweet potato)	5
Carboxypeptidase A (bovine)	7.5
Catalase (bovine liver)	3–10
Cathepsins (liver)	3.5–5
Cellulase (snail)	5
α-Chymotrypsin (bovine)	8
Dextransucrase (*Leuconostoc mesenteroides)*	6.5
Ficin (fig)	6.5
Glucose oxidase (*P. notatum*)	5.6
Lactate dehydrogenase (liver)	8 (forward reaction)
Lipase (pancreatic)	7
Lipoxygenase-1 (soybean)	9
Lipoxygenase-2 (soybean)	7
Pectin esterase (higher plants)	7
Pepsin (bovine)	2
Peroxidase (fig)	6
Polygalacturonase (tomato)	4
Polyphenol oxidase (peach)	6
Rennin (calf)	3.5
Ribonuclease (pancreatic)	7.7
Trypsin (bovine)	8

[a]The pH optimum will vary with source of enzyme and experimental conditions. These pH values should be taken as approximate values. From ref. 2, p. 274; courtesy of Marcel Dekker, Inc.

1994a). When the reaction time is longer, at t_2, the observed activity deviates from the solid line at 40ºC, reaches a maximum at 50ºC and then decreases. When samples are taken at a shorter time, at t_1, the activity deviates at 50ºC, reaches a maximum at ~55ºC and then decreases at higher temperatures. These deviations from the solid line are due to temperature inactivation of the enzyme. The rate of inactivation of enzymes at different temperatures is important for preservation of food quality during heat processing. Rate constants determined at different temperatures can be used to determine the type of ionizing groups in the active site. (Ref. 2, p. 312.)

Figure 12.11 shows changes in activation energy along the reaction pathway during conversion of substrate A to product P in an enzyme-catalyzed reaction. The left hand peak shows the activation energy required to form the enzyme·substrate complex, E·A. Note the $(E\cdots A)^{\ddagger}$ transition-state complex at the top of the energy peak. The second (large) peak shows conversion of the enzyme·substrate complex (E·A) to the transition state complex $(E{\cdot}A\cdots EP)^{\ddagger}$ and dissociation of E·P via the transition state $(E\cdots P)^{\ddagger}$ in the third step. Step two ($E{\cdot}A\cdots EP$) requires the highest energy. Therefore, step two is the rate-determining step in the reaction.

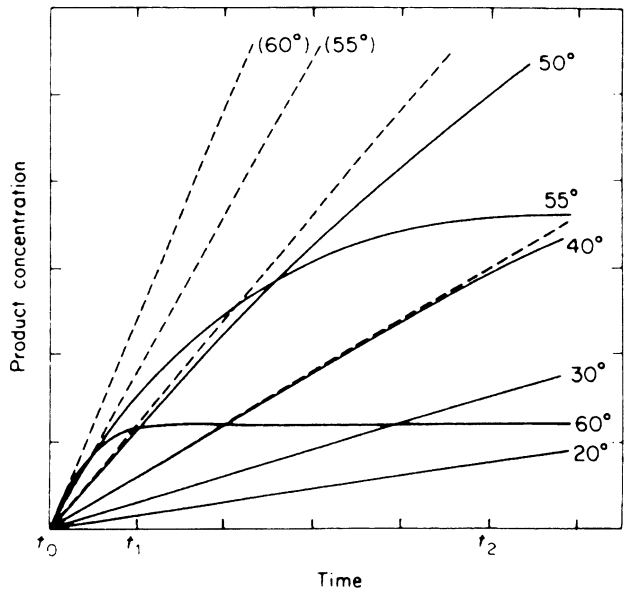

Fig. 12.10 Schematic effect of temperature on rate of product formation at 20°, 30°, 40°, 50°, 55° and 60°C. The solid lines are experimental data; the dashed lines indicate initial velocity, v_o (Whitaker 1994a, page 303 (ref. 2); courtesy of Marcel Dekker, Inc.).

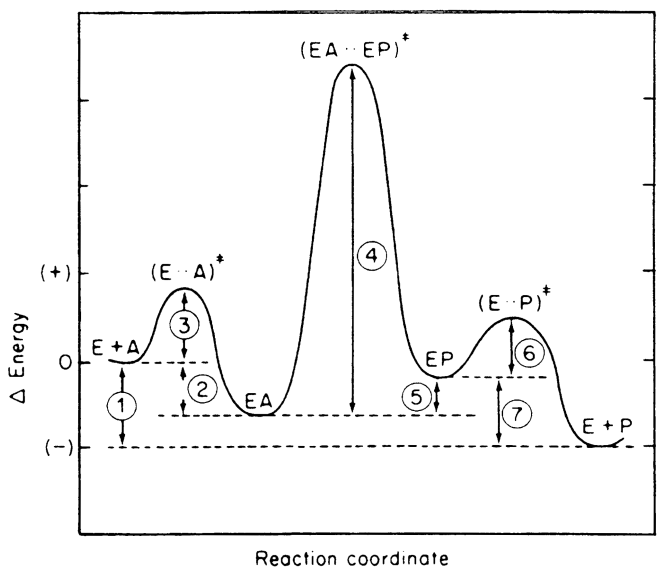

Fig. 12.11 Change in energy along the pathway of conversion of substrate A to product P in an enzyme-catalyzed reaction (ref. 2, page 319; courtesy of Marcel Dekker, Inc.).

12.4 Future trends

Many advances have been made in understanding enzymes, how they function and their biological activities in recent years. Less progress has been made in their applications in food processing. On the positive side is the use of amylase, glucoamylase and xylose (glucose) isomerase in conversion of starch to glucose and fructose, a process that produces millions of pounds of sweeteners per year at a price about 70% of that sucrose production from cane and sugar beets. Amino acid acylases for separation of D- and L-amino acids are used in large amounts by Ajinamoto Company for producing L-amino acids for further fabrication into many products. Crude preparations of pectinases are used in large amounts in increasing the juice yield (10–20%) from grapes, apples and other fruits. Chymosin from calf stomachs to make casein-based cheese is also used on a large scale. Large amounts of papain are used in chill proofing of beer, i.e., to hydrolyze proteins to prevent sedimentation of protein-pectin complexes. Speciality lipases are used for transesterification of triglycerides. But there are still many needs not yet met.

12.4.1 Enzyme indicators of heat inactivation

It was shown as early as 1928[15] that heat treatment (blanching) of fruits and vegetables and their juices to 65–85ºC, cooling and freezing stabilized the products for several months (currently up to two years). Peroxidase inactivation is generally used as indicator of adequate blanching (see below). Color, taste, texture (in some cases) and nutritional quality approximates that of the fresh product. Pasteurization of cow's milk at 71–73ºC for 1–4 min. preserves it for two to three weeks at 5ºC. Inactivation of alkaline phosphatase is the indicator used. However, there is less certainty of which enzyme to use for blanching (70–105ºC) of fruits and vegetables. Peroxidase is used most frequently, but other enzymes could be better used for some vegetables as demonstrated by Williams *et al*.[5]

Numerous studies of enzyme activity are available using enzyme chips.[16] Detection of blanching and pasteurization end points can be done more efficiently on line by computer chips. But, because of economic issues, most of the investigations using computer chips are for detecting enzyme levels in patients with various types of diseases. These same products can be used to monitor inactivation of enzymes in food product manufacture.[17] Wagner[18] has described the state of the art in the use of biosensors for food analysis.

12.4.2 Enzyme stability

The relative instability of enzymes in industrial processes is a limiting factor. This applies to both temperature and time of the process. Temperature stability of enzymes varies widely; some are inactivated near 0ºC (cold-sensitive) and others such as ribonuclease and thermolysin are stable at 100ºC up to 30–60 minutes. Most enzymes are inactivated around 40º to 50ºC.

Some important studies have been done to stabilize enzymes, especially subtilisin which is a protease added to laundry detergents. Changing specific amino acids, such as methionine near the active site or cross-linking the enzyme via additional disulfide bonds increased the stability about twofold. Enzymes bound to matrices such as cellulose are more stable and can be used repeatedly. Extremophilic enzymes from organisms growing at high temperatures (hot springs) or at high pressures (at great depths in the ocean) generally have more stability.

Other methods for improving heat stability are being studied. Synzymes are laboratory synthesized compounds that contain the active site (binding and catalysis components) but are much smaller and more stable than the enzymes.[19] The active site must be three-dimensional. Abzymes are antibodies produced *in vivo* which have binding specificity for specific substrates, but do not have the catalytic groups.[19] These catalytic groups are added by the chemist. Polystyrene polymers can be 'imprinted' to contain the three-dimensional shape of the active site.[20, 21] The binding and catalytic groups then are added. Such catalysts are very stable.

DNA shuffling is a relatively new method for increasing the stability and activity of enzymes.[19] Libraries of RNAs can be generated by in vitro transcription of DNA by PCR with random priming. Selection steps are applied to the RNA pool to isolate variants having the targeted functional property. The few best RNA molecules are subjected to iterative cycles of amplification, mutation and selection until the best-fit molecules are identified. Enzymes, such as α-amylase, have been increased 100–200 fold in specific catalytic activity. This technique is frequently called 'gene farming'.

12.4.3 Enzyme efficiency

The hydrolytic enzymes are relatively inefficient enzymes (turnover number k_o $\sim 10^1$ to 10^2 sec^{-1}) relative to peroxidase and catalase (k_o $\sim 10^7$ sec^{-1}). Can enzymes be made more efficient? Directed evolution can be done in the laboratory by several techniques shown to be successful in improving the catalytic efficiency and stability of some enzymes. The original technique called DNA shuffling[22] involves random digestion of a pool of DNA sequences with DNase I to give a mixture of small DNA fragments. These fragments are reassembled by DNA polymerase, relying on self-priming of homologous end sequences, and recombination or crossover occurrences by template switching. The thousands of products formed are screened to isolate the best product(s) for the targeted properties (stability, pH optimum, superior activity, size, etc). The original DNA-shuffling technique subsequently has been modified to give the 'family shuffling' technique (two or more related DNAs used as starting material,[23] 'staggered extension process recombination',[24] 'random chimeragensis',[25] 'incremental truncation' for creation of hybrid enzymes,[26] 'exon shuffling'[27] and 'transmutation' of function.[28]

Current recombinant DNA technology now can be used to introduce foreign genes or to change endogeneous genes to redesign metabolic pathways in microbial, plant and animal cells. This permits manipulation of genes to tailor the chemical composition and physical properties of various food components, to enhance characteristics best suited for food formulation and processing, to improve nutritional values, to remove or reduce levels of antinutrients, and to produce value-added nonfood products.

Enzymes have been produced with 10^2–10^3 higher activity rates. Many years of fundamental studies of these accomplishments at the molecular level need to be done to maximize and understand these results.

12.4.4 Enzyme purity

For medical and analytical uses, the enzyme required must be pure and devoid of any other enzymatic activities and other proteins. Pure enzymes are expensive. Therefore, to date, pure enzymes are not used in food applications. The enzyme preparations used to produce food products often contain less than 1% of the enyzme needed. Several other enzymes with different activities are in the crude material. How much these impurities contribute to the overall results obtained is not known. But there are numerous reports of the inconsistent performance of different batches of the same product. These inconsistent performance products give a bad name to enzyme use in some food products. With the very good protein separation systems available today, better quality enzyme preparations can be made available. Consistent performance of an enzyme may be worth the additional price. Even a tenfold increase in purity may be sufficient to eliminate much of the undesirable enzymes and other compounds. Perhaps the DNA shuffling techniques mentioned above can contribute to higher purity enzymes.

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13

Detecting proteins with allergenic potential

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13.1 Introduction

Food allergy is defined as an immunologically based adverse reaction towards certain foods and foodstuffs. IgE-mediated reactions (Type I) are the most prevalent, other adverse food reactions include non-IgE-mediated primary immunological sensitivities (Type III), non-immunological food intolerances, and secondary sensitivities. Food allergy is of both industrial and public interest as 0.3–7.7% of individuals including young children show allergic reactions to distinct foods (Taylor, 1985; Sampson, 1999). Clinical symptoms associated with food allergies range from itching, sneezing, asthma, swelling of the tongue and mouth, atopic dermatitis, eczema, nausea, abdominal cramps to anaphylactic shock or death in the most severe cases. Industry and consumers alike require sound analytical methodology to reliably detect proteins with allergenic potential in foods to ensure correct labelling or in order to avoid the respective food. These methods have to be highly specific and sensitive since already small amounts of the offending food, in the lower mg range can elicit reactions (Yeung *et al*., 2000; Taylor *et al*., 2002).

At present, in the detection of proteins with allergenic potential, methods based on immunological techniques are mostly employed. Several applications of SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblot and other blotting techniques as well as rocket immunoelectrophoresis have proven their potential in the analysis of allergenic proteins, however, only enzyme-linked immunosorbent assays (ELISA) methods have reached commercialisation. There is still a great need for fast and easy-to-use tests for allergenic proteins with sufficient sensitivity. Polymerase chain reaction (PCR) is a valuable tool for the identification of different food species on a molecular biological level

and has proven very useful for the detection of genetically modified foods. So far, only a few methods using PCR for allergen detection have been published. This chapter reviews the analytical methods available for the determination of food allergens and gives an outlook for future trends.

13.1.1 Range of allergenic food proteins

The allergens present in food which cause allergic reactions in humans are naturally occurring proteins that stimulate abnormal immunological responses in sensitive individuals (Taylor *et al*., 1987). They are usually proteins or glycoproteins with a molecular mass ranging from 10 to 70 kDa (Hefle, 1996; Lehrer *et al*., 1996). Only a very small percentage of natural food proteins can be regarded as allergenic proteins yet they stem from different protein families (Aalberse, 2000; Breiteneder and Ebner, 2000). Most animal derived major allergens causing respiratory sensitisation belong to the family of proteins called lipocalins. Other allergenic proteins are derived from protein families such as chitinase (from avocado, banana, and chestnut), thaumatin-like proteins (from cherry and apple), Betv1 related proteins (from celery and apple), lipid transfer proteins (e.g. in peach and maize), actin-regulating profilins (from celery, apple, soybean), seed storage proteins (nut proteins), enzyme inhibitors (from cereals, soybean, egg white), proteases (from fruits and egg white), parvalbumins (fish), serum albumins (beef, egg yolk), and tropomyosins (crustaceans, molluscs) (Besler, 2001). Members of an allergen group that have more than 67% amino acid sequence homology are called isoallergens.

The Food and Agricultural Organisation (FAO) of the United Nations (UN) has designated the following foods as the most common causes of food allergy world wide: Cereals containing gluten (wheat, rye, barley, oats, spelt or their hybridised strains and products), crustacea (shrimp, crab, lobster, crayfish), eggs, fish (all species of finfish), peanuts, milk, tree nuts (almonds, walnuts, pecans, cashews, Brazil nuts, pistachios, hazelnuts, pine nuts, macadamia nuts, chestnuts, and hickory nuts), and soybeans and all of their products. These commodities have also been termed 'The Big Eight'. Allergic reactions are subject to local food preferences: in the US, peanuts belong to the most important allergenic foods being responsible for severe allergic reactions mostly due to hidden allergens. In Europe, hazelnut and celery allergies are most prevalent, while Asians are sensitive to buckwheat and products thereof.

An allergen nomenclature has been adopted recently, where allergens are assigned the first three letters of the genus, followed by the species and then an Arabic number. The numbers refer to the identification order and the same number is usually used to designate homologous allergens of related species. For example, the first allergen described in brown shrimp, *Penaeus aztecus*, is designated *Pen a* 1, while the homologous molecule from Indian shrimp, *Penaeus indicus* is *Pen i* 1. Furthermore, allergens are commonly described as *major* or *minor*. Major allergens are proteins for which 50% or more of the allergic patients studied have specific IgE (King *et al*., 1994). Examples of

major allergens are Ara h 1 from peanuts, ovalbumin, ovomucoid, and ovotransferrin from eggs, and Pen a 1 from shrimp. Minor allergens may be the result of experimental artefacts or may contain similarities in structure to major allergens that allow for IgE binding, but do not have the conformation necessary to elicit histamine release.

13.2 Methods of analysing allergenic proteins

Labelling of food products is the most effective way for food-allergic consumers to avoid causative proteins. However, avoidance is only an effective measure if it can be assured that the allergen in question is absent in food where it is not expected to occur. So-called 'hidden allergens' are a coincidental or intended contamination of safe food. This occurs when manufacturers use the same equipment to make different products without adequate cleaning of the equipment or use previously manufactured products for manufacturing a secondary product. Currently, some ingredients are added for a specific application, e.g., as emulsifier, that must not be labelled as they are present at less than a specific percentage of the total product. In Europe it is not obligatory at the moment to label components of compound ingredients that make up less than 25% of the final food product. Recently the EU proposed an amendment to the food labelling Directive (EU-Directive, 2000) in order to abolish this '25% rule' requiring the labelling of allergenic proteins at any level (Byrne, 2002). To ensure correct labelling, manufacturers depend strongly on robust and reliable analytical methods. So far, no methods have been designated for regulatory purposes. Likewise, the protection of consumers requires specific and sensitive methods for the detection of trace amounts of potentially allergenic proteins.

13.2.1 Influence of food processing

Allergen specific immunoglobulin E (IgE) binds to certain antigen determinants of the offending protein. Antigen determinants (epitopes) are divided in sequential epitopes according to a certain amino acid sequence and conformational or sterical epitopes according to the configuration of the polypeptide chains (Bleumink, 1970). The conformational epitopes are sometimes denatured during food processing, while sequential epitopes are more stable. Denaturing effects are observed during heating, the most important food processing technique, as well as mechanical processing or irradiation. Sometimes, separation processes lead to the production of allergen-free produce as for example highly refined vegetable oil, which does not exhibit allergenic potential due to the removal of offending protein fractions.

The influence of food processing on allergenic potential has been assessed for a number of foods (Vieths *et al*., 1994). Examples of foods with stable allergenic proteins are almonds (Roux *et al*., 2001), peanuts, celery, and tomato, while food with labile allergenic proteins are, e.g., apple and cabbage. It is often thought

that heating generally denatures offending proteins; it must, however, be kept in mind that in some cases also the contrary can take place, i.e., enhancement of allergenicity during heating processes (Davis *et al*., 2001). What are the implications of food processing for the analysis of the proteins in question? In foods containing mostly stable proteins, specific marker proteins are useful, that exhibit low or no diminution of reactivity after harsh processing conditions. For example, the almond major protein, armandin retained >60% of its pretreatment reactivity after roasting or blanching (Roux, 2001). It is apparent that in such cases the detection of the major almond allergen is of advantage in order to reduce cross-reactivities related to methods based on whole almond extracts. Labile proteins that have been denatured during food processing are not detected with common allergen-assays. As a consequence, for immunological analytical methods, antibodies against the denatured proteins, such as those from roasted peanuts, have to be raised.

13.2.2 Requirements for analytical methods

Analytical methodology is sought that provides specific, sensitive, and rapid detection of offending foods or allergenic proteins. The desired technology should show the following characteristics:

- sufficient specificity to allow the detection of the analyte in question in a wide variety of matrices including processed foods
- satisfactory sensitivity to allow the detection of residues at least down to 1 mg/kg
- capability to rapidly detect contaminations so that corrections can be made before generation of large volumes of mislabelled produce or to give the consumer a yes/no decision within an adequate time span
- easy to use so that less experienced persons can also utilise the assay
- for industrial purposes, continuous sampling could also be of advantage.

13.2.3 Sample preparation

Although food extraction does not follow regulatory methods, the most commonly employed method for food of plant origin is the low temperature extraction developed by Clements (Clements, 1965), which has been modified by Vieths (Vieths *et al*., 1992). Briefly, the food is homogenised with cold acetone at −40 ºC for matrix removal and the precipitated proteins are washed, lyophilised and extracted with buffered aqueous solutions (Hird *et al*., 2000; Moutete *et al*., 1995). Other applications use organic solvents such as petroleum ether (Olszewski *et al*., 1998) or rely solely on the extraction with buffers such as 0.05–0.5 M phosphate buffered saline (PBS) or 0.1 M NH_4HCO_3. Special products for specific applications, e.g., extraction of ovalbumin of ovomucoid from egg are commercially available (Breton *et al*., 1988, Ebbehoj *et al*., 1995).

In most cases, no further purification is necessary because a separation step such as electrophoresis or chromatography precedes the determination step. In cases of very complex matrices such as chocolate or nuts containing a high percentage of fat, additional defatting procedures using organic solvents are unavoidable. It should be mentioned at this point that a number of foods of plant origin such as vegetable or fruit are prone to loss of allergenicity due to enzymes such as protease, peroxidase stemming from the plant matrix after homogenisation. This risk can be minimised, however, by use of additives such as enzyme inhibitors.

13.3 Methods of detecting food allergens

At present, immunochemical techniques are the preferred method for the determination of allergenic proteins in food. However, only a few validated methods are available. SDS-PAGE immunoblot, rocket immunoelectrophoresis and enzyme-linked immunosorbent assays have been applied. Although most of these methods are considered as rapid, there is still great demand for easy-to-use immunochemical tests for the detection of traces of proteins with allergenic potential in food. Research in novel assay designs such as dip-stick or lateral flow devices is still limited, and only singular diagnostic tools have been published (Mills *et al*., 1997; Baumgartner, 2002a; see Table 13.1). Common bioanalytical methods for the structural determination of proteins are also used in the determination of allergenic proteins. Although still limited, applications of molecular biological techniques such as PCR are already established in the analysis of food allergens. The most important detection methods for food allergens are summarised in Fig. 13.1.

13.3.1 Separation techniques

Electrophoretic separation of food protein extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is based on the migration of charged particles in an electrical field. Size and charge of the particles result in different electrophoretic mobility. Mixtures of food proteins are therein separated in different zones. Zone electrophoresis using gels as support, isoelectric focusing using pH-gradients or capillary zone electrophoresis are most commonly employed in the analysis of allergenic proteins (De Jong *et al*., 1998; Scheibe *et al*., 2001). Further separation can be achieved by combining SDS-PAGE with IEF to achieve 2-dimensional (2D-) electrophoresis enabling the identification of isoallergens with the same molecular mass but differing isoelectric points due to minor differences in amino acid sequence. Specific identification of the protein in question is then performed by use of antibodies raised against the respective protein. Techniques obtained by combination of the separation of antigens with the precipitation with antibodies in agarose gels, are called immunoelectrophoresis. The analysis of peanut and hazelnut allergens has

Table 13.1 Selected immunological methods for the detection of food allergens

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Malmheden Yman, 1994	Food	Ovalbumin, casein, corylin, peanut	Tris buffer, pH 8.6	Rocket immunoelectrophoresis, rabbit and sheep antiserum	Ovalbumin 25–200μg/mL Casein 25–100μg/mL Corylin 25–420μg/mL Peanut 25–420μg/mL
Holzhauser *et al*., 1998	Food	Peanut	8 mM Tris buffer	Rocket immunoelectrophoresis	550μg/kg protein in food
Schäppi *et al*., 2001	Food	Peanut allergens	6 M urea buffer	Dot-blot NC-membrane, patient sera, goat-anti-human IgG, POD – 4-chloro-1-naphthol	
Baumgartner *et al.*, 2002b	Food	Egg white	PBS buffer, pH 7.4	Dip-stick immunoassay, rabbit IgG, POD	20 mg/kg–2μg/kg
Mills *et al*., 1997	Food	Peanut conarachin	50 mM Tris-HCI, 0.2 M NaCl, pH 8.2	Dip-stick immunoassay (rapid sandwich ELISA with paddle type dipstick), rabbit IgG, biotin-streptavidin, POD, TMB	0.01%–0.1%

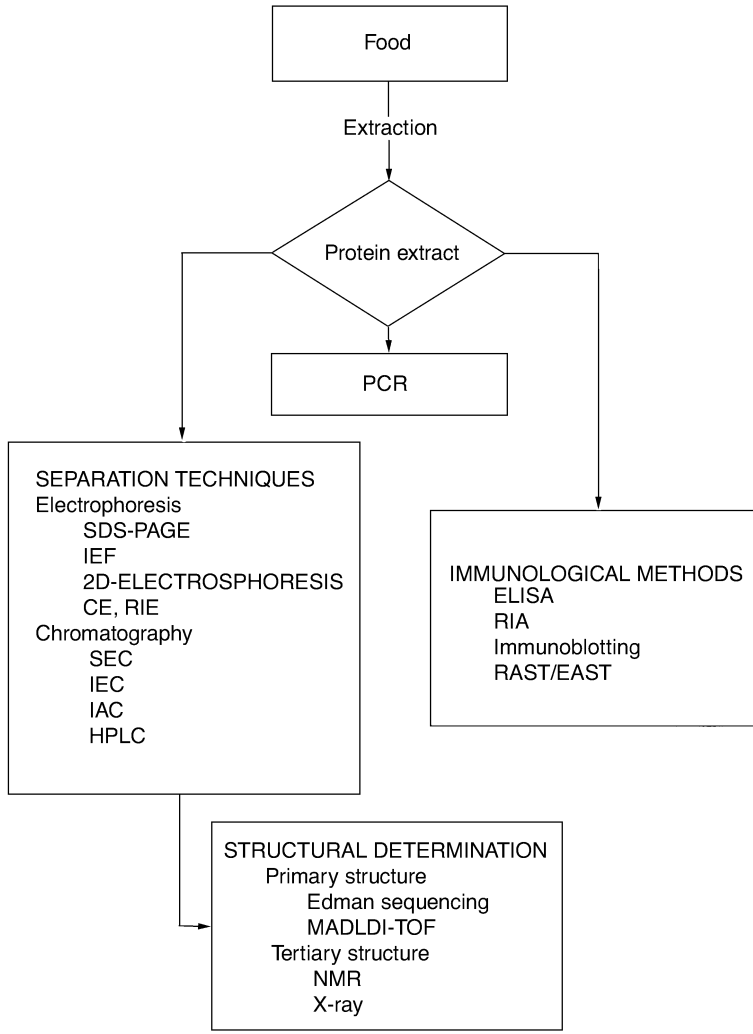

Fig. 13.1 Analytical methods for the determination of allergenic proteins in food (abbreviations are explained in the text).

used protein denaturing electrophoretic techniques. However, also native 2-D gel electrophoresis was applied (Hird *et al*., 2000), overcoming the problem of hindered identification of common features of allergen native structure and the identification of discontinuous epitopes – epitopes formed through folding of the polypeptide chains of proteins in their native conformation.

Milk proteins have successfully been separated by capillary electrophoresis (Recio *et al*., 1997). Disk electrophoresis on polyacrylamide gel for the determination of certain proteins has frequently been used in the past (Silano *et al*., 1968), and has proven valuable for the differentiation between non-meat proteins (casein, egg white albumin and soy protein) in meat products (Ring *et*

al., 1982). Although its mention in the literature is now scant, the principle of disk electophoresis is still used in advanced methods such as SDS-PAGE. Rocket immunoelectrophoresis (RIE) has been used lately for the determination of allergenic peanut proteins in processed foods (Yman, 1994; Holzhauser *et al*., 1998. See also Table 13.1). This analytical method is based on the migration of sample proteins through an antibody-containing gel. Migration is determined by electrophoretic mobility until the antigen-antibody complexes formed in the gel precipitate and build rockets at a constant antigen/antibody ratio. These precipitates are visualised by either Coomassie staining or the more sensitive immunostaining. Thus, this method combines a separation technique with immunological determination. However, difficult practical procedures of gel preparation and immunostaining are disadvantages of this method.

Chromatographic separation is often preparative in order to subject the collected fractions either to electrophoresis and Western blotting or to elucidate tertiary structure by means of NMR or X-ray-structure analysis. Reversed phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography (SEC), ion exchange chromatography (IEC) are all attributed an important role in sample preparation in the determination of potential allergenic proteins.

13.3.2 Immunological methods

Highly specific and sensitive methods are required for the detection of proteins in food matrices. Immunochemical techniques, based on antibody-antigen interactions are the major choice for the identification of the proteins in question. Mostly, tests based on the specific serum IgE from sensitised individuals are used for the determination of allergens. Although unavoidable and essential for the characterisation of allergenic proteins, this approach is unsuitable for the identification of allergens in food matrices due to two severe disadvantages. Firstly, the specificity of IgE from sensitised persons varies considerably and the amount of serum is also limited. Secondly, cross-reactivities to more than one allergenic food may occur in serum from individuals sensitive to more than one potential allergen. Additionally, the simple determination of the presence of potentially allergenic proteins in food using patient sera cannot be ethically justified. Nevertheless, serological tests based on human IgE are most important for determining a patient's allergenicity to a certain protein and will therefore also be dealt with in this chapter.

For the reasons given above, immunological tests based on antisera from animals have been developed. Polyclonal antibodies are raised in rabbits or bigger animals such as sheep or goats in order to obtain larger volumes of antisera. The disadvantage of antibodies recovered from mammalian serum lies in the restricted amount. This problem can be overcome by the production of monoclonal antibodies after the immunisation of mice, enabling continuity of supply of specific antibodies. Even egg yolk antibodies (IgY) have been successfully used for the detection of peanut protein in food (Blais and Phillippe,

2000). Now the question should be asked, whether to raise antibodies against a specific allergenic protein for its application as an 'allergen-assay' or a range of soluble proteins of the respective food for its use as a 'protein-assay'. The two approaches complement each other since an immunoassay developed against the soluble proteins may detect the presence of the more abundant proteins at a very low level as crucial information for allergic persons but gives no reliable information of the allergenicity of the food. On the other hand, the immunoassay developed against the isolated allergen of enriched allergen fraction will give an indication of the likelihood that the food will trigger an allergic reaction. The allergenicity of all the isolated soluble proteins have to be verified with immunoblots using sera from allergic patients before presentation for antibody production. Cross-reactivity is generally a problem of antibody-based methods. Testing for unwanted cross-reactions can be carried out using double immunodiffusion. The specificity of antibodies depends largely on the respective animal, however, the purity of the protein or protein fraction used for immunisation also contributes to the overall result.

RAST inhibition/ELAST inhibition

The radioallergosorbent test or RAST involves the use of human blood serum collected from allergic patients. In this procedure, a food extract is bound to a solid phase such as microcrystalline cellulose. The blood serum containing IgE antibodies specific for the allergens in the extract can be reacted with the solid phase. If the serum is preincubated with a varying amount of food extract, then the protein interferes with the binding of the serum to the adsorbed proteins. This procedure is called the inhibition assay. The percentage of binding of the IgE to the immobilised proteins can be detected in a last step using radioactively labelled anti-human IgE (RAST) or enzyme-labelled anti-human IgE (ELAST or EAST inhibition). While the latter assay overcomes the disadvantages associated with radioactive labelling, it is less sensitive.

Immunoblotting

After employing a separation technique such as SDS-PAGE, immunoblotting with patient sera is performed to allow the identification of allergenic proteins. The recently described dot immunoblot method on polyester cloth uses chicken antibodies for the determination of peanut proteins in various food products (Blais and Phillippe, 2000). Examples for SDS-PAGE and succeeding immunoblotting in the literature are numerous (see Table 13.2), although the combination of these two techniques is time consuming and only denatured proteins are detected.

Double immunodiffusion (Ouchterlony)

This technique developed by Ouchterlony in 1948 is the simplest technique for the identification and distinction of proteins within a very short time span. It allows the distinction of identical, partially identical and non-identical proteins by immunodiffusion from pockets on agarose plates. For the determination of

Table 13.2 Selected SDS-PAGE applications for food allergens

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Hird *et al*., 2000	Extracts from nuts	Peanut and hazelnut protein	Acetone powder, 5 mM TEA pH 8.5, 2% CHAPS, 20% TMS, 2 mM EDTA	Native 2D PAGE, Immunoblotting with patient sera, MALDI-TOF-MS	
De Jong *et al*., 1998	Crude peanut extract	Ara h1, Ara h2		SDS-PAGE, Immunoblotting with patient sera, POD – chloronaftol/DAB	
Burks *et al*., 1991	Peanut extracts	Ara h1	Peanut powder, 20 mM Na-phosphate, 1 M NaCl, 8 M Urea,	AX-chromatography, SDS-PAGE, Immunoblotting with patient sera, AP – BCIP/NBT	
Scheibe *et al*., 2001	Chocolate	Hazelnut, almond	25 mM Tris-HCl, pH 8.8, 2% SDS, 0.5% DTT, 10% glycerol, 0.005% bromophenol blue dye	SDS-PAGE, Immunoblotting with patient sera, AP – BCIP/NBT	500–0.5 mg/kg
Moutete *et al*., 1995	Peanut kernels and shells	HPLC fractions	Acetone powder, diethyl ether, 0.1 M Na-bicarbonate pH 8.0, TCA precipitation	Allergens immobilised to epoxy-Sepharose, human sera, ^{125}I-anti-IgE	

Table 13.2 Continued

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Reese *et al*., 2001	Brown shrimp	Recombinant peptide library and synthetic overlapping peptides		Cellulose membrane, Immunodetection with patient sera, 125I-horse-anti-IgE, or monoclonal AP-anti-human-IgE	
Olszewski *et al*., 1998	Peanut oil	Peanut allergens	Petroleum ether, 0.1 M Na-bicarbonate pH 8.0, TCA precipitation, 62.5 mM Tris-HCl	SDS-PAGE, Western blot with patient sera, ^{125}I-anti-IgE	
Wigotzki *et al.*, 2001	Chocolate, nougat, crème, cookies, muesli, croquant	Hazelnut allergens	0.01 M K-phosphate, 0.13 M NaCl pH 7.4	SDS-PAGE, Immunoblotting with patient sera, EAST-inhibition, anti-human-IgE AP – PNPP	
Schäppi *et al*., 2001	Food	Peanut allergens	6 M urea buffer	Dot-blot NC-membrane, patient sera, goat-anti-human IgG, POD – 4-chloro-1-naphthol	

Schocker *et al*., 2000	Native and roasted hazelnut	Allergens	0.1 M NH_4CO_3, pH 8.0	SDS-PAGE, Immunoblotting patient sera, anti-human-IgE AP	
Koppelman *et al*., 1999	Food	Hazelnut proteins	20 mM Tris, pH 8.2	SDS-PAGE and Immunoblotting with patient sera	LOD 5000 mg/kg
				ELISA, polyclonal rabbit antiserum, POD phenyldiamine	LOD 1000 mg/kg (50 μg/mL)
Parra *et al*., 1993	Pistachio nuts	Pistachio proteins	PBS, pH 7.3	SDS-PAGE, Immunoblotting with patient sera	
Bauer *et al*., 1996	Celery, Mugworth pollen, Birch pollen	Celery, Mugworth pollen, Birch pollen	10 mM K-phosphate pH 7.0, 2% PVPP, 2 mM EDTA, 10 mM DIECA, 3 mM Na-azide	SDS-PAGE, Immunoblotting with patient sera	
Ebbehoj *et al*., 1995	Egg	Ovomucoid, ovotransferrin, ovalbumin, lysozyme, egg-white extract	Purified Sigma products	SDS-PAGE, Immunoblot with patient sera, RAST	
Arshad *et al*., 1991	Brazil nut	Brazil nut	0.125 M Na_4HCO_3	SDS-PAGE, Immunoblot with patient sera, RAST	

Table 13.2 Continued

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Jankiewicz *et al*., 1997	Celery roots, salad, pickled celery	Celery protein	0.01 M phosphate buffered saline pH 7.4	SDS-PAGE, Immunoblot with patient sera, Biotin-streptavidin, POD	
Möller *et al*., 1997	Kiwi fruit	Kiwi allergen Act c2	-40 ºC acetone powder, double distilled water	SDS-PAGE, Immunoblot with patient sera	
Fritsch *et al*., 1997	Roasted pumpkin seed	Pumpkin seed	NA	SDS-PAGE, Immunoblotting with patient sera	

food proteins, the double immunodiffusion by Ouchterlony is employed for the detection of hidden allergens, e.g., the determination of albumin from eggs or wheat gluten in meat products (Brauner-Glaesner and Ristow, 1990). Additionally, it is useful for the screening of antisera in regard to cross-reactivity to other potential allergens (Brehmer and Schleicher, 2000). Although only qualitative and usually of poor sensitivity the easy procedure makes up for these disadvantages.

Immunochemical assays

ELISA

ELISAs have become popular in the 1980s and have been widely used in biochemical and biomedical applications since then (Samarajeewa *et al*., 1991; Skerrit and Hill, 1991). Despite their high analytical potential – specificity of the reaction, sensitivity, high screening capacity – ELISA applications for the detection of allergenic food proteins are limited to a few allergens. Nevertheless, ELISAs seem to be the most important tool of analysis of allergenic proteins. Most ELISAs are developed in academic laboratories, but a number of ELISA kits are now commercially available. The great advantages of ELISA techniques include the full automation of the test procedure by means of robots and the resulting extensive screening potential, the high sample throughput and the easy operation. ELISAs involve antibodies against specific proteins or a range of proteins raised in mammals such as rabbits, goats, sheep or, more recently, chickens (Blais and Phillippe, 2001). Monoclonal antibodies which overcome the disadvantages associated with animal sera and guarantee long-term supply, which is often seen as a prerequisite for commercial assays, are still scarce in the analysis of allergenic proteins. ELISAs have been developed for the most important food allergens such as almond, peanut, hazelnut, egg, soy proteins, etc. Practically all ELISA formats have been used but the sandwich ELISA has been most commonly employed (see Fig. 13.2). Within this format, antibodies are adsorbed to the bottom of microtiter plate wells. They are reacted with the food extract and specifically bind proteins. After this antibody-antigen interaction, bound proteins are visualised by another antibody labelled with an enzyme. This antibody, however, has to be raised in animals of a different species. A summary of non-commercial ELISAs is given in Table 13.3.

Most commercially available ELISA test kits for the quantitative determination of food allergens reach detection limits up to the desired 1 mg/kg, ranging mostly between 0.5 to 2 mg/kg but also higher detection limits can be encountered as 10 mg/kg for egg, milk, and peanuts (Table 13.4). Assays developed in academic laboratories may show detection limits even down to the low ng/kg range, such as described by Mariager *et al*. in 1994. The limits of detection are of crucial importance for successful test kits. In a statistical evaluation the predicted doses for allergic reactions may be induced by quantities as low as, e.g., 0.7 μg of peanut for one per million of population (Bindslev-Jensen *et al*., 2002). As threshold levels vary strongly from patient to patient, it is difficult to assess limits of detection for a regulatory basis.

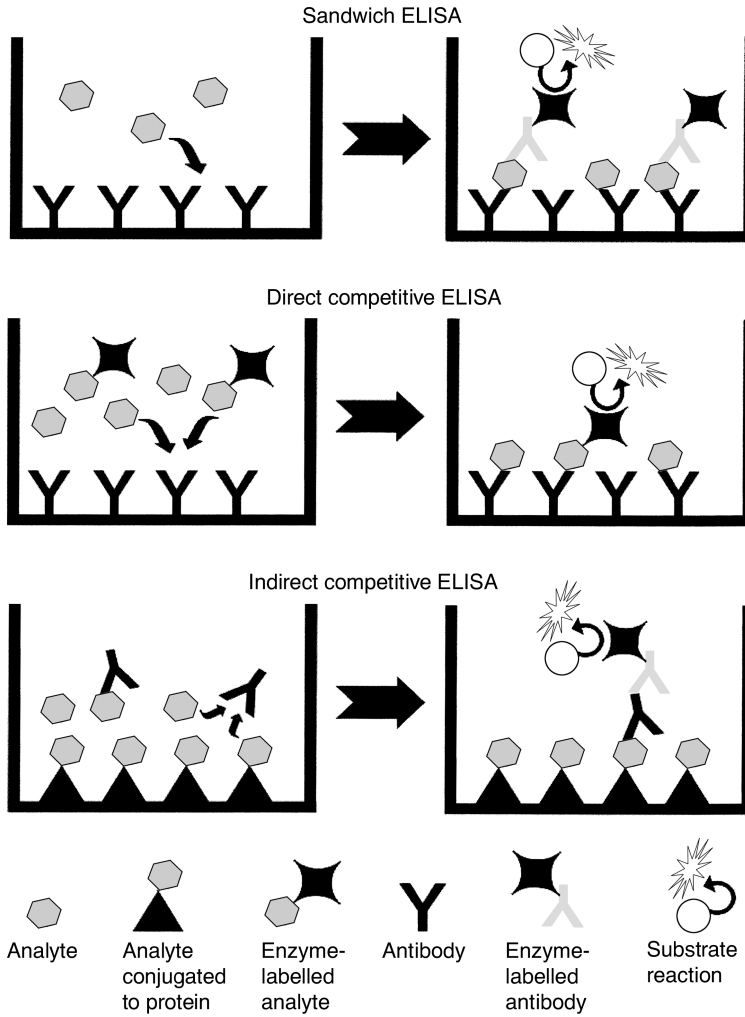

Fig. 13.2 Principles of immunoassays: Sandwich ELISA (non-competitive); direct competitive immunoassay; indirect competitive immunoassay.

However, the statistical evaluation shows that test kits with LODs even in the low μg/kg-range are required.

ELISAs also require sufficient specificity to allow the detection of residues of specific allergenic proteins. Therefore, the selection of antibodies is of crucial importance. In order to obtain sufficient specificity, antibodies binding with high affinity are preferred. The advantages and disadvantages of human IgE have already been discussed in section 13.3,2. It must however be kept in mind, that also antibodies raised in animals may exhibit multiple sensitivities and/or cross-

Table 13.3 Selected ELISA applications for the detection of food allergens

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Blais and Phillippe, 2001	Food	Hazelnut protein	PBS, 0.1% Tween 20, 4% Carnation skim milk powder	ELISA, polyclonal IgY, Streptavidin-POD - TMB	1–0.03 μg/mL
Koppelman *et al*., 2000	Food extracts	Hazelnut protein	20mM Tris pH 8.2	ELISA/polyclonal rabbit IgG, POD	5 ng/mL–1 μg/mL
Leduc *et al*., 1999	Meat	Egg-white	10 m M Na_2HPO_4, 150 mM NaCl, pH 7.2	ELISA, rabbit anti-hen's egg-white antiserum, mouse anti-rabbit IgG AP, NBT-BCIP	0.125%–0.03%
Acosta *et al*., 1999	Commercial food products	Almond (purified major protein)	0.02 M Tris-HCl pH 8.1, anionic exchange (DEAE), gel filtration	ELISA non-competitive, AP goat anti-rabbit IgG, PNPP	1–100 ng/mL
				ELISA competitive	> 300 ng/mL
Holzhauser and Vieths, 1999b	Commercial food products	Hazelnut protein		ELISA, hazelnut-specific polyclonal rabbit antiserum, hazelnut-specific polyclonal sheep antiserum, POD-labelled rabbit anti-sheep IgG	3–360 ng/mL

Table 13.3 Continued

Reference	Matrix	Assayed subst.	Extraction	Detection	Range
Keck-Gassenmeier *et al*., 1999	Food	Peanut proteins	0.05 M Tris/0.2 M NaCl pH 8.2 (Cortex) 0.1 M Tris/0.4 M NaCl pH 8.2, 12.5% fish gelatine	ELISA (Cortex) non-competitive sandwich type ELISA with biotin-avidin enhancement POD TMB, IgG against ConA	5–179 ng/mL
Newsome and Abbott, 1999	Chocolate	Peanut proteins		Immunoaffinity column, ELISA	0.1 μg/g
Yeung and Collins, 1997	Food	Soy protein	PBS	ELISA, polyclonal rabbit IgG, anti-rabbit IgG, POD	3–170 ng/mL
Richter *et al*., 1997	Goat and ewe milk cheese	Bovine milk and bovine caseinate	PBST, Na_2CO_3/$NaHCO_3$ pH 8.3	Indirect competitive ELISA, AP	LOD 0.5 μg/mL
Yeung and Collins, 1996	Food	Peanut protein	20 mM NaH_2PO_4, 1M NaCl, 0.1% Tween20 pH 7.0	Sandwich ELISA, POD	400 μg/kg
Hefle *et al*., 1994	Food	Peanut proteins	20 mM PBS	Sandwich ELISA with monoclonal IgM and rabbit IgG	40 μg–2 mg/mL
Pressi *et al*., 1994	Pasta	Egg yolk	0.05 M carbonate buffer pH 9.6	Indirect ELISA, rabbit IgG, avidin streptavidin-POD	200 g/kg

Stevenson *et al*., 1994	Raw meat mixtures	Chicken meat	7 M urea	Heterologous non-competitive solid-phase ELISA	1%
Turin and Bonomi, 1994	Dairy-based food	Ovalbumin	1 + 10 dilution with 50 mM phosphate, pH 6.8	Competitive ELISA, rabbit anti-ovalbumin serum, goat anti-rabbit IgG, AP, 4-nitrophenylphosphate-diethanolamine	0.2–10 μg
Meisel, 1993	Milk and milk proteins	Soybean glycinin A	PBS	Indirect ELISA with IgY, rabbit anti-chicken IgG-POD	0.5–256 μg/mL
Meisel, 1990	Milk, cheese	Lactoferrin	PBST, 8 M Urea	Indirect ELISA with IgY, rabbit anti-chicken POD	LOD 4 ng/mL, 15–2000 ng/mL
Breton *et al*., 1988	Egg	Ovalbumin	Purified Sigma product	ELISA competitive assay, rabbit IgG, POD	

Table 13.4 Commercially available ELISA testkits for quantification of food allergens

Company	Test kit trademark	Allergen	Detection range	LOD (if available)
ELISA Technologies, Inc. of Alachua, Florida, USA www.elisa-tek.com/peanut.htm	ELISA-TEK, non-competitive, sandwich-type EIA utilising biotin-avidin enhancement	Peanut, Conarachin A	0.5–2 mg/kg	100–150 pg/mL
		Gluten	20 mg/kg	
		Casein, Whey	25 mg/kg	
		Soybean		
Neogen Corporation, Lansing, MI, USA www.neogen.com	Veratox® for Egg Allergen, Sandwich-ELISA	Egg	2.5–25 mg/kg	
	Veratox® for Milk Allergen, Sandwich-ELISA	Milk	2.5–25 mg/kg	
	Veratox® for Peanut Allergen, Sandwich-ELISA	Peanut	2.5–25 mg/kg	
r-Biopharm, Darmstadt, Germany, Europe www.r-biopharm.com	Ridascreen Egg Protein Assay	Egg white	Standards 0–54 mg/kg	5 mg/kg
	Ridascreen Gliadin, monoclonal antibodies	ω-gliadin fraction	Standards 0–320 mg/kg	5 mg/kg
	Ridascreen Peanut, Sandwich ELISA	Peanut protein	Stds 0–90 mg/kg	< 2.5 mg/kg
	Ridascreen Fast Gliadin	Gliadins from wheat and prolamins from rye and barley	10–80 μg/kg	5 mg/kg
	Ridascreen Hazelnut, Sandwich ELISA	Hazelnut protein	Std 0–160 mg/kg	10 mg/kg
	Ridascreen β-Lactoglobulin, competitive ELISA	β-Lactoglobulin	Std 10–810 μg/kg	5 mg/kg
	Ridascreen Histamin, competitive ELISA	Histamin	Std 0–4050 μg/kg	0.5–2.5 mg/kg

ELISA Systems, Australia www.elisas.com.au		Milk proteins Egg Allergen Seafood Allergen Soy Allergen		
Tepnel Biosystems Ltd., Flintshire, UK www.tepnel.com	BioKits Peanut Protein Test Kit, non-competitive sandwich type EIA	Peanut, Conarachin A	1–10 ng/mL	< 0.1 mg/kg
	BioKits Soya Protein Assay Kit, indirect competitive ELISA	Soya, denatured, renatured protein	< 0.5– > 20%	< 0.5%
	BioKits Casein Assay Kit, indirect competitive ELISA	Bovine Casein	25–500 mg/kg	< 5 mg/kg
	BioKits β-LG Assay Kit, indirect competitive ELISA	β-lactoglobulin	25–500 mg/kg	< 7.5 mg/kg
	BioKits BSA Assay Kit, indirect competitive ELISA	Bovine Serum Albumin	25–500 mg/kg	< 10 mg/kg
	BioKits Sesame Protein Test Kit, non-competitive sandwich-type ELISA	Sesame proteins	5–100 mg/kg	< 1 mg/kg
	Gluten Home Test Kit, dry-strip immunochemistry format	ω-gliadin		50–200 mg/kg, high processed flours 200–1200 mg/kg

reactivities to several allergenic foods. The purity of the immunogen is a decisive factor and also whether a purified protein is used for immunisation or a crude protein extract. Although most ELISAs are highly specific (Yeung and Collins, 1996), several common cross-reactivities have been described (Acosta *et al*., 1999, Holzhauser and Vieths, 1999a, 1999b).

Other assays

Other immunochemical assays of great importance are the radioimmunoassay (RIA), the immunofluorescence assay and the luminoimmunoassay (LIA), which have all been used in the determination of allergenic proteins. Some disadvantages however, such as the use of radioactive labels have favoured the use of the ELISA.

13.3.3 Molecular biological methods – PCR

The Polymerase Chain Reaction (PCR) is mainly used and routinely applied for the detection of microorganisms and for authenticity testing of genetically modified organisms like transgenic soybean and maize in various food products. Otherwise, the use of the PCR method in the food industry and especially for the determination of food allergens is still very limited. So far, only two methods have been published. The PCR permits the amplification of extremely small amounts of DNA (as little as one DNA molecule). If the sequence of at least a part of a DNA segment is known, cloning can be facilitated by amplifying the DNA segment. Two oligonucleotides have to be synthesised. Each has to be complementary to a short sequence in one strand of the desired DNA segment and positioned just beyond the end of the sequence to be amplified. These synthetic oligonucleotides are used as primers for replication of the DNA segment *in vitro*. DNA strands are separated by heating, then annealed to an excess of short synthetic DNA primers that flank the region to be amplified. Then the heat-stable DNA polymerase and the four deoxynucleoside triphosphates are added, and the primed DNA segment is selectively replicated. After polymerisation, the process is repeated for 25 to 30 cycles. The thermostable DNA polymerase TaqI (from *Thermus thermophilus*) is not denatured by the heating steps. After 25 cycles, the target sequence has been amplified about 10^6-fold and the resulting products are separated according to size by agarose gel electrophoresis. The gel is stained with ethidium bromide, a fluorescent dye, which intercalates with the DNA and glows orange under UV light.

The use of the PCR method in food analysis is afflicted with a number of problems. Extreme fragmentation of DNA through mechanical, thermal and enzymatic influences or acidic pH as well as PCR-inhibitors from different food products are just a few examples. Furthermore, DNA is extremely difficult to extract in the presence of polysaccharide-containing thickeners. If DNA-free protein is added to a food product the detection of a specific allergenic protein could lead to false negative results.

One publication (Holzhauser *et al*., 2000) shows a successful PCR amplification with a 182 bp (base pairs) fragment of Cor a 1 (hazelnut allergen). No cross-reactivities to 30 different fruits, nuts, beans and other ingredients were detected. Chocolate bars, chips, flakes, muesli and peanut paste were tested as food samples and the detection limit for hazelnut was 10 mg/kg. For wheat, the wheat prolamin (ω-gliadin) was amplified and showed no cross-reactivities to oats. In another study (Koeppel *et al*., 1998), different oat samples, flakes and grains, and processed oat products were tested. The limit of detection for wheat flour was 10–100 mg/kg and for gliadin 400 μg/kg.

In 2002, Holzhauser (Holzhauser *et al*., 2002) published a comparison of a protein sandwich ELISA and a DNA PCR-ELISA (SureFood-Allergen Hazelnut Kit No. S3002, CONGEN Biotechnology, Berlin, Germany) for the detection of hazelnut residues in food. Again, the recombinant hazelnut allergen rCor a 1.0401 was chosen but the primer pairs were optimised and therefore the newly created 152 bp-PCR generated no artefacts. The combination of PCR DNA amplification, the immobilisation of the PCR products on a solid-phase, the hybridisation and the detection with enzyme-conjugated antibody describes a simple system to be performed in contrast to real-time PCR, where sequence verification and an expensive equipment is needed. It was successfully demonstrated that allergen monitoring at a level below 10 mg/kg is possible.

Compared to the ELISA method the PCR method detects the presence of DNA from a specific source but not a specific protein from a food sample. The test development for PCR methods is very quick (within a few days), if the referring DNA sequence is known. DNA stability as well as protein stability for the ELISA is a critical point for food testing. Particularly in cases where no promising immunogen can be established, the PCR method is a powerful and specific alternative analytical tool for the detection of allergenic proteins in food.

13.3.4 Structural determination

Apart from the identification and the determination of potentially allergenic proteins by immunological, electrophoretical, or biomolecular methods, the elucidation of primary and tertiary structure is also very important. Prior to such determinations is usually a separation technique as discussed in section 13.3.1, which effectively separates or purifies the proteins based on different physical or chemical properties of the proteins such as pH, molecular weight, or charge. Edman sequencing is still most frequently used for the determination of the primary structure. It is a cyclic process, based on splitting off the N-terminal amino acid from one end of the peptide chain in each reaction cycle and a subsequent identification. The three-step-process coupling to phenyl-thiohydantoin-derivatives, cleavage, and conversion has been automated in order to minimise loss in handling the sample. This sequenator does not require more than a 5 nmol sample for the determination of amino acid sequences.

Recently, sesame seed allergens have been successfully identified by Edman sequencing (Beyer *et al*., 2002). Determination of the molecular mass of

allergenic proteins and peptides is performed by mass spectrometry. The introduction of gentle ionisation techniques such as ESI (electrospray ionisation) and MALDI (matrix assisted laser desorption/ionisation) in the late 1980s has made mass spectrometry the most important tool for the analysis of peptides and proteins. MALDI uses cinnamic acid dihydroxy benzoic acid as matrix for the sample. Apple and peach allergens are examples of plant-food proteins, which have been characterised by MALDI analysis (Sanchez-Monge *et al*., 1999). However, highly purified proteins are required for such a characterisation.

Other techniques for the bioanalytical characterisation of allergenic proteins are molecular mass fingerprinting, internal sequencing and post-translational modification as well as epitope identification. For the elucidation of the tertiary structure, separated or purified proteins are subjected to nuclear magnetic resonance spectroscopy (NMR) and X-ray structure analysis. Those two techniques are still the only methods for the determination of structure of macromolecules like proteins or nucleic acid on an atomic level. Both methods have been successfully applied in the elucidation of allergenic proteins (Fukush *et al*., 1998, Betzel, 2001).

13.4 Developing new rapid tests: dip-sticks and biosensors

Besides the common ELISA procedures there is an increasing demand for immunoassay techniques for field and home use offering protocols for quick and reliable results without expensive laboratory equipment and reagents. One of these diagnostic tools is the dip-stick immunoassay, resembling ELISA constructs: instead of microtitre plates, carrier membranes (usually polyvinylidene difluoride (PVDF), nylon and nitro-cellulose (NC)) are used to immobilise either the antibody or the antigen. Depending on the entire test format, one to three working steps have to be performed, requiring a total of about 30 min. to three hours for obtaining the test results. These dip-stick assays which provide qualitative and semi-quantitative results do not require a profound analytical knowledge, but are intended to be used by following simple instructions.

Another interesting alternative rapid assay format for the detection of trace proteins in food is provided by lateral flow devices. These one-step tests take only a couple of minutes (2–3 min.) to perform. At the moment, not much information and published data about commercially available dip-sticks applied in the food industry can be found. In the literature, one article deals with the rapid dip-stick detection of histamine in tuna (Hall *et al*., 1995). This non-immunological assay, based on an enzyme reaction of diamine oxidase and histamine, was sensitive to 0.07 mM histamine. In Europe, the detection of peanut allergens reaches a greater demand than histamine. With the combination of rabbit anti-conarachin IgG, biotinylated rabbit anti-conarachin IgG, avidin-horseradish peroxidase and a paddle-type dip-stick, a rapid test was achieved with a detection limit of 0.01% of unprocessed peanut in marzipan (Mills *et al*., 1997), which corresponds to a

detection limit of 0.0007–0.001% of conarachin. The principle of the lateral flow device is used with the commercially available Gluten Home Test Kit, a dry-strip immunochemistry format from Tepnel Biosystems Ltd. (Flintshire, UK) for the qualitative determination of ω-gliadin. Detection limits of 50–200 ppm are reached for cakes for example and 200–1200 ppm for highly processed flour. Although currently a lot of academic research can be observed towards the development of dip-sticks for the determination of allergenic proteins, none is suitable for industrial purposes so far.

Other alternatives are immunoassays which are capable of allergen-antibody binding, but without any enzymatic labelling for the detection. These immunological biosensors comprise three components: a biological receptor of appropriate specificity for the analyte, a transducer to convert the recognition event into a suitable signal and a detection, analysis, processing and display system that is normally electrical. The signal can be acoustic, electrical, mechanical or optical. Many technologies are currently used for biosensing, e.g., amperometric sensors, potentiometric sensors, light-addressable potentiometric sensors, fluorescence evanescent-wave sensors or surface plasmon resonance (SPR) sensors (Malan, 1994). The latter uses an electron charge density wave phenomenon that arises at the surface of a metallic film when light is reflected at the film under specific conditions. The resonance is a result of energy and momentum being transformed from incident photons into surface plasmons, and the resonance is sensitive to the refractive index of the medium on the opposite side of the film from the reflected light. Briefly, protein binds to immobilised antibodies on a gold film surface. The antigen-antibody interaction induces a change in the refractive index, which can be measured. The SPREETA^{TM} immunoassay (Texas Instruments) for the detection of peanut allergen achieved a detection limit of 0.7 μg peanut/mL extract (Mohammed *et al*., 2001).

The SPR technology of BIACORE (Uppsala, Sweden) is a label-free technology for the monitoring of such biomolecular interactions. Data has been published mainly for clinical purposes and, e.g., for the detection of nonmilk proteins in milk powder with a direct biosensor immunoassay (Haasnoot *et al*., 2001). Kits for the determination of vitamins, veterinary drugs and antibiotics are commercially available. The determination of protein quality or food allergens is also pursued at the moment (Biacore, 2002).

Other microchip-techniques, e.g., immobilisation of proteins on gel pad arrays (Arenkov *et al*., 2000), agarose-film coated glass slides for covalent linking of proteins through reactive (terminal) NH_2 groups (Afanassiev *et al*., 2000), electrospray deposition of dry proteins or covalent linking of proteins from dry deposits to a dextran-grafted surface (Avseenko *et al*., 2002), immobilisation of biomolecules within a porous reaction layer on a semiconductor device that employs complementary metal oxid semiconductor (CMOS) technology to create high-density arrays of microelectrodes with parallel addressing within the array (Dill *et al*., 2001) have been published and the list with many different techniques is not finished yet. Further development of the techniques of microarrays or microchips for the determination of proteins

in general will facilitate the research and method development in, e.g., food allergen monitoring applications.

13.5 Future trends

It can be summarised that immunoassays are specific, sensitive and rapid methods which enable the detection and quantification even of trace amounts of allergens in raw materials as well as in intermediate and finished food products. However, routine application is still limited to the detection of a few allergens. Food manufacturers are recommended to use only validated and, if available, standardised tests to monitor food allergens. There is still a great need for cost-effective screening methods which can rapidly detect minute amounts of allergens. PCR methods are a promising alternative for food allergen detection at much lower detection levels. Major disadvantages are, however, DNA denaturation during food processing and matrix-dependent sensitivities (Besler, 2001). The use of innovative techniques, such as SPR, which allows sensitive detection of molecular interactions in real time without the use of labels, may also be an interesting future alternative. Likewise, the use of advanced array technology which allows a screening of many different food allergens on one chip within a relatively short time span does have great potential to become a key technology in the area of food monitoring.

13.5.1 Reference materials

The quantitative detection of food allergens in food is dependent on the development procedure of the immunoanalytical tests, in particular against which extracts of the offending food antibodies are raised. Often the food component detected is not the offending allergen and therefore the test results do not reflect the allergenic potency of the food in question. There is a great need for the production and certification of well defined food allergen reference materials which can be used to validate tests for food allergens and provide well characterised allergen preparations for the study of food processing on the allergenicity of foods. Reference allergen preparations are also required for the standardisation of the various tests for food allergens particularly in proficiency testing and also as a source material for evaluating food processing techniques to reduce allergenicity of food products.

The availability of purified natural and/or recombinant allergens is a good starting point for the development of new certified reference materials based on cocktails of the pure food allergens. However, the use of recombinant allergens in the preparation of the reference material should be viewed with caution since post-translational modification to these proteins have recently been shown to have a significant bearing on the allergenic nature of the protein in the food and the presence of glycosylation in many indigenous allergens will certainly affect the fate of the allergen during food processing and digestion

13.5.2 The AllergenTest project

At the beginning of 2002, the 3.5 years research project 'AllergenTest' (EU Project, 2000) with a total financial volume of 1.3 million Euro has been launched by the European Commission. The required work is carried out by nine project partners from six different countries including an immunoassay producer, a food producing company, two hospitals providing sera from allergic patients and a consumer protection organisation. The major goal of this project is the development of rapid easy-to-use immunochemical tests for the detection of traces of proteins with allergenic potential in food. Peanut and hazelnut proteins are used as model substances. The test format should allow the detection of the soluble proteins of hazelnut or peanut ('protein-assay'). In addition, for some selected allergenic protein fractions of peanut and hazelnut, specific assays ('allergen-assay') are developed for the detection of a certain allergen (e.g., Ara h 2).

The developed tests are intended to be used as a diagnostic tool to avoid contamination, check uncertain food and foodstuffs and aid further research in this important area. The main part of the project deals with analytical work, which poses high challenges due to the complex matrices of food and foodstuffs. An important step towards the development of the proposed easy-to-use test systems are laboratory based ELISAs using, e.g., conventional microtitre plates. Their suitability is tested for the analysis of unprocessed and processed food products before being passed on for further development of the rapid easy-to-use test systems. For that purpose novel assay designs are currently being developed including dip-sticks and lateral flow devices (Baumgartner *et al*., 2002b).

13.6 Sources of further information and advice

Useful web pages which could be accessed April 2003: *http://ambl.lsc.pku.edu.cn*, *www.foodallergy.com*, *www.medizinfo.com/allergy* – mainly from the medical point of view, *www.anaphylaxis.org.uk*, *www.foodallergy.org*, *http://allergy.mcg.edu/links.html*, *www.food-allergens.de*, *http://allergies.about.com*, *http://www.ifr.bbsrc.ac.uk/protall/*, *http://www.informall.eu.com/*

13.7 References

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14

The extraction and purification of proteins: an introduction

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14.1 Introduction

Protein purification is an art which has been refined over the last four decades such that excellent techniques are now available that simplify or enhance the recovery and homogeneity of protein products in relatively short periods of time. This chapter will discuss some of the methods that can be used to extract proteins to produce heterogeneous mixtures suitable for use in food systems. These mixtures have substantially higher protein contents than the starting materials. Methods that can be used to purify food proteins to homogeneity for detailed structure-function studies will also be described. The initial section discusses some of the factors that influence extraction, composition, and quality of the protein products. This is followed by a discussion of various extraction agents such as water, aqueous solvents, salt solutions, and buffers that are used to obtain enriched protein products. The later sections describe purification methods such as column chromatography and electrophoresis that can be used to separate a protein of interest from a mixture of proteins. The chapter concludes with a brief look at some of the future trends in protein extraction and purification.

14.1.1 Objectives in extraction and purification

In order to study the structure-function properties of a particular protein, it is necessary to purify it, i.e., separate it from other protein and non-protein components present in the cell. An initial step in protein purification is to extract the raw material with a suitable solvent (usually aqueous) with the aim of producing an enriched protein product. Enriched protein products are also used

in food formulations where they perform several functional properties such as water absorption, emulsification of fats, foaming, flavour binding, and gelation. Organic solvents are rarely used to extract food proteins due to the negative effect on protein structure and functionality, especially solubility in food systems. Aqueous extraction is usually followed by centrifugation to separate the solubilized proteins (supernatant) from insoluble materials (precipitate). Since the aqueous solution often extracts other biopolymers apart from the protein of interest, it is necessary to subject the supernatant to further treatments that will remove the impurities. Therefore, the supernatant may be dialyzed or treated with dilute acid solution to precipitate most of the protein of interest and leave the impurities in solution. Similarly, the impurities could be precipitated while the protein of interest remains in solution. The type of products obtained from this type of extraction and precipitation process are called protein concentrates and protein isolates. Concentrates have a minimum protein content of 65%, dry weight basis (dwb) while isolates have a minimum protein content of 90% (dwb) (Uzzan, 1988). Protein concentrates and isolates are common ingredients in the manufacture of various food products.

The protein concentrates and isolates can be processed further by various purification methods to isolate individual protein components with high degree of homogeneity. The purification process involves separation of proteins based on their molecular properties such as size, hydrophobicity, ionic properties, and affinities for certain ligands. Each successive step in the purification process is called fractionation and results in increased homogeneity of the protein molecule. Fractionation serves two purposes; removal of contaminating material and enrichment of the fraction that contains the protein of interest. The enriched fraction can be subjected to yet another round of fractionation until the desired purity is achieved. Once a food protein is purified, it is possible to establish its unique functional activities (gelation, foaming, etc.) as a function of amino acid composition and molecular conformation (secondary and tertiary structures). Purified proteins can be used to determine amino acid sequence, which establishes a unique primary structure for the protein. The primary structure data is an important tool in determining the quantitative structure-activity relationships of proteins (Siebert 2001; Nakai *et al*., 2003). Purification is also required in order to study the 3-dimensional structure of proteins by X-ray diffraction, as demonstrated by characterization of an amaranth globulin (Vasco-Mendez *et al*., 1999). A simplified overview of protein extraction and purification processes is shown in Fig. 14.1.

14.2 Factors affecting extraction

14.2.1 Source of raw material

Generally, raw materials that contain high levels of oil must be defatted before protein isolation is carried out. Removal of the oil will prevent emulsion formation during protein extraction and produces oil-free protein materials that

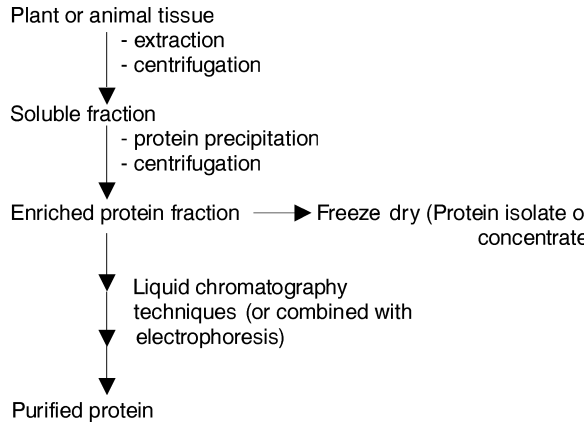

Fig. 14.1 Overview of the protein extraction and purification processes.

are compatible with subsequent purification protocols that employ mostly aqueous solutions. Fat-free samples are obtained by defatting the raw materials with solvents such as hexane (Abbot *et al*., 1991; Kumagai *et al*., 2002) and petroleum ether (Sathe *et al*., 2002) or by mechanical press (Shrestha *et al*., 2002). Raw materials such as animal muscles that contain high levels of moisture and fat should first be ground and freeze-dried (to preserve native protein structure) before the lipids are extracted with hexane or petroleum ether.

14.2.2 Nature of raw material

Some seed proteins contain high levels of phytate, phenolic compounds and other contaminants that may be toxic, interfere with protein isolation (reduction in yield), or contribute to discoloration, off-flavour, or reduced functionality of the isolated protein. For example, phenolic acids can reduce protein-protein interactions that are required for emulsifying and gelation properties in food systems (Arntfield, 1996; Rubino *et al*., 1996). Therefore, such materials undergo preliminary treatments to remove the undesirable contaminants before protein isolation is carried out. Phenolic compounds in sunflower seed proteins were removed using 80% aqueous methanol without negative effects on extractability and native structure of the proteins (Gonzalez-Perez *et al*., 2002). Repeated extraction of defatted sunflower meal with 50% aqueous ethanol at pH 5.0 was also used in subsequent production of a low-phenolic protein isolate suitable for incorporation into wheat bread (Yue *et al*., 1991).

A low-phytate rapeseed protein isolate was produced by initial extraction of the meal at pH 4.0; the extraction removed about 70% of the phytate and 18% of the protein present in the meal (Blaicher *et al*., 1983). The meal residue obtained after the pH 4.0 extraction was extracted with aqueous solvent at pH 11.0 followed by acid precipitation of the proteins at pH 4.7. The protein isolate contained only 0.2% phytate, which is similar to the 0.1% level found in

commercial soybean protein isolate (Blaicher *et al*., 1983). In comparison rapeseed protein isolates prepared without the initial phytate extraction at pH 4.0 contained phytate levels that range from 4.3 to 9.8% (Blaicher *et al*., 1983).

The potential for browning in potato products means that isolation of its proteins must be carefully controlled to prevent polymerization of phenolic acids, which may interact with the proteins to reduce yield and discolour the isolate. Potato proteins were extracted with water that contained an antioxidant (sodium metabisulfite) to prevent the browning reaction. After centrifugation, the supernatant was filtered and kept in contact with Amberlite XAD-4 resin under mechanical stirring to remove the phenolic acids (Ralet and Gueguen, 2000). This was followed by dialysis against water before the proteins were freeze-dried.

The presence of phytate and flatulence-producing oligosaccharides in peas requires additional processing before the protein products can be incorporated into infant formulas. Flatulence factors were removed from pea protein isolate by an ultrafiltration process that effectively partitioned the low molecular weight oligosaccharides into the permeate while the proteins remained in the retentate (Fredrikson *et al*., 2001). Addition of phytase enzyme to the retentate reduced the phytate level of the protein isolate to trace levels to produce a high quality pea protein product suitable for use in infant foods (Fredrikson *et al*., 2001).

Preparation of protein isolates from plant materials that contain high levels of cell wall materials involves the use of carbohydrases to break down the cell wall materials and facilitate extraction of proteins. For example, rice bran contains proteins with protein efficiency ratio similar to that of casein, and the protein isolate could be used to fortify various foods (Wang *et al*., 1999). To produce a rice bran protein isolate, the defatted rice bran is mixed with water, adjusted to pH 5.0 and treated with phytase and xylanase to degrade phytic acid and break down the cell walls, respectively (Wang *et al*., 1999). The digested bran mixture is then adjusted to pH 10.0 to inactivate the enzymes and extract the proteins. After centrifugation the supernatant is adjusted to pH 4.0, precipitate is neutralized to pH 7.0 and freeze-dried as the rice bran protein isolate (Wang *et al*., 1999).

14.2.3 Type of protein (solubility properties)

The solubility properties of proteins most often differ in different solutions, depending on the pH and ionic strength. Therefore, enriched protein fractions can be obtained by extracting the sample with specific solutions designed to remove the protein of interest while other proteins are left in the residue. Proteins may be fractionated using a modification of the traditional Osborne procedure as follows (Betschart *et al*., 1977): sample is extracted successively with distilled water (albumins), 5% NaCl (globulins), 60–90% ethanol (prolamins or gliadins), and 0.4% NaOH (glutelins). Each extract is centrifuged, filtered through a suitable filter paper, and dialyzed against distilled water. The contents of the dialysis bag are centrifuged and the supernatant freeze-dried to obtain the various protein fractions.

Differential solubility of whey proteins in trichloroacetic acid (TCA) has been used to separate glycomacropeptide (GMP), which can be further purified by column chromatography techniques (Stan *et al*., 1983; Neeser *et al*., 1988; Olieman and van Riel, 1989; Nakano *et al*., 2002). A solution of the whey powder (5g in 25 ml of water) was mixed with an equal volume of 20% (w/v) TCA solution at 4ºC, which precipitates all proteins except the GMP (Nakano *et al*., 2002). The supernatant obtained after centrifugation was neutralized with NaOH and dialyzed at 4ºC against water using membrane with molecular weight cut-off of 6000–8000; the contents of the dialysis bag was then freeze-dried as the GMP protein isolate (Nakano *et al*., 2002).

Preparative scale isolation of β-lactoglobulin from whey protein isolate (WPI) was achieved by taking advantage of the solubility of the protein at pH 2.0 in the presence of 7% (w/v) NaCl; at these conditions other whey proteins are insoluble (Mate and Krochta, 1994). A 15% (w/w) solution of WPI in deionized water was adjusted to pH 2.0 by addition of 2 M HCl followed by gradual addition of solid NaCl until the concentration reached 7% (w/v). After centrifugation of the resultant slurry, the precipitate (containing contaminating proteins) was discarded while the supernatant (containing β-lactoglobulin) was adjusted to either pH 5.0 or 7.0 and the salt removed by diafiltration (Mate and Krochta, 1994). The material obtained from diafiltration was freeze-dried and contained >98% protein, of which >95% was β-lactoglobulin.

The storage proteins (SP) and non-storage proteins (NSP) of cottonseed are soluble at pH 4.0 and pH 7.0, respectively; each group of protein is over 90% soluble when the other is least soluble (Uzzan, 1988). Therefore, cottonseed protein isolates consisting of each protein fraction can be prepared by taking advantage of the marked difference in solubility properties. The defatted cottonseed flour is first extracted with water and the extract acidified to pH 4.0 to precipitate the NSP. The residue from the first extraction is then extracted with 0.015 M NaOH and the extract acidified to pH 7.0 to precipitate the SP (Uzzan, 1988). After centrifugation, each precipitate is then freeze-dried. An alternative method is to extract the defatted cottonseed meal with 0.034 M NaOH, centrifuge and acidify the supernatant to pH 7.0, which precipitates the SP, after centrifugation to remove the SP; the supernatant is further acidified to pH 4.0, which precipitates the NSP (Uzzan, 1988).

Phosvitin was isolated from hen's egg yolk using the differences in the solubility properties of yolk proteins. The egg yolk is diluted with water and incubated for 6 hr at 4ºC followed by centrifugation, which partitions the water soluble proteins in the supernatant while the phosvitin is partitioned in the granules that constitute most of the precipitate (Losso and Nakai, 1994). The precipitate is washed with hexane to remove lipids and the residue extracted with 1.7 M NaCl (pH 7.0), which solubilizes most of the phosvitin in the granule but not the lipoproteins. The saline extract is then dialyzed against water to remove salts before the phosvitin extract is freeze-dried (Losso and Nakai, 1994).

14.3 Extraction and fractionation methods

During extraction the objective is to prepare a clarified sample through removal of particulate matter or other contaminants that could interfere with subsequent fractionation and purification steps. This is particularly important during purification of proteins from plant and animal materials where the target protein may be present within a large number of other unwanted biological materials. For example, proteases may need to be inactivated or removed quickly from the sample in order to prevent degradation of the molecular structure and conformation of the target protein. During extraction, protease inhibitors can be added to prevent degradation of proteins or reducing agents (mercaptoethanol and dithiothreitol) added to prevent sulfhydryl-induced polymerization of the proteins. Metal chelators such as ethylene diamine tetracetate (EDTA) may also be added to prevent oxidation of proteins by metal ions. Release and/or solubilization of proteins from food sources is performed mostly using mechanical disruption (homogenization, blenders, and ultrasound). Once extracted, the protein solution must be kept cold (4ºC) to prevent denaturation (if native structure is required for protein functionality).

14.3.1 Water

Water-soluble proteins like albumins can be isolated by aqueous extraction of the sample. The procedure involves mixing the sample with distilled water for a period of time (30 min.–2hr), preferably at 4ºC to prevent proteolysis (Sathe and Salunkhe, 1981; Neves and Lourenco, 1995; Sathe *et al*., 2002). After centrifugation to remove insoluble materials, the supernatant is dialyzed against distilled water to eliminate residual salt and precipitate contaminating salt-soluble proteins (globulins). This is because during extraction with water, salts that are present in the sample will enhance solubilization of some of the globulin molecules. A low molecular weight cut-off membrane (<8000) should be used for dialysis to prevent loss of proteins of interest. Upon completion of dialysis, the contents of the dialysis bag is centrifuged (or filtered) and the supernatant (or filtrate) is freeze-dried as the albumin isolate (Sathe *et al*., 2002).

Sunflower protein isolate was prepared by extraction of the defatted dephenolized meal with water, though pH was maintained constant at 9.0 by addition of 1 M NaOH (Gonzalez-Perez *et al*., 2002). The soluble proteins were recovered in the supernatant after centrifugation and subjected to diafiltration using a 10 000 molecular weight cut-off cartridge. The retentate was freeze-dried as the sunflower protein isolate.

Protein concentrates may be produced by extraction of the sample with acidified water (pH adjusted to the isoelectric point of the proteins), which removes soluble materials such as sugars, some minor proteins as well as odour and flavour substances (Uzzan, 1988). At the pH of extraction, most of the proteins along with polysaccharides and some minerals are insoluble and remain

in the residue. The extracted residue is neutralized with an alkali solution and freeze-dried as the protein concentrate (minimum 65% protein content).

14.3.2 Aqueous alcohol

Seed protein concentrates can also be prepared by extraction of the flour with 50–60% alcoholic solution (usually ethanol) followed by centrifugation to separate the soluble matter (mostly sugars, and odour and flavour substances) from the insoluble residue (Uzzan, 1988). The extracted residue is desolventized at temperatures not exceeding 50–55ºC in an inert gas environment or under vacuum to remove alcohol and water as well as maintain protein quality (Uzzan, 1988). After desolventization, the residue is dried as the protein concentrate (minimum 65% protein content). This process produces a bland and creamy protein product that is suitable for incorporation into food products. However, the protein concentrate produced by alcoholic washing has less biochemical quality and reduced nitrogen solubility index when compared to the concentrate produced by acid water washing (Uzzan, 1988). This is due to the fact that the alcohol solution produces some degree of denaturation of the native protein during the washing process.

Aqueous alcoholic solutions can be used as precipitating agents in the production of protein isolates. Potato protein isolate (PPI) was prepared by adjusting the ethanol concentration of potato fruit juice to 20% (v/v) with 95% (v/v) ethanol at 4ºC and adjusting the pH to 5.0. The precipitated proteins were recovered by centrifugation, washed with ammonium acetate that contained 20% (v/v) ethanol, dispersed in water, adjusted to pH 7.0 with NaOH and freeze-dried as the PPI (van Koningsveld *et al*., 2002).

Corn protein consists of high levels of prolamins called zein, which are soluble only in aqueous alcoholic solvents. Therefore, preparation of zein isolates involves extraction of corn meal with aqueous ethanol solution followed by dilution of the extract to precipitate the proteins. A typical process involves pre-treatment of corn meal with water that contains lactic acid and sodium metabisulfite in order to prevent formation of zein aggregates (Parris *et al*., 2002). After filtration, the residue meal is extracted with 70% ethanol, the extract is diluted to 40% ethanol with water, centrifuged and the precipitate freeze-dried as the zein isolate (Parris *et al*., 2002).

14.3.3 Buffers

All proteins have an isoelectric point (pI), a pH at which they carry no net charge. Proteins are least soluble at their pI because there are no electrostatic repulsions between them. Since the pI of proteins differ, initial fractionation can utilize a buffer at pH value that solubilizes mostly the protein of interest while unwanted proteins are left in the residue. After centrifugation, the supernatant can be used as a concentrated source for isolation and purification of the protein of interest. For example, cowpea globulins were extracted by mixing ground

flour with 0.25 M Tris-HCl buffer, pH 8.0 containing 0.5 M NaCl (Aluko and Yada, 1995). After centrifugation, the supernatant was dialyzed against water to remove salts and precipitate the salt-soluble proteins (globulins), which were removed and freeze-dried as the globulin isolate. A similar method (phosphate buffer, pH 7.6 containing 0.4 M NaCl) was used to isolate mungbean globulins (Mendoza *et al*., 2001). Chickpea albumins were isolated by extraction of the flour with 0.1 M borate buffer, pH 8.3 followed by centrifugation and the supernatant dialyzed against citrate buffer, pH 4.6 (Vioque *et al*., 1999). After centrifugation of the dialyzed extract, the supernatant, which consisted of albumin molecules, was then freeze-dried.

14.3.4 Salts

Ammonium sulfate precipitation

Proteins can be fractionated based on their solubilities in varying concentrations of ammonium sulfate. This is because solubility of a protein is sensitive to the concentration of salts present in the medium. At low ionic strength, protein solubility increases with the salt concentration and is known as 'salting in'. However, at high ionic strength, protein solubility decreases (salting out). Therefore, ammonium sulfate concentration in a solution containing several proteins can be adjusted to just below the precipitation point of the protein of interest. This will precipitate most of the unwanted proteins while the protein of interest will remain in solution. Conversely, the salt concentration can be adjusted to just over the precipitation point of the protein of interest, while most of the unwanted proteins remain in solution. The ammonium sulfate can be removed from the protein by dialysis, buffer exchange on Sephadex G25, or during hydrophobic interaction chromatography. The major globulin fraction in cowpea was partially purified by adjusting an aqueous extract (pH 8.0, 0.4 M NaCl) of the flour to 80% ammonium sulfate saturation, which precipitated the albumins (Aluko *et al*., 1997). After centrifugation that removed the albumins as a precipitate, the supernatant was further brought to 100% ammonium sulfate saturation to precipitate the globulins. Ammonium sulfate was removed from the isolated globulins by dialysis against water and the salt-free globulins fractionated by size exclusion chromatography (Aluko *et al*., 1997). A globulin crude extract from defatted mungbean flour was fractionated into 11S and 8S globulins by addition of saturated ammonium sulfate solution to 60% saturation (Mendoza *et al*., 2001). The supernatant contained only 8S globulin while the precipitate contained 11S and some 8S globulins (Mendoza *et al*., 2001). The ammonium sulfate fractionation enhanced purification of the 8S globulin by ion exchange chromatography.

