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**STUDIES ON HEAT- AND PRESSURE-INDUCED
INTERACTIONS OF MILK PROTEINS**



Massey University

**A thesis presented in partial fulfilment of the requirements
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ABSTRACT

The present study was aimed at understanding the high pressure (HP) processing-induced interactions of milk proteins in whey protein concentrate (WPC) solutions, in skim milk and in pure protein systems. The changes in milk proteins induced by heat treatments in the same systems under selected conditions were also evaluated.

The main approach taken was to elucidate changes in the whey proteins in heat- and pressure-treated samples from common aliquots, under identical conditions, using various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques in the absence or presence of a disulphide bond reducing agent. In some instances, the samples were also analysed using small deformation rheology, size exclusion chromatography (SEC) and transmission electron microscopy (TEM).

The results of the present study indicated that, in general terms, heat treatment and HP treatment had common effects, i.e. denaturation and subsequent aggregation of whey proteins. Both heat treatment and HP treatment generated disulphide-bonded and hydrophobically bonded aggregates of whey proteins. However, the sensitivities of each of the whey proteins to heat treatment [immunoglobulin (Ig) > lactoferrin (LF) > bovine serum albumin (BSA) > β -lactoglobulin B (β -LG B) > β -LG A > α -lactalbumin (α -LA)] and pressure treatment (β -LG B > β -LG A > IgG > LF > BSA > α -LA) were considerably different. Also, HP treatment generated a comparatively greater proportion of smaller aggregates than did heat treatment.

The effects of protein concentration, intensity of pressure treatment, holding time and pressurising temperature on whey protein aggregation in WPC solutions were investigated. The rate of aggregation of whey proteins increased with an increase in the concentration of protein in the WPC solution and the pressurising temperature. The combination of low protein concentration, mild pressure treatment (200 MPa) and low pressurising temperature (20°C) led to minimal loss of native-like and SDS-monomeric β -LG, whereas the combination of high protein concentration, severe pressure treatment (600 MPa) and higher pressuring temperature (40°C and higher) led to significant loss of both native-like and SDS-monomeric β -LG. The sensitivity of pressure-resistant

whey proteins, such as α -LA and BSA, to the aggregation was significantly increased at pressurising temperatures of 40°C and higher. Self-supporting gels were formed when 8 or 12% (w/v) WPC solutions were pressure treated at 600–800 MPa, 20°C. Detailed analysis of the behaviour of the proteins during the formation of these gels revealed a novel pathway, suggesting that intermolecular disulphide bond formation occurred at high pressure but that hydrophobic association became important after the HP treatment.

In the later part of the study, heat- and HP-induced interactions of caseins and whey proteins were studied in a more complex system, i.e. skim milk. With the application of modified PAGE techniques, it was possible to show that the high molecular weight disulphide-bonded aggregates that were formed by HP treatment of skim milk contained disulphide-linked complexes consisting of α_{s2} -casein (α_{s2} -CN) as well as κ -CN, β -LG and other whey proteins. The results showed that the effects of heat treatment and HP on the interactions of the caseins and whey proteins in milk were significantly different. The accessibility of α_{s2} -CN and the formation of complexes involving α_{s2} -CN, κ -CN and whey proteins in the HP-treated milk, as demonstrated using the modified 2D PAGE technique, and as explained by possible proposed reactions of the caseins and whey proteins in pressure-treated milk, was an important finding of the present study.

Finally, a study on the effects of HP treatment in model systems using pure proteins in solution, both singly or in binary and ternary combinations, generated very useful information and clarified the role of each protein in pressure-induced aggregation and interactions of milk proteins in complex systems such as WPC and milk. It was found that the reactions of β -LG were not significantly affected by other proteins such as α -LA or BSA, but that the presence of β -LG in the system catalysed the reactions of other proteins such as α -LA or BSA.

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CHAPTER 1

Introduction

Heat treatment is one of the most widely used conventional methods of food preservation. In the dairy industry, heat treatments are employed mainly to improve the keeping quality of milk and milk products by the destruction of microorganisms or to modify the functional properties of milk products.

The proteins of milk play an important role in the heat-induced functionality of many dairy products. Some well-known examples of the beneficial effects of the heat-induced functionality of milk proteins, which have recently been reviewed, include heat-induced denaturation, aggregation and gelation of whey proteins (Singh & Havea, 2003), heat stability of milk (O'Connell & Fox, 2003), protein stability in sterilised milk and milk products (Nieuwenhuijse & Van Boekel, 2003), functional properties of milk powder (Kelly et al., 2003) and improvement in yoghurt texture (Lucey & Singh, 2003). To understand the mechanisms of the heat-induced functionality of milk products, various aspects of the heat-induced denaturation, aggregation and gelation of whey proteins (e.g. Donovan & Mulvihill, 1987; McSwiney et al., 1994a, 1994b; Gezimati et al., 1996, 1997; Prabhakaran & Damodaran, 1997; Manderson et al., 1998, 1999; Havea et al., 1998, 2000, 2001, 2002; Schokker et al., 1999, 2000; Hong & Creamer, 2002) and their interactions with casein micelles (Singh & Fox, 1985a, 1985b, 1987a, 1987b; Dalgleish et al., 1987, 1997; Haque et al., 1987; Haque & Kinsella, 1988; Hill, 1989; Singh & Creamer, 1991, 1992; Singh, 1995; Corredig & Dalgleish, 1996, 1999; Holt & Horne, 1996; Oldfield et al., 1998, 2000; Anema & Li, 2003a, 2003b; Cho et al., 2003) have been studied extensively using model systems consisting of pure protein(s), solutions of commercial whey protein concentrates (WPCs), and isolates (WPI), and fresh or reconstituted skim milks. Most of these studies concluded that thiol/disulphide interchange reactions, leading to the formation of intermolecular disulphide bonds, play an important role in the heat-induced aggregation of β -lactoglobulin (β -LG) and its interaction with other proteins, including caseins. Some of these studies also reported that, as well as the aggregation by intermolecular covalent (disulphide) bonds, non-covalent interactions (such as hydrophobic or ionic interactions) are also involved in the heat-induced interactions of milk proteins.

Although heat treatment is an essential unit operation in the manufacture of milk and milk products, severe heat treatments often cause unwanted changes to the sensory and nutritional quality of the final products. Moreover, non-thermal technologies, such as high pressure (HP) processing, are gaining popularity and wide acceptance for food processing and preservation, because of increased consumer demand for microbiologically safe, nutritious and 'fresh-like' food products with an acceptable shelf life. In addition to food preservation, HP processing has also been recognised as a physical tool for the modification of proteins (Balny & Masson, 1993; Buchheim, 1998; Balci & Wilbey, 1999), and has the potential to produce new products with modified textures and functional properties (e.g. Balny & Masson, 1993; Datta & Deeth, 1999, 2003; Huppertz et al., 2002). Most of the previous research on the effects of HP on milk proteins has been concentrated on denaturation kinetics, rheological or microstructural aspects of individual proteins (mainly β -LG) in various systems, including model systems consisting of pure proteins, solutions of WPC and milk (e.g. Dumay et al., 1994a, 1994b, 1998; Funtenberger et al., 1995, 1997; Van Camp & Huyghebaert, 1995a, 1995b; Hinrichs et al., 1996; López-Fandiño et al., 1996; Anema et al., 1997; Iametti et al., 1997; Jegouic et al., 1996, 1997; Van Camp et al., 1997a, 1997b; Walkenström & Hermansson, 1997; Kanno et al., 1998; Botelho et al., 2000; Needs et al., 2000a, 2000b; Huppertz et al., 2004a-d,). Another well-studied aspect is the effects of HP processing on casein micelles and the properties of milk (Desobry-Banon et al., 1994; Lee et al., 1996; López-Fandiño et al., 1996, 1997, 1998; Anema et al., 1997; 2005a-2005c; López-Fandiño & Olano, 1998a, 1998b; Arias et al., 2000; Huppertz et al., 2002; 2004a-d; 2005; 2006a-c). However, no specific studies have been reported on the detailed characterisation of the pressure-induced aggregation or interaction products and the type of molecular forces involved in their stabilisation in complex systems (such as in WPC or in milk). Thus, the pathways of the pressure-induced aggregation of whey proteins and their interaction products remain largely unclear.

Various two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques were found to be useful tools in the characterisation of the heat-induced aggregates and for understanding the mechanism of the aggregation and gelation of whey proteins (Havea et al., 1998, 2000, 2001). Therefore, the present study was aimed at providing greater understanding of HP-induced interactions of milk proteins in various systems, including WPC solutions, milk and pure protein systems by successful applications of such 2D PAGE techniques in combination with other available techniques.

CHAPTER 2

Literature Review¹

2.1 Introduction

Heat treatment of milk is one of the most widely used unit operations in the manufacture of dairy and food products. The reason for the heat treatment of milk or milk products usually falls into one of two groups – microbiological (destruction of microorganisms for food safety or shelf-life extension) and technological (functionality or product quality) by selectively denaturing the whey proteins (Jelen & Rattray, 1995; Singh, 1995). Some examples of the heat treatments commonly used in dairy processing are listed in Table 2.1 (Jelen & Rattray, 1995; Singh, 1995).

Table 2.1. Common heat treatments employed in the commercial processing of milk and milk products (adapted from Jelen & Rattray, 1995)

| Heat treatment | Conditions |
|---|--------------------|
| Thermisation | 65°C/30 s |
| Pasteurisation | 72°C/15 s |
| Preheat treatment for milk powder | 80–120°C/2–10 min |
| Preheat treatment for yoghurt manufacture | 90°C/5–10 min |
| UHT sterilisation | 140°C/3–20 s |
| Sterilisation (batch/in-container) | 110–120°C/5–20 min |

The heat-induced interactions of milk proteins are of considerable importance and have an impact on the functionality of the final products. For example, preheat treatments are important in the manufacture of yoghurt and in the manufacture of heat-stable milk powders intended for use in recombined evaporated milk, bakery and confectionery products. In studies of heat-induced functionality, the effects of heat treatments on milk proteins (denaturation, aggregation and gelation of whey proteins) and protein–protein interactions, including interactions of caseins and whey proteins, have been studied in great detail over the last 5–6 decades and the subject has been reviewed several times

¹ Part of the content presented in this chapter has been published as a peer-reviewed review paper in *Innovative Food Science and Emerging Technologies* (2007) **8**, 1-23.

(e.g. Mulvihill & Donovan, 1987; de Wit, 1990; Jelen & Rattray, 1995; Singh, 1995; O'Connell & Fox, 2003; Singh & Havea, 2003).

However, heating may also affect the textural, nutritional as well as changes in organoleptic quality (taste, flavour and colour) of the final product. The latter effects are often considered to be disadvantages of heat treatment. The application of high pressure (HP), rather than heat, to food enables the destruction of microorganisms without causing significant changes to the colour, flavour and nutritional attributes of the food. HP offers uniform and instant treatment of the product, and has the potential for the design of new products due to the creation of new textures and functional properties (Datta & Deeth, 1999; 2003). Considering this advantage, HP has been successfully applied to the commercial processing and preservation of fresh fruit juices, jams, jellies, salad dressings etc. (Mertens & Deplace, 1993; Huppertz et al., 2002; Trujillo et al., 2002; Claeys et al., 2003). Recently, HP has also been considered for the processing of dairy products, and thus the modification of the protein structure and the functional properties as a result of HP processing is particularly important for its application to dairy products (Needs et al., 2000a, 2000b).

The aim of the present chapter is to review relevant aspects of the effects of heat treatment and pressure treatment on bovine milk proteins, focusing mainly on the effects of HP on the pressure-induced denaturation, aggregation and gelation of whey proteins, and their interactions with casein. General aspects of milk and milk proteins are summarised in the initial part, then, in the latter part of this review, the focus is narrowed to discuss more specific subjects related to the effects of heat and HP on milk proteins. This review covers primarily the literature reported prior to 2003, when this project was initiated. However, relevant recent work is discussed in appropriate results and discussion chapters.

2.2 Composition of milk

Milk is a complex biological fluid containing relatively high amounts of proteins and minerals. The major components of milk are water, fat, lactose, proteins and minerals in order of decreasing proportion. Water is the continuous phase in which other constituents are either dissolved or suspended. Lactose and a portion of the mineral salts

are found in solution; proteins and the remainder of the minerals are found in a colloidal suspension; and fat (98% triglycerides) is found as an emulsion stabilised by a phospholipid and glycoprotein membrane. The average composition of raw bovine milk is presented in Table 2.2.

Table 2.2. Average composition of raw bovine milk (adapted from Walstra & Jenness, 1984)

| Component | % (w/w) in milk |
|-----------------|-----------------|
| Water | 87.30 |
| Fat | 4.60 |
| Lactose | 3.90 |
| Proteins | 3.25 |
| Casein proteins | 2.60 |
| Whey proteins | 0.65 |
| Minerals | 0.65 |
| Organic acids | 0.18 |

2.3 Milk proteins

2.3.1 Protein structure and stabilising forces

The proteins are composed of amino acids. The particular sequence of amino acids in a protein determines its structure, conformation and properties. Depending on the amino acids present, different conformational forms, which are stabilised by various molecular forces, are possible (Figure 2.1) (Privalov & Gill, 1988; Paulsson, 1990; Singh & Havea, 2003). The basic level or primary structure is the specific amino acid sequence along the covalently linked polypeptide chain. As a result of molecular forces being created between the amino acid side chains, the primary structure will fold in an ordered fashion, forming the secondary and tertiary structures that give rise to a uniquely folded native structure that possesses the lowest feasible free energy. The most abundant regular secondary structures found in proteins are α -helix and β -pleated sheet. The α -helix is emerged by a helical coiling of the amino acid chain and is stabilised by hydrogen bonding between the atoms of the peptide bonds. β -Sheets are formed by a linear alignment of some arrays of the amino acid chain. This structure is also stabilised

by hydrogen bonding between the strands. The tertiary structure is the three-dimensional arrangement of the several arrays that are present within the protein. The tertiary structure of a globular protein refers to the three-dimensional chain fold, with structural domains as spatially separated entities. The contributions of inter- and intramolecular interactions are balanced in such a delicate manner that a three-dimensional structure is formed. The energetic contributions to protein stability involve intermolecular forces acting between groups within the protein and interaction with water. These determine the unique three-dimensional structure. The existence of a three-dimensional, folded protein structure depends on several forces. These include hydrogen bonding, hydrophobic interactions, van der Waals' forces and electrostatic interactions (Figure 2.1).

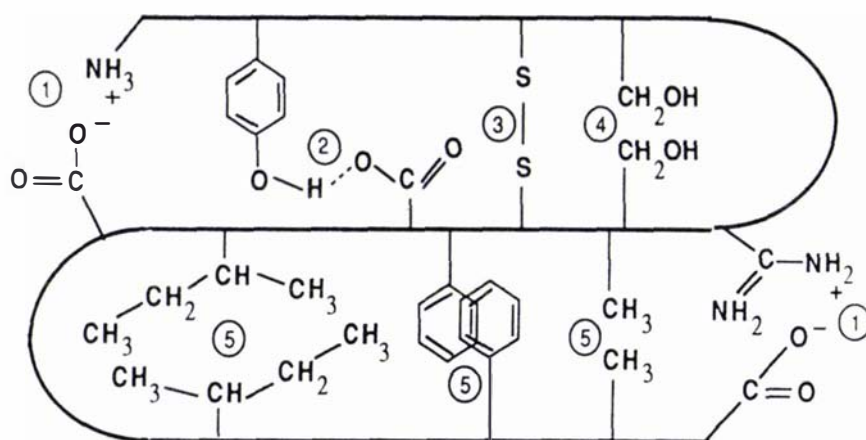


Figure 2.1. Schematic diagram showing stabilising forces in proteins: (1) electrostatic interactions; (2) hydrogen bonds; (3) disulphide bonds; (4) dipole-dipole interactions; (5) hydrophobic interactions (adapted from Paulsson, 1990).

Some of the residues of the amino acids may exhibit a hydrophobic character; electrostatic forces are based on interactions between charged residues. Some proteins contain two or more polypeptide chains. The result of interactions between these components is the quaternary structure, which under normal physiological conditions, many a time referred to as the 'native state'. Thus, the quaternary structure refers to a super-assembly of individual protein molecules, which is formed by the spatial arrangement by non-covalent interactions into a multimeric protein (Paulsson, 1990).

Milk proteins influence the behaviour and properties of dairy or food products and are generally of great importance to human nutrition. Normal bovine milk contains 30–35 g protein/L and can be broadly classified into two main categories, namely casein proteins and whey proteins (Walstra & Jenness, 1984; Fox, 2003). The relevant aspects of casein and whey proteins are discussed here under:

2.3.2 Caseins

Casein proteins (CNs) account for about 80% of the total milk protein. Originally defined as the phosphoproteins precipitated from milk at pH 4.6, casein are generally classified into four different types: α_{S1} -CN, α_{S2} -CN, β -CN and κ -CN (Swaisgood, 2003); each of which consists of a single polypeptide chain (Horne, 2003) and represents a different gene product. Each casein has a different sequence and different properties from the other caseins and, within each casein category, there are a number of separate protein species. This microheterogeneity is caused by various levels of post-translational phosphorylation of serine (or threonine) and/or glycosylation of threonine residues, by various mutational changes or genetically controlled amino acid substitution (genetic polymorphism) in the casein genes, by proteolysis by the indigenous milk enzymes or by oxidation of cysteine's sulphhydryl groups to disulphide bonds (Fox, 2003; Swaisgood, 2003). Most notable are their various levels of phosphorylation and the proteolytic action of plasmin on β -CN, yielding γ -CN and proteose-peptones (Swaisgood, 2003). All caseins are phosphorylated but to various extents. Genetic variants of κ -, α_{S1} - and β -CN usually contain a characteristic number of phosphoserine residues, namely 1, 8 or 9 and 4 or 5 respectively (Whitney, 1988; Farrell et al., 2004). The binding of Ca^{2+} to caseins is mainly through phosphoserine residues and also via the carboxylic acid side chains. Many of the technologically important properties of milk, such as heat stability, rennet coagulability and the strength and syneresis properties of rennet gels are strongly influenced by Ca^{2+} . Another post-translational modification of interest is the formation of disulphide bonds. The two cysteinyl residues in α_{S2} -CN occur in both intramolecular and intermolecular disulphide bonds, aligned in either an anti-parallel configuration or a parallel configuration (Rasmussen et al., 1992a, 1992b, 1994). The disulphide bonds in κ -CN are intermolecular, resulting in the formation of polymers (Groves et al., 1992, 1998). Caseins are hydrophobic, have a relatively high charge and contain many proline

residues but few cysteine residues (Swaisgood, 2003). All the caseins have molecular weight in the range of $\approx 20\text{--}24$ kDa.

In fresh milk, the caseins are present in the form of essentially spherical particles and exist primarily in colloidal form, containing many protein molecules and ranging in size from 15 to about 1000 nm (with an average range of 50–300 nm) in diameter. These particles are known as casein micelles. Casein micelles are colloidal aggregates of the caseins and mineral calcium phosphate. The dry matter of the micelles is $\approx 94\%$ protein and 6% colloidal calcium phosphate; generally known as CCP (Fox, 2003). Apart from κ -CN, which is mostly on or near the external surface of the micelle, the caseins appear to be more or less evenly distributed within the micelle (Creamer, 2003). The surface of the micelle consists primarily of κ -CN, with the hydrophilic C-terminal region of κ -CN protruding from the surface, as a polyelectrolyte brush, which provides electrostatic and steric repulsion, thereby preventing inter-micellar aggregation (de Kruif & Zhulina, 1996). The enzymatic hydrolysis or the acid-induced neutralisation of the proteins in this polyelectrolyte brush allows the conversion of milk into a cheese curd or a yoghurt gel respectively. A detailed overview of the structure and properties of casein micelles has been published recently (de Kruif & Holt, 2003).

Various models of casein micelle structure have been proposed since the initial reports (Rose, 1969; Garnier & Ribadeau Dumas, 1970; Garnier, 1973; Waugh et al., 1970); the models have been progressively refined and reviewed as new research data has become available. Some of these models include subunit models where that the subunits are linked only through CCP (Schmidt, 1982) or are held together by both hydrophobic bonding and CCP (Rollema, 1992). A subunit model of Walstra (1999) based on Waugh (1970)'s model proposed that, instead of subunits linked by CCP, as in earlier models, the CCP is located as a number of discrete packages within the subunits. Horne (1998) proposed that the network is cross-linked by hydrophobic bonding between casein chains and salt bridging between the calcium-sensitive casein and CCP. Several different criticisms were associated with the earlier models and it seems that these earlier models were not consistent with the dissociation behavior of the casein micelles resulting from the addition of urea or excess κ -CN (Horne, 1998; Walstra, 1999; Horne, 2003). Other models proposed in the interim include the Holt model and the dual binding model for micelle assembly and structure. All these models proposed for casein

micelle structure have been reviewed in detail (Holt et al., 1998; de Kruif & Holt, 2003; Fox, 2003).

It has been suggested that the framework of the casein micelles is formed by so-called nanoclusters (Holt et al., 1998). Certain highly phosphorylated phosphopeptides derived from the calcium-sensitive casein will combine with amorphous calcium phosphate to form defined chemical complexes called calcium phosphate nanoclusters. The nanoclusters are composed of an amorphous CCP core, with a radius of ≈ 2.3 nm and surrounded by a shell of phosphopeptides (caseins), which contribute phosphoseryl clusters (Holt et al., 1998). A micelle with a radius of 100 nm and a mass of 10^6 kDa contains nearly 800 nanoclusters (Holt et al., 2003). The nanoclusters are distributed more or less homogeneously throughout the micelle and are linked presumably through hydrophobic bonding and electrostatic interactions.

2.3.3 Whey proteins

Whey proteins or milk serum proteins are the proteins that remain soluble after the isoelectric precipitation of casein at pH 4.6 at 20°C or after the coagulation of casein by limited proteolysis with rennet (Donovan & Mulvihill, 1987; Jelen & Rattray, 1995) to give acid whey or sweet whey respectively. The average compositions of these two whey types are shown in Table 2.3.

Whey proteins represent $\approx 20\%$ (i.e. 5–7 g/L) of the total nitrogenous material in bovine milk. The principal whey proteins are β -lactoglobulin (β -LG), α -lactalbumin (α -LA), bovine serum albumin (BSA) and immunoglobulins (Igs) in decreasing order of whey protein concentration (Singh & Havea, 2003). Whey also contains various other components including lactose, lipids and minerals. Whey proteins are mainly globular with a rather uniform distribution of hydrophobic/hydrophilic amino acids along their polypeptide chains (in contrast to caseins) and lack the amphiphilic nature of casein monomer subunits, a feature that confers on them many unique functional properties (Mulvihill & Donovan, 1987). The substantially lower proline content of the whey protein molecules permits a globular conformation with a large amount of helical content, which again explains their strong susceptibility to denaturation by heat (Hambling et al., 1992; Sawyer, 2003).