Salt-soluble proteins such as globulins can be extracted using dilute solutions of NaCl, followed by centrifugation and dialysis of the supernatant (extract) against water. Dialysis (at 4ºC to prevent proteolysis) is required to separate the globulins from the albumins, which are also extracted by the salt solution. During dialysis, the NaCl is removed and results in the precipitation of

globulins, while the water soluble albumins remain in solution. The globulins from cowpea were extracted using 0.5 M NaCl solution at 22ºC for 135 minutes. The extract was centrifuged at 20 000*g* for 20 min and the supernatant dialyzed against distilled water at 4ºC for 24 hr to precipitate the salt-soluble proteins (Sefa-Dedeh and Stanley, 1979). The precipitate can be recovered by centrifugation, freeze-dried and used as raw material for further purification. Mendoza *et al*. (2001) showed the potential for purification of mungbean globulins on the basis of their solubility in NaCl solutions. For example, basic globulin proteins can be extracted by 0.15 M NaCl and 11S globulin by 0.35 M NaCl (Mendoza *et al*., 2001).

A protein product called micelle isolate was prepared from canola meal by extraction with 0.1 M NaCl, centrifugation and dilution of the supernatant with four volumes of cold water to precipitate the extracted proteins (Murray *et al*., 1985). The proteins were recovered by centrifugation and freeze-dried as the micelle isolate. This protocol uses mild processing conditions and produced a less denatured canola protein isolate, when compared with canola protein isolate prepared by isoelectric precipitation. The micellization technique was also used to prepare fababean protein isolate that contained less than 5% of the antinutritional factors (hemagglutinin, phenols, phytate, vicine, convicine) present in the air classified protein concentrate (Arntfield *et al*., 1985).

Isoelectric precipitation

Most food proteins are insoluble at their pI and this is often used to differentially precipitate proteins of interest from an extract containing a mixture of proteins. For liquid foods like milk, the major proteins (caseins) are isolated by acidification followed by neutralization of the precipitate to obtain caseinate (Lucey *et al*., 2000; Bazinet *et al*., 2000). Solid samples such as defatted seed meals or freeze-dried animal products are first mixed with an aqueous solution (water, buffer, dilute sodium hydroxide, polyphosphate, or salt solution) to extract the protein of interest; this process also extracts contaminating proteins. After clarification by centrifugation or filtration, pH of the extract is adjusted to the pI of protein of interest (usually by acidification), which results in precipitation of the proteins.

The precipitate contains mostly the protein of interest and is collected by centrifugation (Thompson *et al*., 1982; Sefa-Dedeh and Stanley, 1979; Dev and Quensel, 1988; Aluko and Yada, 1997; Bazinet *et al.*, 1997; Qi *et al*., 1997; Kumagai *et al*., 2002). Acidification can also be effected by dialyzing the extract against a suitable buffer, e.g., chickpea globulins were isolated by dialyzing a buffered extract against citrate buffer at pH 4.6 (Clemente *et al*., 2000). The protein isolate may be neutralized to pH 6.9–7.2 with dilute alkali (usually NaOH) to give a more soluble product called ‘proteinate’. The supernatant that remains after precipitated protein isolate has been removed contains soluble proteins that can be recovered by membrane processing. The supernatant is processed by ultrafiltration and diafiltration to remove low molecular weight substances, especially salts and peptides to produce a retentate, which is freeze-

dried as the soluble protein isolate (Tzeng *et al*., 1990; Xu *et al*., 2003). The membrane process increased recovery of proteins from the raw materials (Tzeng *et al*., 1990; Xu and Diosady, 1994a) and the soluble protein isolate possessed better solubility and foaming properties than the precipitated protein isolates (Xu and Diosady, 1994b).

If a protein concentrate is the desired product (instead of a protein isolate), the precipitation step is omitted. Instead, the aqueous extract is exhaustively dialyzed against water to remove salts and other low molecular weight compounds before it is freeze-dried (Sathe *et al*., 1982). A combination of acid and calcium chloride or magnesium sulfate has also been used to precipitate seed proteins; the aqueous protein extract is adjusted with acid to pH above the pI followed by addition of calcium chloride or magnesium sulfate (Soetrisno and Holmes, 1992). The yield of protein isolate was better with acid (only) precipitation than with the combination of acid and salt (Soetrisno and Holmes, 1992).

Fractionation of the 7S and 11S of soybean can be achieved by taking advantage of the differences in their pI. Defatted soybean meal is extracted with Tris-HCl buffer, pH 8.0 and the extract adjusted to pH 6.4 with dilute HCl solution followed by centrifugation; the 11S globulin is collected in the precipitate while the 7S globulin remains in the supernatant (Thanh and Shibasaki, 1976). The supernatant is adjusted to pH 4.8 to precipitate the 7S globulin, which is then recovered by centrifugation.

Edible proteins can be recovered from animal tissues such as lung and stomach by alkaline extraction at pH 10–11 followed by precipitation at pH 4.5. Protein extraction can also be carried out using sodium dodecyl sulfate (SDS) and the protein-SDS complex is precipitated with 0.005–0.05 M ferric chloride (Ellison *et al*., 1980). The precipitate is then washed with 40% methanol or 60% acetone in the presence of 10% potassium chloride to remove the SDS and produce a protein isolate. The protein isolate produced by SDS extraction had higher levels of residual iron but possessed better functional and nutritional attributes when compared to the protein isolate produced by alkali extraction (Ellison *et al*., 1980).

14.4 Purification techniques

Initial steps in the purification process involves extraction of the material with a suitable solvent followed by crude fractionation through selective precipitation to obtain an enriched protein fraction. The protein fraction is further processed by one or more chromatographic or electrophoresis techniques until the protein of interest is obtained in a homogeneous preparation, i.e., purified (Fig. 14.1). Most purification techniques involve chromatography, a group of protocols that are used to separate solution mixtures of molecules as they pass through a porous matrix. The solvent that dissolves the proteins is called the mobile phase while the matrix is the immobile phase. The rate of movement of proteins

through the column depends on degree of interaction with the matrix; the greater the interaction with the matrix the longer it takes for the protein to move through the column. As the solvent emerges from the end of the column, it is collected into fractions which separates the proteins according to their residence time on the column. Collection of fractions in about 1 ml aliquots ensures that the bulk of the protein of interest will land in two or three tubes, which reduces contamination from other proteins. Proteins with least interaction with the matrix will emerge out of the column first followed by proteins that interacted more with the matrix.

In order to prevent clogging and ensure maximum performance of the matrix it is important to clarify the sample before it is applied onto the column. Clarification can be achieved by centrifugation (10,000–50,000 $\times$ g for 15–30 min. depending on sample), ultrafiltration (1,000–30,000 molecular weight cut-off membranes), and membrane filtration (0.22 μm filter is recommended for sterile filtration or extra clean samples). During membrane filtration it is important to check recovery of the target protein using a test run because some proteins adsorb non-specifically to filter surfaces. Low protein binding membrane filters are available to reduce the risk of losing large of amounts of target protein during clarification.

In general, the best way to start a purification process is to use high capacity/low resolution methods that can be carried out fast and with ease, followed by high resolution/low capacity methods. Capacity is the total amount of proteins that can be accommodated on the column while resolution is the ability of the column to separate the protein of interest from contaminants. As the resolution of the purification method increases, the time required to complete the protocol increases and the protein capacity decreases. Exceptions to this rule include gel filtration, which is not a high resolution technique but requires longer periods to complete and has low capacity. The type of ligand bound to the column determines the capacity and resolution of affinity chromatography; some ligands provide high resolution as well as high capacity. High performance liquid chromatography (HPLC) can substantially increase resolution of the chromatographic technique, but capacity is much lower than other techniques such that it is used mostly in the later stages of purification. This can be illustrated as follows. Capacity: Differential solubility > Ion exchange > adsorption > hydrophobic interaction > electrophoresis > HPLC. The order for resolution, time and effort is the reverse of the order for capacity. Table 14.1 shows a list of the basic protein purification techniques and the molecular properties that are involved.

Dilute protein solutions are usually obtained from various chromatographic techniques, which often necessitates concentration of the sample between chromatographic steps or after purification is completed. Water can be removed by lyophilization (freeze-drying), which may increase the salt concentration. The protein solution can be enclosed in a dialysis bag and covered with solid high molecular weight polyethylene glycol, which causes water and buffer components to diffuse out of the bag. Ultrafiltration can also be used to

Table 14.1 Purification techniques and related molecular properties of proteins

Technique	Molecular property of protein
Gel filtration (size exclusion chromatography) SEC	Size (effective radius)
Ion exchange IEC	Charge
Hydrophobic interaction (HIC) and reverse-phase (RPC)	Hydrophobicity
Affinity (AC)	Ligand recognition
Chromato-focusing	Isoelectric point
Foam fractionation	Surface activity

concentrate the protein solution by forcing it through a membrane with defined molecular weight cut-off such that the protein molecules are retained whereas water and buffer components are expelled. Ultrafiltration can be performed using a special apparatus such as the Amicon stirred cell set-up or by centrifugation using a disposable filter cartridge that fits into a centrifuge tube. The protein solution is placed into the cartridge and centrifuged until the protein solution, which remains above the filter, is at the desired volume.

After the protein has been purified, qualitative analysis should be performed by gel electrophoresis to confirm purity. A pure protein should exhibit a single band when analyzed by non-denaturing (native) polyacrylamide gel electrophoresis (PAGE). Determination of molecular weight of the polypeptide chains (subunit size) of a protein uses denaturing or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, the protein is denatured by heating it in a detergent (SDS) and a thiol reductant (e.g., 2–mercaptoethanol and dithiothreitol), which breaks the disulfide bonds. Since most proteins bind 1.4 g SDS/g protein with very little variation, all the subunits have a uniform negative charge, which ensures that the polypeptides separate only according to size during electrophoresis. The high molecular weight subunits do not migrate far and will stay near the top of the gel while smaller subunits move down the gel. Polypeptides of known molecular weights are run on the same gel and used to plot a calibration curve, which can be used to determine the size of the unknown proteins.

14.4.1 Basic rules in protein purification

The following rules have been suggested as important components of a successful processing design aimed at purification of a protein of interest from a multi-protein solution (Asenjo and Patrick, 1990; Prokopakis and Asenjo, 1990; Lienqueo *et al*., 1999).

1. Choose the separation based on the different physicochemical properties, such as surface charge (titration curve), surface hydrophobicity, molecular weight and isoelectric point values. Biochemical properties such as

biospecificity with different ligands, stability at different temperatures and pH values are also important considerations.
2. Eliminate those proteins and compounds that are found in greater percentage first.
3. Use a high resolution step as soon as possible. The following chromatographic techniques are ranked according to their decreasing efficiency: affinity, ion exchange, hydrophobic interaction, gel filtration.
4. Do the most arduous purification step at the end of the process (final polishing).

14.4.2 Size exclusion chromatography (SEC)

This procedure is also called gel filtration and involves separation of proteins according to their molecular weights, i.e., physical interaction with the column matrix. Since there is no chemical interaction with the matrix, SEC can be considered as a very 'gentle' method of protein purification (Amersham Pharmacia, 1999). The column is packed with a solid matrix (beads) that contain pores of various sizes around a mean pore size. The size range of proteins that can be separated is determined by the distribution and mean pore size of the beads. The beads are made up of insoluble but highly hydrated polymers such as dextran or agarose or polyacrylamide. Sephadex, Sepharose, and Bio-gel are commonly used commercial preparations of these beads, which are typically 0.1 mm in diameter. During migration of the proteins through the column the probability of entering the pores of the matrix is inversely proportional to the molecular weight (or size) of the protein. As a result, smaller proteins will enter the pores of the beads, which impedes the rate of flow and increases the elution time. In contrast, large proteins with sizes bigger than the pores sizes are excluded from entering the beads and are eluted faster from the bottom of the column (Fig. 14.2).

Protein molecules that are completely excluded from the beads will elute in the void volume, which is approximately equal to one-third of the column volume. Protein molecules that are completely included within the pore sizes of the beads will elute in the included volume, which is approximately equal to two-thirds of the column volume. Protein molecules with molecular weights between the extremes will elute between the excluded and included volumes. The elution volume of a protein is inversely proportional to the log of the molecular weight. Therefore, SEC can be used to determine the molecular weight of a particular protein if appropriate standards are available (Clemente *et al*., 2000; Mendoza *et al*., 2001; Ooi *et al*., 2002). The molecular weight of the protein of interest should fall within the molecular weight range of the standard proteins. Samples are eluted using isocratic conditions, i.e., single buffer, no gradient (Amersham Pharmacia, 1999). In order to obtain the highest possible resolution the sample volume must not exceed 5% of the total column volume. Therefore, SEC is best used at the end stages of protein purification when the sample can be readily concentrated into a small volume. The longer the column

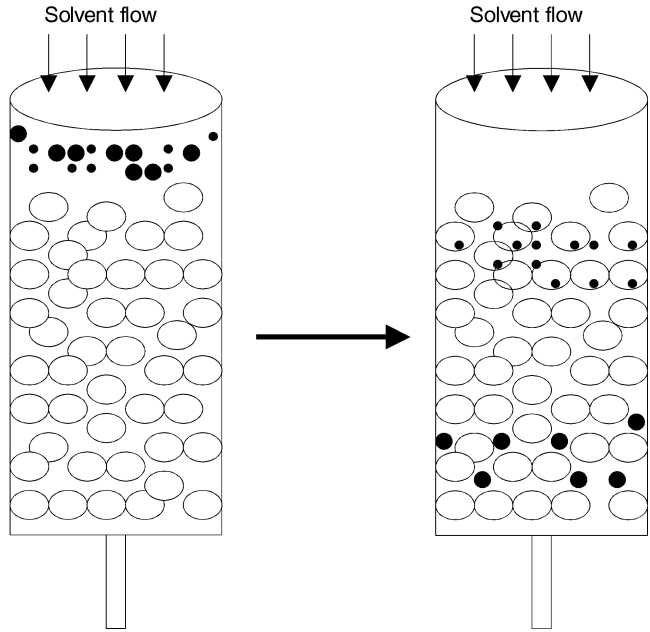

Fig. 14.2 Gel filtration chromatography. The gel beads (O) have pores of defined size range that allows smaller molecules (•) to enter and move slowly through the column. Larger molecules (●) are excluded from the beads and move rapidly through the column.

length the higher the resolution obtained; however, if protein is unstable the higher running time on a longer column can increase degradation or denaturation of the protein.

If native structure of protein is required for food functionality, separation should be carried out at cold temperatures; this also helps to reduce diffusion of the sample during the run, which improves resolution. The technique is independent of sample concentration, though at 50 mg/ml protein viscosity may cause 'fingering'; therefore, viscous samples should be diluted before loading onto the column (Amersham Pharmacia, 1999). Examples of protein purification by SEC include cowpea (Aluko *et al*., 1997), chickpea albumins (Clemente *et al*., 2000), and mungbean globulins (Mendoza *et al*., 2001).

14.4.3 Ion exchange chromatography (IEC)

In this procedure, proteins are separated based on differences in charge. The column is packed with beads that contain bound groups with positive (anion exchanger) or negative charges (cation exchanger). Strong ion exchangers are fully charged over a broad pH range, while weak ion exchangers cover a narrow pH range. Unlike SEC columns, ion-exchange columns are short and fat in dimensions. A list of the most commonly used ion exchange resins is shown in Table 14.2. The material to be fractionated is dissolved and moved

Table 14.2 Chemical properties of some ion exchange resins

Type and functional group	Name	Abbreviation
Strong cation		
$-SO_3-$	Sulpho	S-
$-CH_2SO_3-$	Sulphomethyl	SM-
$-CH_2-CH_2-CH_2-SO_3-$	Sulphopropyl	SP-
Strong anion		
$-CH_2N^+(CH_3)_3$	Trimethylaminoethyl	Q-, TAM-
$-C_2H_4N^+(C_2H_5)_3$	Triethylaminoethyl	TEAE-
$- C_2H_4N^+(C_2H_5)_2CH_2CH(OH)CH_3$	Diethyl-2-hydroxypropylaminoethyl	QAE-
Weak cation		
-COO-	Carboxy	C-
$-CH_2COO-$	Carboxymethyl	CM-
Weak anion		
$-C_2H_4N^+H_3$	Aminoethyl	AE-
$-C_2H_4NH(C_2H_5)_2$	Diethylaminoethyl	DEAE-

through the column in a low ionic strength solvent (buffer) whose pH promotes binding of some of the target protein to the beads (Fig. 14.3). The net charge of a protein is dependent on the type of amino acids present in the molecule. In turn, charges on the amino acids are dependent on the pH of the medium. Although the protein is neutral at the isoelectric point, charged regions within the protein can allow it to bind to the beads via non-covalent ionic (salt-bridge) interactions. Proteins have net negative charges at pH values above the isoelectric point and will bind to an anion exchanger. In contrast proteins have net positive charges at pH values below the isoelectric point and will bind to a cation exchanger. When the buffer pH is slightly below the pI, the protein will have a weak overall positive charge and will only bind to a strong cation exchange resin. When the buffer pH is significantly below pI, the protein will have a moderately strong positive charge and will bind to both a weak or strong cation exchange resin (use a weak cation exchange resin). Similarly, proteins with overall moderately strong negative charges (buffer pH is significantly above pI) will bind both weak and strong anion exchanger (use a weak anion exchanger resin) whereas a strong anion exchanger resin will be used for proteins with weak overall negative charges (buffer pH is slightly above pI). Knowledge of the pI is, therefore, helpful in the design of a purification protocol that uses ion-exchange resins. However, if the pI is unknown, different ion-exchange resins can be tried to determine the most suitable for the desired level of resolution. A strong ion exchanger is recommended for purification of proteins that have not been fully characterized, so that method development can be performed over a wide range of pH (Amersham Pharmacia, 1999).

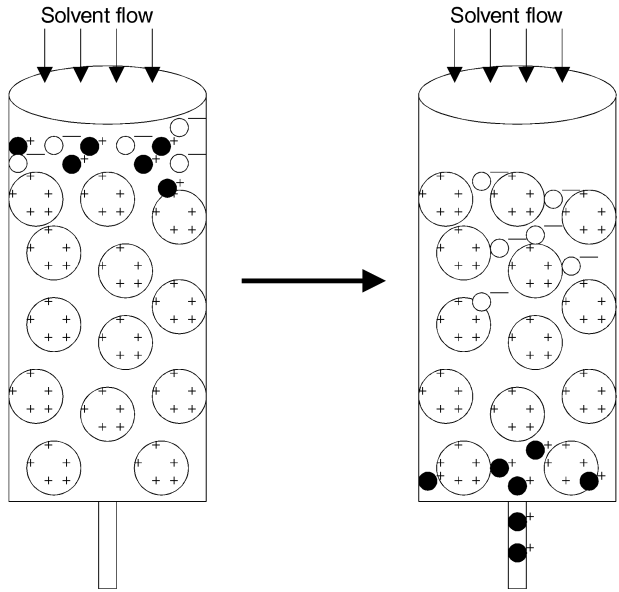

Fig. 14.3 Ion exchange chromatography. The column is packed with an anion exchanger, i.e., positively charged groups are attached to the surface of the gel beads. Negatively charged protein molecules (O^-) bind to the gel while positively charged protein molecules ($\bullet^+$) are free and wash off the column. The bound protein molecules can then be eluted by increases in ionic strength or decrease in the pH of the buffer.

Unbound proteins are quickly eluted during sample loading and washing while the bound proteins can be sequentially displaced and eluted later by increasing the ionic strength and/or pH of the buffer. Proteins that are tightly bound to the charged beads will be displaced at high ionic strengths while low ionic strengths will displace proteins that are weakly bound. Elution of bound proteins is generally carried out using two types of methods: gradient and step-wise. Gradient elution refers to a smooth transition of salt concentration (from low to high) in the elution buffer (Fig. 14.4a).

Most automated protein purification systems contain a gradient maker that produces a linear salt or pH gradient (over the total volume of the gradient) from two containers, one contains the low salt (or low pH) buffer and the other contains the high salt (or high pH) buffer. Stand-alone gradient makers may also be used to produce a linear gradient that is passed through the column by a peristaltic pump. Step-wise method involves sequential buffer elution with discrete concentrations of salt (Fig. 14.4b); the protein of interest will elute over a volume range that contains a fixed amount of salt. Step-wise elution is useful if the concentration range of salt over which a protein of interest will elute is known and when contaminants elute at a significantly different salt concentration. The step-wise elution method is faster to run and the protein is eluted in a smaller overall volume when compared to gradient elutions.

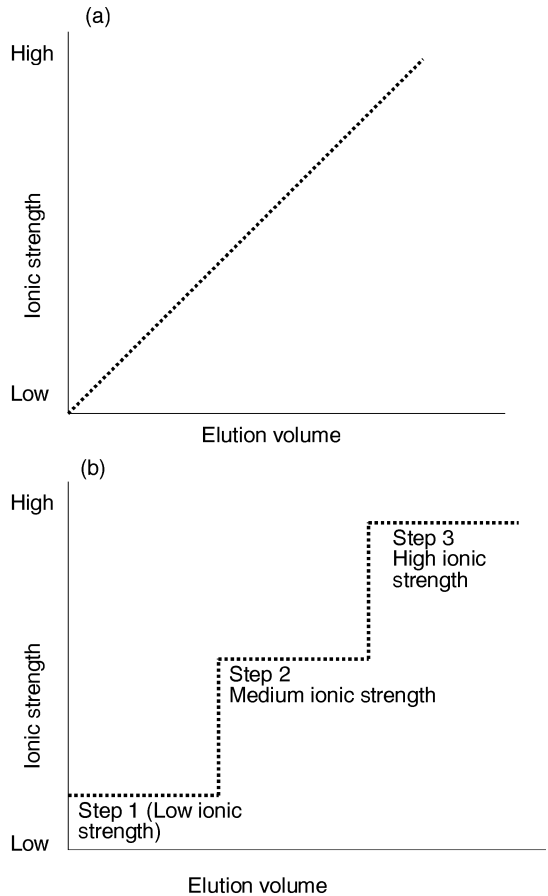

Fig. 14.4 Linear(a) and step-wise (b) elution methods used in ion-exchange chromatography. The number of steps could be more than the three shown in (b).

IEC permits high sample loading and provides high resolution separation of a wide variety of proteins (Amersham Pharmacia, 1999). Separation of proteins by IEC is independent of sample volume because the process involves binding to the solid matrix. However, the ionic strength during sample application must be low and the target protein must be highly charged to facilitate binding to the oppositely charged matrix. Each column has a total binding capacity (TBC), which must not be exceeded during sample loading while gradient elution should not exceed one fifth of the TBC in order to obtain optimal separation of the protein fractions (Amersham Pharmacia, 1999). Because of its high capacity, ion exchange can be used as an initial step in the purification of proteins.

After the IEC step, the protein of interest may be in a high salt buffer, which may be undesirable. Dialysis using a semi-permeable membrane can be used to get rid of salt in the protein; the protein sample is placed in a dialysis tubing and put into water or the desired low salt buffer. The membrane is permeable to the

small salt molecules, which diffuse out into the surrounding water or low salt buffer, while the protein is retained in the dialysis bag. It is essential to use a membrane that will not interact with the protein of interest. At equilibrium most of the salt in the protein will have moved out into the surrounding liquid. However, an increase in the volume of sample as a result of water molecules moving into the dialysis bag may lead to a decrease in the protein concentration. To avoid rupture of the bag due to increased volume during dialysis, it is recommended not to fill the bag completely, but leave a void to allow for potential swelling. The protein sample can also be desalted using the gel filtration method (SEC); the small salt molecules enter the pores of the gel and are retained while proteins are excluded from the pores and elute in the void volume. Desalting by SEC is faster than the dialysis method.

14.4.4 Hydrophobic interaction chromatography (HIC)

HIC involves the use of columns that are packed with beads linked to hydrophobic side chains that will bind proteins containing exposed hydrophobic regions; therefore, proteins are separated based on differences in hydrophobicity. Proteins with net charges or exposed hydrophilic regions will not bind to the beads and are eluted first from the column. In contrast, proteins with exposed hydrophobic surfaces will interact with the hydrophobic surface of the column matrix; this interaction is enhanced by high ionic strength buffers. First, the sample is dissolved in a high ionic strength buffer and loaded onto the column, which allows target proteins be concentrated as they bind to the matrix. Salt ions that fall towards the lyotropic end of the Hofmeister series (salting out ions) are used to increase binding to and retention of proteins on the HIC column, whereas 'salting in' ions (chaotropic) decrease affinity of the protein to the column (Shukla *et al*., 2002). The column is then washed with the sample buffer to elute unadsorbed protein contaminants.

In order to reduce the number of chromatographic steps in the downstream purification process and increased eluate purity, chaotropic agents may be added to the wash buffer prior to elution of the protein of interest. For example, a wash buffer containing sodium thiocyanate (0.05 M), glycerol (5%, v/v), and urea (1.5 M) was shown to substantially increase the yield and purity of a recombinant protein from *Escherichia coli* (Shukla *et al*., 2002). Second, the bound proteins are desorbed and eluted in a purified and concentrated form from the column by using stepwise or continuous decreases in salt concentration, e.g., a decreasing gradient of ammonium sulfate. Thus, HIC may also be used to desalt protein samples. For example, the high salt protein sample obtained from IEC can be loaded directly onto a hydrophobic column and eluted with low salt buffer. Elution of the adsorbed protein can also be carried out using reduced eluent polarity (up to 50% ethylene glycol gradient), addition of chaotropic agents (urea or guanidine hydrochloride), detergents, and changes in temperature and pH.

Tight binding of highly hydrophobic proteins to similarly hydrophobic ligands in a column may require the use of harsh conditions such as chaotropic

salts and detergents to desorb and elute the proteins from the column. Such conditions may severely alter the structural conformations of food proteins accompanied by poor functional performance in food systems. Moreover, the use of detergents will require further treatments aimed at producing a detergent-free protein that is suitable for incorporation into foods. Therefore, when working with proteins of unknown charge characteristics it is recommended to screen several hydrophobic media starting with the low hydrophobic types. The medium that gives the best resolution and loading capacity at low salt concentration is recommended for purifying the food protein. Typically, the binding strength of ligands increases in the following order: ether, isopropyl, butyl, octyl, phenyl (Amersham Pharmacia, 1999). Similar to IEC, HIC is based on binding of proteins to the ligands attached to the matrix and therefore, mostly independent of sample volume. Also the total amount of protein loaded and bound to the column should not exceed the TBC of the column (Amersham Pharmacia, 1999). Gradient elution should use approximately one fifth of the TBC of the column to obtain optimal separations.

14.4.5 Reverse-phase chromatography (RPC)

This technique is based on reversible interaction of proteins with the hydrophobic surface of a chromatographic medium. The proteins are separated according to differences in their hydrophobicity, which determines the strength of the interaction with the medium. Proteins become bound to the medium as they are loaded onto the column. The conditions are then altered so that the bound proteins are eluted differentially (Amersham Pharmacia, 1999). The strong hydrophobic nature of the reversed phase medium causes a very strong interaction with proteins and usually requires the use of organic solvents for elution. Increasing the acetonitrile concentration is the most common method used for elution in RPC. RPC is ideal for analytical separation of peptides but is not recommended for protein purification if return to native structure is required for optimal functionality of food systems. This is because many proteins are denatured in the presence of organic solvents. Available hydrophobic ligands, in increasing hydrophobicity include C4, C8 or C18 n-alkyl hydrocarbon ligands. Therefore, highly hydrophobic proteins will bind tightly to C18, for example, and will require high concentrations of organic solvents to elute from the column. Such a drastic elution condition could damage protein structure resulting in poor functional properties. In contrast, low hydrophobic column media should be used for purification when the native protein structure is important for functional performance in food systems. Since RPC is a binding technique, it is often independent of sample volume.

14.4.6 Affinity chromatography

In this procedure, specific ligands are covalently attached to beads of the resins. The protein of interest is separated according to its ability to bind non-covalently

to the ligand, while unwanted proteins are washed out through the column. Ligands can be small molecules (ATP, dye, amino acids, cofactor, and ions), nucleic acids, sugars, steroids, fatty acids, proteins, or antibodies. Binding of the proteins produces a concentrated and purified form of the protein. The bound protein is eluted from the column by addition of a solution that contains free ligands or by changing the ionic strength, pH, or polarity of the solvent. Protein recovery can be difficult if affinity to the column ligand is very high. The technique is independent of sample volume subject to conditions that favour strong interactions between the protein and matrix ligand (Amersham Pharmacia, 1999). All commercially available affinity media have defined total binding capacity.

In order to ensure strong interactions between proteins and matrix ligands, it is important to remove particulate matter and other contaminants (e.g., lipids) that may bind non-specifically to the matrix. The use of solvents that may damage the ligand of an affinity column should be avoided. Affinity chromatography has good to excellent resolution and scales very well, but it needs to be tailored for each protein of interest. Figure 14.5 shows the chromatogram demonstrating purification

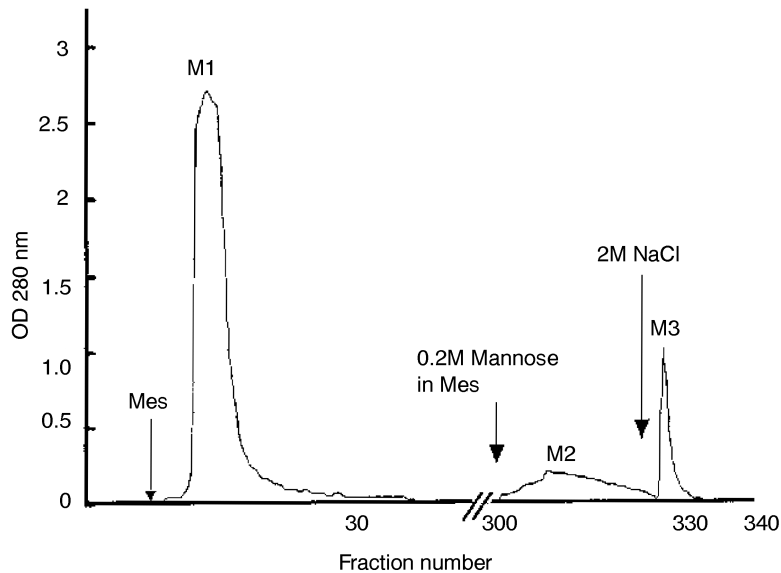

Fig. 14.5 Affinity chromatography of Allium tuberosum (JH cultivar) extract on a mannose-agarose column (1.5 × 8 cm) that has been equilibrated and was eluted with Mes buffer (20 mM, pH 6). After unadsorbed materials (M1) had been eluted completely with the buffer (until there was no more absorbance at 280 nm), the adsorbed materials (M2 and M3) were eluted with 0.2 M mannose in Mes buffer and 2 M NaCl, respectively. Because there was only a minute amount of M2 and M3, the crude powder was repeatedly loaded on the column and eluted 8 times (designated as // to indicate the pool of 8 eluants) in order to obtain a substantial amount of M2 and M3 fractions for further purification. The arrows indicate the point at which the buffers were changed. (reproduced from Ooi *et al*., 2002 with permission of American Chemical Society).

of a mannose-binding protein (lectin) from Chinese chive (an edible vegetable that is closely related to garlic and onion) using a mannose-agarose column (Ooi *et al*., 2002). The first step involved loading the column (equilibrated with same buffer used to dissolve the sample) with the sample solution. In the second step, the column was eluted with the sample buffer and unadsorbed proteins were removed from the column (peak M1). In the third step, proteins that were adsorbed onto the column were eluted consecutively with buffers containing 0.2 M mannose and 0.2 M NaCl to desorb, respectively, the weakly (peak M2) and strongly (peak M3) adsorbed proteins (Ooi *et al*., 2002).

14.4.7 Membrane chromatography (MC)

This method is similar to the conventional chromatography methods described above because it is based on adsorption of protein molecules to a solid support. However, instead of gel beads, MC uses thin microporous membranes as a support for chromatography (Charcosset, 1998); thus pressure drop is not a limitation. The internal pores of the membranes contain adsorptive groups that bind the target protein. The advantages of MC include high velocities and short residence times because the sample flows through a membrane, which provides a very short, wide bed (Charcosset, 1998). Adsorptive separation of proteins is typically one-tenth of the time for packed gel columns due to elimination of diffusion resistance (Briefs and Kula, 1992; McGregor *et al*., 1986; Prpic, 1993).

The binding capacities of adsorptive membranes are comparable to those exhibited by packed columns and proteins of interest can be rapidly concentrated 10-fold or more with 85–100% recovery (McGregor *et al*., 1986; Champluvier and Kula, 1991; Krause *et al*., 1991; Tennikova *et al*., 1991; Lutkemeyer *et al*., 1993; Luska *et al*., 1994; Charcosset *et al*., 1995; Wang *et al*., 1995). Convection through the micrometre-sized pores of the membranes reduces or eliminates mass transfer limitations, which produces an increase in binding capacity and throughput (Yang *et al*., 2002).

In order to produce protein separations that are equivalent to those of column chromatography methods, MC uses stacks of membranes in conjunction with gradient elution (Josic *et al*., 1992; Tennikova and Svec, 1993; Charcosset, 1998). Commercially available membranes include flat sheet systems, membrane stacks, radial flow cartridges and hollow fibre modules; they contain chemically reactive sites that can be covalently linked to ligands or they are supplied with bound ligands (Charcosset, 1998).

Application of MC to food proteins was illustrated by the separation of lysozyme from egg white by dye membrane affinity chromatography (Grasselli *et al*., 1999). The support membrane system consisted of hydrophylised polyethylene hollow-fibre membranes (0.3 μm pore size) to which a ligand of Red HE-3B (dye) was covalently linked. The membrane was equilibrated with 25 mM sodium phosphate buffer, pH 7.0 before sample was loaded. Egg white (100 ml) was loaded onto the membranes in the cross-flow mode followed by

washing with water in three steps: through the lumen side to remove particulate materials, through the shell inlet and through the membrane. Sample elution was carried out using two aliquots (100 ml each) of 25 mM sodium phosphate buffer, pH 8.0 containing 0.6 M NaCl that was pumped through the membrane. Enzyme activity was used to detect lysozyme in the eluates and HPLC showed purity of the lysozyme to be 88% with a 92% recovery (Grasselli *et al*., 1999). Membranes were regenerated by washing with 10 mM and 100 mM NaOH solutions, each containing 1 M NaCl. The authors showed that the highest binding capacity of 26 mg/ml obtained with the hollow fibre affinity membranes was similar to that obtained using commercial gel beads during column chromatography separation of lysozyme (Chase, 1984).

14.4.8 Isoelectric focusing

The method of isoelectric focusing (IEF) involves mixing a protein sample with desired pH range carrier ampholyte, or other carrier buffers, in a focusing cell (Issaq *et al*., 2002). Application of an electric potential to the focusing cell forces the proteins to migrate to a position in the established pH gradient that is equivalent to their respective pI; the end result produces proteins that are focused in narrow bands at their pI values. An advantage of the liquid-phase IEF is that a complex mixture of proteins can be fractionated according to their pI in a nongel medium and the fractions collected and analyzed further (if necessary) by electrophoresis or chromatography (Issaq *et al*., 2002). Disadvantages of IEF include (Issaq *et al*., 2002):

- precipitation of high concentrations of proteins at their pI (because of the net neutral charge), which can cause overlap between fractions.
- The ampholytes that are used to establish the pH gradient may interfere with subsequent analysis such as electrospray ionization-mass spectrometry. To avoid this problem, the protein fractions can be dialyzed or passed through a size exclusion gel chromatography to remove the ampholytes.
- Highly hydrophobic proteins may be lost in sample preparation or during focusing when proteins reach their pI.

Several IEF devices are available for preparative fractionation of complex mixtures of proteins: (a) Rotofor cell apparatus (Kachman *et al*., 2002); (b) multi-component electrolyzer with isoelectric membranes (Herbert and Righetti, 2000); (c) recycling IEF (RIEF) (Bier, 1998); (d) free-flow IEF (FFIEF) (Hannig, 1982). RIEF recycles the sample medium through a cooling chamber, while FIEF involves continuous injection of samples into a carrier ampholyte solution (Issaq *et al*., 2002).

14.4.9 Off-gel electrophoresis

Off-gel electrophoresis is a free-flow protein purification method that is based on isoelectric electrophoresis (Ros *et al*., 2002). However, unlike IEF

purification is achieved without the need for carrier ampholytes. The protein solution is passed under an immobilized pH gradient (IPG) gel through which an electric field is applied perpendicular to the direction of flow (Ros *et al*., 2002). Proteins with pI values that are close to the pH of the gel in contact with the flow chamber have neutral net charge and will stay in solution. However, other proteins that have net charges will be extracted into the gel by the electric field. The method is suitable for proteins with known pI and can separate molecules whose pI differ by as little as 0.1 pH unit (Ros *et al*., 2002).

14.4.10 Foam fractionation

Foam fractionation is part of a group of processes known as adsorptive bubble separation techniques that isolate molecules depending on surface activity (Lockwood *et al*., 2000). The method employs an apparatus that consists of (a) a glass tube where the protein solution is held and (b) a means of introducing an inert gas stream. Introduction of the inert gas causes the proteins to adsorb to the air-water interface of the bubbles (Lockwood *et al*., 2000). The bubbles will rise to the surface of the liquid to form a foam that can be collected to give an enriched form of the protein (foamate).

In order to use this method for protein isolation and purification effectively, the protein of interest must either be the most surface active or the least surface active among the macromolecules present in the mixture. If the protein of interest is the most surface active, then it will be found in the foamate while the less surface active contaminants will remain in the liquid pool (retentate) that is left in the glass column. A potential disadvantage of this mode of operation is that not all food proteins can form a stable foam that will enhance their recovery in the foamate. In contrast if the contaminants are more surface active, they will be collected in the foamate while the protein of interest will be left in the retentate. This mode of operation is suitable for isolation of proteins that have poor foaming properties.

Previous studies have used the technique of collecting the protein of interest in the foamate (Ramani *et al*., 1993; Varley and Ball, 1994; Vazquez and Varley, 1996; Bhattacharjee *et al*., 1997; Poxson and Hughes, 1999). Performance of foam fractionation may be assessed by the separation ratio of proteins in the mixture or by the recovery of proteins of interest (Lockwood *et al*., 2000).

14.5 Future trends

The integrated process for isolation and purification is an integrated approach that combines ultrafiltration membranes with chromatographic resin beads in one device and has been proposed for the isolation and purification of proteins (Dai *et al*., 1999). The technique utilizes commercial hollow fibre ultrafiltration or microfiltration cartridge with appropriate adsorbent beads packed in the shell

(permeate) side. The sample solution is passed through the lumen of the hollow fibre for a given period of time in such a way that the solution pressure on the lumen side is greater than that on the shell side; this ensures that the permeate flows out in the same direction as the feed solution and exits the shell side through an outlet (Dai *et al*., 1999). Proteins that are smaller than the pore size of the membrane will pass into the permeate and become adsorbed on the resin beads packed on the shell side, depending on the pH and ionic strength. It is necessary to use conditions that ensure that any bioproduct in the permeate will become adsorbed to the beads. When flow of sample into the fibre lumen ends, the proteins adsorbed on the shell side beads are eluted with a suitable solvent that is introduced through the lumen inlet on the shell side (Dai *et al*., 1999). Particles larger than the membrane pore size are retained in the lumen and exit the cartridge with the retentate stream.

Magnetic affinity support (MAS) is a novel magnetic support material that consists of Cibacron blue 3GA dye (CB) coupled to magnetic particles (ferric oxide molecules entrapped in polyvinyl alcohol) and has been shown to have better protein binding capacity than other types of magnetic supports (Tong *et al*., 2001). For example the CB-MAS ligand system had a binding capacity for lysozyme of up to 76.5 mg/g, which is three times greater than that of CB-Sepharose at a similar ligand coupling density (Tong *et al*., 2001). Unlike the normal ferromagnetic materials that are used in biochemical separations, the polyvinyl entrapped magnetic material is superparamagnetic and does not cause permanent magnetization that is responsible for particle aggregation (Tong *et al*., 2001).

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15

The use of genetic engineering to modify protein functionality: molecular design of hen egg white lysozyme using genetic engineering

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15.1 Introduction

Since lysozyme is easily obtained in a purified form, it has been studied comprehensively as a model protein for structure, dynamics and folding. The recent development of recombinant techniques has enabled the elucidation of the molecular mechanism of structural and functional properties of lysozyme. In early studies, recombinant lysozyme was investigated in the *E. coli* expression system. However, since the prokaryotic cells have a different secretion system from eukaryotic cells, the correctly folded lysozyme could not be expressed. It seems likely that the folding of a disulfide-rich protein such as lysozyme is difficult in *E. coli* because of the absence of endoplasmic reticulum (ER) in which proteins are posttranslationally folded. Although the enzyme is obtained as an inclusion body, the yield of refolding is at a very low level. The N-terminus of recombinant lysozyme obtained from *E. coli* is methionine, while that of native lysozyme is lysine.

Since the N-terminus lysine is essential for correct folding, substitution with other amino acid results in a significant decrease in the stability of lysozyme. Kumagai *et al*. (1987) found that hen egg white lysozyme was correctly processed and folded in *S. cerevisiae*. The N-terminus of recombinant lysozyme is lysine and the stability is the same as the native lysozyme. Since *S. cerevisiae* is a typical eukaryotic cell with an ER system, lysozyme is correctly processed and folded in the yeast. Thus, the relationship between the functional and structural properties of lysozyme have been investigated at a molecular level using protein engineering.

Although the catalytic sites of lysozyme were confirmed by chemical modifications, these sites were easily reconfirmed by the site-directed mutagenesis of responsible amino acids. The mutation of Glu35 to Asp or Asp52 to Glu in hen (Imoto, 1990) or human (Muraki *et al*., 1991) resulted in inactivation of the enzyme. These observations indicate that the catalytic groups are strictly defined and that the replacement of even one methylene group is not allowed. Taniyama *et al*. (1992) reported the folding mechanism of disulfide bond-deficient human lysozyme C77/95A mutant secreted in *S. cerevisiae*. Although the deficient mutant of other disulfide bonds could not be secreted, the only C77/95A mutant secreted eight-fold greater than wild-type in *S. cerevisiae*. The stability of C77/95A mutant greatly decreased despite the correct folding. These observations show that the disulfide bond Cys77–95 contributes to the stabilization of the folded form of human lysozyme. Thus, the relationship of the structural and functional properties of lysozyme has been clearly elucidated by genetic engineering. However, the molecular design and construction of a protein with a novel function is the ultimate goal of protein engineering. For this purpose, we used lysozyme as a model protein for designing a functionally novel protein. Our strategy was the enhancement of heat stability and bactericidal action. The former was performed with the hyperglycosylation in yeast (*S. cerevisiae*) expression system and the latter was done by the genetic fusion of hydrophobic peptide to C-terminus of lysozyme.

15.2 Lysozyme-polysaccharide conjugates

We have reported (Kato *et al*., 1990; 1991) that Maillard-type lysozyme-polysaccharide conjugates can be efficiently prepared during storage of the freeze-dried powders of lysozyme-polysaccharide mixtures under a controlled dry state. The Maillard reaction between the ϵ-amino groups in protein and the reducing-end carbonyl group in polysaccharide is accelerated at a low water activity. The rate of reaction of the conjugate formation seems to depend on the rigidity of protein conformation. The casein-polysaccharide conjugate is formed only within one day, while it takes long time to form the lysozyme-polysaccharide conjugate. It seems likely that the rigid structure of protein may suppress and the unfolded structure may promote the conjugate formation, because of the exposure of lysyl side chain upon unfolding. The binding mode was investigated using lysozyme-polysaccharide conjugates (Nakamura *et al*., 1991; Shu *et al*., 1996).

The SDS polyacrylamide gel electrophoretic patterns demonstrate a broad single band for protein and carbohydrate stains near the boundary between stacking and separating gels, indicating the formation of the conjugate between lysozyme and polysaccharide. The increase in molecular weight and the decrease in free amino groups of protein-polysaccharide conjugates

suggest that about two moles of polysaccharide are bound to lysozyme. The limited number of bound polysaccharides may come from the steric hindrance of attached polysaccharide. This limitation is suitable for designing the functional properties of proteins, because the functions of proteins deteriorate if most lysyl residues are masked by small saccharides as observed in the conjugates of proteins with monosaccharides and oligosaccharides. Both protein and polysaccharide have a role in the stabilization of oil in water emulsions. Proteins adsorb at the oil-water interface during emulsification to form a coherent viscoelastic layer. On the other hand, polysaccharides confer colloid stability through their thickening and gelation behavior in the aqueous phase. Therefore, the protein-polysaccharide conjugates are expected to exhibit the good emulsifying properties. As expected, the dramatic enhancements of emulsifying properties were observed in protein-polysaccharide (Kato *et al*., 1990; Nakamura *et al*., 1991).

The conjugates of proteins with polysaccharide revealed much better emulsifying activity and emulsion stability than the control mixtures of proteins with polysaccharides. The emulsifying property of the conjugate of lysozyme with galactomannan was the best of various proteins. The similar excellent emulsifying properties were obtained in the conjugates of lysozyme with other polysaccharides. The use of galactomannan is desirable for food ingredients, because it is not so expensive and already utilized as a thickener, binder and stabilizing agents in food. In order to evaluate the potential to industrial applications, the emulsifying properties of lysozyme-polysaccharide conjugates were compared with commercial emulsifiers (Nakamura *et al*., 1992). The lysozyme-galactomannan conjugate was much better than that of commercial emulsifiers (sucrose-fatty acid ester and glycerin-fatty acid ester). In addition, the emulsifying properties of the conjugates were not affected in acidic conditions, in the presence of 0.2M NaCl and by heating of the conjugates. Since high salt conditions, acidic pH, and/or heating process are commonly encountered in industrial applications, the lysozyme-galactomannan conjugate may be a suitable ingredient for use in food processing. Since the commercial mannase hydrolysate (galactomannan) of guar gum is contaminated with considerable amounts of small molecular carbohydrates, thereby resulting in deterioration of emulsifying properties, the low-molecular weight of galactomannan should be removed prior to the preparation of the lysozyme-polysaccharide conjugate. By screening various polysaccharides, galactomannan (Mw 15,000–20,000) obtained from mannase hydrolysate of guar gum was found to be suitable for polysaccharides. When glucose is attached to proteins in a similar manner, the function of protein is adversely affected causing detrimental effects, such as browning.

As expected, the heat stability of lysozyme was also dramatically increased by the conjugation with polysaccharide. No coagulation was observed in the lysozyme-polysaccharide conjugates, while nonglycosylated lysozyme formed the insoluble aggregates during heating. These results suggest that the attachment of polysaccharide causes proteins to form a stable structure.

Upon heating in aqueous solution, the protein molecule partially unfolds and results in the aggregates due to the heat-induced disruption of the delicate balance of various noncovalent interactions. This process may be reversible in the protein-polysaccharide conjugates, because of the inhibition of the unfolded protein-protein interaction due to the attached polysaccharide. This resistance to heating is favorable to food applications, because the heating is essential for pasteurization of food ingredients. Therefore, the approach of protein-polysaccharide conjugates can be useful for the developments of various functional food proteins.

15.3 Constructing polymannosyl lysozyme using genetic engineering

The dramatic improvement of the functional properties of protein by glycosylation is very interesting when remodeling proteins using genetic engineering. We attempted to construct the glycosylated lysozymes in yeast expression system using genetic engineering (Nakamura *et al*., 1993a; Kato *et al*., 1994). In yeast cells, the proteins having Asn-X-thr/Ser sequence are N-glycosylated in the endoplasmic reticulum and the attached oligosaccharide chain can be elongated with further extension of a large polymannose chain in the Golgi apparatus, as shown in Fig. 15.1. For this reason, we attempted to construct the yeast expression plasmid carrying lysozyme cDNA.

The mutant cDNAs of hen egg white lysozyme having N-glycosylation signal sequence (Asn-X-Thr/Ser) at positions 49, 67, 70 and 103 were inserted into *Sal* 1 site located downstream GPD promoter in pYG-100 (Fig. 15.2). The expression vectors were introduced into *S. cerevisiae* AH22. The yeast *S. cerevisiae* carrying mutant lysozyme cDNAs was cultivated at 30 ºC for five days and then glycosyl mutant lysozymes secreted in the medium were isolated by a CM Toyopearl, Sephadex G-50 and concanavalin A-Sepharose column chromatography. Glycosylated lysozyme was obtained only from G49N mutant lysozyme, while it was not from the mutants, G67N, P70N and M105T, as shown in Fig. 15.3. It is probable that the N-linked glycosylation seems to be inhibited by the steric hindrance around positions 67, 70 and 103. The SDS-PAGE patterns indicated that a large molecular size of polymannosyl lysozyme was secreted and stained strongly with carbohydrate reagent (right panel in Fig. 15.3).

A large molecular size of N-glycosylated lysozyme with a polymannose chain was predominantly expressed in the yeast carrying the G49N lysozyme expression plasmid. The secreted amount of polymannosyl lysozyme was much higher than that of the oligomannosyl lysozyme. The mutant G49N lysozyme was secreted in the two types of glycosylated forms, a small oligomannose chain ($Man_{18}GlcNAc_2$)-linked form and a large polymannose chain ($Man_{310}GlcNAc_2$)-linked form, according to the carbohydrate analysis. Both types of glycosylated lysozymes were susceptible to endo β-N-

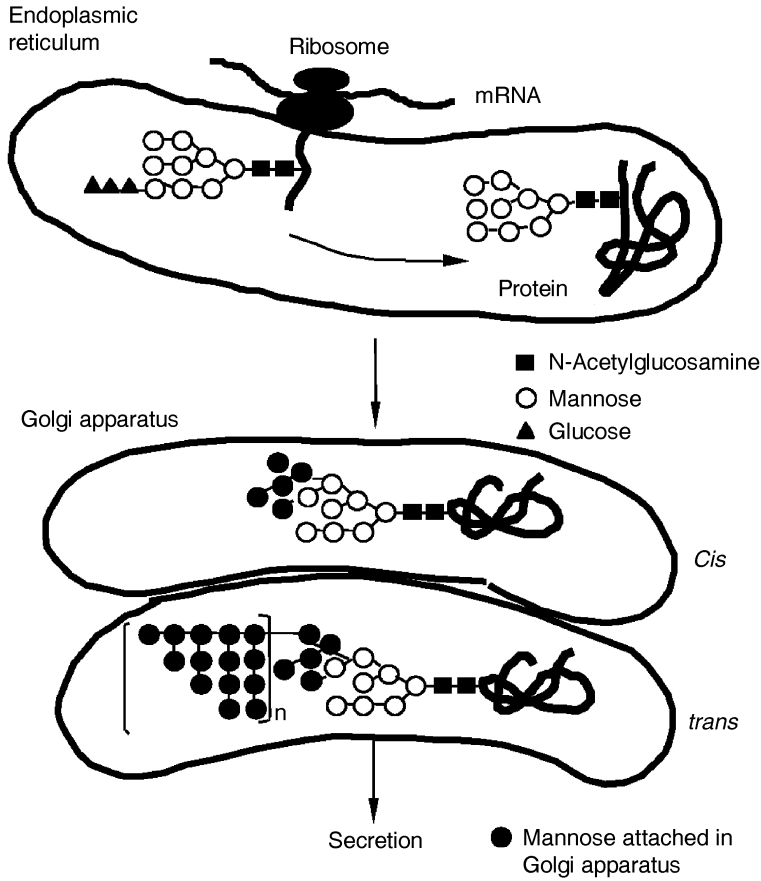

Fig. 15.1 Glycosylation processing of protein in ER and Golgi apparatus in yeast. Glc_3-Man_9-$(GlcNAc)_2$ oligosaccharide unit attaches to asparagines in protein. After carbohydrate processing, polymannosylation occurs in Golgi apparatus by polyammosyl transferase.

acetylglucosaminidase cleavage of their carbohydrate chains. The average molecular weights of oligomannosyl and polymannosyl lysozymes were 18 and 71kDa, respectively. The length of the polymannose chain was found to be 200–350 residues/molecule of lysozyme according to the estimation of the molecular mass distribution by the low angle laser light scattering measurement. The protein conformation estimated by CD analysis was completely conserved in these glycosylated lysozymes. The enzymatic activities of oligomannosyl and polymannosyl lysozymes were 100 and 91%, respectively, of wild-type protein when glycol chitin was used as a substrate.

Further, we successfully constructed another polymannosyl lysozyme R21T in which arginine 21 was substituted with threonine (Kato *et al*., 1994). In addition to these mutants having single glycosylation site, the mutant (R21T/

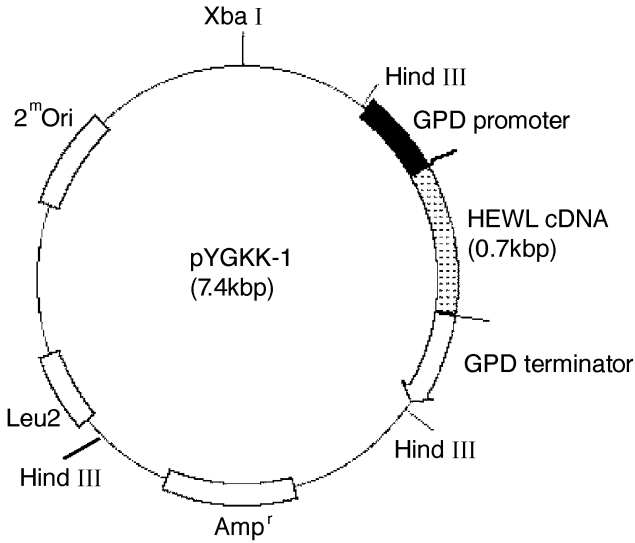

Fig. 15.2 Insertion of lysozyme cDNA into pYG100 (pYGKK-1). Lysozyme cDNA is inserted downstream of GPD promoter.

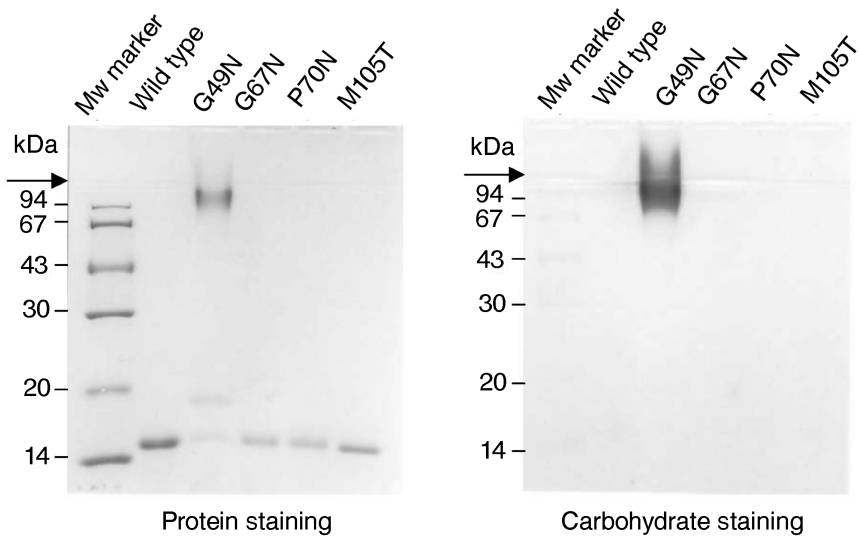

Fig. 15.3 SDS-PAGE patterns of wild-type and mutant lysozymes. Left panel, protein staining; right panel, carbohydrate staining. G49N, Glycine 49 was substituted with asparagines; G67N, Glycine 67 was substituted with asparagines; P70N, Proline 70 was substituted with asparagines; M105T, Methionine 105 was substituted with Threonine.

Table 15.1 Carbohydrate composition of glycosylated lysozymes

Mutant lysozymes	Contents (moles/mole lysozyme)	
	N-acetylglucosamine	Mannose
G49N		
Polymannosyl	2	310
Oligomannosyl	2	18
R21T		
Polymannosyl	2	338
Oligomannosyl	2	14
R21T/G49N		
Polymannosyl	4	290

G49N) having double glycosylation site was also constructed. The oligomannosyl and polymannosyl lysozymes were secreted in the yeast carrying cDNA of R21T, G49N and R21T/G49N mutants. The carbohydrate contents of these glycosylated lysozymes are shown in Table 15.1. The carbohydrate content in double glycosylated mutant R21T/G49N was almost the same as that in single glycosylated mutant, G49N and R21T. This suggests that further extension of mannose residues may be suppressed in yeast cells. Although many polymannosyl proteins were known to secrete in *S cerevisiae*, the number of polymannosyl residues was usually 50–150 (Tanner and Lehle, 1987). Why was the length of polymannose residues of lysozyme much longer than that of general mannoproteins? The glycosylated lysozyme may be retained in the Golgi apparatus longer than other proteins and result in hyperglycosylation. The hyperglycosylated lysozyme was an interesting novel protein constructed by genetic engineering. We could elucidate the role of carbohydrate chains in the

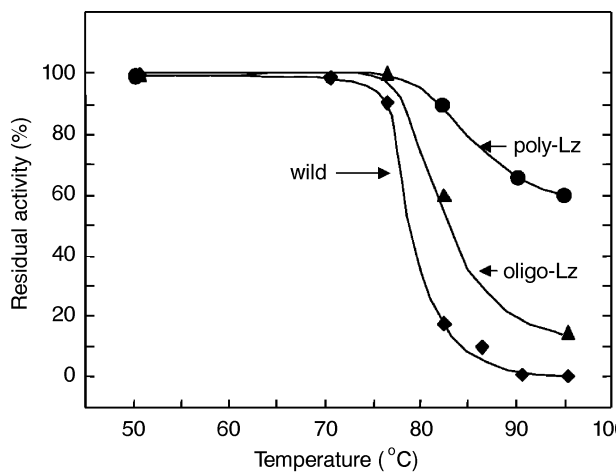

Fig. 15.4 Thermal stability of glycosylated lysozyme (G49N) during heating. ◆ wild type lysozyme; ▲ oligomannosyl lysozyme; ● polymannosyl lysozyme.

functional properties of proteins using single polymannosyl and oligomannosyl lysozymes, and double glycosylated lysozyme.

15.4 Improving functional properties of lysozymes

15.4.1 Heat stability

The enzymatic activity of glycosylated lysozymes during heating was measured as an indication of the apparent heat stability (Fig. 15.4). As expected, the thermal stability of polymannosyl lysozyme was much higher than that of oligomannosyl lysozyme. No coagulation was observed in the polymannosyl lysozyme on heating up to 95 ºC, whereas the wild-type lysozyme completely coagulated at more than 85 ºC. In addition, about 60% of the residual enzymatic activities were retained in the polymannosyl lysozyme after heating up to 95 ºC, while about 15% of residual enzymatic activities were retained in oligomannosyl lysozyme. This result suggests that the attachment of polysaccharide is much more effective for the improvement of thermal stability of proteins than that of oligosaccharide. It is probable that polysaccharide attached to lysozyme may confer stability on the aqueous phase around protein molecules and protect the intermolecular interaction with unfolded molecules, providing stabilization of protein structure against heating.

To understand the role of polyglycosylation in protein stability, the thermodynamic changes in the denaturation of various polymannosyl lysozyme mutants (R21T, G49N, R21T/G49N) constructed by genetic modification were analyzed using differential scanning calorimetry (Kato *et al*., 2000). As shown in Fig. 15.5, the denaturation temperature (Td, peak) and the enthalpy change (ΔH, peak area) for unfolding of the lysozymes were reduced with an increase in the length of polymannose chain and the number in the binding site to a protein, although the polymannosyl lysozymes revealed apparent heat stability in that no aggregation was observed and the enzymatic activity was conserved under conditions in which the wild-type lysozyme coagulated. Despite the destabilization of the lysozyme by attachment of the carbohydrate chain, the polymannosyl lysozyme revealed an apparent heat stability.

In order to elucidate the contradiction, the reversibility of the denaturation was investigated by DSC analysis which compared the DSC curves of the unheated polymannosyl lysozymes with those of the heat denatured ones. DSC measurement was again carried out using the sample solution immediately cooled after attaining or surpassing the Td. The DSC curves were drawn at pH 4.0 where was the critical point to maintain the solution without aggregation during the denaturation. As shown in Fig. 15.6, the peak area of the DSC curve for the wild-type lysozyme was significantly reduced by reheating after denaturation (Fig. 15.6(a)). On the other hand, the DSC curve of the polymannosyl lysozyme (G49N) was overlapped by reheating after attaining the Td (Fig. 15.6(b)), and that of the double mannosyl lysozyme (R21T/G49N) was also overlapped by reheating after denaturation (Fig.

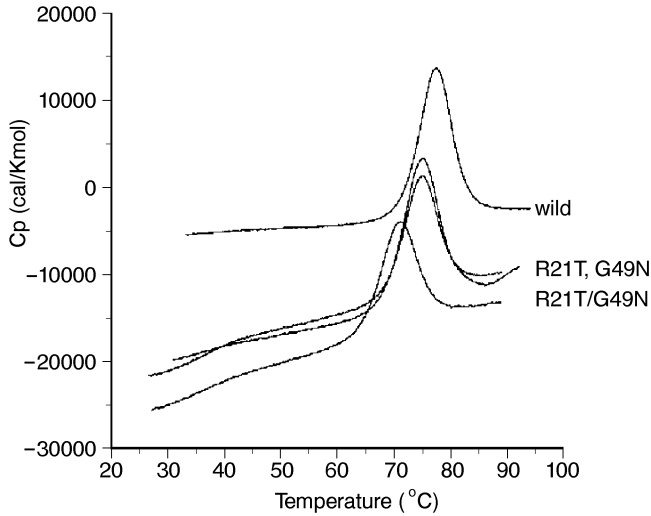

Fig. 15.5 Typical excess heat capacity curves of polymannosyl lysozymes at pH4. Wild, wild-type lysozyme; R21T, mutant polymannosyl lysozyme in which arginine 21 was substituted with threonine; G49N, mutant polymannosyl lysozyme in which glycine 49 was substituted with asparagines; R21T/G49N, mutant double polymannosyl lysozyme in which arginine 21 and glycine 49 were substituted with threonine and asparagine, respectively.

15.6(c)). This result indicates that during the process of refolding the polymannosyl chain contributes to proper folding and enhances the reversibility of refolding. Based on these results, the polymannosyl lysozyme seems to easily refold due to the excellent reversibility of denaturation, despite the decreases in enthalpic stabilization due to the strain in the protein molecule by the introduction of a polysaccharide chain.

15.4.2 Emulsifying properties

The emulsifying properties were greatly increased by the attachment of polymannose chains (Fig. 15.7), as expected from the data of Maillard-type lysozyme-polysaccharide conjugate mentioned above. The polymannosyl lysozyme constructed by genetic engineering showed much higher emulsifying properties than the oligomannosyl lysozyme (Nakamura *et al*., 1993b; Kato *et al*., 1996). This suggests that the length of polysaccharide is very important and critical for the emulsifying property of protein-polysaccharide conjugates. It is interesting that the polymannosyl lysozyme shows better emulsifying properties than commercial emulsifiers. It is probable that the role of polysaccharide in the stabilization of emulsion is performed as follows. The hydrophobic residues in protein molecule are anchored in the oil droplets during emulsion formation. The polysaccharide moiety orients in the aqueous outer layer of oil/water emulsion acting as hindrance layer as well as decelerator of migration of oil

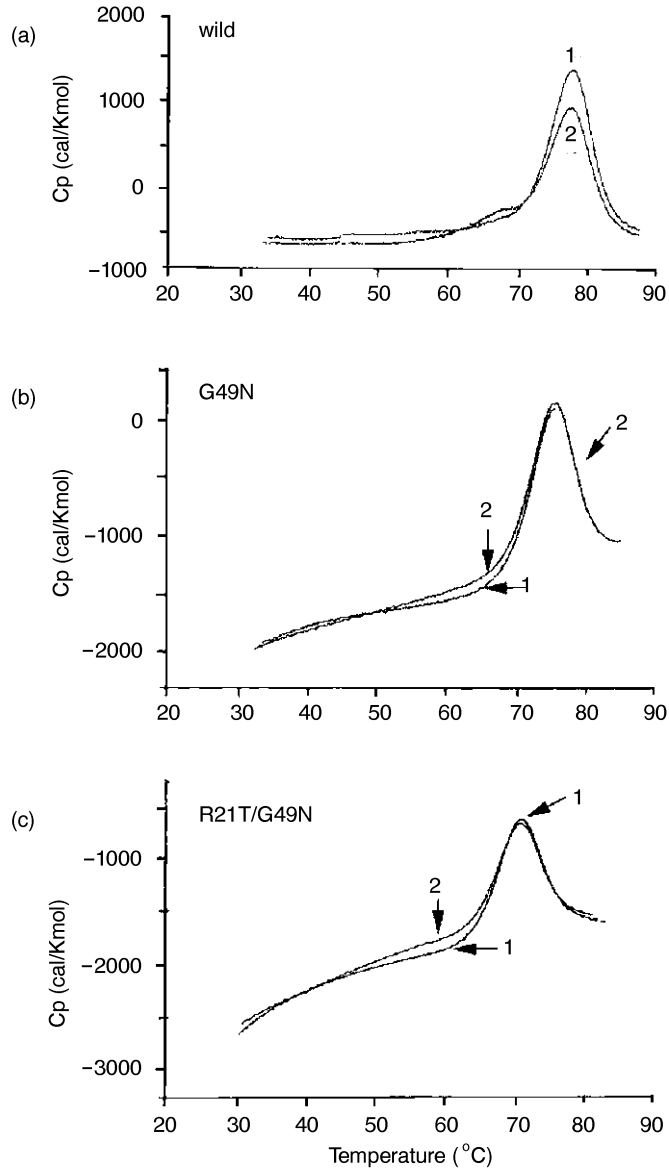

Fig. 15.6 Reversibility of the denaturation of wild-type (a), G49N (b), and R21T/G49N (c) lysozymes. DSC measurement was again carried out. Using the sample solution cooled immediately after attaining (b) or surpassing (a and c) the Td. 1, first measurement of DSC; 2, second measurement of DSC after cooling the same solution of first measurement.

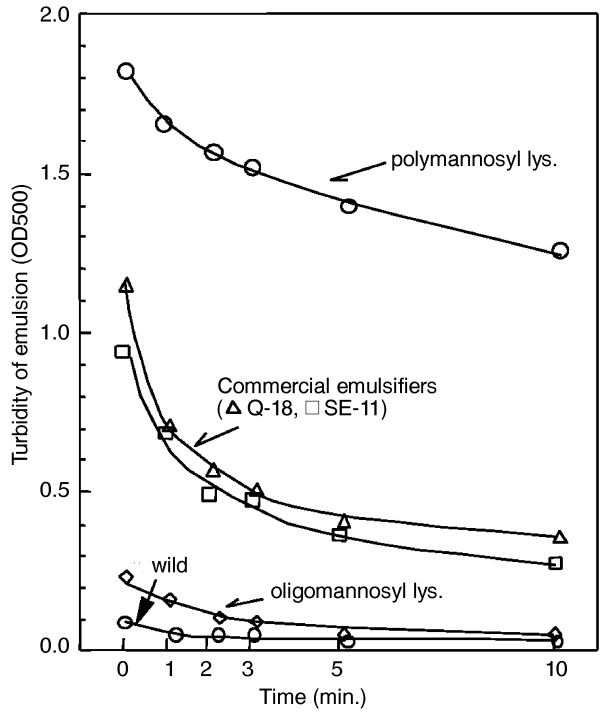

Fig. 15.7 Emulsifying properties of oligomannosyl and polymannosyl lysozyme (G49N). The turbidity of emulsion homogenized a mixture of 1 ml corn oil and 3 ml water (0.1% glycosyl lysozyme or emulsifier) at 12,000 rpm for 1min. was followed for 10 min.

droplets for their coalescence due to high surface viscosity. Thus, it was evidenced on the molecular basis that the dramatic improvements of the thermal stability and emulsifying property of lysozyme are brought about by the attachment of polysaccharide but not oligosaccharide.

Another subject to consider is the effect of the number of glycosylation sites in proteins on the functional properties of polymannosyl lysozyme. This can be explained only by genetic protein glycosylation, because the chemical glycosylation of proteins including Maillard-type glycosylation cannot control the number of glycosylation sites. In order to solve this problem, the single polymannosyl mutants (R21T, G49N) and double mutant (R21T/G49N) were successfully constructed by genetic engineering. Double polymannosyl lysozyme revealed much higher emulsifying properties than single polymannosyl lysozyme (Kato *et al*., 1996). This suggests that the formation of a thick steric stabilizing adsorbed layer around the emulsion is further enhanced and the coalescence of oil droplets is further effectively inhibited by increasing the number of glycosylation sites.

The novel and promising approaches were described here to improve the functional properties such as heat stability and emulsifying properties of hen

egg white lysozyme by genetic engineering. In order to understand the molecular mechanism of the dramatic improvement of functional properties of lysozyme, the oligomannosyl and polymannosyl lysozymes were constructed in a yeast expression system using genetic engineering. The importance of the size of the saccharide chain in the effective improvement of the functional properties was proved by the genetic modification of lysozyme.

15.4.3 Modification of antimicrobial action of lysozyme

The bactericidal action of lysozyme is stronger to Gram-positive bacteria than to Gram-negative bacteria, because the cell envelope consisting of lipopolysaccharide of Gram-negative bacteria obstructs the access of lysozyme to peptidoglycan layer. We reported that the covalent attachment of fatty acids (myristic and palmitic acids) to lyszoyme broadened the bactericidal action to Gram-negative bacteria (Ibrahim *et al*., 1991). The lipophilized lysozyme facilitates the access to invasion of the outer membrane to hydrolyze the peptideglycan, thereby killing the Gram-negative bacteria. Based on the observation, the molecular design of lysozyme was attempted to attach a hydrophobic pentapeptide for anchoring of lysozyme in the outer membrane to extend the action to Gram-negative bacteria. First, we attempted to attach the hydrophobic pentapeptide (Phe-Phe-Val-Ala-Pro) in milk casein to the C-terminus of lysozyme. This peptide readily forms β-strand in an almost similar length of miristic acid. The ribbon model structure of the hydrophobic pentapeptide-fused lysozyme (H5) is shown in Fig. 15.8. The H5 lysozyme revealed strong bactericidal action against *E. coli* (Ibrahim *et al*., 1994).

The different lengths of hydrophobic peptides were attached to the C-terminus of the hen egg white lysozyme to investigate the most effective length of the hydrophobic peptides for killing bacteria (Arima *et al*., 1997). The oligonucleotides encoding Phe-Val-Pro (H3), Phe-Phe-Val-Ala-Pro (H5) and Phe-Phe-Val-Ala-Ile-Ile-Pro (H7) were fused to the C-terminus Leu 129 of lysozyme cDNA. The reconstructed cDNAs were inserted into the yeast expression vector. The hydrophobic peptide-fused lysozymes were secreted in the yeast carrying the reconstructed cDNA. The hydrophobic peptide-fused lysozymes retained 75–80% lytic activity of the wild-type protein. The bactericidal action against *E. coli* of the tripeptide-fused lysozyme slightly increased compared to the wild-type lysozyme, while that of the pentapeptide- and heptapeptide-fused lysozymes greatly increased (Fig. 15.9). These results suggest that the pentapeptide is long enough to kill Gram-negative bacteria. The hydrophobic pentapeptide attached to the C-terminus of lysozyme may contribute to the penetration into the outer membrane of the bacteria.

Ibrahim *et al*. (1994) indicated that the enhanced bactericidal action of H5 lysozyme to *E. coli* is due to the disruption of the electrochemical potential of the inner membrane in cooperation with the inherent function of lysozyme to the outer membrane and peptideglycan using model experiments with *E. coli* phospholipid liposome. Thus, the bactericidal action of lysozyme was exerted

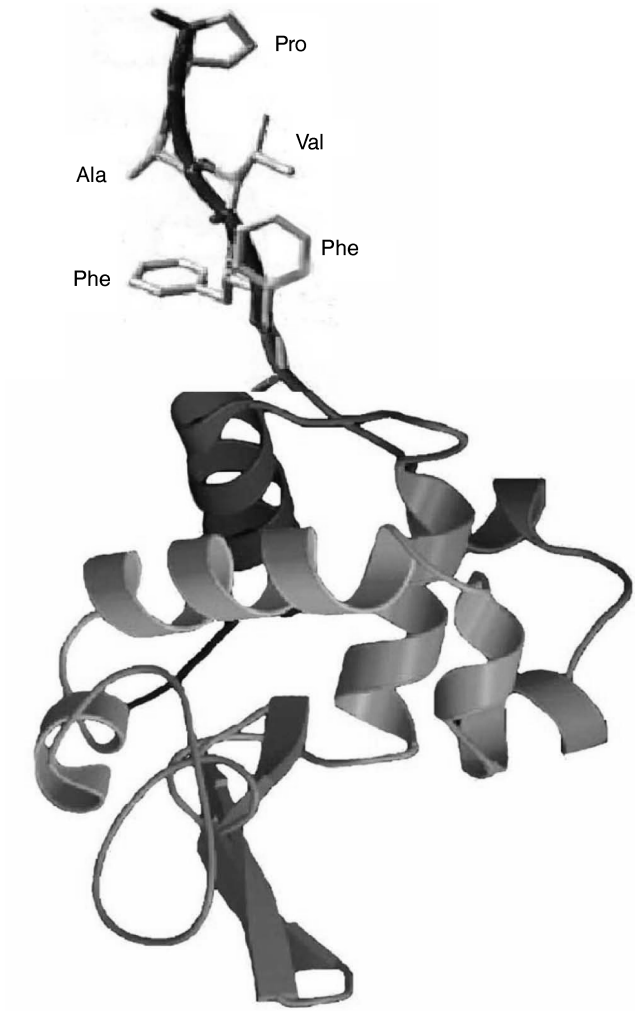

Fig. 15.8 Ribbon model structure of hydrophobic pentapeptide-fused lysozyme. The hydrophobic pentapeptide, Phe-Phe-Val-Ala-Pro, was attached to the C-terminus of lysozyme. The peptide is easy to form as a β-strand.

to both Gram-negative and Gram-positive bacteria. The bactericidally converted lysozyme can be used for industrial applications. However, the secretion amount of H5 lysozyme was very low in *S. cerevisiae*. In order to overcome the low secretion, we successfully increased 400 times the secretion amount of H5 lysozyme in *Pichia pastoris* expression system (Liu *et al*., 2003). Thus, it has been shown that lysozyme can be converted to have the bactericidal action against both Gram-positive and Gram-negative bacteria by genetic modification.

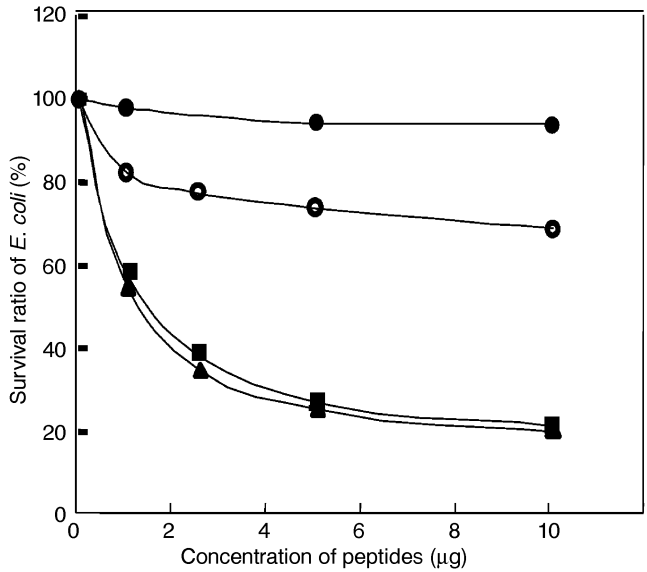

Fig. 15.9 Bactericidal action of wild and hydrophobic peptide-fused lysozymes to *E. coli* cells. The different concentrations of wild lysozyme (•), Phe-Val-Pro(H)-lysozyme (o), Phe-Phe-Val-Ala-Pro (H5)-lysozyme (■), Phe-Phe-Val-Ala-Ile-Ile-Pro (H7)-lysozyme (▲) were incubated with the *E. coli* cells (10^5 cells/ml) at 37 ºC for 30 min. in 50 mM acetate buffer, pH5.5.

15.4.4 Construction of transgenic tobacco with inserted mutant lysozyme genes

We were successful in designing and creating mutant lysozymes with a novel function using genetic engineering. These glycosylated (G49N) and bactericidal switching mutant (H5) lysozyme genes were inserted into plant cells to improve the bactericidal action. The bactericidal action of the modified lysozyme against general plant mold diseases was confirmed in transgenic tobacco with and without inserted H5 and G49N genes. The cDNA of modified lysozymes (H5 and G49N) were engineered into tobacco through *Agrobacterium*-mediated transformation. As shown in Fig. 15.10, the transgenic tobacco with inserted genes of wild, G49N and H5 mutant lysozyme showed an apparent bactericidal action against gray mold bacteria. A leaf of tobacco was infected with the agar containing gray mold bacterium by putting the agar (diameter 2 mm) containing the bacteria on the leaf of tobacco, and then the infected diameter was measured after one week. The result is summarized in Table 15.2. The transgenic tobacco with inserted glycosylated lysozyme (G49N) and hydrophobic peptide (H5) genes showed much stronger resistance against infection using concentrated bacteria than that with wild lysozyme.

Similarly, the strong resistance of transgenic tobacco with inserted G49N and H5 lysozyme genes against powdery mildew disease were observed as shown in

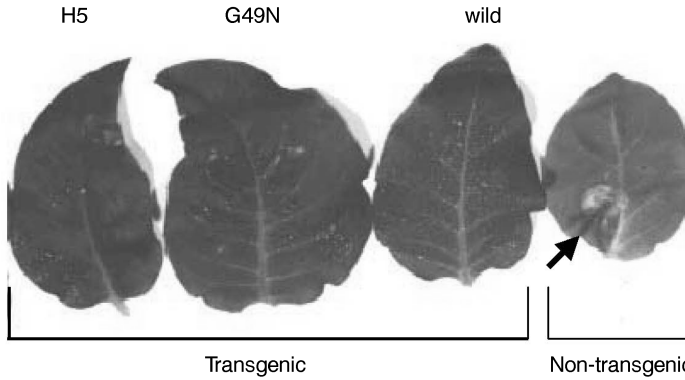

Fig. 15.10 Bactericidal action of tobacco leaf with and without inserted glycosylated mutant (G49N) and hydrophobic pentapeptide-fused (H5) lysozyme genes against gray mold bacterium. The bacterium was infected by putting an agar disk of 2 mm diameter containing gray mold on the surface of the tobacco leaf. The infected diameter was measured after one week.

Fig. 15.11. Apparent resistance against infection was shown in the transgenic tobacco leaf inserted with G49N and H5 genes. The percentages of withered leaf infected with powdery mildew were further decreased in the transgenic tobacco inserted with G49N and H5 lysozyme genes than that inserted with wild lysozyme (Fig. 15.12). The bactericidal actions of tobacco inserted with mutant lysozyme genes against gray mold and powdery mildew bacteria were much higher than that of tobacco inserted with wild-type lysozyme genes. It seems likely that the mutant lysozymes are localized in the plant cell membrane to protect the insertion of bacteria. This observation suggests that bactericidal transgenic plants can be readily constructed by the insertion of glycosylated or H5 lysozyme genes.

In summary, genetic modification is a very powerful method for improving the functional properties such as heat stability, emulsifying properties, and

Table 15.2 Bactericidal action against gray mold disease of transgenic tobacco with inserted wild-type, H5 and G49N lysozyme genes

Tobacco	Diameter of infected symptom (mm)	
	Weak infection	Strong infection
Wild-type tobacco	4.80	∞
Transgenic tobacco		
Lysozyme	2.50	∞
H5 lysozyme	2.17	3.60
G49N lysozyme	2.20	4.36

Weak infection was carried out using diluted gray mold bacteria, while strong infection was done using ten times concentrated bacteria. Gray mold was infected by putting agar disk (diameter 2 mm) containing bacteria on the surface of tobacco leaf. The measurement of infected symptom was carried out two days after infection. The mark, ∞, indicated the withered leaf.

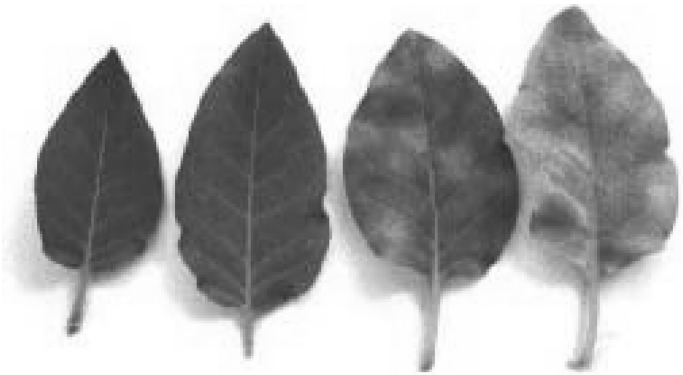

Fig. 15.11 Bactericidal action of tobacco leaf with and without inserted glycosylated mutant (G49N) and hydrophobic pentapeptide-fused (H5) lysozyme genes against powdery mildew bacterium.

antimicrobial action that might be useful in industrial applications. The progress of yeast expression system would allow the design and creation of new functional proteins. We have previously used the expression 'system' with *Saccharomyces cerevisiae* to secrete various proteins, but the yield of the target proteins was very low. Recently, a *Pichia pastoris* expression system was introduced to increase secretion of heterologous proteins, thereby enabling the investigation of characteristic functions of the newly modified proteins.

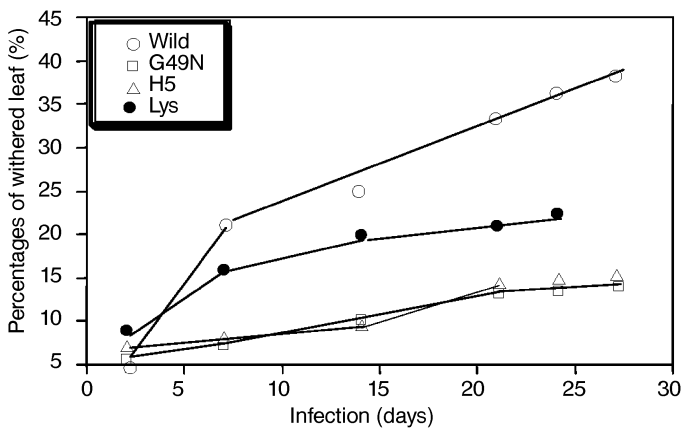

Fig. 15.12 Time-course of bactericidal action of tobacco leaf with and without inserted glycosylated mutant (G49N) and hydrophobic pentapeptide-fused (H5) lysozyme genes against powdery mildew bacterium. o Non-transgenic tobacco, • Transgenic (wild lysozyme), △ Transgenic (H5 lysozyme), □ Transgenic (G49N lysozyme).

15.5 Acknowledgement

I would like to express my appreciation to Professor Soichiro Nakamura, Shimane University, Japan, and Professor Hisham Radwan Ibrahim, Kagoshima University, Japan for their excellent work during my PhD course at Yamaguchi University. This review was mainly written on the basis of their work. The work on transgenic plants was carried out in cooperation with Professor Michiyo Kato, Numazu College, Japan.

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16

Modifying seeds to produce proteins

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16.1 Introduction

Seeds contain nutrients for the new plantlet in different forms, e.g., as starch, proteins and/or lipids. The protein content of seeds of the major crop plants varies between 10 and 50%, occurring mainly in the form of storage protein. Storage proteins have no enzymatic activity; they simply provide a source of amino acids, nitrogen and carbon for the developing seedling. Storage proteins are deposited in the seed in an insoluble form in protein bodies and survive desiccation for long periods. Traditionally, these proteins were classified into albumins, globulins, prolamins and glutelins according to their extraction and solubility in a series of solvents (Osborne 1924). The major form of storage proteins in legumes and other dicotyledonous plants are the globulins, but in cereals the major storage proteins are prolamins and glutelins.

Most globulins fall into two distinct structural groups with sedimentation coefficients of 11S and 7S. These groups are legumins (11S) and vicilins (7S). The sedimentation coefficients are usually expressed in Svedberg units (S), defined as the velocity divided by the centrifugal field. The sedimentation coefficient is dependent on the mass, the shape and the density of a particle (Stryer 2000). For example, the molecular weights of 11S storage globulins range from 320 to 400 kDa and those of 7S globulins from 145 to 190 kDa (Shotwell and Larkins 1989). Globulins have a nutritionally rather well balanced amino acid composition, except for relatively low levels of the sulfur-containing amino acids, methionine and cysteine.

Prolamins are the main storage proteins in wheat, barley and maize. Prolamins generally contain relatively little lysine. The maize prolamins, zeins, have high sulfur contents. The different molecular weight zeins (10, 14, 15, 19,

22 and 27 kDa) vary in their sulfuric amino acid compositions: the 15 kDa zein is rich in methionine and the 10, 15 and 27 kDa zeins are rich in cysteine (Pedersen *et al*. 1986, Hoffman *et al*. 1987).

The high-protein foods that are consumed in the human diet originate both from plant and animal sources. Plant proteins account for approximately 30% of the dietary protein supply in Europe and in the United States, 47% in Japan, and 80–90% in other Asian countries and in Africa (Utsumi 1992). Nutritional properties of food proteins are determined by their content of essential amino acids. Plant proteins are abundant but are usually inferior to animal proteins in terms of nutritional value. Humans and livestock require eight essential amino acids in their diets. Most cereal seeds are deficient in lysine, threonine and tryptophan, whereas most legume seed proteins are deficient in the sulfur-containing amino acids, methionine and cysteine, and in threonine and tryptophan (Utsumi 1992, Habben and Larkins 1995). Consequently, diets based only on cereal or legume species are likely to result in amino acid deficiencies. This problem can be reduced to some extent by using a mixed diet of cereal and legume seeds. However, improvement of the nutritional quality of protein in legumes and cereals has been an important aim of plant breeders. Recent developments in the molecular biology of seed proteins offer breeders new tools for this work.

Transformation methods have been available for dicotyledonous plants since the 1980s, and the current transformation methods for most cereals were developed during the 1990s. In recent years, methods for modification of seed protein composition have been developed. These include various efficient seed-specific promoters from legumes and cereals, which have been cloned and used for expression studies. Using genetic engineering it has been possible to enhance the nutritional and functional properties of seed proteins. For example, various approaches have been proposed for improvement of the amino acid balance of seed proteins by genetic engineering. One possibility is to substitute the particular storage protein that is deficient in an amino acid by a protein of higher nutritional quality. Another possibility is to alter the amino acid composition of the deficient protein (John 1992). Seed proteins also affect the functional properties of foods such as dough formation, gelation, emulsification and foaming, e.g., breadmaking quality is correlated with the presence of HMW subunits of gluten. Methods for modification of seed proteins with respect to their functional properties are discussed in more detail in sections 16.2 and 16.3.

Although seeds are mainly used as food or feed, they are also used as raw materials for various other industrial applications. Many industrial processes involve enzymes degrading plant cell walls and starch. These industries include the food and feed, paper and pulp, and brewing industries. The processing quality of seeds can be modified by altering the structural grain constituents or the enzyme activities that mobilize storage reserves of the seeds. Various heterologous enzymes have been expressed in seeds in order to improve their processing quality. Modern molecular biological techniques can also be applied to plant seeds in order to develop novel expression systems for the production of

heterologous proteins. For example, expression of bioactive or therapeutic compounds in seeds would give added value to seeds as industrial raw material. The various applications of protein expression in seeds, i.e. enhancement of nutritional and functional quality as well as expression of industrial and therapeutic proteins, will be reviewed in section 16.3.

16.2 Methods of seed modification

16.2.1 Genetic engineering of plants

The first transgenic plants were produced by *Agrobacterium*-mediated gene transfer already in the early 1980s (Zambryski *et al*. 1983, De Block *et al*. 1984, Horsch *et al*. 1984). However, the initial successes were limited to *Solanaceae*. Especially monocotyledonous plants proved to be very recalcitrant to *Agrobacterium* gene transfer, and alternative methods were developed. The situation changed during the next decade and by the end of the 1990s it was possible to transform a wide range of species, including many agronomically important crops. The first transgenic cereals were produced using electroporation or particle bombardment, e.g., maize (Rhodes *et al*. 1988), rice (Toriyama *et al*. 1988, Zhang *et al*. 1988, Zhang and Wu, 1988), wheat (Vasil *et al*. 1993), and barley (Jähne *et al.* 1994, Ritala *et al*. 1994, Wan and Lemaux, 1994). Only in recent years have methods for *Agrobacterium*-mediated gene transfer to cereals been developed (e.g., Chan *et al*. 1993, Cheng *et al*. 1997, Tingay *et al.* 1997). For most crop plants, gene transfer has now become a standard procedure, and attention has been shifted more towards studying the regulation and enhancement of gene expression, and optimization of production of desired proteins.

16.2.2 Seed-specific promoters

Gene expression is regulated by a promoter. Depending on the type of the promoter, gene expression can be constitutive, tissue specific, temporal or inducible. The first transgenic plants contained the gene of interest mainly under the regulation of a constitutive promoter. Many of the constitutive promoters are also strongly expressed in seeds and therefore have been used for expressing proteins in seeds. For example, high levels of avidin, β-glucuronidase and aprotinin have been produced in maize seeds using the maize ubiquitin promoter. Although the ubiquitin promoter is considered to be constitutive, in all these cases the majority of the recombinant protein was present in the embryo, thus showing a strong tissue preference in the seed (Hood *et al*. 1997, Witcher *et al*. 1998, Zhong *et al*. 1999). However, in most cases it is beneficial to use a spatially and developmentally regulated promoter to drive the gene of interest. Therefore, regulatory sequences of many seed specific promoters have been isolated in order to express proteins specifically in seeds. Most of these promoters are from seed storage proteins. In many dicotyledonous plants (e.g.

legumes) the storage proteins are mainly expressed and accumulated in the embryonic axis and cotyledons. However, in monocotyledonous plants, especially cereals, the storage proteins are mainly expressed in the endosperm (i.e. starchy endosperm, sub-aleurone layer and aleurone layer). In general, dicot promoters have been used to drive expression in dicots and monocot promoters to drive expression in monocots.

Seed-specific promoters cloned from dicotyledonous plants include the promoters for β-phaseolin (Hoffman *et al*. 1987), soybean lectin (Guerche *et al*. 1990), unknown seed protein (USP) from *Vicia faba* (Bäumlein 1991a), legumin B4 (LeB4) from *Vicia faba* (Bäumlein *et al*. 1986, 1991b, Fiedler *et al*. 1997), pea lectin (De Pater *et al*. 1996), helianthin (Nunberg *et al*. 1995), and arcelin (Goossens *et al*. 1999). The expression levels obtained with various promoters vary considerably, ranging from 0.0 to 8.0% of total extractable seed protein (Table 16.1). The seed-specific promoters differ in strength and temporal activity during seed development. For example, the USP promoter from *Vicia faba* is active during early stages of seed development, whereas the LeB4 promoter becomes active during the middle to late stages (Fiedler *et al*. 1997).

Several seed-specific promoters have also been cloned from cereals (Table 16.1). In cereals the expression can be restricted to embryo, to aleurone layer or to starchy endosperm. For example, the β-amylase promoter is active in the embryo, the aleurone and the starchy endosperm, but the hordein B and D promoters are active only in the starchy endosperm (Kreis and Shewry 1992). For applications based on malting (i.e. germination), α-amylase promoter, which is active only during germination, has been used (Nuutila *et al*. 1999).

Leite *et al*. (2000) used a γ-kafirin promoter cloned from a monocot plant, sorghum (de Freitas *et al*. 1994) to drive the expression of a recombinant human growth hormone in tobacco seeds. A few copies of this gene in the sorghum genome are responsible for producing up to 5% of the total storage protein. In transgenic tobacco the expression was first detected 21 days after anthesis and the recombinant protein accumulated during the late stages of seed development. The expression level varied from 0.07% to 0.16% of total soluble seed protein. The authors concluded that a higher expression level could be obtained in a monocot plant.

16.2.3 Modification of seed proteins

Prior to modifying the genes encoding the seed storage proteins, several factors should be taken into consideration. Storage proteins have very ordered structures, pathways of synthesis and deposition, and therefore great care should be taken in modifying the primary amino acid sequence (Shotwell and Larkins 1991). For example, the packaging and deposition of storage proteins is dependent on their secondary structure, i.e., the path that the polypeptide backbone of the protein follows in space. The most commonly found repeating conformations of polypeptide chains are the α-helix and the β-pleated sheet, and short segments of both of these are found in many proteins. These secondary

Table 16.1 Examples of seed-specific promoters from dicots and monocots

Promoter	Source plant	Host plant	Reference
AT2S1	*Arabidopsis thaliana*	*Brassica napus*	De Clerq *et al*. 1990a, b
	Arabidopsis thaliana	*Nicotiana tabacum*	De Clerq *et al*. 1990a, b
	Arabidopsis thaliana	*Arabidopsis thaliana*	De Clerq *et al*. 1990b
Napin (napA)	*Brassica napus*	*Nicotianan tabacum*	Stålberg *et al*. 1993
Lectin	*Glycine max*	*Brassica napus*	Guerche *et al*. 1990
	Glycine max	*Glycine max*	Philip *et al*. 2001
	Glycine max	*Nicotiana tabacum*	Philip *et al*. 1998
β-Conglycinin	*Glycine max*	*Arabidopsis thaliana*	Naito *et al*. 1995
Helianthin (HaG3-A)	*Helianthus annuus*	*Nicotianan tabacum*	Nunberg *et al*. 1995
β-amylase	*Hordeum vulgare*	*Hordeum vulgare*	Kihara *et al*. 1997
α-amylase	*Hordeum vulgare*	*Hordeum vulgare*	Nuutila *et al*. 1999
Hordein B1	*Hordeum vulgare*	*Hordeum vulgare*	Cho *et al*. 1999
	Hordeum vulgare	*Hordeum vulgare*	Patel *et al*. 2000
Hordein D	*Hordeum vulgare*	*Hordeum vulgare*	Cho *et al*. 1999
	Hordeum vulgare	*Hordeum vulgare*	Horvath *et al*. 2000
Chi26	*Hordeum vulgare*	*Oryza sativa*	Hwang *et al*. 2001b
Lto1	*Hordeum vulgare*	*Oryza sativa*	Hwang *et al*. 2001b
Glutelin A-3	*Oryza sativa*	*Nicotiana tabacum*	Yoshihara *et al*. 1966
Glutelin B-1	*Oryza sativa*	*Hordeum vulgare*	Patel *et al*. 2000
	Oryza sativa	*Oryza sativa*	Hwang *et al*. 2001a

Table 16.1 Continued

Promoter	Source plant	Host plant	Reference
Glutelin B-2	*Oryza sativa*	*Oryza sativa*	Hwang *et al*. 2001a
Gt3	*Oryza sativa*	*Oryza sativa*	Hwang *et al*. 2001a
Prolamin (PG5a)	*Oryza sativa*	*Oryza sativa*	Hwang *et al*. 2001a
Prolamin (RP6)	*Oryza sativa*	*Oryza sativa*	Hwang *et al*. 2001a
α-globulin (Glb)	*Oryza sativa*	*Oryza sativa*	Hwang *et al*. 2001a
Glutelin (Gt1)	*Oryza sativa*	*Oryza sativa*	Lucca *et al*. 2001
β-phaseolin	*Phaseolus vulgaris*	*Nicotiana tabacum*	Hoffman *et al*. 1987
	Phaseolus vulgaris	*Nicotiana tabacum*	Voelker *et al*. 1989
	Phaseolus vulgaris	*Brassica napus*	Altenbach *et al*. 1992
PHA-L	*Phaseolus vulgaris*	*Nicotiana tabacum*	Voelker *et al*. 1989
Arcelin-5 (arc5)	*Phaseolus vulgaris*	*Phaseolus acutifolius*	Goossens *et al*. 1999
	Phaseolus vulgaris	*Arabidopsis thaliana*	Goossens *et al*. 1999
Vicilin	*Pisum sativum*	*Lupinus angustifolius*	Molvig *et al*. 1997
γ-kafirin	*Sorghum tuberosum*	*Zea mays*	de Freitas *et al*. 1994
	Sorghum tuberosum	*Nicotiana tabacum*	Leite *et al*. 2000
Glutelin (Bx7)	*Triticum aestivum*	*Oryza sativa*	Hwang *et al*. 2001a
Legumin B4	*Vicia faba*	*Vicia narboensis*	Pickardt *et al*. 1995
	Vicia faba	*Vicia narboensis*	Saalbach *et al*. 1995
	Vicia faba	*Nicotiana tabacum*	Saalbach *et al*. 1995
USP	*Vicia faba*	*Arapidopsis thaliana*	Bäumlein *et al*. 1991a
	Vicia faba	*Nicotiana tabacum*	Bäumlein *et al*. 1991a

structures are prerequisites of aggregation (Stryer 2000). Insertion or replacement of amino acids could prevent the formation of these secondary structures by preventing the formation of disulfide or hydrogen bonds or other necessary interactions. This would, in turn, interfere with the tight packaging of the polypeptides, which may be required for deposition in the storage protein bodies (Shotwell and Larkins 1991). In some cases the storage protein subunits are also assembled into oligomers prior to deposition (Nielsen 1990). The effect of protein modifications on protein stability is also an important consideration, especially if the protein is to be deposited in a lysosomal compartment such as the protein body (Hoffman *et al*. 1988). Some of the various aspects that should be considered prior to sequence modifications are presented in Table 16.2.

For improvement of the amino acid balance of seed storage proteins by genetic engineering, one possibility is to alter the amino acid composition of the deficient protein (John 1992). For example the 11S/12S globulins generally contain inserts of polar amino acids. These inserts vary considerably in length, amino acid composition and location in the subunit molecule, both within species and between species. This variation makes these insertion regions attractive targets for the genetic engineering of altered amino acid sequences (Shotwell and Larkins 1989, Nielsen 1990). The 2S albumins also have a region, between the sixth and the seventh cysteine residues, which varies in both length and sequence in different species. It has been shown that this region will tolerate changes in its sequence (De Clerq *et al*. 1990b). Sometimes a model system for testing the modified genes with respect to expression and stability of the modified protein can be useful. Beachy *et al*. (1985) mutated a soybean storage protein gene in order to alter its amino acid composition. They used transformation of petunia to test *in vivo* the effect of these mutations on the

Table 16.2 Factors to consider prior to sequence modification of proteins

Factor	Possible effects	Reference
Secondary structure	Defective secondary structure can interfere with deposition into protein bodies	Shotwell and Larkins 1991
Assembly of oligomers	Changes in subunits can interfere with assembly into oligomers	Nielsen 1990 Dickinson *et al*. 1990
Proteolytic cleavage sites	Modifications in proteolytic cleavage sites can interfere with post-translational processing of protein precursors	Dickinson *et al*. 1989
Intracellular transport	Many storage proteins carry intracellular targeting signals that direct the protein to ER, and from there to protein bodies. Changes in the targeting signals interfere with intracellular sorting.	Frigerio *et al*. 1998
Stability of protein	Protein stability can be sacrificed by changes in structure, assembly and deposition of the modified protein	Hoffman *et al*. 1988

storage protein structure and function. In this way the modification could be evaluated before transferring the modified genes back into agronomically valuable plants, which are usually more difficult to transform.

Some of the storage proteins, especially prolamins, are encoded by large multigene families (Shotwell and Larkins 1991). For example, there are many genes encoding zeins in the maize genome; the α-class zein genes alone exist in the genome as 25 to 50 copies. Therefore, simple manipulation of a few genes is not likely to result in a drastic change in the composition of zein protein fraction (Ueda and Messig 1993).

16.2.4 Modification of enzymes

Modification of enzymes is sometimes needed in order to enhance their characteristics and commercial potential. For example the three-dimensional structures of barley (1,3)-β-glucan endohydrolases and (1,3–1,4)-β-glucan endohydrolases are essentially identical, but there are differences in substrate specificity and stability (Varghese *et al*. 1994). Varghese *et al*. (1994) noted that these differences in substrate specificity and function were achieved without any major changes in the conformation of the polypeptide backbone. In fact, a relatively small number of amino acid differences were responsible for the distinct substrate specificities of the enzymes. Steward *et al*. (2001) attempted to increase the thermostability of barley (1,3–1,4)-β-glucanase isoenzyme EII by introducing amino acid substitutions into the wild-type form of the enzyme. They used site-directed mutagenesis of a cDNA encoding the enzyme. The amino acid substitutions were based on structural comparison of the barley (1,3)- and (1,3–1,4)-β-glucanases and other higher plant (1,3–1,4)-β-glucanases. Eight amino acid substitutions were designed, and three of the resulting mutant enzymes showed increased thermostability. The mutant enzyme retained its activity five times longer than the wild-type enzyme in a simulated mashing experiment at 65 ºC.

16.2.5 Expression of heterologous proteins in seeds

In addition to expressing modified seed storage proteins, seeds can also be used to express heterologous proteins. A heterologous protein is a product of a gene that has been isolated from a different species than that in which it is expressed. For example, expression of a fungal gene in a plant would result in the production of a heterologous protein in the plant. Expression of heterologous proteins can result in improved nutritional or functional properties of the seed protein fraction, or in production of novel proteins. Plant seeds have been used to produce heterologously various enzymes and different types of pharmaceuticals; peptides, blood proteins, and hormones. For this type of production seeds have some advantages over using green plant tissue. They are likely to have fewer phenolic compounds and a less complex mixture of proteins and lipids than green leaves, which is an advantage in purification. Leaves

especially are not a suitable tissue for heterologous protein accumulation because of their high water content and hydrolytic activity, which result in low stability of the recombinant protein (Leite *et al*. 2000). However, seeds are storage organs that accumulate protein efficiently, and are specialized for long periods of storage without protein degradation. Plant seeds also possess the capacity to fold and assemble correctly multimeric proteins. Furthermore, scale up is easy (simply by increasing the planted acreage) and there already exist established practices for harvesting. Thus, the production can rather easily meet industrial demands.

Methods for expression of heterologous proteins in seeds have been studied extensively in recent years. In the case of heterologous expression many of the same factors play an important role as in the modification of proteins (see Table 16.2). The level of expression and the functionality of the protein are strongly dependent on whether the post-translational modifications are performed correctly and on the intracellular trafficking of the (pre-)protein. In recent years many advances have been made in the enhancement of heterologous protein stability and expression levels (see section 16.2.6).

Heterologous proteins have also been expressed in seeds as fusion constructs. In constructing a fusion protein it is important to select the insertion site so that it does not interfere with the secondary structure and/or the stability of the protein, or with the specific processing sites of the flanking sequence. For example, the insertion of leu-enkephalin into 2S albumin was performed in the region between the 6th and 7th cysteine residues, which probably forms a structurally independent loop in the mature protein (Vandekerckhove *et al*. 1989). Fusion constructs have also been used to reduce the potentially expensive purification steps. For this purpose oleosin fusions have been used in the production of hirudin, an anticoagulant used to treat thrombosis (Parmenter *et al*. 1995, van Rooijen and Moloney 1995a, b, Chaudhary *et al*. 1998).

16.2.6 Enhancement of protein expression in seeds

Protein expression can be enhanced by exploiting the protein sorting and targeting mechanisms that plant cells use to target host proteins to organelles. Significant increases in yield have been observed when the recombinant proteins (e.g. antibodies) have been targeted to the secretory pathway. However, the highest yield of functional protein is currently obtained by endoplasmic reticulum (ER) retention, the increase being 10–100 fold in target protein yield when compared to expression in the secretory pathway (Conrad and Fiedler, 1998). Furthermore, targeting to the ER may also ensure correct folding of the recombinant protein. Leite *et al*. (2000) used a *Coix* prolamin signal sequence (Ottoboni *et al*. 1993) to target the expressed recombinant human growth hormone to the endoplasmic reticulum in transgenic tobacco seeds. The expressed recombinant protein was shown to be identical to the native mature human growth hormone and the signal peptide mediated the secretion into the apoplast region.

Recombinant protein expression can be further optimized by using stronger promoters, by improving the stability of transcripts, by using a range of leader and polyadenylation signals and by optimizing the codon usage for plants (Gallie 1998, Mason *et al*. 1998, Richter *et al*. 2000, Streatfield *et al*. 2001, Tuboly *et al*. 2000). There are indications that plant-derived transcription terminators could enhance gene expression considerably. For example Ali and Taylor (2000) showed that a 3′ non-coding region of the *Me1* gene improved gene expression from promoters of different classes. The transcription-enhancing effects of the 3′ non-coding region were related to its effectiveness at terminating transcription. The applicability of this region in monocot plants remains to be seen.

Incorporating introns into transgenes also has an enhancing effect on gene expression (Koziel *et al*. 1996). However, the mechanism underlying the phenomenon is still unclear, although introns and their positions have been shown to affect the translational activity of mRNA (Bourdon *et al*. 2001). Furthermore, 5′ untranslated leaders (Koziel *et al*. 1996) and conserved nucleotides downstream of the start codon (Sawant *et al*. 2001) have been shown to enhance expression. The conserved nucleotides also improve protein stability. Modification of bacterial genes toward plant gene codon usage has been shown to increase the expression of transgenes in plants (Perlak *et al*. 1991, Perlak *et al*. 1993). For example, the codon usage of a hybrid bacterial gene encoding a thermostable (1,3–1,4)-β-glucanase was modified to match that of the barley (1,3–1,4)-β-glucanase isoenzyme EII gene. When introduced into barley aleurone protoplasts, only the gene with adapted codon usage directed the synthesis of heat stable (1,3–1,4)-β-glucanase, whereas activity of the non-modified heterologous enzyme was not detectable (Jensen *et al*. 1997). In general, there are several factors that need to be considered in transgene design, i.e., types and locations of *cis*-acting regulatory elements and how and where integration may affect transgene expression (Gallie 1998).

Plant viruses can also be used as gene vectors in order to achieve efficient and rapid production of heterologous proteins in plants. The most frequently used viruses are the tobacco mosaic virus (TMV; Yusibov *et al*. 1997, Modelska *et al*. 1998) and the cowpea mosaic virus (CPMV; Brennan *et al*. 1999, Dalsgaard *et al*. 1997). However, until now, viral vectors have not been utilized in protein expression in plant seeds, due to the fact that they are more suitable for use in leaf tissues.

Furthermore, genetic elements derived from plant viral genomes have been used to regulate gene expression in transgenic plants. For example, the 5′ untranslated regions (5′ UTRs) of many capped and uncapped RNAs of plant viruses enhance the expression of chimeric genes *in vitro* and *in vivo* (Gallie 1996, 1998). Most of the *in vivo* studies have been made in cells of dicot plants using 5'UTRs of dicot specific viruses. However, the few existing reports on the effects of these leader sequences on gene expression levels in monocots indicate that there are compatibility differences between dicots and monocots. Even though no reports on the use of viral enhancer elements in transgenic plant seeds

hitherto exist, this could be a viable option for expression enhancement in the future.

16.3 Application and use of modified seeds for protein production

16.3.1 Expression of modified proteins in seeds

Improvement of the amino acid balance of seed storage proteins has been attempted by altering the amino acid composition of the deficient protein. De Clerq and co-workers (1990b) modified a poorly conserved region of 2S albumin with methionine-rich sequences, resulting in the methionine-enriched 2S albumins.

A second possibility for improvement of the amino acid balance of seed proteins is to substitute the particular storage protein that is deficient in an amino acid by a protein of higher quality. For example, chimeric genes encoding the methionine-rich 2S albumin of Brazil nut have been introduced to *Vicia narbonensis* (Pickardt *et al*. 1995, Saalbach *et al*. 1994) and to *Brassica napus* (Altenbach *et al*. 1992), in order to improve the amino acid balance of seeds. More examples of seed protein composition modification by expression of high nutritional or functional quality proteins in seeds are presented in Table 16.3.

Food proteins also affect functional properties such as dough formation, gelation, emulsification, and foaming (Utsumi 1992). Breadmaking quality of flour is strongly dependent on the visco-elastic gluten. Although gluten is also present in barley and rye flour, only wheat flour possesses the appropriate properties for breadmaking. Breadmaking quality is correlated with the presence of the HMW subunits of gluten (John 1992).

The composition of high molecular weight (HMW) subunits of glutenin determines the gluten strength and influences the bread baking quality of wheat. The production of transgenic wheat lines differing in their HMW subunit composition has been used to study the effects of individual subunits on glutenin structure and on the technological properties of wheat gluten (Altpeter *et al*. 1996, Barro *et al*. 1997, Blechl *et al*. 1996). The transgenic wheat line containing additional genes encoding the HMW subunit 1Dx5 resulted in a fourfold increase in the proportion of total HMW subunits and total glutenins. This was associated with a dramatic increase in dough strength (Rooke *et al*. 1999).

Altpeter *et al*. (1996) introduced the HMW glutenin subunit 1Ax1 gene into a wheat variety in which it is not present in nature. The amount of heterologous protein produced in transgenic lines varied from 0.6% to 2.3% of total protein, resulting in an increase of up to 71% in total HMW glutenin subunit proteins. When these two HMW subunit encoding genes were separately introduced into two near-isogenic wheat lines differing in their HMW subunit compositions and mixing properties, the over-expression accounted for 50–70% of HMW subunits. Glutenin properties were much more affected by expression of the

Table 16.3 High nutritional quality proteins expressed in seeds

Target plant	Expressed gene	Result	Reference
Arabidopsis thaliana *Brassica napus* *Nicotiana tabacum*	modified (methionine-enriched) 2S albumin	up to 1–2% of total protein in high-salt seed extract[a]	De Clerq *et al*. 1990b
Brassica napus	Brazil nut 2S albumin	up to 0.06% of total seed protein[a]	Guerche *et al*. 1990
Brassica napus	Brazil nut 2S albumin	33% increase in seed methionine content	Altenbach *et al*. 1992
Lupinus angustifolius	sunflower seed albumin	94% increase in seed methionine content, 12% reduction in cysteine content	Molvig *et al*. 1997
Nicotiana tabacum	maize 15 kD zein	test system to study feasibility of zein expression in dicot seed; up to 1.6% of the total seed protein	Hoffman *et al*. 1987
Oryza sativa	pea legumin	up to 4.2% of total seed protein[a]	Sindhu *et al*. 1997
Oryza sativa	bean β-phaseolin	up to 4.0% of total seed protein[a]	Zheng *et al*. 1995
Vicia narboensis	Brazil nut 2S albumin	up to 1–4.8% of total SDS-soluble seed protein, 3-fold increase in seed methionine content	Pickardt *et al*. 1995 Saalbach *et al*. 1995

[a] No data on amino acid composition.

1Dx5 transgene, which resulted in increased cross-linking of glutenin polymers (Popineau *et al*. 2001). This confirmed that transformation can indeed modify the technological properties of gluten proteins and may help to develop new uses for wheat either in the food industry or in non-food applications.

16.3.2 Expression of enzymes in seeds

Many industrial processes are based on the use of enzymes. If the raw material is of plant origin, the endogenous enzymes can be utilized. However, on many occasions the endogenous enzymes do not necessarily have optimal properties for the process in question. The processing quality of seeds can be modified by altering the enzyme activities that mobilize the storage reserves of the seeds. Examples of enzymes expressed in seeds for food, feed and industrial uses are presented in Table 16.4.

In barley, improvement of the malting quality has been attempted by the expression of heterologous β-glucanase in seeds (Jensen *et al*. 1996, Nuutila *et al.* 1999). The endogenous barley (1,3–1,4)-β-glucanases are rapidly inactivated at elevated temperatures, thus resulting in incomplete hydrolysis of (1,3–1,4)-β-glucans. Thus, a (1,3–1,4)-β-glucanase with enhanced heat stability would be useful in overcoming filtration problems and other difficulties encountered in the malting and brewing industries. In a study by Nuutila and co-workers, a gene encoding a thermotolerant fungal endo-1,4–β-glucanase (EGI) was introduced into two barley cultivars, Golden Promise and Kymppi. The gene was expressed in the seeds during germination, thus providing a thermotolerant enzyme that is active under mashing conditions. The amount of thermotolerant β-glucanase produced by the seeds (ca. 0.025% of soluble seed protein) was shown to be sufficient to reduce wort viscosity by decreasing the soluble β-glucan content.

Feed quality can also be enhanced by enzyme expression in seeds. β-Glucanase and xylanase have been expressed in barley for feed use (Patel *et al*. 2000, von Wettstein *et al*. 2000). Addition of β-glucanase barley to chicken feed resulted in a slight weight gain and reduced the amount of adhering sticky droppings (von Wettstein *et al*. 2000). Phytase has been expressed in tobacco seeds, and the milled seeds were tested *in vivo* in broilers. A diet supplemented with transgenic seeds resulted in a significantly higher growth rate of broilers than the control diet (Pen 1996).

Seeds can also be used for production of industrial enzymes. Active α-amylase and phytase can be stored in stable form in tobacco seeds for at least one year (Pen 1996). For example, milled tobacco seeds containing transgenic α-amylase can be used directly in liquefaction of corn and potato starch (Pen *et al*. 1992).

The relatively high cost of enzymes for industrial use can be significantly reduced if the enzyme can be recycled in the industrial process. This can be achieved in some cases by immobilizing the enzyme onto a matrix. Liu *et al*. (1997) utilized the existing oleosin fusion expression system (e.g. van Rooijen and Moloney 1995) for production of recombinant xylanase in Canola seeds.

Table 16.4 Enzymes expressed in seeds for food, feed and industrial uses

Protein	Production host	Level in seeds	Reference
α-Amylase	*Nicotiana tabacum*	0.3% of total soluble protein	Pen *et al*. 1992
	Vicia narboensis	5 U/kg seed	Czihal *et al*. 1999
	Pisum sativum	8000 U/kg seed	Saalbach *et al*. personal comm. cited by Biesgen *et al*. 2002
β-Amylase	*Hordeum vulgare*	n.a.	Kihara *et al*. 1997
β-Glucanase	*Hordeum vulgare*	n.a.	Jensen *et al*. 1996
	Hordeum vulgare	0.025% of soluble protein	Nuutila *et al*. 1999
	Hordeum vulgare	5.5% of soluble protein	Horvarth *et al*. 2000
β-Glucuronidase	*Brassica napus*	n.a.	Rooijen and Moloney 1995
	Brassica napus	0.4–0.7% of water-soluble protein	Witcher *et al*. 1998
Phytase	*Nicotiana tabacum*	0.5% of soluble protein	Pen *et al*. 1993
	Glycine max	n.a.	Denbow *et al*. 1998
	Oryza sativa	9.4 U/kg seed	Lucca *et al*. 2001
Xylanase	*Brassica napus*	300–2000 U/kg seed	Liu *et al*. 1997
	Hordeum vulgare	1.6–6.6 U/grain	Patel *et al*. 2000

n.a. not analyzed.

The characteristics of the immobilized xylanase were similar to those of the free enzyme derived from microbial fermentation. Furthermore, xylanase on the oil-bodies could be recovered by flotation after use in a reaction and recycled many times without loss of activity. The procedure developed thus offers economic advantages in a variety of industrial processes.

16.3.3 Expression of nutraceuticals and therapeuticals in seeds

In recent years plants have been used to produce different types of pharmaceuticals: peptides, blood proteins, cytokines, hormones, immunogens and antibodies (e.g. Ma *et al*. 1998, Gómez *et al*. 1998, Mahon *et al*. 1998, Tackaberry *et al*. 1999, Terashima *et al*. 1999). The production of these therapeutic recombinant proteins has until now been limited to a number of plants only, e.g. tobacco and potato, and expression has been achieved mainly in organs other than seeds. However, seeds are an attractive alternative for this type of production as they are likely to have fewer phenolic compounds and a less complex mixture of proteins and lipids, which will be an advantage in purification.

Plant seeds have been used to produce some therapeuticals, e.g. peptides, blood proteins, and hormones (see Table 16.5). Pharmaceutical proteins and peptides have also been expressed in seeds as fusion constructs. Vandekerckhove *et al*. (1989) produced leu-enkephalin, a neuropeptide displaying opiate activity, as part of a chimeric plant seed storage protein (2S albumin). An anticoagulant hirudin was produced using oleosin as a carrier (Parmenter *et al*. 1995, van Rooijen and Moloney 1995a, b, Chaudhary *et al*. 1998). Oleosins are a class of seed proteins associated with the oil-body membranes in embryos of monocot and dicot plants. The fusion construct was designed to contain an endoprotease recognition site between the hirudin and oleosin genes for the recovery of the hirudin. Oleosin-hirudin fusion proteins were isolated with oil-bodies, from which they were separated by floating centrifugation. An additional advantage is that the oleosin-hirudin fusion is inactive and hirudin is activated only after purification. This system is currently in use in Canada for commercial production of hirudin (Giddings *et al*. 2000).

Seeds as production hosts have several advantages, e.g., the relatively low cost of production and the absence of animal and human pathogens. However, it is also recognized that the production of foreign proteins, particularly the production of biologically active compounds, might require efficient isolation from conventional food and feed crops. In most cases, this can be achieved simply by the use of physical isolation, but biological segregation may also be beneficial. For example, molecular farming of hirudin, a thrombin inhibitor in transgenic *Brassica carinata*, is an attractive alternative to the use of *Brassica napus*. *B. carinata* is a self-pollinating crop which is not conventionally grown in North America (Chaudhary *et al*. 1998).

Although glycosylation of proteins in plants differs from that in mammals due to the presence of α(1,3)-fucose and β(1,2)-xylose and the absence of

Table 16.5 Expression of therapeutic proteins in seeds

Protein	Therapeutic use	Production host	Production level in seeds	Reference
Aprotinin	Acute pancreatitis, prevention of fibrinolysis, pediatric cardiac operations	*Zea mays*	0.1% of extractable protein	Zhong *et al*. 1999
Avidin	Diagnostics	*Zea mays*	1.5 to 3% of extractable protein	Hood *et al*. 1997
Hirudin	Blood anticoagulant for treatment of thrombosis	*Brassica napus*	Oleosin-hirudin fusion ca. 1% of total seed protein	Parmenter *et al*. 1995
		Brassica carinata	n.a.	Chaudhary *et al*. 1998
Human growth hormone	Growth hormone deficiency in children	*Nicotiana tabacum*	Up to 0.16% of total soluble protein	Leite *et al*. 2000
Leu-enkephalin	Neuropeptide displaying opiate activity	*Arapidopsis thaliana*	206 nmol peptide/g seed corresponding to 0.1% of total protein	Vanderkerckhove *et al*. 1989
		Brassica napus	10 to 50 nmol peptide/g seed	Vanderkerckhove *et al*. 1989

n.a. not analyzed.

terminal sialic acid residues (Cabanes-Macheteau *et al*. 1999), no detectable human *anti*-plant antibody response has been observed in clinical trials (Bardor *et al*. 1999, Ma *et al*. 1998, reviewed in Peeters *et al*. 2001). On the other hand, the xylosyl and fucosyl residues of plant glycans have been shown to be responsible for the allergenicity of plant glycoproteins (Garcia-Casado *et al*. 1996, reviewed by Doran 2000). Of course, glycosylation is not an issue for non-glycosylated proteins and peptides and may be a problem only in therapeutic glycoproteins dosed by injection. However, it is very unlikely that a differently glycosylated plant-produced antibody would trigger a response in our immune system, as humans are continuously exposed to plant sugars in food (Doran 2000, Peeters *et al*. 2001). Nevertheless, it might be necessary to develop strategies by which the carbohydrate profiles of plant produced antibodies could be made structurally more consistent with those obtained from mammalian cells, i.e., the formation of possibly immunogenic plant glycans could be prevented by retention of glycoproteins in the endoplasmic reticulum, thus bypassing the plant-specific modification in the Golgi (Doran 2000).

16.4 Future trends

As discussed in this chapter, there are many application areas in which genetic engineering could be used to modify seed proteins, i.e., enhancement of nutritional and functional quality as well as expression of industrial or therapeutic proteins. The first transgenic plant varieties with improved nutritional quality are slowly emerging on a global scale. Golden Rice with high levels of beta-carotene and carotenoids, precursors to vitamin A, is the best known example of these. It is clear that in the future the potential exists to produce varieties with improved nutritional or functional quality. Gene technology will be able to facilitate certain compositional changes in seeds that would be difficult, if not entirely impossible, to achieve through conventional breeding. Whether this potential will be realized in the future, will be at least partially dependent on how the regulatory environment develops. At the moment the regulations on the cultivation and use of transgenic plants vary considerably between different countries. Furthermore, there is also a need for regulation of plant-derived therapeuticals, which are currently being developed. Only if these regulatory issues can be resolved and harmonized to a certain extent, can the full potential of plant seeds as production hosts be realized.

In addition to regulatory issues, there are also other areas in need of more research. For example, the expression stability of plant-based, edible vaccines in seeds should be studied extensively in order to ensure that the exact desired dose will be delivered every time. Under-dosing could possibly result in development of oral tolerance, and thus needs to be avoided. In the case of purified proteins, the cost of downstream processing can be significant. Hence, by developing uses for the by-products of purification, these costs can be lowered, and the process

made economically more viable. Yet another area in need of more research is the use of plant seeds as production hosts for various biopolymers, such as biodegradable plastics or spider silk, which could create added value for seeds.

Developments in the technology, i.e., molecular biology, genetic engineering, genomics and proteomics, will offer new tools for seed modification. For example, increasing knowledge of the factors that control homologous recombination in plants will enhance the likelihood of more targeted gene insertion events. Additionally, developments in the activation or repression of gene expression by chemical regulation will provide sophisticated ways to control seed specific expression.

In the future, in the case of medical or high-tech applications, there could be a need for superior purity of the product and enhanced containment of seed lots. Some approaches to resolve these questions have already been investigated. One solution could be that the therapeutic products would be produced in an inactive form, and activated only after purification. A second option is the use of germination-specific promoters. In the transgenic, ungerminated seeds no heterologous proteins are present. Production is induced during germination, and the product can be recovered from the germinated seeds. A third alternative would be to use non-food plants as production hosts. This would decrease the possibility of accidental mixing of transgenic seeds containing therapeutic products with non-transgenic seeds intended for food use. Furthermore, for therapeutic and high-value products the use of artificial growth chambers or greenhouses would provide an alternative and/or additional way of containment.

It is clear that modification of seed proteins holds great promise for the future, with a potential for enhanced nutritional and functional quality and expression of industrial or therapeutic proteins in seeds. However, there are also many unanswered questions which need to be addressed before this promise can be realized.

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17

Processing approaches to reducing allergenicity in proteins

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17.1 Introduction: food allergens

In order to take a systematic approach as to how allergens may be removed from foods using approaches such as raw material selection or physical processing it is important to have a clear understanding of what constitutes an allergen. Taken in its strictest sense an allergen is defined as a macromolecule to which immunoglobulin E (IgE) binds. IgE responses are generated as part of the normal immune reaction to parasitic infections, but for reasons that are only partly understood can also be generated following exposure to environmental agents, such as pollen, dusts, and foods. On a subsequent encounter the allergen is able to cross-link surface bound IgE molecules located on basophile or mast cells, causing them to release preformed inflammatory mediators, including histamine. It is these mediators which actually cause the symptoms manifested in an allergic episode which usually occurs quite rapidly following exposure and is consequently classified as a Type I hypersensitivity reaction. Such reactions are distinct from food intolerances, which are reproducible, sometimes non-immune-mediated, reactions whose symptoms can take days to manifest themselves. An example of such a condition is the gluten intolerance syndrome, Coeliac disease, and whilst the gluten proteins which trigger it are not IgE-binding proteins, some workers refer to them as allergens, and they are treated as such in the WHO Codex Alimentarius Commission Labelling regulations.

Almost all allergens are proteinaceous in nature, as other polymers, such as polysaccharides, usually generate only poor antibody responses. There is

limited evidence that plant polysaccharides can act as allergens, although it is often unclear whether such reactions result from protein contamination, as some plant cell-wall associated proteins have been found to be allergens (Lunardi *et al*., 2000). IgE binding *per se* is not indicative of an allergen functioning to elicit an allergic response. Thus, peanut allergic patients have serum IgE which cross-reacts with soy proteins but generally suffer no allergic reaction when they eat soya containing foods (Eigenmann *et al.,* 1996). Multivalency is essential for an allergen to trigger release of histamine and other inflammatory mediators and it is thought that whilst carbohydrate determinants are widely found and can elicit IgE responses, they are largely monovalent and hence are generally unable to trigger histamine release (Alberse, 1998). On the basis of the frequency of reaction, allergens have also been classified as being either major (an allergen recognised by IgE from 50% of a group of at least ten allergic patients), or minor (Larsen and Lowenstein, 1996). However, such a classification gives no indication of allergenic potency and is questioned by some (Alberse, 2000).

Only around eight types of foods are responsible for causing the majority of food allergies, including cow's milk, egg, peanuts, tree nuts, wheat, fish, shellfish and soya (Bush and Hefle, 1996). What follows is a brief overview of these allergens, followed by a summary of their properties which are relevant to the issues of reducing their levels in foods.

17.2 Protein allergens of animal origin

17.2.1 Cow's milk

Both whey proteins, which include β-lactoglobulin and α-lactalbumin, and caseins have been characterised as allergenic fractions (Wal, 1998). β-lactoglobulin is a member of the lipocalin superfamily, to which a number of other inhalant allergens belong (Mantyjarvi *et al*., 2000). It is highly resistant to proteolysis and is taken up in an intact form by the gut in experimental systems, a factor which is thought to contribute to its allergenicity. The IgE epitopes have been identified in β-lactoglobulin with four main IgE binding regions being located on the more mobile surface loops of β-lactoglobulin. Caseins include the α_{s1}-, α_{s2}-, β- and κ-caseins, which have a low level of sequence identity but are all relatively unstructured phosphoproteins with a high proline content. Caseins retain their IgE reactivity irrespective of their phosphorylation state. The IgE binding regions have been identified in caseins with six major and three minor epitopes found for α_{s1}-casein (Chatchatee *et al*., 2001a), four major and six minor epitopes found for α_{s2}-casein (Busse *et al*., 2001), six major and three minor epitopes for β-casein and eight major epitopes for κ-casein (Chatchatee *et al*., 2001b). These epitopes are thermostable, possibly a reflection of the rheomorphic character of this protein. IgE binding to two epitopes on α_{s1}-casein, one on β-casein and two on κ-casein correlated with persistent cow's milk allergy (Jarvinen *et al*., 2002).

17.2.2 Egg

The major allergens of egg originate from the white and include the glycoproteins ovomucoid (*Gal d 1*) and ovalbumin (*Gal d 2*), which make up 10% and 50% of egg white protein respectively, together with two minor allergens, ovotransferrin (*Gal d 3*) and lysozyme (*Gal d 4*). Whilst around 25% of the mass of ovomucoid comprises carbohydrate, none of the seven IgE binding regions encompass glycosylation sites. The nature of the IgE epitopes appear to change during the development of egg allergy, with conformational epitopes appearing in newly acquired sensitivities and linear epitopes being more important in long-standing egg allergy. Seven IgE-binding regions have been identified in ovalbumin clustering at the *N*- and *C*-terminal regions, which are resistant to enzymic digestion and denaturation (Cooke and Sampson, 1997).

17.2.3 Fish

The major fish allergen is parvalbumin (*Gad c 1)*, a calcium buffer protein in fast muscle unique to fish and amphibians (Bugajska-Scretter *et al.,* 1998). It is highly conserved across fish species, a factor responsible for the cross-reactive nature of allergens in fish such as cod, salmon, mackerel, herring and plaice. Parvalbumin is a member of the EF-hand superfamily of proteins, as it contains an EF-hand calcium binding motif. *Gad c 1* has five IgE-binding regions evenly distributed along the length of the protein, one of which encompasses one of the Ca^{2+} binding sites, and indeed the holo-form is more IgE-reactive. Like other calcium binding proteins it is thermostable, its stability increasing when it is binding Ca^{2+}.

17.2.4 Shell-fish and seafood

Another heat-stable muscle protein, tropomyosin, is the major allergen in crustacea and other shell-fish. As well as being found in cooked meat, the allergen is highly soluble and leaches into cooking water, or even spray found in fish markets. Tropomyosins show a high degree of homology across commonly edible crustaceans, with lower homology to vertebrate tropomyosins. There is cross-reactivity between the tropomyosins of crustacea and mollusca (Leung *et al*., 1996). Two main linear IgE-binding sites were identified in the shrimp allergen, *Pen i 1* (Shanti *et al*., 1993). The *N*-terminal epitope showed very little identity with vertebrate tropomyosins while the C-terminal epitope was similar except for first two residues, which are conserved as FL in arthropods and HI in vertebrates, and were thus suggested to be crucial for IgE binding (Leung *et al*., 1996).

More recently, studies of Pen a 1 have defined eight partially overlapping epitopes (Reese *et al*., 1997; Ayuso *et al*., 2002). These epitopes show some correlation with the imperfect repetitions in the sequence of tropomyosin. The first epitopes of Pen a 1 and Pen i 1 overlap while the initial phenylalanine of the second epitope of Pen i 1 is the last residue of the fourth epitope of Pen a 1.

Ayuso *et al*. (2002) noted that the epitope regions of Pen a 1 are well conserved in the tropomyosins of other invertebrates and suggested that this causes the clinically relevant cross-reactivity between various types of seafood including shrimps, lobsters, crab, squib and abalone, as well as inhalant allergens from insects such as cockroaches. By contrast, there is no cross-reactivity between IgE from shell-fish allergic individuals, and vertebrate muscle tropomyosins.

17.3 Protein allergens of plant origin

17.3.1 Wheat

Whilst there is a widespread perception that wheat is responsible for a large proportion of food allergies, the role of cereal proteins in food allergy is poorly characterised, although their role in occupational allergies such as baker's asthma and in coeliac disease is well defined. Most wheat allergens are either prolamin storage proteins or belong to the family of α-amylase/trypsin inhibitors. The former are an extensive family of proteins characterized by their solubility in aqueous alcohols and insolubility in dilute salt solutions, M_rs range from about 10,000 to 100,000 and are rich in proline and glutamine (Shewry and Tatham, 1990, 1999). Almost all contain regions that consist either of repeated peptide motifs or are enriched in specific amino acids (especially proline and glutamine) and are usually accompanied by one or more non-repetitive domains that contain most or all of the cysteine residues. Allergens belonging to the prolamin seed storage proteins have been identified, particularly in relation to atopic dermatitis (Maruyama *et al.*, 1998; Takizawa *et al*., 2001; Tanabe *et al*., 1996b) including a γ-gliadin and an α-gliadin involved in exercise induced anaphylaxis (Palosuo *et al.,* 1999). Allergens belonging to the cereal inhibitor family can be involved in both respiratory and food allergies, the most active in baker's asthma being the glycosylated forms of subunits of tetrameric α-amylase inhibitors (Carbonero and García-Olmedo, 1999). The same allergens have been characterised in orally sensitised wheat allergic individuals, although only one M_r ~15,000 subunit was involved (James *et al.,* 1997).

17.3.2 Peanuts, nuts, seeds and soybean

The major allergens in these plant-based foods are generally the seed storage proteins, and comprise the 11S-12S (legumin-like) and 7S (vicilin-like) globulins, and the 2S albumins. The globulins have homologous three-dimensional structures and are members of the cupin superfamily, sharing a β-barrel structure (Lawrence *et al*., 1994, Dunwell, 1998). Oligomeric proteins, produced from multigene families, legumin-like globulins have M_rs of about 300,000–450,000 and consist of six subunits of M_r about 60,000, associated by non-covalent forces. Each subunit is post-translationally processed to give rise to acidic (M_r about 40,000) and basic (M_r about 20,000) chains, which are linked

by a single disulphide bond. In contrast the 7/8S globulins are typically trimeric proteins of M_r about 150,000–190,000, with subunit M_rs ranging from about 40,000–80,000 but usually about 50,000. Whilst the 11/12S globulins are rarely, if ever, glycosylated, the vicilins frequently are.

Major allergens belonging to this group of proteins include the 11S and 7S globulins of soy (Shibasaki *et al.,* 1980, Burks *et al.,* 1988; Rabjohn *et al.,* 1999) peanut (Burks *et al.,* 1991, 1992), together with the 7S globulins of walnut (Teuber *et al.,* 1999), sesame (Beyer *et al.,* 2002a), cashew nut (Ana c 1) (Wang *et al.,* 2002), hazelnut (Beyer *et al*., 2002b) and one of the subunits of the proteolytically processed 7S globulin of lentil (Sánchez-Monge *et al.,* 2000). The 11S globulins have also been confirmed as an allergen in almond (also known as almond major protein, AMP) (Roux *et al.,* 2001) and implicated as allergens in coconut and walnut (Teuber and Petersen, 1999).

2S albumin storage proteins may accompany the globulins but are much smaller proteins. Synthesised as single chains of M_r 10,000–15,000 they are post-translationally processed to give small and large subunits which usually remain joined by disulphide bonds. 2S albumins were among the first plant proteins to be described as allergens, over 50 years ago (Youle and Huang, 1981), they have been identified as allergens from mustard seed, Sin a 1, (Menendez-Arias *et al.,* 1988), Bra j 1e (Monsalve *et al.,* 1993), peanut (Burks *et al.,* 1992), Brazil nut (Pastorello *et al.,* 1998b), almond (Poltronieri *et al.,* 2002), sunflower (Kelly *et al.,* 2000), walnut (Teuber *et al*., 1998) and, most recently, sesame (Pastorello *et al.,* 2001b). It has also been reported that the 2S albumins of soy (Shibasaki *et al.,* 1980) and chickpea (Vioque *et al.,* 1999) are allergens.

The non-specific lipid transfer proteins (ns LTPs) are also members of the prolamin superfamily and have been characterised as food allergens from several nut and seed species, including peach, apple, hazelnut and sesame (Pastorello *et al*., 2001a; Sánchez-Monge *et al*., 1999). Whilst not especially abundant in fruits or seeds, ns LTPs can become concentrated in epidermal tissues and associated with the waxy layers (Douliez *et al.,* 2000). They are thought to play a role in the synthesis of the waxy cuticle of fruits and have been designated pathogenesis related (PR) proteins as they form part of a broad spectrum of proteins, which are synthesised in plants in response to infection, damage or stress.

The cysteine protease superfamily includes a major soybean allergen. This is the 34 kDa protein, which was originally misidentified as an oil body-associated protein, and is known variously as Gly m Bd 30K, Gly m 1, or P34 (Ogawa *et al.,* 1993). Gly m Bd 30K is probably not an active protease but may be part of the PR system of soya (Ji *et al*., 1998). However, Act c 1 is the major allergen from kiwi and is an active cysteine protease (Pastorello *et al*., 1998a). Gly m Bd 30K exists as complex of molecular weight >300,000, as it becomes disulphide-linked to the α, α' subunits of the soya globulin β-conglycinin (Ogawa *et al*., 1993; Samoto *et al.,* 1996) and is *N*-glycosylated (Bando *et al.,* 1996).

17.3.3 Allergens involved in cross-reactive allergy syndromes

Individuals sensitised to inhalants such as pollen and latex may suffer from food allergies as a result of IgE developed to pollen or latex proteins then binding to homologous proteins found in foods. These have been well characterised for pollen-fruit/vegetable and latex-fruit allergies (Breiteneder and Ebner, 2000) as well egg allergies in bird fanciers who become sensitised to feathers (Anibarro *et al*., 1991) and dust mite allergies which can result in reactions to arthropod and mollusc foods such as snails (Leung *et al*., 1996).

Pollen-fruit/vegetable allergy syndrome

The major birch pollen allergen, Bet v 1, is related to a class of putative plant defence proteins (PR10) and results in dietary reaction to proteins in a range of fruit (including Mal d 1 of apple and Pru av 1 of cherry) and vegetables (including Api g 1 of celery, Dau c 1 of carrot). Although the biological role of PR10 proteins has not been established, they are presumed to be defensive (Hoffman-Sommergruber 2002). A further type of PR protein (PR-5) which is related to the sweet protein thaumatin is an allergen in fruits such as apple (Mal d 2), cherry (Pru av 2). A second major pollen allergen, Bet v 2, is a profilin, a protein which binds to the actin cytoskeleton of cells. Sensitisation to this pollen protein results in allergic reactions to proteins in various plant foods including hazelnut, peanut (Ara h 5), soybean (Gly m 3), celery (Api g 4) and pear (Pyr c 4).

Latex-fruit allergy syndrome

Inhalation of two other PR-type proteins from latex can also result in dietary allergy to related proteins in fruit and vegetables. These are PR-2/Hev b 2 (β1,3–glucanase) and PR-3/Hev b 6 (class II chitinases) which result in cross-reactive allergies to foods such as avocado, chestnut, banana, fig, kiwi and other foods.

17.4 General properties of protein allergens: abundance, structural stability and epitopes

Allergens found in foods that cause allergies as a result of oral sensitisation seem in general to possess two properties, abundance in the food and/or structural stability. Thus many of such food allergens are found in major foods which comprise a large proportion of the human diet (eggs, milk) or are abundant proteins in a food. Thus, the major allergens of milk, egg, fish, and peanuts are all highly abundant, comprising 25–50% of the protein in the original food. Many allergens are stable proteins, resisting thermal denaturation and/or proteolysis and include many cupin allergens (Mills *et al*., 2003), members of the prolamin superfamily such as the ns LTPs (Douliez *et al*., 2000), cow's milk β-lactoglobulin (Wal, 1998), cod parvalbumin (Bugajska-Scretter *et al.,* 1998) and hen egg ovomucoid (Cooke and Sampson, 1997). Whilst many food allergens share both these attributes, some such as the caseins, are abundant but

proteolytically labile proteins, whilst the ns LTPs are not especially abundant but are remarkably stable.

One reason underlying such common characteristics, may be that for a food allergen to sensitise an individual via the gastrointestinal tract it must possess properties which preserve its structure from the degradation in the gastrointestinal tract, such as resistance to low pH, proteolysis, and surfactants such as bile salts. This is required to ensure that sufficient intact (or semi-intact) protein survives to be taken up by the gut and sensitise the mucosal immune system. However, allergens involved in cross-reactive allergy syndromes do not share such properties, but have more in common with inhalant allergens, being readily soluble in physiological buffers, being more thermolabile and readily digested proteins. Consequently, whilst they are able to elicit an allergic reaction they are often unable to sensitise an individual through ingestion. Susceptibility to digestion by protease has become a matter for debate in recent times and has been reviewed elsewhere (Mills *et al*., in press). Thus, fruit allergens belonging to the Bet v 1 family of proteins which are involved in the pollen-fruit allergy syndrome are considered to be 'incomplete' allergens, as ingestion of fruit itself does not stimulate an IgE response, but does trigger histamine release from mast cells loaded with IgE to the birch pollen allergen Bet v 1 (Alberse, 2000).

17.4.1 Epitopes and allergenicity of food

Allergic reactions to foods are in fact allergic reactions to individual food components, in most cases proteins (Lehrer *et al*., 1996), but sometimes also carbohydrates (Jankiewicz *et al*., 1997). Allergens are often proteins with molecular masses as determined by SDS PAGE of 8–70 kDa. They may occur as larger multimers such as the peanut allergen *Ara* h 1 (Lehrer *et al*., 1996; Hefle, 1996; Taylor and Lehrer, 1996). Small regions of allergens, called epitopes (also known as B-cell epitopes), provoke the IgE-mediated allergic response (Lehrer *et al*., 1996; Hefle, 1996; Taylor and Lehrer, 1996; Taylor and Hefle, 2001).

Food allergens and their epitopes may be relatively resistant and able to withstand the effects of food processing and digestion (Taylor *et al*., 1987, Taylor and Hefle, 2001). The integrity of conformational epitopes is not exclusively determined by their primary structure. These epitopes are also characterised by the tertiary structure of a protein, and their integrity therefore depends on the thermodynamics that determines tertiary protein structure (Lehrer *et al*., 1996).

Despite the relative stability of allergens and epitopes to digestion and processing, knowledge of the epitope structure and the factors that determine its chemical and physical stability can lead to the design of specific and well directed approaches to decrease food allergenicity. Structural similarities between allergens and their epitopes may lead to the development of more generic approaches to alter their allergenicity. In this context, it is of paramount importance that better knowledge is gained on structural similarities between

allergens from various origins, for instance as described by Mills *et al*. (2003). Plant allergens can be classified in a relative limited number of groups, based e.g. on their biological function as storage proteins (peanut, soy, many tree nuts) or as PR-related proteins (Midoro-Horiuti *et al*., 2001; Hoffmann-Sommergruber, 2002). The emerging genomics and proteomics technology is likely to accelerate the structural study of allergens.

In addition to the B-cell epitopes which correspond to the sites on a protein which are recognised by an antibody, another set of epitopes, known as T-cell epitopes are necessary for generation of humoral immune responses. They are generally short continuous sequences of 12 to 18 residues (Lehrer *et al*., 1996, Matsuda and Nakamura, 1993) and are diverse in nature (Cooke and Sampson, 1997; Mizumachi and Kurisaki, 2003). As a consequence of their short length food processing is much less likely to affect them than the IgE (B-cell) epitopes, and this is borne out, at least for enzymatic hydrolysis which can destroy B-cell but not T-cell epitopes in, for example, peanut proteins (Hong *et al*., 1999). However, it is clear that chemical modification of protein will reduce the number of possible T-cell epitopes, since the HLA molecule binds most modified peptides only weakly, and hence processing induced changes, such as Maillard modifications, may have the potential to inactivate some T-cell epitopes.

17.5 Factors affecting protein allergenicity in raw foods

17.5.1 Genetics and breeding for low allergen foods

As the major food allergens of animal origin seem to be highly conserved across the limited number of species which are consumed, breeding is not a viable option for reducing the levels of such allergens. Thus β-lactoglobulin is similar in cow's, goat's and sheep's milk and triggers reactions in cow's milk allergic individuals. Similarly the conservation of major fish and shell-fish allergens means that in general individuals become allergic to many fish species (Pascual *et al*., 1992).

The situation is more promising with plant-based foods. A number of plant protein allergens belong to multigene families where a number of isoforms differing in only a few amino acid residues may be present. In addition the expression of different isoforms may vary between cultivars. Substitution of Ser^{111} with a Pro residue removed a pollen-fruit cross-reactive epitope in the apple allergen *Mal d 1*, indicating that point mutations in critical regions can be highly effective in removing IgE reactivity from an allergen (Son *et al*., 1999). An analysis of IgE-reactivity showed that Mal d 1 IgE binding was demonstrably higher in certain apple cultivars and that one variant, *Mal d 1a,* had a higher IgE-binding capacity than another variant *Mal d 1b*, which differed by only 15 amino acid residues. In kiwi fruit the levels of actinidin have been shown to vary widely between cultivars, suggesting that breeding may be a way of reducing the allergens in these fruits (Nishiyama and Oota, 2002), although

there appears to be little cultivar variation in Gly m Bd 30K in soybeans (Yaklich *et al.*, 1999).

An extensive screen of peanut accessions from S America and the USA for IgE-binding protein content, using pooled human allergic sera showed a wide variation in allergen levels. Two accessions were identified with lower allergen contents, although no 'null' allergen lines could be identified (Dodo *et al*., 2002). Thus there is undoubtedly the potential in the future for plant breeders to select for reduced allergenicity in raw plant-based foods.

17.5.2 Low-allergen plant foods produced by genetic modification

In the past few years, a number of examples have been described of successful attempts to reduce allergenicity via the application of molecular genetic techniques. Almost a decade ago, the major allergen from rice, an α-amylase inhibitor, was knocked out by means of antisense-technology (Adachi *et al*., 1993; Tada *et al*., 1996). The newly acquired trait was stably inherited in at least three generations (Tada *et al*., 1996).

In many cases, cross-reactions between respiratory and food allergies exist. In the case of rice pollen allergy, immunoblotting revealed major allergens of 16, 26 and 32 kDa (Tsai *et al*., 1990). Also situation cross-reactivity may be a significant factor in the pathology, as cross-reactivity between major allergens from rice and rye grass (*Lolium perenne*) has been demonstrated (Xu *et al*., 1995). The 16 kDa allergen may be a connecting factor in allergic cross-reactions between various members of the cereal Poaceae family, including maize (Urisu *et al*., 1991; Yamada *et al*., 1991). Recently, a genetically engineered variety of *Lolium* was described, in which the Lol p 5 allergen was knocked out via an antisense construct. Hypoallergenicity was demonstrated with immunoblots and low IgE-binding (Bhalla *et al*., 1999).

To decrease the allergenicity of soy, both mutagenesis and genetic modification have been applied successfully. The major allergens in soy are the storage proteins Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K. The allergenicity of soybean and soybean products was reduced by a combination of mutation breeding, physico-chemical treatment and enzymatic digestion. Gly m Bd 60K and Gly m Bd 28K were eliminated by development of a mutant line, while the strongest allergen, Gly m Bd 30K was salted out or destroyed by enzymatic digestion. Approximately 80% of soy sensitive patients could ingest products that were produced from this hypoallergenic soy, without adverse reactions (Ogawa *et al*., 2000).

Very recently, a hypoallergenic soy variety was developed making use of antisense technology, in which Gly m Bd 30K was knocked out. Although preliminary tests with patient's sera could not detect the allergen in knockout beans, more tests are needed to investigate the claimed hypoallergenicity, or decreased allergy-causing. The yield of the new variety, based on cultivar 'Jack', looked normal. The plants developed normally, the seeds set at a normal rate, and seemed to have the same protein and oil composition suggesting that

silencing P34 does not interfere with the plant's agronomic characteristics (Suszkiw, 2002).

For peanut, attempts have been made to reduce the allergenicity of Ara h 1 via replacement of epitope-located amino acids (Burks *et al*., 1999). Indeed reduced IgE-binding was achieved. As most allergenic epitopes are located in the hydrophobic regions of Ara h 1 that are involved in the integrity of the trimeric structure, the possibility cannot be ruled out that the change in epitope structure affects the physiological functioning of the protein (Bannon *et al*., 1999). In addition, peanut allergy will remain a notoriously recalcitrant food allergy since peanut contains a number of other major allergens that are recognised by large groups of patients (Kleber *et al*., 1999).

From the foregoing examples, the conclusion can be drawn that DNA technology can be successfully applied for reduction of allergenicity of raw materials for food production. However, for foods where there are multiple allergens which are highly abundant in edible plant tissues, the costs of producing the hypoallergenic food may be prohibitive, and there would likely to be regulatory hurdles as such a modified food will, of its very nature, not be 'substantially equivalent' and hence undergo even closer scrutiny in the regulatory process before it could reach the market place.

17.5.3 Post-harvest storage

Many plant-based foods are stored prior to further processing or consumption. This is particularly important in fresh fruits and vegetables. Allergen levels appear to increase in apples following harvest and during storage at 4ºC over 130 days probably because of their association with the ripening process, and the fact that they are 'PR' proteins and hence synthesised in response to environmental stress. This is especially well characterised in apples, where ripening has been shown to result in an increase in allergens (Vieths *et al*., 1993). Storage under modified atmospheres can reduce these increases in allergen levels, as might be anticipated from its ability to delay fruit ripening. Such storage was also found to result in changes, especially in the thaumatin-like protein, a minor apple allergen which is also a PR-protein (Li-Shan *et al*., 1995). Recent work in our laboratory has shown that levels of the apple allergens Mal d 1 and Mal d 3 (a ns LTP allergen) both decrease on storage of fruits for 3–5 months under modified atmosphere. However this may not extend to all climacteric fruits, as a limited study on the storage of mango fruits showed no alteration in levels of allergens (Paschke *et al*., 2001).

17.6 Reducing protein allergenicity during food processing

17.6.1 Removal of allergens

Processing has the potential to reduce the allergenicity (IgE-reactivity) of foods in two ways. Firstly, in plant-based foods where allergens may only be

expressed in certain tissues, the allergen load of a final processed food can be reduced by removing the tissue. This has been employed to particular effect in reducing the allergenicity of peach juice. Ns LTPs allergens normally find their way into juices as the peel is included in the juicing process. However, by first removing peach skins, the IgE reactivity of juices could be significantly reduced. This approach was particularly effective when a chemical lying procedure was employed (Brenna *et al*., 2000). *Psylium* husk was used as a functional ingredient in cereals because of its ability to lower cholesterol, but caused adverse reactions in individuals who had become sensitised to it through its use in bulk laxatives. By modifying the milling process to remove the parts of the seed coat which contain the allergens, the problem was overcome (Simmons, 1993). Lastly, such an approach has been used, in combination with proteolysis, to produce improved hypoallergenic infant formulas (van Beresteijn *et al*., 1994). Residual intact allergen in such preparations can cause reactions in some milk-allergic infants, but by employing an ultrafiltration, larger immunologically active allergen fragments can be removed.

17.6.2 Modification of allergens by thermal treatments

The second way in which processing (particularly thermal) can remove allergens is through denaturing the allergen, in particular by destroying conformational IgE epitopes. However, it is emerging that thermal process can both reduce and increase food protein allergenicity, depending on the food concerned. There may also be thermostable IgE epitopes in food proteins which are unaltered by thermal processing. One example of these is the carbohydrate residues on glycoproteins which remain unaltered by thermal processing. However, there is some doubt over the importance of carbohydrate determinants in allergy because their sparse nature means they do not offer the polyvalency necessary IgE cross-linking on mast cells and subsequent histamine release (Alberse, 1998).

A number of food proteins do not adopt a compact globular three-dimensional structure, but are instead highly mobile, adopting an ensemble of conformations and their secondary structure does not melt on heating in the same fashion as globular proteins. These include the prolamin (gluten) proteins from wheat and caseins from milk. The IgE-epitopes on such proteins tend to be linear in nature and will therefore be less affected by thermal treatments than the conformational epitopes which tend to predominate on globular proteins. In addition, as summarised in section 17.1, many food allergens tend to have very stable three-dimensional structures, a property which includes thermostability. There is also evidence that whilst many of highly disulphide bonded plant proteins, like ns LTPs and 2S albumins, unfold on heating they refold on cooling to an almost native structure. Hence many allergenic proteins may be able to retain both linear and conformational epitopes following mild heat-treatments below 60 ºC, and their ability to trigger an allergic reaction in a sensitised individual will be essentially unaltered. This applies to many allergens, including those from soya, egg and shell-fish meat, which form the aggregates

on heating which become incorporated into heat-set gel networks. However, due to the difficulties of working with such insoluble proteins, the IgE binding of such aggregates has not been widely studied.

For example, Ara h 1 of peanut is remarkably thermostable, unfolding to only a limited extent with many core elements in the structure remaining, as is observed for other seed storage globulins (Koppleman *et al*., 1999). IgE binding to wet or dry-heat denatured protein was essentially unchanged leading to the suggestion that either Ara h 1 does not contain any conformational epitopes, or that they are restricted to the thermostable regions of the structure. The secondary structure of another allergen, patatin (Sola t 1, previously Sol t 1) from potato, was also essentially unaltered following heating to 65 ºC, as even after denaturation, it can refold on cooling (Koppleman *et al*., 2002). Biophysical studies showed that the loss of IgE-binding capacity on cooking results from aggregation with other potato proteins (protease inhibitors). This illustrates the importance of taking account of the behaviour of food allergens in the food matrix itself, as other components may affect the behaviour of the allergens themselves. However, as well as removing IgE epitopes, the unfolding and subsequent aggregation processes which occur in many food proteins on thermal processing, offers the possibility of introducing new epitope sites.

In summary, many of the cross-reactive allergens, such as the Bet v 1 homologues involved in the pollen-fruit allergy syndrome, are labile proteins and are generally destroyed by cooking; allergic reactions being generally confined to raw produce. There are exceptions to this, notably a soybean homologue, SAM22, has been found to cause allergies in a processed soya-based food supplement (Kleine-Tebbe *et al*., 2002). Reports of sensitivity only towards processed foods are rare. One example of processing induced increases in allergenic activity was described early in the history of food allergy research, when Kustner reported his own sensitivity towards cooked but not raw, fish (Prausnitz and Kustner, 1921). In general individuals are sensitive towards both the raw and processed foods, but thermal processing may modify the degree of reactivity. Thus heating has been found to reduce the allergenicity of beef and purified bovine allergens (Fiocchi *et al*., 1995).

Food proteins also undergo chemical modification during thermal treatments, in particular the formation of Maillard browning adducts following severe, dry heat treatments, and during ageing of foods, which may affect food protein allergenicity (Davis *et al*., 2001). However, the technical difficulty of working with large aggregated protein systems has meant that the characterisation of such processing-induced epitopes has been difficult. Maillard modified peanut allergens *Ara h 1* and *Ara h 2* become cross-linked to form high molecular weight aggregates which bind IgE more effectively than unmodified allergens, and are also more resistant to gastric digestion (Maleki *et al*., 2000a). The allergic IgE binding towards peanut allergens Ara h 1, 2 and 3 appears to be lower in boiled and fried, compared with roasted peanuts (Beyer, 2001) indicating that certain types of thermal

processing can introduce additional IgE binding sites. There is also a case report of pecan nut allergenicity, where the allergenic reactivity of the nut increased over time, and whilst Maillard adducts were not identified as the causative agents, it seems likely that these, or some other ageing related oxidative reaction increased the allergenicity of the nut on storage (Berrens, 1996). Other thermally induced modification, such as lactosylation of milk proteins, may play a part in altering the allergenic activity of dairy products, as the allergenic activity of β-lactoglobulin has been found to increase 100–fold following heating in the presence of lactose (Bleumink and Berrens, 1966). Thus, whilst processing of allergenic foods may not remove their allergenicity, it would increasingly appear that there are ways of reducing the allergenic potential of foods, such as peanuts, through the application of specific processing methods.

17.6.3 Novel processing

High-pressure processing (100–400 MPa) was used to decrease the allergenicity of rice. Under these conditions, polished rice grains released a considerable amount of proteins (0.2–0.5 mg per gram of grains). The major proteins released were identified, with SDS-PAGE and immunoblotting, as 16 kDa albumin, α-globulin, and 33 kDa globulin, which were known as major rice allergens. Scanning electron microscopic study of rice grains pressurised at 300 MPa demonstrated morphological changes in endosperm cells. No apparent structural changes in protein bodies were detected. The levels of the allergenic proteins mentioned by these workers were decreased by pressure treatment. Pressurisation processing in the presence of a proteolytic enzyme led to almost complete removal of the allergens. The effects can be explained by pressurisation-induced partial destruction of endosperm cells leading to enhanced permeation of the surrounding solution into rice grains. As a result, a part of the proteins is solubilised and released into the surrounding solution (Kato *et al*., 2000).

That high-pressure treatment is not a guarantee of success is illustrated by the unsuccessful attempt (600 MPa, 20 ºC) to reduce the IgE-binding capacity of Api g 1 from celery. Neither pulsed electric field treatment (10 kV, 50 Hz), nor γ-irradiation (10 kGy), were effective in this respect (Jankiewicz *et al*., 1997).

Apparently, a complex of factors such as specific requirements for allergen structure, processing effects on tissue integrity and embedding of the allergens in the tissue matrix, determine the potential effectiveness of novel processing methods. Physical processing holds promise to reduce allergenicity in a number of cases. Better understanding of the structural relationships between various food allergens on the one hand, and respiratory and food allergens on the other hand, has the potential to rationalise and target the design of novel processing strategies for allergenicity reduction.

17.7 Reducing protein allergenicity using enzymatic processing

17.7.1 Removal of epitopes

Prerequisites for effective reduction of allergenicity via proteolytic enzymatic treatment are sufficient contact between enzyme and target (usually implying a largely destroyed macrostructure in the food product) and sufficient substrate specificity of the protease. An important factor to take into account is to minimise the potential decrease of desirable sensory and processing properties, such as, e.g., foaming or baking properties, of the product.

Examples of unsuccessful proteolytic treatment are the difficulties encountered in reducing the allergenicity of the peanut allergen Ara h 1 (Maleki *et al*., 2000b) and the major peach allergen (Brenna *et al*., 2000). In both cases, insufficient contact between allergen and protease explains the lack of success either because of the compact allergen structure and/or in the case of peach, intact tissue structure. Also, allergen specific characteristics may play a role in both studied cases. Ara h 1 carries at least 23 different relatively short epitopes (Burks *et al*., 1997), making it complicated to identify a suitable protease or protease cocktail that destroys sufficient allergenic epitopes. The major peach allergen (variously named Pru p 1 or Pru p 3) is a lipid transfer protein, a group of proteins characterised by a compact structure, which is difficult for proteases to access.

More success has been achieved in reducing wheat flour allergenicity with bromelain (Tanabe *et al*., 1996a; Watanabe *et al*., 1995). An important factor was the proper match between epitope structure (Q-X-Y-P-P) and protease specificity. Side effects on processing properties were manageable, as it was still possible to produce muffins from this flour (Tanabe *et al*., 1996a). Protease specificity also was a key factor in the treatment of soybeans with proteases, to reduce allergenicity. Of eight proteases tested two (Proleather and Protease N) were found to be effective in reducing IgE binding in soybean allergic patients. Immunoblotting with a monoclonal antibody showed that at least a major allergen (*Gly m* Bd 30K) was affected (Yamanishi *et al*., 1996). As the experiments were performed on autoclaved soybeans, next to substrate specificity, also parameters such as tissue penetration and diffusion characteristics may have played a role.

Another success story is the enzymatic reduction of rice allergenicity. Watanabe *et al*. (1990a) and Watanabe (1993) used actinase, in combination with glycerin monooleate to facilitate endosperm penetration. The treatment resulted in a negative RAST-test, and no symptoms in six out of seven patients to which the product was clinically administered. Treatment with hydrochloric acid resulted in improved colour, while a favourable texture (stickiness/hardness score) was maintained (Watanabe *et al*., 1990b).

Trypsin, elastase, and a protease mixture were successfully used to decrease the allergenicity of hazelnut allergens in a crude protein extract, as demonstrated via immunoblot and EAST-inhibition. Pancreatin and pepsin appeared less

effective in this process (Wigotzky, 2001), again suggesting that a good match between protease and allergen is important.

The conclusion seems obvious that there is a potential in protease treatment to reduce allergenicity. It is difficult to draw abstract conclusions at this stage, because of the variety in allergens and proteases on which data was reported. Additionally, factors such as ease of penetration, diffusional properties, protease specificity, etc., are to be considered. Other enzymes that have the potential to be used for this purpose are various oxidases, which contribute for instance to reduction of apple allergenicity (Wichers *et al*., publication in preparation).

17.7.2 Masking of epitopes?

Cross-linking of entire proteins, e.g., enzymatically, has resulted in the production of less allergenic food materials. Yamauchi *et al.* (1991) patented the cross-linking of α_{s1}-casein by transglutaminase treatment. The cross-linked product was claimed to be less allergenic. Also wheat flour was successfully treated with transglutaminase for this purpose (Watanabe *et al*., 1994). Although the mechanism by which the reduction in allergenicity was achieved is not *a priori* clear, it can be speculated that the cross-linking process will result in the 'embedding' of epitopes in the polypeptide chain, thus making them less accessible to the immune system.

17.8 Future trends: low allergen proteins

With rapid progress in the new research fields of genomics and in particular proteomics, it can be envisaged that our understanding of allergen structure and relationships between allergens from various sources will rapidly improve. This will shed light on allergic cross-reactions, for instance between respiratory and food allergies, and allergies for different food products. More knowledge will allow us to make better predictions of potential allergenicity.

Better understanding of allergen structure will also open up the possibility to screen in for instance germplasm collections *in silico* for varieties with improved properties from an allergy perspective. Such an approach might in certain cases allow avoiding the use of genetic modification, despite the promising potential of this discipline for production of hypoallergenic foods (see above). It is of note in this respect, that in many cases proteins do not lose their biological function when an antigenic or allergenic epitope is modified (Burks *et al*., 1999; Soler-Rivas and Wichers, 2001), at least suggesting that there is a fair chance that agronomical traits do not necessarily have to be influenced negatively if hypoallergenic varieties are selected.

In addition, improvements in the understanding of the relationships between protein structure and processing behaviour will allow the development of better directed food technology, also towards reduction of allergenicity. It is questionable whether food technology as such, even when better directed

technologies can be developed with the help of improved raw material knowledge, will be sufficient in all cases to design hypoallergenic foods. However, better understanding of allergen structure and the impact of processing thereon, will also allow the development of better analytical systems to monitor the allergenic nature of food products throughout the food (processing) chain, and thus to better quality control and labelling.

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Part III

Applications

18

Using proteins as additives in foods: an introduction

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18.1 Introduction

Proteins are being used as ingredients in man-made food products because they contribute to one or more of the desired characteristics of that food product. These characteristics might be consumer related (e.g. texture, mouthfeel, appearance, taste) or technology related. The latter includes both storage (shelf life, palatability) and processing (e.g. mixing behaviour, foam, emulsion or gel formation). Proteins contribute to one or more of these characteristics because of their functional properties. This term is mostly used to indicate physico-chemical properties that govern the performance and behaviour of a protein in food systems during preparation, processing, storage and consumption. (Kinsella and Whitehead, 1989). Because proteins often are also added to foods to improve the nutritional quality and, in fewer cases, to exert a physiological activity, protein properties relevant for these functionalities belong by definition also to these properties. A short overview of the different functionalities and examples of them is given in Table 18.1.