Table 2.3. Protein compositions of sweet whey and acid whey (adapted from Mulvihill & Donovan, 1987; Pearce, 1989; Jelen & Rattray, 1995)

| Protein | % of total whey protein | |
|--------------------------------|-------------------------|------------|
| | Acid whey | Sweet whey |
| β -Lactoglobulin | 54 | 45 |
| α -Lactalbumin | 23 | 18 |
| Bovine serum albumin | 6 | 5 |
| Immunoglobulins | 6 | 5 |
| Casein-derived peptides | 2 | 20 |
| Enzymes | 2 | 2 |
| Phospholipid–protein complexes | 5 | 5 |

Most of the whey proteins are sold as commercial products, such as whey powders, whey protein concentrates (WPCs) and whey protein isolates (WPIs). WPC and WPI are regarded as valuable ingredients in the food industry because of their high nutritional value and important functional properties, such as emulsification, solubility and ability to form heat- or pressure-induced gels (Mulvihill & Fox, 1989; de Wit, 1990; Mulvihill, 1992). The average chemical composition of commercial WPCs varies widely (Morr & Foegeding, 1990; Huffman, 1996), as affected by various factors such as seasonal variation, the type of whey (whey source) and the processing methods used to manufacture the WPC. The average concentrations of the protein fractions in WPCs manufactured from acid whey and sweet whey are shown in Table 2.4.

Table 2.4. Average concentration of protein fractions (% of total protein in dry matter) in WPC80 manufactured using cheese (sweet whey) and casein (acid whey) (adapted from Huffman, 1996)

| | Cheese WPC | Casein WPC |
|------------------------|------------|------------|
| β -Lactoglobulin | 52 | 65 |
| α -Lactalbumin | 15 | 21 |
| Bovine serum albumin | 2 | 4 |
| Immunoglobulins | 5 | 10 |
| Glycomacropeptide | 26 | 0 |

β -Lactoglobulin

β -LG is a typical globular protein, and represents \approx 50% of the whey proteins (Table 2.3). It contains 162 amino acid residues, has a monomeric molecular weight of \approx 18 300 Da and is usually present as a stable dimer between pH 5.5 and pH 7.5 (Hambling et al., 1992; Sawyer, 2003). It is well known that, depending on the pH, ionic strength and temperature, β -LG may exist as a monomer or as a dimer and also undergoes reversible and irreversible conformational changes (Nielsen et al., 1996). Because of its dominant role in denaturation, aggregation and gelation, a great deal of research has been done to elucidate the properties of β -LG, which is considered to be representative of WPC or WPI and therefore is considered to be an ideal model whey protein for gaining a better understanding of the whey proteins in general; thus, the physico-chemical properties of β -LG have been reviewed several times in the last 3–4 decades (e.g. Tilley, 1960; Townsend et al., 1969; McKenzie, 1971; Lyster, 1972; Kinsella & Whitehead, 1989; Hambling et al., 1992; Fox, 1995; Sawyer, 2003).

Native β -LG contains two disulphide bonds and a free thiol group, which is buried within the protein structure (Hambling et al., 1992; see also Figure 2.2). It has been confirmed that one disulphide bond occurs between residues 106 and 119 and the free thiol occurs at residue 121 (Creamer & Sawyer, 2003). The other disulphide bond is between residues 66 and 160 (Figure 2.2A). The presence of disulphides and a single sulphhydryl has important consequences from both a structural viewpoint and a functional viewpoint. β -LG has also been reported to be a member of a family of lipid-binding proteins (Frapin et al., 1993; Flower, 1996; Sawyer, 2003).

Several polymorphic forms of β -LG have been reported (Creamer & Harris, 1997; Sawyer, 2003), of which the A and B variants are the most common in western breeds (Ng-Kwai-Hang & Grosclaude, 2003; Farrell et al., 2004) and occur at almost equal frequency (Hambling et al., 1992). β -LG A and β -LG B differ at positions 64 and 118, where Asp and Val in β -LG A are replaced by Gly and Ala in β -LG B (Hambling et al., 1992; Farrell et al., 2004).

The first medium resolution three-dimensional structure of β -LG at 2.8 Å was reported by Papiz et al. (1986). The structure was subsequently refined considerably by

Brownlow et al. (1997) to 1.8 and Jayat et al. (2004), who reported resolution to 1.95 Å and provided an independent view of the dimer structure.

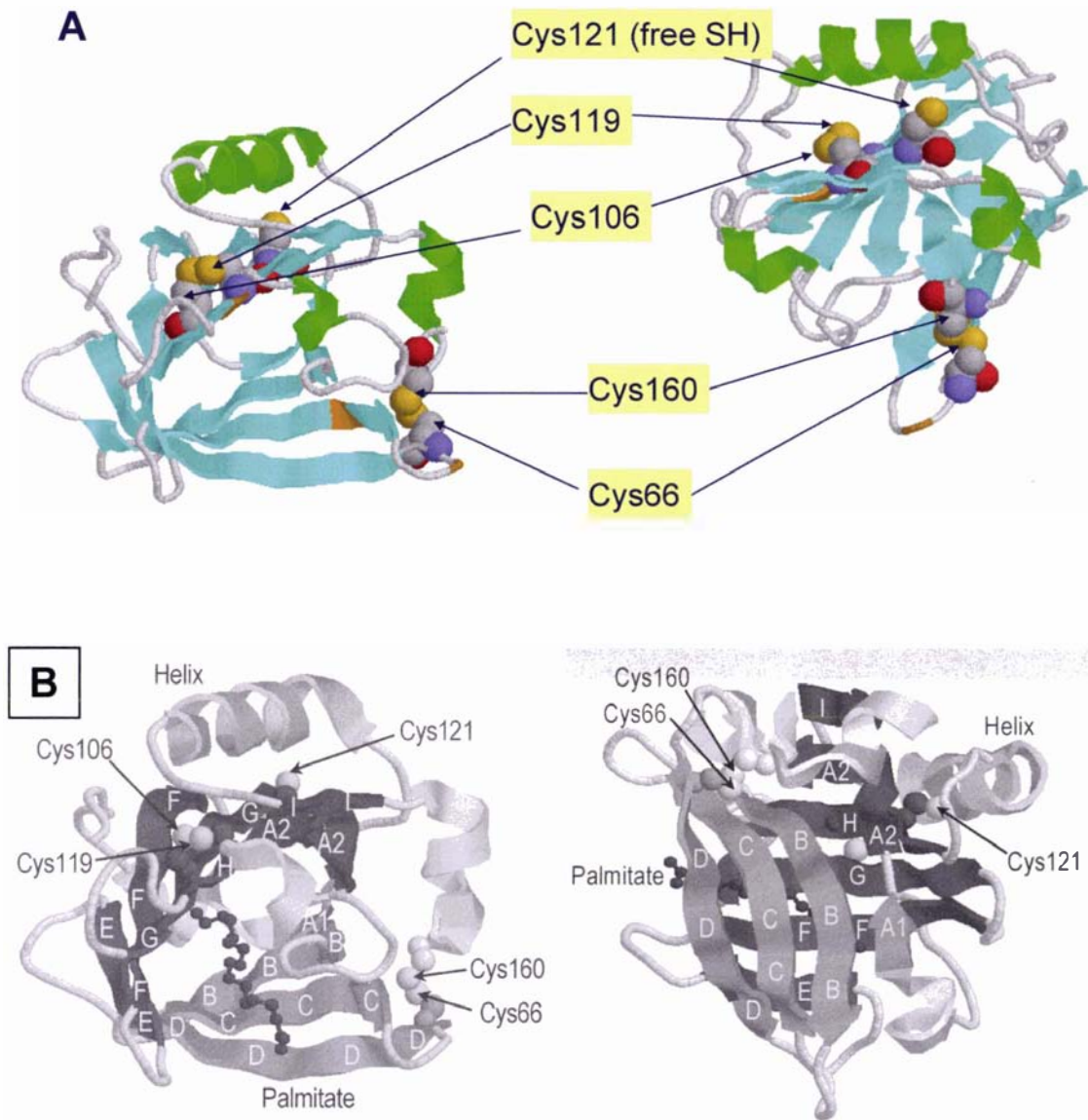


Figure 2.2. Diagram of the three-dimensional structure of β -LG, showing the relative positions of the five Cys residues (Figure A) and the bound palmitate (Wu et al., 1999). The helix and the strands that constitute sheets 1 and 2 are also labeled (Figure B). The diagram was drawn from the PDB file 1GXA using RASMOL Version 2.6.

Native β -LG (see Figure 2.2B) has nine strands that are folded into two β -sheets: sheet 1 contains strands B, C and D and part of strand A (A1); sheet 2 contains strands D, E, F, G and H, part of strand A (A2) and strand I. One side of sheet 1 is hydrophobic and the other side is hydrophilic. Sheet 2 is also hydrophobic on one side and faces the hydrophobic side of sheet 1, thus creating a very hydrophobic cavity, which is filled with water. There is also another hydrophobic region on the side of sheet 2, where the three-turn helix lies above it and along strands F, G and H. The α -helix covers the Cys residue, providing it remains packed against the exterior of the calyx. Subjecting β -LG to different denaturing conditions, e.g. heat, pressure, urea etc., exposes the Cys¹²¹ residue and initiates a chain of reactions involving sulphhydryl–disulphide interchange.

α -Lactalbumin

α -LA, the second major bovine whey protein (\approx 20% of the total whey proteins), has been physically and chemically well characterised (Fox, 1989; Brew & Grobler, 1992; Brew, 2003). α -LA is a nearly spherical, monomeric, compact, globular protein at neutral and alkaline pH and consists of 123 amino acid residues, with a monomeric molecular mass of 14200 Da. It is stabilised by four disulphide bonds and does not contain a free thiol group (Brew & Grobler, 1992; Brew, 2003). However, one of the disulphide bonds (Cys6–Cys120) is more sensitive to cleavage than the other three because of its lower inherent stability (Kuwajima et al., 1990).

α -LA binds calcium very tightly, which stabilises its conformation in a complex structure and thereby has a major effect on its thermal stability (Hiraoka et al., 1980; Bernal & Jelen, 1984; Brew, 2003). α -LA exists in a number of environment-dependent conformations, including the apo (calcium-depleted) and holo (native, calcium-bound) forms; the holo form is the major form in milk. The holo form is converted to the apo form when α -LA is subjected to low pH (1.7) dialysis (Baumy & Brule, 1988). In milk, a proportion of the molecules are glycosylated (Kinghorn et al., 1995; Slangen & Visser, 1999). α -LA has been studied extensively in recent years as a model for protein folding studies, particularly because of its ability to form molten globules (Chrysina et al., 2000; Griko, 2000; Wijesinha-Bettoni et al., 2001) and its close structural similarity to lysozyme.

High tryptophan and aspartate levels, the presence of single arginine and methionine residues, four intrachain disulphide bonds, the absence of phosphoryl and free sulphhydryl groups, and amino acid sequences similar to those of lysozyme are the notable features of the primary structure of α -LA (McKenzie & White, 1991; Brew & Grobler, 1992; Brew, 2003). Analyses of circular dichroism (CD) spectra indicate that α -LA contains 20% α -helix, 14% β -sheet and 60% unordered structure; this is in good agreement with the secondary structure indicated using X-ray crystallography (Robbins & Holmes, 1970). NMR studies of folding indicate that calcium has a role in facilitating rearrangements in the molten globule form to generate the native tertiary structure (Forge et al., 1999). Crystallographic structures of the apo and holo forms of bovine α -LA at 2.2 Å resolutions have been reported (Zhang & Brew, 2003).

Bovine serum albumin (BSA)

Bovine milk contains approximately 0.4 g (\approx 10% of whey proteins) BSA/L (Peters, 1985). BSA contains 582 amino acids and has a calculated molecular weight of 66 267 Da and 17 intramolecular disulphide bonds that hold the molecule in a structure consisting of nine loops. It contains one free thiol group at position 34 (Carter & Ho, 1994). BSA exists in a multi-domain structure with complex ligand-binding specificities (Hsia et al., 1984) and is characterised by an overall oblate shape. It consists of three domains (I, II and III), each stabilised by an internal network of disulphide bonds (Carter & Ho, 1994). Each of the domains contains two large double loops and one small double loop, and the domains differ in hydrophobicity, charge and ligand binding. BSA has been reported to have 5–12 binding sites at neutral pH (Brown, 1977; Peters, 1985), which has the capacity to bind several ligands, apparently at different sites, among which fatty acids and cations (especially Cu^{2+} and Ni^{2+}) are most significant (Fox, 1989). BSA is a monomer. However, the appearance of several bands in isoelectric focusing patterns indicates that considerable microheterogeneity may exist due to post-translational modification of amino acid side chains and/or disulphide bond isomerisation (Spencer & King, 1971; Foster, 1977), some of which is due to ligand binding and post-secretion modifications.

It is estimated that the secondary structure of BSA consists of \approx 66% helix, 10% turn and 23% extended chain, and no β -sheet (Reed et al., 1975; Wetzel et al., 1980; Carter

& Ho, 1994; Gelamo & Tabak, 2000; Gelamo et al., 2002). The numerous disulphide bonds impose conformational restrictions and probably reduce the helical content of the molecule but, because the molecule contains no long distance disulphides, it is relatively flexible. Denaturation by dissociating agents is reversible, as is heat denaturation up to a certain point, but excessive heating, especially at elevated pH, is irreversible (Fox, 1989). BSA is a well-known transport protein for fatty acids in the blood circulatory system (Walstra & Jenness, 1984). Any specific function that it may have in the mammary gland or in milk is not yet known. Treatment of BSA to rupture its intramolecular disulphide bonds increases its flexibility, allowing the exposure of normally buried hydrophobic side groups to the aqueous phase and therefore enhancing its emulsifying ability.

Immunoglobulins

Immunoglobulins (Igs) are a group of complex large glycoproteins that possess antibody activity. They are present in milk at low concentrations (0.06–0.10% w/v), representing \approx 10% of the whey proteins, but are found in colostrum at much higher levels. Five distinct classes of Igs occur in bovine milk (Butler, 1983; Larson, 1992; Hurley, 2003; Farrell et al., 2004), all with similar basic structures as well as amino acid sequences and carbohydrate groups: IgA, IgM, IgE, IgD and IgG, the last class being subdivided into IgG₁ and IgG₂. The molecular weight of Igs varies between \approx 150 and 900 kDa and the amino acid sequence is variable. IgG₁ is the main class in bovine milk and represents \approx 80% of the total Igs (Farrell et al., 2004). As a group, the Igs are either monomers or polymers of a four-chain molecule consisting of two light (IgL) polypeptide chains (molecular weight 22 kDa) and two heavy (IgH) polypeptide chains (molecular weight 50–70 kDa) (Mulvihill & Donovan, 1987). A disulphide bond connects each of the light chains to a separate heavy chain, and the heavy chains are held together by disulphide bonds (Larson, 1992). Igs can be easily denatured by heat-treating milk above 60°C (Lyster, 1972; Li-Chan et al., 1995).

Other minor whey proteins

Minor whey proteins include β_2 -microglobulin (a small cell-surface protein with a molecular weight of 11630 Da and 98 amino acid residues), lactoferrin (LF) and serotransferrin (iron-binding protein). The strong iron-binding capacity of LF indicates two roles: iron absorption and antibacterial activity and protection against enteric infection in the neonate (Walstra & Jenness, 1984; Fox, 1989).

2.4 Effects of heat treatment on milk proteins

Protein structures and various molecular forces that stabilise the native structures of proteins has been discussed earlier (see Section 2.3.1). The stability of the 'ordered' native state is marginal because the free energy change from various stabilising forces/interactions (see Figure 2.1) just counterbalances the large conformational entropy of the 'disordered' random state. The net free energy differences that stabilise the native conformation against transitions to other forms are small and may be overcome by marginal alterations in the surrounding medium. Therefore, protein structures can be readily destabilised from their native state by introducing relatively minor changes in environmental conditions (Mulvihill & Donovan, 1987). Application of heat, application of pressure or changes in other conditions cause proteins to lose their native structure and the compact molecule begins to unfold into a disordered, random structure. The 'denaturation or unfolding of proteins' is thus characterised as the breaking of stabilising forces in the native structure, and conversion to a less ordered conformation (Privalov & Gill, 1988).

Thermo-denaturation of whey proteins occurs when hydrogen, hydrophobic or covalent bonds are affected (Mulvihill & Donovan, 1987). Denaturation of whey proteins often exposes hydrophobic amino acid side chains that are normally buried within the native three-dimensional structure and thus causes an increased reactivity of such groups. Through sulphhydryl–disulphide interchange and hydrophobic interactions, the unfolded protein molecules may associate with each other to form aggregates, which will become insoluble as they grow in size. Severe heat treatments can lead to interactions with other protein molecules, which result in intermolecular association and aggregation and finally precipitation or gelation (see Table 2.5 for a detailed description of these terms),

depending on the protein concentration, heating and cooling rates, pH and ionic strength (de Wit, 1984, 1989; Mulvihill & Donovan, 1987; Paulsson, 1990; Singh, 1995; Singh & Havea, 2003), which are usually accompanied by specific changes in their structure and properties.

The structure of whey proteins undergoes temperature-dependent changes over a wide temperature range. The transformation from the initial native state to the unfolded state is co-operative and reversible. Heat-induced unfolding of globular proteins is accompanied by an endothermic heat effect and a change in enthalpy (Singh & Havea, 2003). Heating a globular protein molecule in water increases the thermal motion of numerous structural elements in the polypeptide chain. These movements are all related to the energy supplied in the form of heat, which leads to rupture of intermolecular and intramolecular bonds, van der Waals' and electrostatic interactions, and disulphide bonds (Privalov & Gill, 1988).

Table 2.5. Description of possible heat-induced changes in the milk proteins (adapted from Messens et al., 1997)

Protein denaturation is any modification in secondary, tertiary or quaternary conformation that is not accompanied by the rupture of peptide bonds involved in primary structure. The final conformation after denaturation can correspond to a totally (random coil) or partially unfolded polypeptide structure.

Association refers to changes occurring at the molecular level (e.g. dimerisation) that are characterised by weak bonds at specific interaction sites.

Aggregation or polymerisation: contrary to association, the terms aggregation or polymerisation, precipitation, coagulation and flocculation refer to unspecified protein-protein interactions that result in the formation of large complexes with higher molecular weights.

Gelation is an orderly aggregation of native and/or (partially) denatured proteins, forming a three-dimensional network structure in which protein-protein and protein-solvent interactions are balanced to produce a well-ordered matrix that is capable of holding significant amounts of water.

As discussed in many reports (e.g. Mulvihill & Donovan, 1987; de Wit, 1990; Roefs & de Kruif, 1994; Singh & Havea, 2003), the denaturation of whey proteins is generally assumed to be at least a two-step process. Initially, the native protein unfolds cooperatively to expose side chain groups originally buried within the native structure. The second step involves aggregation of unfolded protein molecules through thiol–disulphide interchange reactions, hydrophobic interactions and ionic linkages. There may be several intermediate conformations between the native state and the final unfolded state (Haynie & Freire, 1992; Griko & Kutishenko, 1994), some of which may not be picked up by using just one method of detection. It is also important to point out that the general term ‘denaturation’ may encompass quite a number of denatured forms of a protein, varying from slight changes in the tertiary structure without changes to the secondary structure (e.g. non-native form), to major changes in tertiary structure and slight unravelling of the secondary structure.

The denaturation and aggregation of whey proteins has been studied extensively over the last 5–6 decades, in different systems, using a wide range of methods, for example, using differential scanning calorimetry (DSC) (Ruegg et al., 1977; de Wit & Klarenbeek, 1981; Park & Lund, 1984; Imafodin et al., 1991), immunodiffusion (Lyster, 1970), serological activity (Baer et al., 1976), solubility at pH 4.5, specific optical rotation (Harwalkar, 1980a, 1980 b), electrophoretic techniques (Hillier & Lyster, 1979; Dannenberg & Kessler, 1988a; McSwiney et al., 1994a, 1994b; Manderson et al., 1998; Havea et al., 1998, 2001, 2002), fast protein liquid chromatography (Manji & Kakuda, 1986) and various other methods (Manji & Kakuda, 1986; Donovan & Mulvihill, 1987; Dannenberg & Kessler, 1988a; Kessler & Beyer, 1991; Parris & Baginski, 1991, Parris et al., 1991, 1993; Regester et al., 1992; Anema & McKenna, 1996; Oldfield et al., 1998; Manderson et al., 1999; Anema et al., 2000; Oldfield et al., 2000). The susceptibility to and the rates of denaturation of various whey proteins in whey or in buffer, as influenced by many factors such as pH, time and temperature of heat treatment, total solids, protein concentration and ionic strength, have been reported (e.g. Elfagm & Wheelock, 1978; Hillier & Lyster, 1979; Hillier et al., 1979; Smits & van Brouwershaven, 1980; de Wit & Klarenbeek, 1984; Bernal & Jelen, 1984; Mulvihill & Donovan, 1987; Dannenberg & Kessler, 1988a; Kinsella & Whitehead, 1989; Griffin et al., 1993; Hines & Foegeding, 1993; Parris et al., 1993; Oldfield et al., 1998, 2000; Havea, 1998; Havea et al., 1998, 2001, 2002; Anema, 2000). However, the results and the data available are not directly comparable and are sometimes contradictory because

of different experimental protocols, such as: heating medium, heating methods, protein concentration and pH etc. (Mulvihill & Donovan, 1987; Mulvihill & Kinsella, 1987).

There are distinct differences in the denaturation behaviours of individual whey proteins. Different whey proteins are reported to have different thermal transition temperatures (Table 2.6) and different heat treatments are reported to have different effects on individual whey proteins.

Because of the heterogeneity of the whey protein system, and because the individual proteins exhibit different responses towards heat, the thermal denaturation and aggregation of the total whey proteins reflects the collective response of the component proteins (de Wit & Klarenbeek, 1984).

Table 2.6. Thermal denaturation temperatures and enthalpies of whey proteins (adapted from de Wit, 1984; Kinsella & Whitehead, 1989; Jelen & Rattray, 1995)

| Whey protein | Td (°C) | Ttr (°C) | ΔH (kJ/mol) |
|--------------|---------|----------|-------------|
| β-LG | 78 | 83 | 311 |
| α-LA | 62 | 68 | 253 |
| BSA | 64 | 70 | 803 |
| Ig | 72 | 89 | 500 |

Td = the initial denaturation temperature; Ttr = the temperature at the DSC peak maximum; ΔH = the enthalpy of denaturation.

To understand the heat-induced aggregation of whey proteins in whey products such as WPC and WPI, the responses of purified individual proteins and mixtures of these proteins to heat have been studied extensively and their main effects on the structures of β-LG, α-LA, BSA and their binary and ternary combinations have been presented herein.

2.4.1 Studies on heat-induced changes in individual whey proteins

Effects of heat treatment on β-LG

Studies on the effect of heat treatment (denaturation, aggregation and gelation) on native β-LG showed that a range of different physical techniques, such as light

scattering (Griffin et al., 1993; Gimel et al., 1994; Roefs & de Kruif, 1994; Qi et al., 1995; Elofsson et al., 1996; Hoffman et al., 1996, 1997), CD spectroscopy (Griffin et al., 1993; Matsuura & Manning, 1994; lametti et al., 1996, 1998; Prabakaran & Damodaran, 1997; Qi et al., 1997; Manderson et al., 1999), intrinsic protein fluorescence (Mills, 1976; lametti et al., 1996; Manderson et al., 1999), hydrophobic probe (Elofsson et al., 1996), DSC (Huang et al., 1994a, 1994b; Qi et al., 1995; Rüegg et al., 1977), microcalorimetry (Qi et al., 1995) and NMR (lametti et al., 1995, 1997; Belloque & Smith, 1998; Edwards, 2002), have been used. Numerous studies have been reported on changes in the behavior of β -LG during heat treatments. However, only selected reports have been considered in the present review to provide general overview and other relevant reports will be discussed appropriately in the results sections of the respective chapters.