All proteins are built of amino acids in a certain sequence, determined by their encoding DNA. Many proteins are post translationally modified (e.g. glycosylated, phosphorylated, proteolytically processed) before they acquire their mature, native form. It is the exact composition, spatial structure and size that determines how proteins 'act' in food products, thus how they function. Extrinsic factors (like pH and temperature), the presence of other constituent (salts, surfactants, gums, water, etc.) and process treatments (also in the past) all can alter the performance. Therefore the functionality of a protein is largely affected by circumstances. The behaviour in model systems, as mostly discussed in books and papers, can be different from that in real food products. Reasons for this discrepancy are:

Table 18.1 An overview of the different classes of functionality. Adapted from van der Ven (2002)

Functional property	Type of property	Examples
Techno-functional	Solubility	Solubility Precipitation
	Bulk rheological	Thickening Gelling Texturing
	Surfactant (interfacial rheological)	Emulsifying Foaming
	Sensorial	Taste Bitterness
Bio-functional	Nutritional	Digestibility Hypoallergenicity Antimicrobial activity
	Physiological	ACE inhibition Opioid activity

- The interactions of the proteins with other components in the food product.
- The use of water by other components, so less water is available for the protein.
- Usually mixtures of proteins are being used. Most industrial protein preparations derived from a particular source (e.g. soy, egg, and milk) are mixtures of different types. Furthermore, in food different protein sources are often intentionally mixed, mostly for nutritious or economic reasons (Comfort and Howell, 2002).
- The purification or isolation process used may have irreversibly affected the behaviour of the protein.
- The structure of the food may cause local differences in composition.
- The exact treatment during processing may be inhomogeneous; an example of this is the effect of product size on the local temperature during heating.

Furthermore, the use of a protein in a product is governed by on the one hand economic considerations (costs of protein preparation and their handling, their availability and constancy in quality, etc.) and on the other hand by technological ones. Regarding the latter, the protein should contribute as much as possible to the optimal combination of functional properties that is required to reach the full pallet of desired product characteristics. For example, egg white, a rather expensive protein preparation, is used in meringues primarily because of its excellent foaming properties. However, its good gelling properties are also of major importance for this application. The combination of functional properties should be optimal; each separate functionality need not to be maximal. Some examples of techno-functionalities, typical food products in which these functionalities are exploited and examples of proteins that can exert the desired functionalities are listed in Table 18.2.

Table 18.2 An overview of the different techno-functionalities and examples in food systems

Functionality	Example of typical food product	Example of typical protein
Solubility	Infant formula	Whey protein concentrate
Thickening	Yoghurt, drinks, sauces	Caseinate
Gelling	Meat, bakery	Egg white
Emulsifying	Dressing, mayonnaise, ice cream, desserts,	Caseinate
Foaming	Whipped topping, desserts, ice cream	Chicken egg white
Taste	Coffee whitener	Na-caseinate

In this chapter we will discuss the properties of proteins involved in the different techno-functionalities. Successively we will pay attention to rheological properties, surfactant or interfacial-rheological properties and protein–flavour interactions (sections 18.2–4). In section 18.5 the relation between the different functionalities and protein structure (including modification thereof) is discussed. Protein properties largely depend on circumstances like temperature, pH, ion strength, other constituents as well as the process of purification. Other chapters in this book pay attention to these more product specific items.

18.2 Rheological properties of proteins

Rheology is the study of the deformation and flow of matter (Bourne, 2002). Rheological properties of protein dispersions address the behaviour regarding viscosity, shear thinning, deformability, gel stiffness and strength, fracturability, etc. These types of properties are important both for the behaviour of food during eating, as well as for food processing and handling.

Proteins, like other polymers, have the ability to thicken food products by increasing the viscosity or producing a gel, thereby increasing the texture of the foods as well as providing certain stability and water binding capacity. The exact behaviours of the proteins however differ enormously, primarily due to differences in iso-electric point, molecular weight and in volume occupied. The behaviour depends not only on the specific protein and the amount present, but also on environmental properties like the temperature, the pH of the product, the ionic strength and other constituents present. Below we will give a general overview on the rheological behaviour of protein dispersions, with special attention to the effect of concentration. Because solubility is of prime importance in determining these properties, attention will also be given to this functionality.

Other chapters in this book review the special properties of individual proteins and their reactions to (changing) circumstances. Historically much has been published on the properties of dairy proteins (e.g. Kinsella, 1984). Recently reviews have been presented on vegetable proteins with emphasis on soy, wheat (van Vliet *et al*., 2002) and legume proteins (Schwenke, 2001).

18.2.1 Solubility

The solubility of proteins is often a prerequisite for being functional. Theoretically, solubility is defined as the concentration of a pure compound in a saturated solution containing crystals. However, for practical reasons this condition is seldom met in the determination of the solubility of proteins. Therefore, solubility is not a well-defined property; it largely depends on the method used for its determination. The solubility of a protein strongly depends on the pH, temperature, ionic strength and the concentration. Near the iso-electric point (pI) proteins are least soluble because the net overall charge is close to zero and attraction between opposite charged parts of different protein molecules occurs. By screening the charges by increasing the ionic strength the solubility is enhanced; the so-called salting in effect. At very high ionic strength the opposite occurs. Solubility decreases due to changes in solvent quality thereby increasing hydrophobic interactions (salting out). Furthermore, protein properties are involved; aggregates of proteins often show decreased solubility in comparison to their non-aggregated forms. Also unfolding of the protein, occurring for instance during increase in temperature, decreases its solubility because of enhanced possibilities for hydrophobic interactions. The solubility of proteins can be enhanced by:

- adjusting the circumstances (pH, salt, etc.)
- adding polysaccharides, for example sugar
- changing the history of the proteins like the process of isolation
- modifying the protein, especially using hydrolysis to decrease the molecular weight (see also section 18.5).

18.2.2 Viscosity

Proteins, like all other macromolecules, have the ability to alter quickly (this means at low concentrations) the rheological properties of the systems. Ross-Murphy (1995) has given a general overview of the effect of biopolymers on the rheological properties of dispersions. In dilute dispersions, that is for very low concentrations, the proteins can be regarded as non-interacting separate particles. Generally the viscosity of a dispersion depends on the viscosity of the continuous phase and the total volume of particles. The latter depends on the concentration and the size of the proteins. From this relation it can be understood that the molecular weight (Mw), protein swelling and water binding are important. The intrinsic viscosity is affected by changing Mw, molecular structure or circumstances like pH, ionic strength, protein charge and solvent quality. The effects of extrinsic factors and treatments on the intrinsic viscosity have been published for different proteins, e.g. ovalbumin (Monkos, 2000), whey proteins (Rattray and Jelen, 1995), caseinate (Kinsella, 1984; Gup *et al*., 1996; Carr *et al*., 2002), bovine serum albumin (Monkos, 1996).

Above a certain concentration, the increase in viscosity with increasing concentration is much steeper, see Fig. 18.1. This concentration is called the

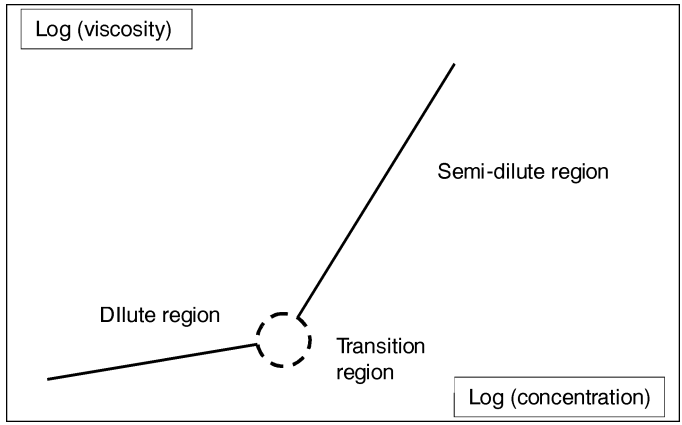

Fig. 18.1 The different regions illustrate the effect of concentration on the viscosity of protein dispersions.

critical concentration. Data for different proteins and extrinsic circumstances can be found in the literature. In practice, this critical concentration is difficult to define and as a result we have to deal with a transition region. The most important reason for this broadness of the transition is the range of molecular properties within a certain protein preparation, especially the molecular weight distribution.

At protein concentrations higher than the critical concentration, the protein molecules start to interpenetrate and form entanglements. This concentration region is called the semi-diluted region. Because of the interactions between the protein molecules, the viscosity behaviour of the dispersions become shear thinning. At higher rates of deformation the dispersions become apparently thinner. Both concentration dependency and shear thinning effects are more pronounced for proteins with higher molecular weights. At still higher concentrations, the proteins form gels in the system and the viscosity is no longer the most important property.

18.2.3 Gelling and texturing

A gel is a continuous three-dimensional network of connecting molecules or particles that entrap water (van Vliet *et al*., 1991). The structural network retains shape and has some mechanical strength (Kinsella and Whitehead, 1989). It is predominantly elastic on deformation during a relevant time-scale. The elastic modulus is relatively low, i.e., below about 10^6 Pa.

A general theory often presented to describe the different steps leading to gelation of protein dispersions starts with the unfolding of the protein, followed by irreversible denaturation and aggregation. During the first transition, functional groups become available for further reactions, because they become more exposed. The extent of this unfolding depends on the type of protein, but in

general the protein size and shape are minimally disturbed and affected. In the next stages the proteins interact with each other to form aggregates and ultimately networks, which may result in further loss of spatial structure. Recently Goscal and Ross-Murphy (2000) summarised the different discussions on the events involved in the gelation of proteins and the description of the gel structures involved. Most food protein gels are formed during a heating process or as a result of the action of enzymes. Other types of gelation, like cold gelation, have been reported recently (i.e. Alting *et al*., 2002).

Gels can be very different in nature. We can roughly distinguish between gels of flexible macromolecules and particle gels. Most protein gels are particle gels. The particles may consist of single molecules as well as of large aggregates of 100 up to 1000 molecules (van Vliet, 2000; Goscal and Ross-Murphy, 2000). During particle aggregation clusters with a fractal structure are often formed. The use of this concept for understanding the behaviour of the protein gels and the effect of changing circumstances is found to be very useful. Examples are described in the literature, e.g., soy protein isolates (Renkema, 2001) and casein (Bremer *et al*., 1989). Only some specific protein gels can be considered as polymer gels, an example is gelatin. Flexible molecular chains form the bridges between the interaction points. Some protein types can show both types of structure, the structure of egg white protein gels for instance depends on circumstances, mainly the pH and the ionic strength (Mine, 1995; Weijers *et al*., 2002).

In general the shear modulus (G) of the gels increases with increasing concentration as $G \propto c^n$. The parameter n gives information on the structure of the gel (van Vliet, 2000; Goscal and Ross-Murphy, 2000; Weijers *et al*., 2002) and can depend on the concentration range used. Gelation time usually decreases with increasing concentration.

Different types of interactions between protein molecules or particles can be distinguished; physical interactions (i.e. non covalent like electrostatic, hydrophobic) and chemical interactions (covalent like disulphide bridges for instance). The type of interaction gives an insight into the effect of changing circumstances. For example, when electrostatic interactions are most important, salts, which shield the local charges on the protein molecules, definitely affect aggregation and gelation. Renkema *et al*. (2000) and Renkema (2001) studied the effect of pH on soy protein isolates. They found that the solubility at pH 7.6 was higher than at pH 3.8, resulting in a lower stiffness at the same overall protein concentration. The strands of protein aggregates in the gels for pH 7.6 were finer. Also the colour and shininess of the gels was affected by pH. Differences could be explained by the effect of shielding of charges and the possibility for disulphide bridging of β-glycinin, one of the types of protein in soy protein isolate.

The behaviour of food products during eating, especially the sensory texture, is governed by the large deformation behaviour (van Vliet *et al*., 1991; van Vliet and Luyten, 1995) where yielding and fracturing are important. In general, particle gels are more brittle than gels of flexible macromolecules. Also other

structural features can explain the differences in large deformation behaviour. Renkema *et al*. (2000) for instance, found that gels of soy protein isolate were more stiff and brittle at pH 3.8 than at pH 7.6. The latter gels showed finer and more curved strands of aggregates. Because of the bending and stretching possibilities these gels are easier to deform than the gels at a lower pH where the strands were found to be coarse, thick and straight.

Increasing the protein concentration can produce systems that are stiffer than gels. The behaviour of such more concentrated protein systems is governed by the mobility of the molecules. In general, more mobile polymers give products that are easier to deform and less stiff. By using general polymer theories it is possible to understand the behaviour of proteins in products like powders, extrudates, crusts, films and coatings. The mobility of the protein is affected by the presence of plasticisers, in food systems the most important plasticiser is water, but the presence of other constituents can affect this mobility. The glass transition temperature, i.e., the temperature where one can observe a more or less sharp increase in molecular mobility, is influenced by both the macromolecular structure and the presence of other constituents. Models to calculate these effects are available (for example by Matveev *et al*., 2000).

18.3 Surfactant properties of proteins

Proteins are often used in food systems to stabilise the co–existence of two or more phases. Foams, for example, consist of a water phase with air bubbles that will spontaneously separate when no stabiliser is added. In most food emulsions small oil/fat droplets are distributed through the water phase. When no stabiliser is added, the oil droplets will flow together and form an oil layer (Walstra, 1987). The role of the proteins that act as surfactants is to prevent the separation process and to retain fine oil droplets or air bubbles (Dickinson, 1992; Friberg, 1997; Tornberg *et al*., 1997). Different proteins are used in food systems for this functionality. Numerous studies have been reported on this subject, mostly concerning the effects of changing circumstances like the pH, the presence of salts, etc. (Dickinson *et al*., 1998; Dickinson 1999 and 2003). Furthermore, proteins are often modified to improve the surfactant properties (Panyam and Kilara, 1996; see also section 18.5).

18.3.1 Different mechanisms involved

The interface of a foam or emulsion is composed of surface-active molecules that are amphiphilic. This means that part of the molecules is attracted to the hydrophilic phase, and part of the molecules to the non-polar phase. The latter being the air (foam) or the fat phase (emulsion).

Many different processes are involved in the preparation and (in)stability of foams and emulsions. Each process requires other functionalities of the surfactant. The different requirements a protein has to meet can be understood

when regarding the different processes involved. Whether the protein functionality is good depends also on the process considered. This can be understood by carefully studying the constraints of a process; these will differ for stability and for the preparation of a structure. The ability to create foams and emulsions has been correlated to the interfacial tension, and especially to the rate of decrease in interfacial tension (Wilde, 2000). Because both emulsion formation and foaming are rapid processes, the surfactants must be able to adsorb quickly to the newly formed interfaces to show their required functionality in time.

The most important aspect for the stability of emulsions is the prevention of coalescence (Dickinson, 1992). For foams, the prevention of growth of bubbles at the expense of smaller bubbles, i.e., disproportionation, is most important (Dickinson, 1992). Repulsion between adjacent droplets or bubbles due to charge or steric hindrance can prevent these phenomena. The design and properties of the adsorbed layer as well as the distance between the droplets or bubbles are important in this respect. For more concentrated systems (smaller distances), the reaction of the interfaces after (small) disruptions or after changes in interfacial size or shape is the most important aspect for stability. Interfacial rheology is the science that describes this behaviour both for fast (for instance emulsification) and slow (stability) changes (Bos and van Vliet, 2001). Gelling of the adsorbed protein layer causes a more elastic behaviour of the interface with both stabilising as well as yielding and fracture phenomena (Martin *et al*., 2002).

The viscosity of the bulk phase also plays a role in stabilizing emulsions and foams. The rate of creaming or sedimentation is directly related to this viscosity. As a result of creaming or sedimentation, the part of the product containing droplets or bubbles becomes smaller, thereby decreasing the mean distance between the droplets or bubbles, and enhancing coalescence and disproportionation. Coalescence and disproportionation phenomena depend on surface rheological properties as well as bulk rheological behaviour. In the presence of proteins the bulk rheological properties are relatively less important than in case of low molecular weight surfactants (Wilde, 2000). Recently Martin *et al*. (2002) showed that the adsorption rate and the ability to unfold at the interface are important factors for foam formation, while stability against disproportionation was regulated by surface rheological properties. Conformational changes of the proteins during and after foaming or emulsifying may stabilise the interface. The extent of the changes depends on the protein and on the properties of the phases on both sides of the interface. Some authors use the term interfacial denaturation to describe conformational change. A well-known example of this phenomenon is the irreversible change when whipping egg white.

The total size of the interface in emulsions and foams is large, the total interfacial area in an emulsion can be tens of square metres per litre, for instance, in milk the natural milk fat globules have a total interface of about 80 m^2/l (Walstra and Jennes, 1984). The amount of adsorbed protein is only a few milligrams per square metre (Bos and van Vliet, 2001), a typical value is 2–3

mg/m^2. This implies that in a lot of food systems only a small amount of the protein is present at the interface.

The ability of proteins to contribute to foam or emulsion stability may differ. This is most clear when changing the protein properties by proteases, thereby decreasing the molecular weight. In general it is known (van der Ven *et al*., 2002a) that an optimum in the functionality for foaming properties can be found at higher molecular weights than the optimum functionality for emulsion properties.

18.3.2 Food systems

Understanding protein functionality towards surfactant properties is very difficult because of the many processes involved, the fact that properties can have opposite effects depending on the process, and – still – a lack in understanding. Therefore the effect of intrinsic and extrinsic factors on foaming and emulsification behaviour is studied frequently. Some general remarks can be made.

- The effect of the protein source can be partly understood by the molecular structure. Important aspects are hydrophobicity/hydrophilicity and distribution, charge and distribution, Mw, history of purification, etc. A further explanation is given in section 18.5.
- Small changes in the amino acid composition can cause large differences in surfactant functionality, as was demonstrated for some genetic variants of β-lactoglobulin (Wilde, 2000).
- Extrinsic factors, like ionic strength, types of salt present, pH, temperature, etc. largely affect the performance of the protein molecule.
- The presence of other constituents, especially other surfactants, like low molecular weight ones, have a large effect. When both are present, competition often weakens the interfacial structure and stability, thereby decreasing the stability of emulsions and foams compared to those of individual surfactants (Wilde, 2000). There can also be a competition for a place in the interface, as will be discussed in section 18.5.
- Also desorption of the protein is possible. Bos and Van Vliet (2001) give an overview.
- The rheological behaviour of the bulk phase of the food product affect both the formation and especially the stability of emulsions and foams.

An overview of the effects of molecular structure of the proteins, composition and structure of the interface, and interfacial rheology has been published recently (Wilde, 2000).

Because of the large number of processes involved and the complexity of these processes, testing the functionality of proteins towards surfactant related functionalities and the relation towards the actual performance in food systems, is very difficult. However, progress is being made. Van der Ven (2002) and Van der Ven *et al*. (2002a and 2002b) very recently studied the relation between biochemical and functional properties of milk protein hydrolysates. Reversed

phase chromatography was very suitable to correlate different molecular properties with emulsion and foam related functionalities. This relation demonstrated the importance of both molecular weight distribution and surface hydrophobicity. Fourier transformed infra-red spectra showed good results as a fast fingerprinting method for establishing surfactant functionality of protein hydrolysates.

18.4 Protein-flavour relationships

In addition to the effects of proteins on the texture and the mouthfeel of food products, the complex conformational structure of proteins creates an important source for interactions with flavour compounds influencing the flavour perception of food products. In general, proteins themselves have little flavour of their own. However, the complex conformational structure of proteins creates an important source for (ir)reversible interactions with flavour compounds. This ability is exploited in several ways, both with respect to enhancing and masking the flavour of food products. Flavour compounds are often deliberately added to food to enhance its smell and taste. Proteins may then serve to bind these compounds, in other words to act as a reservoir. In this context another application of proteins can be mentioned. Proteins (e.g. gelatin) are used to encapsulate flavour compounds to protect them from deterioration during processing. The latter usage may even result in controlled release of the flavour compound, for instance during chewing (Taylor and Linford, 1996).

As already mentioned, proteins are also used to mask off-flavours. Compounds responsible for off-flavours may be inadvertently added to a food product because of their presence in one of the constituents (e.g. in fats and oils). Because of their binding capacity, the use of proteins may then result in reduction or complete masking of the off-flavour. Next to the binding of added (off-) flavour compounds, proteins can interact with flavour compounds that originate from degradation reactions occurring during processing and storage, such as Maillard reactions and oxidation of lipids. Products of these reactions might have a bitter taste or an off-flavour; they may contribute to the desired flavour, or have a precursor function in the (off-)flavour formation during food storage. In Table 18.3 an overview is presented of the different aspects involved in flavour perception of food products by proteins. The most important process in terms of the influence of protein on flavour in food systems is the (ir)reversible binding of flavour components.

Important for the interactions between proteins and flavour constituents are both the nature of the protein as well as the type of solvent (water and/or lipids) (Franzen and Kinsella, 1974). Maier and Hartmann (1977) showed that the building blocks of the proteins, the amino acids, show large differences in their affinity for volatiles and thus for different flavour components:

- Lysine adsorbs best and this adsorption is often irreversible.
- Arginine, histidine, phenylalanine, tryptophan, prolin, valin, leucin and isoleucin show high binding affinities.

Table 18.3 An overview of the different aspects of added proteins influencing the flavour of food systems

Aspect	Example
Protein itself	Not important for food proteins
Accompanying compounds	Soy proteins
Binding/adsorption	β-lactoglobulin
• masking flavours	Na-caseinate in coffee whiteners
• controlled release	Soy proteins as encapsulation material
Chemical reactions	
• degradation products (odour active)	General: storage of proteins
• specific reaction products of proteins (e.g. Maillard-reaction)	Strecker-reaction of amino acids Metabolism products (microorganisms)

- Cysteine can form thiazolidinecarboxylic acids with certain aldehydes and/or ketones.
- Tyrosine and hydroxyproline adsorb less than the corresponding amino acids without the hydroxyl group (i.e. phenylalanine and proline).

Possible sources for flavour compounds are other ingredients added to the food systems and/or odour active compounds, which are formed during storage or processing. Manufacturers use this protein-flavour interaction to mask certain flavours and to protect flavour compounds during the processing of the food.

The interactions between proteins and flavour compounds are based on physical surface effects (resulting in reversible binding, e.g., van der Waals interactions) and/or chemical reactions (irreversible, e.g., covalent or electrostatic). The interactions themselves are largely affected by extrinsic factors; e.g., pH, ionic strength or water content (Fisher and Widder, 1997). According to Lubbers *et al*. (1998), these effects are responsible for the decrease or increase of aroma active volatiles in the headspace.

Another important aspect that has to be taken into account is the rheological behaviour of the system. A high protein concentration can lead to gel network formation as is discussed in section 18.2. This may reduce the release of flavour, probably because of a limited diffusion. Furthermore, the presence of a protein layer in oil-water emulsions will also influence the volatility of the flavour compound present in the oil phase (Dickinson *et al*., 1994).

In general, the internal region of a protein, being rather hydrophobic, is able to bind non-polar flavour compounds, while the hydrophilic surface of the protein enables interactions with polar compounds. Small increases in the protein content can significantly decrease the perception of certain flavour compounds (Hansen and Heinis, 1991) and therefore change the sensory properties of a given food to a large extent. Besides sensory experiments these effects are mostly determined by gas chromatographic methods after collecting the samples in the headspace, i.e., the measurement of volatile concentration in the headspace above a given protein (solution). One approach is the use of high

performance liquid chromatography and immobilized proteins (Sostmann and Guichard, 1998).

18.4.1 Examples: whey and soy proteins

Two examples, whey and soy proteins, demonstrating the importance of flavour-protein interaction will now be discussed.

Whey proteins

Whey proteins are a byproduct of the cheese industry, generated in large amounts. They are added to food systems because of their good rheological and surfactant functionalities. Another important reason for the use of whey proteins is their ability to form fat-like systems. In this way protein-based fat substitutes are used to simulate the mouthfeel of fat. Whey proteins are able to bind various types of compounds. Specifically β-lactoglobulin is known for its interactions with a large variety of hydrophobic ligands (Lee *et al*., 1996; de Wolf and Brett, 2000; Muresan *et al*., 2001). This protein has been used by several research groups as a model macromolecule for flavour release measurements (Guichard and Langourieux, 2000; van Ruth and Villeneuve, 2002). It was found that the release of flavour depended on protein concentration. Also these researchers showed that flavour compounds within the same chemical class had an increase in binding affinity with an increase in chain length. This effect was also observed by Landy *et al*. (1995) for a homologous series of ethyl esters in the aqueous phase of sodium caseinate.

During the production and storage of whey protein concentrate (WPC) the formation of an off-flavour is possible. Among others, lipid oxidation may result in formation of short chain aldehydes that have a high affinity for long-term binding by covalent interactions with whey proteins. Flavour formation is of special importance when whey proteins are stored at a high water activity, resulting in an increase in the formation of aldehydes, ketones, furans and sulphur-containing compounds (Lee *et al*., 1996). The strongest binding affinities were observed for WPC, while the separate proteins α-lactalbumin and β-lactoglobulin did not contribute that much to the flavour-binding capacity of the total whey protein, due their low binding affinities (Jasinski and Kilara, 1985).

Recent studies on flavoured whey protein gels (Wheel *et al*., 2002) showed that the nosespace flavour concentration is independent of the gel hardness or water-holding capacity. However, changes in flavour intensity were perceived by the panellists. The authors concluded that perception of flavour intensity is determined by texture rather than in-nose flavour concentration.

In food product development the different effects mentioned above are mostly used to reduce negative sensory attributes. A very common example is the usage in coffee whiteners. Sodium caseinate is often used in these products. The actual content in the recipe varies depending on the fat content because in low fat products it is also used as a fat replacer. Due to the high content of fat or

protein these coffee additives change completely the perception of the coffee beverage (Bücking and Steinhart, 2002).

Soy proteins

Soybeans provide a high-quality protein, but the demand for this protein is hampered because soy protein preparations are often associated with an undesirable flavour (Kinsella, 1979). In contrast to alcohols, which do not interact with soy protein, aldehydes, especially unsaturated aldehydes, react with the protein (Gremli, 1974). This reaction is at least partly irreversible. The binding constant increases with the chain length by three orders of magnitude (Damodaran and Kinsella, 1981a). Specifically the medium-chain aldehydes (e.g. hexanal) largely contribute to the 'beany' and 'grassy' off-flavour of soy protein (Maheswari *et al*., 1997). Aqueous solution of soy-protein isolates showed in their headspace beside the aldehydes also sulphur-containing odour active compounds like dimethyl trisulfide. Also these constituents contribute to the well-known unpleasant odour of this protein (Boatright and Lei, 2000). Because the soy protein fractions 11S and 7S showed different binding affinities, with a different temperature dependency, it is possible to remove certain off-flavours by reversibly altering the quaternary structure (Damodaran and Kinsella, 1981b). This process could be exploited for soy protein, food processing and the formulation of soy-protein flavourings. Knowledge of the exact interactions and binding processes allow food technologists to increase the usage possibilities for soy proteins. In food processing, the different flavour binding abilities are also important for the right choice of encapsulation material. Kim and Morr (1996) and Kim and Min (1996) showed that soy protein isolate was most effective while whey protein isolate was least effective for retaining orange oil during spray-drying of the liquid orange oil emulsions.

In the future novel and functional foods will play important roles in our daily lives. Therefore ingredients with new sensory attributes and/or improved nutritional value will be introduced to the market. For example proteins like Thaumatin, Monellin and Miraculin have an exceptional position because of their sweet taste attributes. Traditionally they have been used by West Africans for flavour improvement and bitterness suppression. Since consumer demand for more natural ingredients is increasing, taste-modifying proteins might be an alternative as long as they have similar properties to food products (Witty, 1998). These proteins are amenable to genetic engineering, i.e., they have a special potential for improvements in taste intensity, aftertaste and taste profile.

Furthermore, certain protein preparations could be used not only for optimizing sensoric properties but also the nutritional value of food products. Gonzalez-Martinez *et al*. (2002) showed that whey powder that was used to replace milk powder in yogurt was preferred by sensory panellists. In addition to this preference whey powder is an important source for lactose, calcium and soluble vitamins, i.e., the nutritional value of this food product was increased.

18.5 Protein structure and techno-functionality

Several structural features of proteins determine their ability to perform their techno-functional properties. Examples of such features are conformation, molecular mass, charge and charge distribution. For the latter two, the influence, which is very much dependent on extrinsic factors such as pH and ionic strength, has, for instance, clearly been demonstrated by adsorption experiments on solid surfaces using proteins of similar mass and dimensions (Arai and Norde, 1990). In this discussion emphasis will be on the effects of structural features that are less dependent on extrinsic factors, i.e., protein conformation (especially flexibility) and molecular mass. Furthermore, attention is given to methods for deliberately changing the protein structure by modification.

18.5.1 Conformation

The unfolding of the three-dimensional structure or conformation of a protein plays an important role in its functional behaviour. By unfolding, the hydrophobic core of a protein becomes exposed. Furthermore, the accessibility of reactive side chains of amino acids is increased. Both these changes favour protein-protein interactions and thus the formation of aggregates. This is especially true when free thiol groups become exposed as unfolding could result in the formation of tightly bound aggregates. The unfolding and aggregation of a protein usually has a negative effect on its solubility/dispersability, a prerequisite to exert techno-functional properties such as gelling, emulsification and foaming. However, during execution of these functions the picture changes; unfolding is often required. Excessive unfolding is a prerequisite for the formation of gels; after unfolding the proteins interact to form a gel (see section 18.2). The contribution and desired extent of unfolding is much less clear with respect to interfacial properties such as foaming and emulsifying. Generally, it is accepted that upon adsorption to an apolar interface (such as the oil-water and air-water interface) the conformation of a protein molecule will change.

The conformation of proteins is only marginally stable. Important structure stabilising factors are the hydrophobic interaction between the apolar amino acid residues in the interior of a globular protein molecule. These interactions originate from the favourable dehydration of the apolar side chains of these residues. However, after adsorption part of the aqueous environment surrounding the protein molecule is replaced by an apolar one. Hence, intramolecular hydrophobic interactions become less important and apolar parts from the interior of the protein may become exposed to the apolar phase or, in the case of fluid interfaces, even protrude into this phase without making contact with water. This may result in conformational changes, which can be very drastic, resulting in surface coagulation and irreversible denaturation (Henson *et al*., 1970).

Conformational changes due to adsorption at interfaces have been studied quite extensively using solid surfaces (Norde, 2000). These changes can occur

very quickly. The partial unfolding of α-lactalbumin to a molten globule-like state on an apolar surface (polystyrene nanospheres) has a rate higher than the unfolding induced by the addition of 5.4 M guanidinium hydrochloride (Engel *et al*., 2002). At liquid interfaces much less is known about conformational changes.

The use of spectroscopic techniques such as Front-Face Steady-State Fluorescence (Castelain and Genot, 1994) and Fourier Transform Infra Red Spectroscopy (Fang and Dalgleish, 1997, 1998) has shown that conformational changes occur after adsorption of bovine serum albumin (BSA), β-lactoglobulin and α-lactalbumin at the oil-water interface. Recent studies using circular dichroism in refractive index matched emulsions showed that the effects on the secondary structure are rather small; the tertiary structure appears to be more affected (Husband *et al*., 2001). Similar findings were reported with respect to conformational changes of proteins at the air-water interface (Clarkson *et al*., 1999).

18.5.2 Molecular mass

Next to well-known effects on bulk rheological properties such as viscosity, the molecular mass is also expected to influence the interfacial properties, especially in the initial stage of adsorption. This stage is to a large extent diffusion controlled. Therefore it is to be expected that the smaller proteins, which have the higher diffusion rate, migrate faster to the interface than the larger ones. This has been demonstrated using blood proteins and solid surfaces (Norde, 2000). However, at liquid interfaces the sequence in adsorption rate for globular proteins was found to be BSA $\geq$ ovalbumin > lysozyme (Benjamins, 2000, de Feijter and Benjamins, 1987), in contrast to their molecular mass sequence (about 69 kD, 45 kD and 14.5 kD resp.). The non-globular, very flexible β-casein (MW 24 kD) had, despite its lower molecular mass than BSA and ovalbumin, an adsorption rate even higher than that of BSA. This indicates that at liquid interfaces other structural features also play a prominent role or that diffusion is of less importance during the emulsification process (high convection).

In later stages of adsorption proteins having a higher affinity for the interface, e.g., proteins having a higher (potential) exposed hydrophobicity, may displace the initially adsorbed proteins. Last but not least it should kept in mind that in practice usually more than one type of protein is present. The various proteins compete with each other for adsorption at the interface. It is to be expected that molecules that have the highest rate of arrival will first cover the interface. These are the ones most abundant in solution or the ones having the lower molecular mass or other beneficial structural features.

18.5.3 Modification

As is evident from the above paragraphs, the structure of a protein is an important determinant for its functionality. Changing the protein structure may

improve the functionality. The structure can unintentionally be changed during processing (e.g. Maillard reactions, oxidation of cysteine) or deliberately by so-called modification reactions. The three main methods that have been used for the latter purpose are physical, chemical and enzymatic modification (Vojdani and Whitaker, 1994). Physical methods (e.g. heat, shear and pressure) are usually aimed at changing the conformation of proteins without affecting their polypeptide backbone. In contrast, the other two methods are directed at changing this backbone. The remainder of this section will focus on these two methods. Some examples of these modifications and the primary intended change in structural features of the protein are presented in Table 18.4.

Chemical modifications are not used very much in the food industry because of cost-performance ratio, the expected low acceptability of consumers and regulatory issues. Chemical methods that are commercially exploited include extensive hydrolysis and deamidation. Extensive hydrolysis by boiling with acids results in free amino acids and very small peptides. This modification is used for the preparation of H(ydrolysed) V(egetable) P(rotein), which is utilised, for instance, to fortify and enhance the aroma of soups. Deamidation (the conversion of the amino acids glutamine and asparagine to their acidic counterparts) is aimed at changing charge. This modification is used for alteration and extension of the range of functional properties of wheat gluten (Anteunis, 1998). Wheat gluten has a very high glutamine content (about 30% of its constituting amino acids), so even a low percentage of deamidation has large effects on charge and functionality.

Most studies on modifying the polypeptide backbone of food proteins have focused on enzymatic modification, especially on the use of proteases and transglutaminases. Numerous publications on the enhancement of solubility, foaming and emulsifying properties by (limited) proteolysis are available (Panyam and Kilara, 1996). The changes in these properties are attributed to changes in charge, hydrophobicity and molecular mass in going from protein to peptide mixtures. A comprehensive study regarding the relation between the molecular mass distribution of the peptides in hydrolysates and their foaming

Table 18.4 Examples of chemical and enzymatic modifications

Method	Type of reaction	Intended change in structural feature
Chemical	Deamidation	Increased negative charge
	Succinylation	Increased negative charge
	Esterification	Increased positive charge and hydrophobicity
	Reductive alkylation	Increased hydrophobicity and negative charge
	Alkylation of SH groups	Increased hydrophobicity
	Cross-linking	Increased molecular mass
Enzymatic	Proteolysis	Decreased molecular mass
	Cross-linking	Increased molecular mass
	Phosphorylation	Increased charge

and emulsifying properties has recently been published (van der Ven *et al*., 2002a and 2002b). Enhancement of the gelling properties of proteins can also be achieved by limited proteolysis (Otte *et al*., 1999). Last but not least, proteases are extensively used for enhancing the nutritional quality of proteins (for use in, e.g. baby and clinical foods, sport drinks).

Another enzymatic modification that attracts much attention, is modification by transglutaminase (Nielsen, 1995; Motoki and Seguto, 1998). This enzyme catalyses the reaction between the γ-carboxyamide group of protein-bound glutaminyl residues and various substrates including water and primary amines. Transglutaminase can be used for deamidation (reaction with water) but is more frequently used for attachment of amino acids and especially cross-linking (reactions with primary amines). Cross-linking results in the formation of bonds between glutaminyl and lysyl residues from the same protein molecule (intramolecular) or from two separate molecules (intermolecular). By this intermolecular cross-linking covalent interactions between proteins are brought about resulting in, for instance, enhanced gel strength or network formation in meat and bakery products (Nielsen, 1995). Cross-linking of proteins can also be achieved by the use of oxidative enzymes such as peroxidases (Faergemand *et al*., 1998). Besides cross-linking, these enzymes can also be used for the attachment of carbohydrates to proteins (Oudgenoeg *et al*., 2000 and 2002).

In addition to these more classical approaches to modify proteins a new one emerging is site-directed mutagenis by genetic modification. However, to exploit the high potential of this approach, much more knowledge is required on the relationship between the functionality of proteins and their structure.

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19

Edible films and coatings from proteins

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19.1 Introduction

There has been ongoing interest in the development of films and coatings, including edible packaging materials, from renewable biopolymers. Opportunities for adding value to underutilized agricultural materials and concerns over the potentially adverse environmental impact of synthetic packaging materials are two major drivers for such interest. The terms films and coatings are often used interchangeably. Normally, films are stand-alone, self-supporting structures that are pre-formed and then placed on or between food components. Coatings are thin layers of material formed directly (by dipping, spraying, or panning) onto the surface of the food product they are intended to protect or enhance in some manner (Krochta, 2002).

In general, the purpose of edible films and coatings is to inhibit the migration of moisture, gases, aromas, and lipids; to carry food ingredients (e.g., antioxidants, antimicrobials, and flavors); and/or to improve the mechanical integrity or handling characteristics of foods (Krochta and De Mulder-Johnston, 1997). Proteins, which are abundantly available from sustainable resources of plant or animal origin, can be used as raw materials for the formation of edible films and coatings. A number of published reviews have covered the formation, characterization, and uses of edible films and coatings (Kester and Fennema, 1986; Guilbert and Gontard, 1994, 1995; Koelsch, 1994; Cuq *et al*., 1995; Martin-Polo, 1995; Baldwin *et al*., 1997; Callegarin *et al*., 1997; Gennadios *et al*., 1997; Krochta 1997a; Krochta and De Mulder-Johnston, 1997; Debeaufort *et al*., 1998). Also, Gennadios *et al*. (1994b), Torres (1994), Krochta (1997b), and Cuq *et al*. (1998) have reviewed protein-based films and coatings, in particular. This chapter presents an overview of protein-based films and coatings

discussing formation methods, functional properties, and commercialized or envisioned applications.

19.2 Materials and methods used in protein film formation

19.2.1 Proteinaceous materials used for film formation

Naturally, proteins that are by-products or side-products of food processing have attracted considerable interest as film-forming materials. Table 19.1 lists the major film-forming proteinaceous materials along with published reviews focusing specifically on films and coatings from these proteins. Proteins that are less available from a commercial standpoint, such as feather keratin, sorghum kafirin, peanut protein, rice protein, and pea protein, have also been studied for their film-forming properties (Park *et al*., 2002a). In addition to protein isolates and protein concentrates, isolated protein fractions that are not commercially available have received attention as film-formers. Examples include the 7S and 11S fractions of soy protein (Okamoto 1978; Kunte *et al*., 1997; Subirade *et al*., 1998), the gliadin fraction of wheat protein (Sánchez *et al*., 1998; Mangavel *et al*., 2001), and the β-lactoglobulin fraction of whey protein (Maté and Krochta, 1996; Sothornvit and Krochta, 2000a). Also, Sothornvit and Krochta (2000b, c) studied films from hydrolyzed whey protein.

19.2.2 Plasticizers

Protein films and coatings need to have good strength and flexibility to prevent cracking during handling and storage (Torres, 1994). Therefore, plasticizers of relatively low molecular weight are typically added to protein film-forming preparations to increase the flexibility and 'soften' the structure of formed films. Generally, plasticizers act by entering between polymeric molecular chains,

Table 19.1 Commercially available proteinaceous materials that have been studied extensively for the formation of edible films and coatings

Proteinaceous material	Published reviews
Corn zein	Gennadios and Weller (1990); Padua and Wang (2002)
Wheat gluten	Gennadios and Weller (1990); Guilbert *et al*. (2002)
Soy protein isolate	Gennadios and Weller (1991); Park *et al*. (2002b)
Cottonseed flour	Marquié and Guilbert (2002)
Whey protein concentrate/isolate	McHugh and Krochta (1994a); Chen (1995); Pérez-Gago and Krochta (2002)
Casein	McHugh and Krochta (1994a); Chen (1995, 2002)
Egg albumen	Lim *et al*. (2002)
Collagen/gelatin	Arvanitoyannis (2002)
Fish myofibrillar protein	Cuq (2002)

physicochemically associating with the polymer, reducing cohesion within the film network, and effectively extending and softening the film structure (Banker, 1966). Unfortunately, plasticizers generally also decrease the ability of films to act as barriers to the transport of moisture, gases, and aroma compounds (Krochta, 2002).

The amount of plasticizer added into protein film-forming preparations varies widely within the range of 10 to 60% by weight of the protein. The polyols glycerol and sorbitol are the most commonly used plasticizers for protein-based films (Gennadios *et al*., 1994b). Other hydrophilic molecules that have been used as protein film plasticizers include triethylene glycol, polyethylene glycol, propylene glycol, glucose, saccharose, and sucrose (Cherian *et al*., 1995; Cuq *et al*., 1996; Di Gioia *et al*., 1998; Gueguen *et al*., 1998; Sánchez *et al*., 1998; Krochta, 2002). Also, fatty acids, such as oleic, palmitic, stearic, and linoleic acids, have been used to plasticize zein films (Lai *et al*., 1997; Santosa and Padua, 1999). In fact, water itself depresses the glass transition temperature of protein films acting as an effective plasticizer (Gontard and Ring, 1996). Thus, film moisture content, as affected by the relative humidity (RH) of the surrounding environment, largely affects protein film properties (Krochta, 2002).

19.2.3 Film formation by skimming

Edible soy-protein-lipid films have traditionally been produced in Eastern Asia on the surface of soymilk heated (85 to 98 ºC) in open, swallow pans (Gennadios *et al*., 1994b). Such films are known as 'yuba' in Japan, 'kong kook' in Korea, 'tou-fu-pi' in China, and 'fu-chock' in Malaysia (Snyder and Kwon, 1987). They are considered a specialty item rather than a daily food source since their production is expensive. Films are successively formed on and removed from the surface of the heated soymilk until protein in the solution is exhausted. Upon drying, yuba films are shaped into sheets, sticks, and flakes for further use as wrappers of meats and vegetables in cooking (Snyder and Kwon, 1987). Besides soymilk, protein-lipid films have also been prepared on the surface of heated peanut milk (Wu and Bates, 1973; Aboagye and Stanley, 1985; Del Rosario *et al*., 1992), cottonseed milk (Wu and Bates, 1973), and winged bean milk (Sian and Ishak, 1990). Furthermore, heat-catalyzed protein polymerization through surface dehydration has been studied with heated solutions of proteins containing no lipids or carbohydrates (Wu and Bates, 1973; Okamoto, 1978).

19.2.4 Film formation by solvent casting

Self-supporting protein-based films are typically formed by casting and drying dilute (usually 5–10% w/v) protein solutions in water or in aqueous ethanol, depending on the protein. For example, films from soy protein isolate (Park *et al*., 2002b), whey protein (Pérez-Gago and Krochta, 2002), fish myofibrillar protein (Cuq, 2002), and egg white protein (Lim *et al*., 2002) are typically cast

from aqueous solutions. Other proteins that have higher contents of non-polar amino acids, such as zein (Padua and Wang, 2002) and wheat gluten (Guilbert *et al*., 2002) are typically cast from solutions in aqueous ethanol. The use of other organic solvents, such as acetone, methanol, and isopropyl alcohol, for preparing protein-based film-forming solutions has also been reported. For example, zein films have been cast from acetone-water mixtures (Yamada *et al*., 1995). However, use of such solvents would inherently render the cast films inedible.

The development of film structures during the drying of cast protein solutions involves formation of hydrophobic, hydrogen, and ionic bonds. Also, it is believed that covalent intramolecular and intermolecular disulfide bonds play a major role in film formation from sulfur-containing proteins (e.g., wheat gluten, soy protein isolate, whey protein, and egg albumen) (Okamoto, 1978; Gennadios *et al*., 1994b; McHugh and Krochta, 1994a; Handa *et al*., 1999a). The pH of protein film-forming solutions is usually adjusted away from the protein's isoelectric point to avoid protein precipitation. For example, films from wheat gluten, which has an isoelectric point of 7.5 (Wu and Dimler, 1963), have been cast from film-forming solutions of pH 9 to 13 (Gennadios *et al*., 1993b) or 2 to 6 (Gontard *et al*., 1992; Gennadios *et al*., 1993b).

The drying temperature and RH, which determine the drying rate of cast solutions, can affect the structure and properties of protein films. In general, rapid drying of cast polymer solutions limits the development of intermolecular associations within the film structure as solvent removal restricts the mobility of the molecular chains (Banker, 1966). Alcantara *et al*. (1998) studied the effect of drying rate on the properties of cast films from whey protein isolate. Rapid drying at 95 ºC and 30% RH yielded films that were thinner, stiffer, stronger, and less extendable than films dried more slowly at 21 ºC and 50% RH. Also, films dried at 95 ºC and 30% RH had lower water vapor permeability than films dried at 21 ºC and 50% RH (Alcantara *et al*., 1998). Similarly, cast peanut protein films dried at 90 ºC had lower water vapor permeability and greater tensile strength than films dried at 70 or 80 ºC (Jangchud and Chinnan, 1999). Recently, Kaya and Kaya (2000) reported the use of a microwave oven to rapidly dry (5 min. versus 18 h. at room conditions) cast whey protein film-forming solutions. Microwave drying and drying at room conditions resulted in films of similar water vapor permeability, tensile strength, and elongation values. However, films dried in the microwave oven were less hazy and glossier than films dried at room conditions (Kaya and Kaya, 2000).

19.2.5 Film formation by extrusion

Thermoplastic extrusion could be an attractive method for large-scale commercial production of protein films, especially since it eliminates the need for solvent addition and evaporation (Krochta, 2002). Collagen casings are produced commercially by extruding viscous, aqueous suspensions of acidified collagen (Osburn, 2002). The formation of extruded films from other types of proteins has also been reported in the patent and scientific literature. For

example, Naga *et al*. (1996) described such an extrusion process using soy protein, plasticizer (e.g., polyhydric alcohols), and water as the main formulation ingredients. They used pressure of 5 to 50 kg/cm^2 and temperature of 110 to 180 ºC in the extruder to knead and melt the raw material. Frinault *et al*. (1997) developed a wet spinning process to prepare films from acid casein. Their process involved extrusion of an acid casein solution into a coagulating bath containing sodium hydroxide, followed by hardening, rinsing, and drying. The harsh chemicals used in the coagulation and hardening steps would likely render such films inedible. A similar process for producing casein films by using mild chemicals was described in a patent (Metzger, 1997).

Rampon *et al*. (1999) described film preparation by spinning soy protein isolate in a coagulating buffer. Specifically, they prepared a film-forming dope by mixing soy protein isolate, water, and 1.3 M sodium hydroxide solution in a cutter to a homogeneous, viscous, alkaline (pH = 12.5) mass. This dope was poured into a cylinder and forced through a filter using compressed air. The dope was then extruded through a rectangular nozzle immersed into a coagulating bath of acetate buffer. The buffer had a pH of 4.7, which corresponded to the isoelectric region of soy protein isolate. Upon contact with the buffer, the proteins in the dope coagulated into a wet film that was collected on a polyethylene roller and dried overnight at 15 ºC (Rampon *et al*., 1999).

19.2.6 Film formation by thermal compaction

Another technique for preparing protein films was described by Ogale *et al*. (2000) and Cunningham *et al*. (2000). They thermally compacted mixtures of soy protein isolate and glycerol at a temperature of 150 to 160 ºC and pressure of 10 MPa. Processing temperatures above 180 ºC could not be used due to substantial thermal degradation of the protein films as evidenced by thermogravimetric analysis (Ogale *et al*., 2000). Compression-molding of whey protein films has also been investigated (Sothornvit *et al*., 2003).

19.3 Properties of protein film

19.3.1 Tensile properties

Tensile strength (TS) and percentage elongation at break (E) are the two most commonly measured mechanical properties of protein films. Published TS and E values for protein films range widely from 0.5 to 17 MPa and from 1 to 260%, respectively (Cuq *et al*., 1998; Krochta, 2002). These TS values approach those of 9 to 17 MPa for low-density polyethylene (LDPE) and 17 to 35 MPa for high-density polyethylene (HDPE) (Briston, 1988). However, LDPE and HDPE have E values of 500 and 300%, respectively (Briston, 1988), which are greater than those of protein films. Typically, polysaccharide films, such as those from cellulose ethers and amylose, have TS values of 56 to 70 MPa (Cuq *et al*., 1998; Krochta, 2002), which are greater than those of protein films. Comparisons

among tensile property data reported in the literature for biopolymer and synthetic films require caution due to potential differences in specimen conditioning procedures, testing instruments, and testing parameters.

19.3.2 Water vapor barrier properties

Krochta (2002) compiled water vapor permeability (WVP) values reported in the literature for several protein-based films. Overall, protein films exhibit high WVP values, approximately two to four orders of magnitude greater than the value of 2.6–3.5 $\times$ 10^{-3} g·mm/m^2·h·kPa for LDPE (at 38 ºC, 0/90% RH gradient, and 25 μm film thickness) reported by Briston (1988). The limited resistance of protein films to water vapor transmission is attributed to the substantial inherent hydrophilicity of proteins and to the significant amounts of hydrophilic plasticizers incorporated into protein films to impart adequate flexibility. Published WVP values for protein films should be cautiously compared to consider varying plasticizer contents. Furthermore, temperature, RH gradients applied across films, and film thickness affect WVP values of hydrophilic protein films (McHugh *et al*., 1993; Gennadios *et al*., 1994a).

19.3.3 Oxygen and carbon dioxide barrier properties

The good oxygen barrier properties at low RH of films from collagen (Lieberman and Gilbert, 1973), wheat gluten (Aydt *et al*., 1991; Gennadios *et al*., 1993a, Park and Chinnan, 1995, Gontard *et al*., 1996), corn zein (Aydt *et al*., 1991, Gennadios *et al*., 1993a, Park and Chinnan, 1995), soy protein isolate (Brandenburg *et al*., 1993), whey protein (McHugh and Krochta, 1994b), and fish myofibrillar protein (Gontard *et al*., 1996) have been documented. Protein films also have low carbon dioxide permeability (CDP) (Gontard *et al*., 1996; Mujica-Paz and Gontard, 1997). In general, at low to intermediate RH, protein films have lower oxygen permeability (OP) values than LDPE and HDPE, which are not good oxygen barriers (Krochta, 2002). In fact, the OP values of protein films at low RH approach those of the best synthetic oxygen barriers, ethylene-vinyl alcohol copolymer (EVOH) and polyvinylidene chloride (PVDC). Similar to the hydrophilic EVOH, at higher RH conditions, protein films are plasticized by absorbed moisture and their OP increases drastically (Gontard *et al*., 1996; Mujica-Paz and Gontard, 1997). The effect of increasing RH is even more pronounced on the CDP of protein films (Mujica-Paz and Gontard, 1997). The selectivity ratio (CDP/OP) of wheat gluten films at high RH was very high compared to conventional synthetic films, thus showing potential for modified atmosphere packaging applications (Guilbert *et al*., 2002).

19.3.4 Aroma barrier properties

The aroma permeability of protein films has not been studied extensively. A wheat gluten film was a better barrier to 1-octen-3-ol (mushroom aroma) than

LDPE or methylcellulose films (Debeaufort and Voilley, 1994). Also, a whey protein film was better than co-VDC and was comparable to EVOH as barrier to limonene (citrus aroma) (Miller and Krochta, 1998).

19.3.5 Oil barrier properties

Grease resistance is an important characteristic of packaging materials used with products containing fat or oil. Very limited quantitative data are available regarding the grease barrier properties of protein films. Inherently, protein films are expected to be highly grease-resistant due to their substantial hydrophilicity. This was confirmed with zein (Trezza and Vergano, 1994; Parris *et al*., 2000) and whey protein (Han and Krochta, 2001) coatings on paper. Also, soft gelatin capsules and gelatin microcapsules are used extensively for encapsulating oily fills.

19.4 Treatments used for modifying the functional properties of protein films and coatings

Various physical, chemical, and enzymatic treatments have been employed for modifying the functional properties of protein films and coatings. Most of these approaches aim to promote cross-linking within the film structure and are briefly discussed below.

19.4.1 Heat curing

A few studies reported substantial changes in the properties of preformed protein films that were thermally treated within the temperature range of 55 to 140 ºC for exposure times ranging from a few minutes to 24 h. Thermal treatments of proteins promote formation of intramolecular and intermolecular cross-links, which mainly involve lysine and cystine amino acid residues (Cheftel *et al*., 1985). Films from soy protein (Gennadios *et al*., 1996; Rangavajhyala *et al*., 1997; Rhim *et al*., 2000; Kim *et al*., 2002), wheat gluten (Ali *et al*., 1997; Micard *et al*., 2000), whey protein (Miller *et al*., 1997), and peanut protein (Jangchud and Chinnan, 1999) have been subjected to heat curing treatments. In general, heat-treated films had increased TS and reduced E, water solubility, and WVP.

19.4.2 Enzymatic treatments

Use of enzymes that promote protein cross-linking to improve film properties appears feasible. Stuchell and Krochta (1994) incubated (37 ºC for 24 h.) soy protein isolate film-forming solutions with horseradish peroxidase prior to casting. Treatment with horseradish peroxidase, which catalyzes the oxidation of tyrosine residues, did not affect film WVP, but it increased film TS and protein

solubility. Because of the increase in protein solubility of enzyme-treated films, it was concluded that the enzyme caused protein degradation in addition to cross-linking (Stuchell and Krochta, 1994).

Transglutaminase (TG), an enzyme isolated from cattle blood plasma and guinea pig liver, catalyzes the formation of ϵ-(γ-glutamyl)-lysyl cross-links in proteins (Nielsen, 1995). Whey protein films have also been prepared via cross-linking with TG (Mahmoud and Savello, 1992, 1993). Such films, although insoluble in sodium dodecyl sulfate and in β-mercaptoethanol, were digestible by proteolytic enzymes. Increased TS and moisture resistance as a result of treatment with transglutaminase have been reported for films from casein (Motoki *et al*., 1987b), egg white protein (Lim *et al*., 1998), gelatin (Lim *et al*., 1999), and deamidated wheat gluten (Larré *et al*., 2000). Also, heterologous biopolymers have been formed through TG-induced cross-linking from combinations of soy protein and casein (Motoki *et al*., 1987a) or 11S soy protein fraction and whey protein (Yildirim *et al*., 1996; Yildirim and Hettiarachchy, 1997, 1998). High cost and limited availability have restricted the use of TG for cross-linking protein films. However, a less costly TG of microbial origin that functions independently of calcium cations has recently been commercialized. This microbial TG may stimulate interest in enzymatic cross-linking of protein films and coatings.

19.4.3 Irradiation

Reportedly, aromatic amino acids, such as tyrosine and phenylalanine, can absorb UV radiation and recombine to form covalent cross-links in proteins (Tomihata *et al*., 1992). Films from soy protein isolate, which has a high content of aromatic amino acids, that were subjected to UV irradiation at a wavelength of 253.7 nm had increased TS and reduced E compared to control non-irradiated films (Gennadios *et al*., 1998b). Also, the UV-irradiated soy protein isolate films had lower solubility in water, but similar WVP, compared to non-irradiated films (Rhim *et al*., 2000). In another study, UV treatment reduced the solubility in water of films from sodium caseinate and egg white, while also increasing film yellowness (Rhim *et al*., 1999). However, exposure to UV radiation did not affect the mechanical properties of films from pea protein (Gueguen *et al*., 1998) or wheat gluten (Micard *et al*., 2000). Thus, it appears that the effect of UV radiation treatments on preformed protein films depends largely on the aromatic amino acid content of the protein.

Mixed results have also been reported regarding the effect of γ-irradiation on the properties of preformed protein films. Soy protein isolate films subjected to γ-irradiation (5, 10, 20, or 30 kGy) did not differ in film TS and E from control non-irradiated films (Ghorpade *et al*., 1995). In contrast, sodium caseinate films irradiated at doses greater than 12 kGy had higher puncture strength than control films (Brault *et al*., 1997). Similar, γ-irradiation at 10 to 40 kGy increased slightly the TS of wheat gluten films (Micard *et al*., 2000). Sodium caseinate films subjected to γ-irradiation also showed substantial reduction in solubility in

water (Brault *et al*., 1997). In fact, at an irradiation dose of 32 kGy, about 70% of the caseinate film matter remained insoluble after boiling in water for 30 min. followed by soaking in water at ambient temperature for 24 h. (Lacroix *et al*., 1999). The cross-linked caseinates had a molecular size of up to 100 times that of monomer casein as shown by size exclusion chromatography (Lacroix *et al*., 1999). Furthermore, the γ-irradiated caseinate films were more resistant to spoilage microorganisms (i.e., *Pseudomonas aeruginosa*) than control caseinate films, but they were still susceptible to microbial degradation (Mezgheni *et al*., 1998; Ressouany *et al*., 2000). In contrast, γ-irradiation did not affect the solubility in water of wheat gluten films (Micard *et al*., 2000).

19.4.4 Composite films

Composite films of proteins and lipids are meant to combine the good structural and oxygen barrier properties of protein films with the good water vapor barrier characteristics of the hydrophobic lipids. One approach is to directly incorporate lipids into protein film-forming solutions, which are subsequently cast and dried to prepare bi- or multi-component films. Emulsifiers are typically added to allow dispersion of the lipid material in the solution (Krochta, 2002). Alternatively, molten lipids can be deposited (laminated) onto preformed protein films to prepare bi- or multi-layer films. Several studies reported that protein-lipid films had notably lower WVP than control protein films. Examples include films from sodium caseinate and beeswax (Avena-Bustillos and Krochta, 1993), wheat gluten and beeswax or diacetyl tartaric ester of monoglycerides (Gontard *et al*., 1994, 1995), zein and fatty acids (Park *et al*., 1994b), soy protein isolate and fatty acids (Gennadios *et al*., 1998a), whey protein and various waxes (Shellhammer and Krochta, 1997; Pérez-Gago and Krochta, 1999), zein and grain sorghum or carnauba wax (Weller *et al*., 1998), and egg white protein and fatty acids (Handa *et al*., 1999b).

Besides lipids, proteins have also been combined with polysaccharides to form composite films. Examples include films from zein and methylcellulose (Park *et al*., 1994b), soy protein isolate and propylene glycol alginate (Shih, 1994), protein isolate of *Pistacia terebinthus* with hydroxypropyl methylcellulose (Ayranci and Cetin, 1995), whey protein or caseinate with alginate or pectin (Parris *et al*., 1995), zein and starch (Parris *et al*., 1997), and sodium caseinate and starch (Arvanitoyannis and Biliaderis, 1998).

19.4.5 Chemical treatments

The ability of mono- and, particularly, bi-functional aldehydes to promote covalent intermolecular and intramolecular cross-linking of proteins is well documented (Feeney *et al*., 1975). Several studies have focused on the effects of formaldehyde and glutaraldehyde on protein films from cottonseed flour (Marquié *et al*., 1995), soy protein (Rhim *et al*., 2000), zein (Parris and Coffin, 1997), pea protein (Gueguen *et al*., 1998), whey protein (Galietta *et al*., 1998),

and wheat gluten (Micard *et al*., 2000). In these studies, protein exposure to the aldehydes was achieved by soaking the films in aldehyde solutions, by exposing the films to aldehyde vapors, or by directly incorporating aldehydes into the protein film-forming solutions. In general, the aldehyde treatments increased film TS and reduced E and solubility in water. With soft and hard gelatin capsules carrying drug formulations, dissolution failures can occur because of inadvertent cross-linking of gelatin shells by trace aldehydes present in the excipients (Gennadios, 2002; Bowman and Ofner, 2002). Undoubtedly, the toxic nature of aldehydes limits aldehyde-treated films and coatings to non-edible applications. Besides aldehydes, other chemical agents have been used to modify protein film properties such as epichlorohydrin (Parris and Coffin, 1997) and sodium dodecyl sulfate (Fairley *et al*., 1996; Rhim *et al*., 2002).

19.5 Commercial applications of protein films and coatings

19.5.1 Collagen casings and films

Natural casings from beef, pork, or lamb intestines to restructure comminuted meat have been one of the earliest uses of protein films, and edible packaging materials in general. Subsequently, artificial collagen casings made from extruded collagen fibers supplanted the natural casings. Fabricated collagen casings allow meat processors to manufacture portion-controlled, value-added products from meat trimmings on high-speed automated equipment. Hood (1987) and Osburn (2002) discussed in detail the manufacture of collagen casings from the regenerated corium layer of food-grade beef hides through a wet or a dry extrusion process referred to as North American or European process, respectively. The preformed, cylindrical, dry casings are subsequently stuffed with meat batter and the formed sausages are thermally processed on smoking/cooking equipment. As an alternative to preformed casings, a technology was developed in the 1980s that allows the co-extrusion of collagen casing and meat batter in a continuous processing system (Smits, 1985). Co-extruded collagen casings have better eating qualities than preformed casings. Such co-extrusion systems are capital intensive and, therefore, suitable for high-volume processing plants.

Besides collagen casings, collagen edible films have been used commercially since the 1980s as overwraps for boneless meat products that are heat-processed in nettings. Such films, marketed by Brechteen (Mt. Clements, MI) under the trade name Coffi^{TM}, aid removal of the non-edible netting after heat processing and improve product appearance (Farouk *et al*., 1990).

19.5.2 Zein coatings

Zein-based coating formulations have long been commercially available as finishing agents imparting surface gloss on confectionery products (Boutin, 1997). They can also function as oxygen, moisture, and lipid barriers on nuts (Andres, 1984; Anonymous, 1997). Conventional panning techniques are typically used to

apply these coatings. However, such coating formulations are ethanol-based, an unattractive attribute considering the strict regulations surrounding the emission of volatile organic compounds (Trezza and Krochta, 2002).

19.5.3 Gelatin capsules

The film-forming ability of gelatin and the thermo-reversibility of gelatin gels have long been utilized in the manufacture of microcapsules, hard gelatin capsules, and soft gelatin capsules. Gelatin microcapsules are spherical particles ranging in size (diameter) from 1 μm to 2 mm and are typically formed by simple or complex coacervation (Radwick and Burgess, 2002). Hard gelatin capsules are two-piece, powder-filled, oblong-shaped containers (Bowman and Ofner, 2002). The gelatin shells contain no plasticizer, thus having a rigid structure. Soft gelatin capsules (softgels) are one-piece, hermetically heat-sealed gelatin-based shells containing a liquid, a suspension, or a semi-solid (Gennadios, 2002). In contrast to hard gelatin capsules, the gelatin shells in softgels are plasticized, typically with glycerol and/or non-crystallizing sorbitol, thus having a flexible structure. Orally administered softgels can be oblong-, oval-, or round-shaped. Gelatin microcapsules, hard gelatin capsules, and softgels have found wide use in the oral administration of drug substances, dietary supplements, and herbal preparations.

The benefits of hard and soft gelatin capsules over tablets for oral administration of pharmaceutical preparations include taste-masking, ease of swallowing, pharmaceutical elegance, and, often, enhanced bioavailability (Bowman and Ofner, 2002; Gennadios, 2002). In general, commercial applications of gelatin capsules in the food industry have been scarce. For example, the use of softgels as single-use/single-dose packages for food processing ingredients (e.g., cooking oils) has been a niche application (Anonymous, 1992).

19.5.4 Envisioned applications in food processing

Meat coatings

As mentioned, collagen films are used commercially as overwraps for boneless meats. In addition, studies showed that extruded collagen films were as effective as plastic bags in maintaining the quality of beef during frozen storage (Conca, 2002). Films or coatings from other types of proteins (i.e., corn zein, wheat gluten, soy protein, and egg albumen) carrying antioxidants have shown promise for reducing the rate of lipid oxidation in meat products (Herald *et al*., 1996; Wu *et al*., 2000; Armitage *et al*., 2002).

Antimicrobial films and coatings

In recent years, research on antimicrobial food packaging systems, an application of active packaging, has attracted great interest, particularly as a result of concerns over microbial food safety (Han, 2000). Edible films and coatings, including those that are protein-based, can potentially be used to carry

and deliver antimicrobial agents to food products (Cutter, 1998; Han, 2002). The diffusivity of antimicrobial agents, such as nisin, sorbic acid, and potassium sorbate, in various types of protein films has been determined (Torres *et al*., 1985; Redl *et al*., 1996; Ozdemir and Floros, 2001; Teerakarn *et al*., 2002).

Several studies have demonstrated the bactericidal or bacteriostatic activity of protein-based films containing antimicrobial agents based on zone of inhibition assays and/or cell counts of test microorganisms. For example, zein or soy protein films impregnated with nisin, lauric acid, lysozyme, or combinations of nisin and lauric acid or lysozyme had antimicrobial effects, enhanced by the addition of the chelating agent EDTA, on *Lactobacillus plantarum*, *Escherichia coli*, *Listeria monocytogenes*, or *Salmonella enteritidis* (Padgett *et al*., 1998, 2000; Hoffman *et al*., 2001). Pre-cast whey protein films containing p-aminobenzoic acid or sorbic acid were inhibitory to several strains of *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium* DT104 (Cagri *et al*., 2001). Wheat gluten, soy protein, egg albumen, or whey protein isolate films carrying nisin were effective in inhibiting *L. monocytogenes* (Ko *et al*., 2001).

The concept of using protein films as carriers of antimicrobials has also been validated through application research. Whey protein films with or without 1% p-aminobenzoic acid were heat-sealed into casings and used to prepare hot dogs that were cooked and surface-inoculated with *L. monocytogenes* (Cagri *et al*., 2003). Populations of *L. monocytogenes* remained relatively unchanged during 42 days of storage at 4 ºC in the case of whey protein casings containing p-aminobenzoic acid. In contrast, populations of *L. monocytogenes* increased substantially in the case of control whey protein casings without p-aminobenzoic acid (Cagri *et al*., 2003). Also, zein coatings with nisin prevented the growth of *L. monocytogenes* on ready-to-eat chicken (Janes *et al*., 2002).

Edible coatings for fresh produce and nuts

Coating formulations based on lipids (e.g., waxes) and/or polysaccharides (e.g., cellulose ethers) are commonly applied to whole and minimally processed fruits and vegetables to reduce moisture loss and/or to impart gloss (Baldwin, 1994; Grant and Burns, 1994; Baldwin *et al*., 1995, 1997). Proteins have not been used as ingredients of fresh produce coatings, mainly because of their hydrophilicity. In general, the successful selection of edible, protective coatings for fresh produce to obtain the same effect as modified atmosphere packaging is challenging (Park, 1999). Cisneros-Zevallos and Krochta (2002) used steady-state mathematical models to study the influence of RH on the performance of hydrophilic films formed as coatings on fruits.

Empirical research has examined the possible utility of protein coatings in prolonging the shelf life of minimally processed produce. For example, zein coatings delayed color change and loss of firmness and weight in whole tomatoes stored at 21 ºC (Park *et al*., 1994a). Cherry tomatoes dipped in wheat gluten film-forming solutions retained better texture (less soft and soggy) than uncoated samples (Tanada-Palmu *et al*., 2000). Broccoli florets packaged in zein films maintained their original firmness and color after six days of refrigerated storage

(Rakotonirainy *et al*., 2001). Caseinate or whey protein coatings delayed oxidative browning of potato and apple slices by acting as oxygen barriers and/or as scavengers of reactive oxidative species (Le Tien *et al*., 2001). Caseinate-lipid emulsion coatings decreased moisture loss from peeled carrots (Avena-Bustillos *et al*., 1993) and zucchini (Avena-Bustillos *et al*., 1994). Whey protein coatings substantially reduced oxygen uptake and rancidity of roasted peanuts (Maté *et al*., 1996) and walnuts (Maté and Krochta, 1997) at low RH conditions. Baldwin and Baker (2002) and Trezza and Krochta (2002) reviewed published research on the use of protein-based coatings for fresh produce and nuts, respectively.

Miscellaneous applications

Several other potential applications for protein-based edible films and coatings have been discussed in the literature. Examples include protein-based coatings for popcorn (Wu and Schwartzberg, 1992), frozen fish (Stuchell and Krochta, 1995), shell eggs (Wong *et al*., 1996; Xie *et al*., 2002), chocolate (Lee *et al*., 2002b), and deep-fat fried foods to reduce oil uptake during frying (Mallikarjunan *et al*., 1997; Huse *et al*., 1998; Rayner *et al.*, 2000).

19.6 Future trends

19.6.1 Sensory properties

The voluminous published research on protein films, and biopolymer films in general, includes several application studies. Researching potential edible packaging applications is crucial to further advancing the commercialization of protein-based films and coatings. Undoubtedly, such research is bound to continue. However, in most of the published application studies, the assessment of protein film/coating functionality did not include sensory evaluation. Tolerability/desirability by consumers is ultimately essential for the commercialization of edible packaging materials. Therefore, it is imperative that sensory evaluation is integrated into edible packaging research to detect the introduction of any adverse organoleptic effects by the tested films and coatings. In fact, a few recent studies reported on the sensory properties of foods treated with protein-based coatings, such as zein-coated cooked turkey meat (Herald *et al*., 1996), zein-coated cooked sweet corn (Carlin *et al*, 2001), whey protein-coated peanuts (Lee *et al*., 2002a), soy protein-coated potato fries (Rayner *et al*., 2000), and chocolate-covered almonds coated with whey protein/lipid formulations (Lee *et al*., 2002b). Also, the sensory attributes of stand-alone emulsion films from whey protein isolate and candelilla wax have been determined (Kim and Ustunol, 2001).

19.6.2 Scale-up of film/coating production and application

Published studies on protein-based films and coatings have primarily used simple, laboratory-scale approaches to prepare protein-based films and coatings

(e.g., solvent casting) and to apply them on food items (e.g., hand-wrapping or manual dipping in film-forming solutions). Such crude techniques, while useful for gleaning insights on protein film formation and application, are largely impractical and unsuitable for scaling-up. For example, the production of stand-alone films via solvent casting, although practiced for selected synthetic polymers on a limited basis, is quite costly and time-consuming. Further research on formation of stand-alone protein films via extrusion is certainly needed. To this effect, a few recent studies have focused on rheological and flow properties of 'malleable,' plasticized protein preparations (Di Gioia and Guilbert, 1999; Redl *et al*., 1999a, b). In regards to protein-based edible coating formulations, their effective, economical, and microbiologically safe application on various food items on a commercial scale presents challenges.

19.6.3 Physical and chemical stability of films and coatings

Inherently, edible packaging materials are themselves susceptible to biodegradation over time, similarly to the foods that they are meant to protect. The expected biodegradability of protein-based films by common microorganisms has been demonstrated (Garcia-Rodenas *et al*., 1994; Park *et al*., 2000; Marquié and Guilbert, 2002; Rhim *et al*., 2003). Therefore, the stability of protein-based edible packaging over time under the intended conditions of use merits investigation. For example, protein films and coatings may function as substrates for microbial growth. Also, protein films and coatings applied on food products that carry proteolytic enzymes, such as meat products, may degrade prematurely, thus losing their utility. Chemical stability is another parameter that can affect the functionality of protein films and coatings. For example, the properties of such films can be substantially affected by Maillard (non-enzymatic browning) reactions with reducing carbohydrates present in foods. Maillard reactions can also change the color of protein films (Cuq *et al*., 1996; Trezza and Krochta, 2000).

19.6.4 Dietary and regulatory restrictions

Potential applications of edible protein-based packaging materials should carefully consider any implications arising from allergic reactions, intolerances, or religion-based dietary restrictions that may be linked to proteins. For example, proteins from several food sources (e.g., peanuts, milk, and eggs) have been known to induce allergic reactions when consumed (Taylor, 1992). Wheat gluten is linked to a form of dietary intolerance known as celiac disease, non-tropical sprue, or gluten-sensitive enteropathy (Skeritt, 1988). The use of proteins of animal origin, such as porcine or bovine gelatin, may be disagreeable to Jewish, Muslim, and/or Hindi consumers. Regenstein and Chaudry (2002) comprehensively discussed the kosher and halal issues pertaining to the use of edible films and coatings.

Finally, consumers embracing a vegetarian lifestyle may also object to the presence of animal protein-based films or coatings in foods of plant origin.

Therefore, the need for properly declaring film-forming ingredients on product labels is obvious. In addition, the business rationale, including business ethics dimensions, of using protein-based films and coatings on products that do not typically contain these particular proteins merits assessment on a case-by-case basis. Labeling alone may not shield consumers from exposure to protein-based packaging materials in the case of bulk, institutional, or food-service packaging. Finally, the regulatory status of 'edible' films and coatings lacks clarity. Several film ingredients and processing techniques mentioned in the research literature would, undoubtedly, raise interesting questions from a regulatory acceptance standpoint.

19.6.5 Cost

Effectively extending the shelf life of food products is not the only requirement for the wide commercialization of protein-based edible packaging. Cost is also a major factor, particularly in comparison to the cost of typical synthetic packaging materials. In general, the cost of proteins and other renewable biopolymers, with the exception of high amylose starch, can be as much as eight-fold higher than the cost of raw synthetic packaging plastics (Fishman, 1997). Blending with inexpensive starch can, perhaps, improve the economic viability of protein-based films and coatings.

19.7 Sources of further information and advice

Academic research on protein films, and edible films in general, remains particularly active in recent years. The output of this research activity has been a large body of articles that interested readers can monitor in peer-reviewed journals in the broad fields of food science and technology. For example, based on a cursory review of volume contents, the *Journal of Food Science*, published by the Institute of Food Technologists (IFT), has printed 13, 12, and 18 articles on edible films and coatings in 2000, 2001, and 2002, respectively. More specifically, 6, 10, and 12 of those articles, respectively, have dealt with protein-based films and coatings. The annual meetings of IFT also present a forum for the dissemination of edible packaging research. A cursory review of the IFT annual meeting programs in 2000, 2001, and 2002, shows that 38, 39, and 29 presented papers, respectively, pertained to edible films and coatings. These numbers included 21, 25, and 14 papers, respectively, focused on protein-based films and coatings. The *Journal of Agricultural and Food Chemistry*, published by the American Chemical Society, has been another rich source of peer-reviewed research on edible films and coatings.

Information related to edible films from whey proteins can be accessed at the non-profit research and education Wed site 'Incredible Edible Films' sponsored by the California Dairy Research Foundation (*http://www.ediblefilms.org*). The patent literature, which serves as a proxy for state-of-the-art developments in

edible packaging in the industry, is another important source of information. An on-line searchable database of US patents is available without charge by the US Patent and Trademark Office at *http://www.uspto.gov/patft/index.html*.

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20

Protein gels

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20.1 Introduction

Nature has two main modes of immobilising large quantities of water at ambient temperature that are relevant to foods: cells and gels. A gel has been defined as a 'form of matter intermediate between a solid and a liquid' (Tanaka, 1981). Gels are also regarded as *soft solids* and ubiquitously present in high moisture foods, as such (e.g., jello, puddings) or as inclusions or matrices in composite foods (Aguilera, 1992). Gels show the following microstructural features that distinguish them from other biomaterials: (i) A continuous network of interconnected material (molecules or aggregates), that spans the whole volume; (ii) the presence of a few restriction points in the network that hold the chains together avoiding flow, and (iii) a large proportion of liquid phase that swells the polymeric network.

Food protein gels may be classified in various ways. According to the supramolecular structure they may be either true cross-linked polymer networks or particle gels consisting of strands or clusters of aggregated protein (Fig. 20.1). Gels can be *thermoreversible*, melting on heating and gelling upon cooling, or *thermoset*, meaning that once formed by heating they will not melt. From the viewpoint of their physical properties they may be brittle or ductile, opaque or translucent, and so on. Protein gels are ubiquitous in our daily foods: boiled eggs, yoghurt, frankfurters, flans, crab leg analogs, etc. Also, they form part of the exquisitely shaped structures of gastronomy recipes, terrines, mousses, etc.

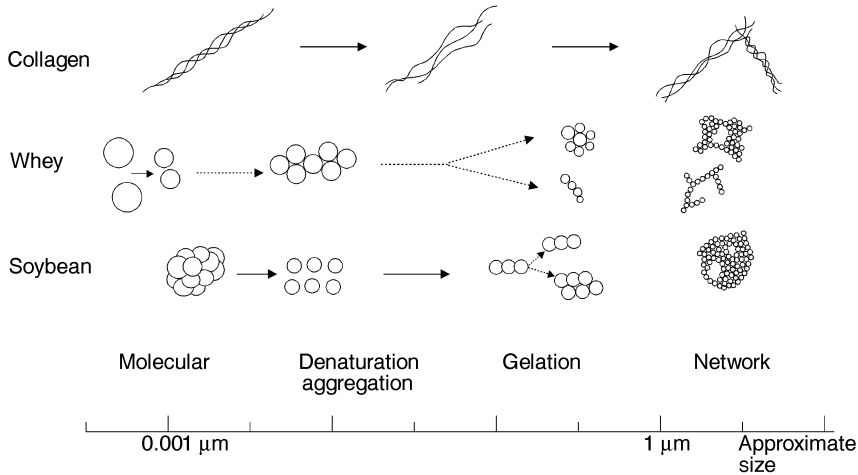

Fig. 20.1 Structural elements formed during gelation of different proteins (scale only as reference).

20.2 Food proteins and their gels

20.2.1 Casein

Casein, with its four main fractions α_{s1}-, α_{s2}-, β- and κ-casein, represents almost 80% of the milk protein and is involved in nearly all structural transformations from liquid milk to milk products exhibiting viscoelastic properties, e.g., yogurt, cheese. Structurally, casein in milk is a suspension of roughly spherical, highly hydrated clusters of 50–300 nm in diameter, called micelles. Micelles are composed of sub-micelles with a diameter of 10–20 nm and have porous, open structures. Casein molecules are strongly hydrophobic, so sub-micelles are held together by hydrophobic bonds and salt bridges and assembled into micelles by colloidal calcium phosphate (CCP). Micelles are stabilised against aggregation by protruding chains of κ-casein, having a hydrophilic C-terminus (the so-called caseinomacropeptide, CMP) and occupying mainly the surface of the micelles. Enzymatic hydrolysis of κ-casein by rennet (containing the proteolytic enzyme chymosin) releases CMP and causes the micelles to aggregate leading to rennet gelation. Aggregation of rennet-altered micelles can be described by the von Smoluchowski theory for the diffusion-controlled aggregation of hydrophobic colloids, provided that a sufficient concentration of particles capable of aggregation has been hydrolysed (Overbeek, 1952; Dickinson, 1992; Walstra, 2003). Structurally, rennet gels are particle gels consisting of strands of more or less spherical casein micelles. The thickness of the network strands is approximately five times the diameter of casein micelles (Fox and Mulvihill, 1990). Regularity and pore size of the network is determined by the rate of the enzymatic reaction and the aggregation process.

Compared to most other proteins, the caseins are very heat stable at their physiological pH (>6.5) and therefore, do not normally form thermally-induced gels. On the other hand, they are insoluble at pH-values around their isoelectric points (pH 4.5 to 4.9) which is the basis of the acid-induced gelation. Upon acidification, CCP in the micelles is solubilised and aggregation of the partly disintegrated micelles occurs due to charge neutralisation. At temperatures above 10 ºC a three-dimensional network structure is formed consisting of multiple strands with chain junctions appearing as clusters of micelles and leaving pores in which the serum is immobilised (for TEM micrographs see Kalab *et al*, 1976). Theoretical approaches to describe the aggregation reactions of destabilised casein micelles and the morphological structure of the network have been made using the adhesive sphere model (De Kruif, 1999) and the fractal models (Walstra, 2003), respectively.

20.2.2 Whey proteins

Acid or sweet dairy whey is the soluble fraction separated from the casein curd during cheese manufacture. Whey proteins is a generic name for many different proteins present in whey among which the globular species β-lactoglobulin and α-lactalbumin comprise almost 50 and 20% of the total protein content, respectively.

Solutions of whey proteins in their undenatured form have the ability to form rigid irreversible gels when heated above *c.* 75 ºC. Heat-induced gelation of whey proteins is typical of globular proteins (such as those from soybean and egg albumin) and proceeds through a series of transitions (Aguilera, 1995): (i) denaturation (unfolding) of native proteins; (ii) aggregation of unfolded molecules; (iii) strand formation from aggregates, and (iv) association of strands into a network. Aggregates are formed in the presence of salts that screen electrostatic repulsion between molecules. Structurally, most whey protein gels are particle gels in which the units forming network are protein aggregates (0.5–2 μm in diameter) associated as a string of beads or in clusters. It is not yet clear which forces stabilise the gel structure but it appears that disulfide bonds, hydrophobic interactions and ionic interactions play a significant role. The microstructure of gels (and their physical properties) depends on the environmental conditions (e.g., pH and ionic strength) and the heating rate under which the gel is formed (Fig. 20.2). For example, transparent fine stranded gels of β-lactoglobulin (strands in the order of nm) are formed above pH 6 and below pH 4, in the absence of salt, otherwise opaque particle gels are formed (Langton and Hermansson, 1992). The water-holding capacity of gels exhibiting a dense network (e.g., fine stranded) is superior to those with an open microstructure having large pores (Hermansson, 1994).

20.2.3 Egg proteins

The liquid part of eggs consists of about 67–70% egg white (albumen) and 30–33% egg yolk. While egg albumen can be regarded as a solution of globular

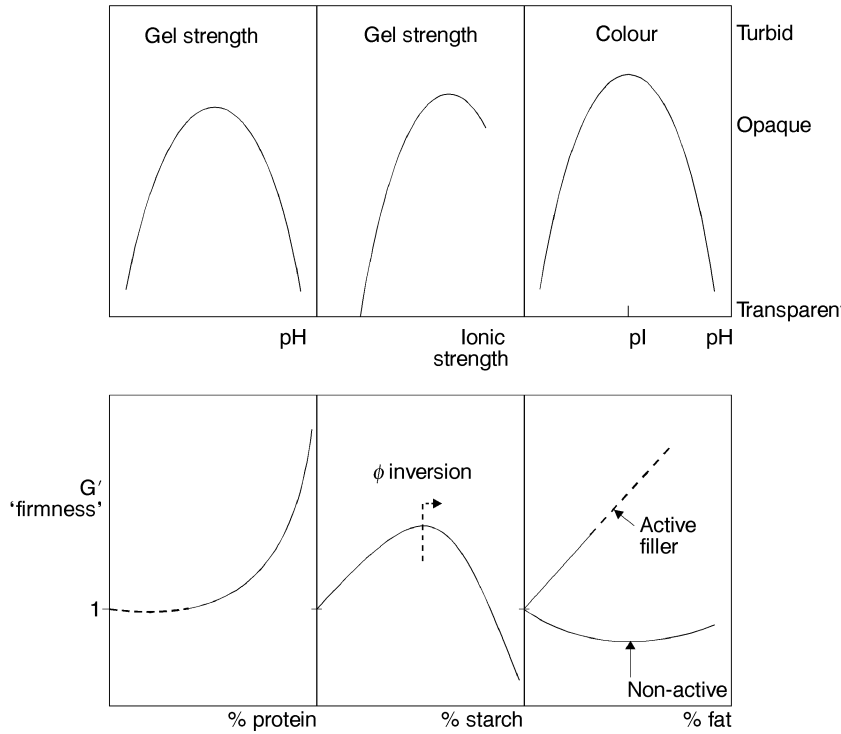

Fig. 20.2 General relation between gel properties and process variables.

proteins containing ovomucin fibres, egg yolk is a very complex structural system. In egg yolk several types of particles like spheres, granules or low-density lipoproteins, are suspended in a protein solution or plasma (for detailed information about the chemistry see Chapter 15). Both fractions of liquid eggs have the capacity to form gels upon heating. Nevertheless for heat-induced gel preparation egg white has been preferred over egg yolk one reason being that egg white is more stable because it contains no lipids. Gel formation of egg white is similar to whey proteins in that a two-step process of denaturation followed by aggregation of denatured proteins takes place as temperature increases. At temperatures above 61 ºC egg white begins to lose fluidity initiated by the denaturation of conalbumin being the least stable protein fraction at native pH (Montejano *et al*., 1984) and followed by denaturation of lysozyme and ovalbumin. Denaturation of ovalbumin, being the predominant protein fraction in egg white, determines the optimum temperature for gel formation and contributes to the increase of gel strength at temperatures above 80 ºC. Exposure to higher temperatures leads to higher rates of gelation resulting in stronger gels. However, temperatures above 90 ºC or excessive heating times may lead to over-processing resulting in a decrease in gel strength, shrinkage of the gel and syneresis.

Depending on the environmental conditions the structure of egg-white gels can range from fine-stranded to particle gels and from transparent to opaque structures (Fig. 20.2). Similar to gels of most other globular proteins the functionality of egg white gels is strongly influenced by pH and ionic conditions since these variables determine the relation between rates of denaturation and aggregation reactions. This is especially important since the pH of egg white increases during storage from approx. 7.6 to 9.4. In addition, the influence of pH has to be considered if egg white is to be used in complex food systems. Gels of good quality concerning firmness and water-holding capacity are obtained at pH 9 due to the high degree of cross-linking, minimal pore size and uniformity of network structure (Woodward and Cotterill, 1986).

20.2.4 Gelatin

Gelatin is widely used in the food industry because of its unique rheological properties (Clark and Ross-Murphy, 1987) and typical melt-in-the-mouth-texture. Gelatin is derived from the ubiquitous protein collagen, the main constituent of connective tissue, by controlled acid (Type A, isoelectric point 6.5 to 9.0) or alkaline hydrolysis (Type B, isoelectric point 4.8 to 5.0) that destroys the quaternary, tertiary and secondary structure of collagen. Gelatin properties are derived from ability to regenerate the triple helical molecular structure of tropocollagen, and the proprotion of proline and hydroxyproline. The type of collagen and the extraction conditions determine the properties of the gelatines derived therefrom, e.g., a lower extraction pH results in gelatines of lower strength. Gelatin swells and adsorbs up to ten times its weight in hot or cold water, and when heated to temperatures above the melting point, gives a viscous solution of random-coiled linear polypeptide chains.

On cooling to temperatures below 20 ºC a gel is formed by partly stacked reformed triple helices of peptide chains (i.e., microcrystallites), stabilised by hydrogen bonding between the amino acids and nearby C-H groups (Johnston-Banks, 1990). Minimum concentration for gel formation is 0.5 to 1.0 %. Gelatin gels are built at relatively low temperatures compared to polysaccharides (e.g., room temperature) and melt at body temperature. The gelation is completely thermoreversible but depending on the cooling rate considerable hysteresis occurs between heating and cooling curves. Factors affecting the gel properties are the concentration, pH, time and temperature of set and the presence of low-molecular weight solutes, i.e., salts and sugars. Main applications of gelatin are confectionery, meat products and chilled dairy products (Johnston-Banks, 1990).

20.2.5 Myosin gels

Heat-induced gelation of myofibrillar proteins plays a major role in the water and fat retention capacity during meat processing, directly affecting process yields and sensory properties. Myosin consists of a mixture of elongated helical chains and compact globular heads. Actin *in situ* consists of a double stranded

'string of pearls' helical fibrous structure formed by polymerised actin monomers. The gelling of myosin is induced by heating above 60 ºC, and it strongly depends on pH and ionic strength. Myosin can form stranded as well as aggregated gels (Hermansson, 1988). Non-filamentous myosin gels made by heating solutions at temperatures above 60 ºC are strong and elastic. The mechanism is predominantly tail-to-tail association via non-covalent bonding. Gels formed from myosin filaments at low ionic strength result in very large aggregates cross-linked via the head groups. Actin does not gel when heated but when added to myosin it enhances the strength of heat-induced gels (Zayas, 1997). Other myofibrillar proteins and muscle constituents can influence the gelling properties as well (Lefevre *et al*., 1999). Fat droplets in meat emulsions and batters appear to be stabilised by an interfacial protein film around the droplets as well as the gel matrix that restricts their movement.

20.2.6 Surimi gels

'Surimi' is a Japanese term for the wet protein concentrate resulting from water-washed, mechanically deboned (minced) fish. Leaching with water in the presence of salt (NaCl) solubilises the myobrillar proteins (myosin, actomyosin and tropomyosin) and removes undesirable compounds such as water-soluble proteins (sarcoplasmic proteins), pigments, digestive enzymes and fat (Lanier and Lee, 1992). After refining and dewatering, the recovery of minced fish is around 20% of the raw material (Ohshima *et al*., 1993). The known commercial form of surimi is the so-called 'frozen surimi', a mixture of washed fish mince and cryoprotectants added to retain the undenatured state and the gel-forming properties of proteins.

Myofibrillar proteins in fish form strong gels upon heating that are generically called *kamaboko.* The gelling properties of proteins in surimi have been utilised commercially in the imitation of high-value marine products: crab meat, scallops and shrimp. Different fish species present different gelling ability attributable to the cross-linking of the heavy myosin chains (Chan *et al*., 1992). Detailed and updated information on surimi processing, regulations and formulations can be found in the book edited by Park (2000).

Gelation of surimi proceeds through several stages during heating (Niwa, 1992; Stone and Stanley, 1992). When fish mince reaches 50 ºC a soft gel network or *suwari* is formed by the interaction of actomyosin and myosin molecules and stabilised by hydrophobic forces between the tails of adjacent myosin molecules. This process, referred to as setting, is highly species dependent. As the temperature increases (e.g., to 52–64 ºC, depending on the species) the structure of suwari is weakened and the softer network of *modori* is formed. This gel-weakening effect has been attributed to the action of alkaline proteases or a thermally driven mechanism (Stone and Stanley, 1992). Further heating (e.g., > 60–70 ºC) induces aggregation of protein molecules until a firm and elastic gel known as *kamaboko* is formed. In kamaboko the stabilising mechanism responsible for gel strengthening is mediated by the globular head

portion of myosin, possibly involving S-S interactions. Upon cooling hydrogen bonding may occur reinforcing the rigidity of the gel.

20.2.7 Soybean protein gels

Although legumes and oilseeds play an important role as foods worldwide, only soybeans proteins are consumed as food gels to a major extent. Tofu, a highly hydrated soy protein gel, is the most important of the soybean products in the Orient (Smith and Circle, 1972). Gelation in tofu manufacture is achieved by heating soybean milk followed by addition of salt (e.g., Ca^{2++} or Mg^{2++}) to form a curd, although acidification (e.g., with glucono-δ-lactone) also induces aggregation of denatured protein molecules as negatively charged groups become neutralised by protons.

Heat-induced gels from soybeans derive their properties from the interaction of two globulin (acid-precipitated) fractions accounting for more than 50% of the protein in soybeans: the 11S fraction (glycinin) and the 7S fraction (β-conglycinin) which vary in a ratio from 0.5 to 1.7 depending on the cultivar. Molecular phenomena involved in heating of soybean proteins and their relation to gelation have been reviewed by Utsumi *et al*. (1997) and Yamauchi *et al*. (1991).

Glycinin is an hexamer (six sub-units consisting of acidic and basic polypeptides linked through disulfide bonds) with a molecular mass of 300–380 kDa and in the native state is found as a two-layered structure. No gels are formed at the PI of the acidic subunits (pH 4.75–5.40). Gelation proceeds first by thermal unfolding and association-dissociation of subunits followed by aggregation involving, at least partly, sulfhydryl-disulfide exchange (Yamauchi *et al*., 1991). At pH 6.0 a coarse aggregated gel is formed while fine stranded gels are formed around pH 7.0. Reversible gels are formed by heating glycinin in the non-denatured state to 80 ºC while gelation at 100 ºC show no such thermoreversibility.

The glycoprotein β-conglycinin is a trimer of 150–200 kDa composed of four subunits. It is described as a flat disc about 8.5 nm in diameter and about 3.5 nm thick (Lampart-Szczapa, 2001). Gelation temperatures of β-conglycinin are lower than those of glycinin and more dependent on protein concentration. Glycinin gels are stiffer than those of β-conglycinin under similar pH and protein concentration, possibly due to differences in network structure and stronger interactions between the protein molecules. Interestingly, mixtures of the 7S and 11S fractions or soy protein isolates perform better in gelling than the individual fractions, denoting that thermal interactions between them are likely to occur (Renkema *et al*., 2001). The maximum strength of soybean gels is achieved around neutral pH (Zayas, 1997).

20.3 Mechanisms of protein gel formation

The different mechanisms of protein gel formation can be classified basically between physically induced (heat, pressure) and chemically induced (acid, ionic,

enzymatic) gelation reactions. In general the gelation of proteins requires a driving force to unfold the native protein, followed by an aggregation process giving a three-dimensional network of aggregates or strands of molecules cross-linked by non-covalent bonds or less frequently by covalent bonds, e.g., disulphide bridges (Totosaus *et al*., 2002).

20.3.1 Heat

Heat-induced gelation is probably the most important method to obtain gels since processing of many foods include a heat treatment or the food is to be heated during preparation. Generally it is accepted that heat-induced gelation is a two-step process. Firstly, an unfolding or dissociation of the protein molecule due to the energy input takes place which may expose reactive sites of the molecule for further reaction. The second step is the association and aggregation of unfolded molecules which leads to the formation of higher molecular weight complexes. Gelation follows if the protein concentration is above a critical value, if at least part of the protein has been heat denatured and if environmental conditions are adequate. The first step may be reversible, the second is usually not.

The overall reaction rate can be determined either by the unfolding or by the aggregation reaction depending on the ratio of the reaction rates of the single steps in a particular temperature range (Kessler, 2002). Starting with the same protein solution, provides different structures especially in respect to particle size and porosity of the aggregates. The rate of heating of the protein solution is another factor which particularly influences the particle size. Whereas fast heating gives small particles a slow rate of heating results in bigger particles. The situation becomes even more complex because heat-set gels are generally made of protein mixtures (whey, soy, egg white) rather than pure fractions of proteins where the various species react in a different way during heat treatment. The bonds involved in the gel network are of various natures. Presumably, -S-S-bridges and hydrophobic interactions often play major roles.

20.3.2 High pressure

High pressure offers an additional degree of freedom in modifying functional properties of proteins since high pressure can be applied as single process or in combination with others, in particular increased temperatures. In general, high pressure favours reactions which lead to a reduction of the overall volume (suspended particles plus solvent) of the system. This becomes important in the case of water. Due to the fact that the formation of an ion in solution is accompanied by an electrostriction of the solvent, the formation of a monovalent ion leads to a negative volume change. As a consequence pressure causes water to dissociate and the pH becomes more acid under pressure (Heremans, 1995).

The same applies for pressure-sensitive buffers like citrate and phosphate solutions. Under pressure, hydrogen bonds especially are favoured due to a

negative reaction volume while hydrophobic interactions are weakened. Concerning proteins, high pressure causes conformational changes which are stabilised by covalent disulfide bonds, e.g., denaturation of whey proteins, or by non-covalent bonds, e.g., dissociation of caseins, leading to coagulation and gelation if the protein concentration is high enough. High-pressure induced denaturation and aggregation show time-dependent behaviour and are to some extent reversible, as was demonstrated for the aggregation of β-lactoglobulin (Dumasy *et al*., 1994). Significant differences in appearance and rheological properties between pressure- and heat-induced gels were reported by several authors but due to differences in the protein type and process parameters applied general guidelines cannot be derived.

20.3.3 Ionic

Enhancing the ionic strength in protein solutions, e.g., by adding calcium or sodium chloride, can shield electrostatic charges on the surface of molecules or aggregates. As a result, the electrostatic repulsive forces between the molecules are reduced or neutralised and gelation can occur. In addition, divalent cations like calcium can promote aggregation of proteins by formation of intermolecular calcium bridges provided that the pH is above the pI. Ionic-induced gelation has been reported for pre-denatured whey proteins and in contrast to heat-induced gelation it is named *cold gelation* (Bryant and McClements, 1998; Alting *et al*., 2002). The two-step process starts by the unfolding of the globular proteins in solution at low ionic strength by heat, pressure or enzymatic means leading to the formation of aggregates. In the second step gels are formed by adding a salt or lowering the pH, to favour the interaction between aggregates. Ionic-induced gelation is of greater importance in polysaccharide gels, e.g., alginate, pectin or carrageenan. The typical egg-box of alginate is formed by junction zones linked by ionic complexation, in which a divalent cation (e.g., Ca^{++}) bridges two strands of the polymer.

20.3.4 Acid

Changes in pH due to the addition of acids or microbial fermentation change the net charge of the protein molecule and therefore alter the attractive and repulsive forces between protein molecules as well as the interactions between protein and solvent, i.e., hydration properties. The net charge of a protein at its isoelectric point is zero and therefore repulsive forces are minimal which favours hydrophobic interactions and aggregation of the molecules. pH-values far away from the isoelectric point prevent gel formation. In addition the solubility of salts changes with pH which may contribute to gel formation as is the case for the acid coagulation of casein micelles in milk due to the solubilisation of colloidal calcium phosphate with decreasing pH. The mechanism of acid gel formation could be explained by the fractal aggregation theory which assumes that spherical particles of a determined radius can move by Brownian motion

and can aggregate when they encounter each other. The aggregates then can further aggregate with each other leading to fractal clusters which may be considered as the building blocks of the gel (Lucey and Singh, 1998).

20.3.5 Enzymatic

Enzyme-induced gelation is based on the introduction of artificial covalent cross-links into food proteins. Amongst others reactions catalysed by trans-glutaminase (TG; EC 2.3.2.13), peroxidase (POD; EC 1.4.3.13) and polyphenol oxidase (PPO; EC 1.14.18.1) are suitable for cross-linking of proteins. Especially transglutaminase has found numerous applications within the food industry supported by the fact that TG possesses a broad specificity for acceptor substrates and can be used for many protein sources, e.g., meat products, fish, milk and soy proteins. TG catalyses acyl transfer reactions between γ-carboxyamide groups of glutamine residues in proteins with primary amines. When the ε-amino group of lysine acts as the acyl receptor, the reaction results in inter- and intramolecular cross linking via ε-(γ-glutamyl)-lysine linkages. Depending on the enzyme concentration, the incubation time and the type and concentration of proteins available functional benefits of TG-treatment are the gelation of proteins, the improvement of gel strength and elasticity and water-holding capacity. By combining incubation with TG with a high-pressure treatment substrates can be cross-linked which are normally not susceptible to TG, e.g., β-lactoglobulin (Lauber *et al*., 2003).

20.4 Mixed gels

A large proportion of natural and fabricated foods are multicomponent systems containing considerable quantities of water. It is not surprising then that improved performance of gels and closeness to real food mimicry is achieved by mixing gelling polymers and/or combining them with other structural elements (particles, droplets, etc.). Five cases in which improved performance is achieved by using mixed gel systems follow. The microstructure of some mixed gels is schematically depicted in Fig. 20.3.

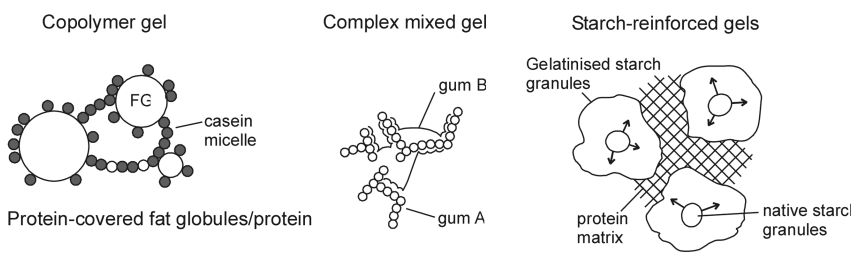

Fig. 20.3 Examples of mixed gels in foods.

20.4.1 Starch-reinforced protein gels

Starch is a widely used filler in commercial surimi-based products to strengthen the mechanical properties of gels as well as to reduce costs (Verres-Bagnis *et al*., 1993). The role of starch granules is to withdraw water from the system as they swell and imbibe water during gelatinisation (ca. 65 ºC), as shown schematically in Fig. 20.3. The net result is that the effective concentration of the protein solution increases and when it gels at a higher temperature (e.g., 75 ºC for whey proteins) a strong protein matrix is formed around gelatinised starch (Aguilera and Baffico, 1997). Excess of starch favours phase inversion and formation of a weak matrix of gelatinised starch in which packets of protein gel are embedded resulting in a weaker gel (see bottom half of Fig. 20.2).

20.4.2 Emulsion gels (copolymer gels)

Fat plays an important role in nutrition, texture and as carrier of fat-soluble colours and micronutrients. Incorporation of a dispersed fatty phase (e.g., fat globules) to an otherwise aqueous matrix (a protein gel) generally affects adversely the physical properties of the emulsion gel. However, filled gels containing protein-covered fat droplets exhibit enhanced stiffness if the adsorbed protein layer interacts with the protein gel matrix (see bottom half of Fig. 20.2). In fact, these emulsion gels containing protein-covered oil droplets are stronger than whey or soybean isolate gels having the same protein concentration (Aguilera and Kessler, 1989; Kim *et al.*, 2001).

20.4.3 Phase-separated gels

Concentrated solutions of mixed polymers (e.g., proteins and polysaccharides) almost always lead to phase separation and de-mixing. In segregative phase separation each phase is enriched in one of the polymers while in associative phase separation one of the phases is enriched in both polymers. If a mixture of gelatin and a non-gelling polysaccharide such as dextran is allowed to phase-separate (gelatin being the continuous phase) and later to gel, the modulus of the mixed gel will be much higher than for a gelatin solution of the same overall concentration. Water partition after phase separation increases the effective concentration of gelatin in the continuous phase and therefore results in a stronger gel (Zasypkin *et al*. 1997).

20.4.4 Kinetically induced gels

As seen before, protein gelation may occur by various mechanisms involving different conditions. A case in point is whey proteins that gel by heating (under specific pH conditions) and casein micelles that form gels by acidification. Co-gels of whey protein and casein micelles can be formed if an acid-producing compound is decomposed in the mixed protein solution during heating, promoting formation of casein strands. Mixed gels of these two protein sources

have been produced using glucono-δ-lactone, a food grade ingredient that releases gluconic acid when hydrolysed by heat (Aguilera and Kinsella, 1991). Kinetics also plays a key role in phase-separated gels as the morphology of the final gel structure will depend on the extent of phase separation when the gel is formed.

20.4.5 Coupled networks

When a favourable interaction exists between chains of two polymers intermolecular binding and gelation may be possible. A classical example is κ-carrageenan and κ-casein above pH 4.4 where both molecules carry the same charge and Ca ions are supposed to link both molecules together. Complex gels are also formed by gelatin and sodium alginate at pHs from 2 to 4.5 where biopolymers bear unlike net charges. This type of synergic interaction is more typical among binary polysaccharide systems

20.5 Conclusion and future trends

Traditional applications of gel-forming proteins in foods are likely to increase as refined or modified protein fractions become commercially available. Increased basic understanding of the relations between protein structure, transformations and interactions with factors controlling the gelation process will result in improved functionality of gels. Some of the areas where protein gels are likely to find future applications are as follows.

- In the development of low-calorie analogs where the singular ability of gels to entrap large quantities of water while imparting desirable textural or functional properties are unsurpassed by other food materials. Phase-separated binary gels trapping a liquid phase inside a continuous gel matrix increase the possibilities of creating tailor-made structures for specific food applications.
- In foods having delicate textures or containing heat-sensitive components the novel process known as cold gelation may reinforce texture and provide extra water-binding capacity.
- Specific functionality may be affected by mechanical shear so that structural modifications of aggregates and gel dispersions result in modified rheological behaviour, for example, to be used as fat replacers (Walkenström *et al*., 1998).
- Novel foods may make use of transparent or translucent food gels made by fine strands which allow light to pass through. Eventually, collapsible gels that absorb or expel water in response to small changes in pH or temperature may be developed following trends in synthetic polymer gels.

20.6 Acknowledgement

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21

Proteomics: examining the effects of processing on food proteins

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21.1 Introduction

For the large part, nutritionists have valued the proteins in the foods we eat primarily as sources of amino acids (1, 2), i.e., viewing them as the building blocks for new proteins to be synthesized in our bodies, rather than as molecules that might have biological or functional importance in their unhydrolyzed states. Those who process the foods had much the same perspective – processed food products naturally low in essential amino acids such as methionine or lysine were supplemented with the deficient amino acid(s) to overcome this deficit (3, 4). No doubt this view is nutritionally correct from the perspective of the macro components. Indeed, historically, societies where a staple food, such as corn, provided a limited set of amino acids, fermentation of the staple produced a new set of foods supplemented by microbial protein synthesis and an improved amino acid composition (5).

Due to recent advances in research in the food industry as well as in basic science, it is clear that food proteins are more than just sources of individual amino acids. Individual proteins in a food may have critical importance in both their intact forms (6) and as partially hydrolyzed products (7, 8). In many cases, this understanding evolved due to identification of the toxic or allergic properties of certain proteins (9) – which therefore created the need to get rid of them. Beyond this rationale, however, there has been a growing realization that the peptides derived from food proteins may have biological activities once ingested (10). Thus it is important to identify these peptides as well as the *proteome* in foods.

A *food proteome* is the set of proteins that constitute the food item. The importance of knowing the *proteome* is not just restricted to nutritionists and

consumers, but also to farmers and food processors. For farmers, regardless of whether they use genetically modified (GM) crops or those grown *organically*, questions such as how the protein content depends on the growing conditions and the timing of harvesting are important to optimize the yield, or to enrich for a particular composition. Similarly, they want to know are animals fed on different foods going to yield the same protein quality? Although there are little data on this, it seems intuitively obvious that there would be variation depending on the nature of the diet and its components. Similarly, the preparation of a protein-enriched product will result in selective retention (or loss) of the proteins as well as chemical modifications of individual proteins and amino acids. Indeed, it is not hard to envisage a situation in the near future where it would be necessary to show that a processed food claiming to be derived from a particular food contains a protein profile consistent with that claim. A further issue, largely in the hands of the consumer, but also the responsibility of the food manufacturer, is how are the proteins in a food modified during storage and use, such as cooking? These are all becoming important questions for the 21st century food industry.

The science of *proteomics* has received a great deal of attention because of its impact on the biomedical research arena. In fact, all the methods developed in biomedicine and other areas of biology are just as appropriate and applicable to agriculture, food processing and food manufacturing. The goal of this chapter is to introduce the terminologies of *proteomics* and describe and appraise the analytical methods that can be used to determine the *food proteome*. Proteomics has gained enormously from the systems biology approach to science much heralded by the publication of genomic sequence information from humans (11, 12) to sea squirts (13) to mice (14) to plants such as rice (15, 16) and the mustard plant *Arabidopsis* (17).

Computationally, it is possible to identify the complete list of genes that are translated into proteins. Proteins, never previously identified, are suddenly revealed from the genomic information. To quantify the amounts of each of the proteins, it is essential first to separate them both from the matrix they are in and from each other. Because of the complexity of the *proteome*, this requires the use of high *resolution*, often *multi-dimensional* protein separation techniques, exploiting individual characteristics of the proteins (charge, hydrophobicity and molecular weight). Once separated, the process of identification can begin. This is carried out by *mass spectrometry* (MS). To facilitate analysis, *bioinformatics* techniques are applied to the MS-derived data. Finally, statistical techniques appropriate for *high dimensional systems* are essential to interpret the data fully. The type of sample that can be analyzed is highly variable. A key element is the reduction of the sample proteome into a microfluidics analysis prior to mass spectrometry. Whereas the mass spectrometer used to be the limiting step, this is no longer true. Heavy investment in computers is needed to keep up with the dataflow from modern mass spectrometers. Statistical help is critical to identify the important features of the data. Bioinformatics support is essential to catalog the resulting data and to convert it into practical information for the nutrition

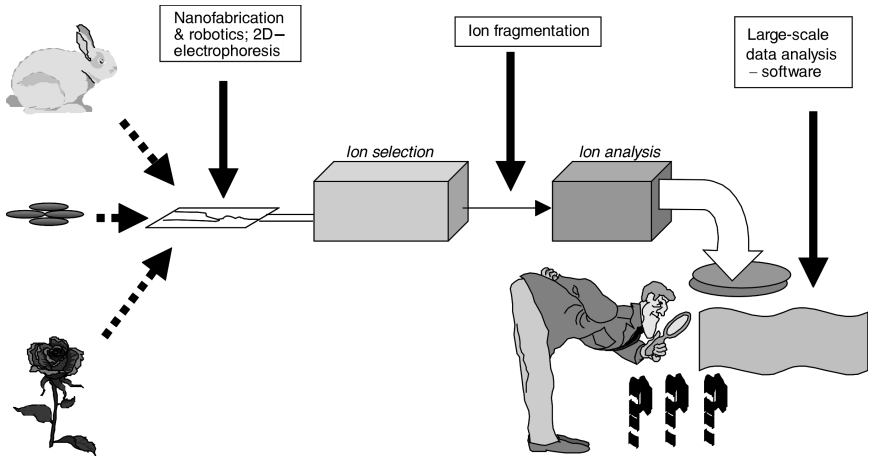

Fig. 21.1 Cartoon of the major components of the analysis of the proteome using mass spectrometry.

scientist and food processing expert. The remainder of the chapter is focused on these topics (Fig. 21.1).

It should be noted that mass spectrometry has been used to study individual food proteins on many occasions (18–21). The advantages of using mass spectrometry are well summarized in the review by Alomirah *et al*. (22). These include the ability to determine the molecular weight of the protein, its posttranslational modifications (both in the organism from which the food is derived and during processing), sequences of protein isoforms, and protein structure. Similarly, 2D-gel analysis of food proteins has been frequently reported (Table 21.1 – refs 23–36). What is relatively new to the food industry is the combination of these two techniques. This chapter provides not only a definition of the terms used in this new science but also advice on the methods that can be used to study foods in a whole new way.

21.2 Protein separation techniques

Proteins are made by cells and are found in every part of a cell. Some are naturally soluble in water, others are insoluble and form part of the matrix that holds cells and tissues together, whereas certain others are imbedded in the cell membranes. Some proteins are small (the really small ones are called *oligopeptides* and may have a role in signaling processes), whereas others can have molecular weights in excess of 200,000 daltons (Da). Although separating them is a daunting challenge, the first problem is recovering them from food or plant matrices.

There are two major approaches to extraction that can be taken. In the first, the proteins are kept in their original state. Plant or food matrices in which there

Table 21.1 Use of two-dimensional electrophoresis for the study of proteins found in foods

Food type	Investigation	Reference
Bird's Nest	Identification	23
Isolated soy proteins	Comparison of processing methods	24
Rice (*Oryza sativa*)	Leaf, root and seed proteomes	25
White sesame proteins	Identification of allergen	26
Yam bean seed proteins (*Sphenostylis stenocarpa)*	Acceptability as a food	27
Food microorganisms		
Bacillus cereus	Heat adaptation	28, 29
Lactobacillus rhamnosus	Heat and osmotic stress response	30
Listeria monocytogenes	Effect of cold shock	31, 32
Listeria monocytogenes	Resistance to divercin-41	33
Listeria monocytogenes	Resistance to salt stress	34
Propionibacterium freudenreichii	Acid adaptation	35
Vibrio parahaemolyticus	adaptation to acid shock	36

are intact cells are homogenized in an aqueous isotonic buffer. Because of the plant cell wall, procedures such as the Hughes press, in which the cells at liquid nitrogen temperature are forced through a small gap and undergo shearing, must be used. The isotonic buffer prevents lysis of internal organelles that may otherwise immediately begin degrading the protein. It may also be necessary to add inhibitor cocktails to this buffer to prevent cellular proteases from degrading the proteins. The homogenate can be subjected to differential centrifugation to separate out proteins associated with the nuclear fraction (< 1,000 × g), chloroplasts, mitochondria and membrane fractions (10,000–30,000 × g), the endoplasmic reticulum (<100,000 × g), and a soluble fraction. The individual organelle fractions can be further resolved by sucrose density gradient centrifugation and by free flow electrophoresis. The latter technique has been recently shown to be particularly useful for purifying mitochondria (37). The methods for fractionating plant proteins have been well reviewed by Pasquali *et al*. (38).

In the second method, all the proteins are solubilized by use of a strong chaotropic agent (7 M urea-2 M thiourea) in a reducing buffer – this reagent breaks intermolecular bonds and unwinds the complex tertiary and secondary structure of a protein (39, 40). This method of solubilization can also be used for the proteins in the subcellular particulate fractions noted above. It is also the preferred method for the proteins in foodstuffs since they have usually been denatured by processing and cooking procedures.

To analyze all the proteins that are in a proteome or sub-proteome, there are two main techniques. First, where the intact proteins are resolved intact by a combination of isoelectric focusing (IEF) and sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) (41). Secondly, where the protein is first hydrolyzed to peptides and then analyzed by LC-mass spectrometry, MUlti-Dimensional Protein Identification Technology (MUDPIT)(42) or other 2D-LC techniques (43).

In biomedical research, the very wide dynamic range of the protein content of blood is a serious issue. However, in foods, dynamic range is of lesser importance unless a minor food component is toxic or has an unusually strong bioactive property. For the food industry, most interest is in the abundance and forms of the major proteins that characterize the properties and appearance of food products, or undergo digestion when consumed. In particular, in food processing one would want to know which proteins were preferentially lost or retained by a procedure used to manufacture a commercial product. In addition, the stability of the proteins during storage and cooking is important. Proteins may also undergo extensive posttranslational modifications both in the original tissue and during processing, storage and cooking.

21.2.1 Principles of two-dimensional electrophoretic separation of proteins

Two-dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-IEF/SDS-PAGE) is very popular and has been in use for nearly 30 years since its introduction by O'Farrell (41). It is limited to proteins in the molecular weight range from 10–250 kDa – however, this includes a large part of the proteome. Its main advantage is that the proteins are analyzed intact so that protein isoforms (where individual amino acid residues are substituted by other amino acids), as well as posttranslationally modified proteins, are physically separated in 2D-space (Fig. 21.2). Solubilized proteins are first subjected to IEF to resolve them with respect to their isoelectric points (pI). In a second orthogonal step, the proteins are separated by SDS-PAGE on the basis of their molecular weights. Generally, isoforms of a protein have similar molecular

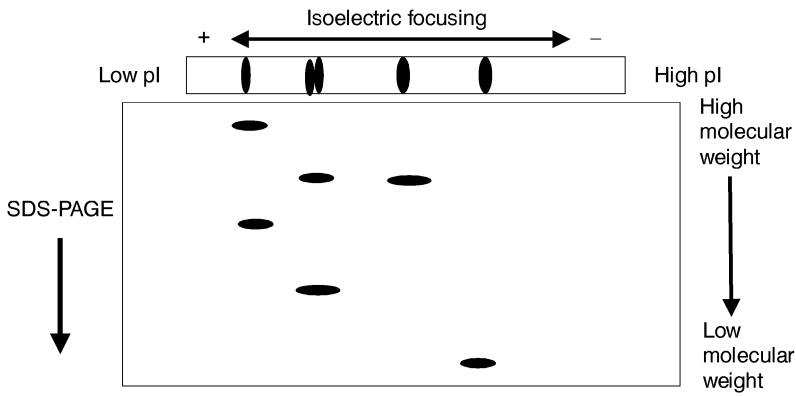

Fig. 21.2 Elements of 2D-IEF/SDS-PAGE separation of proteins.

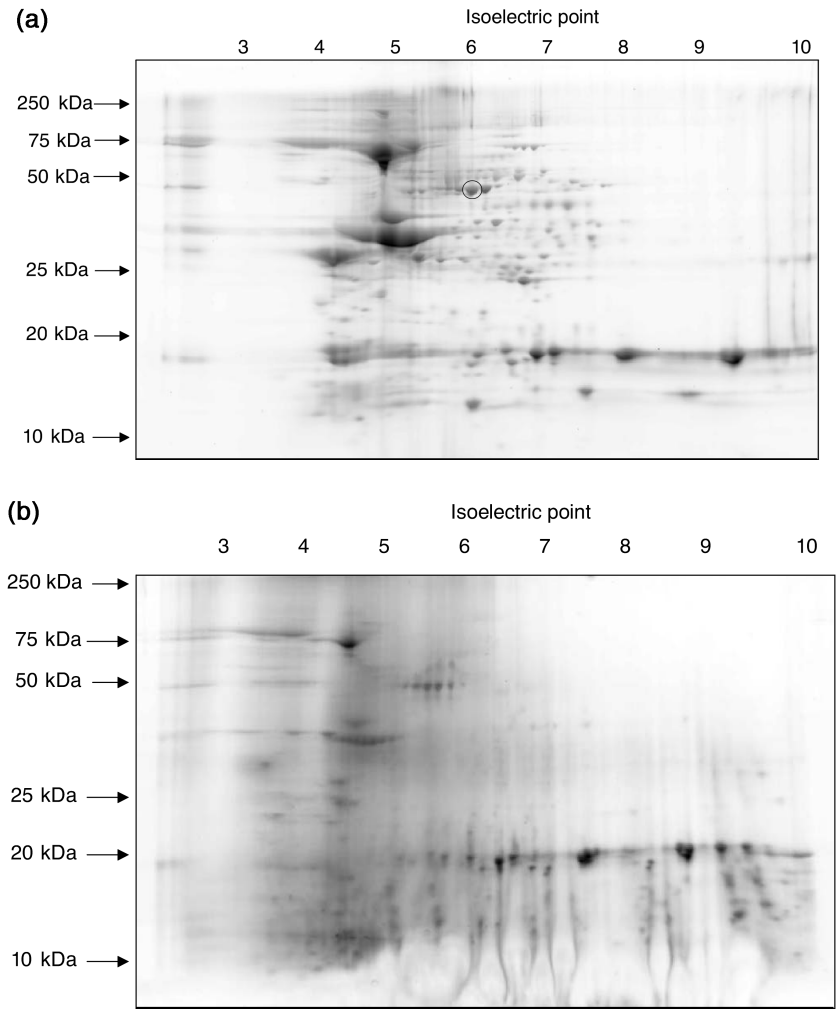

Fig. 21.3 2D-IEF/SDS-PAGE analysis of soybean cotyledon proteins (A) and isolated soy protein (B).

weights and are not separated by the SDS-PAGE step, but rather have different isoelectric points and are thereby separated in the IEF step.

By comparing the positions of proteins in the 2D-picture from a pre-processed sample and from a processed sample, the effect of processing can be readily determined. As illustrated in Fig. 21.3, the proteins were solubilized from each sample by homogenization in extraction buffer (7 M urea-2 M thiourea, 40mM Tris-HCL, pH8.8, 2% pH 3–10 ampholytes, 4% CHAPS and 2 mM tributyl-phosphine). The homogenate was allowed to stand at room temperature for 10 min. It was ultracentrifuged at 100,000× g for 10 min. at 22 ºC to remove particulate matter. The protein concentration was determined using the Bradford assay using

bovine serum albumin as the protein standard. An aliquot of the sample (200 μg protein) was diluted into the IEF buffer (7 M urea, 2 M thiourea, 2% pH 3–10 ampholytes, 4% CHAPS, 5 mM tributylphosphine and 0.1% bromophenol blue) and used to rehydrate individual IPG pH 3–10 strips (Amersham Biosciences) overnight. Following rehydration, the strips were electrophoresed on a cooled (20 ºC) Multiphor II flatbed apparatus (Pharmacia Biotech) at a linear gradient from 1–300 V for 1 min, 300–3500 V for 1.5 h, followed by a final phase at 3500 V for 4.5 h. After IEF, the strips were stored at −80 ºC for at least 16 h. The strips were then equilibrated by gentle shaking for 20 min. at room temperature in SDS-equilibration buffer containing 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS and bromophenol blue. The strips were loaded onto 8–16% acrylamide gradient Criterion Tris-glycine gels (BioRad) and second dimension electrophoresis (SDS-PAGE) was carried out in the Criterion DODECA cell (BioRad) at 200 V for one hour. Gels were then fixed for 30 min. in 40% (v/v) ethanol and 10% (v/v) acetic acid with gentle shaking followed by staining in SYPRO Ruby (Molecular Probes) for 3h-overnight at room temperature.

Following staining, the gels were destained in 10% (v/v) methanol, 7% (v/v) acetic acid for 2 × 2 h and scanned using the FX Pro-Plus fluoroimager (BioRad). Molecular mass standards (broad range, Bio Rad) were electrophoresed next to the IPG strip. The introduction of strips which contain immobilized pH gradients has led to a considerable improvement in the reproducibility of the 2D-gels and therefore the ability to detect protein changes.

2D gel proteomics can be enhanced with the use of Cy-dyes (44). In this method, the lysine groups of the processed sample are minimally (1–3%) labeled with a fluorescent dye (green). The untreated sample is similarly labeled with a second fluorescent dye (red) whose spectral characteristics are distinct from the first dye. The two samples are mixed and electrophoresed on the same 2D-gel. This method takes into account any slight differences from gel to gel in the position of a protein. Unchanged proteins will appear yellow (red/green mixture) and proteins that changed position will be pure red or green. The two samples illustrated in Fig. 21.4 to be compared are reacted individually with one of two different color fluorescent dyes (red for the original specimen before processing and green for the processed food). These label approximately 1% of the protein (on lysine residues). The two samples are then mixed together and run on the same gel – this eliminates gel-to-gel variation in spot movement. In the cartoon, protein spots are depicted where (1) there is no change and both colors (red and green) are present – the spot appears yellow; (2) red spots are proteins eliminated by processing; and (3) green spots are protein forms that were formed by processing.

Another advantage of this method is that for proteins with molecular weights greater than 20 kDa, there is no need to stain the gel after electrophoresis. For the smaller proteins, the increased molecular weights caused by reaction with the Cy-dyes may lead to a signification separation from the unreacted protein; thus, post staining may be required.

Careful analysis of 2D-IEF/SDS-PAGE gels is best carried out with digital imaging devices and image analysis software. The human eye has only limited

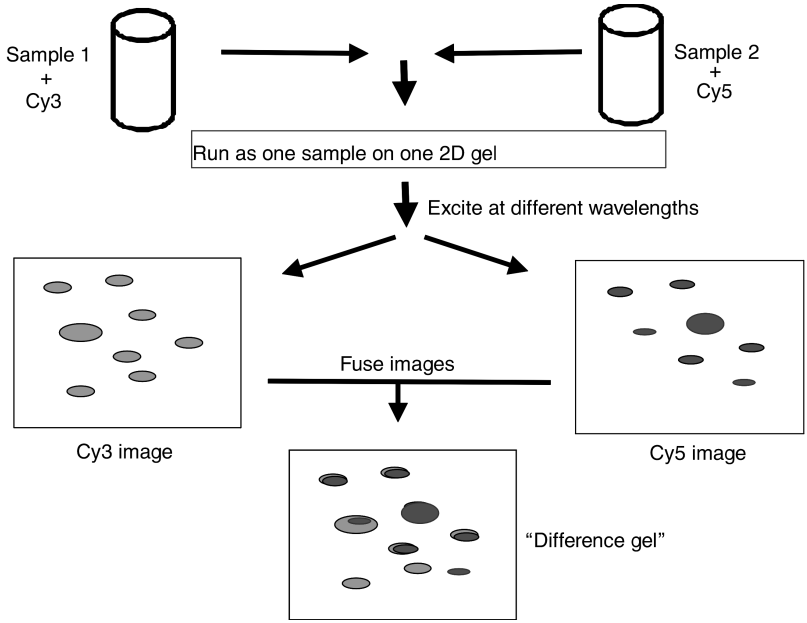

Fig. 21.4 Principles of difference gel electrophoresis.

capability for detecting changes. Although relatively inexpensive software is available (Melanie and PDQuest) for image analysis, more sophisticated (and much more expensive) image analysis software is available to carry out this most time-demanding aspect of proteomics. The reader is encouraged to review a recent evaluation of the commercially available image analysis software (45). Once protein spots of interest have been identified, they can be removed from the gel either manually or by using spot-picking robots. The latter are needed to ensure that the analysis is carried out under *clean* conditions. The much more accurate control provided by the spot picking robot permits selection of regions containing the highest protein concentration and thereby enhances signal to noise. The sensitivities of the techniques described in this chapter are high enough to detect contaminant proteins, particularly keratins, that may be introduced anywhere during processing or from the analyst. As a word of caution, protein spots that are detected by fluorescent sypro Ruby dye should be removed robotically – it is very dangerous for the eyes to be exposed to UV light for even short periods of time. Operators should, in any case, use safety glasses.

21.3 Using mass spectrometry to identify and characterize proteins

To identify a protein spot on a 2D-IEF/SDS-PAGE gel, the region of interest is cut out of the gel (manually or robotically), carefully washed to remove the

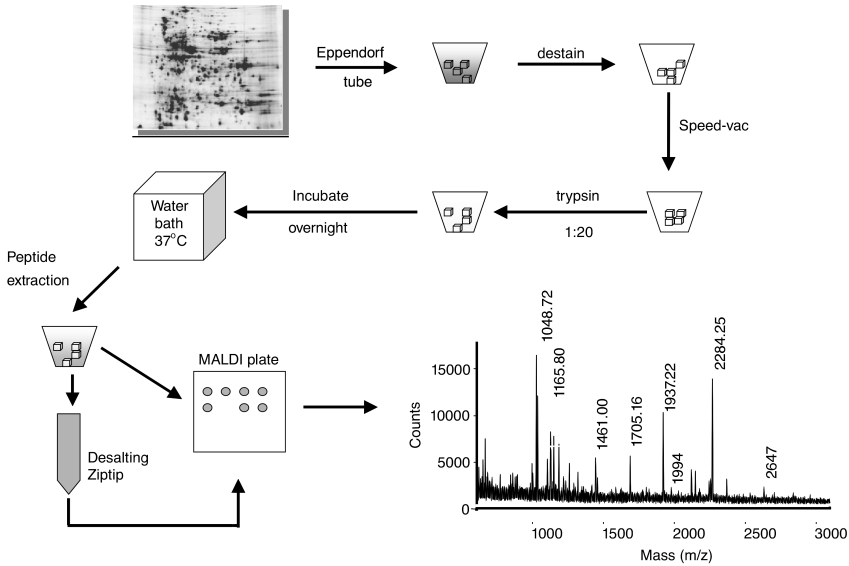

Fig. 21.5 Steps used in tryptic mass fingerprinting and MALDI-TOF mass spectrometry.

electrophoresis buffer and any removable stain, dried, reconstituted in digestion buffer, and then treated with the appropriate peptidase. In Fig. 21.5 the image of the 2D-IEF/SDS-PAGE can be examined by inspection. However, for systematic and unbiased analysis of gels with a very large numbers of spots, powerful imaging software is essential. Once selected, the protein spots of interest are excised from the gel. Note that use of fluorescent dyes to visualize the proteins represents a potential danger to the analyst due to prolonged exposure to UV light. Safety glasses and full coverage of exposed skin are essential if this part of the procedure is carried out manually. It is highly recommended that spot picking robots are used instead. They are far more accurate than the analyst and can pick the part of the spot where the protein concentration is highest, thereby increasing the signal-to-noise. The excised gel pieces are destained to remove the dye and buffers used in the SDS-PAGE step.

After drying, they are rehydrated in 25 mM NH_4HCO_3 buffer, pH 8, containing trypsin, covered to prevent evaporation and incubated overnight at 37 ºC. The peptides in the buffer and those retained in the gel piece are recovered by addition of acetonitrile (final concentration, 50%). The combined extracts are evaporated to dryness and reconstituted in 5% formic acid-50% aqueous acetonitrile (10 μ l). An aliquot of this sample (1 μ l) is mixed with an equal volume of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile. This mixture (1 μ l) is spotted onto the MALDI target plate and allowed to dry. Once in the mass spectrometer MALDI-TOF analysis is performed (see Fig. 21.6).

Proteins, like DNA, are characterized by having a particular linear amino acid sequence. In the denatured state following SDS-PAGE electrophoresis, they are

susceptible to enzymatic hydrolysis, producing sets of peptides whose sequences can be predicted by simple computer algorithms (46). The most common enzyme used for protein digestion is trypsin (which targets Lys and Arg groups), although Glu-C, Arg-C, chymotrypsin and pepsin are also used. After an overnight incubation, the resulting peptides are recovered first by aspiration and then by washing the gel pieces with dilute formic acid or aqueous acetonitrile. If necessary, the solvent is removed by evaporation. The aqueous solution of peptides is cleaned up by passage over a Zip-tip reverse-phase column (Fig. 21.5). The peptides are bound to the column because of their hydrophobicity. Inorganic electrolytes and neutral materials do not bind and any traces in the column are removed by careful water washing. The peptides are eluted with a small volume of methanol or acetonitrile (Fig. 21.5).

Each member of the set of peptides has a particular molecular weight that is determined by its amino acid composition. The peptide *mass fingerprint* is characteristic of a given protein. The fingerprint can be compared against a virtual set of peptides from proteins predicted *in silico* by computers, using proteome databases that are either publicly available or proprietary (Table 21.2).

So, how can we measure the molecular weights of the peptides accurately and quickly? The answer to this question is to use Matrix-Assisted Laser Desorption Ionization (MALDI) to 'evaporate' the peptides, and time-of-flight (TOF) mass spectrometry to determine their masses (Fig. 21.5). The peptide mixture from a protein spot is mixed with a UV-absorbing matrix in a saturated solution (α-cyano-4-hydroxycinnamic acid or sinapinic acid in 50% aqueous acetonitrile) and 1 μl spotted onto a target plate. The solution is allowed to dry slowly so that crystals of the matrix (now containing the peptides) are formed. Once in the MALDI-TOF mass spectrometer, each spot in turn is irradiated with short nanosecond UV laser light pulses, thereby evaporating the matrix and dragging peptide ions into the gas phase. The positively charged ions formed in this initial step are focused by an electric field and then injected into the TOF analyzer by applying an accelerating potential (20 kV). The ions 'drift' down the flight tube of the TOF analyzer and by timing their arrival at a distal photodiode detector, their 'time-of-flight' and hence their mass-to-charge (*m/z)* ratio can be determined (Fig. 21.6). Since they are singly charged, they take the form of $[M+H]^+$ molecular ions.

Table 21.2 Software and websites for protein identifications from MALDI-TOF mass spectra

Program name	Function	URL
MASCOT	Peptide fingerprinting	*http://www.matrixscience.com*
MS-Fit	Peptide fingerprinting	*http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm*
PepMapper	Peptide fingerprinting	*http://wolf.bms.umist.ac.uk/mapper/*
PepSea	Peptide fingerprinting	*http://195.41.108.38/PepSeaIntro.html*
PeptIdent	Peptide fingerprinting	*http://us.expasy.org/tools/peptident.html*
ProFound	Peptide fingerprinting	*http://prowl.rockefeller.edu/*

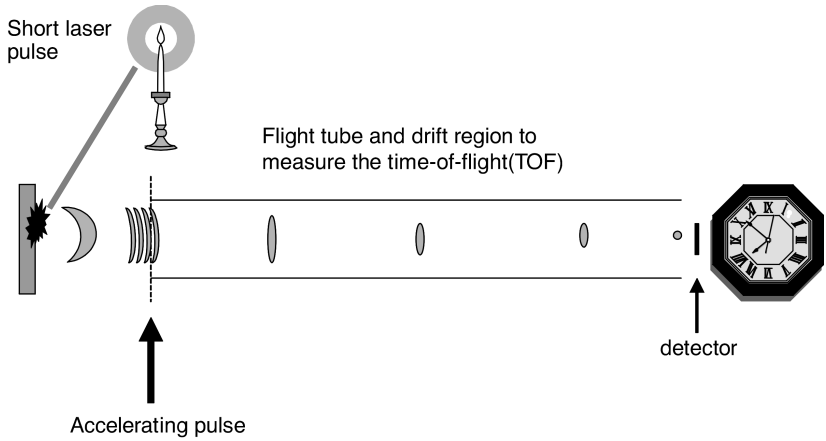

Fig. 21.6 Cartoon of the MALDI-TOF mass spectrometry analysis of peptides.

The peptides in the matrix crystals on the target plate are evaporated by a short (1 nsec) laser pulse. Typically a nitrogen laser operating at 337 nm is used. The resulting ions are allowed to focus at a grid and then are injected with a 20 kV accelerating pulse into a drift-free zone. The time of transit (time-of-flight) to the detector is proportional to the square root of the molecular weight. The data from 100 laser shots are accumulated and processed to obtain the MALDI mass spectrum.

By inspecting the mass spectrum of a sample, the ions that can be attributed to monoisotopic peptide peaks are selected (Fig. 21.7) and these are analyzed by search engines such as MASCOT and MS-FIT to identify the protein that they are derived from. These are both available to academic users at free websites (*http://www.matrixscience.com* for MASCOT and *http://prospector.ucsf.edu/*

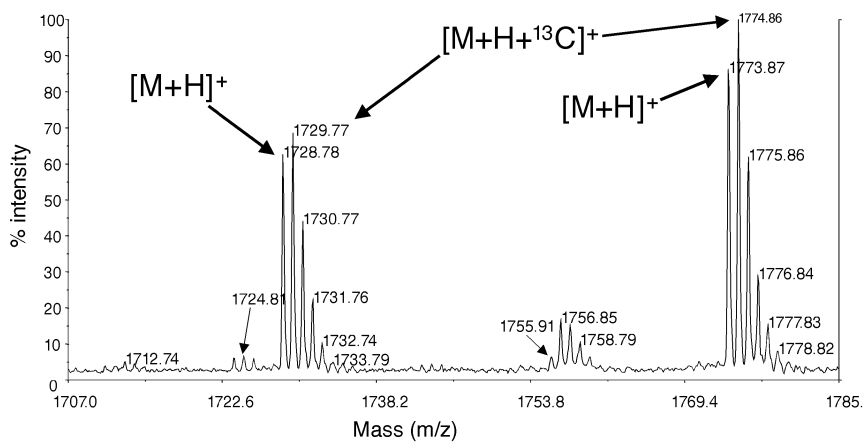

Fig. 21.7 Identification of the monoisotopic peaks of tryptic peptides.

ucsfhtml4.0/msfit.htm for MS-FIT). These software programs are available for in-house analysis for those needing to protect proprietary data. Searching of a protein through data derived from entire proteomes in general takes less than a few seconds, thereby providing identification of proteins at mind-boggling rates. In new TOF-TOF-MS instruments, MALDI spectra can be obtained at a rate of at least 1,000 per hour.

Search engines require the *m/z* of the monoisotopic $^{1}H/^{12}C$ ion for each peptide. At lower masses, this ion is the most abundant one (A). However, from *m/z* 1500–3000, the most abundant ion contains one ^{13}C atom. By spreading out the mass spectrum (B), the isotopic distribution can be readily determined by inspection. Some software carries out the task of doing so automatically.

It should be noted that the protein 'identified' by peptide fingerprinting is the most likely protein out of all possible proteins considered. The fit is statistical in nature as opposed to being absolutely known. The importance of the fit is given by statistical scoring (for MASCOT, the unit of fitting is the MOWSE score). This value increases with more peptides that are identified and/or the accuracy of the observed masses versus the calculated masses. It is often useful to use software to predict the expected set of peptides for the protein in question. There are several websites where this can be carried out (see Table 21.3). It is also helpful to evaluate how other proteases cut the protein; dependent on the sequence, there may be a better choice than trypsin.

To positively identify the protein, it is necessary to determine the amino acid sequence of the observed peptides. Sequencing used to be carried out by chemical methods, but now can be carried out very rapidly with high sensitivity by tandem mass spectrometry. There are three mass spectrometry methods that can be used to acquire MS-MS data – a quadrupole ion trap, a triple quadrupole

Table 21.3 Software and web sites from predicting masses of proteolytic fragments from identified proteins

Program name	Function	URL
MS-Digest	Lists peptides formed by selected protease(s)	*http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm*
PeptideCutter	Lists peptides formed by selected protease(s)	*http://us.expasy.org/tools/peptidecutter/*
PeptideMass	Lists masses of peptides (and their modifications) formed by selected protease(s)	http://us.expasy.org/tools/peptide-mass.html

Table 21.4 Software for using tandem mass spectrometry data for peptide identification and sequencing

Program name	Function	URL
MASCOT	MS-MS interpretation	*http://www.matrixscience.com*
MS-Seq	MS-MS interpretation	*http://prospector.ucsf.edu/ucsfhtml4.0/msseq.htm*
MS-Tag	MS-MS interpretation for beginners	*http://prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm*
PepFrag	MS-MS interpretation	*http://prowl.rockefeller.edu/*

or a TOF-TOF. In each case, guided by the ions observed in the MALDI-TOF experiment and thought to be a member of a set of peptides for the protein of interest, molecular ions are individually isolated and then collided with a neutral gas (hydrogen or argon) generating many fragment ions.

For the quadrupole ion trap and the triple quadrupole, the peptides ions are generated by another procedure, *electrospray ionization* (Fig. 21.8). The very fine spray emerging from the tip of the positively charged spraying needle is directed off-axis (not at) the orifice of the mass spectrometer. It forms a Taylor cone wherein the liquid droplets repel each other. As the spray moves towards the mass spectrometer, rapid evaporation occurs, helped by the warm dry curtain gas. The charged peptide ions in the droplets create an outward columbic pressure as the droplets become smaller. This eventually causes the droplets to burst and thereby release the peptide ions into the gas phase. These ions move orthogonally down the potential gradient between the spraying needle and the orifice of the mass spectrometer. Once in the mass spectrometer they are accelerated which completes the 'drying' of the peptide ions in readiness for analysis.

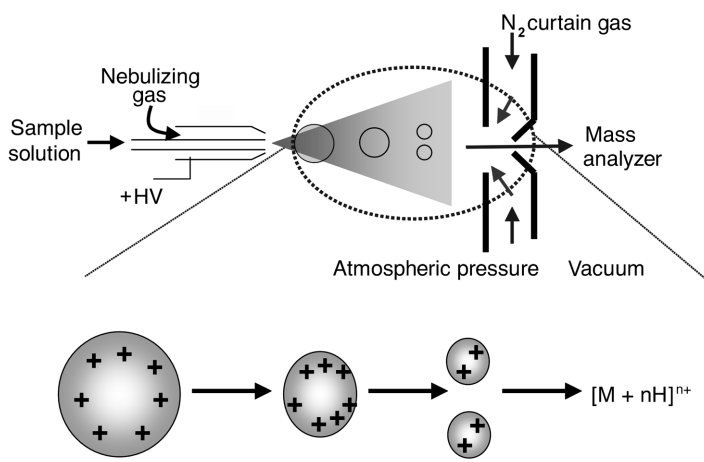

Fig. 21.8 Cartoon of electrospray ionization.

Peptides, either in aqueous solution or in mobile phase solvent following LC separation, are sprayed at atmospheric pressure from a needle set at a voltage (usually 2,500–5,000 V) relative to the orifice leading into the mass spectrometer. The direction of the spray is offset to miss the orifice. The ions that separate from the sprayed droplets as the latter evaporate, move orthogonally towards the orifice and enter the mass spectrometer down the potential gradient. They are further dried by increasing their energy and interacting with a flow of warm, dry curtain gas coming in the opposite direction (Fig. 21.8).

The quadrupole ion trap mass spectrometer 'captures' molecular ions in an ion trap where they can either be successively released from the trap to obtain a mass spectrum, or individual ions are isolated and subsequently caused to undergo gas collisions within the trap. These are in turn are released from the trap to yield a tandem mass spectrum. This type of mass spectrometer is moderately cheap and has modest, but nonetheless, highly usable mass accuracy. It is a particularly sensitive instrument, product of trapping the ions before analysis – tandem mass spectra can be obtained with as little as 10 fmol of a peptide.

In the triple quadrupole mass spectrometer, three quadrupole mass analyzers are arranged in a head-to-tail fashion. The first quadrupole selects the molecular ion of interest (usually the corresponding doubly charged ion) and filters out all the other ions. The selected ions undergo collision in the second quadrupole and then enter the third quadrupole for analysis of their *m/z* values.

The collisions in both the ion trap and triple quadrupole mass spectrometers cause fragmentation of the peptide backbone at the peptide bonds resulting in daughter ions (b and y ions) in which successive losses of individual amino acid residues can be observed (Fig. 21.9). In triple quadrupole instruments, the peptide molecular ions are selected one at a time to undergo collision with inert gas (argon). The energies generated in the collisions induce fragmentation of the ion at its peptide bonds. The principal mechanism is the generation of so-called 'b' and 'y' daughter ions. The 'b' ions originate from the N-terminus and can be written in the general form $[(\text{amino acid residue})_n + 1]^+$, where the amino acid residue takes the form of NHCHRC=O (it is also the molecular weight of the amino acid minus a molecular of water). The 'y' ions originate from the C-

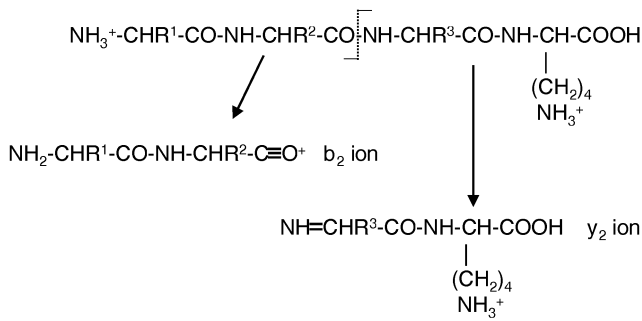

Fig. 21.9 Fragmentation of peptides by collisional dissociation.

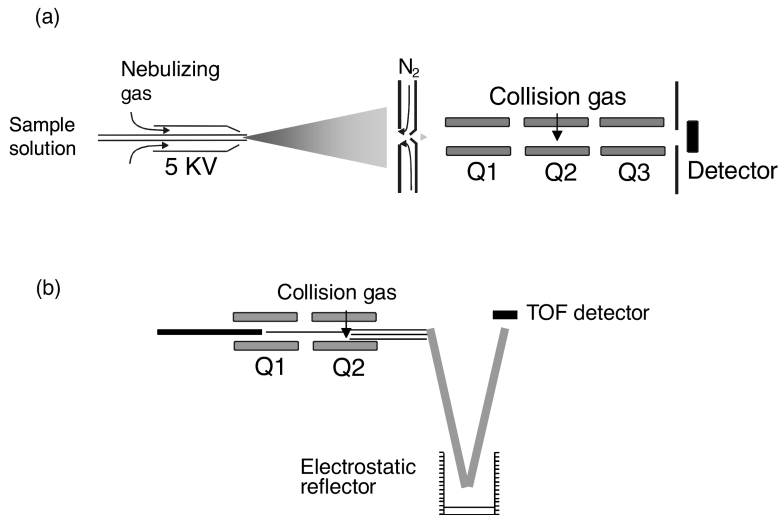

Fig. 21.10 Tandem mass spectrometry.

terminus and are written in the general form of $[(\text{amino acid residue})_n + 18 + 1]^+$. Other fragmentation processes can also occur leading to the loss of $-NH_3$, H_2O, CO and the R side chain.

With experience, these MS-MS spectra can be interpreted by hand. However, computers can do this job much faster. If a sequence is suspected, the *m/z* values of the daughter ions in MS-MS spectrum can be predicted (but not their abundances) and compared to the observed sequence. An excellent description of the expected fragments ion can be found at http://www.matrixscience.com/help/fragmentation/help.html.

Since this last step in a triple quadrupole analysis is very inefficient (the analyzer selects one *m/z* value at a time and in doing so discards large amounts of information on the other ions in the MS-MS spectrum), an orthogonal TOF detector is used instead (Fig. 21.10). This hybrid instrument is known as a Qq-tof. It is substantially more sensitive than the triple quadrupole instrument and has the additional advantage that the mass accuracy of ions in the daughter ion MS-MS spectra is 5 ppm or better (if careful temperature control of the flight tube is maintained). This is important since it increases the quality of the identification of the peptide and has the power to also distinguish between a glutamine (128.05858 Da) and a lysine (128.09496 Da) residue.

In triple quadrupole instruments (Fig. 21.10a), the molecular ions are isolated by the quadrupole filter (Q1) and then collided with argon gas in Q2. The resulting fragment ions are analyzed in Q3. The disadvantage of this arrangement is its low sensitivity as well as its low mass accuracy. The ion intensity at each *m/z* value is measured by scanning. Since one ion is measured one at a time, most of the potential signal is discarded. However, in 2003 sensitivity been substantially improved by using a linear ion trap. The Qq-

TOF instrument (Fig. 21.10b) has the advantage that the TOF analyzer measures *all* the fragment ions. It also produces a very high (~ 5 ppm) mass accuracy.

The sensitivity possible with electrospray ionization depends on the concentration of the peptides in the spraying solvent. The consequence of this is that by carrying out the LC analysis in columns with smaller internal diameters (and hence lower flow rates) considerable improvements in sensitivity can be obtained. The sensitivity increases with the inverse square of the internal diameter of the LC column. So, if the sensitivity limit for analysis on a 2.1 mm i.d. narrow-bore column is 5 pmol injected, then for a 75 μm i.d. column of the same material, the sensitivity increases by $(2.1/0.075)^2$, i.e., 784 times. Thus, on a so-called nanocolumn, the predicted sensitivity limit is 6.4 fmol. For this reason, reverse-phase columns operating at flow rates of 200 nl/min or less have become routine in proteomics analysis. To maximize sensitivity, the reverse-phase HPLC of the peptides arising from a trypsin (or other protease) digest is typically carried out using columns with an inner diameter of 15–75 μm. (Fig. 21.11). The flow rate through these columns is in the range of 20-400 nl/min. The column eluent is passed without splitting into the nanoelectrospray ionization interface. Considerable care is needed to prevent small particles blocking the flow in this arrangement.

In the quest for faster analysis, the TOF-TOF mass spectrometer offers significant gains. This instrument is similar to the MALDI-TOF instrument described earlier. Its distinguishing feature is that it has an accurate timed ion selector gate positioned in the flight tube. Using a brief opening of the gate, ions of a particular *m/z* value are allowed to enter a collision chamber and generate

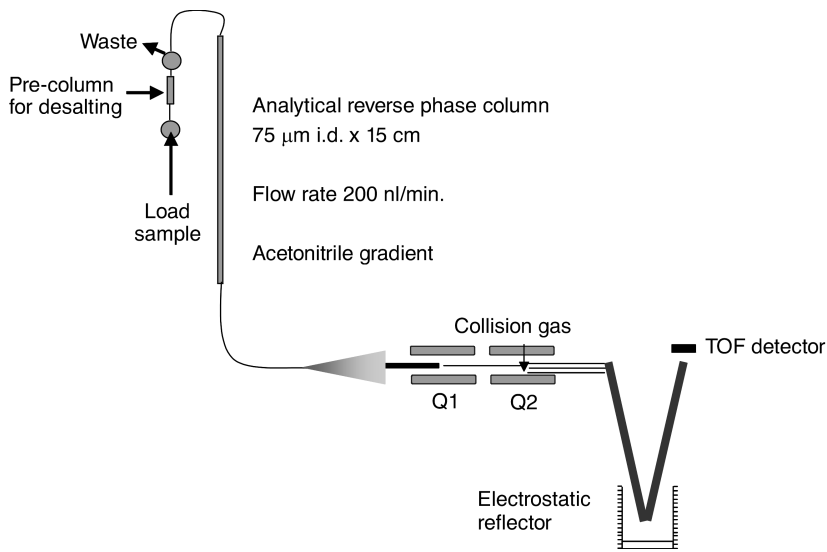

Fig. 21.11 Nano LC-ESI tandem mass spectrometry.

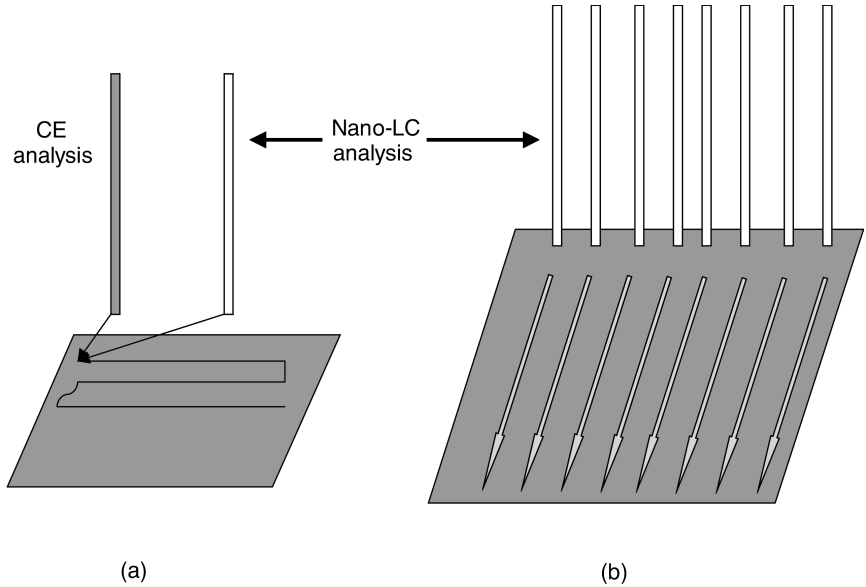

Fig. 21.12 Use of deposition of eluants onto a coated surface for MALDI-TOF analysis.

daughter ions. These are focused and re-accelerated down the flight tube for TOF analysis. Using this instrument, 500 MS-MS spectra/hour can be performed, albeit that this places considerable stress on the data reduction system. It is best suited to peptide fingerprints from proteins that have undergone substantial purification prior to MS analysis. It is possible to lay down the eluate of a nano-reverse phase column or capillary electrophoresis onto a pre-coated MALDI plate that is placed on a moving stage (Fig. 21.12). The nl/min. flow rates associated with nano LC and capillary electrophoresis permit the deposition of their eluants directly onto the surface of a pre-coated MALDI plate (Fig. 21.12a). The plate is moved using a stepping motor that can trace a serpentine track. The advantage of this method is that the material deposited on the track can be studied off-line using MALDI-TOF analysis, and if required, the analysis can be repeated. In another application (Fig. 21.12b), the eluants from eight different nano LC columns can be deposited onto a Maylar film coated with MALDI matrix. In order to measure the eight channels simultaneously, the tracks are subjected to MALDI analysis using a laser operating at 1000 Hz. MALDI-TOF analysis can be successively performed by moving along the trail deposited on the MALDI plate (47). When combined with TOF-TOF analysis it is a particularly powerful technique (48).

Since processing of foods may lead to degradation of proteins and since there are also short peptides (up to 60–80 amino acids) that naturally occur in foods, there is a need to fractionate food proteins so as to recover the fraction with molecular weights below 5,000 Da. Since these short polypeptides may bind to the larger proteins, it is necessary to dissociate them using chaotropic agents (8

M urea or 6 M guanidinium HCl) prior to ultrafiltration. These peptides can be analyzed after careful desalting by either LC-ESI-MS or by TOF-TOF.

21.3.1 Multi-dimensional LC analysis for peptides and proteins

A limitation of the 2D-IEF/SDS-PAGE method concerns proteins whose isoelectric points (pIs) lie outside of the pH range of commercial IEF gels, those that are too little or too big, and those that are insoluble in the IEF buffers. A popular method to overcome these problems is to ignore separating the proteins, but instead use one or more peptidases to turn the entire proteome into peptides. Of course, this makes an even more complex analytical matrix. The protease digests of unfractionated proteins may contain in excess of 10,000 different peptides. To reduce this complexity, the peptides are first absorbed onto a cation exchange resin in H^+ form. They are desorbed in groups using increasing step concentrations of either ammonium acetate or KCl. The desorbed peptides are captured on a reverse-phase column. They are then eluted with a linear (5–50%) gradient of acetonitrile in 0.1% formic acid and analyzed by tandem mass spectrometry using a Qq-TOF or an ion trap mass spectrometer. Both produce very large amounts of MS-MS data that have to be analyzed using automated computer approaches. Although a two-column arrangement is shown in Fig. 21.13, a single column method is widely used. The ion exchange material is stacked above the reverse-phase material. In this arrangement, only ammonium salts can be used to elute peptides from the ion exchange phase. To make the separation of the peptides and their identification tractable, the peptides are subjected to 2D-LC. In the MUDPIT approach developed by Yates and his group (42), the peptides are first retained on a strong cation exchange matrix

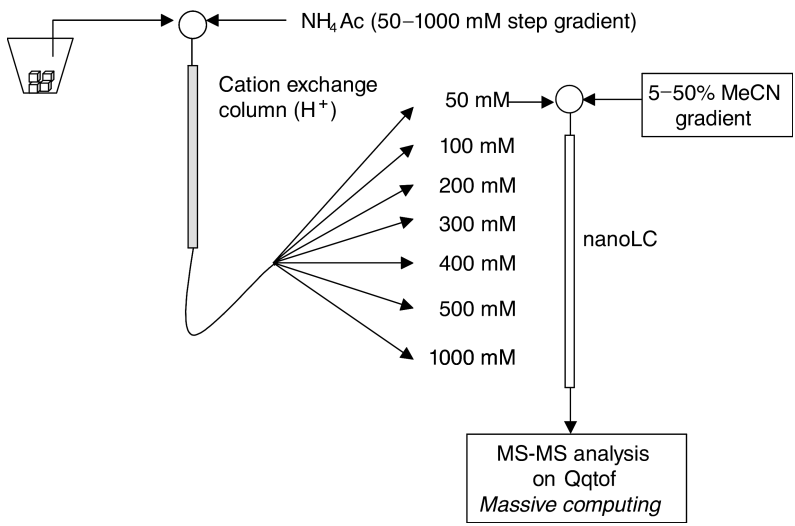

Fig. 21.13 Two dimensional LC analysis of peptide mixtures.

(Fig. 21.13). They are then eluted in 6–12 different fractions by adding ammonium acetate or KCl in concentration steps from 50 mM to 1000 mM. The increasing concentrations of the ammonium or K^+ ions differentially displace the peptides from the cationic matrix that are then immediately captured on an analytical reverse phase column (either in the same column or a separate column – the use of KCl is restricted to multi-column analysis). The peptides in this fraction are eluted by a slow, linear gradient of acetonitrile in 0.1% aqueous formic acid and individual peaks analyzed by ESI-quadrupole ion trap or ESI-Qq-tof to obtain peptide MS-MS spectra. These spectra can be analyzed via the internet by MASCOT software at *http://www.matrixscience.com*, or locally using a licensed version of MASCOT or SEQUEST (a ThermoFinnigan software program). This method of analysis can be fully automated. A typical 2D-LC run for a given sample may take 8–12 hours depending on the number of ammonium acetate elution steps. The computer requirements for analysis of these data are substantial. Many users employ Beowulf clusters for the computer analysis.

In another type of 2D-LC analysis, emphasis is placed on the separation of proteins entirely in the liquid phase (43). Proteins are separated by ion-exchange or IEF and then fractions are passed on for reverse-phase analysis using a pellicular column packing (this prevents the proteins penetrating into the packing and thereby being lost by irreversible absorption). The separated proteins are hydrolyzed as described earlier and analyzed by MALDI-TOF or LC-ESI-MS-MS.

21.3.2 Quantitation and protein mass spectrometry

Once a protein has been identified, it is important to determine how much of it remains following processing of a food. The difference gel electrophoresis method mentioned in section 21.2.1 is the best method for whole proteins. Those using 2D-LC-ESI-MS techniques have taken advantage of the fact that 85% of the proteins in a proteome contain at least one cysteine group. The Cys – SH group is reacted with the so-called ICAT (isotope-coded affinity technology) reagent (49). The ICAT reagent consists of three regions – a part that reacts with the −SH group, an alkyl bridge group that can be labeled with deuterium or ^{14}C, and a biotin group. The proteome sample from the control group in the experiment is reacted with a 'light' ICAT that has natural isotope labeling, whereas the treated sample is reacted with 'heavy' $^{13}C_4$- or D_8-labeled ICAT. Following these reactions, the two proteome samples are mixed and digested with trypsin or another protease, ensuring equal digestion and recovery from that point on in the analysis.

To reduce substantially the total number peptides that need to be resolved, the ICAT-peptides are then captured on a StrepAvidin column (the underivatized peptides do not bind) followed by elution with biotin. Since difficulties were encountered in quantitatively recovering low abundance peptides, an acid-labile group is now included in the ICAT reagent (50). When analyzed by LC-ESI-MS,

a peptide is observed as two sets of ions – the lower *m/z* ion is from the peptide from the control experiment, whereas for the one from the treatment experiment the *m/z* value is 8 amu higher. By comparing the ratios of intensities of these two sets of ions for each of the peptides that can be detected, investigators can determine which proteins are unchanged and which are significantly increased or decreased. The issues at the statistical level are very similar to those that have been encountered in DNA microarray analysis.

There are other methods to label proteins isotopically and hence produce quantitative data. In one, the two sets of proteins are digested in either $H_2{}^{16}O$ or $H_2{}^{18}O$ (51). The ^{18}O is added to the peptide formed in the proteolysis. This is more generally useful for all peptides, but of course creates a major problem in that all the peptides in the proteome have to be separated and analyzed. The isotope shift (+2 Da) results in overlapping isotope profiles, and the need for two separate proteolysis reactions, introduces error into the analysis. For those studying protein synthesis and turnover, the treatment group (plants or cultured cells) is administered ^{15}N-labeled amino acid (52). The $^{15}N/^{14}N$ ratio can be determined for peptides containing that amino acid. Of course, this method may be affected by metabolism of the labeled amino acid, although the large unlabeled sink of related compounds makes it unlikely that the label will reappear in other amino acids.

Finally, the question of peptide quantification can be approached using a triple quadrupole multiple reaction ion monitoring (MRM) method. It is based on the selection of the *m/z* for the molecular ion of the peptide of interest, collision with argon gas, and then selection of a specific daughter ion. MS scans are not obtained in this method; instead, several channels, each with its own parent/daughter ion combination, are monitored in turn for 50–100 msec periods on a revolving basis. Peptide mixtures are analyzed by acetonitrile gradient reverse-phase LC as described elsewhere in this chapter. Individual ion chromatograms are produced and the areas under the single peak determined by integration. By adding a known amount of a synthetic ^{13}C-labeled peptide corresponding to the peptide of interest to the digest, the amount of the biologically derived peptide can be calculated. Although widely used for the quantitative analysis of small molecules (xenobiotics, nutrients in foods, metabolites) (53), its application to proteomics has generated a new acronym AQUA (*A*bsolute *QU*antitative *A*nalysis) (54).

21.3.3 Bioinformatics, proteomics and mass spectrometry

The power of proteomics and mass spectrometry is such that in a very short period of time a spot on a gel can be associated with a GI|number of a protein. What is this GI|number? It is a record number of a file on that protein at bioinformatics sites (Table 21.5). The best known of these are the National Center for Bioinformatics (NCBI), part of the National Library of Medicine in the USA, and **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy) at the Swiss Bioinformatics Institute. The ExPASy site also has a section dedicated to 2D-gels of numerous proteomes (although not one

Table 21.5 Bioinformatics programs to pursue newly identified proteins

Program name	Function	URL
Entrez	Locates publication site and sequence information for the protein – also permits BLAST and Blink searches	*http://www.ncbi.nlm.nih.gov/entrz/*
ExPASy	A well annotated site for protein information. Contains 2D-gel images and links to other sites having 2D-gel information.	*http://www.expasy.org*

of soy proteins) and links to many other sites with 2D-gel information (*http://us.expasy.org/ch2d/*). Many of these images are annotated, i.e., by clicking on a spot on the gel image, one is linked to the full record of the protein in the Swiss-Prot or TrEBML databases. Unfortunately, there is no equivalence of accession numbers between the different databases. When using several programs, it is better to do this all at one bioinformatics site.

21.4 The impact of food processing on soy protein

21.4.1 Identifying proteins that are lost or chemically changed during food processing

It is highly probable that food processing alters the complement of proteins that were present in the unprocessed raw material. An example of this is the changes that occur in the manufacturing of soy protein isolate from soybean cotyledons. The process involves initial delipidation of crushed cotyledon with hexane, solubilization of the de-fatted proteins in an alkaline buffer (to remove insoluble complex carbohydrate material), and finally precipitation of the proteins at approximately pH 5. This last step removes soluble materials, mostly carbohydrates, but also some proteins. In addition, the mild alkaline treatment could lead to hydrolysis in sensitive regions of the proteins. The net result is that the protein profile of the soy isolate is different from the whole soybean (24). As a consequence, attempts to purify soy into different fractions to identify the substances that lead to its alleged beneficial health effects may be compromised by the changing protein composition background in each fraction. As noted earlier, the role of short peptides, either naturally short (up to 10 kDa) or produced from partial hydrolysis of soy proteins during processing or fermentation, is newly appreciated in nutrition science.

There are marked differences in protein composition of soy cotyledon and isolated soy protein (Fig. 21.3a and b). In isolated soy protein, there is a

substantially increased number of protein spots below 25 kDa. The latter is consistent with protein degradation. In addition, most of the proteins with molecular weights between 35–65 kDa and pIs from 6–7 are absent in the isolated soy protein.

The soy cotyledon contains large amounts of the storage proteins, beta-conglycinin and glycinin (55). Beta-conglycinin is the predominant protein in the 7S fraction of soy protein, whereas glycinin is the predominant protein in the 11S fraction. Beta-conglycinin consists of α, α' and β subunits (56). The β subunit of beta-conglycinin has a predicted average molecular weight of 47,776.5 Da and pI of 5.57. In the 2D-electrophoretic image of soy cotyledon proteins, there is a cluster of 8 spots (Fig. 21.3a) with these parameters. There is an apparent molecular weight difference between spots 1–4 and spots 5–8. The lateral separation within each of these groups is suggestive of individual isoforms (substitution of lysine or arginine groups by neutral or acidic amino acids would lower the pI), or there are posttranslational modifications such as phosphate groups on serine, threonine or tyrosine residues. The latter may give rise to apparent molecular weight shifts that are larger than the 80 Da of the phosphate group.

As an illustration of how proteomics can be applied to food protein analysis, the beta-conglycinin β subunits were then subjected to the various bioinformatics and mass spectrometry methods described in the previous sections. First, each spot was cut out of the gel, and subjected to trypsinolysis. The tryptic peptides were analyzed using MALDI-TOF mass spectrometry (Fig. 21.14a and b). Note that it is necessary to carefully identify the monoisotopic peak; for peptides with 14–20 amino acids, the abundance of ^{13}C is such that the most intense peak in the isotopic cluster contains one ^{13}C atom (Fig. 21.7). Database searches of the tryptic mass fingerprints for each spot matched tryptic peptides predicted for the beta-conglycinin β subunit with MOWSE scores greater than 150. Using PeptideMass, an EXPASY program, the expected peptide masses of this protein were determined (Table 21.6). By comparing these values with the observed ions in the MALDI-TOF mass spectrum (Fig. 21.14), the sequence coverage is 49%. The protein spot marked with a circle in Fig. 21.4 was digested with trypsin and subjected to MALDI-TOF analysis (a). The region from *m/z* 1200–1650 is shown in an expanded form (b). A list of expected and observed ions corresponding to those without missing tryptic cuts is given in Table 21.6. Ions with missing tryptic cuts are marked with asterisks (*). The sequence coverage in this experiment is shown in Table 21.7. The ions at *m/z* 1618.84 and 1827.90 were not identified. Examination of the MALDI-TOF mass spectra for all eight spots revealed two peptides at *m/z* 1551.73 and 1585.73. The latter (*m/z* 1585.73) corresponds to the N-terminal peptide sequence VREDENNPLYR and the former to the Phe/Leu variant VREDENNPFYR.