Many of the previous studies showed that, when β -LG is heated above 70°C, it denatures and aggregates depending on many factors such as protein concentration, pH, salt concentration and heating temperature. The heat-induced unfolding of β -LG is essentially reversible at lower pH (below 3.0) (Pace & Tanford, 1968; Gimel et al., 1994), whereas irreversible reactions involving thiol-catalysed disulphide bond interchange lead to aggregation at higher pH (Sawyer, 1968; McKenzie, 1971; Creamer, 1995). Upon heating to approximately 70°C at neutral pH, the native dimers progressively dissociate into native monomers (McKenzie, 1971; Hambling et al., 1992), which partially unfold (lametti et al., 1996; Qi et al., 1997); subsequently, a thiol group (lametti et al., 1996; Hoffmann & van Mil, 1997; Prabakaran & Damodaran, 1997) and hydrophobic residues (lametti et al., 1995, 1997; Relkin, 1996) are exposed (become solvent accessible), resulting in formation of reactive monomers. The aggregates are formed via intermolecular thiol-catalysed disulphide bond interchange (Griffin et al., 1993; Cairoli et al., 1994; McSwiney et al., 1994a; Roefs & de Kruif, 1994; lametti et al., 1995, 1997; Qi et al., 1995; Gezimati et al., 1996, 1997; Hoffmann et al., 1997; Havea, 1998; Havea et al., 1998, 2000, 2001, 2002; Manderson et al., 1998; Verheul et al., 1998, 1999; Schokker et al., 1999, 2000) and to a lesser extent thiol–thiol oxidation (Mulvihill & Donovan, 1987; McSwiney et al., 1994a, 1994b; Hoffmann & van Mil, 1997; Prabakaran & Damodaran, 1997; Qi et al., 1995; Qi et al., 1997; Schokker, 1999), leading to the formation of non-native monomers, dimers, trimers, tetramers and larger

aggregates. The rates and pathways are dependent on the protein concentration, pH, temperature and other factors.

It has been shown that native monomer proteins are converted to non-native (the native fold or one of a range of non-native folds and containing non-native disulphide bonds) monomers (Iametti et al., 1998; Manderson et al., 1998; Schokker et al., 1999), non-native disulphide-bonded dimers, trimers etc., probably via intramolecular thiol–disulphide bond interchange (Iametti et al., 1997; Havea, 1998; Manderson et al., 1998; Schokker et al., 1999, 2000; Havea et al., 2001, 2002; Cho et al., 2003). Some of these monomers participate in intermolecular thiol–disulphide bond interchanges to give covalently bonded aggregates (Manderson et al., 1998) and some form hydrophobically associated products with the covalently bonded aggregates (Havea et al., 2001). In addition to disulphide bond interchange reactions, when 10% solutions of β -LG are heated, stable non-covalently bonded aggregates appear to form (McSwiney et al., 1994a, 1994b; Havea et al., 1998, 2001), indicating that two major aggregation features, or possibly mechanisms, are related to hydrophobic association and disulphide bond interchange reactions. Many different proposals have been offered for the reaction order of β -LG denaturation and aggregation, and it appears that this area has yet to be fully clarified. However, the presence of non-native monomers and dimers and their role as intermediates in the heat-induced denaturation process of β -LG appear to be of vital importance (De la Fuente, 2002). It has also been suggested that β -LG also forms an intermediate that has similarity to the ‘molten globule’ state (Cairolì et al., 1994; Huang, 1994a, 1994b; McSwiney et al., 1994b; De la Fuente et al., 2002). The initial stages of heating would result in a reversible unfolding of the native structure to form an unfolded monomeric β -LG. Irreversible aggregation would follow to form β -LG aggregates. A number of different polymeric aggregates could be formed (Roefs & de Kruif, 1994).

Effects of heat treatment on α -LA

The heat-induced interactions of α -LA have been studied extensively as a model for protein folding studies mainly because of its ability to form a molten globule (e.g. Wijesinha-Bettoni et al., 2001).

When α -LA is heated in solution using differential scanning calorimetry (DSC), it can reversibly undergo thermal transitions. α -LA has been shown to undergo conformational changes at about 64°C (Rüegg et al., 1977). In contrast, when β -LG is treated similarly, it irreversibly undergoes transitions up to about 73 °C (Rüegg et al., 1977). However, α -LA does not readily form aggregates or undergo disulphide bond interchange when heated alone at up to 80°C, pH 6.70 (Rüegg et al., 1977; Eigel et al., 1984; Paulsson et al., 1986; Matsudomi et al., 1992, 1993, 1994; Calvo et al., 1993; Hines & Foegeding, 1993; Dalgleish et al., 1997; Gezimati et al., 1997; Schokker et al., 2000) partly because of the lack of a free thiol group in the α -LA monomer structure (Calvo et al., 1993). Also, there is evidence that α -LA is readily heat denatured but has a much greater tendency to renature rather than to form aggregates (Rüegg et al., 1977). In some of the studies, α -LA has been shown to form disulphide-linked polymers as well as modified monomers when it is heated under severe conditions (100°C for 30 min) and these are probably in the molten globule state (Kuwajima, 1989; Hirose, 1993). Also, when α -LA is heated at 100°C for 30 min (Schnack & Klostermeyer, 1980), there is significant loss of disulphide bonds and a group similar to the thiol group is formed simultaneously, which has been suggested to be responsible for the molecular disulphide interactions of α -LA.

The heat-induced interactions of α -LA are pH dependent and Ca^{2+} ions are important. It has also been reported that, when α -LA binds less than one mole of calcium (or other divalent cation) per mole of protein, the thermal transition temperature of this apo- α -LA decreases to about 35°C (Relkin, 1996). Addition of calcium ions to apo- α -LA increases the transition temperature to about 66°C (Relkin et al., 1993). When holo- α -LA (with bound calcium) is extensively heated by itself at 80°C, it slowly forms non-native monomers and dimers; apo- α -LA is noticeably more reactive (Hong & Creamer, 2002). Extensive heating (100°C for at least 10 min) in the absence of extra calcium gives rise to disulphide-bonded α -LA dimers, trimers etc. as well as some non-native monomeric protein (Chaplin & Lyster, 1986).

Effects of heat treatment on BSA

Many studies have been reported on the heat-induced interactions of BSA, the most heat sensitive of the major whey proteins when heated alone (Clark et al., 1981; Takeda et al., 1989; Yamasaki et al., 1991) at neutral pH (de Wit & Klarenbeek, 1984). Many of these studies reported that BSA does not denature at up to 40°C. The conformational changes are reversible between 42 and 50°C, but unfolding of the α -helices of BSA is irreversible between 52 and 60°C. Temperatures of 60°C and above show unfolding of BSA and thiol-catalysed aggregation and gel formation occurs at temperatures above 70°C (Clark et al., 1981; Takeda et al., 1989; Yamasaki et al., 1991). Kang & Singh (2003) have described the DSC profile of intact BSA, which does not adequately fit a two-state model, with two peaks at 61 and 66°C.

BSA has been found to form a self-supporting gel when heated at 80°C for 30 min (Paulsson et al., 1986; Matsudomi et al., 1993; Gezimati, 1995). As the temperature increases, some molecular regions become accessible to new intermolecular interactions, producing soluble aggregates through disulphide and non-covalent bonds (Wang, 1999). The mechanism of interaction of BSA with other whey proteins is probably similar to that of β -LG (McSwiney et al., 1994a, 1994b; Havea et al., 2001), except that BSA forms aggregates and gels at lower temperature. This observation was confirmed using native- and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Gezimati, 1995), and may be explained on the basis that both β -LG (Cys121) (Hambling et al., 1992) and BSA (Cys34) (Carter & Ho, 1994) contain free thiol groups.

Effects of heat treatment on Igs

Igs are very heat labile. Richardson (1991) studied the heat-induced interactions of IgG heated at 95°C and found that the elution profiles of IgG heated alone indicated the formation of large complexes through covalent interactions, possibly through thiol–disulphide interchange. Li-Chan et al. (1995) suggested that commercial pasteurisation processes should not result in complete destruction of IgG. An analysis of Igs in commercial dairy products (reconstituted skim milk powder and whey) revealed that, although between 60 and 75% of the original Igs amount present in the milk samples were retained after pasteurisation, the levels of IgG in UHT or canned (evaporated) milk were negligible (Li-Chan et al., 1995).

2.4.2 Studies on heat-induced changes in whey proteins involving binary and ternary mixtures

Effects of heat treatment on binary mixtures of α -LA and β -LG

Many studies have reported on the effects of heat treatment on mixtures of α -LA and β -LG in model systems (Noh et al., 1989; Calvo et al., 1993; Hines & Foegeding, 1993; Qi et al., 1995; Dalgleish et al., 1997; Gezimati et al., 1997; Havea, 1998; Havea et al., 1998, 2001; Schokker et al., 2000; Hong & Creamer, 2002). Many of these studies concluded that, besides self-aggregation, β -LG can form heat-induced complexes with α -LA. α -LA does not readily form aggregates or undergo disulphide bond interchange when heated alone at up to 80°C, pH 6.70, partly because of the lack of a free thiol group in the α -LA monomer structure (Calvo et al., 1993). However, in the presence of β -LG, aggregation of α -LA occurs readily (Calvo et al., 1993; Dalgleish et al., 1997) to form non-native monomers, dimers, trimers and larger aggregates of both α -LA and β -LG. This probably occurs through the interaction of a free thiol on a non-native β -LG monomer via a thiol-induced disulphide bond interchange reaction with one of the surface-located cysteine residues of α -LA (Livney et al., 2003).

Almost all of the β -LG was incorporated into soluble aggregates via disulphide bonds (Calvo et al., 1993) and to a considerable extent via hydrophobic interactions (Calvo et al., 1993; Dalgleish et al., 1997; Havea et al., 1998, 2001; Oldfield et al., 1998, 2000; Schokker et al., 2000), when mixtures of α -LA and β -LG was heat treated. It has been reported that the homopolymers (Dalgleish et al., 1997), as well as heteropolymers (Gezimati et al., 1997) of α -LA and β -LG. As well as formation of disulphide-bonded α -LA dimers, 1:1 α -LA: β -LG dimer aggregates and non-native monomers and dimers etc., of both α -LA and β -LG have been reported (Havea et al., 2001; Hong & Creamer, 2002). The rate of loss of native α -LA from the mixture is rapid and comparable with that of β -LG under the same conditions. The presence of α -LA diminishes the proportion of smaller aggregates and increases the number of very large aggregates. Preheating β -LG decreases the extent of loss of α -LA from the mixtures, suggesting that the Disulphide bond shuffling that occurs during heat treatment is enhanced by the formation of molten globule intermediates and by thiol catalysis (Hong & Creamer, 2002).

Further, it has been reported that, at constant protein concentration, mixtures of α -LA and β -LG give firmer gels than β -LG by itself (Hines & Foegeding, 1993; Gezimati, 1995, Gezimati et al., 1997), probably because of a greater number of inter-protein disulphide bonds between α -LA and β -LG. Hines & Foegeding (1993) suggested that the interaction of denatured α -LA with denatured β -LG molecules is kinetically or thermodynamically more favourable than renaturation. In the presence of α -LA, β -LG is converted into a series of disulphide-bonded and hydrophobically associated aggregates. These patterns are similar to those when β -LG solution is heated on its own. These results indicate that the mechanism of aggregation of an α -LA/ β -LG mixture is governed by β -LG (Schokker et al., 2000; Hong & Creamer, 2002). Addition of α -LA to β -LG solutions prior to heat treatment affects the pathway of aggregation to favour greater disulphide-bonded aggregates, but does not alter the initial rate of loss of native-like β -LG from the mixtures of α -LA and β -LG, indicating that the modifications to aggregation introduced by α -LA are after the initial heat-induced intramolecular reorganisation of the β -LG disulphide-bonding patterns. The inaccessible disulphide bonds of α -LA react with the free sulphhydryl groups of the 'unfolded' β -LG molecules, after which they can catalyse intermolecular disulphide bond interchange (Schokker et al., 2000).

Effects of heat treatment on binary mixtures of BSA and β -LG

It has been reported that the thermal transition temperature of BSA is lower than that of β -LG (de Wit & Klarenbeek, 1984), and both β -LG and BSA have a free cysteine (Carter & Ho, 1994). BSA apparently responds to heat treatment almost similarly to β -LG, except that BSA forms aggregates at a lower temperature (Gezimati et al., 1996). The rate of interaction and aggregation of β -LG and BSA is dependent on the temperature. When mixtures are heated at temperature less than 70°C, the number of β -LG molecules that undergo the thermal transition is less than the number of BSA molecules and hence the role of BSA is dominant in the aggregation and gelation (Matsudomi et al., 1994; Gezimati, 1995). In contrast, at temperatures > 75°C, both proteins form aggregates and the aggregation rates of both proteins are comparable (Gezimati, 1995). The formation of soluble aggregates of β -LG:BSA occurs through disulphide cross-links (Matsudomi et al., 1994).

It has been suggested that, although BSA aggregation appears to be unaffected by β -LG, the rate of aggregation of β -LG is enhanced by the presence of BSA (Hines & Foegeding, 1993; Gezimati et al., 1996), and the gels from the mixture are stronger, indicating a synergistic effect (Matsudomi et al., 1994). Similar findings were reported by Havea et al. (2001) using binary and ternary mixtures of α -LA, β -LG and BSA. Hines & Foegeding (1993) reported that native β -LG aggregates with BSA with a second order rate constant when they are mixed in a 1:1 molar ratio. The rate is seven times greater than when β -LG is alone in solution. In addition, the rate of aggregation of BSA is unaltered by β -LG.

Effects of heat treatment on binary mixtures of BSA and α -LA

The concentrations of BSA and α -LA are important as a good correlation between BSA concentration and aggregate (α -LA:BSA) formation in milk ultrafiltrate (α -LA, 1.5 mg/mL; BSA, 0.3–10.0 mg/mL) heated at 90°C was found (Calvo et al., 1993). Thiol–disulphide interchange was involved in aggregate formation between 0.2% α -LA and 0.2% BSA heated at 80°C for 30 min (Matsudomi et al., 1993). A significant increase in gel hardness was observed by the addition of $\geq 3\%$ α -LA to 6% BSA solution, which may be attributed to the formation of a more uniform and finer gel matrix (Matsudomi et al., 1993); in addition to the thiol–disulphide interchange reaction, BSA was involved in the formation of hydrophobically associated aggregates (Matsudomi et al., 1993; Havea et al., 1998, 2001), whereas there was no evidence of α -LA being incorporated into hydrophobic aggregates. Soluble aggregates of α -LA and BSA were formed (when heated in 100 mM phosphate buffer, 80°C for 30 min, pH 6.80) through thiol–disulphide interchange reaction during gel formation and these aggregates had lower molecular weights than those formed by BSA alone (Matsudomi et al., 1993).

Havea et al. (2000) studied the formation of new protein structures in heated mixtures of BSA and α -LA. Heating the individual proteins alone showed BSA only containing large disulphide-bonded BSA aggregates. Combining α -LA and BSA (1:1; w/w) led to the production of larger disulphide-bonded aggregates and SDS-monomeric BSA and SDS monomeric α -LA. On lowering the concentration from 5 to 2%, fewer larger disulphide-bonded aggregates were evident and there was less SDS-monomeric α -LA and BSA. This study concluded that BSA forms disulphide-bonded aggregates that

contain available thiol groups that can catalyse the formation of differently structured α -LA monomers, dimers, higher polymers and adducts of α -LA with BSA. It has been suggested that the exposed thiol group of BSA molecules/aggregates can react via thiol–disulphide interchange with the disulphide of α -LA, thus generating α -LA:BSA adducts and α -LA dimers (Havea et al., 2000).

Effects of heat treatment on Binary/ternary mixtures of BSA, β -LG and/or α -LA

The heat-induced interactions of β -LG, α -LA and BSA have been studied in model systems (Hines & Foegeding, 1993; Matsudomi et al., 1994; Gezimati, 1995; Gezimati et al., 1996, 1997; Havea et al., 1998, 2000, 2001; Schokker et al., 2000) and in the milk/whey system (Hillier & Lyster, 1979; Havea, 1998; Havea et al., 1998, 2001, 2002).

BSA formed polymers prior to the unfolding of either α -LA or β -LG at 75°C (Gezimati et al., 1996, 1997). The heat-induced aggregation and gelation of a 10% (w/w) solution of a 1:1 mixture of β -LG and BSA (Gezimati et al., 1996) or β -LG and α -LA (Gezimati et al., 1997) in a buffer simulating the WPC environment was studied and it was found that both covalent bonding and non-covalent bonding were involved in the interaction. Hines & Foegeding (1993) studied the interactions of α -LA and BSA with β -LG in thermally induced gelation and found that the rheological transitions and the properties of gels made with mixtures of β -LG and BSA were dependent on the ratio of the proteins in the mixture. Addition of α -LA to β -LG or BSA solution markedly enhanced the gel strength of the heat-induced gel (Matsudomi, 1991 1993, 1994).

More recently, it has been shown that heating α -LA in the presence of BSA or β -LG in WPC permeate (Havea et al., 2001) can induce the formation of α -LA dimers and 1:1 aggregates of α -LA with β -LG and possibly with BSA. When all three whey proteins are present (α -LA, β -LG and BSA), BSA is more effective than β -LG in catalysing the formation of α -LA polymers (Havea et al., 2001). This may be related to the differences in thermal denaturation between the protein species as discussed earlier. Thus BSA will begin to unfold and aggregate before β -LG.

2.4.3 Effects of heat treatment on caseins and casein micelles

The major proteins of milk, the caseins, possess low levels of secondary and tertiary structures, a feature that contributes to their remarkable stability at high temperatures. However, when subjected to severe heat treatment, casein undergoes changes, such as dephosphorylation and proteolysis (Fox, 1982; Singh & Creamer, 1992; Singh, 1995). Polymerisation of casein can occur as a result of condensation reactions, e.g. Maillard-type reactions and the formation of lysinoalanine (Lorient, 1979). The changes in casein micelles upon heat treatment include increase in hydrodynamic diameter, decrease in zeta potential and hydration, and dissociation of caseins from micelles (Fox, 1982; Singh & Creamer, 1992), which have been reviewed in detail (Singh, 1995; De Kruif & Holt, 2003).

2.4.4 Heat-induced interactions of whey proteins with casein micelles

On heating milk, the denatured whey proteins can interact with each other and with the casein micelles. Considerable research has been conducted on deciphering the specific reactions that occur, the composition of the reaction products and the sequence of events involved in the heat-induced aggregation reactions. Non-covalent interactions and disulphide bonds (via thiol–disulphide interchange reactions) are known to be involved in the interactions. The interactions between β -LG and κ -CN in heat-treated model systems have been reported and the results show that two disulphide bridges and a free sulphhydryl group present in the native structure of β -LG play an important role in its heat-induced interactions with κ -CN (Morr, 1985; Haque et al., 1987; Haque & Kinsella, 1988; Hill, 1989; Noh et al., 1989; Dalgleish, 1990; Jang & Swaisgood, 1990; Singh, 1995; Corredig & Dalgleish, 1999; Cho et al., 2003). Interestingly, α_{s2} -CN occurs at the same concentration as κ -CN, and has one disulphide bond, but has not normally been reported to interact with β -LG in milk systems. It can be speculated that α_{s2} -CN is inside the micelle and therefore that β -LG cannot reach it, but equally valid is that α_{s2} -CN is a particularly stable entity, especially as the dimer (Rasmussen et al., 1992a, 1992b). Only a few studies suggesting heat-induced interactions of α_{s2} -CN with whey proteins have been reported (Snoeren & Van der Spek, 1977).

2.5 High pressure processing

2.5.1 Introduction

Although thermal processing is effective, economical and readily available, in many cases, it has undesirable effects on the sensory and nutritional quality of food (Balny & Masson, 1993; Trujillo et al., 2002). Moreover, during the past few years, growing consumer demand for minimally processed, high quality and safe foods has led to increased interest in non-thermal processes for food preservation. HP processing is considered to be an emerging non-thermal technology and has been proposed as an alternative to established thermal processing; it can be used for the production of a range of safe and nutritious foods with minimal effects on the sensory or nutritional quality of food products (Hayashi, 1988; Farr, 1990; Hoover, 1993; Datta & Deeth, 1999, 2003; Needs et al., 2000a, 2000b; Velazquez de la Cruz et al., 2002).

More than a century ago, Hite (1899) used HP with the objective of developing an alternative preservation treatment to heat pasteurisation. He demonstrated that microbial spoilage can be delayed and that the shelf life of milk can be extended significantly by pressure treatment. A few years later, the pioneering work of Bridgman (1914) demonstrated the effects of HP processing on the denaturation and the functional properties of proteins, by pressurising egg proteins. However, it was not until 1990 that equipment advances and consumer demand for minimally processed, high quality foods led to considerable research interest in HP processing technology. Reports have indicated that HP has the potential for both preservation and modification of various aspects of foods, including alteration to their functional properties (Ohmiya et al., 1989; López-Fandiño et al., 1996; García-Risco et al., 1998, 2000), and the subject has been reviewed several times (e.g. Heremans, 1982; Balny et al., 1982, 1989.; Masson, 1992; Weber & Drickamer, 1983; Balny & Masson, 1993; Mozhaev et al., 1994; Johnston, 1995; Messens et al., 1997; Heremans et al., 1997; Datta & Deeth, 1999; 2003; Farkas & Hoover, 2000; Huppertz et al., 2002; Trujillo et al., 2002; Claeys et al., 2003).

Today, HP processing is considered to be a possible alternative to heat treatment and has reached the consumer with a variety of products, such as HP-treated commercially sterilised fresh fruit jams, jellies, juices, salad dressings, rice, cakes and guacamole

(Farr, 1990; Earnshaw, 1992; Cheftel, 1992; Hoover, 1993), but no HP-treated commercial dairy products are available as yet. However, as there is increased interest in the HP treatment of dairy products, it is currently a major focus of investigation.

2.5.2 Effects of HP processing on protein structure and stabilising forces

The effects of HP processing on protein stability are governed by Le Chatelier-Braun's principle, which predicts that, when a system is subjected to a constraint, it will adapt to minimise this constraint. Consequently, when applying HP, the constraint is volume. In other words, whenever a stress is applied to a system in equilibrium, the system will react so as to counteract the applied stress. This implies that an increase in pressure will shift the equilibrium towards the state that occupies a smaller volume. Thus, under pressure, reactions with a negative volume change will be enhanced and reactions with a positive volume change will be suppressed (Buchheim & Prokopek, 1992; Johnston, 1995). Such reactions may result in inactivation of microorganisms or enzymes and denaturation and aggregation of proteins, and thus may bring about textural changes in foods (Huppertz et al., 2002; Balci & Wilbey, 1999).

Various stabilising forces and their role in maintaining the organisational structure of proteins at different levels have been described in detail earlier in this chapter (see Section 2.3.1), which suggests that the secondary, tertiary and quaternary structures of the proteins are stabilised by various types of interactions. As with heat treatment, HP processing is known to induce protein denaturation by altering the delicate equilibrium between the interactions that stabilise the folded conformation of native proteins (Johnston et al., 1992a, 1992b; Masson, 1992; Balci & Wilbey, 1999). Pressure also acts as a physico-chemical parameter that alters the balance of intramolecular, solvent-protein interactions, and brings about changes in conformational volume and changes in solvation volume (Balny & Masson, 1993; Heremans, 1992; Messens et al., 1997; Hendrickx et al., 1998; Claeys et al., 2003). However, HP treatments have been reported to have slightly different effects on protein structure compared with heat treatments (Mozhaev et al., 1996).

In contrast to heat treatments, where covalent as well as non-covalent bonds are affected, it has been reported that HP processing at room temperature ($\approx 20^\circ\text{C}$) disrupts only relatively weak bonding such as intramolecular hydrophobic and electrostatic

interactions (Balny & Masson, 1993; Silva & Weber, 1993), whereas hydrogen bonds are relatively insensitive to pressure, suggesting that HP affects the tertiary and quaternary structure of globular proteins and has little effect on their secondary structure. There are views that covalent bonds are largely insensitive to pressure treatment, because HP is not sufficient to cause a breakdown of covalent bonds (energy of disulphide bonds: $213.1 \text{ kJ mol}^{-1}$) and thiol–disulphide reactions are not affected (Hayakawa et al., 1996). Reversible effects such as dissociation of polymeric structures or partial unfolding are observed below 100–200 MPa (Jaenicke, 1991a, 1991b; Silva & Weber, 1993). Higher pressures (>200 MPa) cause irreversible and more extensive effects on proteins. These include unfolding of monomeric proteins and aggregation (Zipp & Kautzmann, 1973; Heremans, 1982; Weber & Drickamer, 1983; Silva & Weber, 1993), as well as the formation of gel networks (Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1997a, 1997b). Subsequent re-formation of intra- and intermolecular bonds within and between protein molecules, leading to the formation of oligomers and polymers, linked by hydrophobic interactions and disulphide bridges, occurs (e.g. Hoover., 1993; Cheftel, 1992; Masson, 1992; Galazka et al., 1996; Messens et al., 1997; Trujillo et al., 2002).

Changes in the solvation volume are mainly caused by pressure-induced ionisation, changes in solvent exposure of amino acid side chains and peptide bonds, and diffusion of water into cavities located in the hydrophobic core of the protein (Balny & Masson, 1993; Heremans, 1982; 1992; Messens et al., 1997; Hendrickx et al., 1998; Claeys et al., 2003). Protein compressibility is governed by the compression of the internal cavities, which depends on the internal packing (Mozhaev et al., 1996). The hydrophobic core of a protein is less stable at HP, because the loss of cavity volumes is a major factor contributing to the partial molar volume change upon the unfolding of a protein (Weber & Drickamer, 1983; Frye & Royer, 1998). With respect to changes in the conformational volume, the primary protein structure (the amino acid sequence) will remain intact because covalent bonds are almost unaffected by HP at relatively low temperature. The secondary structure (α -helices or β -sheets) will be affected at HP, resulting in irreversible unfolding. Changes in the tertiary structure (three-dimensional configuration held together mainly by hydrophobic and ionic interactions) occur at pressures above 200 MPa and lead to irreversible unfolding. At relatively low pressures (<150 MPa), the quaternary structure (the spatial arrangement by non-covalent interactions into a multimeric protein) is disrupted into subunits. However, during the

pressure release phase and after pressure treatment, new intermolecular interactions are formed.

2.5.3 Effects of HP processing on milk proteins

High pressure can alter the protein conformation and this can lead to protein denaturation, aggregation and gelation (Tedford et al., 1999; Fertsch et al., 2003), depending on the type of protein, protein concentration, pH, ionic strength, the applied pressure and pressurising temperature, and the duration of the pressure treatment etc. (Masson, 1992; Messens et al., 1997; Fertsch et al., 2003; Huppertz et al., 2004a, 2004b). Thus whey protein denaturation and aggregation has been studied extensively under various conditions and in systems such as milk (Felipe et al., 1997; Law et al., 1998; Arias et al., 2000; Hinrichs, 2000; Scollard et al., 2000; Huppertz et al., 2002, 2004a, 2004b; Anema et al., 2005a), WPC (Van Camp et al., 1997a, b; Johnston et al., 1992a, 1992b), WPI (Hinrichs et al., 1996 a, 1996b; Hinrichs, 2000; Michel et al., 2001) and isolates of the individual whey proteins, e.g. β -LG (Dumay et al., 1994, 1998; Funtenberger et al., 1995; Jegouic et al., 1996; Galazka et al., 1996; Olsen et al., 1999), and is reviewed in the subsequent sections of this chapter.

HP processing has been found to have different effects on proteins when analysed at high pressure ('in situ' analysis) and when analysed after pressure release. With increasing pressure, protein molecules undergo a sequence of conformational changes because of the alterations in stabilising interactions (Johnston et al., 1992). However, during the pressure release phase and after pressure treatment, new intermolecular interactions are formed and the proteins may be newly structured (Fertsch et al., 2003).

As the present study explores the interactions of milk proteins after pressure release, the majority of the reports included in the subsequent sections of the present review deal with the interactions of proteins after pressure release.

2.5.4 HP-induced denaturation and aggregation of whey proteins

Effects of pressure treatment on β -LG

β -LG appears to be the most sensitive of the major whey proteins to HP (Stapelfeldt et al., 1996) and dominates the pressure-induced denaturation, aggregation and gelation of the entire whey system (Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1996, 1997a, 1997b; Kanno et al., 1998; Belloque et al., 2000). Therefore, the majority of previous studies have concentrated on the effects of HP on β -LG. The major changes that occur in the structure of β -LG include monomerisation of the dimeric state (Iametti et al., 1997), a decrease in α -helix and β -sheet content (Hayakawa et al., 1996; Panick et al., 1999) and irreversible changes involving the formation of intermolecular disulphide bonds (Funtenerger et al., 1997; Iametti et al., 1997; López-Fandiño et al., 1997; Moller et al., 1998). In the pressure-induced mechanism proposed (Iametti et al., 1997), release of monomers represents one of the earliest events, whereas association of transiently modified monomers stabilises the denatured forms of the protein.

The reactivity of the free SH group of β -LG increases with pressure up to 150 MPa (Tanaka et al., 1996), whereas pressures ranging between 0 and 140 MPa do not affect β -sheets (Subirade et al., 1998). The results of this study (Subirade et al., 1998) suggested that, in spite of having a similar overall conformation, the structure of β -LG before and after HP are stabilised by slightly different interactions. It has been reported that pressures of 100 MPa resulted in some unfolding of β -LG but allowed the core to remain structured. Increasing the pressure between 100 and 200 MPa initiated structural changes (Moller et al., 1998; Belloque et al., 2000). β -Sheets formed by FGH strands were the strongest portion of β -LG after pressurisation at 200 MPa for 5 min (Belloque et al., 2000), thus indicating that they are the last part to unfold during pressure processing. Subsequent increases in pressure (300 and 400 MPa) showed high flexibility of the entire structure of β -LG. Pressure treatment of the individual variants indicated that the structure of the core of β -LG A becomes flexible more rapidly than that of β -LG B (Belloque et al., 2000). It was speculated by Belloque et al. (2000) that the intermolecular disulphide bonds, formed by the sulphhydryl–disulphide interchange reaction, are likely to involve Cys66–Cys160 and Cys121 rather than Cys106–Cys119. Inter- and intramolecular reactions of SH groups were suggested by Tanaka et al. (1996)

as the main cause for the pressure-induced irreversible denaturation of β -LG at 400 MPa at pH 6.8. These findings were supported, demonstrating that most disulphide bonds resulted from sulphhydryl–disulphide interchange reactions rather than oxidation of SH groups (450 MPa, 25°C, pH 7.0; Funtenberger et al., 1997). This is in agreement with Valente-Mesquita et al. (1998), who suggested that the correct refolding of the native β -LG dimer is hampered by the formation of non-native disulphide bonds. This instability of β -LG at high pressure (150–200 MPa) is due to a single large loss of partial molar volume upon local unfolding and is likely to be a consequence of the proximity of the hydrophobic core to the large water-accessible cavity (Kuwata et al., 2001).

It has been found that HP-induced denaturation of β -LG is partially reversible at lower (2.5%) protein concentration but that the denaturation is irreversible and aggregation occurs at higher (5.0%) concentration (Dumay et al., 1994). The progressive formation of intermolecular disulphide-bonded dimers to hexamers or higher polymers of β -LG (pH 7.0) has been reported to be a function of the pressure level and of the buffer type and molarity (Funtenberger et al., 1995). Funtenberger et al., 1995 also suggested that HP induced the formation of intermolecular S–S bonds, especially at neutral pH. Tanaka & Kunugi (1996) used hydrogen / deuterium (H/D) exchange reactions under pressure (200 MPa) and analysed samples by Fourier transform near infrared and NMR spectroscopy after pressure release. They suggested an intermediate unfolding stage under moderately high pressures. According to previous reports, pressure-induced denaturation was described as a simple two-state process. However, there were indications (Jonas & Jonas, 1994) of the existence of pressure-induced pre-denaturation transitions, which in turn pointed to step-wise processes. Stapelfeldt & Skibsted (1999) also proposed a three-stage pressure denaturation of β -LG. Moreover, it has been reported that factors, such as protein concentration (Dumay et al., 1994), pH, ionic strength, type and molarity of the buffer used for preparation of the protein solutions (Funtenberger et al., 1995; Cheftel & Dumay, 1996), pressure intensity, pressurising time and pressurising temperature (Yang et al., 2001), affect the pressure-induced changes in the protein structure, and the denaturation and aggregation of β -LG. As described for heat treatment, when β -LG is unfolded as an effect of high pressure, the free SH group of Cys121 (CysH121) has the ability to interact irreversibly with disulphide bonds.

It has been suggested that HP treatments induce β -LG into hydrophobic molten globule structures that remain stable for at least 3 months (Yang et al., 2001). Further, the same authors demonstrated that HP treatment resulted in changes in the hydrophobic calyx and surface hydrophobic sites of β -LG (Yang et al., 2003), showing effects on the ligand-binding properties of β -LG in the molten globule state induced by HP treatment. Hummer et al. (1998) examined the pressure dependence of hydrophobic interactions with pressure denaturation of proteins. They focused on pressure-dependent transfer of water into the protein interior, gradually filling cavities, and eventually breaking the protein structure. They thus examined the effects of pressure on the association of non-polar residues in water. The pressure destabilisation of hydrophobic aggregates was explained using an information theory model of hydrophobic interactions, which accounts for the primitive hydrophobic effects of solvation, association and conformational equilibria of small non-polar solutes in water. Further, the pH dependence of pressure denaturation of β -LG indicated that the Tanford transition has significant implications in terms of stability of the β -LG monomer (Botelho et al., 2000). Raising the pH from 6 to 8 causes the EF loop of β -LG to flip open, exposing Glu89. The opening of the β -barrel is probably accompanied by exposure of buried hydrophobic surface areas, which in turn leads to structural rearrangements that result in an increase in the volume of β -LG monomers (Botelho et al., 2000).

Effects of pressure treatment on α -LA

Comparatively little has been reported on the effects of pressure treatment of pure α -LA solutions. α -LA changes its conformation from molten globule to the unfolded state without volume changes. However, the volume of α -LA changes at the transition from a native state to a molten globule state (Kobashigawa et al., 1999). It has been shown that the tight packing is lost from the molten globule of α -LA, but that a hydrophobic core exists, which disappears at the transition from the molten globule state to the unfolded state (Semisotnov et al., 1987; Baum et al., 1989; Alexandrescu et al., 1992, 1993). Further, it has also been demonstrated that the interior of the molten globule state of α -LA is highly hydrated (Kharakoz & Bychkova, 1997). Recently, Lasselle et al. (2003) reported that, not only heat but HP also had the similar effects on α -LA, supporting the view that the molten globule state is stabilised by hydrophobic interactions. α -LA has been shown to be resistant to denaturation (López-Fandiño et al., 1996; Scollard et al.,

2000) or denatures reversibly (Tanaka & Kunugi, 1996; Tanaka et al., 1996) at pressures up to 400 MPa, but forms high molecular weight disulphide-bonded oligomers at high pressure (1000 MPa) and the oligomers of α -LA are stabilised mainly by non-native interchain disulphide bridges (Jegouic et al., 1996).

A comparison of pressure-induced changes of two bovine proteins, α -LA and β -LG, at neutral pH showed that the structural changes of α -LA were practically reversible up to 400 MPa (Tanaka et al., 1996), whereas those of β -LG became irreversible at 150 MPa or lower. Fluorescence measurement of dansylated (prepared at atmospheric pressure) proteins, especially the energy transfer from the intrinsic Trp residue to the dansyl group, showed that the protein structure was deformed by pressure and that the energy transfer facility of the two proteins was differently affected by HP, probably reflecting the degree of compactness of their pressure-perturbed structures (Tanaka et al., 1996).

Effects of pressure treatment on BSA

Compared with β -LG, few studies have been reported on the effect of pressure on pure BSA. Unlike heat treatment, BSA has been found to be quite resistant to pressure treatment. Pressure resistance (100–400 MPa) has been demonstrated by Hayakawa et al. (1992), showing that pressure does not affect the α -helix and does not impart sufficient energy to disrupt disulphide bonds, thus maintaining the molecular structure of BSA. The greater stability of BSA is probably related to the fact that this molecule, through its 17 intramolecular disulphide bonds and the presence of several separate domains, has an extremely rigid molecular structure (López-Fandiño et al., 1996).

Effects of pressure treatment on binary mixtures of α -LA and β -LG

Mixed aggregates of denatured α -LA and β -LG have been found in whey protein solutions prepared in buffer (Jegouic et al., 1997). As with heat-induced denaturation of α -LA, pressure-induced denaturation and aggregation of α -LA occurs only in the presence of reactive thiol groups (Jegouic et al., 1996, 1997). It is likely that the critical α -LA/ β -LG interactions take place in a hydrophobic environment. However, in an HP situation, very few hydrophobic environments will arise, partly because $\Delta V/\Delta P$ is much less for α -LA (Lassalle et al., 2003) than for β -LG (Royer, 2002) and thus α -LA retains

most of its structure at HP. On increasing the pressure to 800 MPa, β -LG has been shown to be > 80% irreversibly denatured whereas only 40% of α -LA is denatured. This has been attributed to the conformational stabilising bonds inherent to the structure of each protein (Hinrichs et al., 1996b; Messens et al., 1997).

Effects of pressure treatment on binary or ternary mixtures of BSA and/or α -LA and/or β -LG

No specific studies on the effects of pressure on binary mixtures of BSA and β -LG or BSA and α -LA or ternary mixtures of BSA, β -LG and α -LA have been reported.

2.5.5 HP-induced gelation of whey proteins

Studies on pressure-induced gelation of whey proteins

At high protein concentration (10%), intermolecular interactions and irreversible aggregation are favoured (Wong & Heremans, 1988; Dumay et al., 1994). HP treatment of concentrated (80–160 g/kg) β -LG isolate solutions (pH 7.0) prepared in water or various buffers induces β -LG gelation at low temperature (Dumay et al., 1998;; Zasytkin et al., 1996). The decreasing solubility (in various dissociating media) of the protein constituents of pressure-induced gels as a function of storage time after pressure release suggests that the aggregation and gelation result from hydrophobic interactions and also disulphide bonds and that a progressive build-up of these interactions takes place after pressure release (Dumay et al., 1998). It was postulated that β -LG plays a major role in the aggregation and gel formation of WPC under pressure (Van Camp et al., 1997a, 1997b), which suggested that the major whey protein component in WPC primarily determines its functional behaviour under HP. However, it was suggested that some additional studies will be needed to confirm this hypothesis, as well as to deduce the role of other whey proteins (i.e. α -LA, BSA and Ig) in gel formation (Van Camp & Huyghebaert, 1995b; Van Camp et al., 1996).

At protein concentrations sufficiently high enough for gel formation, WPC has been found to produce pressure-induced gels in the pressure range 200–400 MPa (Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1996). However, most of the earlier

studies have been concentrated on the rheological and microstructural properties of pressure-induced WPC gels. Protein concentration, applied pressure, holding time and pressurising temperature have been found to be major factors in the formation of pressure-induced gels and the properties of the gels (Van Camp & Huyghebaert, 1995a). The gel strength (Van Camp & Huyghebaert, 1995a; Van Camp et al., 1996; Kanno et al., 1998; Fertsch et al., 2003), the storage modulus G' and the loss modulus G'' (Van Camp & Huyghebaert, 1995b) and the breaking stress of whey protein gels (Kanno et al., 1998) increase with increasing protein concentration because of increased probability of interactions between denatured proteins (Van Camp et al., 1996; Dumay et al., 1998). The ratio G''/G' decreases with increasing protein concentration and the gels become more elastic (Van Camp & Huyghebaert, 1995a). In addition, the gel strength also increases with increasing pressure and prolonged holding time (Van Camp & Huyghebaert, 1995a; Van Camp et al., 1996). Longer pressure holding times may improve the strength of the gel network, stimulating the formation of more intensive intermolecular interactions (Van Camp & Huyghebaert, 1995a). In contrast to heat-induced gels, pressure-induced gels of β -LG underwent mechanical and protein solubility changes when stored at 4°C following pressure release, clearly indicating a time-dependent strengthening of protein–protein interactions. It appears that primary aggregates of β -LG further aggregated during storage through hydrophobic interactions and disulphide bonds (Dumay et al., 1998).

The hardness and the breaking stress of pressure-induced WPI gels increase with increasing WPI concentration (12–18%) and pressure intensity (Kanno et al., 1998). WPI gels have a porous network microstructure, whereas WPC gels are irregular particulates. β -LG, α -LA and BSA participate in pressure-induced aggregation and gelation through disulphide bonding. Moreover, it has been reported that the number of stabilising disulphide bonds directly influences the texture properties of HP-induced whey protein gels (Keim & Hinrichs, 2004).

Studies on comparison of heat- and pressure-induced whey protein gels

Studies have demonstrated that significant differences in protein denaturation and aggregation induced by heat compared with HP occur in a number of food proteins (Heremans et al., 1997). Considering this, it may be expected that gels produced from

they proteins by HP treatment may have different properties from those made by heat treatment.

Pressure-induced as well as heat-induced gel formation within the neutral pH range is explained by the build-up of intermolecular disulphide bonds, which connect the whey proteins to form a network structure (Van Camp & Huyghebaert, 1995b; Tanaka & Kunugi, 1996; Van Camp et al., 1997a; Stapelfeldt et al., 1996, Stapelfeldt & Skibsted, 1999). It has been suggested that the structure and properties of heat-induced and pressure-induced whey protein (β -LG) gels are different (Van Camp & Huyghebaert, 1995a; Van Camp et al., 1996). Van Camp & Huyghebaert (1995a, 1995b) showed that WPC gels produced by HP (400 MPa for 30 min) at protein concentrations ranging from 110 up to 183 g/L differ significantly from heat-induced protein gels (80 °C for 30 min) with respect to gel strength and appearance. It was found that heat-set gels, for equal protein concentrations, are firmer than pressure-induced gels (Van Camp & Huyghebaert, 1995a, Zasytkin et al., 1996). HP treatment generates gels with a more porous structure and lower firmness (Van Camp & Huyghebaert, 1995b; Zasytkin et al., 1996; Dumay et al., 1998), and that are weaker, less elastic and more exudative, than heat-induced gels (Cheftel & Dumay, 1996,; Dumay et al, 1998).

β -LG gels made by pressure treatment also exhibit different rheological properties from those made by heating (Dumay et al., 1998). Electron microscopy also suggested a higher level of cross-links in the heat-induced gels; HP generated a more porous network with a lower level of intermolecular cross-links (Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1996). Pressurisation of β -LG isolate solutions (70 g/kg protein, pH 7.0) at 450 MPa and 25°C for 30 min formed gels with a sponge-like texture and a porous microstructure that was prone to exudation (Zasytkin et al., 1996). The rigidity and elasticity of pressure-induced gels increased with the concentration of β -LG, but remained lower than that of heat-induced gels (87°C for 40–45 min) at the same protein concentration (Zasytkin et al., 1996). These heat-induced gels also displayed a finely stranded network and high water retention. Some of the previous studies also suggested that, at neutral pH, β -LG forms transparent, fine-stranded heat-induced gels (Paulsson, 1990; Stading & Hermansson, 1991; Stading et al., 1993; Langton & Hermansson, 1992; Foegeding et al., 1995). These results suggested that comparatively weaker intermolecular or interparticulate forces are formed by pressure treatments.

Many such studies on microstructure and rheological analysis of pressure-induced gels have been reported. However, no study has characterised the interactions or aggregates of whey proteins in the pathways of pressure-induced gel formation, and the composition of the protein aggregates formed during pressure-induced WPC gel formation has not been determined.

Factors affecting pressure-induced gel formation of whey proteins

It has been reported that factors such as pH and use of combinations of pressure and different temperatures (Walkenström & Hermansson, 1997), pHs (Van Camp & Huyghebaert, 1995b; Arias et al., 2000) and calcium contents (Van Camp et al., 1997b) affect the aggregation behaviour, pressure-induced functionality such as gel formation, and physical, rheological and microstructural properties of whey proteins. Protein–protein interactions are favoured near the isoelectric point of the whey proteins, and neutral and alkaline pHs stimulate the formation of intermolecular disulphide bonds (Van Camp & Huyghebaert, 1995b). Pressure-induced β -LG denaturation increased considerably at alkaline pH and decreased at acidic pH (Arias et al., 2000). Further, it has been reported that the role of calcium in the pressure-induced aggregation and gelation of whey proteins may be explained in a similar manner to the heat-induced effects on whey proteins (Mulvihill & Kinsella, 1988; Kinsella & Whitehead, 1989; Van Camp et al., 1997b). However, there is little information available on the effects of pressurising temperature on the whey protein aggregation and gelation.

2.6 Effects of HP on milk

Studies on the effects of HP on milk can be broadly grouped into several topics, including the effects of HP on casein micelles, changes in the appearance of pressure-treated milks, effects of pressure treatment on the dissociation of casein micelles, protein particle size, pressure-induced denaturation of whey proteins in milk and interaction of whey proteins with the casein micelles, effects of HP on milk from various species, effects of pressurising temperature on micelle size and turbidity of pressure-treated milk and effects of pH on pressure-induced solubilisation of the casein micelles, some of which have been reviewed (Huppertz et al., 2002).

2.6.1 Changes in the opacity or lightness of HP-treated milks

On pressure treatment, skim milk becomes translucent or semi-transparent with a slightly yellow hue. A similar change can be obtained by adding a calcium chelator or dissociating agent such as urea. If the milk is held at refrigerator temperature ($\sim 5^{\circ}\text{C}$), it will retain the semi-transparent appearance for several days. However, if it is held at room temperature, it becomes progressively more turbid. Warming samples from 4 to 43°C returned the colour values of HP-treated milk towards those of the untreated milk, although not to the same initial point (Needs et al., 2000a, 2000b; Trujillo et al., 2000).