Searches for beta-conglycinin β subunit sequences at the Swiss-Prot/TrEMBL database (*http://us.expasy.org/cgi-bin/sprot-search-ful*) revealed three entries for this protein (GLCB SOYBN, P25974; O22121; Q93VL9). These also appeared as the most homologous proteins when a BLAST search was conducted against this database. An additional sequence (PDB1IPJ) was

Table 21.6 Expected and observed tryptic peptides from the beta-conglycinin beta subunit from *Glycine Max*

Expected	Observed	Residue #s	Amino acid sequence
4415.02	–	158–194	YDDFFLSSTQAQQSYLQGFSHNILETSFHSEFEEINR
4270.11	–	332–370	AELSEDDVFVIPAAYPFVVNATSNLNFLAFGINAEN NQR
3276.79	3276.75	74–102	IVQFQSKPNTILLPHHADADFLLFVLSGR
2657.38	–	269–292	DLDIFLSSVDINEGALLLPHFNSK
2181.11	2181.12	126–144	IPAGTTYYLVNPHDHQNLK
2093.22	–	293–312	AIVILVINEGDANIELVGIK
1773.88	1773.86	387–402	QVQELAFPGSAQDVER
1728.79	1728.79	38–52	SSNSFQTLFENQNGR
1439.68	1438.71	409–420	ESYFVDAQPQQK
1407.68	1407.68	232–243	TISSEDEPFNLR
1372.62		114–125	DSYNLHPGDAQR
1330.57	1330.58	28–37	EDENNPFYFR
1256.61		320–329	QEEEPLEVQR
1243.66		03–113	AILTLVNNDDR
1235.59		195–204	VLFGEEEEQR
1229.67		205–215	QQEGVIVELSK
1153.56		246–255	NPIYSNNFGK
1134.62		429–439	GPFPSILGALY
1094.67		148–157	LAIPVNKPSR
1010.52		256–263	FFEITPEK
956.52	956.53	63–70	SPQLENLR

discovered by carrying out a BLAST search on the NCBI database. The four sequences were converted to FASTA format and aligned using CLUSTALW (Table 21.8). This analysis revealed that there are four residues where amino acid heterogeneity apparently occurs (36 – Phe/Leu; 61 – Val/Gly; 166 – Gly/Ser; 197 – Phe/Leu; 267 – Leu/Pro). However, none of these amino acid residue heterogeneities would be expected to alter the pI and thereby generate the spot pattern observed in Fig. 21.6.

It is therefore reasonable to propose that the differences in pI values are due to phosphorylations of serine, threonine or tyrosine residues. Although this has yet to be established for the beta-conglycinin β subunit, it has been previously shown that two serines in the beta-conglycinin α subunit are phosphorylated by yeast cAMP-independent protein kinase (57). It should, however, be noted that these residues are not present in beta-conglycinin β subunit.

21.4.2 Other chemical changes that occur during processing and/or storage of food proteins

The aldehyde forms of glycosyl residues in the carbohydrates in foods can react with lysine residues on a protein. This initially results in the formation of Schiff

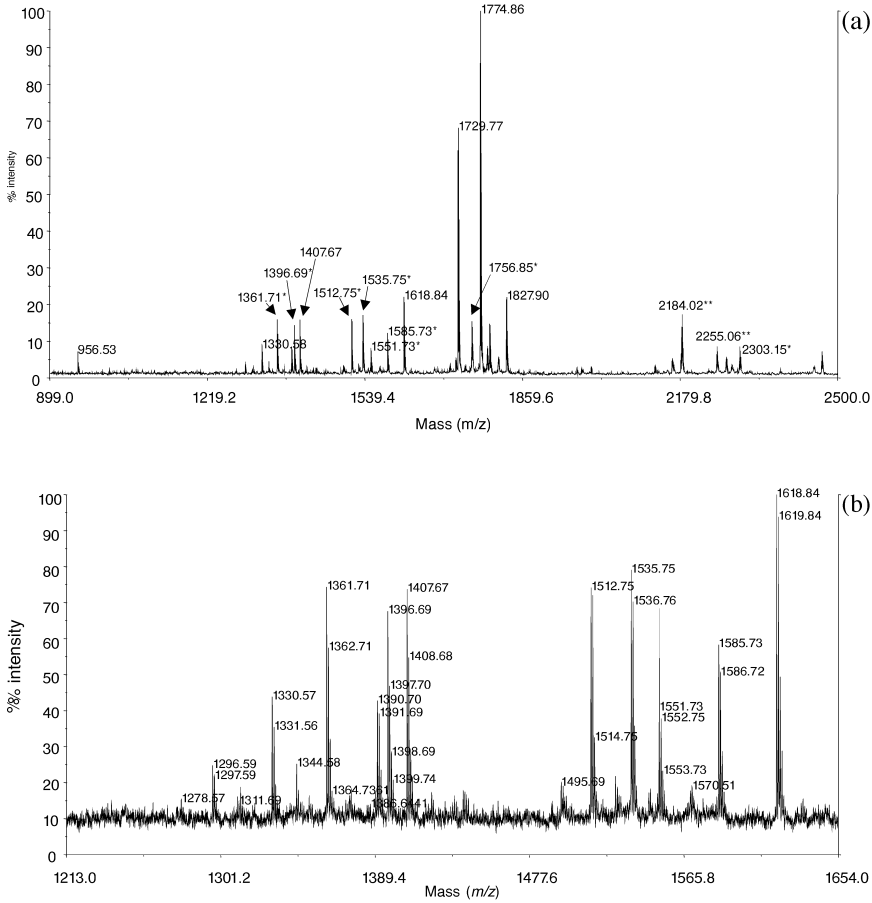

Fig. 21.14 MALDI-TOF analysis of a soybean β-conglycinin β-subunit.

bases, which then undergo further oxidation leading to advanced glycation end (AGE) products. These are a particular problem for the food industry in the form of the Maillard reaction (58), the browning of foodstuffs that occurs during cooking (acceptable) and storage (unacceptable). Formation of AGE products also occurs physiologically, being particularly important in diabetic patients who have elevated blood glucose concentrations (59), in ageing (60), and in certain organs such as the lens protein in the eye where protein turnover is very low (61).

The preparation of a purified protein product such as an isolated soy protein represents an advantage in that the carbohydrate content is substantially reduced compared to the soy cotyledon and other soy protein products. Since the formation of AGE products occurs on lysine residues, this can interfere with peptide mass fingerprinting. A solution to this problem is to utilize proteases such as chymotrypsin or Glu-C that cut proteins at other amino acid residues. In

Table 21.7 Amino acid sequence of pro-beta-conglycinin beta subunit*

1	MMRVRFPLLV	LLGTVFLASV	CVSLKVREDE	NNPFYFRSSN	SFQTLFENQN	GRIRLLQRFN
61	KRSPQLENLR	DYRIVQFQSK	PNTILLPHHA	DADFLLFVLS	GRAILTLVNN	DDRDSYNLHP
121	GDAQRIPAGT	TYYLVNPHDH	QNLKIIKLAI	PVNKPSRYDD	FFLSSTQAQQ	SYLQGFSHNI
181	LETSFHSEFE	EINRVLFGEE	EEQRQQEGVI	VELSKEQIRQ	LSRRAKSSSR	KTISSEDEPF
241	NLRSRNPIYS	NNFGKFFEIT	PEKNPQPRDL	DIFLSSVDIN	EGALLLPHFN	SKAIVILVIN
301	EGDANIELVG	IKEQQQKQKQ	EEEPLEVQRY	RAELSEDDVF	VIPAAYPFVV	NATSNLNFLA
361	FGINAENNQR	NFLAGEKDNV	VRQIERQVQE	LAFPGSAQDV	ERLLKKQRES	YFVDAQPQQK
421	EEGSKGRKGP	FPSILGALY				

*The first 25 residues are predicted to be a leader sequence, with the mature protein N-terminal amino acid being Val26. The predicted molecular weight (average mass) of the mature protein is 47,776.5 Da and its predicted pI is 5.57.
The shaded residues represent tryptic peptides with no missing cuts that were detected by MALDI–TOF mass spectrometry analysis (see Table 21.6); the underlined residues are from tryptic peptides with one missing trypsin cut. The observed peptides accounted for 237 out of the 414 residues (57.2%).

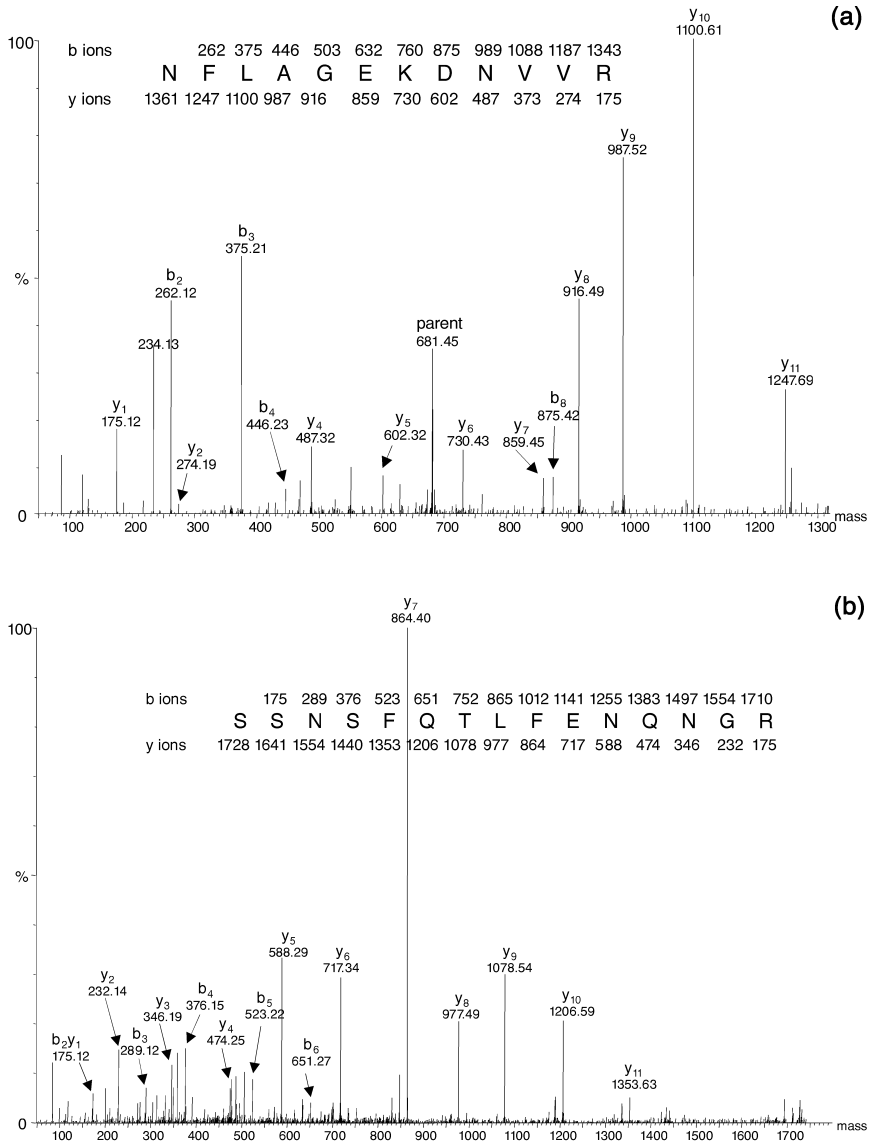

Fig. 21.15 Tandem mass spectroscopy of two tryptic pesticides from the soybean β-conglycinin β-sununit. In (a), the tandem mass spectrum of NFLAGEKDNVVR is shown; in (b) it is SSNSFQTLFENQNGR.

the case of the beta-conglycinin β subunit, Glu-C generates the same number of peptides with molecular weights between 800–3000 Da as trypsin. MALDI-TOF mass spectrometry has been utilized to identify AGE products formed in the reaction between lysozyme and D-glucose (62).

Table 21.8 Clustal analyses of β-conglycinin beta subunit sequences from the Swiss–Prot/TrEMBL and NCBI databases

```
sp|P25974|   MMRVRFPLLVLLGTVFLASVCVSLKVREDENNPFYFRSSNSFQTLFENQNVRIRLLQRFN   60
tr|Q93VL9   MMRVRFPLLVLLGTVFLASVCVSLKVREDENNPFYFRSSNSFQTLFENQNGRIRLLQRFN
tr|O22121   — ——————————————————LKVREDENNPFYFRSSNSFQTLFENQNGRIRLLQRFN
pdp|1IPJ                          LKVREDENNPFYLRSSNSFQTLFENQNGRIRLLQRFN

sp|P25974|   KRSPQLENLRDYRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHP   120
tr|Q93VL9   KRSPQLENLRDYRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHP
tr|O22121   KRSPQLENLRDYRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHP
pdp|1IPJ    KRSPQLENLRDYRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNNDDRDSYNLHP

sp|P25974|   GDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYDDFFLSSTQAQQSYLQGFSHNI   180
tr|Q93VL9   GDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPSRYDDFFLSSTQAQQSYLQGFSHNI
tr|O22121   GDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYDDFFLSSTQAQQSYLQGFSHNI
pdp|1IPJ    GDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYDDFFLSSTQAQQSYLQGFSHNI

sp|P25974|   LETSFHSEFEEINRVLFGEEEEQRQQEGVIVELSKEQIRQLSRRAKSSSRKTISSEDEPF   240
tr|Q93VL9   LETSFHSEFEEINRVLFGEEEEQRQQEGVIVELSKEQIRQLSRRAKSSSRKTISSEDEPF
tr|O22121   LETSFHSEFEEINRVLFGEEEEQRQQEGVIVELSKEQIRQLSRRAKSSSRKTISSEDEPF
pdp|1IPJ    LETSFHSEFEEINRVLLGEEEEQRQQEGVIVELSKEQIRQLSRRAKSSSRKTISSEDEPF
```

Table 21.8 Continued

sp|P25974|	NLRSRNPIYSNNFGKFFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSKAIVILVIN	300
tr|Q93VL9	NLRSRNPIYSNNFGKFFEITPEKNPQPRDLDIFLSSVDINEGALLLPHFNSKAIVILVIN	
tr|O22121	NLRSRNPIYSNNFGKFFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSKAIVILVIN	
pdp|1IPJ	NLRSRNPIYSNNFGKFFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSKAIVILVIN	
sp|P25974|	EGDANIELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLA	360
tr|Q93VL9	EGDANIELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLA	
tr|O22121	EGDANIELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLA	
pdp|1IPJ	EGDANIELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLA	
sp|P25974|	FGINAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQPQQK	420
tr|Q93VL9	FGINAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQPQQK	
tr|O22121	FGINAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQPQQK	
pdp|1IPJ	FGINAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKKQRESYFVDAQPQQK	
sp|P25974|	EEGSKGRKGPFPSILGALY	439
tr|Q93VL9	EEGSKGRKGPFPSILGALY	
tr|O22121	EEGSKGRKGPFPSILGALY	
pdp|1IPJ	EEGSKGRKGPFPSILGALY	

The light gray region (residues 1–25) is the predicted leader sequence. The darker gray represents those residues where there is apparent heterogeneity.

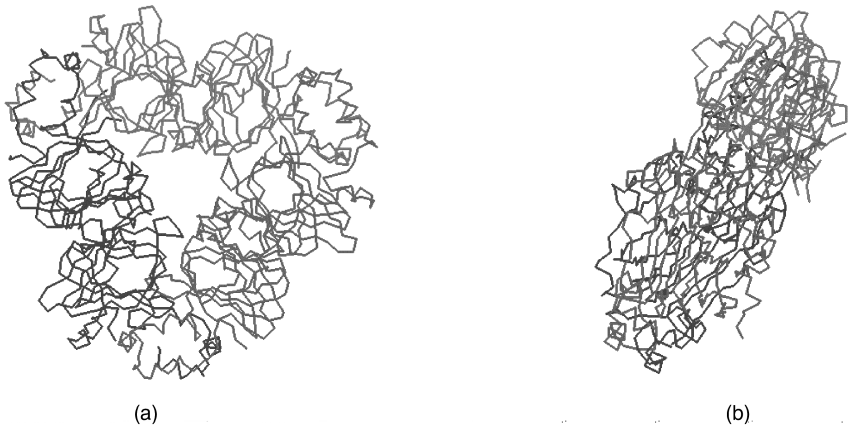

Fig. 21.16 Crystal structure of soybean β-conglycinin β-subunit.

The advantage of mass spectrometry over analysis based on the fluorescence of the AGE products is that it can simultaneously observe individual chemical forms. With the resolving power and high mass accuracy of Fourier Transform-Ion Cyclotron Resonance (FT-ICR) mass spectrometry, 20 glycated peptides were identified from the reaction between albumin and glucose (63). Given the complexity of the AGE products, procedures based on LC-tandem mass spectrometry are expected to provide invaluable insights into the chemistry of these compounds.

21.5 Conclusion

Modern proteomics provides very powerful technologies to carefully examine the effects of processing on food proteins. Although not widely applied yet by the food processing industry, it is likely that it will become commonplace to both identify the effects of widely accepted food processing procedures and to establish better methods of standardization. These are essential if the food industry wishes to make health claims for their products. Interestingly, the protein fingerprint of a food and all the potential induced modifications associated with a standardized procedure may also enable the regulatory authorities to ascertain how the food was made and where it came from.

21.6 Acknowledgements

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22

Texturized soy protein as an ingredient

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22.1 Introduction: texturized vegetable protein

22.1.1 Definition

Texturized vegetable protein, once associated with 1970s mystery meat, is today one of the hot lists of ingredients for its ability to contribute to two top food trends – the continued quest for high quality, low fat foods and the thriving field of functional and nutraceutical foods. There are several different definitions of texturized vegetable protein in the literature depending upon the sources.

Texturized protein products have been defined as 'fabricated palatable food ingredients processed from an edible protein source including among others soy grits, soy protein isolates, and soy protein concentrates with or without suitable option ingredients added for nutritional or technological purposes.' They are made up as fibers, shreds, chunks, bits, granules, slices or other forms. When prepared for consumption by hydration, cooking, retorting or other procedures, they retain their structural integrity and characteristic 'chewy' texture (Anon, 1972).

The United States Department of Agriculture has defined texturized vegetable protein products for use in the school lunch program as 'food products made from edible protein sources and characterized by having a structural integrity and identifiable structure such that each unit will withstand hydration and cooking, and other procedures used in preparing the food for consumption' (USDA, 1971).

In generic terms, texturized soy protein 'TSP' (TSP is a copyrighted trademark of the PMS Foods, (now Legacy Foods), Hutchison, KS), typically means defatted soy flours or concentrates, mechanically processed by extruders to obtain a meat-like chewy texture when re-hydrated and cooked. Texturized

vegetable protein 'TVP' is a registered trade mark for texturized soy proteins produced by the Archer Daniel Midland (ADM) company, Decatur, Illinois, USA.

22.1.2 Background

Recent developments in nutrition, production agriculture and world markets have resulted in an increased interest in texturized vegetable proteins in recent years. The approval of a health claim for soy-based foods by the Food and Drug Administration in the United States has resulted in an increased interest in texturized soy-based products. On the other hand, the advent of genetically modified soy varieties which greatly assist farmers in production of soybeans, but have raised product safety questions, have caused increased interest in non-soy proteins such as wheat gluten, pea proteins, and bean proteins. In general, the complexity of the food market worldwide has greatly increased and resulted in an increased demand for more sophisticated texturized protein products.

The term 'texturized vegetable food proteins' has been loosely applied to include a broad range of product categories made from flours or further refined non-meat proteins. These categories include those following below.

Spun protein isolates

Spinning is not necessarily an extrusion cooking process, but does involve dissolving precipitated vegetable proteins that then pass through a spinneret into a precipitating bath (Smith and Circle, 1980). The process is complicated, laborious and cost intensive. This method is essentially the same as that used for spinning textile fibers, except that fat, flavor, color and fiber binding agents are incorporated into the fibers during processing. The bundles of fibers resulting from this process possess the required texture to simulate meat, but lack an acceptable appearance and flavor. After compacting, shaping and flavoring, the product is cooked and/or dried and packaged to simulate the meat of mammals, fowl or shellfish (Campbell, 1981).

Formed meat analogs

Various protein sources (isolates, glutens, albumin, extrusion cooked vegetable proteins) are blended with oil, flavors and binders prior to forming or sheeting into disks, patties, strips and other shapes. These fabricated products have become increasingly popular with the advent of de-boned meats and other foods formerly considered by-products of the meat industry.

Extrusion cooked meat extenders

Meat extenders produced from the extrusion processing of defatted soy flour or flakes and soy concentrates constitute the largest portion of texturized vegetable food proteins. These products are re-hydrated to 60–65% moisture and blended with meat or meat emulsions to extend to levels of 20–30% or higher.

Extrusion cooked meat analogs

An extrusion process utilizing a specially configured extruder with a special die can be used to convert vegetable protein sources directly into simplified varieties of meat analogs that have a remarkable similarity in appearance, texture and mouthfeel to meats. These products, often called structured meat analogs, have a striated, layered structure similar to that found in muscle tissue.

22.2 Texturized vegetable protein: raw material characteristics

Traditionally, the most popular raw material for production of texturized vegetable proteins in an extrusion system has been defatted soy flour. This material was preferred due to its ability to be controlled through the extrusion system in order to produce a quality meat extender. New developments in extruders, improved extruder designs and operation techniques in processing texturized vegetable protein have made it possible to use a wider range of raw materials characteristic for texturization at present. Therefore, several raw materials can be used for protein texturization at present. At the same time, there are several important raw material characteristics one should keep in mind before selecting any raw material for texturization. These characteristics include protein level, protein quality, oil level, fiber level, sugar level and type, and particle size of the raw material. The following is a brief discussion regarding the effect of each characteristic on protein texturization.

Raw materials containing higher protein levels are more easily texturized with an extruder at lower levels of energy input. In addition, higher protein levels tend to result in products having tougher and firmer textures as shown in Fig. 22.1. At the same time protein quality of the raw material is also very important. For soy based raw materials, protein quality is usually measured by the protein dispersibility index (PDI; AOCS Official Method Ba-10–65, 1993) or nitrogen solubility index (NSI; AOCS Official Method Ba-11–65, 1993). Both the PDI and NSI tests indicate the level of protein solubility in water. These indices will reflect the heat treatment history of the raw material throughout the raw material preparation process. Higher levels of heat treatment will result in lower PDI or NSI values. The PDI rapid stir method uses a blender, whereas the NSI slow-stir method uses a laboratory stirrer. In

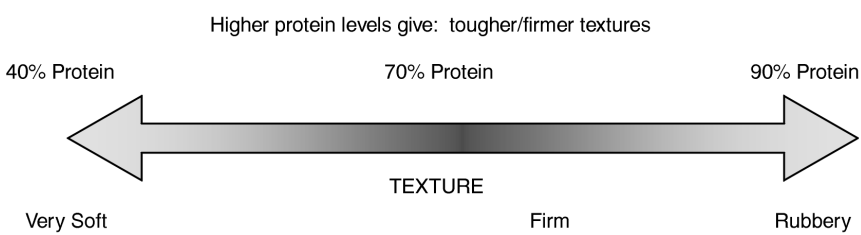

Fig. 22.1 Effect of protein level on the texture of the protein. Courtesy of Wenger Manufacturing, Sabetha KS.

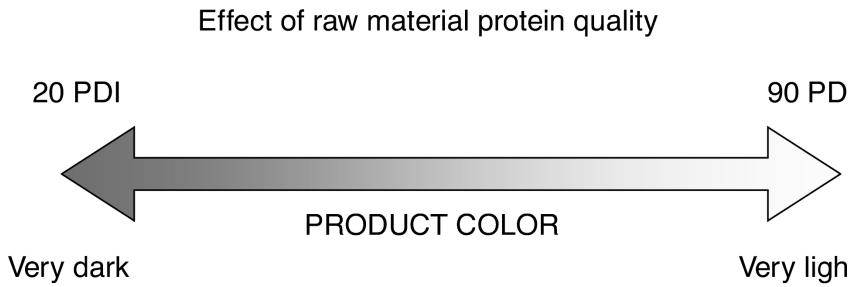

Fig. 22.2 Effect of PDI on protein quality. Courtesy of Wenger Manufacturing, Sabetha KS.

both methods the protein or nitrogen that is leached into the liquid phase is compared with total protein or nitrogen in the sample by kjeldahl analysis. In general, the PDI test will give higher results than the NSI test (Lusas and Riaz, 1995a). Raw materials having a lower PDI value will require more mechanical energy in the extruder to effectively texturize the protein (Fig. 22.2).

22.2.1 Oil

Raw materials from which the protein sources are derived usually contain some quantity of oil. For example, whole soybeans contain approximately 18–20% oil contents. During the process of texturization, oil acts as a lubricant within the extruder barrel and interferes with the addition of mechanical energy to the product. Therefore, raw materials containing a higher level of oil will require an alteration of the screw and die configuration of the extruder in order to effectively texturize them. In addition to the lubrication effect, a higher level of oil in the raw material will also dilute the protein level. Oil contents can be measured by two different methods. The most common method to analyze the oil content is petroleum ether extract. This method will detect oil that is not complexed in some fashion with the carbohydrate or protein fraction of the material. Another method is the acid hydrolysis method, which will detect all the oil, even that which is complexed with proteins or carbohydrates. The acid hydrolysis method will always give 3–4% higher results than the ether extract method. The range of oil content in the raw material for texturization is usually 0.5 to 7%.

22.2.2 Fiber

Fiber is another important constituent of the raw material. It is usually concentrated in the hull or pericarp portion of the seed. Fiber has a negative effect on texturization of protein. A high level of fiber content will interfere with the texturization process by diluting the protein level and causing discontinuities in the texturized matrix. During the texturization process, fiber partially blocks some of the cross-linking of the protein macromolecules, which can affect

structure and texture. To overcome this problem raw material can be ground very finely, so the fiber content will not interfere with the protein texturization.

22.2.3 Sugar

Sugars are naturally present in most of the raw materials used for texturization. If these sugars are not removed from the raw materials, they too act as a diluent and lower the protein content. In addition, many of these sugars ferment in the lower gastrointestinal tract, resulting in digestion problems and flatulence. For these reasons, they are removed from some raw materials, especially those that are soy based.

Particle size of the raw materials is very important in maintaining proper textural properties in texturized vegetable protein. Large particles are difficult to hydrate and may require additional preconditioning or additional mechanical energy input in order to plasticize and disperse the entire particle. In some cases, very fine, floury particles are detrimental because they tend to agglomerate in the preconditioner and then these agglomerates are difficult to re-disperse in the extruder barrel. Particle size has a very wide range. Some raw materials can be as fine as 38 micron or as coarse as 180 microns. The ideal size of the particle should be 90% through #100 U.S. standard sieve.

22.3 Soy based raw materials used for extrusion texturization

Soybean proteins are the single largest source for the manufacturing of texturized protein products worldwide. This is because of the simple economic law of supply and demand. Worldwide soybean supply is plentiful, and overall it is cheap and a relatively easily managed source of protein.

Soybeans and soy products have been used for centuries in Eastern Asia as a staple and highly nutritious food source. Presently, 159 million metric tones of soybeans are grown worldwide. The United States produced 44% of the total soybean production in the world (Golbitz, 2003). After the US, Brazil (22%), Argentina (15%), China (8%), Paraguay (2%), European Union (1%) and all other countries grow about 5% (Fig. 22.3). On an average, there is 40% protein, which means 63.6 million tonnes of soy protein is available for consumption. Soy protein supplies all nine essential amino acids and provides many functional benefits to the food processors and for a healthy diet. Soy ingredients promote moisture and flavor retention, aid emulsification, and also enhance the texture of many foods from a variety of meats to peanut butter, frozen desserts and even cheese. For food manufacturers, soy protein contributes valuable functional characteristics in processing systems as well as offering full digestibility. Both isolated and concentrated soy proteins are easily digested by humans and equal the protein quality of milk, meat and eggs. Moreover, soy proteins are acceptable in almost all diets containing virtually no cholesterol and being lactose free. The nutritional value of soy

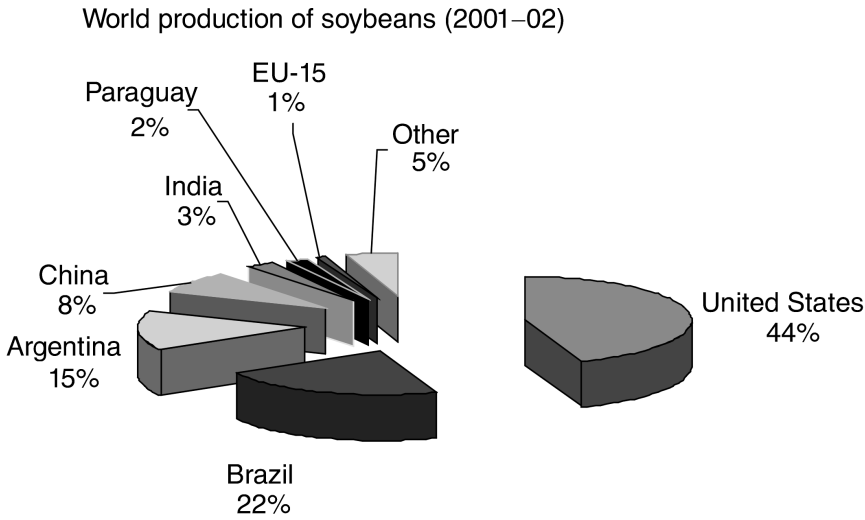

Fig. 22.3 World production of soybeans for 2001-02.
Source: Soya & Oilseed Bluebook 2003.

protein provides the consumer with a cholesterol-free, lower-fat alternative to animal protein.

Soybeans contain about 35–47% protein, 18–22% oil, 4–6% crude fiber, 4–6% ash, and 9–12% moisture (Fig. 22.4). From these whole soybeans, a number of raw materials can be made for use in extrusion texturization. These raw materials include defatted soy flour, soy grits, soy flakes, soy protein concentrate, soy protein isolate, and mechanically expelled soy flour.

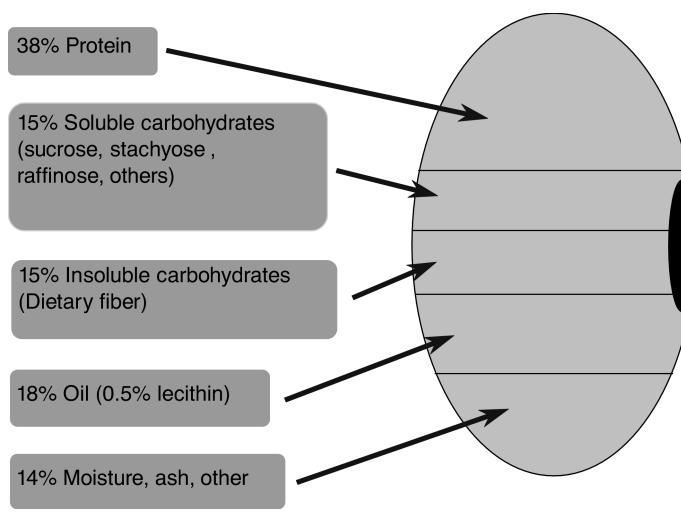

Fig. 22.4 A typical composition of soybean.

22.3.1 Defatted soy flour

Preparation of soybeans for food protein uses differs from processing soybeans for animal feeds. A more throughly cleaned U.S. No. 2 or a U.S. No. 1, soybean is used. Major emphasis is placed on removing splits, which may harbor early lipoxigenase activity that produce a beany flavor. There are several steps which are common to prepare defatted soy flour, grits, flakes, soy concentrates and soy isolates. These steps are briefly discussed here.

Cleaning

Soybean cleaning is important for producing high-quality end products as well as protection of equipment. This is the first step in processing soybeans. Cleaning of soybeans is done on sieves under air aspiration so that dust, plant tissue, pebbles and light contaminating material, as well as bigger impurities, (stones, stems, nails, etc.) are separated. The larger impurities are mostly separated step by step in a destoner and a magnetic separator. After cleaning, soybeans are weighed.

Drying

After cleaning, soybeans are dried in grain driers to a moisture level of 10% which is needed prior to dehulling. The temperature of the dryer should be between 70–76 ºC to achieve the desired moisture level. Uniform drying of the soybeans is very important for removal of hulls, since we need to remove the hulls from every individual bean and not an average. To do a good job of hull removal, it is essential to subject the beans to some kind of thermal impact. This is necessary because the hulls are attached to the bean with a proteinatious material, which when exposed to heat, releases the hulls. After drying the beans, they are stored for tempering for approximately 72 hours to stabilize the moisture contents. Dried beans are usually cleaned to remove as many loose hulls, pods, sticks and other foreign material as possible. This step will increase the efficiency of the cracking rolls and aspirator.

Dehulling of soybeans

The objective of the cracking is to break the soybean into suitable pieces for hull separation and flaking. Dried soybeans are cracked by using cracking mills where seeds are broken into smaller pieces. After cracking, the hulls are separated under airflow and the lighter hulls are withdrawn. It is important at this point to understand exactly how air separation works, since it is presently the basis for all soybean dehulling. With properly dried and cracked soybeans, the cotyledons should separate easily from the hulls. Soybeans contain about 8% of hulls by weight. As a practical matter, it is not possible to get an absolute separation of hulls from the cotyledons.

Conditioning

Cracked and de-hulled soybeans are conditioned in cookers to approximately 70 ºC with steam. Small amounts of water can be added to adjust the moisture to

approximately 11%, which is ideal for flaking. The main function of the conditioning is to facilitate the flaking step.

Flaking

In order to extract the oil from the beans, it is important to destroy the cell structure of the beans. To achieve this, beans are flaked in the flaking machines, since solvents can flow much more readily through a bed of flakes than through a bed of soy meats or fine particles. The ideal thickness of a flake is between 0.25 to 0.35 mm. Thickness of these flakes depends upon the size of the cracked beans, conditioning and the adjustment of the flaking rolls.

Extraction of flakes

Soy flours, soy concentrates and soy isolates typically are made from flakes (also called white flakes). Soy flakes are fed into a solvent extractor, and after sufficient contact time with the solvent (hexane), these flakes are desolventized to remove the residual hexane. There are several different types of extractors available for soybean extraction. This process reduces the residual oil to a low level in flakes. These flakes can be desolventized in a standard desolventizer-toaster (DT) which uses live steam to drive the solvent off. Another system called the flash desolventizer or vapor desolventizer can also be used. This system produces a less heat-treated soy product. Soy flakes processed by flash solvent removing systems have a protein dispersibility index (PDI) as high as 95.

In the flash desolventizer, solvent wet flakes go directly from the extractor into superheated vapor blown at a high velocity through a long tube. Solvent is heated under pressure to 115–138 ºC. As this vapor is blown through the tube, flakes are conveyed in and picked up by the vapor stream. In short seconds, the superheated solvent will flash off the residual liquid solvent leaving desolventized flakes to be collected in the cyclone (Fulmer, 1989). High PDI soy ingredients are more soluble, but at the same time, they also have highly active enzymes and antinutritional factors, which should be deactivated by heat before the final product is consumed.

Expanding of flakes

Recently, several soy flour-, soy concentrates- and isolates-producing companies started using an expander-extruder after the flaking step, and before the extraction. The use of expanders was originally developed in Brazil and was introduced in the US in early 1980. According to Watkins *et al*. (1988), the use of an expander offers several advantages in processing soybeans for extraction.

- The seed is finely homogenized in the expander, freeing the oil for rapid extraction.
- Dense collets are formed (weigh more per cubic foot).
- The collets are porous, and do not restrict percolation of solvent through the extractor bed, as may occur with fragile flakes.
- More oil is recovered by solvent from collets as compared to flakes.

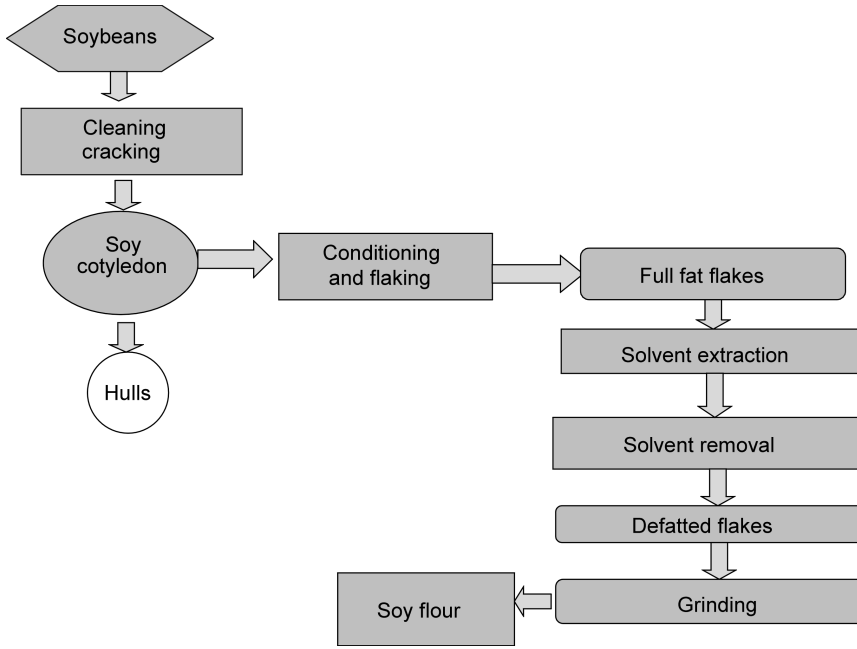

Fig. 22.5 Production of defatted soy flour.

- The solvent drains more completely from the extracted collets, resulting in more complete removal of oil.
- Less energy is required for desolventizing the collets compared to flakes.

22.3.2 Soy flour

Defatted flakes are milled using a hammer mill to produce soy flour. At least 97% of the flour must pass through a U.S. Standard No. 100 sieve. This flour contains 52–54% protein as is. Fig. 22.5 outlines the process for making soy flour from whole soybeans. As reviewed in Table 22.1, the degree of toasting has a large impact on the protein quality as measured by PDI. Defatted soy flour to be used for the extrusion texturization process should have the characteristics

Table 22.1 Effect of toasting on the protein quality as measured by PDI

Type of soy flour	PDI
Negligible toasting	85–95
Light toasting	70–80
Light to moderate toasting	50–60
Moderate toasting	34–45
Toasted	8–20

Table 22.2 Soy flour characteristics for texturization

Soy flour	
Protein	50–55%
Oil (ether extract)	<1%
Oil (acid hydrolysis)	<3%
Crude fiber	<3.5%
% Sugars	12%
PDI	50–70
Particle size	Approx. 100 mesh

shown in Table 22.2. Products made from defatted soy flour will have a flavor that is commonly associated with soy and will tend to cause flatulence problems as evidenced by its high sugar content.

22.3.3 Soy flakes

The main difference in defatted soy flour and soy flakes is grinding. In some cases after desolventizing the raw material (flakes) can be used as it is for texturization. These flakes are chemically the same as soy flour. Physically these are different from flour because of the size. Therefore, more time and energy is required in preconditioning for hydration. A typical composition of soy flakes is shown in Table 22.3.

22.3.4 Soy grits

Grits are chemically the same as soy flour and soy flakes. The only difference between defatted soy flour and soy grits is particle size. Grits are usually larger size particles. Soy flakes are ground to a certain level to produce soy grits for different application. They are classified as coarse (10–20 mesh), medium (20–40) or fine (40–80 mesh) grits according to particle size (Hettiarachchy and Kalapathy, 1999). These soy grits can be used in making texturized soy protein, but will require more energy and time to hydrate in a preconditioner. A typical composition of grits is shown in Table 22.4.

Table 22.3 Soy flakes characteristics for texturization

Soy flakes	
Protein	50–55%
Oil (ether extract)	<1%
Oil (acid hydrolysis)	<3%
Crude fiber	<3.5%
% Sugars	12%
PDI	70
Particle size	As they come from extractor

Table 22.4 Soy grits characteristics for texturization

Soy grits	
Protein	50–55%
Oil (ether extract)	<1%
Oil (acid hydrolysis)	<3%
Crude fiber	<3.5%
% Sugars	12%
PDI	70
Particle size	90% through # 8 U.S. standard screen 10% through # 20 U.S. standard screen

22.3.5 Soy protein concentrates

Defatted flakes or soy flour are used as starting material. Soy protein concentrates are processed selectively by removing the soluble carbohydrates from soy protein flour by either aqueous alcohol or isoelectric leaching. After drying, it is ground to a powder to produce soy protein concentrates having the approximate composition shown in Table 22.5. Soy protein concentrates contain an average 65% protein on moisture free basis (Lusas and Rhee, 1995). During processing of soy concentrates, objectives are to immobilize the protein while leaching away the soluble, removing the strong flavor components and the flatulence sugars (sucrose, stachyose and raffinose). In turn, both protein and dietary fiber contents are increased.

There are several different methods to produce soy concentrates (Lusas and Rhee, 1995).

1. extraction of flakes with aqueous 20 to 80% ethyl alcohol
2. acid leaching of flakes or flour
3. denaturing the protein with moist heat and extraction with water (Ohren, 1981).

Alcohol extraction is considered to produce the blandest products. Mild heat drying conditions are used in an acidic water extraction process to retain high

Table 22.5 Soy concentrates characteristics for texturization

Soy concentrates	
Protein	65–70%
Oil (ether extract)	<1%
Oil (acid hydrolysis)	<3%
Crude fiber	<4%
% Sugars	1%
PDI	Very low
Particle size	100–200 mesh

PDIs. (Lusas and Riaz, 1995b). Because of its blander flavor, soy concentrate is often preferred over soy flour.

In the case of soy concentrates, the sugars have been removed. Therefore, products made from soy protein concentrate are easily digested and contribute less to flatulence problems. Although soy protein concentrate made by this process has a very low protein dispersibility index, it is very easily texturized. Soy protein concentrate usually requires processing at higher moistures and slightly higher mechanical energy inputs than defatted soy flour.

22.3.6 Soy protein isolates

There are several ways for manufacturing isolated soy protein, but the only commercial procedure currently being used is extraction of defatted soy flakes or soy flour with water, followed by centrifugation. In this process, the protein is solubilized at pH 6.8–10 at 27–66 ºC by using sodium hydroxide and other alkaline agents approved for food uses. The protein solution is separated from the flakes or flour by centrifugation. The solids are recovered as a by-product containing 16–36% protein, 9–13% crude fiber and 45–75% total dietary fiber when dried to 6–7% moisture content and have been marketed for food use. The solution is acidified to pH 4.5 by using hydrochloric or phosphoric acid, and the protein is precipitated as a curd. The curd is washed with water and concentrated by centrifugation, and can be neutralized to pH 6.5–7.0 or spray dried in its acidic form (Johnson and Kikuchi, 1989).

Soy isolates are the most concentrated form of soy protein available. Functionally, isolated soy protein has many of the same attributes as soy flour and concentrates. While soy protein isolate can be texturized as the total recipe, this is not commonly done. In some cases it can be added as an ingredient in raw material blends to boost the protein content. Soy protein isolate will contribute to increased chewiness and tougher textures in the texturized product.

22.3.7 Partially defatted soy flour

Partially defatted soy flour is made by a much different process than that used to make defatted soy products. A flow sheet for making partially defatted soy flour is shown in Fig. 22.6. In this process, food grade soybeans are cleaned, cracked, dehulled, and extruded by a dry extruder to free the oil, inactivate anti-nutritional factors and lipoxigenase enzymes. After extrusion, the resulting meal is immediately passed through a mechanical screw type press where oil is removed from the meal. The resulting meal cake is then cooled and ground to prepare it for extrusion.

As with the solvent extraction process, the level of heat treatment (in this case the pre-expelling extrusion step) will determine the PDI of the resulting material. In general, a lower temperature extrusion will result in a higher PDI and higher oil content. On the other hand, a higher temperature extrusion will

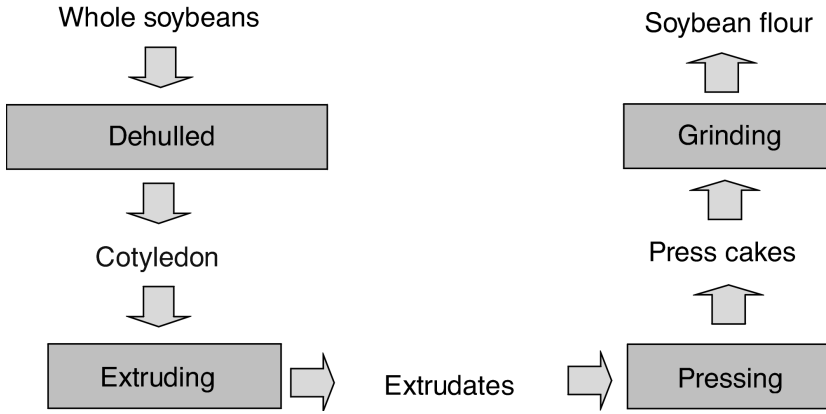

Fig. 22.6 Flow diagram to produce mechanically defatted soy flour.

Table 22.6 Mechanically defatted soy flour characteristics for texturization

Mechanically defatted soy flour	
Protein	40–50%
Oil (ether extract)	7–8%
Oil (acid hydrolysis)	10–12%
Crude fiber	3–4%
PDI	25–30
Particle size	100–200 mesh

produce the opposite result. For best results, it has been found that flour having the characteristics shown in Table 22.6 is most easily texturized. To texturize this material, dramatic adjustments in the screw and die designs are required to overcome the lubricating effect of the high residual oil content and low PDI. More detail about this process is given in the section on low-cost methods to produce texturized soy protein.

22.4 Wheat and other raw materials used for extrusion texturization

22.4.1 Wheat based raw materials

Wheat gluten and wheat starch are economically important co-products produced during wet processing of wheat flour. Wheat gluten is a commodity food ingredient, and its applications are predominantly in baked goods and processed meat products. Recent discoveries found that wheat gluten can be processed into texturized vegetable protein for meat application. The popularity of texturized vegetable proteins that contain wheat proteins is rapidly increasing. Often, these products are extruded in such a way that bundles of long fibers are formed.

The wheat kernel is composed of approximately12–16% protein, 2–3% oil, 2–3% fiber, 1–2% ash, and 8–10% moisture. The wheat milling process produces wheat flour that has most of the fat and ash removed. After milling into flour, wheat gluten, the protein portion of wheat flour, is separated from the starch by one of a number of processes.

Wheat gluten is a protein that has unique properties. When hydrated and mixed, it forms a very extensible, elastic structure that is responsible for the gas-holding ability of bread dough. Wheat gluten can be used in combination with soy-based raw materials, or in combination with wheat flour and other additives to produce a soy-free texturized product. Commercially available wheat gluten is typically 80% protein.

22.4.2 Other raw material sources

In addition to soy and wheat gluten, other raw material sources have been used in the extrusion process for texturization.

Cottonseed

Cottonseed is the world's second major oilseed in tonnage produced and has potential for providing the annual protein needs for approximately 352 million people at 45g/day (Lusas *et al*., 1989). However, cottonseed contains gossypol, a green-brown toxic compound. Options for reducing free gossypol content in cottonseed include: binding gossypol by moist heating of seeds flaked to rupture gossypol 'gland' before solvent extraction, extraction of gossypol using selective solvent systems, physical removal of intact gossypol glands and growing of genetic varieties of 'glandless' cottonseed that do not contain gossypol.

Processes have been developed for making glandless cottonseed flour, concentrates and isolates and roasted kernels. Cottonseed accounts for approximately 60% of the weight of seed cotton, but it provides only about 10–15% of the gross returns to producers. Meat extenders have been extruded from cottonseed protein products. Glandless cottonseed flour containing 65% protein may be used to make an acceptable texturized product when heavy densities are not required. Insoluble carbohydrates tend to prevent a uniformly layered final product. In general, texturized proteins from cottonseed are highly expanded with more cooking loss than similar products made with soy proteins. Availability of glandless cottonseed flour or concentrates is very limited in the market for texturization.

Canola/rapeseed

The problems in canola/rapeseed processing and utilization have included separation of hulls from the small kernels. Texturized rapeseed proteins or canola concentrates possess good fat and water-binding properties. These proteins have excellent nutritional qualities when compared to other vegetable food proteins. Commercially, rapeseed flour or concentrates are very limited and not available for texturization.

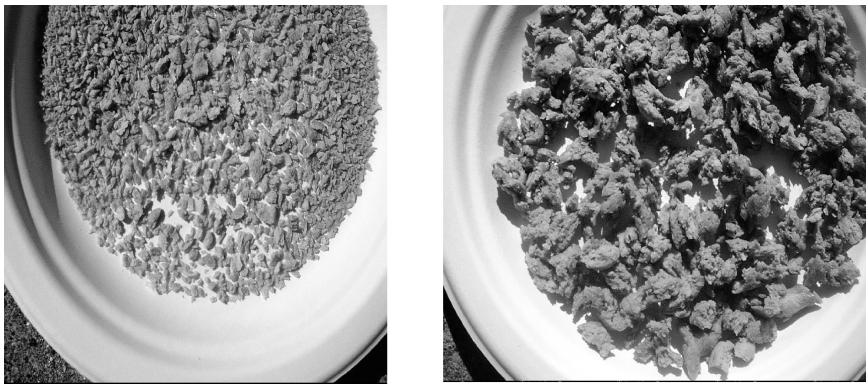

Fig. 22.7 Texturized peanut protein in chunk and shredded for meat application.

Peanuts

Defatted peanut flour has been texturized to produce meat extenders with good taste, but poor color qualities compared with soy-based extenders. The Food Protein Research and Development Center at Texas A&M University has done work on texturization of partially defatted peanut flour. Using the same concept as mentioned above (mechanically defatted soy flour) partially defatted peanut flour can be produced. Defatted peanut flour is available in the market, but it is costly, so making a texturized vegetable protein commercially is not economical. The texturized partially defatted peanut protein is shown in Fig. 22.7.

Sesame

The use of defatted sesame flour has received attention due to its nutritional qualities. This vegetable protein, along with sunflower meal, has occasionally been used to produce satisfactory meat extenders. Again, defatted sesame flour is not available commercially for texturization.

Pea and bean

Pea and bean flour and concentrates have also been used for texturization. These raw materials are somewhat variable and often have been extensively heat treated prior to extrusion and are therefore very difficult to texturize. Pea protein concentrate is available in Canada and Europe and bean flour is available in India. A sample of texturized pea protein is shown in Fig. 22.8, which was made at the Food Protein Research & Development Center, Texas A&M University.

22.5 Effect of additives on texturized vegetable protein

The additions of minor ingredients or chemicals are often used to increase the range of raw ingredients suitable for production of a specific texturized vegetable protein product. It is important to understand the effects of these

Fig. 22.8 Texturized pea protein in chunk.

additives on the texturization of vegetable protein, since these ingredients can improve the final texture and aid in texturization. Some of these additives are food flavor, color, pH modifier, surface active substrates, emulsifiers, wheat gluten and surfactants. These additives can be used to assist the food scientists in controlling the functional properties, structures, mouthfeel and/or density of the processed material. With the addition of minor ingredients or chemical adjustment of the texturized vegetable protein, the raw material can enhance various aspects of the finished product and lessen the specification constraints of some of the raw material (Strahm, 2002).

22.5.1 Texture enhancers and extrusion stabilizers

Several additives are commonly used to enhance the textural integrity of extruded proteins and to stabilize the processing of vegetable proteins.

Color enhancers

When supplementing light-colored meats with meat extenders made from texturized vegetable proteins, it is desirable to bleach or lighten the color of the meat extender. Bleaching agents such as hydrogen peroxide are often used for this purpose. Dosing levels for hydrogen peroxide range from 0.25 to 0.5%. Pigments such as titanium dioxide are also used at levels between 0.5 to 0.75% to lighten color, but at increased levels, pigments will weaken the textural properties of extruded vegetable proteins.

Calcium chloride (CaCl)
Increased textural integrity and smooth product surfaces are the result of incorporating calcium chloride into a texturized vegetable protein product. Dosing levels for CaCl range between 0.5 to 2.0%. With the addition of CaCl and small amounts of sulfur, soybean meal containing 7.0% fiber may be texturized, retorted for one hour at 110 ºC, and still maintain a strong meat-like texture.

Lecithin
Soy lecithin added to formulations of vegetable proteins at levels up to 0.4% tends to assist in a smooth laminar flow in the extruder barrel and die which permits production of increased density soy products. The ability to make dense vegetable protein products is related to the higher degree of cross-linking occurring during the extrusion process.

Sodium chloride (NaCl)
The addition of sodium chloride does not appear to benefit the texture of extruded vegetable proteins. If anything, it tends to weaken textural strength.

Sodium alginate
Addition of sodium alginate increases chewiness, water-holding capacity and density of extruded protein products.

Sugar
Sugar tends to disrupt the textural development of soy proteins.

Sulfur
Known for its ability to aid in the cleavage of disulfide bonding, sulfur assists the unraveling of long twisted protein molecules. This reaction with the protein molecules causes increased expansion, smooth product surface and additional stability to the extrusion process. These benefits however, are not without some undesirable side effects including off flavors and aroma. Normal dosing levels for sulfur or sulfur derivatives are in the 0.01 to 0.2% range.

pH adjustment
Increasing the pH of vegetable proteins before or during the extrusion process aids in the texturization of the protein. Extreme increases in pH increases the solubility and decreases the textural integrity of the final product. Processing at pH 8.0 may also result in the production of harmful lysinoalanines. Lowering the pH has the opposite effect, and decreasing protein solubility makes the protein more difficult to process. Undesirable sour flavors in texturized vegetable protein products may be evident if the pH is adjusted below pH 5.0 (Strahm, 2003).

22.6 Types of texturized vegetable protein

Presently there are four major types of texturized vegetable protein available in the market, 1) meat extenders; 2) meat analog; 3) fibrous protein; and 4) high moisture meat analog. All these texturized protein products are made by using an extrusion process.

22.6.1 Meat extender

These types of products are mixed with meat for further processing, changing the properties of the meat. They can be made in different size ranges from 2–30 mm depending upon the application. Meat extenders are available in chunk form (15–20 mm), in minced form (>2 mm) and in flaked form (>2 mm). Meat extenders can be made from soy flour or soy concentrates depending upon nutrition requirements and protein level in the final products. If made with soy flour, meat extenders can absorb 2.5–3 times their original weight in water, whereas if made from soy concentrates, they can absorb 4.5–5 times their weight in water. Re-hydration rates depend upon the size and surface area of the product. Extender flakes will re-hydrate more quickly than minced products or chunks of meat extender. The raw material properties to make meat extenders are very important in order to absorb the water and oil content when applied in meat systems. Meat extender can be made with soy flour or concentrates. Soy flour should have 60 to 70 PDI, and a protein level of 50%. In the case of soy concentrates, a lower protein solubility material can be used in order to make a good product. The resulting product will have approximately 70% protein. Other raw materials can be added for several reasons in the recipe. Some of the reasons are economics, nutrition balance, to improve the functional properties, to change the color of the final products or to reduce the allergenicity effect of the final products. We can use vital wheat gluten which has high solubility, at least 80% protein, with soy flour or soy concentrate to make meat extenders. When making meat extenders, there are several properties that are kept in mind. For example, water absorption, oil absorption, and meat-like texture. Using proper formulation and processing techniques can help to achieve these properties. Typical chunk and minced style of meat extenders are shown in Fig. 22.9.

22.6.2 Structured meat analog

This type of textured product is remarkably similar to meat in appearance, texture and mouthfeel when properly cooked. A special extruder configuration and die design is used to produce this type of product. Structured meat analog has a striated, layered structure similar to muscle meat as shown in Fig. 22.10. This type of product can be made in different sizes ranging from 6–20 mm depending upon the application. They usually absorb at least three times their weight in water when cooked in boiling water for at least 15 minutes. Raw

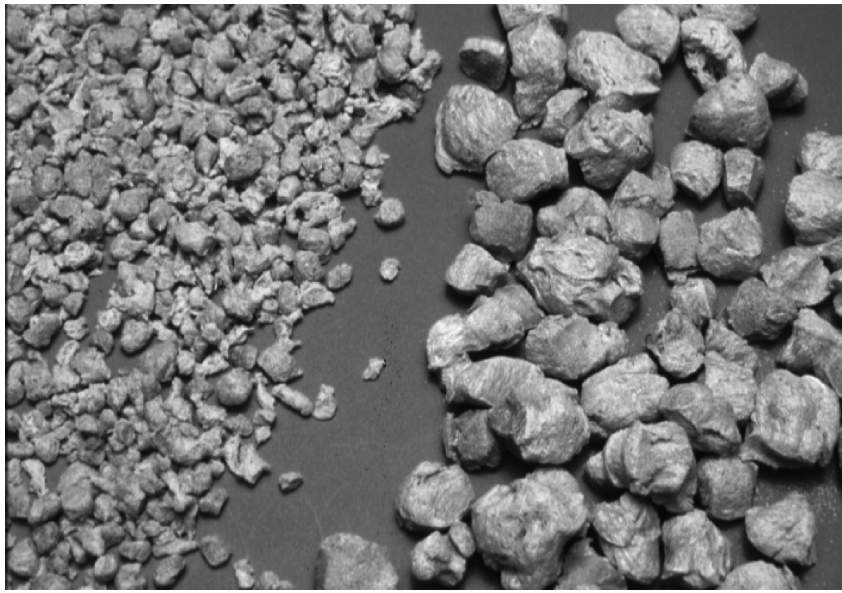

Fig. 22.9 A typical chunk and minced style meat extender. Courtesy of Wenger Manufacturing, Sabetha, KS.

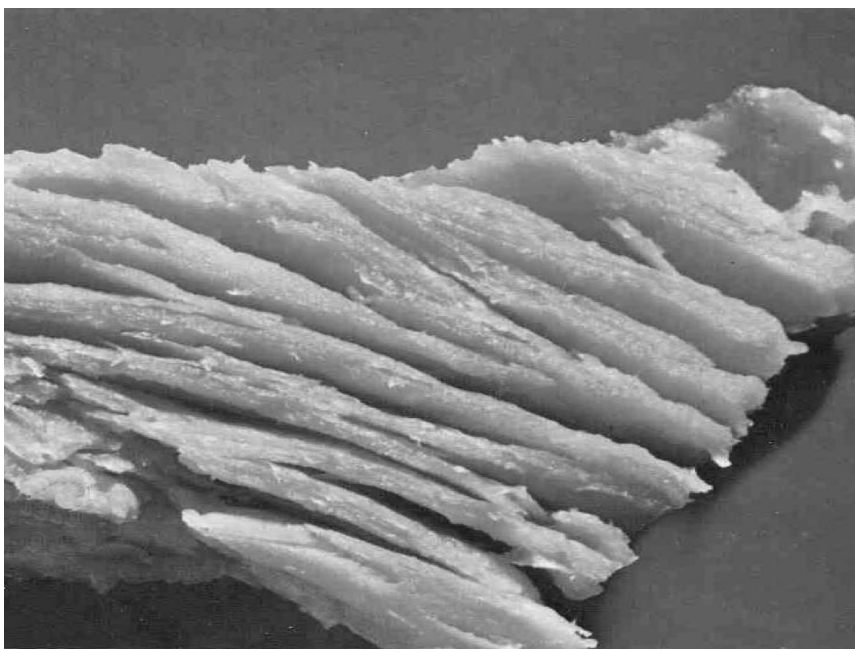

Fig. 22.10 Separating this dehydrated ham analog by knife and fork shows its untwisted layering, a prime characteristic of muscle tissue. Courtesy of Wenger Manufacturing, Sabetha, KS.

Fig. 22.11 A typical structured meat analog. Courtesy of Wenger Manufacturing, Sabetha, KS.

materials and their properties for meat analog are very similar to meat extenders. The only difference between meat analog and meat extenders is how they are texturized by extruders. The same extruder can be used to make both these products. The differences are the configuration of the extruder barrel, die design and processing conditions. The important properties of meat analog are meat-like appearance, meat-like texture, meat-like mouthfeel, water absorption and cooking characteristics should be very similar to meat. A typical structured meat analog is shown in Fig. 22.11.

22.6.3 Fibrous soy protein

This type of textured protein is used to restructure meat analog and to mimic the chicken breast type of meat. It can be processed from 6–20 mm in size and when produced, it will have a fibrous structure. The resulting fibrous protein products are very similar to a spun process. A typical fibrous soy protein is shown in Fig. 22.12. To prepare products for consumption, these textured fibrous soy protein are hydrated and the fibers are extracted by a bowl chopper. Desired flavors, color and binders can be added at this stage. The mass is then reformed into vegetable-based meat analogs such as ham, chicken, beef, etc. These products are very similar to texturized gluten products made by a different process, but

Fig. 22.12 A typical fibrous soy protein. Courtesy of Wenger Manufacturing, Sabetha, KS.

entirely with soy material. It usually absorbs three times the water when re-hydrated. The recipe to make fibrous protein contains soy concentrate (with high solubility and approximately 70% protein), soy isolates (with high solubility and high viscosity) and starch from corn or wheat. A typical formulation for fibrous protein is shown in Table 22.7.

22.6.4 High moisture meat analog

This is a relatively new product produced first in Europe and then in the U.S. Presently there are several pilot and commercial extrusion facilities making these types of products for the human as well as for the pet-food industry. It can be made in various sizes ranging from 12 mm thick and 80 mm wide. This is a layered and fibrous structured meat analog, very similar to real meat in composition and appearance. A typical high moisture meat analog is shown in Fig. 22.13. It contains at least 60–70% moisture, 2–5% oil and 10–15% protein. Once it is made, it must be frozen for storage because of its high moisture content or retorted in cans for longer shelf life. The size and shape of this product is very similar to fresh meat sold at the butchers or at supermarkets. The recipe contains soy concentrates (high solubility and 70% protein), soy isolate (high solubility/high viscosity and 90% protein) starch (corn or wheat) and oil (vegetable source). A typical formulation to make high moisture meat analog is given in Table 22.8.

Table 22.7 Typical formulation for making protein soy fiber

Ingredients	Percentage
Soy isolates	70
Wheat starch	30

Fig. 22.13 A typical high moisture meat analog. Courtesy of Wenger Manufacturing, Sabetha, KS.

Table 22.8 Typical formulation for making high-moisture meat analog

Ingredients	Percentage
Soy isolates	45
Soy concentrates	45
Wheat starch	5
Vegetable oil	5

22.7 Principles and methodology of extrusion technology

A typical process for making texturized vegetable protein is shown in Fig. 22.14. Raw material for making texturized vegetable protein has already been discussed above. A majority of the texturized vegetable protein products are produced using extrusion technology. These products are re-hydrated to 60–65% moisture and blended with meats or meat emulsion which can be extended to a level of 20–30% or higher. For meat extenders a single screw is used, whereas for meat analogs, texturized protein fibers and high moisture

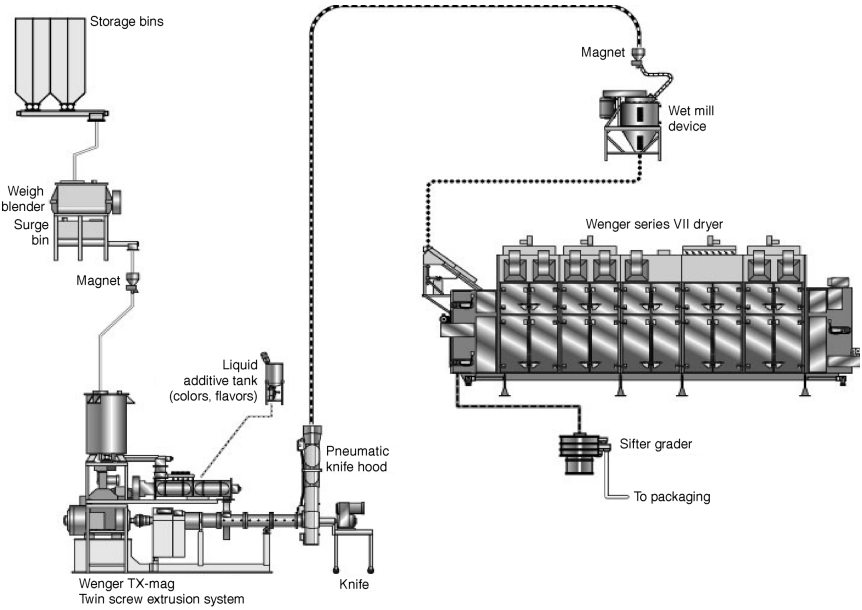

Fig. 22.14 A typical flow diagram for making texturized soy protein. Courtesy of Wenger Manufacturing, Sabetha, KS.

meat analogs a twin screw is used. Therefore, it is necessary to begin with an overview of the extrusion principles and methodology of the process.

22.7.1 Principles of extrusion

Extrusion cooking has been defined as

> the process in which moistened, expansile, starchy and/or proteinaceous materials are plasticized in a tube by a combination of moisture, pressure, heat and mechanical shear. This results in elevated product temperature within the tube, gelatinization of starchy components, denaturization of proteins, the stretching or restructuring of tractile components, and the exothermic expansion of the extrudate (Smith, 1975).

Extrusion is widely used to achieve this restructuring of protein-based raw material to process a variety of texturized protein. During the extrusion process mechanical and thermal energy is applied to the proteinaceous raw material, which makes the macro-molecules in the raw material lose their native, organized structure and form a continuous, visco-elastic mass. The extruder barrel, screws and dies align the molecules in the direction of flow. This realignment 'exposes bonding sites which lead to cross-linking and reformed, expandable structure' that creates the chewy texture in fabricated foods (Harper, 1986). In addition to retexturing and restructuring vegetable proteins, the

extrusion process performs several other important functions (Rokey *et al.*, 1992).

Protein denaturation

Vegetable proteins are effectively denatured during the moist, thermal process of extrusion cooking. Denaturation of protein lowers solubility, increases digestibility and destroys the biological activity of enzymes as well as toxic proteins (Strahm, 2002). At the same time the extrusion process deactivates residual heat labile growth inhibitors native to many vegetable proteins in a raw or partially processed state. These growth inhibitors have a deleterious physiological effect on man or animals as shown by different scientific studies (Rokey *et al*., 1992).

Reducing raw and beany flavor

An extrusion process can control raw or bitter flavors commonly associated with many vegetable food protein sources. Some of these undesirable flavors are volatile in nature and are eliminated through the extrusion and decompression of the protein at the extruder die. The use of preconditioner and an atmospheric venting device in the extruder design may assist in volatilization and removal of off-flavor (Rokey *et al*., 1992).

Homogeneous mixing

Extrusion provides a very homogeneous, irreversible, bonded dispersion of all micro ingredients throughout a protein matrix. This not only ensures the uniformity of all ingredients, such as dyes, flavor and other minor ingredients throughout the product, but also provides a means for minor ingredients to intimately associate with potential reaction sites promoting cross-linking or other desirable chemical and physical modifications (Rokey *et al*., 1992).

Shaping the products

The extrusion process shapes and sizes the final texturized protein in convenient and transportable portions for packaging in retail or bulk containers (Rokey *et al*., 1992).

22.7.2 Methodology of textured vegetable protein

To understand better how the extruder processes textured vegetable protein, it is appropriate to divide the basic components of an extrusion system into three parts: (a) precondition process; (b) the extruder barrel configuration; (c) the die and knife assembly. The design of each of these components is engineered to carry out a particular function in the process of texturizing vegetable proteins (Rokey *et al*., 1992). A typical component of an extruder is shown in Fig. 22.15.

The delivery system (bin/feeder) provides a means of uniformly metering the raw material (flours, grits, meal) into a preconditioner, sometimes called a mixing cylinder, and later on into the extruder barrel itself. This flow of raw

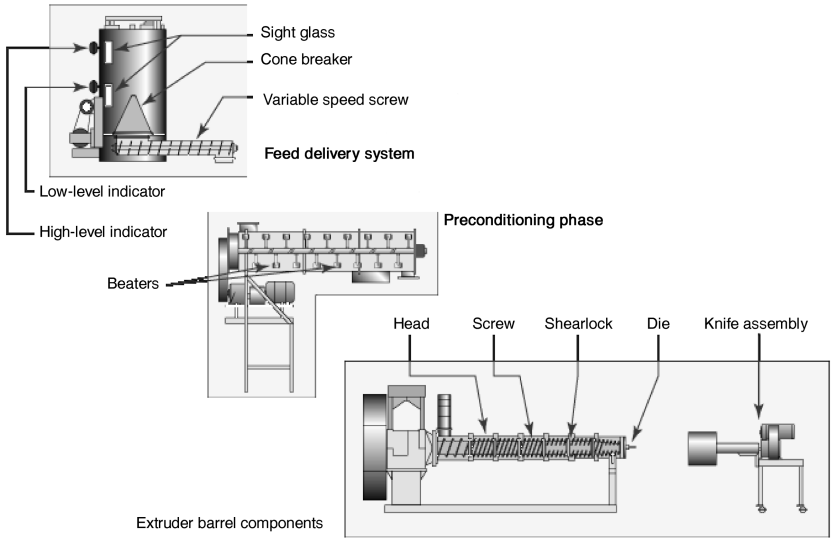

Fig. 22.15 A typical component of an extruder. Courtesy of Wenger Manufacturing, Sabetha, KS.

material must be very uniform and controllable. In the preconditioner, steam and moisture can be injected to increase the temperature of the raw material. It is very helpful where the particle size of the raw vegetable protein is larger (grits, meal or flakes). This initial steam preconditioning hastens moisture and heat penetration of the individual particles resulting in uniform moisture application and increased raw material temperature. The moisture content in the preconditioner should be approximately 20–25% and the temperature of the raw vegetable protein should be in the range of 70–85ºC.

Most of the preconditioners are vented to avoid the excess steam and undesirable volatile flavor components found in the raw vegetable protein. Different color (caramel for beef appearance), flavors and other minor additive or processing aids can be introduced in the preconditioner at this stage for thorough mixing with the raw material entering an extruder barrel. In the preconditioner we can reduce some of the growth inhibitors found in some of the raw vegetable protein. This can be accomplished by introducing steam and increasing the temperature of the raw vegetable protein. Preconditioners are available with a single, double or differential diameter cylinder (DDC). Double or DDC preconditioners provide better mixing and retention time, resulting in a better texturized vegetable protein (Rokey *et al*., 1992).

From the preconditioner, the raw material enters the extruder assembly which consists of the barrel and screw configuration. In this section of the extruder, major transformation of the vegetable protein take place. Extruders can be single or twin-screw in design. For more details about single and twin-screw extruders and their advantages, readers are referred to *Extruders in Food Application*

(Riaz, 2000). In both designs, the impact on the final product texture is affected by screw and barrel profile, screw configurations, screw speed, processing parameters like temperature, moisture, etc., as well as raw material characteristics and die selection (Rokey *et al*., 1992).

The first section of the extruder barrel is designed to act as a feeding or metering zone and simply to convey the raw vegetable protein material away from the inlet portion of the barrel and into the next section called a processing zone. In this zone, amorphous, free-flowing vegetable protein is worked into a colloidal dough. The compression ratio of the screw profile is increased at this stage to assist in blending water or steam with the raw material (Rokey *et al*., 1992).

The temperature of moist, proteinaceous dough is rapidly elevated in the last 2–5 seconds of retention time within the extruder barrel. Most of this heat is from mechanical energy dissipated through the rotating screw and may be assisted from the direct injection of steam or from other external energy sources. The screw profile can be altered by the pitch, flight height and angle and steamlock diameter which affects the conveyance of this plasticized protein material down the screw channel. The net flow patterns of the product within the screw are quite complicated and difficult to understand and describe. Retention times of 10–20 seconds, temperature of 100–180 ºC and moisture levels of 15–30% all influence the protein dough quality just behind the die and the final product expansion (Rokey *et al*., 1992). The temperature at this point actually melts the protein into a visco-elastic, plasticized mass having a very high viscosity. This plasticized material is extruded through the die openings and expansion occurs as the product is released to ambient pressures. Final product density has a direct correlation with temperatures and moisture of extrusion (Rokey *et al*., 1992).

Meat analog, texturized soy fiber and high-moisture meat analog can be made by using extrusion technology. Although the process to produce vegetable protein products is similar to soy extenders, it often employs two single-screw or one twin-screw extruder. Through the special configuration (provided by the extruder manufacturers), dwell time in the extruder is increased, the moisture content increased to 30% and pressure maintained below 150 psig. The

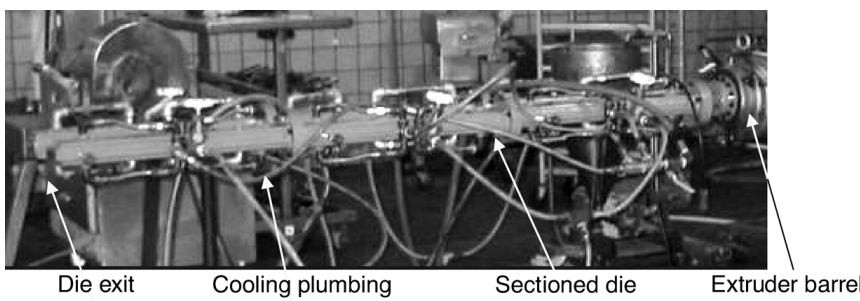

Fig. 22.16 A typical die used for making high moisture meat analog. Courtesy of Wenger Manufacturing, Sabetha, KS.

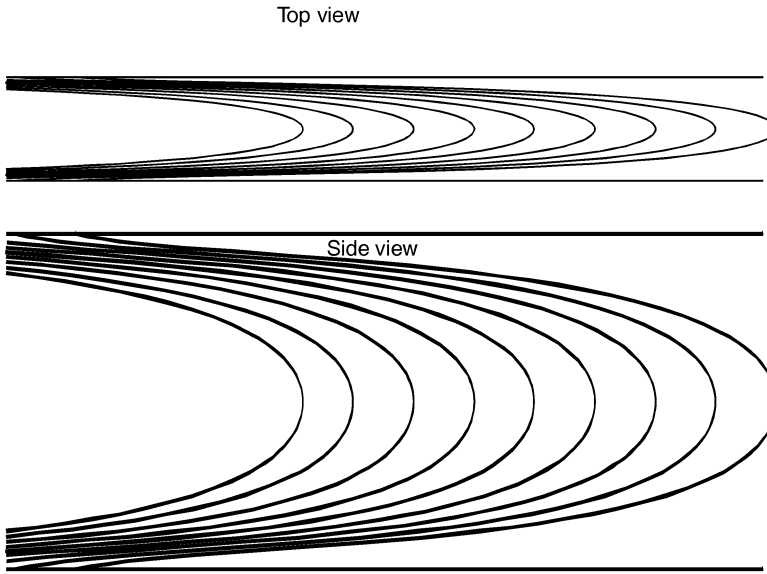

Fig. 22.17 A top and side view of laminar texture development in the die for making high-moisture meat analog. Courtesy of Wenger Manufacturing, Sabetha, KS.

vegetable protein material exposed to this process is stretched, cooled and formed into uniform layers and cut into the desired sizes (Rokey *et al*., 1992). The lower pressure employed inboard of the die reduces product expansion and disruption to the laminar structure. For texturized soy fiber, a stream-line die setup that includes a venturi, followed by a long die spacer, then the final die is required. For making high-moisture meat analog, a special long die is required at the end of the extruder barrel. This die is cooled by water circulation so the product does not expand. A typical die is shown in Fig. 22.16. In this die, the texturization and layer formation take place as shown in Fig. 22.17.

22.8 Processing texturized soy protein: extrusion vs. extrusion-expelling

22.8.1 Current practices

Currently, soybeans are purchased from farmers by buyers such as elevators and commodity traders. They are brought to soybean extraction plants, where they are re-cleaned, tempered with heat and the addition of moisture to about 10–12%, cracked into 4–6 pieces by corrugated rolls, and the hulls removed by aspiration. The cotyledons are then heated to about 82–85 ºC and flaked to about 0.3 mm, extracted with hexane and carefully desolventized to retain protein solubility (determined as PDI, Protein Dispersability Index or NSI, Nitrogen Solubility Index). A typical standard practice for making texturized vegetable protein is shown in Fig. 22.18. The resultant meal is then ground into flour. This

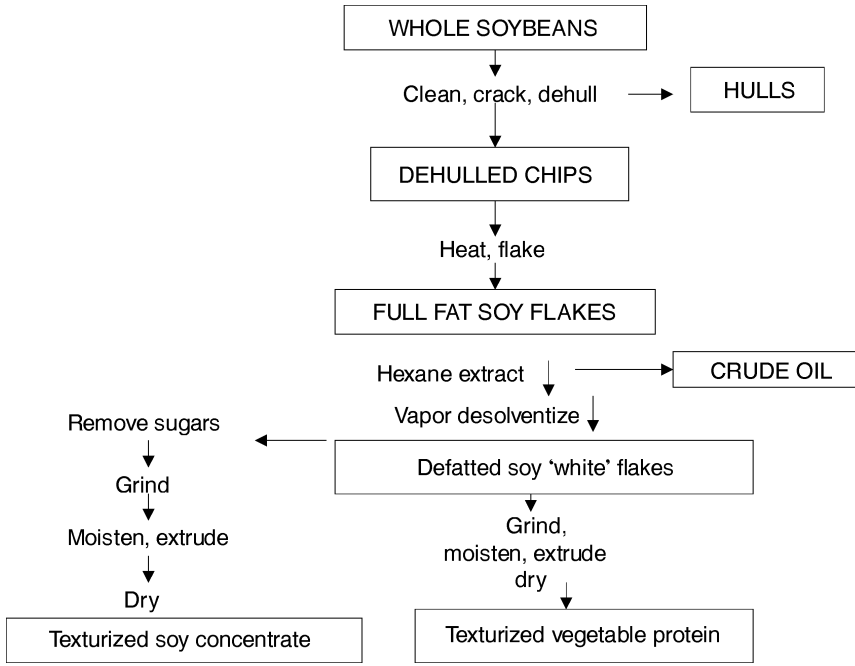

Fig. 22.18 A typical process for making texturized soy protein using defatted soy flour.

soy flour contains 46–48% protein as its basis, which then can be further processed into soy protein concentrate. Both soy flour and concentrate are then processed through the extruder to make texturized vegetable protein.

This process is based on solvent extraction to separate the oil from the meal in soybeans. Solvent extraction technologies thrive on economies of scale. It requires heavy capital investment, high technology and a well-developed infrastructure for collection, storage and distribution of raw materials and finished goods. The main advantage of solvent extraction is the high efficiency of oil extraction and lower cost of production. The major concern for this process is the emission of hexane vapors into the atmosphere which create considerable environmental concerns. Indeed, the solvent extraction industry is under pressure to limit the level of emissions into the environment (Wijeratne, 2000). In this situation, any process that does not use the solvents would find favorable consideration.

22.8.2 Extrusion-expelling of soybeans

Although solvent extraction of soybeans dominates the oilseed crushing industry, direct screw pressing is still practiced in underdeveloped countries. In this process, soybean oil is removed using an expeller by heating the soybeans first, for a long time, and then passing them through the expeller. The

disadvantage of this process is that it gives low extraction efficiency, fluctuation in the quality of protein meal and oil, and high costs of production. Prolonged heat treatment of soybeans prior to pressing, non-uniform heating due to variation of seed size within seed lots, and the need for multi-pass pressing, have adverse effects on the quality of meal and oil. Also, there is not enough heat to destroy all the antinutritional factors causing off flavor to the meal, making it unsuitable for human consumption. Nelson *et al*. (1987), modified this traditional method by adding dry extruders to extract the oil from soybeans as a pretreatment. Dry extruders are single-screw autogenous machines which operate at low moisture and require minimum auxiliary equipment. In this type of extruder, heat is generated by friction and does not require an external source of heat. It has been found that coarsely ground whole soybeans at 10–14% moisture can be extrusion cooked in less than 30 seconds at a temperature of about 130–135 ºC. It was discovered that the semifluid extrudate can be immediately pressed in a continuous screw press to obtain high-quality oil and press cake. The main advantage of adding a dry extruder in this process is that only a single pass is required from the press in order to get oil down to 6% in the meal. Protein content of this meal is in the range of 46–50% and can be used for food or feed application.

Researchers at the Food Protein Research & Development Center, at Texas A&M University, have modified existing technologies for making extruded full-fat and partially defatted soybean meals, and have added an extruder texturizing step to produce mild-flavored texturized soy products which can be made in dispersed locations without solvent extraction plants (Lusas and Riaz, 1996a; Riaz, 1998). This process has been fine tuned using food grade dehulled soybeans to produce soy flour with high protein PDI to make texturized vegetable protein. According to Lusas and Riaz (1996b) good-quality soybean flour was produced using a dry extruder which has PDI of 45 (Heywood *et al*., 2000; Crowe and Johnson, 2000).