In milk, HP may lead to irreversible disintegration of casein micelles into smaller casein particles depending on applied pressure, which may cause a decrease in milk turbidity and lightness (L^*) and an increase in milk viscosity (Johnston et al., 1992a, 1992b; Shibauchi et al., 1992; Desobry-Banon et al., 1994; Anema et al., 1997; Famelart et al., 1997; Gaucheron et al., 1997; Mussa & Ramaswamy, 1997; Schrader & Buchheim, 1998; García-Risco et al., 2000). The effects of HP on the milk from different species have been studied. A decrease in L^* and an increase in greenness ($-a^*$) and yellowness ($+b^*$) were observed when ewe's milk was HP treated (Gervilla et al., 2001). The decrease in L^* value could have been mainly due to disintegration of casein micelles by pressure into small fragments that increase the translucency of the milk (Johnston, 1995; Mussa & Ramaswamy, 1997). However, in skim milk treated at 600 MPa for 15 min, significant changes in L^* , b^* and a^* values, which could also be perceived visually, were observed.

2.6.2 Effects of HP on casein micelles

Casein is present in milk in the form of micelles with CCP playing an important role in maintaining their structural integrity (Horne, 1998; Walstra, 1999). Previous reports suggested that pressurisation of milk causes some irreversible fragmentation of the casein micelles, mainly between 150 and 400 MPa. Reports have indicated that, under pressure, hydrophobic and electrostatic interactions between proteins are disrupted (Buchheim et al., 1996; Mozhaev et al., 1996) and colloidal calcium phosphate (CCP) is solubilised (Buchheim et al., 1996; Schrader et al., 1997; López-Fandiño et al., 1998; De la Fuente et al., 1999); as a result, considerable changes in the size, structure and composition of the casein micelles occur, which affect the turbidity, lightness and

viscosity of milk (Shibauchi et al., 1992; Famelart et al., 1997; Mussa & Ramaswami, 1997; García-Risco et al., 2000). In contrast, the concentration of free Ca^{2+} remains unchanged or increases only slightly (Johnston et al., 1992a, 1992b; Shibauchi et al., 1992; López-Fandiño et al., 1998). This lack of correlation between the loss of CCP and the level of free Ca^{2+} indicates that pressure-induced micelle fragmentation does not result exclusively from the breaking of linkages between caseins and inorganic constituents, but rather results from the weakening or rupture of electrostatic and hydrophobic interactions between casein constituents (Johnston et al., 1992a, 1992b; Cheftel & Dumay, 1996; Messens et al., 1997; De la Fuente, 1999; Claeys et al., 2003) and increased exposure of hydrophobic groups on the surface of the proteins with increasing severity and duration of pressure treatment (Johnston et al., 1992a, 1992b).

2.6.3 Effects of HP on the dissociation of casein micelles

Unlike heated milk, there have been only limited studies on the dissociation of casein micelles in pressure-treated milk. It was reported that dissociation of caseins, particularly κ - and β -CNs, occurs at HP (López-Fandiño et al., 1998). It has also been reported that HP treatment of milk at 100–600 MPa resulted in considerable solubilisation of α_{s1} - and β -CNs, with the extent of solubilisation reaching a maximum at around 250 MPa (Huppertz et al., 2004c). On storage of pressure-treated milk, dissociation of the caseins was largely irreversible at 5°C but considerable re-association of the caseins was observed at 20°C (Huppertz et al., 2004c).

At the natural pH of milk, and under relatively severe centrifugation conditions (100000 g), all caseins were found in the dissociated protein (López-Fandiño et al., 1998; Arias et al., 2000; Huppertz et al., 2004c), although the proportion of κ -CN appeared to be increased when compared with the whole casein (López-Fandiño et al., 1998; Arias et al., 2000). Increasing the pH of the milk to pH 7.1 increased the levels of all caseins dissociated from the micelles, compared with the samples at the natural pH. Decreasing the pH to 6.0 increased the levels of α_s -CN and β -CN dissociated, but decreased the level of κ -CN dissociated, whereas lowering the pH further increased the levels of all caseins dissociated (Arias et al., 2000). However, no study suggesting the pressure-induced dissociation of α_{s2} -CN has been reported.

2.6.4 Effects of HP on whey proteins in milk

Denaturation levels of whey proteins increase with increasing pressure or duration of treatment (López-Fandiño et al., 1996; López-Fandiño & Olano, 1998a, 1998b; Huppertz et al., 2004a). In the case of heat treatment of milk, the minor whey proteins (LF, BSA and Igs) are most labile; however, β -LG is the most pressure-sensitive whey protein, denaturing at pressures as low as 100–150 MPa. In contrast, α -LA and BSA are stable to pressures up to about 400–500 MPa (López-Fandiño et al., 1996; López-Fandiño & Olano, 1998a, 1998b; Huppertz et al., 2002). It has been reported that, compared with β -LG, α -LA (López-Fandiño et al., 1996; Felipe et al., 1997; López-Fandiño & Olano, 1998a; García-Risco et al., 2000; Needs et al., 2000a; Scollard et al., 2000; Huppertz et al., 2002; Huppertz et al., 2004b) or BSA (Hayakawa et al., 1992; Hinrichs et al., 1996b) is more resistant to pressure denaturation in milk and that different whey proteins have different sensitivities to HP treatment.

Several reports suggested that treatment of raw milk at up to 100 MPa does not denature β -LG. However, 70–80% denaturation of β -LG occurs at 400 MPa (López-Fandiño et al., 1996; López-Fandiño & Olano, 1998a; Arias et al., 2000; García-Risco et al., 2000; Scollard et al., 2000). Relatively little further denaturation of β -LG occurs at 400–800 MPa (Scollard et al., 2000). Various studies have reported different extents of denaturation of β -LG following HP treatment at 600 MPa of pasteurised milk (Needs et al., 2000a) or reconstituted skim milk powder (Gaucheron et al., 1997); this may be attributed to the level of denaturation caused by treatments before pressurisation, which may influence the amount of denaturation measured afterwards. The reaction order of HP-induced denaturation of β -LG is 2.5 (Hinrichs et al., 1996b), indicating that the denaturation process is concentration dependent and that a lower initial concentration of native β -LG should reduce the extent of denaturation of β -LG under pressure.

α -LA is very resistant to pressure at ambient temperature and denaturation of α -LA was noted at pressures > 400 MPa for long treatment times (López-Fandiño et al., 1996; Hinrichs et al., 1996b; Hinrichs et al., 1996a, 1996b; Arias et al., 2000; Needs et al., 2000a, Scollard et al., 2000; Huppertz et al., 2002, 2004a). Various studies using raw milk (López-Fandiño et al., 1996; López-Fandiño & Olano, 1998a; García-Risco et al., 2000), reconstituted skim milk (Gaucheron et al., 1997) and pasteurised skim milk

(Needs et al., 2000a) have shown that α -LA is resistant to denaturation at pressures up to 500 MPa.

Several reasons have been proposed for the differences in the sensitivity of these proteins. Differences in the pressure stability of α -LA and β -LG may be linked to the more rigid molecular structure of the former (López-Fandiño et al., 1996; Gaucheron et al., 1997), caused partially by the different numbers of intramolecular disulphide bonds in the two proteins (Hinrichs et al., 1996a, 1996b; Gaucheron et al., 1997), or the lack of a free sulphhydryl group in α -LA (López-Fandiño et al., 1996; Funtenberger et al., 1997), or probably due to differences in the secondary structures of these whey proteins. It has also been commented that the molecular structure of α -LA is more stable than that of β -LG, and that oligomerisation takes place only if, during unfolding, free SH groups are available from other molecules (Hinrichs et al., 1996b; López-Fandiño et al., 1996; Gaucheron et al., 1997; Jegouic et al., 1997). This difference in pressure sensitivity can also be explained by the types of bonds stabilising the conformational structures of β -LG and α -LA (Hinrichs et al., 1996b; Messens et al., 1997).

BSA was also found to be resistant to pressures up to 400 MPa in raw milk (López-Fandiño et al., 1996) or 600 MPa (Hayakawa et al., 1992). The high stability of BSA could be explained by the fact that BSA carries one SH group and 17 disulphide bonds. The energy received under pressure treatment was too small to disrupt all the disulphide bonds and to change the molecular structure of BSA. Also, greater resistance of β -LG and α -LA in whey than in milk may be attributed to the absence of casein micelles and CCP in whey (Huppertz et al., 2004b).

2.6.5 HP-induced interactions of caseins and whey proteins

It was reported that, when mixtures of κ -CN and β -LG were pressure treated at 400 MPa, the presence of β -LG reduced the susceptibility of κ -CN to subsequent hydrolysis by chymosin, indicating interactions between the proteins (López-Fandiño et al., 1997). SDS-PAGE studies of pressure-treated and untreated milks or solutions containing κ -CN or β -LG or both in the presence or absence of denaturing agents showed evidence for the formation of aggregates linked by intermolecular disulphide bonds (López-Fandiño et al., 1997). On HP treatment of milk at 300–600 MPa, β -LG may form small

aggregates (Felipe et al., 1997) or may interact with the casein micelles (Needs et al., 2000a; Scollard et al., 2000). However, the exact mechanism for these processes needs further clarification. Like heat treatment, no study suggesting pressure-induced interactions of α_{s2} -CN with whey proteins has been reported. Also, no studies suggesting pressure-induced interactions of other whey proteins with the casein micelles have been reported.

It has also been shown that the heat-induced interactions of the denatured whey proteins with the casein micelles are strongly pH dependent (e.g. Singh & Fox, 1987a, 1987b; Anema & Klostermeyer, 1997; Anema & Li, 2003a, 2003b). Some these reports suggested that high level (about 80% of the total) of denatured whey protein associate with the casein micelles at pH 6.5, and this level decreases with increasing pH, so that about 30% of the total is associated with the casein micelles at pH 6.7, and even lower levels are observed at higher pH. In contrast to the numerous studies on the distribution of whey proteins between the colloidal and serum phases in heated milk, few studies have examined this distribution in pressure-treated milk. Huppertz et al. (2004a) suggested that the majority of the denatured whey protein was sedimentable, and presumably associated with the casein micelles, under more severe centrifugation conditions than used in the more recent heating experiments. Arias et al. (2000) examined the effect of the pH of the milk at pressurisation (400 MPa for 15 min). However, conclusions on the association of the casein micelles with the whey proteins or the distribution of the whey proteins between the colloidal and serum phases in pressure-treated milk could not be made.

2.7 Conclusions

HP processing is a rapidly growing preservation technology that, even if in the development stage, presents the potential for making products that are microbiologically safe and with improved functional properties (Cheftel, 1992). Although HP processing is currently a focus of major interest (Johnston et al., 1992a, 1992b; Shibauchi et al., 1992; Desobry-Banon et al., 1994), many of the effects of pressure on the components of milk are still not known.

Many studies on HP treatment of dairy systems have focused on the effects of HP on the denaturation and aggregation of the individual whey proteins, particularly β -LG, in pure protein solutions, whey or milk. Also there have been many studies on microstructural or rheological aspects of whey protein gels or effects of HP on protein particle size, casein micelle dissociation, appearance of milk etc., but there is limited information identifying the pressure-induced aggregation products in whey protein solutions or interaction products of casein with whey proteins in pressure-treated milk. Although there is a clear understanding of the effects of heat treatments on the proteins in dairy systems, there is a need for a comparable level of understanding of the effects of pressure treatments on the milk/whey system. A detailed study of the differences between heat- and pressure-induced changes in denaturation, aggregation and interactions of different whey proteins will help to find new applications for HP processing in the food industry.

It is expected that pressure-induced whey protein gels may have different properties from those induced by heat. However, there is limited information on the pathways of pressure-induced whey protein gel formation and on how the protein concentration, pressurising temperature and holding time will influence the denaturation and aggregation of whey proteins. Further fundamental studies are required to understand the pressure-induced aggregation and gelation pathways of whey proteins in detail.

Most of the previous studies concerning interactions of milk proteins have been conducted in model systems (mainly β -LG) and very little has been reported on the pressure-induced changes and interactions that take place during processing in the actual milk/whey system. In addition, various heating methods have been used, including heating samples in glass tubes, capillary tubes immersed in water/oil baths and laboratory-scale heat exchangers. Only a few studies (e.g. Oldfield et al., 2000) on the heat-induced interactions of milk proteins using typical commercial process conditions are available. It is likely that proteins respond differently depending on how the sample is heated, i.e. on the time required to reach the desired temperature, flow conditions, and cooling times and rates etc. Hence, the process of denaturation of the whey proteins on heating milk under industrial conditions is likely to be different from that observed in milks heated in the laboratory.

2.8 Objectives

In the light of all the above, the main objective of the present investigation was to gain greater understanding of the behaviour of various milk proteins and their interactions as affected by HP treatment, by categorising in the following sub-objectives.

- 1 To apply two-dimensional PAGE techniques (with appropriate modifications if necessary) to characterise the pressure-induced aggregation products of whey proteins and the pressure-induced interaction products of caseins and whey proteins.
 - 2 To identify the key differences and similarities (if any) between the heat-induced and the pressure-induced aggregation and interactions of milk proteins using common analytical techniques.
 - 3 To understand the effects of various experimental parameters, such as protein concentration, intensity of pressure, holding time and pressurising temperature, on the pressure-induced aggregation and gelation of whey proteins in solutions of commercial WPC.
 - 4 To study the protein aggregation pathways involved in pressure-induced gel formation in WPC solutions.
 - 5 To study the heat- and pressure-induced interactions of caseins and whey proteins in skim milk under identical conditions (using common aliquots).
 - 6 To clarify the role of different whey proteins in pressure-induced aggregation using model system, i.e. pure proteins, either individual protein systems or mixed protein systems (i.e. in binary and ternary combinations of pure protein solutions).
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CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Whey protein concentrate (WPC)

A 25 kg bulk pack of commercial acid WPC (ALACEN 342), obtained from Fonterra Co-operative Group Ltd, New Zealand, was used throughout this study (Chapters 4–6). The WPC powder was transferred into several small size (about 500 g), light-, oxygen- and moisture-proof aluminium foil bags, which were immediately stored at 4°C to prevent further changes.

This WPC powder was typical of standard commercial product. Analysis showed that the powder contained per kilogram 815 g of total protein, 65 g of fat, 45 g of lactose and 49 g of moisture. Mineral analysis showed that the powder contained per kilogram 1.7, 13.8, 8.0 and 2.5 g of calcium, potassium, sodium and phosphorus respectively. The major proteins of WPC — β -lactoglobulin (β -LG), α -lactalbumin (α -LA), immunoglobulins (Igs) and bovine serum albumin (BSA) — were present in the ratio (w/w) 0.60:0.21:0.12:0.07. This ratio could change with the milk source as well as with the milk and whey processing conditions. The type of WPC used in the current study typically contains β -LG with one lactose molecule attached (Kerianne Higgs, Carmen Norris and Don Otter, Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, 2003, personal communication).

3.1.2 Skim milk

About 40 L of fresh bulk cow's milk (from a morning milking) was obtained, each time, from a dairy farm at Massey University, Palmerston North, New Zealand. The raw milk was warmed to $40 \pm 1^\circ\text{C}$ and skimmed (without pasteurisation) using a pilot-scale cream separator. The fat content of the resultant skim milk was less than 0.1%. The raw skim milk thus obtained was used in subsequent experiments to study the effects of heat and pressure treatments on the proteins in skim milk (Chapter 7).

3.1.3 Pure proteins

The pure protein standards were used for identification of PAGE patterns of individual proteins in native-, sodium dodecyl sulphate (SDS) and alkaline urea (AU) gels. β -LG of approximately 90% purity was made in the laboratory using the method described by Manderson et al. (1998). Bovine α -LA and BSA were obtained from Sigma Chemical Co., St. Louis, MO, USA (purity approximately 85% by electrophoresis). Standard NZMP 7101 lactoferrin (approximately 93% purity), Fonterra Co-operative Group Ltd, New Zealand, was used. Freeze-dried κ -casein (κ -CN), α _S-CN (α _{S1}- + α _{S2}-CN) and β -CN were provided by Skelte Anema (Fonterra Co-operative Group Ltd, Palmerston North, New Zealand); pure α _{S2}-CN and IgG were provided by Don Otter (Fonterra Co-operative Group Ltd, Palmerston North, New Zealand).

3.1.4 Chemicals

The electrophoresis chemicals and molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The reducing agent 2-mercaptoethanol (2-ME) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical grade from BDH Laboratory Supplies, Poole, England. Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and was deionised using a Milli-Q apparatus (Millipore Corporation, Bedford, MA, USA).

3.2 Sample preparation

The common methods generally applicable to most studies are presented in this chapter. Experimental protocols, analytical methods or modifications of methods specific to any particular studies are included in the relevant chapters.

3.2.1 Preparation of WPC solutions

Aliquots of WPC solutions were prepared by dissolving appropriate quantities of acid WPC powder (ALACEN 342) in Milli-Q water so that the final solutions contained the desired concentration of WPC. Generally, either one or more from 0.5, 2, 5, 8 and 12% (w/v) WPC solutions were used in the various experiments (Chapters 4–6) of the

present study. The protein content of each of these solutions was approximately 81.5% (e.g. a 12 % w/v WPC solution had a protein content of about 9.8 %).

The solutions were slowly stirred for 4–5 h at room temperature ($22 \pm 1^\circ\text{C}$) using a magnetic stirrer when a small sample size (up to 1–2 L) was required for laboratory-scale experiments. When a large quantity of WPC solution was prepared for pilot plant trials, an adjustable speed Ultraturrax T25 mixer (Janke & Kunkel GmbH & Co., Staufen, Germany, supplied by Lab Supply Pierce, Auckland, New Zealand) was used to dissolve the large quantity of WPC. The WPC solutions were further held for 5–6 h at 4°C for complete hydration. The pH of the WPC solutions was measured after complete dissolution and just before heat or pressure treatment, and was found to be in the range 6.65 ± 0.05 .

3.2.2 Procedure for obtaining permeate from skim milk

A large quantity (10 L) of reconstituted skim milk was prepared by dissolving 1 kg of low heat skim milk powder (whey protein nitrogen index (WPNI) ≥ 7.0) in Milli-Q water using an adjustable speed Ultraturrax T25 mixer (Janke & Kunkel GmbH & Co., Staufen, Germany). The reconstituted skim milk was stirred in a cold room (4°C) for 10–12 h (overnight) in order to achieve complete hydration and mineral equilibrium.

The skim milk was ultrafiltered using a 1 μm hollow fiber membrane cartridge (molecular weight cut-off 10 000 Daltons (nominal)) (Amicon Division Inc., Beverly, MA, USA) and the associated pumping system with adjustable pressure and flow control (ISMATEC-MV pump system, Glattbrugg, Switzerland). The skim milk was circulated through the system until approximately 1–2 L of clear permeate had been collected. This permeate was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to ensure that it was free from whey proteins. This permeate was used for subsequent sample preparation, mainly for pure protein studies (i.e. Chapter 8).

3.3 Processing

3.3.1 Heat treatment of skim milk or WPC solutions

Aliquots (4 L) of skim milk or WPC solution were heated separately using four different time–temperature combinations (viz. 72°C for 15 s, 100°C for 120 s, 120°C for 120 s and 140°C for 5 s) in a pilot-scale UHT plant, in direct heating (Type D, Alfa Laval, Lund, Sweden) installed at Massey University, Palmerston North, New Zealand. These time–temperature combinations are some of the commercial heat treatments that are used routinely in the dairy and food industries (e.g. pasteurisation, preheat treatments used in the powder manufacturing process and sterilisation). The required holding time was obtained by adjusting the flow of incoming milk or by selecting appropriate holding tubes with different lengths. It should be noted that the raw skim milk was not heat-treated before these heat treatments in order to avoid denaturation of whey proteins. However, WPC was typical standard commercial product, therefore it is expected that the WPC had already undergone standard processing. A photograph of the UHT plant is presented in Figure 3.1.

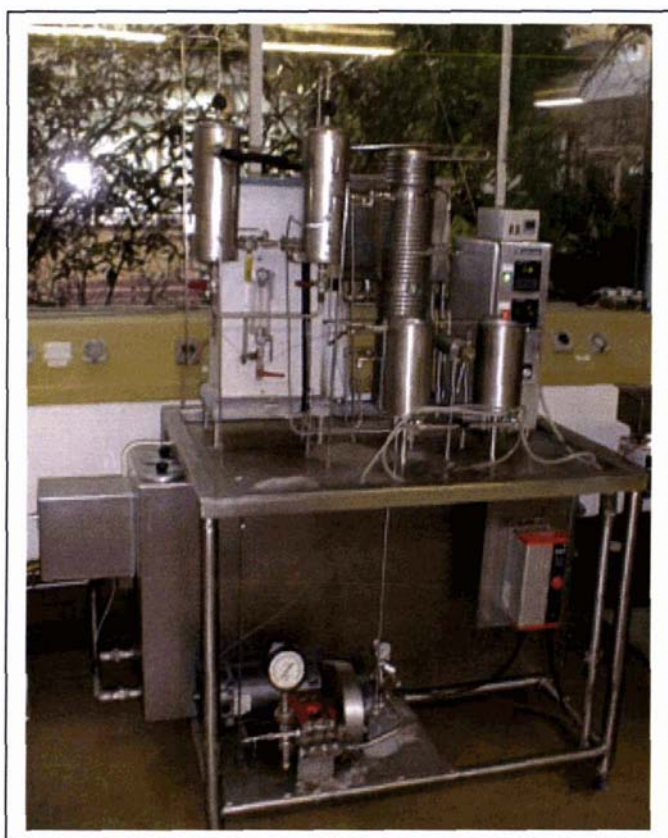


Figure 3.1. Photograph of Alfa Laval UHT pilot plant.

3.3.2 High pressure treatment of skim milk or WPC solutions

A typical high pressure system consists of four main parts: a high pressure vessel and its closure, a pressure-generating system, a temperature control device and a material-handling system. A photograph of a typical laboratory-scale high pressure unit ('Food-Lab' food processor, Model S-FL-085-9-W, manufactured by Stansted Fluid Power Ltd, Stansted, Essex, UK) capable of reaching up to 900 MPa, as used in the present study, and a schematic diagram of pressurisation are presented in Figure 3.2 and Figure 3.3 respectively.

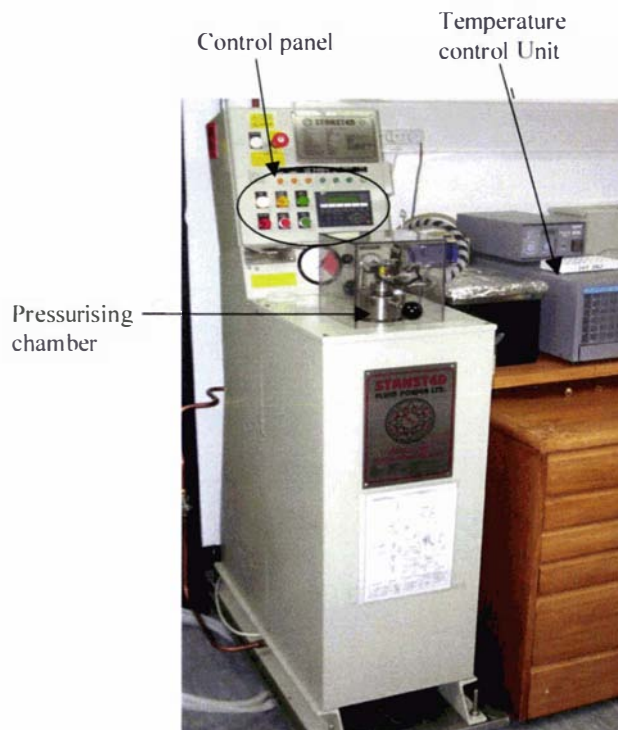


Figure 3.2. Photograph showing a typical laboratory-scale high hydrostatic pressure unit, as used in the present study.

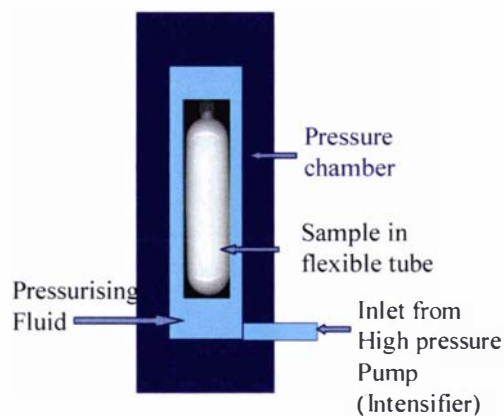


Figure 3.3. Schematic diagram showing pressurisation of a sample in the pressure chamber of a high pressure unit.

Beckman Polyallomer Quick-Seal™ centrifuge tubes (13 mm internal diameter, 15 mm high; Beckman Instruments, Inc., Spinco Division, Palo Alto, CA, USA) were filled with aliquots of skim milk or WPC solution and heat sealed. The tubes were equilibrated at a particular temperature at which they were to be pressure treated for at least 1 h prior to starting the pressure treatment. The sample tubes were then treated in the high pressure unit at a desired pressure and holding time and at a constant temperature of 20°C for most experiments, unless specified otherwise (e.g. effects of different pressurising temperatures were evaluated in the experiments described in Chapter 5). An emulsion, made by homogenisation of 10% vegetable oil in a solution of surfactant and preservative (Tim Carroll, Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, 2003, personal communication), was used as the pressurising fluid in the cylindrical 17 mm × 132 mm high pressure chamber. The pressurisation and depressurisation rates were 5 MPa and 14.5 MPa per second respectively. The average adiabatic heating during pressurisation was 1.4°C/100 MPa. The cooling during depressurisation was 1.3°C/100 MPa. The sample temperature returned to 22°C in about 5 min after pressurisation or depressurisation (Figure 3.4).

For pressure treatment of samples at a desired pressurising temperature (Chapter 5), the method described by Anema et al. (2005a) was followed. The high pressure unit was equipped with a water jacket. The desired pressurising temperature was obtained by heating or cooling the pressurising fluid to the preset temperature using the temperature control unit associated with the high pressure unit, and pumping and recirculating this pressurising fluid in the pressurising chamber. The temperature during pressurisation, depressurisation and the holding period was monitored using the thermocouple associated with the high pressure unit as well as using standard data logging equipment.

It was observed that the temperature of the pressurising fluid and the sample increases (≈ 1.3 – 1.8°C per 100 MPa) during pressurisation as a result of adiabatic heating and decreases (≈ 1.3 – 1.8°C per 100 MPa) as a result of adiabatic cooling (Figure 3.4). The pressure increased at a rate of ≈ 300 MPa per minute (Figure 3.4A). These observations are in agreement with results reported by Anema et al. (2005a) using the same equipment.

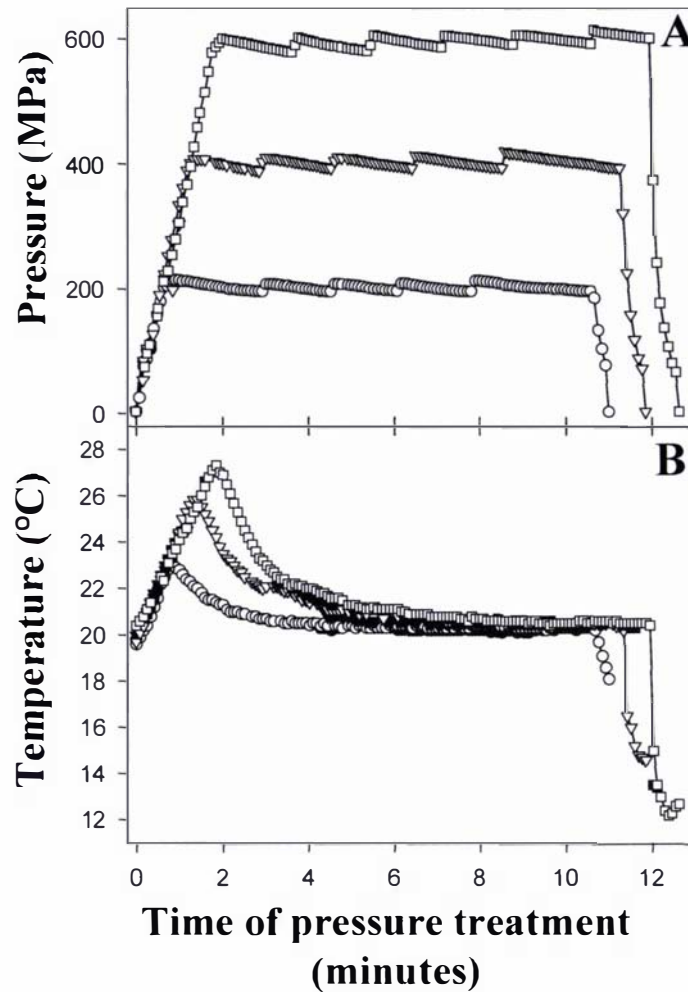


Figure 3.4. Changes in (A) pressure and (B) temperature during typical pressurisation cycles for samples pressure treated at (○) 200 MPa, (▽) 400 MPa and (□) 600 MPa.

3.4 Analysis of samples

After each series of pressure treatments, the Beckman Polyallomer centrifuge sample tubes were removed from the pressurising chamber and the top and bottom sections of the control and pressure-treated sample tubes were carefully cut using a sharp knife. The samples were carefully slid from the tubes in appropriate containers and their contents were analysed using a range of techniques. It is important to mention here that all the samples in the present study were analysed after pressure release. No measurements under pressure 'in situ' were performed.

3.4.1 Changes in colour and consistency

The changes in the colour and the consistency of each sample were noted within 30 min after pressure release, and the samples were then immediately photographed using a Nikon digital camera (3.2 mega pixels), whenever required (e.g. Chapters 6 and 7).

3.4.2 Rheological analysis

Rheological measurements, both rotational and dynamic, of control and pressure-treated samples of concentrated WPC solutions (Chapter 6) were made using a stress-controlled Paar-Physica rheometer model USD200 (Paar-Physica, Stuttgart, Germany), with a cone–plate geometry at a constant temperature of 20°C. The cone had an angle of 2° and a diameter of 50 mm. The flow curves were obtained in steady-state mode and the frequency sweep measurements were obtained at a constant strain of 10%.

3.4.3 Transmission electron microscopy (TEM)

Control and pressure-treated WPC samples were fixed (within 2–3 h after depressurisation), stained and processed as described by Langton & Hermansson (1996) and the transmission electron microscopy images were recorded photographically. The procedure steps followed in the present study are described below.

Fixation: The sample was cut into approximately 1 mm³ cubes (where appropriate) and put into a bijoux bottle containing 6.25% glutaraldehyde in 0.2 M imidazole buffer. This was stored at 5°C for 2 days. The glutaraldehyde solution was rinsed twice with 0.2 M imidazole buffer over 2 h. The buffer was removed and the sample was placed in 1% osmium tetroxide prepared in 0.2 M sodium cacodylate overnight. The sample was rinsed twice with distilled water, placed in 1% uranyl acetate for 30 min and then again rinsed twice with distilled water.

Dehydration: The dehydration process was carried out at 5°C in 25% acetone (15 min) and then in 50, 70 and 90% acetone (for 30 min each) followed by 100% acetone (three changes over 90 min).

Embedding: The acetone was then replaced with Procure 812 embedding resin, and the sample was put on rollers for 24 h. A cube of the sample was placed into a BEEM embedding capsule and cured at 60°C for 48 h.

Sectioning: The embedded samples were sectioned to a thickness of 90 nm using a Leica Ultra cut R microtome. These sections were mounted on 3 mm copper/rhodium grids and stained using lead citrate before examination in a Philips 201C transmission electron microscope (Philips, Eindhoven, The Netherlands) at an accelerating voltage of 60 kV.

3.4.4 Size exclusion chromatography (SEC)

The control and pressure-treated WPC samples (Chapter 6) were diluted by mixing 1 part of sample with 19 parts (w/w) of the elution buffer (20 mM imidazole/HCl, 50 mM NaCl, pH 6.80). The samples that had been pressure treated for more than 60 min were difficult to disperse and often required extensive stirring and agitation using a vortex mixer. The solutions were then filtered through a 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA). These samples were then analysed using a high performance liquid chromatography (HPLC) system, Aligent model 1050 (Aligent Technologies, Palo Alto, CA, USA), fitted with an Aligent 1050 pump and an Aligent 1050 detector set. The HPLC column [150 mm × 30 mm TOSOH TSK-G4000_{PWXL} (Supelco, Bellefonte, PA, USA)] was equilibrated with the elution buffer at a flow rate of 0.2 mL/min. The samples were loaded and eluted at 0.2 mL/min, and the eluates were monitored at 214 and 280 nm.

3.4.5 PAGE analysis

Preparation of native, sodium dodecyl sulphate (SDS) and alkaline-urea (AU) sample buffers

Native sample buffer: The native gel sample buffer (pH 6.8) was prepared by mixing 200 mL native stacking gel buffer (0.5 M Tris adjusted to pH 6.8), 20 mL of 0.4 % Bromophenol blue solution, 600 mL Milli-Q water and 80 mL glycerol.

SDS sample buffer: Similarly the SDS sample buffer consists of 500 mL Milli-Q water, 125 mL of 0.5 M Tris-HCL buffer (pH 6.8), 100 mL glycerol, 200 mL of 10 % w/v SDS solution, and 25 mL of 0.4 % Bromophenol blue solution.

AU sample buffer: AU sample buffer was prepared by dissolving 10.8 g Tris (hydroxymethyl) methylamine base, 360 g urea, 5.5 g boric acid, 0.92 g Ethylene diamine-tetra-acetic acid (EDTA) and 25 mL of 0.4 % (w/v) bromophenol blue solution in about 500 mL water, adjusting the pH to 8.4 with 1 M HCl and making up the final volume to 1 L.

Preparation of sub-samples for polyacrylamide gel electrophoresis (PAGE) analysis

Non-reduced samples: Appropriate quantities (normally 50 or 100 mg depending on the concentration of protein in the original samples) of each of the control and heat- or pressure-treated samples were weighed or transferred accurately into Eppendorf tubes, dispersed into 1.0 mL of sample buffer (native or SDS or alkaline urea sample buffer) and mixed using a vortex mixer. The samples that had been pressure treated for 60 min or longer (Chapter 6) were stirred intermittently with a vortex mixer and often took about 16 h to disperse completely.

Reduced samples: A set of SDS-reduced samples was prepared by heating a mixture of 1 mL aliquot of SDS sub-sample (control and heat- or pressure-treated samples) and 20 μ L of 2-mercaptoethanol (2-ME) at 94°C for 4-5 min. The native- and AU sub-samples were not reduced.

Molecular weight markers

Molecular weight markers (M_r) were also loaded on some of the 1D and 2D SDS-PAGE gels. These M_r were obtained from Bio-Rad and contained the following proteins: phosphorylase (97,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400) and aprotinin (6,500).

One-dimensional (1D) and two-dimensional (2D) PAGE

The control and heat- or pressure-treated samples were analysed using a Mini-Protean II dual cell system (Bio-Rad Laboratories, Hercules, CA, USA) and the discontinuous PAGE system for both 1D and 2D PAGE. The methods described by Havea et al. (1998) and Manderson et al. (1998) were used for 1D and 2D PAGE analysis. The polyacrylamide gel contains two main parts: the resolving gel and the stacking gel (see Figure 3.5). The gels were scanned and photographed, as described by Manderson et al. (1998), using a computing laser densitometer (Molecular Dynamics Model P. D., Sunnyvale, CA, USA) and the integrated intensities of the bands corresponding to β -LG, α -LA and BSA were determined using Molecular Dynamics ImageQuant software (Version 5.0). The experiments were repeated using similar experimental conditions and the variations in the integration results were found to be within 5–10%. The data plotted on the graphs are the averages of two integrations.

Three different types of PAGE systems were used, either singly (1D PAGE) or in combinations (2D PAGE) in the present study. The procedures for preparation of these gels are described below.

Preparation of 1D native-PAGE gels

Native-PAGE resolving gel: An appropriate quantity (2.0 mL) of native resolving gel buffer (stock solution prepared by dissolving 72.6 g of Tris (hydroxymethyl) methylamine base in Milli-Q water, adjusting the pH to 8.8 and finally making up the total volume to 200 mL with Milli-Q i.e. 3.0 M Tris-HCL buffer adjusted to a pH of 8.8), 6.0 mL of water and 8 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide) (Bio-Rad catalogue no. 161-0158)] was accurately transferred to a vacuum flask, mixed well and allowed to stand at ambient temperature (20–23°C) for about 30 min. This mixture was then degassed under vacuum for 20 min with constant stirring of the mixture using a magnetic stirrer. Meanwhile, the gel setting apparatus was assembled using 0.75 mm spacers, following the standard description in the Bio-Rad manual. Immediately after the degassing step and just prior to the gels being poured, 8 μ L of N, N, N', N'-tetramethylethylenediamine (TEMED) and 80 μ L of a 10% pre-made solution of

ammonium persulphate (APS) were added to the mixture to finally give a resolving gel with 15% T and 2.67% C. In preparing acrylamide solution, we used 30 % acrylamide-Bis solution 37.5:1 (2.67 %C) from Bio-Rad. The acrylamide provide gel chain, whereas Bis forms a cross-links between chain to form three dimensional structure. The % T refers to the concentration / strength of acrylamide, whereas % C refers to the % w/w of cross-linking Bis.

From this final mixture, 3.3 mL was transferred between each pair of glass plates. About 0.5–1.0 mL of water was then placed above the resolving gel in the corner and the apparatus was gently tapped to give the resolving gel a smooth surface upon setting. The gel apparatus was set aside at ambient temperature (20–23°C) and left undisturbed for about 30 min for the gels to set. The water from the top layer was then removed completely with the help of a blotting paper wick.

Stacking gel: Similarly, the stacking gel was made from a mixture of 2.0 mL of stacking gel buffer (stock solution prepared by dissolving 30 g of Tris base in 400 mL of milli-Q water, adjusting the pH to 6.8 with 6 M HCL and making the final volume up to 500 mL, i.e. 0.5 M Tris-HCL buffer adjusted to pH 6.8), 5.0 mL of water and 1.0 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide)]. This was then degassed as described above and 40 µL of 10% APS and 8 µL of TEMED were added (to give 3.9% T and 2.67% C gels). This mixture was transferred into the gap between the glass plates, on top of the preset resolving gel, and a 0.75 mm plastic comb with 10 slots was inserted, to form sample loading wells or slots, taking care that no air bubbles were entrained. The gel apparatus was left undisturbed at ambient temperature (20–23°C) for about 1–1.30 h for complete polymerisation of the gels. The entire gel assembly was then transferred into an airtight container containing some distilled water (to prevent drying of the gels), which was then transferred to a cold room (4–6°C) overnight.

Native electrode buffer: The stock solution of native electrode buffer (0.125 M Tris/0.95 M glycine, pH 8.3) was prepared by dissolving 15.0 g of Tris base and 72.0 g of glycine in about 800 mL Milli-Q water and then finally making the volume up to 1 L. In use, this stock solution was diluted 1:4 with Milli-Q water.

Preparation of 1D SDS-PAGE gels

The 1D SDS-PAGE gels were prepared in the same manner as described above for native-PAGE, but different proportions of SDS resolving gel buffer, SDS stacking gel buffer and TEMED, APS and acrylamide were used, and a proportion of Milli-Q water was substituted with stock 10 % (w/v) SDS solution, which is described in detail below:

SDS-PAGE resolving gel: In preparation of SDS-PAGE gels, the resolving gel mixture consisted of 3.75 mL of SDS resolving gel buffer (stock solution prepared by dissolving 18.15 g of Tris (hydroxymethyl) methylamine base in about 60 mL of milli-Q water, adjusting the pH to 8.8 and finally making up the total volume to 100 mL with milli-Q i.e. 1.5 M Tris-HCL buffer adjusted to a pH of 8.8), 3.0 mL of water and 7.95 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide)] was accurately transferred to a vacuum flask, mixed well and allowed to stand at ambient temperature (20–23°C) for about 30 min. This mixture was then degassed under vacuum for 20 min with constant stirring of the mixture using a magnetic stirrer. Meanwhile, the gel setting apparatus was assembled using 0.75 mm spacers, following the standard description in the Bio-Rad manual. Immediately after the degassing step and just prior to the gels being poured, 150 µL of stock 10 % (w/v) SDS solution, 7.5 µL of N, N, N', N'-tetramethylethylenediamine (TEMED) and 75 µL of a 10% pre-made solution of APS were added to the mixture. The remaining steps were same as described for native PAGE resolving gel.

SDS-PAGE stacking gel: Similarly, the stacking gel was made from a mixture of 2.50 mL of stacking gel buffer (stock solution prepared by dissolving 6 g of Tris base in about 60 mL of milli-Q water, adjusting the pH to 6.8 with 6 M HCL and making the final volume up to 100 mL, i.e. 0.5 M Tris-HCL buffer adjusted to pH 6.8), 6.10 mL of water and 1.30 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide)]. This was then degassed as described above and 100 µL of stock 10 % (w/v) SDS solution, 50 µL of 10% APS and 10 µL of TEMED were added. The remaining steps were same as described for native PAGE stacking gel.

Stock SDS electrode buffer: The stock solution of SDS electrode buffer (5X) was prepared by dissolving 15.0 g of Tris base, 72.0 g of glycine and 5 g of SDS powder in

about 800 mL Milli-Q water and then finally making the volume up to 1 L. The pH of the stock solution was in the range of 8.6 ± 0.1 . In use, this stock solution was diluted 1:4 with Milli-Q water.

Preparation of 1D AU-PAGE gels

AU-PAGE resolving gel: For AU-PAGE gels, the resolving gel mixture consisted of 8.90 mL of AU resolving gel buffer (stock solution prepared by dissolving 9.20 g of Tris (hydroxymethyl) methylamine base, 0.8 mL of concentrated HCl, 54.0 g urea in about 120 mL of milli-Q water, adjusting the pH to 8.8 with 1 M HCl and finally making up the total volume to 200 mL with milli-Q in volumetric flask), and 6.00 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide)] was accurately transferred to a vacuum flask, mixed well and allowed to stand at ambient temperature (20–23°C) for about 30 min. This mixture was then degassed under vacuum for 20 min with constant stirring of the mixture using a magnetic stirrer. Meanwhile, the gel setting apparatus was assembled using 0.75 mm spacers, following the standard description in the Bio-Rad manual. Immediately after the degassing step and just prior to the gels being poured, 7.5 μ L of N, N, N', N'-tetramethylethylenediamine (TEMED) and 75 μ L of a 10% pre-made solution of APS were added to the mixture. The remaining steps were same as described for native-PAGE resolving gel.

AU stacking gel: The AU stacking gel was made from a mixture of 8.65 mL of stacking gel buffer (stock solution prepared by dissolving 1.08 g of Tris base, 36.0 g urea, 0.55 g boric acid, 92 mg EDTA in about 70 mL of milli-Q water, adjusting the pH to 8.4 with 1 M HCL and making the final volume up to 100 mL), and 1.30 mL of acrylamide solution [30% stock solution of a 37.5:1 (2.6% C) mixture of acrylamide and N, N'-methylenebis (acrylamide)]. This was then degassed as described above and 50 μ L of 10% APS and 10 μ L of TEMED were added. The remaining steps were same as described for native-PAGE stacking gel.

AU stock electrode buffer: The stock solution of AU electrode buffer (5X) was prepared by dissolving 10.7 g of Tris base, 5.5 g of Boric acid and 0.9 g EDTA in about 800 mL Milli-Q water, adjusting the pH to 8.4 and then finally making the volume up to 1 L. In use, this stock solution was diluted 1:4 with Milli-Q water.

Electrophoresis of the samples on the 1D PAGE gels

After the chamber had been filled with the appropriate electrode buffer (i.e. either the native, SDS or alkaline urea (AU) electrode buffer), the corresponding sub-sample prepared by diluting in the appropriate sample buffer (i.e. the native, SDS or alkaline urea (AU) electrode buffer) were loaded accurately in the sample loading well using a Hamilton syringe. After the samples had been loaded on both gels, the maximum voltage, current, power and time were set at 210 V, 70 mA, 6.5 W and 1.1–1.2 h on the power supply (Bio-Rad model 1000/500) and electrophoresis was commenced. After the bromophenol blue dye band had reached the bottom of the gel, the power was turned off; the gels were carefully removed from the equipment, transferred into a transparent polyethylene container and were then stained, destained and scanned as described below:

Staining and destaining the gels

After electrophoresis, 1D gels were stained using 0.1% (w/v) amido black 10B dye dissolved in 10% acetic acid and destained using a 10% glacial acetic acid solution. It was found that the time and volume of the staining and destaining solutions used were critical for the quantitative determination of proteins. Furthermore, for inter-gel comparison all gels should be treated exactly the same, and stained and destained simultaneously. The following guide-lines were used for staining and destaining the gels:

Staining the gels: The staining step was performed by dispensing 50 mL of Amido Black 10B staining solution (see above) into the container. Set the container on the platform rocker in such a way that the stain covers the entire gel, adjust the time and leave the gel to stain for exactly 1 h.

Destaining: After the gel is stained for 1 h, drain the staining solution from the gel container carefully. Dispense 100 mL of destaining solution and return the gel container to the rocker for 1 h. After exactly 1 h, change the destaining solution by draining-off the used destain and replace with the fresh 100 mL of destain. Leave the container to destain on the rocker for a further 6 h and repeat the procedure at two further 6 h intervals.

Scanning and photographing the gels

The gels were then scanned using a Molecular Dynamics personal densitometer (Model PD-SI computing densitometer. Molecular Dynamics, Sunnyvale, CA, USA). The gels, immediately after completion of the destaining step were scanned at 633 nm with a He/Ne laser with a spot size of 50 μm at a resolution of 100 μm . The scanned images were processed or protein bands were integrated using Molecular Dynamics ImageQuant software, Version 5.0.

The gels were then photographed using a 35 mm camera fitted with both green and orange Hoya filters (to minimise stray light) on to 100 ASA Kodak T-max film, which was then processed in the usual way.

Quantitation of the intensity of protein bands

The total absorbance/integrated intensity of each protein band was determined and compared with the relative intensity of protein bands in untreated samples run on each gel as a control. As mentioned earlier, the small gel-to-gel differences (< 10%) in the absorbance values for the standards on each of the gels were compensated for by comparing the values for each of the standards with the mean value derived from the standard from every gel. A similar compensation factor was calculated, using the control samples on all the SDS-PAGE gels and all the native-PAGE gels, so that the results for SDS-PAGE and native-PAGE could be compared.