A flow sheet for making partially defatted soy flour is shown in Fig. 22.6. Food grade soybeans are cleaned, cracked, dehulled, and extruded by a dry extruder to free the oil. The oil is then removed by screw press. The press cake is ground and adjusted to 21% moisture content, and is formed into shreds or chunks using the same extruder or another extruder. If desired, caramel color or other ingredients can be added to the mix before texturization (Riaz and Lusas, 1997; Riaz, 1999). The extruded products hydrate readily, resemble ground or chunk meat, and retain a chewy texture when cooked. Figures 22.19–21 show different stages of soybeans during processing into texturized products and after hydrating in water.

Advantages

Advantages of the new process include (Riaz, 2001):

1. Soybeans do not have to be first converted into flour or concentrates in centralized oil mills before texturization.

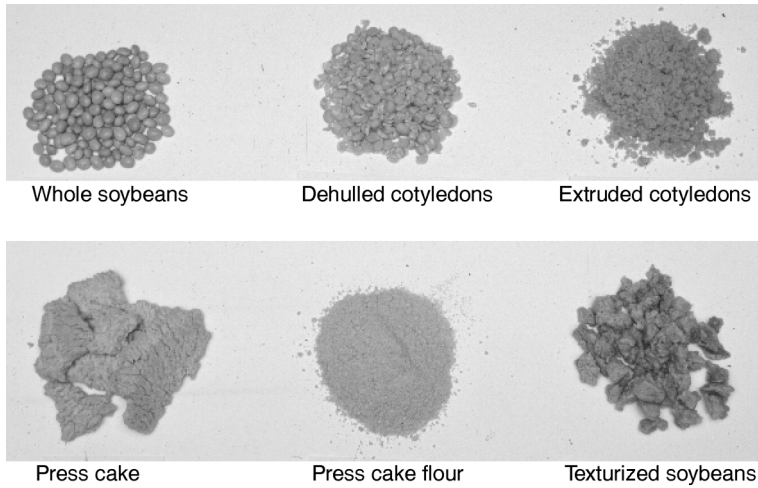

Fig. 22.19 Soybeans during different stages of processing into texturized products.

2. Preparation of texturized soybeans can be done in simple dispersed facilities with small-size extrusion and screw pressing equipment – tractor power take-off-driven if necessary.
3. The texturized product is extremely bland because of early deactivation of lipoxygenases and other enzymes.
4. The pressed oil is more bland than that obtained by typical solvent extraction or screw press processing; it has a nutty flavor, and might be acceptable with minimum processing in developing countries.

Fig. 22.20 Soybeans during different stages of processing into texturized products.

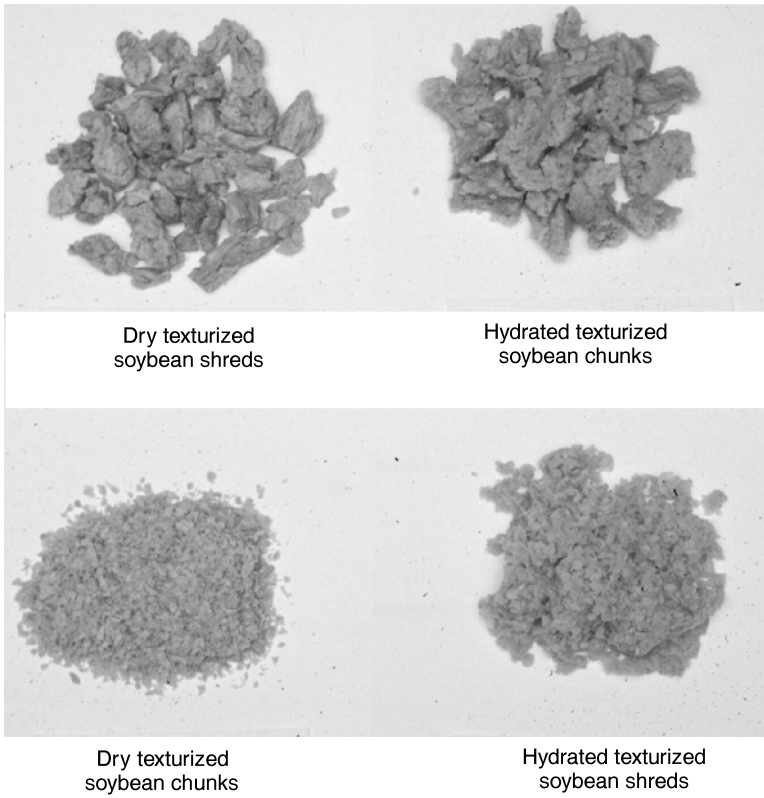

Fig. 22.21 Texturized soy protein made by extrusion-expelling process (ground and chunk) after hydrating with water.

5. Both the texturized soy protein and oil products retain natural antioxidants and have good shelf life.
6. Identity preservation is another factor that is becoming important in soybean processing. This may be due to the need for specific varieties for specific applications, or to separate organically grown varieties or to maintain identity of GMO/non-GMO varieties. The large scale and fully automated structure of the solvent extraction industry does not lend itself to segregation of varieties through the processing cycle. Extrusion-expelling on a small scale can ensure identity preservation and could take advantage of the developing market for identity preserved soybean products.

Although the process is simple in concept, several principles must be considered. 'Beany' and other off-flavors often occur in soy products because of the activity of lipoxygenases and other enzymes. As shown in Fig. 22.22, the lipoxygenases which also probably approximate the activity of other indigenous enzymes, are inactivated at about 105–107 ºC. Yet, at this temperature, the protein

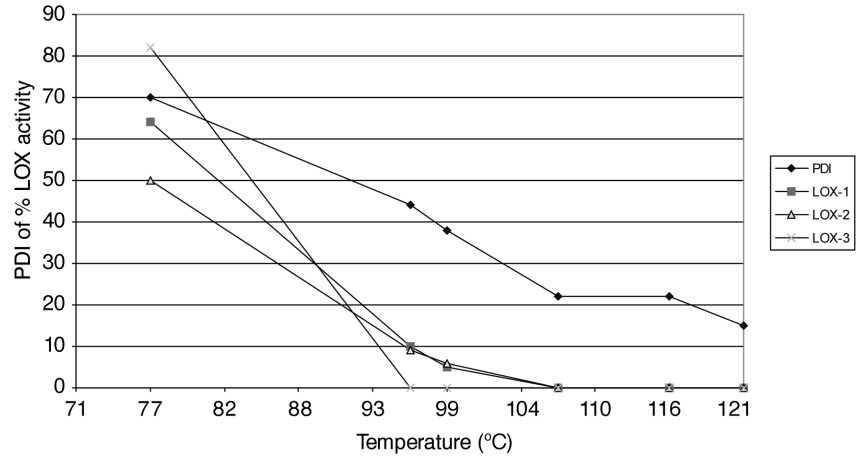

Fig. 22.22 Effect of extrusion temperature on protein dispersibility index (PDI) and lipoxygenase enzyme activity (LOX).

has a PDI of approximately 25 – still enough for later texturization if a high shears extruder is used. As shown in Fig. 22.23, increased moisture also accelerates reduction of PDI, and extrusion cooking at higher than 13% moisture can be detrimental to future texturization (Zhu *et al.*, 1996). Arresting enzymatic activity by essentially instantaneous heating when extruding the soybeans in the extruder is critical to obtaining mild-flavored products.

A variety of screw and die configuration can be used successfully for texturizing the ground press cake. But the extruder must impart enough shear to the proteins to cause their lengthening from globular structures and a long die spacer must be provided for laminar flow to allow the fibers to align in parallel.

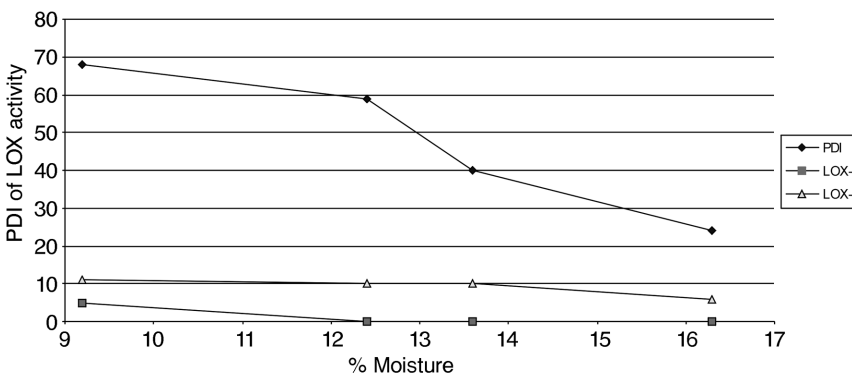

Fig. 22.23 Effect of moisture on protein dispersibility index (PDI) and lipoxygenase enzyme (LOX) during extrusion at 99 °C.

The versatility of the TSP from this process has been emphasized to suggest its potential uses in an area where markets are small, transportation is limited, and dispersed processing is desirable. TSP compares well with texturized soy flours made by traditional processes. The new process is an alternative to defatting by solvent extraction when not required for market acceptance.

22.9 Economic viability of an extrusion processing system for producing texturized soy chunks: an example

Most of this information is provided by Brian Plattner, Wenger Manufacturers, Sabetha, Kansas in a private communication. The following analysis examines the economic viability of an extrusion processing system producing texturized soy chunks. As with any evaluation a number of assumptions must be made before any analysis can be accomplished. This analysis gives a good estimation of costs; however, each individual system should be examined independently to ensure accurate results.

For this example we will examine the operating costs of a Wenger TX-115 Magnum extrusion system. The initial capital investment is outlined in Table 22.9. It will produce a maximum of 1,600 kg/hr of finished product (9% moisture basis). For example, a Wenger Series VII conveyor style dryer will be used to dry the product. The plant will operate approximately 6,000 hours/year (250 days, 24 hours/day). The installation and startup category is used to estimate the costs associated with installing the equipment (plumbing, wiring, labor) and the raw materials and utilities used during startup and commissioning. Typically, these costs run from 15 to 20% of the capital investment but they will vary depending on the experience of the contractors and plant personnel. The plant overheads and construction costs are not included in this analysis.

22.9.1 Fixed costs

To calculate the depreciation and interest expense the following assumptions were made: ten years' equipment life; straight-line depreciation, no salvage value; 10% APR. The costs (in $ per metric ton) are summarized in Table 22.10.

Table 22.9 Capital cost investment

Equipment	Capital investment
Extruder	668,600
Dryer/cooler	323,600
Hammermill	178,500
Installation/startup	200,000
Total	$1,370,700

Table 22.10 Fixed costs

Category	Cost
Depreciation	12.195
Interest	7.652
Misc. capital cost	2.083
Total ($/metric ton)	$21.93

22.9.2 Variable costs

Variable costs can be split into four subcategories: raw materials, utilities, labor, and maintenance/wear. The values used for this analysis are listed below.

Raw materials

We will assume that soy flour can be purchased for $0.27/kg.

Utilities

The following assumptions were used for determining utility costs:

electricity	0.0450	$/kW/h
steam	8.82	$/1000 kg
water	1.06	$/1000 kg

Maintenance/wear cost

The wear cost, which includes extruder screws, barrel sleeves, dies, and knife blades, is shown below. These maintenance costs are based on actual production data; however, they should not be considered as a guarantee.

TX-115 extruder	$4.714/metric ton
Series VII dryer/cooler	$0.25/metric ton

Labor cost

If the total wage package, salary plus benefits, for an operator is $20.00 per hour, and the operator is responsible for one extruder and dryer the direct labor cost is $12.50 per metric ton.

Total production cost

Table 22.11 shows the total production cost. Often the easiest way in which to understand the data shown in Table 22.11, is to examine it in a graphical form. The pie charts in Figs 22.24–26, help one better to understand how each processing cost category affects the overall cost of a system.

By examining Fig. 22.24, we can see that raw materials have an enormous impact on production costs. In fact, 1% change in raw material costs has ten times the impact as a 1% change in variable costs. This amply illustrates the benefit of closely monitoring raw material costs. This ratio increases

Table 22.11 Total production costs ($/metric ton)

Cost category	Cost
Interest	7.652
Depreciation	12.195
Misc. capital cost	2.083
Raw material	270.000
Utilities	11.517
Maintenance	4.964
Labor	12.500
Total	$320.911

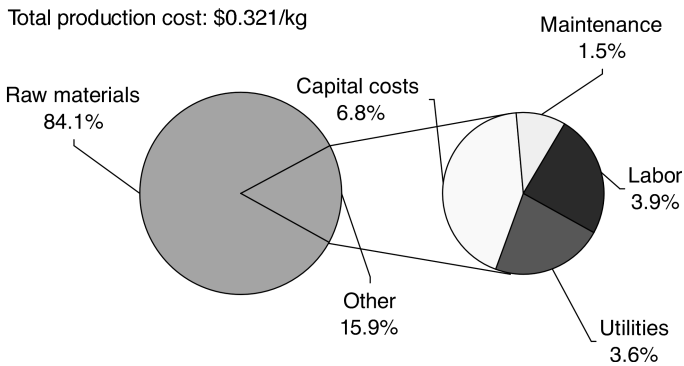

Fig. 22.24 Overall processing costs for TSP system.

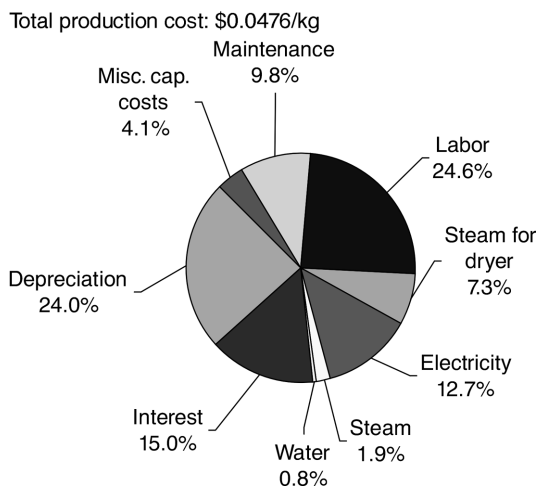

Fig. 22.25 Overall processing cost (not including raw materials).

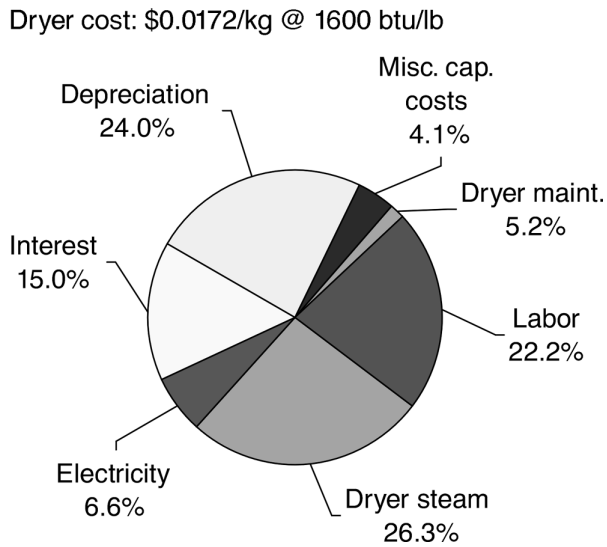

Fig. 22.26 Dryer-related processing costs.

dramatically as we use higher value raw materials. For example, if we use a soy concentrate valued at $1.3 per kilogram, 1% change in raw materials would have forty times the impact as a 1% change in variable costs.

Figure 22.25 shows the result when we remove the raw material costs from our analysis. From this we see that 43% of the costs are associated with capital investment, 9.8% with maintenance, 24.6% with labor, and 22.7% with utilities.

When examining the dryer operational costs, as shown in Fig. 22.26, we can see that utility costs make up 33% of the total operating cost. Thus, an efficient dryer will not only pay for itself in a very short time, but also will greatly increase a plant's profitability.

22.9.3 Moisture uniformity during drying

When examining a dryer, one should consider the moisture uniformity of a dryer. Table 22.12 compares two dryers with different moisture uniformities. The dryers are characterized based on the moisture variation in the product leaving the dryer. For example, dryer A has a moisture variation of ±0.5%. This simply means that if the average moisture content of the product is 8.5%, the maximum measured moisture would be 9.0% and the minimum would be 8.0%.

Notice that dryer A, which operates at a much narrower moisture tolerance, has a discharge rate that is 24 kg/h higher than dryer B. Since the same amount of material is entering the dryer, the additional capacity is all water. This is due to the higher average moisture content of the product leaving dryer A. Therefore, we are now selling 24 kg/h of water at TSP prices. If the dryer is operating 6,000 hours per year and the product is worth $1.00 per kilogram, dryer A would result

Table 22.12 Comparison of two different dryers with different moisture uniformities

	Dryer A	Dryer B
Moisture uniformity at discharge	±0.5	±2.0
Maximum allowed discharge moisture	9.0%	9.0%
Average moisture	8.5%	7.0%
Product into the dryer @ 26%	1800 kg/h	1800 kg/h
Product out of the dryer	1456 kg/h	1432 kg/h

in $144,000 per year of increased sales. Thus, the dryer used in this example would pay for itself in just over two years.

22.9.4 Control systems

In selecting an extrusion processing system, it is often difficult to decide what level of control system should be chosen. Advanced control systems have many advantages including the ability to:

- reduce product waste
- improve product uniformity
- enhance proficiency of operators
- provide process documentation
- reduce utility costs
- improve safety
- provide opportunities for prediction, monitoring, and control of product quality.

The disadvantage of advanced control systems is cost. Advanced control systems will often cost from 15–25% of the initial capital investment. The following analysis examines the possible benefits of using advanced control systems. Table 22.13 lists the parameters used for this analysis. Table 22.14 shows the effect of an increase in efficiency on raw materials saved, increase in production time, and the resulting increase in revenue. For example, a 1% increase in efficiency is equivalent to about 15 additional minutes of producing quality product out of a 24-hour operation day. The increased revenue is based on the ability to produce additional in-spec products. The last two columns show the payback of the two advanced control options. For example, if a 1% efficiency increase was realized the programmable logistic controller (PLC) would pay for itself in 1.28 years and the automatic process management (APM) would pay for itself in 1.68 years.

It is often difficult to estimate the efficiency increase that can be realized by using an advanced control system. In cases where the operators are extremely good, one may see only a slight increase in efficiency. However, in some instances we have seen efficiency increases approaching 15% by allowing a computer to regulate a portion of the process control.

Table 22.13 Parameter used for advanced control system analysis

Control system model parameters	
Raw material cost ($/ton)	270.00
Final product sell price ($/kg)	1.00
Hours/day	24
Days/year	250
Hour/year	6000
Tons/hours	1.60
Manual to AMP upgrade	$161,300
Manual to PLC upgrade	$123,200

Table 22.14 Effect of an increase in efficiency, raw material, production time and revenue

Efficiency increased percent	Raw material saved (tons/year)	Increase in production time (minutes/day)	Increased revenue $/year	PLC payback years	APM payback years
0.25	24.00	3.60	24,000	5.13	6.72
0.50	48.00	7.20	48,000	2.57	3.36
1.00	96.00	14.40	96,000	1.28	1.68
1.50	144.00	21.60	144,000	0.86	1.12
2.00	192.00	28.80	192,000	0.64	0.84
3.00	288.00	43.20	288,000	0.43	0.56

If this example had examined a product having a much higher value, the rate of return would have been much greater (i.e., shorter payback time). On the other hand, if a lower-value product had been examined, we would have seen a much lower rate of return (longer payback time). Thus, it becomes a balancing act in which we have to weigh our potential advantages against the capital investment required.

22.10 Uses of texturized soy protein

Texturized soy protein has been a commercial success for many years because of the development of machinery that is capable of continuously producing texturized vegetable products. The texturization of plant proteins has been a major development in the food industry. Processes, like extrusion, have been developed to impart a meat-like fibrous structure. Once texturized, these plant proteins can be dehydrated for use as an extender of fresh or processed meat. Consumers are becoming increasingly interested in healthy foods and open to soy protein ingredients. Texturization of soy flours into usable meat extenders

and replacers have been economically feasible for some time. Texturized soy products serve a variety of purposes, including alternative protein sources for the manufacture of convenience foods and for centralized feeding of large numbers of people within defined budgets.

Texturized vegetable protein is being used increasingly in North America as an extender of red meat products. Among the low cost vegetable protein products developed for use in foods as meat extenders or replacement, the most rapid growth has been in the area of texturized products manufactured by thermoplastics extrusion. Technology is becoming quite accomplished at creating realistic analogs that equal their meat counterparts in terms of flavor, texture and most importantly, satiety. There are analogs of hamburger, both in patties and ground form, sliced lunch meat, sausages, hot dogs, Canadian bacon, pepperoni, bacon bits, and stuffed turkey. Texture and flavor are the two biggest challenges in developing a meat analog. Vitamins and mineral fortification can be included for school and military luncheon programs.

Some home-made vegetable protein foods are pareve (without meat, milk, eggs and their derivatives) and are of interest to people following Jewish (Kosher) dietary laws. Islam is one of the world's fastest growing religions, and Muslims are today demanding Halal foods. Texturized vegetable foods provide an alternative to animal meat and are accepted as Halal foods (Lusas, 1996).

Texturized vegetable protein from soy concentrate has the advantages of blander flavor and major reduction of non-digestible natural sugars (stachyose and raffinose) which can cause considerable flatulence, abdominal discomfort, and venting in some individuals. Modern texturized soy flours have milder flavors than in earlier years, which are easily masked in highly seasoned foods, like tomato sauces, pizza toppings, and canned chilli. Users of texturized vegetable protein include growing numbers of vegetarians, and people concerned with lowering cholesterol and total fat intake.

Texturized soy protein is not a filler, but can be used as a distinct product, e.g., along with ground beef, as well as simulated products, e.g., a meat analog as a major source of protein in the human diet. When texturized soy protein is used as a substitute for other products, its textural as well as nutritional properties should be similar to those of the product being replaced.

Texturized soy protein can be added to meat as an extender or it can be consumed directly as a meat analog. Breaded chicken patties with as much as 30% of the meat replaced were actually preferred to all meat patties. Meat analogs can be flavored and formed into sheets, disks, patties, strips and other shapes. There are meat-free hot dogs, hamburgers, chicken patties, nuggets, hams, sausages, meat snacks, and loose meat products for chilis, tacos, and spaghetti. It is very difficult to tell the difference between real and texturized soy protein. In India, China, Japan and South Korea texturized soy protein is eaten directly as a flavored or seasoned dish usually as a side or main portion of the meal. A good example of a completely meat-free meat analog is flavored bacon bits. Some of the texturized protein consumption in different parts of the world is based on religious, cultural, or economic reasons. A good example is

vegetarian diets for most Indians (Hindu). Texturized soy protein is widely used in child nutrition programs as well as for worldwide relief agencies to help feed famine plagued people in impoverished countries. Because of their low moisture and water activity, storage, shelf life and handling under poor conditions, do not become problems. Vitamins and minerals can be fortified in texturized soy protein to make it an ideal protein source. It is cholesterol free and can be processed as a low-fat food. Texturized soy products can assist in weight control by providing high-quality protein in a concentrated form, specially designed low-calorie/high-nutrient ready meals can be produced. These can make a significant contribution to weight control products.

Vegetarians have modified many recipes to replace meat with soy proteins. Recipes are available to use texturized soy protein in breakfast foods, appetizers, soups, sandwiches, gravies, desserts, ethnic food and main dishes. Several food items are available with texturized soy protein that are designed to grill or cook in microwave ovens. Sometimes these food items are co-extruded with soy concentrates and wheat gluten. Ingredients, including flavors, and colors and edible adhesives (like soy protein isolates, hydrocolloids, or starch, calcium caseinate and egg whites) are added to hydrated texturized soy protein before forming into patties, freezing and packaging.

The demand for meat extenders and meat analogs will continue to rise. Meat extenders are still the largest segment of the texturized vegetable protein market, however, the use of meat analogs is increasing. We are becoming more aware nutritionally of the foods we eat. Along with the beneficial high protein content of actual meats, there are some disbenefits, namely cholesterol. However, most people still like their meat. Meat analogs have become a viable alternative in offering a nutritionally acceptable meat substitute that in some cases come close to matching actual meat products. Food scientists have made major headway in improving flavor, texture, mouthfeel, appearance and color of meat analog products. In the marketplace, you can see more and more meat analog and meat extender products such as bacon bits, soy burgers, meat-free hot dogs, chicken nuggets, breakfast sausage patties/links and bacon to name a few. Many of these products are even packaged in the same fashion as their meat counterparts.

American consumers' acceptance of texturized vegetable protein has prompted the interest of other countries in this low-cost answer to the desire for foods with more protein. The U.K., South Africa, Japan, Korea, Mexico and India are among the nations that have joined the United States in commercial production.

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23

Health-related functional value of dairy proteins and peptides

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23.1 Introduction

Milk is the main dietary component for over 4,000 species of mammalian neonates. Milk proteins, therefore, are the most widely consumed source of food protein (at least in the early stages of life). Although the majority of protein consumed is of bovine origin other animal sources of dairy proteins may predominate depending on geographical location. Traditionally, milk proteins have been viewed as a source of nitrogen and essential amino acids, and as functional ingredients for use in food products due to their good surface activity and gelling properties (reviewed extensively elsewhere in the literature). However, apart from their nutritional and physicochemical attributes, milk proteins and associated peptides are achieving increased recognition as ingredients for functional foods, i.e., for their beneficial health-related properties. This chapter will briefly review the general nutritional role of milk proteins and will then specifically outline recent developments in immunomodulatory, mineral-binding and hypotensive proteins/peptides.

23.2 Types of milk protein

23.2.1 Caseins

The compositional properties of milk and milk-derived proteins have been previously reviewed in detail (see Chapter 3 or for further information: Swaisgood, 1982; Walstra and Jenness, 1984; Fox and McSweeney, 1998; Fox, 2003). All the milk proteins exhibit genetic polymorphism due primarily to either substitution of specific amino acid residues or to deletion of specific

fragments in their primary structure (Ng-Kwai-Hang and Grosclaude, 2003). Bovine milk contains approximately 30–35 g protein per litre (Swaisgood, 2003). The two main protein groupings present in milk are classified as casein or whey proteins. On a total protein basis, 76–86% of bovine milk proteins are composed of the caseins, i.e., α_{S1}-, α_{S2}-, β- and κ-casein. Incorporation of urea into polyacrylamide gels led to the discovery of genetic variants or polymorphisms in the caseins. One of the defining features of caseins from whey proteins is the presence of phosphate esterified to serine and threonine hydroxyl groups. Nutritionally this is believed to be an important attribute of caseins as the phosphate groups bind bivalent minerals such as Ca^{2+}. Technologically this property may be manipulated to isolate casein, as is the case in Ca^{2+}-induced coagulation of rennet casein (Swaisgood, 2003). The proline content of the caseins is high with α_{S1}-, α_{S2}-, β- and κ-casein containing 17, 5, 17 and 12 mol%, respectively. The caseins are thought to have little secondary structure as a result of this high content of proline. Furthermore, the rather large content of phosphate and proline confers thermal stability to the caseins (Fox and McSweeney, 1998). In milk the majority of the caseins exist as large colloidal self-aggregating particles known as micelles with molecular masses between 10^6 and 10^9 Da (Fox, 2003). Casein micelle structure has been reviewed elsewhere (Swaisgood, 2003).

23.2.2 Whey proteins

Whey proteins are those proteins that remain soluble at pH 4.6 following acidification of milk which leads to isoelectric precipitation of caseins. Whey protein represents 14–24% of the total protein in bovine milk. The main individual protein components of bovine whey are α-lactalbumin (α-La), β-lactoglobulin (β-Lg), bovine serum albumin (BSA) and the immunoglobulins (Ig). Many of the health related functions of whey proteins are linked with the immune or digestive systems (Wong and Watson, 1995). Minor whey proteins such as lactoferrin (Lf), (Lönnerdal, 2003), lactoperoxidase (Pruitt, 2003), lysozyme (Farkye, 2003) and the Igs (Hurley, 2003) are considered anti-microbial proteins.

α-La is a Ca^{2+} metalloprotein, rich in tryptophan, containing four disulphides (Brew, 2003). Of all the whey proteins it is the most stable to thermal denaturation (Fox, 1989; Boye and Alli, 2000). Bovine β-Lg contains two disulphide bonds and exists naturally in milk as a dimer of two non-covalently linked monomers (Sawyer, 2003). β-Lg has the ability to bind retinol and belongs to a 'super-family' of proteins with the ability to bind and transport hydrophobic molecules (Godovac-Zimmermann, 1988; Dufour *et al*., 1990). All milks contain serum albumin and in the case of bovine milk this component is known as BSA. BSA has been shown to be a carrier of small molecules such as metals and fatty acids. The protein contains 17 disulphide bonds and one free thiol group. The exact role of this protein in milk is unclear and it may well be that its presence is simply due to leakage from blood to milk in the mammary

gland. Igs are large globular molecules formed from a minimum of two polypeptide chains, i.e., light (L) and heavy (H) chains. Altogether five classes of Igs exist, only IgA, IgG and IgM are found in milk. The principal Ig in human milk is IgA while in bovine milk it is IgG_1. Physiologically, milk Igs offer the young of a species some degree of protection from pathogenic infection due to micro-organisms. Colostrum, i.e., the milk obtained immediately after parturition may contain as much as 100 times the level of immunoglobulins of mid-lactation milk (Shah, 2000).

23.2.3 Minor milk proteins

Numerous minor milk proteins exist of which many are enzymes such as lactoperoxidase, lipases, phosphatases and proteinases. Two distinct, single polypeptide chain, iron-binding glycoproteins are found in milk. These are transferrin (Tf) a blood plasma protein, and lactoferrin (Lf) which is produced in the mammary gland. The concentration and ratios of both these minor proteins depend on stage of lactation, and from species to species. Tf is capable of binding two moles of Fe^{3+} per mole. Lactoferrins are single chain polypeptides of approximately 80 kDa containing one to four glycans depending on the animal source (Spik *et al*., 1994) with the ability to bind Fe^{3+} (2 moles/mole protein) and other ions. Although bovine and human Lf contains a similar number of amino acids their sequence identity is only 69% (Pierce *et al*., 1991). As a result, the three-dimensional structures of bovine and human Lf are similar but not identical. The level of Lf in milk varies depending on the animal species and stage of lactation.

According to Steijns and van Hooijonk (2000), higher levels of Lf are found in human colostral milk (> 7mg/ml) than mature human milk (>1–2 mg/ml), than bovine colostral whey (> 1.5 mg/ml) and mid-lactation cows milk (>20–200 μg/ml). In human milk, Lf is found in the form of apoLf, i.e., less than 5% iron saturation. Lf is a non-specific anti-microbial component of milk, which exerts a protective function by several mechanisms. The anti-microbial effects can be through direct bacteriostatic and bactericidal activities or indirectly through activation of a complex series of reactions leading to a protective immune response after infection (Bellamy *et al*., 1992; Sánchez *et al*., 1992; Tomita, 1994; Levay and Viljoen, 1995; Lönnerdal and Iyer, 1995; van Hooijonk *et al*., 2000). The bioactivity of bovine and human Lf is comparable although their structure and biochemical properties are quite different as assessed by *in vitro* and *in vivo* model animal studies. Bovine Lf is currently used in commercial food products to increase iron bio-availability, to mimic human milk in infant formulations and to boost the natural defence of an individual against microbial infections (Steijns and van Hooijonk, 2000).

Other minor peptide components found in milk include the proteose-peptones that are N-terminal fragments of β-casein generated by plasmin activity (Swaisgood, 2003). Additionally, rennet whey contains the carbohydrate rich

glycomacropeptide fragment formed by the hydrolysis of κ-casein by chymosin. The milk-fat globule membrane (MFGM) is also a rich source of minor milk proteins (Mather, 1978). Many of the proteins in the MFGM have been isolated and characterised in an attempt to determine their biological functions (Mather *et al*., 1980; Greenwalt, 1993; Wilcox *et al*., 2002). One protein in particular, i.e., CD36, a 75–88 kDa glycoprotein found in MFGM has been suggested to act as a scavenger receptor for low-density lipoproteins. The MFGM also possesses many complex lipids that represent not only nutritionally valuable molecules but also potential anticarcinogenic components including conjugated linoleic acid, sphingomyelin, butyric acid and other lipids (Parodi, 1997; Astaire *et al*., 2003).

23.3 General nutritional role of milk proteins

The nutritive value of milk proteins may be assessed by (i) the energy they supply (ii) their digestibility and absorbability (iii) their content of essential nutrients, i.e., essential amino acids, associated fatty acids, minerals and vitamins, and (iv) their allergenic potential. With allowances for incomplete digestion and the fact that part of the milk protein is converted to and excreted as urea, the metabolically available energy from milk protein was estimated to be 17 kJg^{-1} (4.1 $kcalg^{-1}$) (Walstra and Jenness, 1984). Human and bovine milk have similar overall energy contents, i.e., 2.8–3.0 $MJkg^{-1}$ (670–720 $kcalg^{-1}$), however, on a protein basis human milk protein contributes only 5% of the total energy in contrast to bovine milk protein where it provides 20% of the energy.

Protein quality may be expressed by a number of terms including biological value (BV), protein digestibility (PD), net protein utilisation (NPU), protein efficiency ratio (PER) and protein digestibility corrected amino acid score (PDCAAS). Compared to whole milk and casein, whey protein has higher BV, PD, NPU and PER values (Table 23.1). The nutritive value of the different milk protein fractions primarily depends on their essential amino acid content (Hambræus and Lönnerdal, 2003). Both human and bovine milk proteins are an excellent source of essential amino acids (Table 23.2). Furthermore, due to the fact that caseins are rich in tyrosine and phenylalanine and whey proteins are rich in cysteine and methionine both fractions combined have a greater protein quality than either alone due to their complementary concentrations of amino acids.

23.3.1 Milk protein carriers of vitamins, minerals and essential fatty acids

Trace elements and minerals in bovine milk occur as inorganic ions, salts or in the form of complexes with proteins, peptides, carbohydrates, fats and other molecules. In total, some 20 minerals are considered essential in the human diet (Flynn, 1992). Bovine milk possesses higher concentrations of these minerals compared to human milk (Table 23.3). The main milk proteins with the ability to bind bivalent cations such as calcium are the caseins. Whey proteins also bind

Table 23.1 Nutritional quality values for milk and milk protein fractions. Reprinted from *Nutritional quality of proteins*, European Dairy Association, 1997, with kind permission of the European Dairy Association

Sample	BV	PD	NPU	PER	PDCAAS
Milk	91.0	95.0	86.0	3.1	1.21
Casein	77.0	100.0	76.0	2.9	1.23
Whey protein	104.0	100.0	92.0	3.6	1.15

Abbreviations: BV, biological value; PD, protein digestibility; NPU, net protein utilisation; PER, protein efficiency ratio; PDCAAS, protein digestibility corrected amino acid score.

specific minerals, such as calcium, magnesium, zinc, iron, sodium and potassium (Vegarud *et al*., 2000). Lf is the most notable iron binding protein, however; it may also bind Cu^{2+}, Mn^{3+}, Co^{3+} and Zn^{2+} (Steijns and van Hooijdonk, 2000). Lf has been shown to inhibit the growth of bacteria by scavenging the free iron required for growth (Payne *et al*., 1990; Saito *et al*., 1991). BSA has also been reported to have the ability to bind Zn^{2+} (Lönnerdal, 1985). Due mainly to the fact that whey proteins are not phosphorylated the binding of cationic minerals to whey proteins occurs through a different mechanism than in the caseins (Cayot and Lorient, 1997).

Although milk is an excellent source of vitamins, only a few vitamin-carrying milk proteins have been reported, including cyanocobalamin (vitamin B_{12})

Table 23.2 Approximate contents of essential amino acids in bovine and human milk protein and some dietary recommendations. From *Dairy Chemistry and Physics*, Walstra and Jenness, Copyright © (1984, John Wiley and Sons, Inc.). Reprinted by permission of John Wiley & Sons Inc

	Quantity in protein ($mg.g^{-1}$)		Daily requirement ($mg.kg^{-1}.day^{-1}$)		
Amino acid	Bovine	Human	RDA[a]	Infant[b]	Adult
Histidine	30	23	17	33	
Isoleucine	55	50	42	83	12
Leucine	100	91	70	135	16
Lysine	80	67	51	99	12
Methionine	28	14	26	49	10
Cysteine	9	18			
Phenylalanine	53	36	73	141	16
Tyrosine	44	28			
Threonine	48	42	35	68	8
Tryptophan	14	22	11	21	3
Valine	72	64	48	92	14

RDA, recommended daily allowance.
[a]Estimated pattern of high-quality protein (NRC-NAS, U.S.).
[b]Four to six months old.

Table 23.3 Mean concentration of minerals and trace elements in human and bovine milk (Flynn, 1992)

Component	Human milk	Bovine milk
Arsenic (μg/L)	0.2–0.6	20–60
Boron (μg/L)	60–80	1000
Calcium (mg/L)	280	1200
Chloride (mg/L)	420	950
Chromium (μg/L)	0.27	2
Cobalt (μg/L)	0.1	0.5
Copper (mg/L)	0.25	0.09
Fluoride (μg/L)	16	20
Iodine (μg/L)	64–178	100–770
Iron (mg/L)	0.3	0.5
Magnesium (mg/L)	35	120
Manganese (μg/L)	6	30
Molybdenum (μg/L)	2	50
Nickel (μg/L)	1.2	26
Phosphorus (mg/L)	140	950
Potassium (mg/L)	525	1500
Selenium (μg/L)	16	10
Silicon (μg/L)	700	3000
Sodium (mg/L)	180	500
Zinc (mg/L)	1.2	3.5

binding protein (Holds-Worth and Coates, 1960), folate (vitamin B_9) binding protein (Salter *et al*., 1972; Hansen *et al*., 1978), calciferol (vitamin D) binding protein (Ena *et al*., 1992) and the retinoic acid (vitamin A) carrying protein, β-Lg (Dufour *et al*., 1994; Sawyer *et al*., 1998). The carriers of vitamin B_9, B_{12} and D are minor whey proteins with the ability to resist degradation by a series of proteolytic enzymes in the gastrointestinal tract delivering the vitamins to the small intestine. The physiological significance of these proteinaceous binding systems in milk is unknown but it appears that they play a role in the regulation of these vitamins *in vivo*. Little information is available on the ability of milk proteins to bind/carry essential fatty acids; however, both BSA (Posner and DeSanctis, 1987) and β-Lg (Puyol *et al*., 1993) have been reported to bind fatty acids. Lönnerdal, (1985) and Clare and Swaisgood, (2000), have reviewed the ability of milk proteins to bind dietary components essential to human health.

23.3.2 Anti-cancer properties of milk proteins

Two commonly accepted approaches believed to help avoid certain forms of cancer are early diagnosis and good nutrition. High dietary fat, and low fibre and micronutrient intakes have been identified as risk factors for different forms of cancer, e.g., colorectal cancer (Parodi, 1998; Gill and Cross, 2000). Milk proteins have been identified for their ability to prevent or reduce tumour

development in animals (McIntosh, 1993; Parodi, 1998; Gill and Cross, 2000). McIntosh *et al*., (1998), found that whey protein concentrate (WPC) played a significant part in cancer prevention in rats. Hakkak *et al*., (2000), reported that bovine whey proteins were more effective than soy protein against chemically induced mammary tumours in Sprague-Dawley rats. Furthermore, isolated whey proteins have been reported to possess anti-cancer properties.

Iron-depleted Lf, which has the ability to bind free iron, was shown to reduce the incidence of carcinogen-induced colon adenocarcinoma in rats (Sekine *et al*., 1997; Tsuda *et al*., 2000). Gill and Cross (2000) subsequently, postulated that the reduced incidence of adenocarcinoma in rats may be attributed to Lf preventing oxidant-induced carcinogenesis by free iron. Svensson *et al*. (1999), showed that human α-La induced apoptosis in breast cancer cells with no adverse effect on standard cells. BSA was shown to inhibit the growth of the human breast cancer cell line, MCF-7, when included in cultures of these cells *in vitro* (Laursen *et al*., 1990).

A fatty acid binding protein in bovine whey, termed mammary-derived growth inhibitor (MDGI), was shown to prevent the proliferation of bovine epithelial cell lines *in vitro* (Zavizion *et al*., 1993). Much of the evidence suggesting that milk proteins have anticancer properties has come from *in vitro* studies using tumour cell lines or *in vivo* studies using animal models of tumourogenesis. Both these modes of analysis yield valuable information as to the potential anti-cancer properties of milk proteins, however, caution should be taken when extrapolating from these results as to their potential disease protection properties in humans (Gill and Cross, 2000). As an example, many *in vivo* and *in vitro* studies do not take into account the effect of gastrointestinal proteinase digestion on ingested milk proteins. Such digestive processes may render beneficial ingested proteins/peptides inactive or even more active. Additionally, an anti-tumourigenic response observed in animal-based studies may not translate to an anti-tumourigenic response in humans. Clinical studies on humans using potential anti-cancer milk proteins/peptides are required before definitive claims may be made with respect to their efficacy to treat or prevent cancer.

23.3.3 Milk protein allergenicity

When reviewing the nutritive attributes of milk proteins it is important also to consider their anti-nutritional properties. Some individuals, particularly infants, exhibit an allergic response to ingested bovine milk proteins (Bernard *et al*., 2000; Kaminogawa and Totsuka, 2003). In the case of bovine milk, this condition is referred to as cow's milk protein allergy (CMPA). Allergic responses vary from mild reactions such as rhinitis and diarrhoea to severe responses such as asthma, dermatitis and the potentially fatal anaphylactic shock. These responses may be displayed immediately after exposure to milk protein or alternatively in a time-delayed fashion (Sharma *et al*., 2001). Studies indicate that on average 2–3% of the infant population under one year of age

display an allergy to bovine milk protein (Høst and Halken, 1990). Indeed, such immune mediated hypersensitivity is not restricted to children as a small population of adults may also be vulnerable to this condition (Pelto *et al*., 1998). Wal (1998), reported that the main allergens of milk were whole casein, β-Lg and α-La. However, even proteins that are present in very low quantities in milk such as BSA, Igs and Lf were all capable of eliciting an allergic reaction or hypersensitivity in susceptible humans following oral ingestion. Hypersensitivity reactions are divided into four types. Hypersensitivity Types I-III are mediated by anti-bodies and Type IV by T-cells and macrophages (Pelto *et al*., 1999). Allergic responses are triggered by epitopes or antigenic determinants along the sequence of the milk protein.

In the case of β-Lg, three main peptides have been identified as major epitopes recognised by a large majority of human IgE antibodies (Sharma *et al*., 2001). However, β-Lg like many other milk proteins possesses numerous other epitopes along its amino acid sequence that are capable of eliciting allergenic responses (Järvinen *et al*., 2002). Enzymatic hydrolysis, heat induced denaturation and high hydrostatic pressure modification have been investigated as a means of abolishing the allergenicity of milk proteins (Castro *et al*., 1996; Olsen *et al*., 2003; Sanchez and Frémont, 2003). Such studies have revealed that the allergenicity can be reduced but not abolished, as was the case for heat-denatured β-Lg which exposed a new epitope not previously seen in non-heat denatured β-Lg (Maynard *et al*., 1997). Exl and Fritsché (2001) recently reviewed the current use of moderately and extensively hydrolysed whey protein in infant formulae as a means to prevent allergic reaction to bovine milk protein.

23.4 Milk protein-derived bioactive peptides

Many milk proteins, e.g., immunoglobulins, specific enzymes, along with mineral, fatty acid and vitamin binding proteins, growth factors and anti-bacterial agents have biofunctional roles essential for growth and development. Additionally, milk proteins are a source of other biologically active components, generally referred to as bioactive peptides, which possess the ability to modulate specific systems within the body and may benefit human health. These peptides may act as regulatory compounds with hormone-like activity *in vivo*. The bioactivities associated with these peptides include: opioid, hypotensive, immunomodulatory, anti-microbial, and mineral binding activities. Many of these peptides are multifunctional in nature and may possess two or more bioactive properties. Several milk protein-derived bioactive peptide products are now available commercially as ingredients for functional foods or for use as dietary supplements/nutraceuticals.

Opioid peptides bind to opioid receptors and in doing so may exert an agonist or antagonistic activity. Hypotensive peptides inhibit angiotensin-I converting enzyme (ACE) and reduce blood pressure. Immunomodulatory peptides enhance or suppress the proliferation of lymphocytes and modulate the activity of

macrophages, natural killer cells and granulocytes. Antimicrobial peptides kill sensitive microorganisms. Mineral-binding peptides, in particular the caseinophosphopeptides, complex with bivalent cations such as Ca^{2+} and increase their solubility under physiological conditions. The bioactivities of these peptides are encrypted in the primary structure of milk proteins and generally remain latent until they are activated by enzymatic hydrolysis, e.g., during gastrointestinal digestion and/or food processing (Meisel, 1997a; Meisel and Bockelmann, 1999; FitzGerald and Meisel, 2000; Meisel and FitzGerald, 2000; FitzGerald and Meisel, 2003a). Once formed however, these peptides must still reach their target sites at the luminal side of the intestinal tract or within specific organs.

23.4.1 Immunomodulatory proteins and peptides from milk

The immune system plays a central role in host protection against bacterial, viral, parasitic and fungal infections, and also against cancer (Gill and Rutherfurd, 1998). Deficiencies in any aspect of the immune system may predispose an individual to a greater risk of infection and may increase the severity of the disease. Immunomodulation relates to the enhancement or suppression of the immune system (Meisel, 1997a; Gill and Cross, 2000; Gill *et al*., 2000). Many indigenous compounds are present in the body that play pivotal roles in modulation and regulation of this system. The immunomodulatory properties of milk proteins and peptides are generally determined using *in vitro* or *in vivo* test models.

In vivo studies offer the greatest evidence that the components of interest may modulate the immune system when included in the diet. After consumption of milk proteins/peptides enzymatic hydrolysis may occur during gastrointestinal digestion or as a result of microbial metabolism during transit through the small intestine. These processes may result in the formation of short chain peptides having enhanced or reduced bioactivity (Schlimme and Meisel, 1995). Bovine milk also contains immunomodulatory peptides that require little or no gastric hydrolysis to become biologically active (Guimont *et al*., 1997).

In vitro model studies usually entail the inclusion of milk proteins or their derivatives with target cells such as leucocytes or with immortalised cell lines in culture. Once the milk proteins/peptides are incorporated into cell culture models their effect on different parameters such as cellular proliferation, for example, may be determined. While *in vitro* studies allow a wider range of experimental variables to be measured at a lower cost, the results from such studies only suggest the potential of test components to modulate immune function. A summary of the potential immunomodulatory properties of different milk proteins is presented in Table 23.4.

In vitro *immunomodulation studies with casein*

Immunomodulatory effects have been observed with very low concentrations of milk protein. Carr *et al*., (1990), showed that α_{S1}-casein at a concentration of 10^{-6}

Table 23.4 Immunomodulatory properties of milk proteins and milk protein derived peptides. Adapted from Gill *et al*., 2000 with permission

Protein	Peptide	Preparation	Response	Reference
α_{s1}-Casein	——	——	↑ Proliferation of murine T-lymphocytes *in vitro*	Carr *et al*., 1990
α_{s1}-Casein	——	Pancreatin, Trypsin	↓ Proliferation of murine T-lymphocytes *in vitro*	Otani and Hata, 1995
α_{s1}-Casein	——	Pepsin, Chymotrypsin	No effect of digests on proliferation of murine T-lymphocytes *in vitro*	Otani and Hata, 1995
α_{s1}-Casein	f(194-199)	Trypsin	↑ Anti-body formation *in vitro*	Jollès *et al*., 1992
α_{s1}-Casein	f(194-199)	Trypsin	↓ Infection of mice with Klebsiella pneumoniae *in vivo*	Parker *et al*., 1984
α_{s1}-Casein	f(1-23)	Chymosin	↓ Infection of mice with Staphylococcus aureus *in vivo*	Lahov and Regelson, 1996
α_s-Casein	——	——	↓ phagocytic activity of macrophages *in vitro*	Otani and Futakami, 1994, 1996
α_s-Casein	——	Pepsin	No effect on phagocytic activity of macrophages *in vitro*	Otani and Futakami, 1994, 1996
β-Casein	——	——	↑ Proliferation of ovine B- and T-lymphocytes *in vitro*	Wong *et al*., 1996
β-Casein	——	Pancreatin, Trypsin	↓ Proliferation of murine lymphocytes *in vitro*	Otani and Hata, 1995
β-Casein	——	Pancreatin, Trypsin	↓ Proliferation of rabbit Peyer's Patch cells *in vitro*	Otani and Hata, 1995
β-Casein	——	Pepsin, Chymotrypsin	No effect on murine lymphocytes *in vitro*	Otani and Hata, 1995
β-Casein	——	Pepsin, Trypsin	↓ Proliferation of human lymphocytes *in vitro*	Kayser and Meisel, 1996
β-Casein	f(60-70)	Trypsin	↓ Proliferation of human lamina propria derived lymphocytes *in vitro*	Elitsur and Luk, 1991
β-Casein	f(60-70)	Trypsin	↑ Resistance of mice to Klebsiella pneumoniae infection *in vivo*	Parker *et al*., 1984
β-Casein	——	——	↑ Phagocytic activity of macrophages *in vitro*	Otani and Futakami, 1994, 1996
β-Casein	——	——	↑ Secretion of IL-1 from ovine macrophages *in vitro*	Wong *et al*., 1997a, 1997b Otani *et al*., 1992; Otani and Hata, 1995
κ-Casein	——	——	↓ Proliferation of murine B- and T-lymphocyte *in vitro*	Otani *et al*., 1995
κ-Casein	GMP f (106-109)	Chymosin	↓ Proliferation of murine T-lymphocytes *in vitro*	Otani and Monnai, 1993

——	GMP f (106-109)	Chymotrypsin, Neuraminidase	No effect on murine lymphocytes *in vitro*	Otani and Monnai, 1993
κ-Casein	——	Trypsin, Pronase	↓↓ Proliferation of murine lymphocytes *in vitro*	Jollès-Migliore and Samour, 1986
κ-Casein	f(1-5)	Trypsin	↑ Production of murine antibody formation *in vitro*	Jollès *et al*., 1988
κ-Casein	f(1-105)	Trypsin	↑ Phagocytic activity of human macrophages	Meisel, 1997b
κ-Casein	f(38-39)	Synthetic	↑ Proliferation of human peripheral blood lymphocytes *in vitro*	Otani and Futakami, 1994, 1996
κ-Casein	——	——	↓ phagocytic activity of macrophages *in vitro*	Otani and Futakami, 1994, 1996
κ-Casein	——	Pepsin	No effect on phagocytic activity of macrophages *in vitro*	Torre and Oliver, 1989
Whole whey	——	——	↓ Proliferation of T-lymphocytes *in vitro*	Rejman and Oliver, 1992
Lactoferrin	——	——	↓ Proliferation of bovine T-lymphocytes *in vitro*	Otani and Odashima, 1997
Lactoferrin	——	——	↓ Proliferation of murine T-lymphocytes *in vitro*	Wong *et al*., 1997a
Lactoferrin	——	——	↓ Proliferation of ovine T-lymphocytes *in vitro*	Rejman *et al*., 1992
Lactoferrin (low dose)	——	——	↑ Proliferation of bovine IL-2 dependent T-lymphocytes *in vitro*	Rejman *et al*., 1992
Lactoferrin (high dose)	——	——	↓ Proliferation of bovine IL-2 dependent T-lymphocytes *in vitro*	Otani and Futakami, 1994, 1996
Lactoferrin	——	——	↓ Phagocytic activity of macrophages *in vitro*	Otani and Futakami, 1994, 1996
Lactoferrin	——	Pepsin	No effect on phagocytic activity of macrophages *in vitro*	Shimizu *et al*., 1996
Lactoferrin	——	——	↑ Natural killer cell function of mice/protection against Cytomegalovirus *in vivo*	Otani and Futakami, 1994, 1996
α-lactalbumin	——	——	↑ Phagocytic activity of macrophages *in vitro*	Wong *et al*., 1997a, 1997b
α-lactalbumin	——	——	↑ Secretion of IL-1 from ovine macrophages *in vitro*	Wong *et al*., 1997a
Lactoperoxidase	——	——	↓ Proliferation of ovine T-lymphocytes *in vitro*	Wong *et al*., 1997a

↑ = Enhanced; ↓ = Decreased.

M could enhance the mitogen-stimulated proliferation of murine splenic T-lymphocytes in cell culture. However, later results indicated that specific digestive enzymes might play a critical role in the ultimate immunomodulatory properties of milk proteins. Such was the case when pancreatin and trypsin hydrolysates of α_{S1}-casein were shown to significantly inhibit proliferation of murine splenic lymphocytes (Otani and Hata, 1995). In contrast pepsin and chymotrypsin hydrolysates of α_{S1}-casein had no effect on mitogen-stimulated lymphocytes (Otani and Hata, 1995). Interestingly, the tryptic peptide, f(194-199), from α_{s1}-casein promoted antibody formation in cell cultures (Jollès *et al*., 1992).

Wong *et al*. (1996) demonstrated that β-casein enhanced proliferation of ovine B- and T-lymphocytes in cell cultures in a dose dependent manner. However, pancreatin and trypsin hydrolysates of β-casein were found to significantly inhibit the mitogen-stimulated proliferative responses of murine splenic lymphocytes and rabbit Peyer's patch cells when included in cell culture. No immunomodulatory effect was observed with pepsin and chymotrypsin digests of β-casein (Otani and Hata, 1995). Peptic and tryptic peptides of β-casein were reported to significantly suppress the mitogen-induced proliferation of human lymphocytes (Kayser and Meisel, 1996). Fragment f(60-70) of β-casein (a β-casomorphin opioid peptide) was found to significantly suppress human lymphocyte proliferation at micromolar concentrations (Elitsur and Luk, 1991).

Bovine κ-casein was reported to suppress both murine and rabbit lymphocyte proliferation induced by a range of B- and T-cell mitogens (Otani *et al*., 1992; Otani and Hata, 1995). The immunosuppressive factor responsible was identified as the carbohydrate rich glycomacropeptide (GMP) component of κ-casein. Further analyses revealed that N-acetylneuraminic acid was the specific immunosuppressive component of GMP and that its activity was selectively directed against T-cells (Otani *et al*., 1995). *In vitro* hydrolysis of GMP with chymotrypsin or neuraminidase removed this suppressive potential, however, trypsin, pancreatin and pronase treatment either had no effect or enhanced the degree of suppression exerted by GMP on proliferating cells (Otani and Monnai, 1993).

The tryptic fragment, Phe-Phe-Ser-Asp-Lys corresponding to f(1-5) of κ-casein was reported to promote antibody formation and to increase phagocyte activity of murine and human macrophages (Jollès and Migliore-Samour, 1986; Jollès *et al*., 1988). Meisel, (1997a), demonstrated that Tyr-Gly corresponding to κ-casein f(38-39) could significantly enhance cellular proliferation of human peripheral blood lymphocytes. It was postulated that this dipeptide could be rapidly transported across the small intestine in sufficient quantities to express its immunomodulatory activity. Hata *et al*. (1998, 1999), reported that the caseinophosphopeptides f(1-25).4P and f(1-28).4P of β-casein, f(59-79).5P of α_{s1}-casein and f(1-32).4P of α_{s2}-casein enhanced the production of IgG in mouse spleen cell cultures. Hartmann *et al*. (2000) found that exposure of human peripheral blood lymphocytes to caseinophosphopeptides resulted in a significant increase in IgG production and that the response exceeded that mediated by mitogens.

In vitro *immunomodulation studies with whey proteins*

Whey proteins have also been shown to modulate lymphocyte function *in vitro*. Torre and Oliver (1989), noted that 1.1 ng ml^{-1} of whole whey protein suppressed T-lymphocyte mitogenesis in cell culture. WPC has also been shown to suppress production of the cytokine, γ-interferon, in cell culture (Cross and Gill, 1999). Individual whey proteins have been shown to affect lymphocyte function in different species (Table 23.4). Several studies have shown that the modulatory responses of cultured cells become more pronounced with highly purified milk proteins. In particular, Lf suppressed proliferation of mitogen stimulated T-lymphocytes in cell cultures from cows (Rejman and Oliver 1992; Rejman *et al*., 1992), mice (Otani and Odashima, 1997) and sheep (Wong *et al*., 1997a).

Depending on dosage, Lf was reported to exhibit two different modulatory effects on interleukin-2 (IL-2) dependent proliferating bovine T-cells. At low doses, Lf enhanced proliferation and at higher doses it suppressed proliferation of these cultured cells (Rejman and Oliver, 1992). Furthermore, Lf has been shown to elicit species restricted immunomodulatory responses on cultured cells. For example, Otani and Odashima, (1997), reported that Lf suppressed mitogen induced proliferating murine B- and T-lymphocytes and Wong *et al*. (1997a), reported that lactoferrin could suppress ovine T-cell (but not B-cell) mitogenesis. Both Lf and lactoperoxidase were able to suppress mitogen-induced secretion of the cytokine γ-interferon in cell cultures of ovine lymphocytes (Wong *et al*., 1997a).

The immunomodulatory properties of bovine IgG have also been reported. At a dose of 0.3 mg ml^{-1}, bovine IgG suppressed human lymphocyte proliferative responses to B- and T-cell mitogens when included in cell culture (Kulczycki *et al*., 1987). It was postulated that bovine IgG, which is present in milk at a concentration of 0.6–0.9 mg ml^{-1}, might have the potential to modulate human immune function *in vivo* (Gill and Cross, 2000). Bovine milk derived growth factor was also revealed to be a potent suppressor of proliferating human T-lymphocyte cells (Stoeck *et al*., 1989). Results suggest that a specific combination of milk proteins would have an effect on the ultimate immunomodulatory response observed on cell culture. For example, Lf and lactoperoxidase, are potent suppressors of ovine lymphocyte proliferation when incubated with these cells in isolation of each other. However, when both proteins were incubated simultaneously proliferation of ovine lymphocytes was significantly diminished (Wong *et al*., 1997a). Therefore, an observed immunomodulatory response of cultured cells to whole milk protein may be the sum of different suppressing and enhancing activities.

In vivo *immunomodulation studies with milk proteins*

Studies showed that mice fed a whey protein rich diet had significantly elevated spleen-derived B- and T-lymphocyte proliferative responses to mitogens compared to animals fed diets containing soy or wheat (Wong and Watson, 1995). This highlights the important role milk proteins play in modulation of the

immune system. In many cases, milk proteins, which have been reported as immunosuppressive *in vitro*, display immunoenhancing properties *in vivo* (Table 23.4). Monnai *et al*. (1998), demonstrated that mice fed a diet containing GMP had enhanced T-lymphocyte responses to mitogens. These results conflicted with *in vitro* studies where GMP was shown to suppress lymphocyte proliferation (Otani and Monnai, 1993). Additionally, when β-casomorphin, corresponding to f(60-70) of β-casein, was administered to mice it enhanced their resistance to *Klebsiella pneumoniae* infection (Parker *et al*., 1984). Previous studies by Elitsur and Luk (1991), indicated that the opposite effect may occur *in vitro*. Such discrepancies may result from modification of the proteins by enzymatic hydrolysis during gastrointestinal transit, degradation by enzymes from intestinal microflora or a combination of both.

Gill and Rutherfurd (1998), confirmed that mice fed a diet supplemented with whey protein concentrate (WPC) possessed enhanced gut mucosal antibody responses to orally delivered antigens. This response was not restricted to complex mixtures of milk proteins as co-injection of ovalbumin with β-casein into mice suppressed specific anti-body production (Wong *et al*., 1996). Otani *et al*. (2000), reported that mice fed a diet containing a caseinophosphopeptide preparation had greater levels of serum and intestinal IgA than mice fed a diet with no caseinophosphopeptides. The tryptic peptide f(194-199) of α_{s1}-casein was shown to reduce infection of mice with *Klebsiella pneumoniae* (Parker *et al*., 1984). The chymosin generated peptide 'isracidin' corresponding to f(1-23) of α_{s1}-casein, was shown to protect mice from a lethal infection from a specific *Staphylococcus aureas* strain (Lahov and Regelson, 1996). Furthermore, it was determined that the potency of isracidin was equal to if not greater than some antibiotics.

Little information has been published on the direct influence of bovine milk, or its components, on the human immune system. However, a study with geriatric patients indicated a significant increase in the percentage of phagocytosing peripheral monocytes following daily oral ingestion of UHT milk over a four-week period (Broche and Platt, 1995). The responses observed, however, may not be solely attributed to the proteins in the UHT milk. Oral administration of WPC was shown to increase the number of blood leukocytes in cancer patients (Bounous *et al*., 1993; McIntosh *et al*., 1998).

Immunomodulation of non-lymphoid cells with milk proteins and peptides

Milk proteins also modulate non-lymphoid cells such as macrophages, granulocytes and natural killer cell activities (Table 23.4). Macrophages of which many varieties exist in different tissues are a form of leukocyte and are active participants of the non-specific immune system. Functionally, they are capable of phagocytosis or scavenging of foreign bodies such as microbes, production of immunomodulatory cytokines and the generation of antimicrobial reactive oxygen and nitrogen intermediates. Aberrant macrophage activity has been linked with degenerative diseases such as multiple sclerosis (Minagar *et al*., 2002). *In vitro* cell culture analyses revealed that milk proteins display a

variety of effects on the phagocytic activity of murine macrophages. In particular, α-casein, κ-casein and Lf reduced macrophage activity in a dose-dependent manner, and β-casein and α-La enhanced the phagocytic function of macrophages (Otani and Futakami, 1994, 1996). Following hydrolysis with pepsin, however, the immunosuppressive properties of α- and κ-casein were removed.

These findings illustrate that the modulatory function of milk proteins on the immune system may be altered by exposure to enzymes of the gastrointestinal tract. This may partially explain the differences observed between *in vivo* and *in vitro* model studies. Milk proteins may also modulate the production of cytokines by macrophages. Both β-casein and α-La were shown to enhance secretion of interleukin-1 in ovine macrophages when included in ovine cell culture systems (Wong *et al*., 1997a and 1997b).

Granulocytic cells such as neutrophils are migratory, phagocytic and microbiocidal in nature and are active in localised tissue inflammatory responses. Lf-lactoperoxidase and WPC have been shown to enhance neutrophil oxidative responses in cell cultures (Wong *et al*., 1997a, 1997b). The most conclusive proof of a milk protein-derived component having an impact on immune function was the enhanced T-cell mediated natural killer (NK) cell response of mice following administration of Lf. The enhanced NK cell function was correlated with increased protection against cytomegalovirus infection (Shimizu *et al*., 1996).

Extensive research has demonstrated that bovine milk proteins and their derivatives have the potential to modulate immune function in a number of species (Gill *et al*., 2000; Gill and Cross, 2000). While *in vitro* experiments using cell cultures from different species offer valuable information on the possible effects of milk proteins on the human immune system, they do not offer conclusive evidence as would be obtained from human studies. Furthermore, as already mentioned *in vitro* studies do not yield precise data on the effect of peptides on immunomodulation after GIT transit. Conclusive evidence from clinical trials on the efficacy of milk proteins and their derivatives in modulating the immune system of human subjects is rare. Positive results from such clinical trials are essential before definitive health enhancing claims may be made with regard to milk proteins and their derivatives.

23.5 Mineral-binding properties of milk peptides

Milk contains several proteins capable of binding minerals, including the caseins, α-La, β-Lg, BSA, Lf and Igs (Vegarud *et al*., 2000). Of these proteins, caseins are the most prominent mineral binding protein in milk. Whey proteins bind minerals by a different mechanism than phosphorylated caseins (Baumy and Brulè, 1988; Cayot and Lorient, 1997). The most important mineral binding whey protein is Lf, which has been shown to play a major role in iron uptake in the intestine (Hutchens *et al*., 1994; Viljoen, 1995).

Casein-derived peptides generated during *in vitro* hydrolysis with commercial enzyme preparations or *in vivo* by hydrolysis during gastrointestinal digestion bind minerals via specific and non-specific binding sites. Such peptides may either enhance or diminish the bioavailability of minerals by acting as carriers or chelators. Mineral binding peptides may be used as ingredients to fortify products with minerals for subsequent use in the food and pharmaceutical sectors.

A number of reports detail the ability of whey protein-derived peptides to bind minerals. Pepsin, trypsin and chymotrypsin derived peptides of α-La have been reported to bind copper, calcium, iron and zinc (Hirai *et al*., 1992; Svenning and Vegarud, 1998). Hydrolysis of α-La and β-Lg with pepsin, trypsin and chymotrypsin yielded peptides having a greater affinity for iron than the intact proteins (Svenning and Vegarud, 1998). Large molecular weight peptides from Lf have also been reported to bind iron (Kawakami *et al*., 1993).

The most studied mineral binding peptides are those derived from casein, in particular caseinophosphopeptides (CPPs). CPPs are phosphorylated casein-derived peptides that are capable of binding and solubilising macroelements such as calcium, magnesium and iron in addition to trace elements such as barium, cobalt, chromium, nickel, selenium and zinc. Mellander first introduced the term caseinophosphopeptide in 1950 to describe a group of phosphorylated casein peptides that enhanced bone calcification independently of vitamin D in rachitic children (Mellander, 1950). Milk and dairy products are excellent sources of Ca^{2+} and it is believed that CPPs produced during gastrointestinal digestion of casein enhance the bioavailablity of Ca^{2+} in the diet.

This proposed increase in bioavailability of calcium by CPPs has been attributed to their ability to increase the solubility of Ca^{2+} in the alkaline environment of the small intestine where maximal passive absorption of dietary calcium is thought to occur. CPPs may be naturally produced as part of casein digestion *in vivo* (Naito *et al*., 1972; Meisel and Frister, 1988; Meisel *et al*., 2003) or by *in vitro* hydrolysis of casein by a range of proteinase activities from different sources (Adamson and Reynolds, 1995; McDonagh and FitzGerald, 1998). CPPs have also been identified in fermented dairy products such as cheese (Roudot-Algaron *et al*., 1994). This section will detail the structure, production and activity of CPPs, and their potential commercial application in the food and pharmaceutical sectors.

23.5.1 Structure of CPPs

The structural characteristics of CPPs have been previously reviewed (West, 1986; FitzGerald, 1998; FitzGerald and Meisel, 2003b). Phosphorus is bound to casein molecules via monoester linkages to serine residues. Caseins are phosphorylated to different extents, i.e., bovine α_{s1}-casein may have up to 13 phosphate groups in contrast to κ-casein that has only one phosphate (West, 1986; Reynolds, 1994). A common but not exclusive motif exists termed the triplet anionic region where much of the phosphate is bound to the caseins, i.e., a

Table 23.5 Sequence of different phosphorylated regions found in bovine caseins. Reprinted from FitzGerald, 1998. Copyright (1998), with permission from Elsevier

Casein amino acid sequence	Location
α_{s1}-casein	
Gln-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Glu-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys	f(59-79)
Val-Pro-Asn-Ser(P)-Ala-Glu-Glu-Arg	f(112-119)
α_{s2}-casein	
Glu-His-Val-Ser-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Ile-Ser(P)-Gln-Glu-	f(5-18)
Asp-Pro-Ser(P)-Lys-Glu-Asn	f(29-34)
Gly-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser(P)-Ala-Glu-Val	f(55-64)
Gln-Leu-Ser(P)-Thr-Ser(P)-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-Met-Glu-Ser(P)-Thr-Glu-Val-Phe	f(127-147)
β-casein	
Ile-Val-Glu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Lys	f(12-23)^{A}
Ile-Val-Glu-Ser(P)-Lys-Ser(P)-Glu-Glu-Ser-Ile-Lys	f(12-23)^{D}
κ-casein	
Glu-Ala-Ser(P)-Pro-Glu-Val-Ile	f(147-153)

Ser(P) represents serine phosphate; ^{A}variant A of β-casein ; ^{D}variant D of β-casein.

sequence of three phosphoseryl residues followed by two glutamic acid residues (Table 23.5). Phosphorylation of these regions in the caseins, which occurs in the mammary gland, is catalysed by casein kinase (Mercier, 1981). Each of the casein molecules possesses at least one site for phosphorylation; f(66-70) of α_{s1}-casein, f(8-12) and f(56-60) of α_{s2}-casein and f(17-21) of β-casein represent highly polar acidic domains ideally suited to the binding of cationic bivalent minerals. It should be noted that not all the seryl (or the potentially phosphorylatable threonine) residues are phosphorylated in the caseins. Casein polymorphism also influences the extent of phosphorylation. Trypsin-derived CPPs from the N-terminus of the D and A variants of β-casein possess 2 and 3 phosphate groups, respectively. This difference is a result of the substitution of lysine for serine at position 16 in β-casein variant D (Table 23.5).

The ability of phosphopeptides to bind minerals is directly related to the presence of the highly polar acidic domains as Berrocal *et al*., (1989), revealed when CPPs were dephosphorylated resulting in the loss of mineral binding. The binding and solubilisation of Ca^{2+} in the presence of phosphate prevents hydroxyapatite crystal formation (Holt *et al*., 1996). Ca^{2+}-CPP complexes were reported to exist as tetramers (Reynolds, 1993). With regard to binding abilities, β-casein f(1-25).4P and α_{s1}-casein f(59-79).5P were reported to bind 24 and 17 moles of Ca^{2+} at pH 9.0, respectively. This binding of Ca^{2+} to CPPs occurs with a low binding affinity constant with values reported to be between 10^{2}–10^{3} M^{-1} (Sato *et al*., 1991; Schlimme and Meisel, 1995). Such a low affinity would greatly facilitate the release of Ca^{2+} during intestinal transit. With regard to

other cationic minerals it has been reported that one CPP molecule may bind 6 Zn^{2+} molecules and complex into a structure containing a total of 6 CPP-Zn entities (Reynolds, 1993). Bouhallab *et al*. (1991) reported that the phosphopeptide fragment of β-casein f(1-25) bound 4 mol of iron per mol peptide.

23.5.2 Production of CPPs

Several methods have been published detailing the production of CPPs. The general protocol entails enzymatic hydrolysis of casein, isoelectric precipitation at pH 4.6 to isolate soluble peptides, followed by aggregation of CPPs using mineral salts of barium, calcium, cobalt, copper, iron, magnesium, manganese and zinc (Reeves and Latour, 1958; Manson and Annan, 1971; Brule *et al*., 1982, 1989; Berrocal *et al*., 1989; Juillerat *et al*., 1989; Reynolds, 1992; Adamson and Reynolds, 1995; McDonagh and FitzGerald, 1998). Calcium mineral salts are predominantly used for aggregate formation.

CPPs, once aggregated, are generally precipitated with a hydrophilic solvent such as acetone, butanol, ethanol, methanol or propanol (Reeves and Latour, 1958; Adamson and Reynolds, 1995; McDonagh and FitzGerald, 1998) and subsequently sedimented by centrifugation. At an industrial level solvent precipitation is generally not economically feasible and other methods such as ultrafiltration have been employed to separate Ca^{2+}-induced aggregates of CPPs from non-phosphorylated peptides (Reynolds, 1992). Diafiltration may then be applied to the retained phosphopeptides to remove residual aggregating salt (Reynolds, 1993), followed by a concentration step and spray-drying. Several chromatographic procedures have also been described for the enrichment of phosphopeptides from solvent precipitated aggregates (Berrocal *et al*., 1989; Juillerat *et al*., 1989; Kunst, 1990; Koide *et al*., 1991; Lihme *et al*., 1994). Phosphopeptides are liable to degrade under high heat and alkaline conditions and therefore their production has to be strictly controlled to maintain their structural integrity. Ideally, in the final isolated CPP preparation, all the serine residues should be phosphorylated but in practice this is difficult to achieve (Meisel *et al*., 1991). Yields for CPPs depend on the production protocol and vary from 6% (Gerber and Jost, 1986), 12% (Juillerat *et al*., 1989), 13–16% (Kunst, 1990) and between 3–16% (McDonagh and FitzGerald, 1998).

23.5.3 CPPs and calcium bioavailability

Dairy products including milk are rich sources of minerals and it is reported that certain dairy products provide 75% of the recommended daily allowance of Ca^{2+} (Buttriss, 1990). Humans may absorb Ca^{2+} by a number of means, i.e., a saturable vitamin D-dependent transport system located in the duodenum and jejunum and a passive, vitamin D-independent system located in the ileum and distal small intestine (Bronner, 1987). It is believed that the latter transport route is the major mode of Ca^{2+} absorption from the diet (Schaafsma, 1997).

Additionally, pinocytotic vesicles lining the squamous epithelium actively transport minerals from the diet of infants (Walker and Isselbacher, 1974). The high Ca^{2+} bioavailability of milk and casein derived-products has been attributed to phosphopeptides enhancing the solubility of Ca^{2+} in the small intestine at physiological alkaline pH (Schaafsma, 1997; Bernard *et al.*, 2000). Upon consumption of milk, CPPs were formed in mini-pigs following digestion by gastrointestinal proteinases (Meisel and Frister, 1988). CPPs have recently been identified in the distal ileum of humans (Meisel *et al*., 2003). The presence of phytate, oxalate, phenolic compounds, lactose, phosphorous and vitamin D may affect the absorption of Ca^{2+} from the diet.

Hansen *et al*. (1997a) reported a ~30% increase in calcium and zinc absorption by adult human volunteers from a rice-based infant gruel supplemented with CPPs. However, this effect was not observed in the presence of phytate-containing meals (Hansen *et al*., 1996, 1997b). Mixed results have been obtained from animal based studies with regard to the efficacy of CPPs in enhancing Ca^{2+} absorption from the diet. While some studies show that CPPs enhanced the bioavailability of Ca^{2+} in the intestine of chickens and rats (Naito *et al*., 1972; Mykännen and Wasserman, 1980; Kitts *et al*., 1992), other feeding trials show CPPs were unable to elicit a significant effect on Ca^{2+} absorption in piglets (Pointellart and Gueguen, 1989), rats (Brommage *et al*., 1991), and vitamin D-deficient rats (Scholz-Arhens *et al*., 1990).

Significantly, a rat-based study revealed that a diet containing high levels of CPPs decreased the true fractional absorption of Ca^{2+} (Bennett *et al*., 2000). It is possible that the low absorption of calcium observed in this latter study was due to the chelation of calcium by the presence of high levels of CPPs. More human studies, perhaps using different CPP-food formulations, are required to further unravel the potential roles of CPPs in enhancing mineral bioavailability.

23.5.4 Potential applications of CPPs

The potential ingredient applications of CPPs have been previously reviewed (FitzGerald, 1998) and are summarised in Table 23.6.

Mineral supplementation

CPPs are capable of solubilising a broad range of minerals essential for human health including calcium, magnesium and iron. Adequate supplies of Ca^{2+} are required by all sections of the population, e.g., pre-term infants, adolescents (Sowers *et al*., 2000), post-menopausal individuals and the elderly. Additionally, Ca^{2+} is required for bone recalcification following fracture, and in the prevention and treatment of osteoporosis and rickets. An inadequate supply of dietary Ca^{2+} has been linked with hypertension, colon cancer and kidney stones (International Dairy Federation, 1997). Inadequate Fe^{2+} may lead to anaemia. Poor dietary intake of Zn^{2+} can lead to acrodermatitis enteropathica (symptoms include diarrhoea, hair loss and skin rash), increased susceptibility to infection and to hypogonadism (Salgueiro *et al*., 2002). The human body requires

Table 23.6 Potential applications of caseinophosphopeptides (CPPs). Reprinted from FitzGerald, 1998. Copyright (1998), with permission from Elsevier

Application	Rationale	Product
Prevention of osteoporosis	Increase absorption of Ca by including Ca-CPP as functional food ingredient for target populations, allowing maximum deposition of bone in early life	Ca-CPP complexes
Recalcification of bones after fracture	Increased serosal [Ca], aiding bone mineralisation	Ca-CPP complexes
Calcic addition during treatment of rickets	Increased serosal [Ca]	Ca-CPP complexes
Hypertension	Increased serosal [Ca] may aid reduction of hypertension	Ca-CPP complexes
Prevention of dental caries	Inclusion of Ca-CPP as ingredients in mouthwash and toothpaste	Ca-CPP complexes
Mg deficiency in pregnancy and old age	Aid Mg supplementation	Mg-CPP complexes and ingredients
Anaemia	Aid Fe supplementation, increased serosal [Fe]	Fe-CPP complexes
Humanisation of bovine milk	Increased phosphorous levels	CPPs
Oligoelement supplement	Aid supplementation of Zn, Cu, Cr, Ni, Co, Mn and Se	Oligoelement-CPP complexes

increased levels of Mg^{2+} during stress, pregnancy and old age (Passmore *et al*., 1974; Wasserman and Taylor, 1976; Fairweather-Tait, 1988; Solomons, 1988). Potential food carriers of CPPs include breakfast foods, confectioneries, various desserts, dairy products and sports drinks (Reynolds, 1993; Han *et al*., 1996). CPPs are available from food ingredient suppliers in Europe and Japan.

Anticariogenic potential

Tooth decay or dental caries arises due to demineralisation of the tooth brought about by organic acids produced by dental plaque odontopathogenic bacteria during metabolism of dietary sugars. CPPs may prevent the formation of dental calculus (i.e., calcium phosphate precipitates) by preventing hydroxyapatite formation (Sikes, 1990). This anticariogenic activity of CPPs is associated with their ability to localise calcium phosphate at the tooth surface and as a

consequence their ability to help prevent demineralisation and aid in re-mineralisation of tooth enamel. Additionally, the anticariogenic effects of CPPs may also be related to their ability to deprive plaque-forming bacteria, such as *Streptococcus mutans,* of Ca^{2+} (Rose, 2000). Patent claims have been made for the incorporation of CPPs into dental hygiene products (Reynolds, 1992; Holt, 2001).

Humanisation of bovine milk

The organic phosphorous:total phosphorous ratio of bovine milk is 0.34 whereas human milk has a ratio of 0.83. It has been suggested that supplementation of bovine milk with CPPs may be a means of partially humanising bovine milk (Brule *et al*., 1989).

Calcium and hypertension

An inverse relationship between dietary Ca^{2+} intake and blood pressure has been reported (Sallinen *et al*., 1996; Resnick, 1999). Furthermore, intervention studies have shown that Ca^{2+} supplementation can lower blood pressure in both normotensive and hypertensive humans (Vaughan *et al*., 1997; Resnick, 1999). Although, Kitts *et al*. (1992) concluded that ingestion of CPPs was not a factor in the lowering of blood pressure in spontaneously hypertensive rats, it is possible that CPPs may have a potential antihypertensive role as part of a CPP-induced increase in Ca^{2+} bioavailibility in hypertensive humans (FitzGerald, 1998).

23.6 Hypotensive properties of milk proteins

Angiotensin-I-converting enzyme (ACE) is a critical enzyme in the regulation of peripheral blood pressure (Fig. 23.1). ACE, a key component of the renin-angiotensin system, is a dipeptide-liberating carboxypeptidase (peptidyldipeptide hydrolase, EC 3.4.15.1). ACE converts angiotensin I to angiotensin II, a highly potent vasoconstrictor molecule (Skeggs *et al*., 1956). Additionally, angiotensin II increases vascular tone and promotes volume expansion via sodium retention. ACE also interacts with many different endogenous competitive inhibitors and substrates such as Substance P, enkephalins, β-endorphin and the vasodilatory peptide, bradykinin (Wyvratt and Patchett, 1985).

Bradykinin promotes vasodilation by stimulating the production of nitric oxide, arachidonic acid metabolites and endothelium-derived hyperpolarising factor in the vascular epithelium (Brown and Vaughan, 1998). In specific tissues, bradykinin causes smooth muscle contraction, and increased vascular permeability especially in the kidney where it promotes natriuresis (Fig. 23.1). Inhibition of ACE exerts an anti-hypertensive effect as a consequence of a decrease in the level of angiotensin II and an increase in the level of the vasodilatory peptide, bradykinin (Erdös, 1975). First reports of exogenous inhibitors of ACE displaying an anti-hypertensive effect *in vivo* were from those

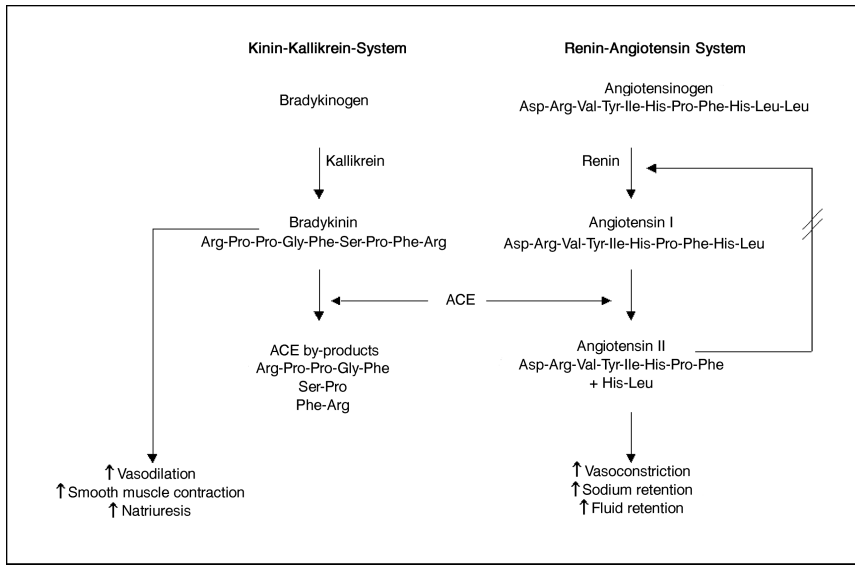

Fig. 23.1 Schematic representation of the role of angiotensin-I-converting enzyme (ACE) in the regulation of the levels of angiotensin II and bradykinin in man.

isolated from snake venom (Ondetti *et al*., 1977). Indeed, from these investigations several orally active pharmacological compounds were developed, one of which, Captopril® exhibits a potent hypotensive effect *in vivo* (Koike *et al*., 1980). Many food proteins such as gelatin (Oshima *et al*., 1979), fish (Ariyoshi, 1993) and milk (FitzGerald and Meisel, 2000) are a source of ACE-inhibitory peptides.