Preparation of 2D PAGE

Native:non-reduced SDS-PAGE or AU:non-reduced SDS-PAGE

After completion of 1D native- and AU-PAGE as described above, the gels were removed from between the glass plates and cut so that each strip contained all the protein bands from a sample, including any material still present in the sample loading well. These sample gel strips were then immersed into SDS sample buffer for 4 min. Each of the sample gel strips was then fitted into the gel apparatus using a 0.75 mm spacer and the SDS resolving and stacking gels were set so that the resolving gel was

below the sample gel strip. The sample gel strip was surrounded by stacking gel and was used as the source of the sample to run on SDS-PAGE in the second dimension (see Figure 3.5).

Once the gels were polymerised, they were run fresh using the same conditions as described for 1D SDS-PAGE. The gels were then carefully removed from between the glass plates and were stained and destained. Two of the 1D PAGE gel strips from the same gel were also stained to confirm that the separation in the first dimension was satisfactory. One of the stained gel strips and the 2D gel including the sample gel strip after completion of staining and destaining were arranged as shown in Figure 3.5. The gels in this arrangement were scanned and photographed (e.g. see 2D gels in Chapter 4 or Chapter 7). This procedure allowed the relationship between the stained spots/bands of the hydrophobic-linked proteins on the vertical pattern and horizontally mounted stained gel strip on the top to be established.

2D SDS then reduced SDS (SDS:SDS^R)-PAGE gels

After 1D SDS-PAGE, the gel was removed from between the glass plates and cut so that each strip contained all the protein bands from a sample, including any material still present in the sample loading well. Each of these unstained strips was placed in a separate test tube containing 10 mL of disulphide bond reducing buffer (a solution of 10 mL of SDS sample buffer containing 200 μ L of 2-ME), and held in a water bath thermostatically controlled at 94°C for 4 min to reduce the covalent disulphide bonds of the protein components.

After 4 min, each strip was removed from its test tube and washed with water to remove excess 2-ME. The washed gel strips were then immersed in SDS sample buffer for 4 min. Each of the treated gel strips (sample gel strips) was then fitted into the gel apparatus using a 0.75 mm spacer and the SDS resolving and stacking gels were set so that the resolving gel was below the sample gel strip. The sample gel strip was surrounded by stacking gel and was used as the source of the sample to run on SDS-PAGE in the second dimension (see Figure 3.5).

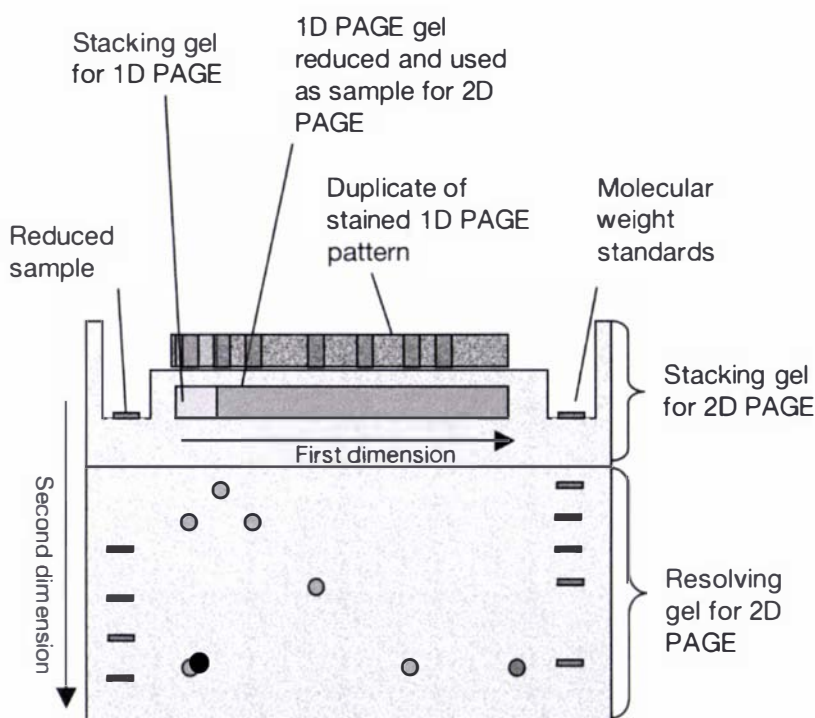


Figure 3.5. Schematic diagram showing the preparation and running of a sample of pressure-treated WPC solution in a 2D SDS:SDS^R-PAGE system.

Once the gels were polymerised, they were run fresh using the same conditions as described for 1D PAGE. The gels were then carefully removed from between the glass plates and were stained and destained. Two of the 1D PAGE gel strips were also stained to confirm that the separation in the first dimension was satisfactory. One of the stained gel strips and the 2D gel including the sample gel strip after completion of staining and destaining were arranged as shown in Figure 3.5. The gels in this arrangement were scanned and photographed (e.g. see 2D gels in Chapter 4 or Chapter 7). This procedure allowed the relationship between the stained spots/bands of the disulphide-bonded proteins and those of the reduced proteins on the vertical pattern to be established.

CHAPTER 4

Effects of Heat and High Pressure Treatments on the Unfolding and Aggregation of Proteins in WPC Solutions¹

4.1 Introduction

Thermal processing is one of the common traditional methods that have been employed to extend the shelf life of milk and milk products or to achieve the desired functional properties in the final products (Mulvihill & Donovan, 1987). Whey proteins are used as functional ingredients in the food industry. The inevitable heat treatments used during the processing and preservation of whey protein products can seriously affect the native state and stability of whey proteins (de Wit, 1990). Many aspects of the heat-induced denaturation, aggregation and gelation of whey proteins have been studied in considerable detail and their mechanisms are well understood, as discussed in Chapter 2. Most of the previous studies on the heat-induced denaturation and aggregation of whey proteins used model systems consisting of pure protein solutions and demonstrated that each of the whey proteins has a different response to heat treatment in terms of conformational changes, altered bonding patterns, formation of inter-protein aggregates via disulphide bond interchange, changed hydrophobic associations etc. (e.g. Gezimati et al., 1997; Manderson et al., 1998; Schokker et al., 2000; Havea et al., 2001; Hong & Creamer, 2002; Cho et al., 2003). Parallel studies using solutions of commercial whey protein concentrate (WPC) and purified β -lactoglobulin (β -LG), α -lactalbumin (α -LA) and bovine serum albumin (BSA) in milk serum (Havea et al., 1998, 2001, 2002) also clarified many important aspects of the heat-induced aggregation and gelation of whey proteins in their natural environment. The findings of all these previous studies have made a significant contribution to a detailed understanding of the mechanisms or pathways of the heat-induced denaturation, aggregation and gelation of whey proteins, but comparatively little is known about the

¹ Part of the content presented in this chapter has been published as a peer-reviewed paper in *Food New Zealand* (2004) 4 (3) 29–35.

The content from this chapter was also presented as a poster at the ADAS-ASAS Joint Annual Meeting, Minneapolis, Minnesota, USA, 9–13 July 2006, and was published in Dairy Foods: Chemistry and Microbiology Section, *Journal of Dairy Science* (2006) 89 (Suppl. 1) 175.

high-pressure (HP)-induced denaturation, aggregation and gelation of whey proteins in WPC systems.

HP processing has been recognised as a physical tool for the modification proteins (Cheftel, 1992; Hayashi, 1992; Balny & Masson, 1993), and it has potential to achieve new products with modified textures and functional properties (Datta & Deeth, 1999, 2003). Previous studies on the effects of HP on milk proteins have focused mainly on denaturation kinetics (e.g. Hinrichs et al., 1996; Hinrichs & Rademacher, 2004, 2005), rheological properties (e.g. Van Camp & Huyghebaert, 1995a, 1995b; Cheftel & Dumay, 1996, Dumay et al., 1998; Famelart et al., 1998) or microstructure (e.g. Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1996; Zasytkin et al., 1996; Walkenström & Hermansson, 1997; Dumay et al., 1998) and other aspects using WPC (e.g. Van Camp et al., 1997a, 1997b; Famelart et al., 1998; Kanno et al., 1998) and individual whey proteins in model systems (e.g. Dumay et al., 1994, 1998; Funtenberger et al., 1995, 1997; Jegouic et al., 1997; Moller et al., 1998). However, little attention has been paid to the characterisation of pressure-induced aggregates and interaction products in a complex whey protein system such as WPC. Also, the previous studies paid little attention to exploring the effects of heat and HP on the unfolding and aggregation of whey proteins using aliquots from the same samples.

Havea et al. (1998) and Manderson et al. (1998) developed various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques that were found to be very useful in understanding the heat-induced interactions of whey proteins and in understanding the heat-induced aggregation pathways in a WPC environment. In the above context, the main purpose of this preliminary work was to apply these PAGE techniques to analyse heat- and pressure-treated WPC solutions. By using the common techniques, we expected to be able to determine basic differences and similarities in the interactions of whey proteins in heat- and pressure-treated WPC solutions.

4.2 Experimental

The general procedures for the preparation and analysis of samples using 1D and 2D PAGE have been described in Chapter 3. A schematic diagram showing details of the experimental protocol used in the present investigation is shown in Figure 4.1.

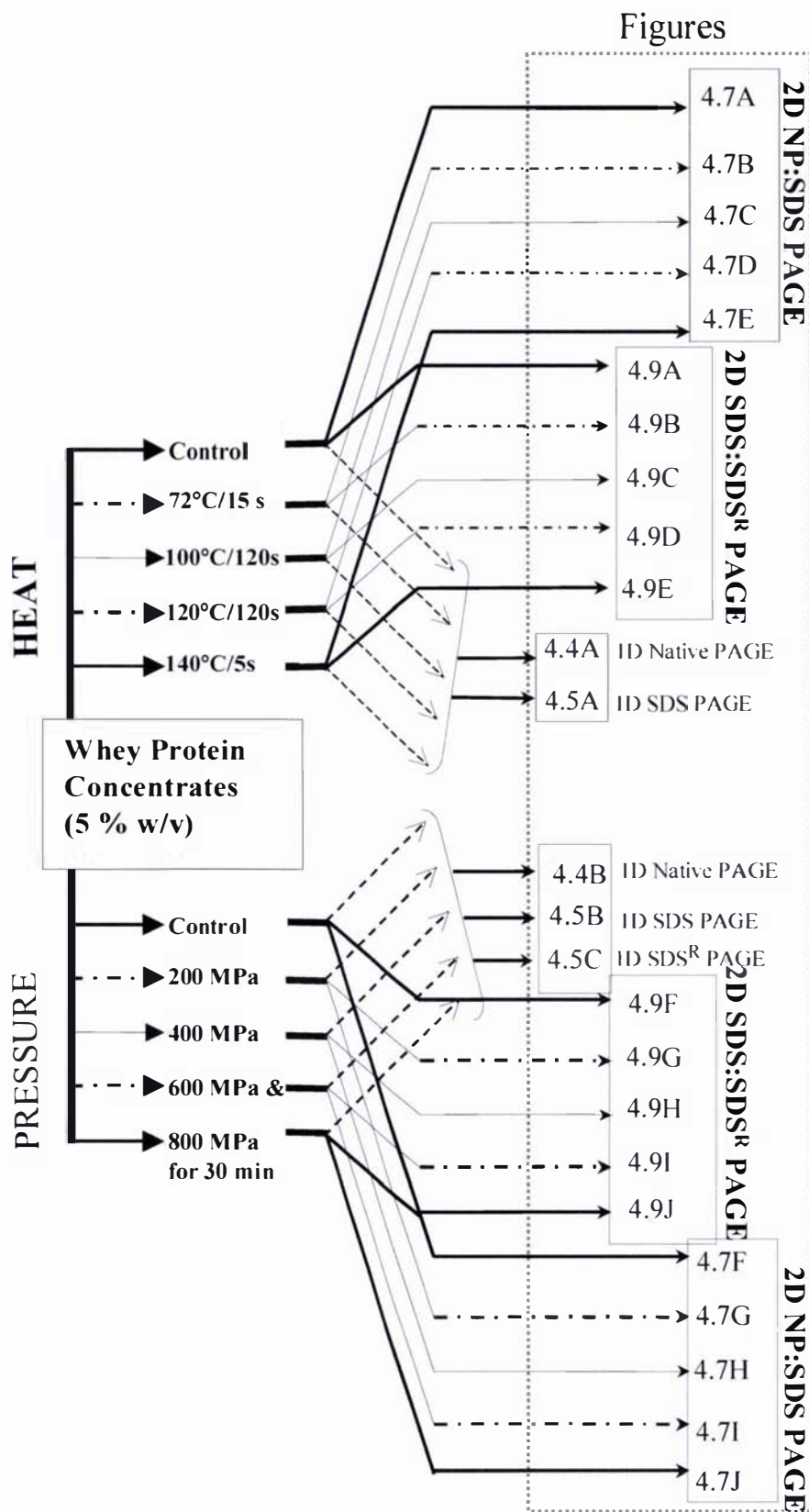


Figure 4.1. Simplified schematic diagram showing the preparation and coding of samples. These samples were subsequently analysed by 1D and 2D PAGE as described in Chapter 3. The PAGE patterns of each of these samples are shown in Figures 4.4–4.9. The abbreviations used are: NP, native-PAGE; SDS, sodium dodecyl sulphate; SDS^R, reduced SDS.

4.3 Results and discussion

4.3.1 1D PAGE patterns of pure whey proteins

The native- and SDS-PAGE patterns of pure whey proteins, including β -LG, α -LA, BSA, immunoglobulin G (IgG) and lactoferrin (LF), are presented in Figures 4.2A and 4.2B respectively. The pure whey proteins were analysed for the purpose of identifying the bands corresponding to individual whey proteins on the native- and SDS-PAGE patterns of mixed whey protein system (WPC).

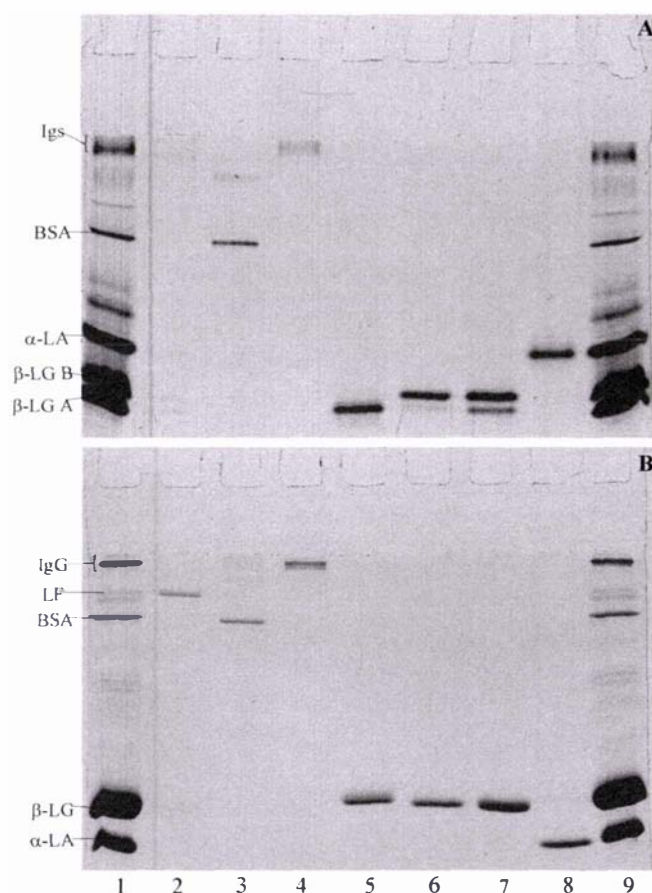


Figure 4.2. Identification of protein bands using pure proteins on (A) native-PAGE and (B) SDS-PAGE. PAGE patterns of: WPC solution (Lane 1), LF (Lane 2), BSA (Lane 3), IgG (Lane 4), β -LG AA (Lane 5), β -LG BB (Lane 6), β -LG AB (Lane 7), α -LA (Lane 8) and WPC solution (Lane 9).

4.3.2 Nomenclature of proteins in control and heat- or pressure-treated samples

The analysis of the control and heat- or pressure-treated samples using native- and SDS-PAGE showed loss of monomeric proteins and the formation of aggregates. Many researchers (McSwiney et al., 1994b; Gezimati et al., 1996, 1997; Havea et al., 1998) have defined and used terms such as native-like protein, SDS-monomeric protein, total reducible protein, hydrophobically linked aggregates, reducible disulphide-cross-linked aggregates etc. to identify different forms of proteins or protein aggregates observed on the native- and SDS-PAGE patterns of heat-treated whey protein solutions. As similar methodologies were used and similar changes were observed on the PAGE patterns of the pressure-treated samples, the same terminology could also be applied to identify and define the changes in the proteins in the pressure-treated samples (Figure 4.3).

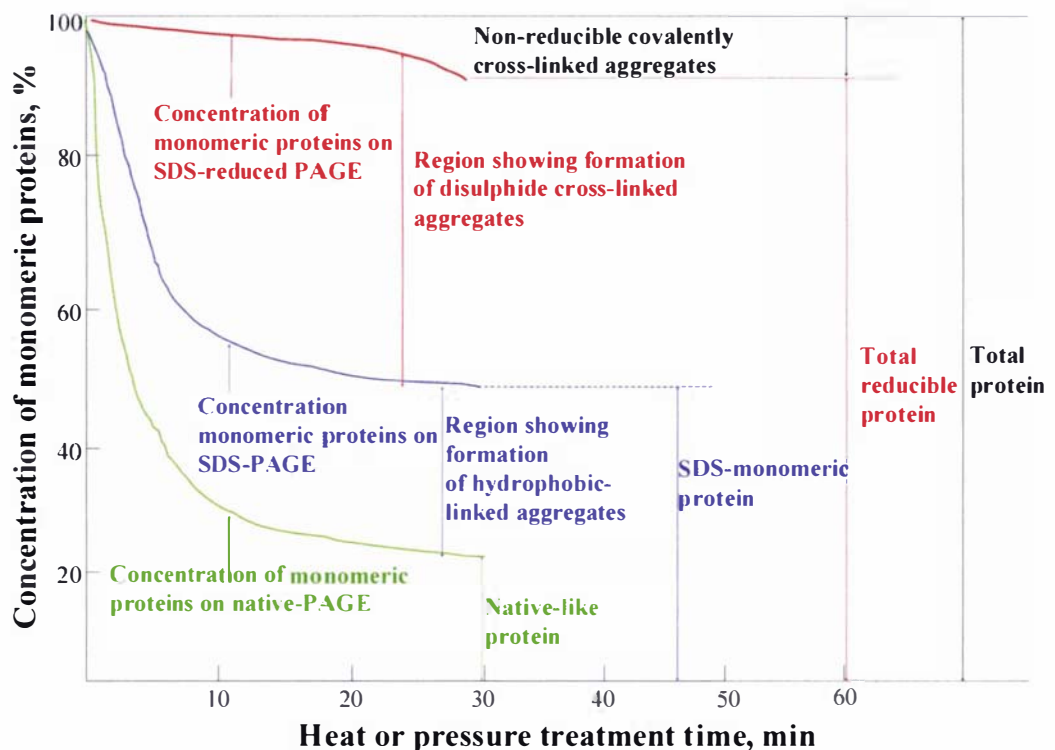


Figure 4.3. Schematic diagram showing a summary of the nomenclature of different forms of proteins and protein aggregates analysed using various electrophoresis techniques. The proteins in control and heat- or pressure-treated samples have been assigned a specific nomenclature, as reported earlier (McSwiney et al., 1994b; Gezimati et al., 1996, 1997; Havea et al., 1998). The figure has been redrawn with the permission of Havea (1998).

Analysis of heat-treated (Lanes 2–5, Figure 4.4A) and pressure-treated (Lanes 2–5, Figure 4.4B) samples by native-PAGE (pH approximately 8.3) gave protein bands that coincided with (resolved almost in the same position as) the native proteins in the untreated samples (Lane 1, Figure 4.4A or 4.4B); these bands could be referred to as ‘native-like’ proteins. On SDS-PAGE (pH approximately 8.3, 1 g SDS/L), the protein bands were more intense than those of the native-like proteins and migrated as though they were monomers. These bands on SDS-PAGE included non-covalent (mainly hydrophobically linked) aggregates, which were dissociated by SDS-PAGE. Therefore, these bands could be referred to as ‘SDS-monomeric’ proteins (Figures 4.5A and 4.5B). When the pressure-treated or heat-treated samples were analysed using SDS-PAGE in the presence of a reducing agent, such as 2-mercaptoethanol (2-ME), which converts disulphide bonds into free sulphhydryl groups, there were even more intense bands that migrated as though they were monomers; these bands could be referred to as ‘total reducible’ protein (Figure 4.5C). The relationships between these different forms of protein and their likely changes during the heat or pressure treatment of WPC solutions are summarised in Figure 4.3.

4.3.3 Identification of protein bands on 1D PAGE patterns of control samples

1D native-, non-reduced SDS- and reduced SDS-PAGE

The bands corresponding to native-like and SDS-monomeric whey proteins (β -LG, α -LA, BSA, IgG and LF) on the native- and SDS-PAGE patterns of the control WPC sample were identified by comparison with the native- and SDS-PAGE patterns of the pure proteins (Figures 4.2A and 4.2B respectively) and were marked appropriately on the left-hand side of the control samples (Lane 1) on the native- (Figures 4.4A and 4.4B) and SDS- (Figures 4.5A and 4.5B) PAGE patterns. The PAGE patterns of these protein bands were also compared with those reported previously (Havea et al., 1998) for WPC solutions analysed using similar methods. Some high molecular weight material that did not enter the gel (marked as X_0 on native-PAGE and \underline{X}_0 on SDS-PAGE) was also present in the PAGE patterns of the control samples (Lane 1, Figures 4.4A, 4.4B, 4.5A and 4.5B). It was noticed from the native-PAGE patterns of the control WPC samples (Figures 4.4A and 4.4B) that the WPC used in the present study contained a small amount of glyco- α -LA (denoted as α -LA*).