Milk-derived ACE inhibitory peptides, like many other bioactivities, are latent or encrypted within the amino acid sequence of the milk proteins (Meisel, 1998; Meisel and Bockelmann, 1999). Proteolysis of food proteins may be controlled to generate a mixture of ACE inhibitory peptides *in vitro* as occurs during processing with commercial enzyme preparations (FitzGerald and Meisel, 1999; Pihlanto-Leppälä, 2001) or by fermentation (Takano, 1998; Sipola *et al*., 2002). Casein- derived peptide inhibitors of ACE are known as casokinins (Meisel, 1993), whereas whey-derived inhibitors are known as lactokinins (FitzGerald and Meisel, 1999).

23.6.1 Structural properties of ACE inhibitory peptides

Casokinin sequences have been identified in α_{s1}-, α_{s2}-, β- and κ-casein, and lactokinin sequences in α-La, β-Lg and BSA from bovine milk (Tables 23.7 and 23.8). Characterisation of ACE inhibitory properties is usually performed on purified, sequenced peptides derived from *in vitro* enzymatically generated hydrolysates of milk proteins or on chemically synthesised peptides corresponding to sequences known to exist in the milk proteins.

The exact substrate specificity of ACE is not fully elucidated, however, notable trends have been observed. There appears to be a strong correlation between the binding of peptides to ACE and the sequence of tripeptides located at the C-terminus of the peptide. Most potent inhibitors or substrates of ACE contain aromatic or aliphatic amino acids at the antepenultimate, penultimate and ultimate C-terminal positions. ACE inhibition studies on a range of synthetic dipeptides revealed that greatest binding to the enzyme was observed when phenylalanine, proline, tryptophan or tyrosine occupied the C-terminal position (Cheung *et al*., 1980). In the majority of cases though, the casokinins have proline, lysine or arginine at their C-terminal position (Table 23.7). The charged group of the latter two amino acids appears to be essential as studies show that removal of the C-terminal arginine of bradykinin resulted in formation of an inactive molecule (Meisel, 1993) therefore structure-activity data indicates that the positive charge on the guanidino or ε-imino group of C-terminal arginine and lysine side chains, respectively, may contribute to inhibitory potency of peptides on ACE activity.

FitzGerald and Meisel, (2000), postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site that is distinct from the catalytic site. Further investigations on the influence of peptide conformation on the mechanism of action of ACE may explain why specific individual milk protein-derived peptides elicit an ACE inhibitory effect.

23.6.2 Hypotensive effects of milk protein-derived peptides

ACE is present in plasma, lung, kidney, heart, skeletal muscle, pancreas, spleen, placenta, arteries, uterus, testes, bone (Haznedaroglu and Öztürk, 2003) and the brain (McKinley *et al*., 2003) of mammals. A membrane bound form of ACE is also found on the brush border of epithelial cells of the human jejunum (Ondetti and Cushman, 1982; Steve *et al*., 1988). ACE inhibitory peptides generated from α_{s1}-, α_{s2}-, β- and κ-casein by the action of trypsin (Maruyama *et al*., 1987b) and by *Lactobacillus helveticus* proteinase (Yamamoto *et al*., 1994, 1999) have been shown to elicit an antihypertensive effect in spontaneously hypotensive rats (SHR) following intravenous and oral administration. In addition, a tryptic digest of whole casein gave an antihypertensive effect in SHR following oral ingestion.

A human study involving normotensive and mildly hypertensive volunteers reported that daily ingestion of 20 g of a tryptic digest of casein over a four-week period produced an antihypertensive effect (Sekiya *et al*., 1992). A placebo-controlled study with hypertensive human volunteers reported a significant reduction in blood pressure following daily ingestion of 95 mL of Calpis sour milk (Hata *et al*., 1996). This sour milk was generated following fermentation of milk with *Lactobacillus helveticus* and *Saccharomyces cerevisiae*. The active peptide components were reported to be Ile-Pro-Pro (β-casein f(74-76) and κ-casein f(108-110)) and Val-Pro-Pro (β-casein f(84-86)) (Nakamura *et al*., 1995a,b). The estimated daily-ingested dose of these ACE

Table 23.7 Bovine casein-derived inhibitors of angiotensin-I converting enzyme (ACE). Adapted from FitzGerald and Meisel, 2000

Protein source	Peptide fragment	Sequence	IC_{50} (μM)	Preparation	Reference
α_{S1}-casein	*f*(25-27)	VAP	2.00	Synthetic	Maruyama *et al*., 1987a
	f(23-27)	FFVAP	6.00	Enzymatic	Maruyama *et al*., 1985
	f(24-27)	FVAP	10.0	Synthetic	Maruyama *et al*., 1987a
	f(194-199)	TTMPLW	16.00	Enzymatic	Maruyama *et al*., 1987b
	f(104-109)	YKVPQL	22.00	Synthetic	Maeno *et al*., 1996
	f(197-199)	PLW	36.00	Synthetic	Maruyama *et al*., 1987b
	f(198-199)	LW	50.00	Synthetic	Maruyama *et al*., 1987b
	f(142-147)	LAYFYP	65.00	Enymatic	Pihlanto-Lappälä *et al*., 1998
	f(23-34)	FFVAPFPEVFGK	77.00	Enzymatic	Maruyama and Suzuki, 1982
	f(157-164)	DAYPSGAW	98.00	Enzymatic	Pihlanto-Lappälä *et al*., 1998
	f(143-148)	AYFYPE	106.00	Enzymatic	Yamamoto *et al*., 1994
	f(28-34)	FPEVFGK	140.00	Synthetic	Maruyama *et al*., 1987a
	f(32-34)	FGK	160.00	Synthetic	Maruyama *et al*., 1987a
	f(27-30)	PFPE	>1000.00	Synthetic	Maruyama *et al*., 1987a
	f(143-147)	AYFYP	>1000.00	Synthetic	Maeno *et al*., 1996
	f(136-139)	LFRQ	17.00*	Enzymatic	Schlothauer *et al*., 1999
α_{S2}-casein	*f*(174-179)	FALPQY	4.30	Enzymatic	Tauzin *et al*., 2002
	f(174-181)	FALPQYLK	4.30	Enzymatic	Tauzin *et al*., 2002
	f(92-98)	FPQYLQY	14.00	Enzymatic	Tauzin *et al*., 2002
	f(182-184)	TVY	15.00	Enzymatic	Tauzin *et al*., 2002
	f(25-32)	NMAINPSK	60.0	Enzymatic	Tauzin *et al*., 2002
	f(81-89)	ALNEINQFY	219.00	Enzymatic	Tauzin *et al*., 2002
	f(81-91)	ALNEINQFYQK	264.00	Enzymatic	Tauzin *et al*., 2002
	f(190-197)	MKPWIQPK	300.00	Synthetic	Maeno *et al*., 1996
	f(198-202)	TKVIP	400.00	Synthetic	Maeno *et al*., 1996
	f(189-192)	AMKPW	580.00	Synthetic	Maeno *et al*., 1996
	f(189-197)	AMKPWIQPK	600.00	Synthetic	Maeno *et al*., 1996

β-casein	f(74-76)	IPP	5.00	Enzymatic	Nakamura *et al*., 1995a
	f(169-174)	KVLPVP	5.00	Synthetic	Maeno *et al*., 1996
	f(84-86)	VPP[¢]	9.00	Enzymatic	Nakamura *et al*., 1995a
	f(177-183)	AVPYPQR	15.00	Enzymatic	Maruyama *et al*., 1987a
	f(49-61)	IHPFAQTQSLVYP	19.00	Synthetic	Kohmura *et al*., 1990
	f(52-61)	FAQTQSLVYP	25.00	Synthetic	Kohmura *et al*., 1990
	f(50-61)	HPFAQTQSLVYP	26.00	Synthetic	Kohmura *et al*., 1990
	f(48-61)	KIHPFAQTQSLVYP	39.00	Synthetic	Kohmura *et al*., 1990
	f(57-61)	SLVYP	40.00	Synthetic	Kohmura *et al*., 1990
	f(56-61)	QSLVYP	41.00	Synthetic	Kohmura *et al*., 1990
	f(59-61)	VYP	44.00	Synthetic	Kohmura *et al*., 1990
	f(55-61)	TQSLVYP	64.00	Synthetic	Kohmura *et al*., 1990
	f(54-61)	QTQSLVYP	73.00	Synthetic	Kohmura *et al*., 1990
	f(53-61)	AQTQSLVYP	76.00	Synthetic	Kohmura *et al*., 1990
	f(177-181)	AVPYP	80.00	Synthetic	Maruyama *et al*., 1987a
	f(58-61)	LVYP	170.00	Synthetic	Kohmura *et al*., 1990
	f(179-181)	PYP[§]	220.00	Synthetic	Maruyama *et al*., 1987a
	f(59-64)	VYPFPG	221.00	Synthetic	Abubakar *et al*., 1998
	f(177-183)	AVPTPNR	274.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998
	f(193-198)	YQEPVL	280.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998
	f(59-61)	VYP	288.00	Synthetic	Abubakar *et al*., 1998
	f(193-202)	YQEPVLQPVR	300.00	Synthetic	Meisel and Schlimme, 1994
	f(177-179)	AVP	340.00	Synthetic	Maruyama *et al*., 1987a
	f(108-113)	EMPFPK	423.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998
	f(140-143)	LQSW	500.00	Synthetic	Maeno *et al*., 1996
	f(60-66)	YPFPGPIP	500.00	Synthetic	Meisel and Schlimme, 1994
	f(80-90)	TPVVVPPFLQP	749.00	Synthetic	Abubakar *et al*., 1998
	f(191-197)	LLYQQPV	>1000	Synthetic	Maeno *et al*., 1996
	f(133-138)	LHLPLP	7.00*	Enzymatic	Schlothauer *et al*., 1999
	f(58-76)	LVYPFPGPIPNSLPQNIPP	19.00*	Enzymatic	Schlothauer *et al*., 1999
κ-casein	f(58-59)	YP[#]	720.00	Synthetic	Yamamoto *et al*., 1999
	f(185-190)[¶]	VTSTAV	30.00*	Enzymatic	Schlothauer *et al*., 1999

[¢]This sequence also occurs in κ-casein f(108-110). [§]This sequence also occurs in κ-casein f(57-59). [#]This sequence also occurs in α_{S1}-casein f(146-147) and f(159-160) and in β-casein f(114-115). IC_{50} = Peptide concentration which reduces the activity of ACE by 50%. [¶]This sequence corresponds to glycomacropeptide f(59-64) of bovine κ-casein. *IC_{50} values quoted are expressed as mg/l.

Table 23.8 Bovine whey protein-derived inhibitors of angiotensin-I converting enzyme (ACE). Adapted from FitzGerald and Meisel, 2000

Protein source	Peptide fragment	Sequence	IC_{50} (μM)	Preparation	Reference
α-Lactalbumin	*f*(104-108)	WLAHK	77.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(99-108)	VGINYWLAHK	327.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(50-52)	YGL	409.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(105-110)	LAHKAL	621.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998
	f(50-53)	YGLF	733.30	Synthetic	Mullally *et al*., 1996
	f(50-51)	YG	1522.6	Synthetic	Mullally *et al*., 1996
	f(52-53)	LF	349.00	Synthetic	Mullally *et al*., 1996
β-Lactoglobulin	*f*(142-148)	ALPMHIR	42.6	Enzymatic	Mullally *et al*., 1997b
	f(102-103)	YL	122.10	Synthetic	Mullally *et al*., 1996
	f(78-80)	IPA	141.00	Synthetic	Abubakar *et al*., 1998
	f(102-105)	YLLF	171.80	Synthetic	Mullally *et al*., 1996
	f(104-105)	LF	349.10	Synthetic	Mullally *et al*., 1996
	f(142-146)	ALPMH	521.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(15-19)	VAGTW	534.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(9-14)	GLDIQK	580.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998

	f(34-40)	LDAQSAPLR	635.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(148-149)	RL	2438.9	Synthetic	Mullally *et al*., 1996
	f(106-111)	CMENSA	788.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(146-148)	HIR	953.00	Synthetic	Mullally *et al*., 1997a
	f(94-100)	VLDTDYK	946.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(81-83)	VFK	1029.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(22-25)	LAMA	1062.00	Enzymatic	Pihlanto-Leppälä *et al*., 2000
	f(146-149)	HIRL	1153.20	Synthetic	Mullally *et al*., 1996
	f(147-148)	IR	695.50	Synthetic	Mullally *et al*., 1996
	f(15-20)	VAGTWY	1682.00	Enzymatic	Pihlanto-Leppälä *et al*., 1998
	f(10-14)	LDIQK	17.00*	Enzymatic	Schlothauer *et al*., 1999
	f(1-5)	LIVTQ	17.00*	Enzymatic	Schlothauer *et al*., 1999
	f(81-82)	VF	19.00*	Enzymatic	Schlothauer *et al*., 1999
	f(7-9)	MKG	24.00*	Enzymatic	Schlothauer *et al*., 1999
Serum albumin	*f*(208-216)	ALKAWSVAR	3.00	Synthetic	Chiba and Yoshikawa, 1991
	f(221-222)	FP	315.00	Synthetic	Abubakar *et al*., 1998
β_2-microglobulin	*f*(18-20)	GKP	352.00	Enzymatic	Abubakar *et al*., 1998

IC_{50} = Peptide concentration which reduces the activity of ACE by 50%.
*IC_{50} values quoted are expressed as mg/l.

inhibitory peptides was in the range of 1.2–1.6 mg per day (FitzGerald and Meisel, 2000). Significantly, systolic blood pressure (SBP) decreased by 9.4 and 14.1 mm Hg after four and eight weeks consumption, respectively, and diastolic blood pressure (DBP) decreased by 6.9 mm Hg, eight weeks after the study began (Hata *et al*., 1996).

More recently, the hypotensive efficacy of Calpis sour milk and milk fermented with *Lactobacillus helveticus* LBK-16H were compared using SHR over a 13-week period (Sipola *et al*., 2002). *L. helveticus* LKB-16H soured milk contained twice the concentration of VPP and IPP than the Calpis product resulting in the SHR consuming 0.4 $mg.d^{-1}$ IPP and 0.6 $mg.d^{-1}$ VPP from *L. helveticus* LKB-16H soured milk and 0.2 $mg.d^{-1}$ IPP and 0.3 $mg.d^{-1}$ VPP from the Calpis product. Significantly, blood pressure decreases of 21 and 10 mm Hg were recorded when SHR consumed *L. helveticus* soured milk and Calpis, respectively (Sipola *et al*., 2002).

A human pilot study using milk fermented with *Lactobacillus helveticus* LKB-16H reported that daily ingestion of 150 ml of this preparation significantly reduced SBP by 14.9 mm Hg and DBP by 8.8 mm Hg after eight weeks consumption (Seppo *et al*., 2002). However, in a later trial where 150 mL of the same fermented beverage was consumed over a 21–week period the SBP and DBP were only reduced by 6.7 and 3.6 mmHg (Seppo *et al*., 2003). A recent human study indicated that SBP and DBP decreased by 11 and 7 mm Hg, respectively, on daily ingestion of 20 g of a hydrolysed whey protein isolate after one week (Pins and Keenan, 2002).

Conflicting reports have been published on the ability of the dipeptide Val-Tyr (IC_{50}, 26 μM) to exert an anti-hypertensive response in humans (Kawasaki *et al*., 2000; Matsui *et al*., 2002). This dipeptide originally isolated from sardine muscle (Matsufuji *et al*., 1994) may also be generated from different milk proteins (β-Lg, β-casein and α_{s2}-casein) as a result of gastrointestinal digestion (Meisel, Walsh and FitzGerald, unpublished data). In a randomised double blind placebo controlled human study, a 100 ml solution containing 3 mg of Val-Tyr was consumed twice daily for four weeks and SBP and DBP decreases of 9.3 and 5.2 mm Hg, respectively, were reported (Kawasaki *et al*., 2000). However, single oral administration of a beverage containing up to 12 mg Val-Tyr to humans did not produce an antihypertensive response after 24 hours (Matsui *et al*., 2002).

Further human studies using higher doses over a longer period of time are required before the potential hypotensive efficacy of Val-Tyr may be fully elaborated. Another dipeptide present in casein, Val-Pro (IC_{50}, 720 μM) was observed to mediate a hypotensive effect in SHR (Yamamoto *et al*., 1999). Tyr-Pro, present in α_{s1}-casein f(146-147) and f(159-160), β-casein f(114-115) and κ-casein f(58-59) was identified in skim milk fermented with *Lactobacillus helveticus* CPN4. The generation of this peptide may be due to the hydrolytic action of starter culture derived post-proline dipeptidyl aminopeptidase activity (Bouchier *et al*., 1999) on casein peptides (FitzGerald and Meisel, 2000).

ACE inhibitory peptides can be produced during the manufacture of a range of dairy products. Several reports have demonstrated that secondary ripening of

cheese produces ACE inhibitory peptides (Meisel *et al*., 1997; Ryhänen *et al*., 2001). Low levels of proteolysis during ripening were associated with low ACE inhibitory index values. Meisel *et al*. (1997), reported that the main ACE inhibitory activity was associated with the low-molecular weight peptides present in cheese. Similar findings were observed for enzymatic digests of whey proteins where most of the ACE inhibitory activity was associated with the peptide fraction having molecular mass less than 3 kDa (Mullally *et al*., 1997a) and 1 kDa (Pihlanto-Leppälä *et al*., 2000).

Different strains of lactobacilli have been reported to be responsible, or partly responsible, for the production of ACE inhibitory peptides during fermentation. These include, *Lactobacillus casei* ssp. *rhamnosus* (Rokka *et al*., 1997), *Lactobacillus helveticus* CPN4 (Yamamoto *et al*., 1999), *Lactobacillus helveticus* LKB-16H (Seppo *et al*., 2002) and *Lactobacillus helveticus* R221 and R389 (Leclerc *et al*., 2002). Rokka *et al*. (1997) identified ACE inhibitory peptides from β-casein, i.e., f(177-183) and f(193-202), in UHT milk pre-fermented with *Lactobacillus casei* spp. *rhamnosus* and subsequently digested with pepsin and trypsin. Pihlanto-Leppälä *et al*. (1998), reported that regardless of prior fermentation of milk proteins with lactic acid bacteria further digestion with pepsin and trypsin was essential for development of ACE inhibitory activity.

In vitro hydrolysis of milk proteins with proteinase preparations has resulted in the generation of many ACE inhibitory peptides having a range of inhibitory potencies (Table 23.7 and 23.8). Enzymatic hydrolysis of whey protein concentrate, α-La and β-Lg with trypsin, chymotrypsin, pepsin and elastase preparations resulted in the generation of ACE inhibitory peptides (Mullally *et al*., 1997a). A tryptic peptide corresponding to β-Lg f(142-148) was reported to have an IC_{50} of 42.6 μM (Mullally *et al*., 1997b). *In vitro* cell culture studies reported that although this peptide was transported intact through a Caco-2 Bbe monolayer, the concentrations transported were probably too low to exert a significant ACE inhibitory activity (Vermeirssen *et al*., 2002). Pihlanto-Leppälä *et al*., (1998, 2000), identified different casein and whey derived ACE inhibitory peptides following digestion of cheese whey, α-La, β-Lg and isoelectric casein with individual enzymes and combinations of pepsin, trypsin, chymotrypsin, pancreatin, elastase and carboxypeptidase.

The ability of milk protein-derived ACE inhibitory peptides to exert an antihypertensive effect *in vivo* is dependent on their ability to reach target sites in sufficient quantities following exposure to numerous peptidases present in the intestine and in plasma (FitzGerald and Meisel, 2000, 2003a). Peptides such as α_{s1}-casein f(23-27) and f(104-109) which demonstrated potent ACE inhibitory activity *in vitro* were subsequently shown to exhibit no hypotensive effect *in vivo* in SHR (Maruyama *et al*., 1987b; Maeno *et al*., 1996). In contrast, peptides such as Ile-Pro-Pro and Val-Pro-Pro were detected in aortal fractions of SHR demonstrating not only that they were transported from the intestine to blood but also that they were stable to peptidase degradation *in vivo* (Masuda *et al*., 1996).

Table 23.9 Examples of multifunctional bioactive peptides encrypted in bovine milk proteins.[1] From Meisel and Bockelmann, 1999. Reprinted with kind permission of H. Meisel and Kluwer Academic Publishers.

Peptide sequence[2]	Fragment	Name	Preparation	Opioid[3] IC_{50}	ACE-inhibitory[4] IC_{50}	Immuno-modulatory[5]	Ca^{2+} binding Kapp $(1.mol^{-1})$[6]
YPFPGPIPNSL	β-Cn f(60-70)	β-casomorphin-11	Intest. Chyme	10	-	-21/+26	-
YPFPGPI	β-Cn f(60-66)	β-casomorphin-7	Trypsin	14	500	-	-
YPFPG	β-Cn f(60-64)	β-casomorphin-5	Trypsin	1.1	0	-	-
RYLGYLE	α_{s1}-Cn f(90-96)	α-casein exorphin	Pepsin	1.2	-	-	-
YGLF	α-La f(50-53)	α-lactorphin	Synthesis	300	733	-	-
YLLF	β-Lg f(102-105)	β-lactorphin	Synthesis	160	172	-	-
YGFQNA	Sa f(399-404)	Serophin	Pepsin	85	-	-	-
SRYPSY.OCH_3	κ-Cn f(33-38)	Casoxin 6	Pepsin	15 (↓)	-	-	-
YIPIQYVLSR	κ-Cn f(25-34)	Casoxin C	Trypsin	50 (↓)	-	-	-
YL	β-Lg f(102-103)	Lactokinin	Synthesis	-	122	-	-
ALPMHIR	β-Lg f(142-148)	Lactokinin	Trypsin	-	43	-	-
VPP	β-Cn f(84-86)	β-Casokinin	Sour milk	-	9	-	-
IPP	β-Cn f(74-76)	β-Casokinin	Sour milk	-	5	-	-
AVPYPQR	β-Cn f(177-183)	β-Casokinin	Trypsin	-	15	-	-
FFVAPFPEVFGK	α_{s1}-Cn f(23-34)	α_{s1}-Casokinin	Trypsin	-	77	-	-
FFVAP	α_{s1}-Cn f(23-27)	α_{s1}-Casokinin	Trypsin + peptidase	-	6	-	-
VAP	α_{s1}-Cn f(25-27)	α_{s1}-Casokinin	Synthesis	-	2	-	-
YQQPVLGPVR	β-Cn f(193-202)	β-Casokinin-10	Synthesis	-	300	−28/+14	-
TTMPLW	α_{s1}-Cn f(194-199)	αs1-Immunocasokinin	Trypsin	-	16	+162	-
PGPIPN	β-Cn f(63-68)	Immunopeptide	Synthesis	-	-	+139	-
LLY	β-Cn f(191-193)	Immunopeptide	Synthesis	-	-	+148	-
YG	α-La f(50-51), f(18-19), κ-Cn f(38-39)		Synthesis	-	>1000	+101	-
YGG	α-La f(18-20)	Immunopeptide	Synthesis	-	-	+35	-
RELEELNVPG EIVES*LS*S*S*EESITR	β-Cn f(1-25)4P	Caseinophosphopeptide	Trypsin	-	-	-	629
DIGS*ES*TEDQAMEDIM	α_{s1}-Cn f(43-58)2P	Caseinophosphopeptide	Trypsin	-	-	-	328
QMEAES*IS*S*S*EEIVPNS*VEQK	α_{s1}-Cn f(59-79)5P	Caseinophosphopeptide	Trypsin	-	-	-	841
FKCRRWQWRMKKLGAPSITCVRRAF	Lf f(17-41)	Lactoferricin	Pepsin	antimicrobial	-	-	-
MAIPPKKNQDK	κ-Cn f(106-116)	Casoplatelin	Trypsin	antithrombic	-	-	-

The stability of these tripeptides to peptidase hydrolysis is not unexpected in light of earlier research which showed that peptides containing C-terminal Pro-Pro were resistant to proline specific peptidases (Yoshimoto *et al*., 1978; Mock *et al*., 1990). In some cases peptidase hydrolysis may result in the beneficial generation of more potent ACE inhibitory peptides. The removal of the C-terminal glutamine from β-casein f(169-175) increased the ACE inhibitory potency of this peptide from 1000 to 5 μM. Both β-casein f(169-175) and f(169-174) exhibited strong antihypertensive effects in SHR (Maeno *et al*., 1996). While *in vitro* analysis yields information on the ACE inhibitory activity of peptides the only true measure of hypotensive efficacy can be obtained from *in vivo* studies.

The milk protein-derived peptides presented in Tables 23.7 and 23.8 do not have ACE inhibitory potencies approaching that of commercial anti-hypertensive medications such as Captopril® (IC_{50}, 0.006 μM), Iisinopril® (0.0012 μM) and Xofenopril® (0.008 μM) (Wyvratt and Patchett, 1985). However, studies have shown that milk protein-derived peptides have the potential to significantly lower blood pressure when consumed over a prolonged period. These peptides, unlike synthetically produced drugs, do not appear to have the side-effects that are associated with some pharmacological antihypertensive drugs such as hypotension, increased potassium levels, reduced renal function, cough, angioedema, skin rashes and foetal abnormalities (Ames, 1983; Seseko and Kaneko, 1985; Nakamura, 1987; Agostoni and Cicardi, 2001).

Hypertension is a risk factor for cardiovascular disease (CVD) and is a widespread condition, currently, for example, reputed to affect over 80 million Americans and Canadians (Pins and Keenan, 2002). The susceptibility to develop CHDs has also been directly related to blood pressure level. Research has shown that for each 5 mm Hg reduction in DBP the risk of developing CHD is reduced by approximately 16% (MacMahon *et al*., 1990; Collins *et al*., 1990). Currently, the annual antihypertensive drug expenditure in the United States is estimated to be approximately $15 billion (Frantz, 2003). Milk protein derived ACE inhibitory peptides have significant potential as natural agents for prevention and control of hypertension and related CVD or renal complications.

Notes to Table 23.9

[1] Details are available within the references used to generate this table: Bellamy *et al*. 1992; Brantl *et al*. 1981; Chiba and Yoshikawa 1986; Chiba *et al*. 1989; FitzGerald and Meisel 1999; Jollès *et al*. 1986; Kayser and Meisel 1996; Loukas *et al*. 1983; Meisel 1986; Meisel and Schlimme 1994; Migliore-Samour *et al*. 1989; Mullaly *et al*. 1996, 1997a; Nakamura *et al*. 1995a; Teschemacher *et al*. 1997.

[2] The one letter amino acid codes were used; S*= Phosphoserine.

[3] IC_{50} values (μM) are given for peptide concentrations inhibiting [3H]-ligand binding by 50%; (↓) indicates antagonistic activity.

[4] IC_{50} values (μM) are given for peptide concentrations inhibiting the activity of angiotensin converting enzyme (ACE) by 50%.

[5] Figures indicate the maximum % stimulation (+) and /or inhibition (−), respectively, in relation to control (=100).

[6] Apparent calcium binding constant (Kapp) were determined with samples obtained from tryptic fractions containing the indicated peptide as the main component.

23.7 Multifunctional properties of milk-derived peptides

Many milk protein-derived peptides reported to date possess multifunctional bioactivities, i.e., a single peptide sequence may possess two or more different biological activities (Table 23.9). The multifunctional peptides derived from milk proteins have been extensively reviewed and further information may be obtained by referral to the following sources: Meisel, 1997a and 1997b; Schanbacher *et al*., 1998; Meisel and Bockelmann, 1999. β-Casein-derived peptides with multifunctional properties have been linked to specific amino acid sequences or 'strategic zones' that are partially protected from proteolytic breakdown (Fiat and Jollès, 1989; Meisel, 1997a; Meisel and Bockelmann, 1999).

23.8 Future trends

As outlined in an earlier part of this chapter, milk proteins possess numerous nutritional attributes by way of their ability to provide essential amino acids, minerals and aid in the transport of certain vitamins. Individual intact milk proteins have demonstrated beneficial health benefits, e.g., the protective effects from bacterial infections associated with Igs and Lf. Furthermore, milk proteins are a source of bioactive peptides that may act as regulatory compounds once liberated by enzymatic hydrolysis. Consumers are becoming more health conscious, paying increased attention to the paradigm of 'prevention is better than cure'. A wide range of foods and beverages containing bioactive peptides are now being designed and marketed to provide specific health benefits. The ability of milk protein-derived peptides to modulate immune function has been demonstrated in both *in vitro* and *in vivo* studies. In some cases the exact mode by which these proteins and peptides alter specific and non-specific immune responses *in vitro* have been identified. However, immunomodulatory responses *in vitro* do not always correlate with those observed *in vivo*. This is reflective of the complex series of modifications orally ingested proteins and peptides experience during gastrointestinal transit and exposure to gut microorganisms.

Future studies may focus on the generation of immunomodulatory peptides that will either be resistant to further degradation *in vivo* or will be activated by endogenous systems once consumed. Beneficial immune responses to improve human health are not exclusively those that enhance the immune system. Both κ-casein and whey-derived proteins have been shown to exhibit immunosuppressive properties and this may be of benefit in the regulation of intestinal inflammation (Cross and Gill, 2000). Recently, probiotic milk products have gained popularity for their reported ability to improve immune function. Enrichment and isolation of immunomodulatory peptides from these products should be an area of interest in the future. Further studies, in particular, clinical studies using enriched fractions of milk proteins/peptides are required before their true benefit to human health is realised.

Studies have demonstrated the ability of CPPs to bind bivalent minerals such as Ca^{2+} and increase their solubility in alkaline conditions. Human studies to date have not conclusively shown the efficacy of CPPs in increasing the bioavailability of dietary Ca^{2+}. This is due, in part, to the complex interactions between dietary minerals and other components in the diet, such as phytate, that strongly influence bioavailability. Recent research shows that CPPs can form nanocluster complexes with minerals (Holt *et al*., 1998) and it may be through further research in this area that the functional food ingredient potential of CPPs can be fully exploited (FitzGerald and Meisel, 2003b).

The ability of casein- and whey protein-derived peptides to inhibit ACE *in vitro* has been well documented in the literature. Considerable developments have been made in this area of bioactive peptides with commercial products being marketed with reported antihypertensive capabilities such as Calpis® (Japan), Evolus® (Finland) and whey derived BioZate® (USA). Some of these products are patent protected. In two of these products, i.e., Calpis and Evolus, the main ACE inhibitory components have been identified as casein-derived tripeptides. The efficacy of these products to lower blood pressure has been attributed to the potency, stability and bioavailability of VPP and IPP *in vivo*. Future studies may focus on the isolation and identification of other potent ACE inhibitory peptides that possess similar attributes to the tripeptides in these commercial products. Basic scientific data on milk protein-derived peptide inhibitors of ACE is required by clinicians, nutritionists and legislators responsible for the introduction of novel, scientifically validated, functional foods/food ingredients for specialist use or general consumption. This is currently being addressed through a European Commission funded shared-cost project within the Fifth Framework Programme: 'Hypotensive Peptides from Milk Proteins (HTMProt)' (EU FPV QLK1-CT2000-00043).

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23.10 References

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24

The use of immobilized enzymes to improve functionality

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24.1 Introduction

The technique of immobilization of enzymes by attachment to a solid surface has been the subject of extensive research for nearly half a century. The topic has been reviewed by many authors; a sampling of reviews include (Mosbach, 1976, 1987a,b; Chibata, 1978; Pitcher, 1980; Swaisgood, 1985, 1991, 2003; Swaisgood and Horton, 1989; Cheetham, 1987; Kennedy, 1987). The many methods of attachment or immobilization that have been developed can be categorized as

1 covalent bonding
2 adsorptive binding, e.g., ionic or hydrophobic attraction
3 entrapment in a polymeric matrix or with a size-exclusion membrane
4 covalent crosslinking with itself or other macromolecules or within the crystal form
5 bioselective adsorption.

The types of solid supports that have been used are as numerous as the methods of immobilization. The choice of support must consider its surface area, its porosity, its stability, its compatibility with the substrate and the enzyme, its resistance to microbial degradation, its regenerative capacity, its fluid flow characteristics and, of course, its cost.

Use of immobilized forms of enzymes offers many advantages over the soluble enzyme. A much greater productivity per unit of enzyme is achieved because the same enzyme is used over an extended period of time so a much larger amount of product is produced from the same amount of enzyme. Immobilization also allows the extent of reaction to be precisely controlled

without the necessity of a downstream inactivation process. This feature would be extremely important for processes modifying protein functionality or flavor development. A corollary of this feature is that the product does not contain the enzyme, which may be of significance as the use of recombinant enzymes increases. Furthermore, most enzyme preparations contain small amounts of other activities that over time will produce undesirable characteristics in the food product. Another industrial advantage is the ability to develop an automated continuous process. Consequently, materials handling and associated labor costs are greatly reduced. Finally, it should be noted that because of some of the features mentioned it may be possible to produce unique products, production of high-fructose corn syrup being the best commercial example, currently. Future use of immobilized enzymes may allow production of proteins with unique functionality or products with unique flavor characteristics.

The economics of an immobilized enzyme process are determined by a number of factors (Pitcher, 1980; Swaisgood, 1985, 1991, 2003; Daniels, 1987). Some important factors are summarized in Table 24.1. As with soluble enzyme processes, the specific activity of the preparation determines the amount of enzyme that must be used and thus the size of the bioreactor. However, the cost of purification usually increases exponentially to achieve the higher ranges of purity so these two factors must be weighed against each other. The surface area

Table 24.1 Some factors associated with the cost of an immobilized enzyme process

Enzyme preparation
• Costs for achievement of desired specific activity
• Costs for removal of undesirable contaminating activities
Immobilization
• Costs of the support material
• Costs associated with and determined by the characteristics of the immobilization process
• The amount of enzyme loading that can be achieved
Operational stability
• The innate stability of the enzyme under the environmental conditions of the process
• The kinetics of enzyme leaching from the support under the process conditions
• The resistance of the support material to attrition
Regenerative capability of the support
• Number of times the activity can be regenerated on the support by removal of inactive enzyme and addition of fresh enzyme
• Number of steps and difficulty of each step of the procedure
Upstream processing requirements
• The number and complexity of the steps
• The costs of the materials added
Sanitation requirements
• Frequency of the required cleaning
• Number and complexity of the cleaning steps
• Costs of the cleaning materials

per unit volume of support will also affect the specific activity of the biocatalyst because this parameter determines the amount of enzyme that can be immobilized per unit volume. Enzyme activity is also affected by the method of immobilization. Because this effect can be peculiar to a particular enzyme, the method chosen should be matched to the enzyme used. For example, some covalent attachment methods or adsorption methods may react with or hinder access to the active site of certain enzymes.

Operational stability is a very important parameter for the economic viability of an immobilized enzyme process. This parameter determines how long a bioreactor can be used and thus the amount of product produced per unit of enzyme. The main components of this parameter are the stability of the enzyme itself, resistance of the support to attrition, and the resistance of the enzyme to leaching from the support. There may be a choice among enzymes with the desired activity so that the enzyme with the greatest stability under the operational conditions can be selected. Also, this area represents opportunity for genetic engineering of the enzyme. To minimize attrition, a support should be selected for its physical stability under the conditions of the desired process. Finally, the method of immobilization should be chosen with the process in mind. For example, covalent immobilization should allow the least leaching; however, regeneration of the biocatalyst activity is not possible. Adsorption of the enzyme on an ion exchange support would permit easy regeneration of the biocatalyst; however, the pH and ionic strength of the product stream would affect the strength of the enzyme binding.

The capability of bioreactor regeneration by removal of inactive enzyme and immobilization of a fresh enzyme preparation would be a definite advantage especially if the support is relatively expensive. Most adsorptive immobilization methods would permit regeneration; thus the deciding factor would be the complexity and cost of the process. Other factors affecting the cost of an immobilized enzyme process include that of any upstream processing requirements and bioreactor sanitation. For example, it may be necessary to remove enzyme inhibitors from the process stream or to add an enzyme cofactor that is not tightly bound.

All of these factors can be summarized in one parameter, namely, the cost per unit of product. This parameter is greatly dependent on one bioreactor characteristic, namely, the productivity, that is the amount of product per unit of biocatalyst per half-life. The viability of the process will depend on the balance of this cost against the value added to the product as viewed by the consumer.

24.2 Modification of carbohydrates

24.2.1 Production of high-fructose corn syrup

The production of high-fructose corn syrup represents the major use of an immobilized enzyme in the world. In the United States for the year 2002, a total

of 9,302,000 metric tons, dry weight, was produced of which 3,640,000 tons were HFCS-42 and 5,662,000 tons were HFCS-55 (USDA). These products, which are used in a variety of foods and beverages, had an estimated value of $2.4 billion. Currently, the major producers are ADM, Cargill and Staley. In 1999, approximately 1500 metric tons of the immobilized enzyme was used for HFCS production (J. Shetty, personal communication). The cost of catalysts for the conversion of starch to HFCS is less than 3% of the product selling price (Wilke, 1999).

The commercial process is comprised of the following steps (White, 1992):

- Starch liquefaction and saccharification using an acid-glucoamylase hydrolysis to give ~94% glucose syrup. Starch liquefaction and saccharification is also achieved using a combined α-amylase-glucoamylase process (Wilke, 1999). During this step, heating and cooling steps, pH adjustments, filtration, ion-exchange and evaporation are required to maximize enzyme efficiency, to decolorize, to protect from microbial contamination, to remove organic and inorganic contaminants, and to concentrate the glucose syrup.
- Isomerization catalyzed by immobilized glucose isomerase (xylose isomerase; EC 5.3.1.5) yielding a 42% fructose syrup. The ion-exchange process removes Cu, Hg, Zn and Ca ions, that inhibit the enzyme, from the glucose syrup; however, Mg^{2+} must be added to ~35 ppm to activate the enzyme. Isomerization is carried out at 60 ºC to favor fructose formation and to control microbial growth.
- Fractionation using moving-bed cation-exchange chromatography is performed to produce an enriched (90%) fructose syrup.
- Blending of the 90% fructose syrup with the 42% syrup is carried out to produce a 55% fructose syrup that has the same sweetness as sucrose.

The forms of commercially available immobilized glucose isomerase have undergone several generations of development. The enzyme has been obtained from *Actinoplanes missouriensis, Micobacterium arborescens, Bacillus coagulans,* and a number of *Streptomyces* sp. (Teague and Brumm, 1992). Initially, the immobilized preparations were simply crosslinked, lysed whole cells that were dried to produce granulated or fibrous forms. The crosslinking agent commonly used was glutaraldehyde. Later, partially purified forms were crosslinked or entrapped in biopolymers, such as gelatin, or organic polymers. More recently, the trend has been towards immobilization of substantially purified forms by adsorption on various resins such as ion-exchange materials or porous inorganic carriers. Gensweet IGI™, the Genencor immobilized enzyme is a substantially purified form that is adsorbed on an ion-exchange resin, crosslinked with polyethylenimine and glutaraldehyde, and granulated by extrusion. Typically, this preparation has a productivity of 12,000–15,000 kg dry substance per kg and a half-life of 80–150 days (J. Shetty, personal communication). Sweetzyme T™, offered by Novozymes, is crosslinked, lysed *Streptomyces murinus* cells that are dried and granulated by extrusion (Pedersen, 1993).

24.2.2 Lactose hydrolysis

Corning Glass Co. was the first to develop a commercial immobilized β-galactosidase (β-D-galactoside galactohydrolase EC 3.2.1.23) using a process of covalent attachment to porous silica (Messing and Weethall, 1970). This technology was used for several years by Nutrisearch to hydrolyze lactose in whey that was then used to produce baker's yeast. However, this venture was not successful, apparently due to problems with microbial contamination and cost.

Possibly the most successful process was developed by Valio, Ltd. in Finland in 1980 (Harju, 1987). The biocatalyst is β-galactosidase from *Aspergillus oryzae* immobilized by adsorption and crosslinking on a food grade resin (Harju, personal communication). Their technology has been used to produce various types of whey syrups for use in whey drinks, ice cream and confectionary products. For example, a typical demineralized whey syrup has 72% of the lactose hydrolyzed and contains 60% solids. The product has increased solubility and sweetness giving it a pleasantly rich taste. Plants producing whey syrups using this process have operated in Norway and England (Dairy Crest) as well as in Finland. A whey feedstock containing about 6% solids is pasteurized and passed through a fixed-bed reactor and then concentrated to the desired solids. A typical reactor has a half-life of 20 months and a productivity of 2000 kg dry matter per kg enzyme. In 2002, Valio and Nordzucker AG, the German sugar and sweetner company, signed an agreement by which Valio will provide their immobilized lactase technology to Nordzucker for production of tagatose, a reduced calorie sweetener.

Hydrolysis of lactose in milk also has been performed commercially using an immobilization process developed by Snamprogetti in which the enzyme is entrapped in cellulose triacetate fibers (Marconi and Morisi, 1979; Swaisgood, 1985, 1991; Uhlig and Linsmaier-Bednar, 1998). The process was used in Italy by Centrale del Latte to produce 10 tons/day of lactose-hydrolyzed milk. A similar process was used in Japan by Snow Brand Milk to produce lactose-hydrolyzed milk with bioreactors containing the Snamprogetti immobilized enzyme (Honda *et al*., 1993). This company's scientists developed a bioreactor sanitation process of immersion in 10% glycerol at 10 ºC that prevented microbial growth.

24.2.3 Starch hydrolysis

Enzymatic hydrolysis of starch is initiated with α-amylase; however, because starch is an insoluble substrate or if gelatinized, highly viscous, use of an immobilized enzyme would most likely be unsuccessful. On the other hand, hydrolysis of the resulting oligosaccharides with glucoamylase can be accomplished with the immobilized enzyme. Glucoamylase (1,4–glucanohydrolase; EC 3.2.1.3) is an exo-acting enzyme that hydrolyzes α-1,4 and α-1,6 glycosidic bonds of α-glucan from the nonreducing ends. Despite numerous studies of immobilized glucoamylase (Reilly, 1980; Lee *et al*., 1976, 1980; Weethall, 1975), widespread adoption of this technology has not occurred

(Reeve, 1992). There are several reasons for this. First, because the reaction is diffusion limited due to relatively large size of most porous supports and the high surface concentration of enzyme, more reversion products form than with the soluble enzyme. Hence, it is difficult to achieve a 95–96 DE product. Secondly, the thermostability of the enzyme constrains the operating temperature to 55 ºC so microbial growth can occur.

However, immobilized glucoamylase can be used to produce high conversion syrups of 60–70 DE. A commercial process has been developed using a high maltose syrup to feed an immobilized glucoamylase bioreactor (Reeve, 1992; Rugh *et al*., 1979). In addition to the advantage of reduced reactor volume and reaction time as compared to the soluble enzyme process, use of the immobilized enzyme allows more accurate control of the carbohydrate product spectrum.

Several recent developments give reason for optimism that a commercial process may be realized in the future. First, the advent of magnetic microbeads, which are very small in size thus eliminating diffusion limitations, and can be retained in a magnetically stabilized fluid bed. Potentially, such a system could simulate a soluble enzyme process. Second, the discovery and cloning of glucoamylases from the extremophiles *Clostridium thermohydrosulfuricum* 39E and *Clostridium thermosacchrolyticum*, the latter having an optimum temperature of 70 ºC at pH 5 (Niehaus *et al*., 1999).

24.2.4 Isomaltulose production

Isomaltulose (6–O-α-D-glucopyranosyl-D-fructose) is a low calorie, noncariogenic sweetener that is growing in use in low calorie foods and beverages as well as a bulking agent in pharmaceuticals. This disaccharide can be produced from sucrose enzymatically using isomaltulose synthase (sucrose glucosylmutase EC 5.4.99.11). It is about one-third as sweet as sucrose and encourages growth of bifidobacteria (Cheetham, 1987). Isomaltulose also is used as the starting material for production of Isomalt or Palatinit, which is obtained by reduction of the keto groups to form a mixture of 6–O-α-D-glucopyranosyl-D-sorbitol and 1–O-α-D-glucopyranosyl-D-mannitol dihydrate. This product is used extensively as a low calorie, noncariogenic sweetener in a variety of foods.

The immobilized enzyme is used commercially, usually in the form of the immobilized cells (Cheetham, 1987). For example, cells of *Protaminobacter rubrum* have been flocculated, extruded into ropes, dried and crosslinked with glutaraldehyde. The immobilized cells were used in a column maintained at 45–60 ºC through which a solution of 45–75% (w/w) sucrose was passed. Another company used *Serratia plymuthica* cells entrapped in calcium alginate and crosslinked with polyethyleneimine and glutaraldehyde.

In a process developed by scientists at Tate and Lyle, *Erwinia rhaponica* cells were entrapped in calcium alginate formed into smooth, spherical pellets with a diameter of 3–5 mm (Cheetham, 1987). Unlike the enzyme from other organisms, the enzyme from *Erwinia* appears to be sucrose specific. Using a

fixed-bed reactor, the immobilized cells exhibited a half-life of about one year with a feed stream of 55% (w/w; 1.6 *M*) sucrose while achieving 99% conversion. Viable cells are not necessary for enzyme activity and, in fact, the half-life for viable cells was only 300 hr. A ten-liter fixed-bed reactor will process 5.2 m^3 of sucrose during one half-life (one year), while producing 1,500 times the biocatalyst weight in crystalline isomaltulose. This corresponds to more than 200 kg product/L of biocatalyst/half-life.

24.3 Production of flavors and specialty products

24.3.1 L-amino acids

L-Amino acids are utilized as pharmaceuticals as well as in foods and animal feeds. L-Lysine, L-tryptophan and L-methionine are commonly used as feed supplements and their use is growing as food supplements. Of course, monosodium glutamate represents a major usage of an amino acid as a flavor enhancer. The world demand for monosodium glutamate in 1999 was about 1.3 million metric tons with almost half of it being used in China. Amino acids are also used in medical infusion solutions and other pharmaceuticals.

Chemical synthesis of amino acids produces a racemic mixture of acyl-D,L-amino acids. The enzyme aminoacylase (N-acylamino-acid amidohydrolase EC 3.5.1.14) asymmetrically hydrolyzes the L-form yielding a mixture of the L-amino acid and acyl-D-amino acid. This mixture can be readily fractionated by charge and solubility characteristics. The acyl-D-amino acid can be racemized by heat treatment to give the racemic mixture that can once more be subjected to the resolution procedure. In 1969, Tanabe Seiyaku Co. Ltd. initiated an immobilized enzyme process with the enzyme immobilized by adsorption on DEAE-Sephadex (Chibata *et al*., 1976, 1987, 1991). Use of this continuous process with a fixed-bed reactor substantially reduced labor costs and increased the yield of L-amino acids. The immobilized enzyme exhibited a half-life of 65 days at 50 ºC. For example, L-methionine was produced using a 1000 L-reactor with a continuous flow rate of 2000 L/hr producing 27 kg of L-amino acid/hr. After crystallization of the L-form, the acyl-D-amino acid was racemized by treatment at 60 ºC with acetic anhydride and passed back through the reactor. The company was able to use the same DEAE-Sephadex for eight years, regenerating the reactor by simply adsorbing fresh enzyme.

Snamprogetti in Italy developed a process for resolution of racemic mixtures of acyl-D,L-tryptophan using their technology of entrapping the enzyme in cellulose triacetate fibers (Marconi and Morisi, 1979). In a pilot plant demonstration using procedures similar to those of Chibata and co-workers they achieved a production of 400 kg L-tryptophan/kg of fiber.

L-Aspartic acid has been produced commercially from ammonium fumarate using immobilized *Echerichia coli* cells that have high aspartase activity (L-aspartate ammonia lyase, EC 4.3.1.1) (Sato *et al*., 1975; 1979; Chibata, 1979). The cells had been treated to inactivate alanine racemase and fumarase. This

commercial application used bioreactors containing whole cells entrapped in polyacrylamide or κ-carrageenan. A procedure for immobilization of the cells in polyurethane also has been described (Fusee, 1987).

L-Alanine is used as a component of infusion solutions and as a flavor enhancer. Scientists at Tanabe Seiyaku developed a two bioreactor process for continuous L-alanine production where the first reactor was the one described above for production of L-aspartate (Chibata *et al*., 1987). The second reactor contained *Pseudomonas dacunae* cells that had high L-aspartate β-decarboxylase activity. The cells were pH-treated to eliminate alanine racemase and fumarase then entrapped in κ-carrageenan and crosslinked with glutaraldehyde to stabilize the activity. To solve the problem of CO_2 gas production the company designed a closed column system operating at a pressure of 10 kg/cm^2. The efficiency of the cells to produce L-alanine increased 1.5 fold as compared to normal pressure conditions. The company has been producing L-alanine by this method since 1982 with a yield of 90% of theoretical from ammonium fumarate.

24.3.2 Flavors

The demand for L-phenylalanine has increased considerably because of its use as a precursor in the production of Aspartame™ (α-L-aspartyl-L-phenylalanine methyl ester). Aside from the immobilized aminoacylase method of amino acid production mentioned above, Purification Engineering developed an immobilized enzyme process based on the high transaminase activity of a mutant *E. coli* (Calton *et al*., 1987; Swaisgood, 2003). Bioreactors with a half-life in excess of eight months were prepared by entrapment of the whole cells in a polyazetidine layer on Amberlite IRA-938 porous beads. The large pore diameter of the beads (2.5–23 μm) allows the microbial cells to access more surface area and facilitates excellent flow properties. A 600-ton per year plant was placed in production using phenylpyruvate as the starting material.

Several industrial immobilized enzyme processes for production of Aspartame™ have been developed (Oyama *et al*., 1987; Scott, 1989; Swaisgood, 2003). These enzymatic methods eliminate the requirement for protection and deprotection of the β-carboxyl group of aspartate in order to prevent the formation of the bitter isomer of Aspartame because the enzyme is specific for the α-carboxyl group. Also, enzymes are specific for the L-forms of amino acids; consequently, the less expensive racemate of phenylalanine methyl ester can be used in the synthesis. Scientists at Toya Soda Co. developed a process using immobilized thermolysin (Thermoase™) to condense carbobenzoxy-L-aspartate and D,L-phenylalanine methyl ester (Oyama *et al*., 1987). The reaction was performed in a biphasic system of water and ethyl acetate that partitioned the product into the ethyl acetate phase thus driving the synthesis of the peptide bond. Another industrial process utilizing the activity of enzymes in immobilized cells to synthesize phenylalanine from cinnamic acid and ammonia followed by direct coupling to acetate has been reported (Scott, 1989).

There is increasing demand for 5′-ribonucleotides for use as flavor enhancers in foods and as precursors in production of pharmaceuticals. A process for hydrolysis of RNA using immobilized 5′-phosphodiesterase has been developed (Keller *et al*., 1987). This enzyme hydrolyzes both RNA and DNA; however, by selection of an immobilization support of the appropriate pore size, the much larger DNA could be excluded from the pore volume where most of the enzyme activity is bound. Thus, the enzyme was covalently immobilized on oxirane acrylic beads (Eupergit C, Röhm Pharma Darmstadt) with an average pore diameter of 350 Å. The activity remained stable even after 500 days of continuous operation with crude RNA/DNA solutions containing 0.1 mM Zn^{2+} at pH 5 and 60 ºC. The 5′-nucleotides resulting from hydrolysis were purified by adsorption on an ion-exchange resin. A pilot scale plant producing ten tons per year of 5′-nucleotides was constructed. The economies that are realized by this process included a great reduction in enzyme costs, simplified product purification, and elimination of the requirement for initial purification of the RNA.

24.4 Modification of lipids

24.4.1 Interesterification of milk fat

Lipases (triacylglycerol hydrolase, EC 3.1.1.3), most of which are specific for the *sn* 1,3 positions of triacylglycerols, can be used to catalyze interesterification of these lipids. In aqueous solutions the hydrolysis is greatly favored over interesterification; thus, water must be present only in limited amounts. Hence, these reactions are performed in organic solvents or in oils. Because of the possible undesirable effects of saturated fats in the diet, there have been numerous studies of various methods to alter the composition of milk fat by enzymatic means. Lipase that is adsorbed to inorganic materials, such as Celite, is very stable in immiscible organic solvents and thus has been used in many of these studies.

Interesterification of milk fat has been demonstrated both in organic solvents and in butteroils. Lipases from *Candida cylindracea* (Kalo *et al*., 1986) and *Pseudomonas fluorescens* (Kalo *et al*., 1990; Lee and Swaisgood, 1997a) have been used to interesterify butteroil in an organic solvent as well as in the absence of solvent (Kalo *et al*., 1990; Elliot and Parkin, 1991; Lee and Swaisgood, 1997a). With the *P. fluorescens* lipase immobilized on Celite, Kalo *et al.* (1990) found that the *sn* 2 position was substituted as well; thus, the triacylglycerol compositions were nearly random. Using the *Rhizopus oryzae* enzyme adsorbed on controlled-pore glass, Oba and Witholt (1994) obtained 50% more oleic acid and significantly less palmitic acid content in milk fat after interesterification (acidolysis) of milk fat and oleic acid in isooctane. Although the reactions occur more rapidly in solvent because of the lower viscosity, most likely the use of butteroils will be preferable because of the elimination of the need to remove the solvent and potential toxic effects can be avoided (Kalo *et al*., 1990; Bornaz *et al*., 1994; Elliot and Parkin, 1991; Lee and Swaisgood, 1997a).

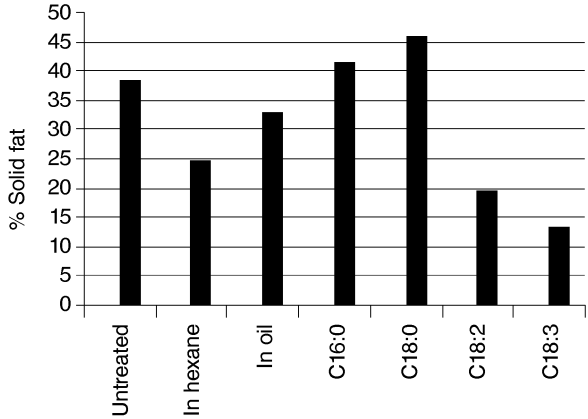

Fig. 24.1 Solid fat content at 20 ºC of butteroil modified by interesterification (acidolysis) with various fatty acids catalyzed by immobilized streptavidin-lipase fusion protein. Adapted from data presented by Lee and Swaisgood (1997a).

In a novel enzyme purification/immobilization approach Lee and Swaisgood (1997a; 1998) specifically adsorbed a streptavidin-lipase fusion protein in a recombinant cell lysate directly on a biotinylated porous inorganic matrix; thus, accomplishing purification and immobilization in a single step. Operation of the bioreactor either with the butteroil dissolved in hexane or with the anhydrous butteroil allowed interesterification among the triacylglcerols or acidolysis with added fatty acids. As would be expected, incorporation of unsaturated fatty acids led to decreased melting points whereas addition of saturated fatty acids increased the melting points (Fig. 24.1).

Lipase from *Candida cylindracea* has been immobilized by adsorption of the biotinylated enzyme on immobilized avidin (adsorbed on biotinylated glass) thus providing a protein spacer between the inorganic matrix and the enzyme (Lee and Swaisgood, 1997b). The interesterification activity, determined with tricaprylin and varying concentrations of oleic acid in hexane, was found to be fourfold higher than for the soluble enzyme. An increase in the esterification activity of a number of lipases entrapped in hydrophobic silica gels (Reetz *et al*., 1996) and *R. miehei* lipase adsorbed on alkylated controlled-pore glass (Bosley and Clayton, 1994) also has been observed.

Hill and co-workers (Garcia *et al*., 2000) have demonstrated the transesterification of butteroil with conjugated linoleic acid. Using a commercial immobilized lipase (Chirazyme L2 from *Candida antarctica*, formerly Novozyme 435 from Novozymes) they were able to achieve an increase of the conjugated linoleic acid content of milk fat from 0.6 to 15 g/100 g of fat. The extent of interesterification was greatest at the lowest water content, 0.15%, with the product containing 91% triacylglycerols. When the water content was increased to 2%, the weight fraction of triacylglycerols decreased to 81%. The effect of water content may depend upon the substrates as the results of such

studies have been variable; however, usually optimum interesterification is achieved between 0.15 and 0.3% (Forsell *et al*., 1993; Safari *et al*., 1993; 1994; Oba and Witholt, 1994; Lee and Swaisgood, 1998).

From the discussion above, it appears that commercial interesterification of milk fat using immobilized lipase to improve its physical and nutritional properties is feasible. Also, several companies, including Novozymes and Amano Pharmaceuticals produce immobilized lipases. To date, however, there has been little study of the functional properties of various modified milk triacylglycerols.

24.4.2 Interesterification of oils

Use of *sn* 1,3 specific lipases, such as those from *Mucor* sp., *Rhizopus* sp., and *Aspergillus* sp., permits more selective interesterification of triacylglycerols. Numerous studies and patents have described use of immobilized lipases for modification of various oils to improve their functional properties. Lipases from *Aspergillus sp.* and *Rhizopus arrhizus* were adsorbed on Celite and used to interesterifiy shea oleine and shea oil dissolved in hexane (Wisdom *et al*., 1984; Lilly and Dunnill, 1987). Similarly, lipase from *Rhizopus delemar* adsorbed on Celite was used to convert olive oil to a cocoabutter type fat by interesterification with stearic acid in hexane (Yokozeki *et al*., 1982).

A similar process was developed and patented by scientists at Unilever to convert the mid-fraction of palm oil to a cocoabutter equivalent (Macrae and How, 1988). Thus, 1,3 specific interesterification of 1,3-dipalmitoyl-2-monoleine, which is the major triacylglycerol of palm oil mid-fraction, in the presence of either stearic acid or tristearin yields products rich in 1-palmitoyl-3-stearoyl-2-monoleine and 1,3-distearoyl-2-monoleine that are important components of cocoabutter. For example, the reaction was catalyzed by *A. niger* lipase adsorbed on Celite with the oil and myristic acid dissolved in petroleum ether using a fixed-bed reactor operating at 40 ºC. Analysis showed that the myristic acid content of the triacylglycerols increased from 1% to 18%. They also described methods for incorporation of stearic acid in the triacylglycerols of shea oil using *M. miehei* lipase and a liquid fraction of palm oil using *R. japonicus lipase* with fixed-bed reactors containing the enzymes adsorbed on Celite.

A process was developed by Eigtved (1989) at Novo Industri for immobilization of *M. miehei* lipase by adsorption on a particulate, macroporous phenol-formaldehyde resin. The enzyme, adsorbed on Duolite S-761, was used to interesterify a mixture of olive oil and decanoic acid in the absence of any organic solvent. The support particle size was chosen to optimize surface area available to the enzyme and fixed-bed reactor back pressure which have opposing relationships to particle size. Operating at 60 ºC, the reactor had a half-life of 3100 h and assuming the reactor to be used through two half-lives, the estimated productivity was 6.5 metric tons product/kg of immobilized enzyme.

24.5 Modification of proteins

The nutritional contribution of proteins or their hydrolysates in foods, which is related to their amino acid composition relative to the essential amino acid requirements for humans, is well recognized. Increasingly, however, proteins are used as ingredients in foods to provide specific functional characteristics such as emulsification, foaming, gelation, viscosity and water-holding capability. Functional behavior is determined by the surface topology of the molecule especially with regard to the distribution of charged, polar and nonpolar groups, its structural stability and flexibility, and the type of secondary structural feature exposed in partially unfolded states. All of these properties can be modified, or possibly even designed, by selective proteolysis with specific proteinases under proper conditions.

24.5.1 Extensive hydrolysis

Extensive hydrolysates of proteins are used as food additives and as pharmaceuticals. Any search of the internet will reveal dozens of companies offering a variety of such products. Extensive proteolysis will eliminate the allergenicity of a protein so this approach has been used to produce hypoallergenic products (Cordle, 1994). In the pharmaceutical industry, extensive hydrolysates are used for oral and intravenous feeding (Schmidl *et al*., 1994). Most likely these commercial products are made with mixtures of soluble enzymes; however, use of immobilized forms would be advantageous because the problem of removing the proteinase to avoid contamination of the hydrolysate would be eliminated.

Studies in our laboratory have led to development of an immobilized proteinase/peptidase system capable of complete hydrolysis of substrate proteins (Church *et al*., 1984; Swaisgood and Horton, 1989). It was shown that this system could be used to determine the amino acid composition of a protein. The advantage over the classical method of acid hydrolysis is obvious; acid-labile amino acids Trp, Asn, Gln, Met, and Cys were more accurately determined (Swaisgood and Horton, 1989). The bioreactor contained Pronase (a commercial mixture of four activities from *Streptomyces griseus*), intestinal mucosal peptidases, prolidase, and aminopeptidase P from *E. coli* each immobilized separately by covalent attachment to succinamidopropyl-controlled-pore glass. Hydrolysis was performed by circulation of the substrate in 2 *M* urea through the reactor at 37 ºC for 24 hours. Comparison of the amino acid composition of carboxymethylated β-lactoglobulin determined by standard chromatographic analysis of the enzymic hydrolysate with that expected from the primary structure showed excellent agreement (Swaisgood and Horton, 1989). Similar results were obtained for an enzymic hydrolysate of lysozyme.

A proteinase that may be very useful in preparation of protein hydrolysates because of its general proteolytic activity was discovered in *Bacillus licheniformis* (Williams *et al*., 1990). This enzyme is capable of degrading

feather keratin. A bifunctional fusion protein was designed to allow one-step purification and immobilization of the keratinolytic activity (Wang *et al*., 2003). The protein, with the C-terminus of keratinase linked to the N-terminus of streptavidin or core-streptavidin, was expressed in *Bacillus subtilis* or *E. coli* and bound to biotinylated beads. The immobilized proteinase exhibited good keratinase activity that was more heat stable than the soluble enzyme.

Nutritional analyses of the protein digestibility of foods and feeds is another potential use of immobilized proteinase/peptidase systems (Swaisgood and Catignani, 1991). Enzymes were covalently immobilized on succinamidopropyl or aminopropyl porous glass using water-soluble carbodiimide to activate the carboxyl groups (Porter *et al*., 1984; Chung *et al*., 1986; Chang *et al*., 1990; Thresher *et al*., 1989). Large pore size (200–300 nm) controlled-pore glass was chosen to allow easy access of large protein complexes to the immobilized enzymes. Two biodigestors were used; one containing pepsin operating at low pH to simulate the stomach and the other containing trypsin, chymotrypsin and intestinal mucosal peptidases operating at pH 7.5 to simulate the intestine. The substrates were treated first in the pepsin bioreactor for 18–20 h at 37 ºC followed by a 20–24 h treatment in the intestinal bioreactor at 37 ºC. Advantages of using the immobilized enzymes for this assay include the prevention of autolysis, the digest is not contaminated with the enzymes or autolysis products, the digest is easily separated from the enzymes, and the reactors can be used for numerous assays. Using this assay, a direct relationship was established between the extent of racemization and crosslinking (lysinoalanine formation) and the degree of Maillard reaction and the digestibility (Chung *et al*., 1986; Culver and Swaisgood, 1989). Digestibilities measured by this method have correlated well with *in vivo* digestibilities (Chang *et al*., 1990). Furthermore, in a cooperative study of digestibilities of various foods this method correlated well with other *in vivo* methods determined in various laboratories (Thresher *et al*., 1989). The method has been further developed by Novus International for analysis of animal feeds. Their results obtained by this method were in good agreement with digestibilities determined *in vivo* with poultry.

24.5.2 Limited hydrolysis

Because limited proteolysis changes the primary structure of a protein, the molecular size, structural stability and flexibility, charge distribution and the distribution of polar and nonpolar surface of the products will be changed (Swaisgood *et al*., 1996). Consequently, the functionality of the product also will be changed. However, desirable functionality usually is only achieved when the proteolysis is limited (Waniska *et al*., 1981; Turgeon *et al*., 1992; Saito, 1994; Swaisgood *et al*., 1996; Huang *et al*., 1996a). Moreover, many proteins have multiple domains of tertiary structure separated by flexible links or, if single domain proteins, flexible loops separating regions of secondary or tertiary structure that are most susceptible to cleavage by proteinases. Hence, limited proteolysis can release oligopeptides having secondary or tertiary structure that

can participate in various interactions of importance to functionality in a food. Use of an immobilized proteinase to liberate these functional peptides has several obvious advantages, namely, easy and precise control of the extent of reaction, elimination of an inactivation step that would also denature the oligopeptides and thus reduce their functionality, and prevention of autolysis and hence autolysis products that would otherwise contaminate the products.

A number of studies have examined the modification of protein functionality by treatment with proteinases (e.g., see Puski, 1975; Waniska *et al*., 1981; Jost and Monti, 1982; Kilara, 1986; Lakkis and Villota, 1990; Turgeon *et al*., 1991, 1992). Despite the advantages of the use of an immobilized proteinase and their remarkable stability, very few studies have employed this form for that purpose (Swaisgood *et al*., 1996; Huang *et al*., 1996a, 1997b, 1999). These studies examined the effect of limited proteolysis by immobilized trypsin on the functional properties of β-lactoglobulin and the whey protein fraction. As a major component of the milk whey protein fraction (50%), β-lactoglobulin, which has an anti-parallel, eight-stranded β-barrel structure, is responsible for most of the observable functionality of this fraction. Controlled limited proteolysis releases a large portion of the β-barrel domain, residues 41–100 linked to 149–162 by a disulfide bond, that retains most of its secondary and tertiary structure (Chen *et al*., 1993). However, the stability of this structure was significantly reduced as shown by a 24 ºC lowering of the T_m measured by DSC and a reduction in the midpoint of the urea transition measured by CD spectra from 5.5 *M* to 3.88 *M*. Hence, the structural flexibility would be greatly increased which should have a large impact on its functionality. Using conditions to optimize the production of the domain peptide fraction (2.4% DH, 50% intact protein remaining), these peptides were isolated and characterized (Huang *et al*., 1996a). The emulsifying activity index (EAI) of the domain peptide fraction was twofold higher than that of the intact protein throughout the pH range of 3 to 9 (Fig. 24.2), the emulsified droplets were smaller, and the emulsion was visibly more stable upon storage.

Limited proteolysis of commercial whey protein concentrate under similar conditions also improved the emulsifying activity of the unfractionated mixture, although to a lesser degree. Furthermore, the foaming properties of the enzyme-treated whey protein were improved if the hydrolysis was limited (Huang *et al*., 1997a). When the DH was 2.8%, the foam overrun increased nearly 1.5 times and the stability was similar to the untreated protein; however, with a DH of 4.3% both the foam overrun and the stability decreased markedly (Table 24.2).

Formation of protein gels results from two molecular steps: first the unfolding of the molecule followed by molecular association to form a three-dimensional network. Change in the relative rates of these steps will affect the type of gel formed and it is believed that a rapid unfolding followed by a slower association leads to fine-stranded gels, whereas the reverse gives particulated gels. Consequently, the presence of the domain peptide fraction in whey protein isolate would be expected to alter the gelling properties because of its less stable, more flexible structure and altered surface characteristics. Comparison of the

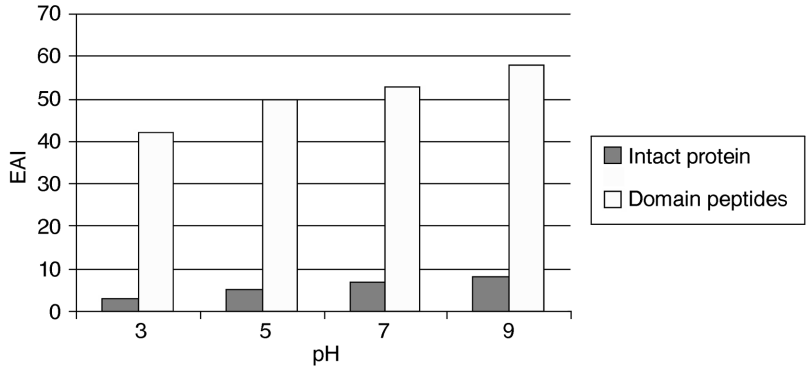

Fig. 24.2 Comparison of the effect of pH on the emulsifying activity of intact β-lactoglobulin and the domain peptides prepared by limited proteolysis with immobilized trypsin. Adapted from data presented by Huang *et al.* (1996a).

thermal gelling properties of 10% protein solutions of enzyme-treated whey protein isolate (EWPI) with untreated whey protein isolate (WPI) showed that the latter formed more rigid and elastic fine-stranded gels, whereas EWPI formed particulated gels (Huang *et al.*, 1999). Furthermore, the EWPI gels exhibited a more sponge-like, porous network that bound water less tightly than the more compact, fine-structured network of the untreated protein gels. Solutions of EWPI exhibited a lower gelation temperature than WPI protein, which began to gel only after holding at 80 °C, but the initial gelation rate was much larger for the WPI solutions (Table 24.3). Results suggested that interaction of the less stable domain peptides with intact protein caused a slight increase in the thermal stability of the protein. These observations are consistent with the lower temperature stability of the domain peptides and suggest their interaction with intact protein led to formation of large aggregates that rapidly associated.

The limited proteolysates used for the characterization described above were obtained with a 500–ml fluidized-bed reactor containing trypsin immobilized on succinamidopropyl-Celite (Huang *et al.*, 1997b). The immobilized enzyme was remarkably stable and the reactor was used over a period of several years

Table 24.2 Foaming characteristics of whey protein isolate (WPI) and enzyme-treated WPI[a]

Protein	Foam overrun (%)	Foam stability (sec)[b]
Whey protein isolate	896	706
EWPI[a] (DH = 2.8)	1232	688
EWPI[a] (DH = 4.3)	647	404

[a]Four ml of 5% protein solutions were evaluated using the micro-scale method developed by Huang *et al.* (1997a). Enzyme-treated WPI: EWPI.
[b]The time for drainage of 50% of the initial weight was used to indicate stability.

Table 24.3 Gelling characteristics of whey protein isolate (WPI) and enzyme-treated WPI[a]

Protein	G′ (kPA)[b] 25 ºC	Gel point (ºC)[c]	Initial gel rate (Pa/min.)	Hardness[d]	Cohesiveness[d]	Gumminess[d]
WPI	7.4	80	89.1	199	0.17	34
EWPI	2.0	77.1	10.7	1608	0.42	717

[a]Adapted from data presented by Huang *et al*. (1999). Data were obtained with 10% protein solutions in 50 m*M* TES buffer, pH 7.0, containing 50 m*M* NaCl.
[b]Gel was formed at 80 ºC and cooled to 25 ºC for this measurement.
[c]The WPI gel formed after holding for 1.4 min. at 80 ºC.
[d]Results from Texture Profile Analysis using a TA-XT2 Texture Analyzer.

without substantial loss of activity. More recently, immobilized tryptic activity was prepared by bioselective adsorption of a bifunctional recombinant fusion protein, trypsin-streptavidin (Clare *et al*., 2001). A bioreactor was prepared by exposure of crude cell extract to biotinylated porous beads; thus, purification and immobilization were obtained in a single step. Characterization of this prototype demonstrates the potential to design recombinant enzymes for single-step purification and immobilization thus greatly reducing the cost of bioreactor preparation and regeneration.

24.5.3 Transglutaminase-catalyzed crosslinking

Proteins can be crosslinked enzymatically using transglutaminase (R-glutaminyl-peptide:amine-γ-glutamyl-transferase, EC 2.3.2.13) to catalyze the acyl transfer between the γ-carboxamide group of glutaminyl residues in proteins and the ϵ-amino group of lysyl residues forming an isopeptide bond. Such reactions can be more extensive than crosslinking by sulfhydryl-disulfide interchange due to the higher frequencies of the former residues in most proteins. It is not possible to limit the degree of crosslinking without some type of inactivation step if the soluble enzyme is used. In many cases, uncontrolled crosslinking is undesirable because of the formation of large protein networks and eventual gel formation. However, limited crosslinking can dramatically affect functionality such as viscosity and gel formation.

For maximum activity of the immobilized guinea pig liver transglutaminase, it was found necessary to include a protein spacer between the surface and the enzyme (Oh *et al*., 1993; Huang *et al*., 1995). For example, the biotinylated enzyme was adsorbed to avidin previously immobilized by adsorption to biotinylated porous glass (Huang *et al*., 1995). Activity was examined with a small peptide substrate and two protein substrates. Because flexibility of the substrate protein is essential for activity, α_S-casein is an excellent substrate; whereas, β-lactoglobulin must be reduced to destabilize its structure. The presence of Ca^{2+} in the reaction mixture causes α_S-casein to exist as large molecular

complexes resulting in pore diffusion limitations in the 200 nm pore diameter beads. Consequently, the catalytic efficiency was significantly reduced with this protein; however, an advantage of the pore diffusion limitation is that the dependence of the activity on pH and temperature is also greatly reduced (Huang *et al*., 1995). Treatment of the protein caused rapid formation of large polymers that could not penetrate standard SDS-PAGE gels and all of the monomer protein had disappeared. Similar results were observed with reduced β-lactoglobulin or WPI as substrate. Analysis of the reaction mixture by size-exclusion chromatography indicated rapid formation of large polymers (Fig. 24.3a and b).

Mammalian transglutaminase also was immobilized by direct bioselective adsorption from crude cell lysates as described above for trypsin-streptavidin (Wilcox *et al*., 2002). The 3′-end of the core streptavidin gene, coding for residues 14–136 that includes the biotin-binding β-barrel region, was linked to the 5′-end of the gene for the mature transglutaminase protein and the fusion protein was expressed in *E. coli*. Thus, the C-terminus of core streptavidin was linked to the N-terminus of transglutaminase in the expressed bifunctional protein. The functionality of commercial WPI following treatment of 10% protein solutions at pH 6.8 in 10 m*M* DTT and 5 m*M* Ca^{2+} at 40 ºC was examined (Wilcox *et al*., 2002). The intrinsic viscosity of the WPI protein increased dramatically with increasing time of crosslinking (Table 24.4). Also, the temperature of initiation of gelation decreased slightly with increasing crosslinking for these limited crosslinking treatments. Recent studies have indicated that more extensive crosslinking actually increases the gelation temperature. Results of vane analysis of the gels formed (Table 24.4) show that dramatically different types of gels can be obtained ranging from brittle, strong gels to weak, flexible gels.

The gene for microbial transglutaminase was not available to the authors (Wilcox *et al*. 2002), so the commercially available microbial transglutaminase was immobilized by adsorption of the biotinylated enzyme to previously immobilized avidin as described above for the mammalian enzyme. The bioreactors prepared were used to crosslink 8% protein solutions of WPI under conditions described previously. Rheological analysis of the crosslinked WPI gave results similar to those obtained with treatment by the immobilized

Table 24.4 Rheological properties of WPI treated with immobilized mammalian transglutaminase[a, b]

Treatment	Intrinsic viscosity (ml/g)	Gelation temperature °C	Vane fracture analysis	
			Stress (Pa)	Deformation
None	0.6	70.3	3047	1.6
1 Hour	36.5	66.3	4096	1.2
2 Hours	50.7	66.2	–	–
4 Hours	81.1	65.9	–	–

[a]Gels were formed with 10% protein solutions.
[b]Adapted from data given by Wilcox *et al*. (2002).

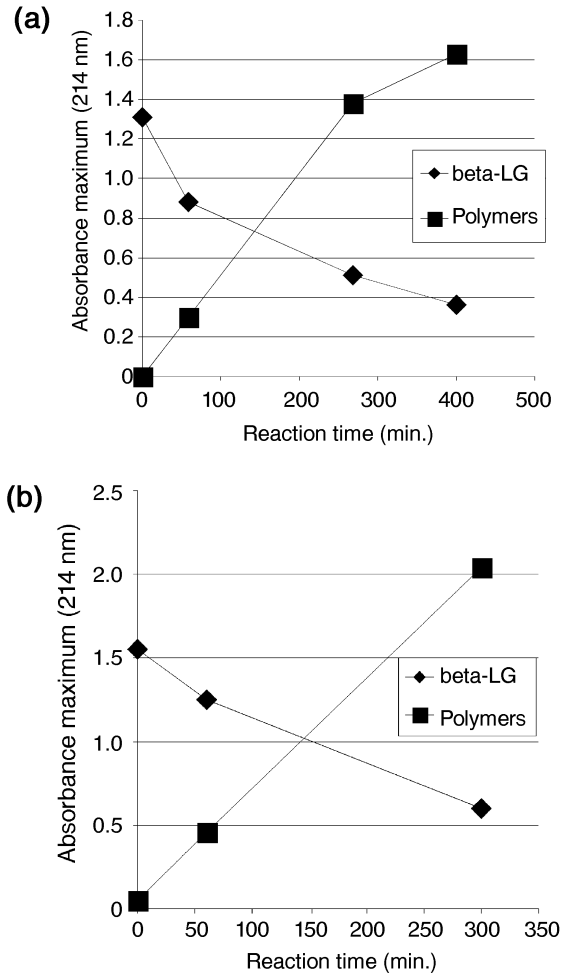

Fig. 24.3 Crosslinking catalyzed by guinea pig liver transglutaminase immobilized by adsorption of the biotinylated enzyme on avidin that had been previously adsorbed to biotinylated 200 nm pore diameter controlled-pore glass. Solutions were prepared in 25 mM imidazole buffer, pH 7.5, containing 10 mM DTT and 5 mM $CaCl_2$ and analyzed by size-exclusion chromatography. (a), Progress curves for the disappearance of native protein (◆) and the appearance of polymers larger than 100 kDa (■) during treatment of β-lactoglobulin. (b), Progress curves for the disappearance of native β-lactoglobulin (◆) and the appearance of polymers larger than 100 kDa (■) during treatment of WPI solutions.

mammalian enzyme (Wilcox and Swaisgood, 2002). Large increases in intrinsic viscosity, decreases in the gelation temperature, and large changes in the gel characteristics were observed that depended upon the length of treatment.

Results of these studies of immobilized transglutaminase demonstrate the advantages of control of the extent of crosslinking. This is readily accomplished with the immobilized form. Under carefully controlled conditions it may be

possible to design the functionality of a protein ingredient for a specific application. Stability of the immobilized activity still remains a problem, which is most likely caused by the large size of the crosslinked protein resulting in increasingly restricted access to the pore volume. This problem may be solved by using very large pore diameter beads or nonporous magnetic beads.

24.6 Future trends

The ability to produce a new product, especially one that cannot be produced of the same quality by other means, is the primary driving force in the development of new immobilized enzyme processes. Modification of protein ingredients to design functionality, such as viscosity, emulsifying, gelling or foaming properties, for specific applications is an area of potential development. Use of an immobilized proteinase to achieve controlled limited proteolysis has unique potential for this application. Choice of the proteinase and control of the extent of reaction without the requirement of a downstream inactivation step that denatures functional peptide structures allows opportunities for designing unique functionalities. Transglutaminase-catalyzed crosslinking dramatically affects viscosity and gel-forming properties of protein ingredients allowing a range of characteristics to be produced if the reaction can be precisely controlled. Some of the examples described above demonstrate these possibilities. A similar argument can be made for flavor development in a food product or ingredient.

Recent discovery and cloning of enzymes from extremophiles (Niehaus *et al*., 1999) has great potential for development of immobilized enzyme processes. For example, a glucose isomerase capable of operating at 80–90 ºC would shift the equilibrium towards fructose and perhaps eliminate the required chromatographic fractionation. Also, many processing operations could be carried out at higher temperatures if the immobilized enzyme was more heat stable, thus eliminating required sanitation steps and increasing the reaction rate, as well as decreasing the pore diffusion limitations by decreasing the viscosity.

The development of recombinant enzymes and the cloning of enzymes from extremophiles into organisms commonly used for fermentations provide the opportunity to design for specific catalytic requirements such as pH and temperature optima and substrate specificity. At the same time it is possible to design for enzyme isolation, purification and immobilization. We have demonstrated that all of these can be achieved in a single step by designing a bifunctional fusion protein with a domain that has high affinity for a covalently immobilized small ligand. Streptavidin was chosen as the affinity domain because of its very high affinity for biotin, which is easily covalently attached to a variety of surfaces (Walsh and Swaisgood, 1994; Lee and Swaisgood, 1998; Koo *et al*., 1998; Clare *et al*., 2001). This technology permits the enzyme to be purified and immobilized from crude cell lysates. When the activity of the bioreactor has decayed, the reactor can be regenerated by desorption of the inactive enzyme and fresh enzyme can be selectively bound (Huang *et al*., 1996b).

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