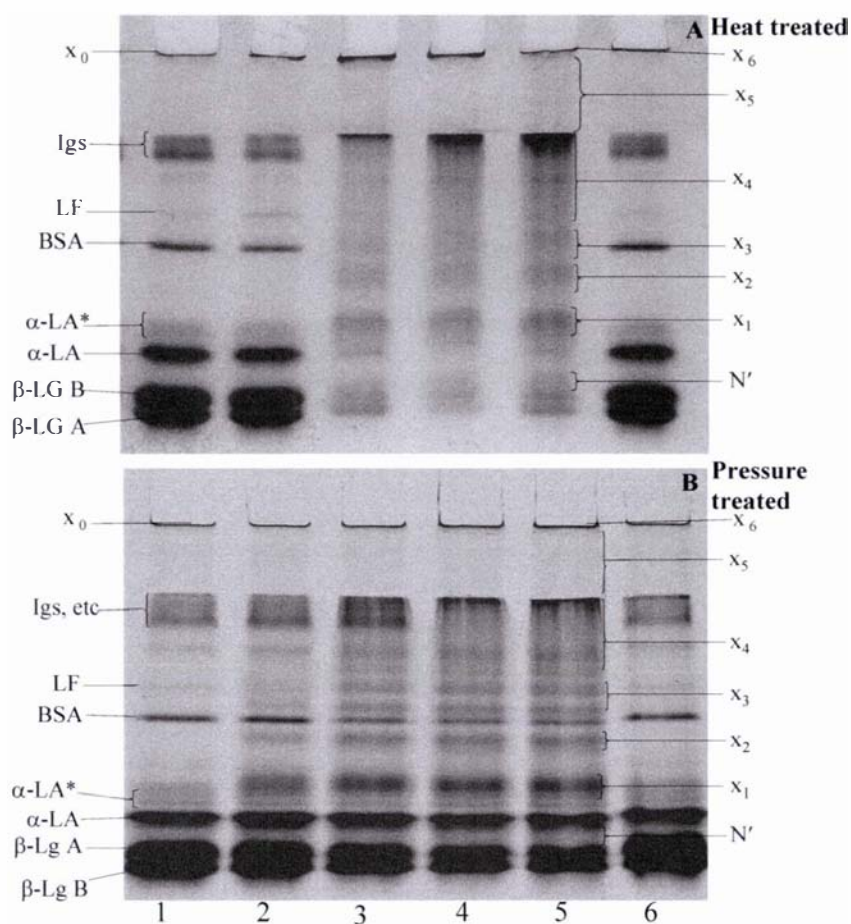


Figure 4.4. Native-PAGE patterns of (A) heat-treated and (B) pressure-treated WPC solutions (5% w/v). For the heat-treated samples (A), the PAGE pattern shows: control sample (Lane 1), samples heated at 72°C for 15 s (Lane 2), 100°C for 120 s (Lane 3), 120°C for 120 s (Lane 4) and 140°C for 5 s (Lane 5), and control sample (Lane 6). Similarly, for the pressure-treated samples (B), the PAGE pattern shows: control sample (Lane 1), samples pressure treated at 200 MPa for 30 min (Lane 2), 400 MPa for 30 min (Lane 3), 600 MPa for 30 min (Lane 4) and 800 MPa for 30 min (Lane 5), and control sample (Lane 6). X₀ stands for material present in the untreated control sample that could not enter the gel. N' indicates non-native monomers, and X₁, X₂ and X₃ correspond to dimers, trimers and tetramers respectively. The regions marked as X₄, X₅ and X₆ correspond to the largest aggregates, i.e. those caught up at the top of the resolving gel, those caught up within the stacking gel and those caught up within the sample well or that could not enter the gel respectively.

The A and B variants of β -LG were partially separated from one another on the native-PAGE patterns (Figures 4.4A and 4.4B), but were indistinguishable and migrated more slowly than α -LA on the SDS-PAGE patterns (Figures 4.5A and 4.5B; also see Figures 4.6A and 4.6B). When the control sample was reduced prior to analysis by SDS-PAGE (Figure 4.5C), the β -LG and α -LA bands were more intense. Also, the material that could not enter the gel or that migrated only a short distance in the non-reduced sample (marked as X_0 in Lane 1, Figures 4.5A and 4.5B) was absent from the PAGE pattern of the reduced sample (Lane 1, Figure 4.5C). The protein bands corresponding to Ig heavy chain (IgH) and Ig light chain (IgL) were clearly identified and marked (Lane 1, Figure 4.5C). This occurred because intra- and intermolecular disulphide bonds had been reduced by 2-ME and thus the proteins migrated as SDS complexes of the monomeric polypeptide chains.

4.3.4 Heat- and pressure-induced changes observed on 1D native- and SDS-PAGE patterns

Native-PAGE – heat treatment

Comparison of the native-PAGE pattern of the control sample (Lane 1, Figure 4.4A) with those of the heat-treated samples (Lanes 2–5, Figure 4.4A) indicated that the intensities of the protein bands corresponding to native-like β -LG AA, β -LG BB, α -LA, BSA, Igs and LF decreased with the severity of the heat treatment. Comparison of the PAGE patterns of the control sample (Lane 1, Figure 4.4A) and the sample heat-treated at 72°C for 15 s (Lane 2, Figure 4.4A) showed that the intensities of the bands corresponding to native-like LF, BSA and Igs (heat-sensitive proteins) decreased slightly in the PAGE pattern of the pasteurised sample, suggesting that mild heat treatment (such as pasteurisation) initiated aggregation of native-like LF, Igs and BSA. However, the intensities of the bands corresponding to native-like β -LG and α -LA appeared to be almost unaffected by this heat treatment (Lane 2, Figure 4.4A).

In contrast, the native-PAGE patterns of the samples heat treated at 100 or 120°C for 120 s and 140°C for 5 s (Lanes 3–5, Figure 4.4A) showed that the bands corresponding to native-like LF and BSA were essentially absent and that the intensities of the native-like β -LG and α -LA bands had decreased significantly. Only faint bands corresponding

to native-like β -LG and α -LA were visible in the PAGE pattern of the samples heat treated at 100°C for 120 s (Lane 3, Figure 4.4A) and 140°C for 5 s (Lane 5, Figure 4.4A). However, the band corresponding to native-like α -LA was absent and the intensity of the band corresponding to native-like β -LG was significantly decreased in the PAGE pattern of the sample heat treated at 120°C for 120 s. The non-native β -LG species that were apparent in pure β -LG systems (Manderson et al., 1998; Considine et al., 2005a, 2005b) were not clearly observed in the native PAGE pattern of the sample heated at 72°C for 15 s (Lane 2, Figure 4.4A). However, faint bands corresponding to such non-native species were evident in the PAGE patterns of the samples heat treated at 100 or 120°C for 120 s and 140°C for 5 s (Lanes 3–5, Figure 4.4A) and are marked as N' on the right-hand side of the PAGE pattern. It seems from these PAGE patterns that the intensity of the band corresponding to β -LG B decreased faster than that corresponding to β -LG A. This was much clearer when lower loadings or lower concentrations were used for PAGE (see Appendix 1).

Comparison of the native-PAGE pattern of the control sample (Lane 1, Figure 4.4A) with that of the severely heat-treated (100 or 120°C for 120 s and 140°C for 5 s) samples (Lanes 3–5, Figure 4.4A) indicated that several new discrete bands appeared on the PAGE patterns of the heat-treated samples. The positions of these new heat-induced bands, corresponding to aggregated proteins, are marked as X₁–X₆ on the right-hand side of the native-PAGE pattern (Figure 4.4A). The large protein aggregates, i.e. those caught up within the sample well (X₆), within the stacking gel (X₅) and at or near the top of the resolving gel (X₄), were high molecular weight protein aggregates consisting of both disulphide-linked and non-covalently associated proteins. The bands marked as X₁, X₂ and X₃ corresponded to intermediate aggregates (e.g. dimers, trimers and tetramers respectively), as identified previously in the PAGE pattern of heat treated whey protein samples (e.g. Havea et al., 1998; Manderson et al., 1998; Schokker et al., 1999).

Native-PAGE – pressure treatment

Similar comparison of the PAGE patterns of control (Lane 1, Figure 4.4B) and pressure-treated (Lanes 2–5, Figure 4.4B) samples also indicated that the intensity of the bands corresponding to native-like monomeric β -LG A, β -LG B, α -LA, BSA, Igs and LF

decreased with the severity of the pressure treatment; however, the order of sensitivity of each of these proteins to pressure treatment was quite different from that to heat treatment.

Comparison of the PAGE pattern of the sample pressure treated at 200 MPa for 30 min (mild pressure treatment) (Lane 2, Figure 4.4B) with that of the control sample (Lane 1, Figure 4.4B) indicated that the intensity of the band corresponding to β -LG decreased slightly, suggesting that the aggregation of β -LG had been initiated by this mild pressure treatment. However, the intensities of the bands corresponding to BSA, LF and Igs remained almost unaffected after this pressure treatment (200 MPa for 30 min) of the sample. The intensities of the bands corresponding to BSA, Igs and LF decreased rapidly as the severity of the pressure treatment increased (greater than 400 MPa for 30 min).

Several new bands also appeared on the PAGE patterns of the pressure-treated samples (Lanes 2–5, Figure 4.4B), compared with the PAGE pattern of the control sample (Lane 1, Figure 4.4B), suggesting the formation of non-native proteins and protein aggregates of different size as a result of the pressure treatment. The positions of the new bands corresponding to these aggregated proteins are marked as X_1 – X_6 on the right-hand side of the native-PAGE pattern (Figure 4.4B). The regions corresponding to X_1 – X_5 were comparatively more intense in the PAGE pattern of pressure-treated samples (compared with the PAGE pattern of heat-treated samples; Figure 4.4A), and corresponded mainly to dimers, trimers, tetramers etc. (X_1 – X_3), as well as to aggregates caught up at the top of the resolving gel (X_4) and to those caught up within the stacking gel (X_5). The proportion of high molecular weight aggregates that could not enter the gel (X_6) was less in the severely pressure-treated samples (Lanes 3–5, Figure 4.4B), compared with the severely heat-treated samples (Lanes 3–5, Figure 4.4A). A detailed description of the differences between these aggregates is given in Section 4.3.5.

SDS-PAGE – heat treatment

The intensities of the bands corresponding to SDS-monomeric whey proteins (β -LG A, β -LG B, α -LA, BSA, Igs and LF) decreased with the intensity of the heat treatment, indicating that the SDS-PAGE patterns of the heat-treated WPC samples (Lanes 2–5,

Figure 4.5A) showed similar trends to the native-PAGE patterns (Lanes 2–5, Figure 4.4A). These SDS-PAGE results confirmed that the aggregation of Igs, LF and BSA was initiated by a mild heat treatment (Lane 2, Figure 4.5A) such as pasteurisation. In contrast, the intensities of the bands corresponding to monomeric β -LG and α -LA appeared to be almost unaffected in the SDS-PAGE pattern of the pasteurised sample (Lane 2, Figure 4.5A).

Comparison of the SDS-PAGE pattern of the control sample (Lane 1, Figure 4.5A) with that of the severely heat-treated (100 or 120°C for 120 s and 140°C for 5 s) samples (Lanes 3–5, Figure 4.5A) suggested that bands corresponding to SDS-monomeric Igs, LF and BSA were essentially absent and that the intensities of the bands corresponding to SDS-monomeric β -LG and α -LA had decreased significantly in the SDS-PAGE patterns of the severely heat-treated samples (Lanes 3–5, Figure 4.5A). It was also observed from these PAGE patterns that the maximum decreased intensity of the bands corresponding to β -LG and α -LA was observed in the PAGE pattern of the sample heat treated at 120°C for 120 s (Lane 4, Figure 4.5A), followed by 100°C for 120 s (Lane 3, Figure 4.5A), 140°C for 5 s (Lane 5, Figure 4.5A) and 72°C for 15 s (Lane 2, Figure 4.5A). These results, together with the native-PAGE results (Figure 4.4A), indicated that the order of severity of heat treatment on the aggregation of the whey proteins was 120°C for 120 s (Lane 4) > 100°C for 120 s (Lane 3) > 140°C for 5 s (Lane 5) > 72°C for 15 s (Lane 2).

SDS-PAGE – pressure treatment

Similar comparison of the SDS-PAGE patterns of the pressure-treated WPC samples (Lanes 2–5, Figure 4.5B) with that of the control sample (Lane 1, Figure 4.5B) indicated many changes in the PAGE patterns of the pressure-treated samples. The intensities of the bands corresponding to monomeric protein decreased and simultaneously several new bands appeared as a result of pressure-induced aggregation of different whey proteins in the WPC solutions. The newly formed bands are marked appropriately as \underline{X}_1 – \underline{X}_6 on the right-hand side of the SDS-PAGE patterns. A detailed description of these bands is given in Section 4.3.5.

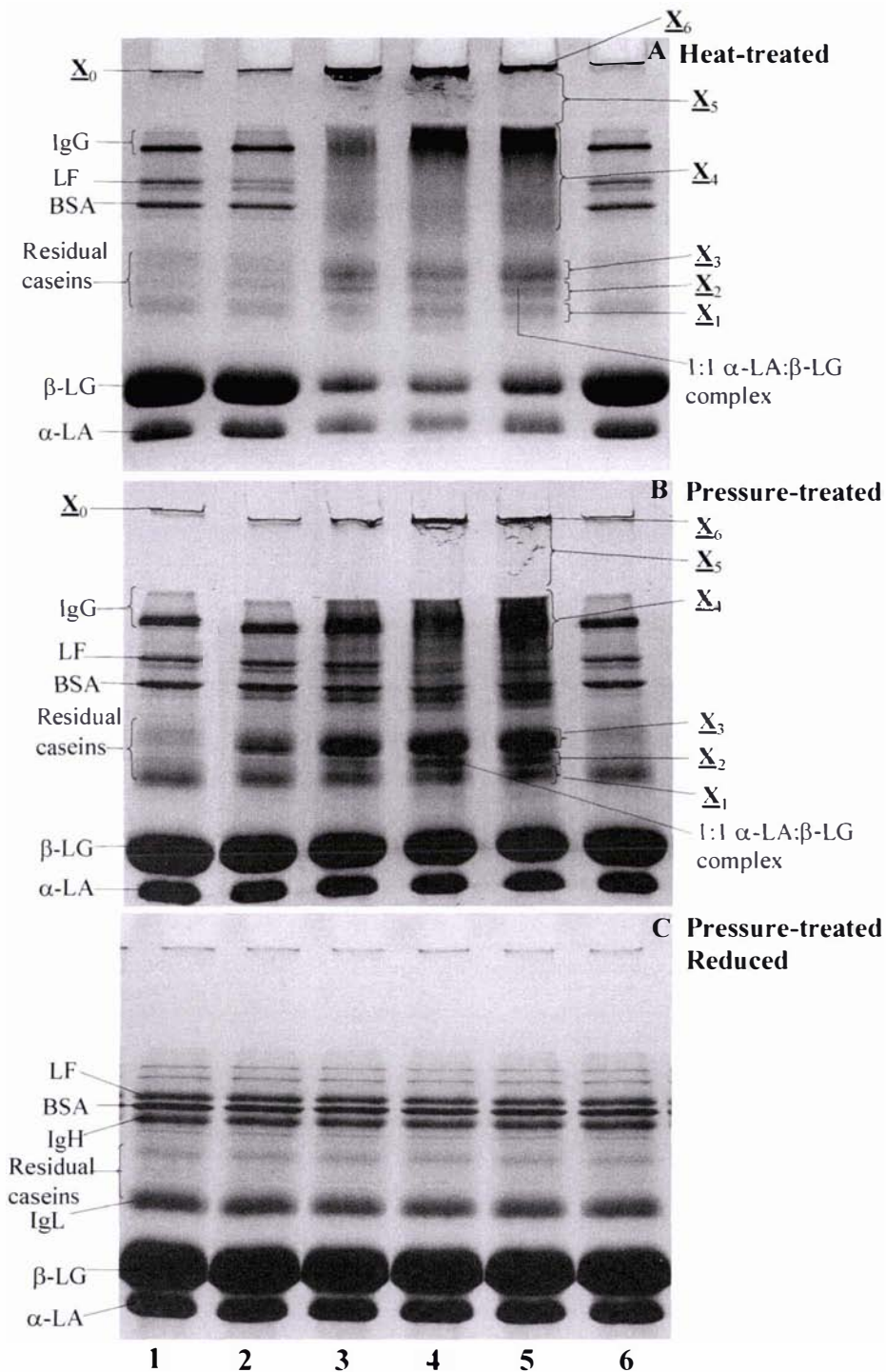


Figure 4.5. SDS-PAGE patterns of (A) heat-treated and (B) pressure-treated WPC solutions. For the heat-treated samples (A), the PAGE pattern shows: control sample (Lane 1), samples heated at 72°C for 15 s (Lane 2), 100°C for 120 s (Lane 3), 120°C for 120 s (Lane 4) and 140°C for 5 s (Lane 5), and control sample (Lane 6). Similarly, for the pressure-treated samples (B), the PAGE pattern shows: control sample (Lane 1), samples pressure treated at 200 MPa for 30 min (Lane 2), 400 MPa for 30 min (Lane 3), 600 MPa for 30 min (Lane 4) and 800 MPa for 30 min (Lane 5), and control sample (Lane 6). (C) SDS^R-PAGE pattern of reduced samples corresponding to the samples shown in Figure 4.5B.

Comparison of the PAGE pattern of the sample pressure treated at 200 MPa for 30 min (Lane 2, Figure 4.5B) with that of the control sample (Lane 1, Figure 4.5B) indicated that the intensities of the bands corresponding to BSA, LF, Igs and α -LA remained almost unchanged, whereas the intensity of the band corresponding to monomeric β -LG decreased slightly, with the simultaneous appearance of a new band (marked as X₃) in the sample pressure treated at 200 MPa for 30 min (Lane 2, Figure 4.5B). These results suggested that this mild pressure treatment partially aggregated β -LG and formed disulphide-bonded dimers of β -LG, but had no apparent effects on LF, Igs or BSA. Such a band corresponding to β -LG dimer was not apparent in the mild heat-treated sample (Lane 2, Figures 4.4A and 4.5A). The same trend continued after pressure treatment at 400 MPa for 30 min (Lane 3, Figure 4.5B), with some effects on LF, Igs and BSA and the formation of strong dimers of β -LG (as indicated by the increased intensity of the band marked as X₃). At this stage, involvement of α -LA in disulphide-linked aggregation was apparent through the formation of a 1:1 adduct of α -LA: β -LG with an approximate molecular weight of 33 kDa (as indicated by band X₂). This is also evident from corresponding 2D-PAGE (see Figure 4.9). The intensities of the bands corresponding to these intermediate-sized protein aggregates (X₁–X₃) increased with the severity of the pressure treatment. In addition (as for the PAGE patterns of the heat-treated samples), some very large aggregates in the sample loading well or at the top of the stacking gel (X₆), within the stacking gel (X₅) and at or near the top of the resolving gel (X₄) were also observed in the severely pressure-treated samples (Lanes 3–5, Figure 4.5B).

The intensity of the band corresponding to β -LG continued to decrease with increasing intensity of the pressure treatment (Lanes 3–5, Figure 4.5B). The intensity of the band corresponding to α -LA also appeared to begin to decrease after a pressure treatment of 400 MPa for 30 min, but these changes were not very clear from the 1D SDS-PAGE pattern (Lane 3, Figure 4.5B). However, it was clear from the corresponding 2D SDS- and then reduced SDS-PAGE pattern (see Figure 4.9H) that disulphide-linked aggregation of α -LA was initiated after pressure treatment of samples at 400 MPa and that disulphide-linked aggregates consisting of β -LG, α -LA, BSA, Igs and LF were formed. Interestingly, the bands corresponding to the heat-sensitive proteins (LF, BSA) were still clearly observable in the SDS-PAGE pattern of the sample pressure treated at 400 MPa for 30 min (Lane 3, Figure 4.5B) with slight decreases in the intensities of

these bands, suggesting that these proteins were only partially aggregated after a pressure treatment of 400 MPa for 30 min. These results suggested that LF and BSA were quite pressure resistant. Further increase in the pressure treatment to 600 and 800 MPa for 30 min (Lanes 4–5, Figure 4.5B) almost completely eliminated the band corresponding to Igs and the intensity of the band corresponding to LF decreased significantly. However, faint bands corresponding to BSA were still present even after a severe pressure treatment of 800 MPa for 30 min (Lane 5, Figure 4.5B), suggesting that BSA was quite resistant to pressure treatment.

From the overall results of native- and SDS-PAGE, it can be summarised that the heat sensitivities and pressure sensitivities of the different whey proteins were quite different; they were in the order $Igs \cong LF > BSA > \alpha\text{-LA} \cong \beta\text{-LG}$ for the heat-treated samples, and in the order $\beta\text{-LG} > Igs \geq LF > BSA > \alpha\text{-LA}$ for the pressure-treated samples.

4.3.5 Formation of high molecular weight protein aggregates by heat or pressure treatment

The decrease in native and SDS-monomeric proteins (as shown by the diminishing band intensities of monomeric proteins in the native- and SDS-PAGE patterns of the non-reduced samples), as a consequence of heat treatment (Lanes 2–5, Figures 4.4A and 4.5A) or pressure treatment (Lanes 2–5, Figures 4.4B and 4.5B), was accompanied by cross-linking of monomeric proteins and the formation of protein aggregates of different sizes.

It was found that many new dark-staining bands appeared in the heat- and pressure-treated samples in the region between the $\beta\text{-LG}$ band and the top of the resolving gel, marked as $X_1\text{--}X_4$ on the right-hand side of the native-PAGE patterns (Figures 4.4A and 4.4B) and as $\underline{X}_1\text{--}\underline{X}_4$ on the SDS-PAGE patterns (Figures 4.5A and 4.5B), and the intensities of these bands increased with the severity of the heat or pressure treatment. The positions of these new bands on the SDS-PAGE gel (based on approximate molecular weight) suggested that various intermediate protein aggregation species, such as disulphide-bonded dimers, trimers etc. of $\beta\text{-LG}$ or $\alpha\text{-LA}$ and/or a 1:1 adduct of $\alpha\text{-LA}:\beta\text{-LG}$, were formed during the various heat and pressure treatments of the WPC

samples. In addition to the intermediate-sized protein aggregates, many high molecular weight protein aggregates, i.e. those present at the top of the resolving gel, those caught up in the stacking gel and those that remained in the sample loading well or could not penetrate the gel (marked as X_4 , X_5 and X_6 respectively on the native-PAGE pattern or as \underline{X}_4 , \underline{X}_5 and \underline{X}_6 respectively on the SDS-PAGE pattern), were also formed in the severely heat- or pressure-treated samples.

Comparison of the PAGE patterns of the pressure-treated (Figures 4.4B and 4.5B) and heat-treated (Figures 4.4A and 4.5A) samples suggested that the proportion of intermediate protein aggregates (such as dimer, trimer etc.) was comparatively greater in the pressure-treated samples (Lanes 2–5, Figures 4.4B and 4.5B) than in the heat-treated samples (Lanes 2–5, Figures 4.4A and 4.5A). Also, the proportion of aggregates that did not migrate far in the resolving gel (X_4 and \underline{X}_4) and that had spread through the stacking gel (X_5 and \underline{X}_5) was also comparatively greater in the severely pressure-treated samples (Lanes 3–5, Figures 4.4B and 4.5B) than in the severely heat-treated samples (Lanes 3–5, Figures 4.4A and 4.5A). These results indicated that, in the severely pressure-treated samples, the aggregates were large but could still enter the stacking gel or migrate a short distance in the stacking gel or the resolving gel. Conversely, in the severely heat-treated samples, the aggregates formed were so large ($> 1 \mu\text{m}$ and having an estimated molecular weight of $> 500 \text{ kDa}$) that they could not enter the stacking gel and remained in the sample loading well and these very large aggregates were subsequently washed away during staining and destaining of the gels. During a set of the present experiments, we attempted to modify the present technique and to develop an improved method for the specific purpose of studying the composition of such very large protein aggregates (that could not enter the gel or were caught up in the stacking gel). This modified PAGE technique (e.g. Figure 4.6A and 4.6B), in which the sample is applied to the sample well in the form of a mixture of sample and stacking gel mixture (polyacrylamide and stacking gel buffer), which then sets as a gel (set sample gel) to trap very large protein aggregates, suggested that there were very large particles (molecular weight $> 500 \text{ kDa}$) in the severely heat-treated samples that could not enter the gel (see Lanes 2–4, Figures 4.6A and 4.6B).

From the above results (Figures 4.4 and 4.5), it is clear that pressure treatment generated a comparatively greater proportion of intermediate-sized protein aggregates [marked as X_1 – X_4 on native-PAGE (Figure 4.4B) and \underline{X}_1 – \underline{X}_4 on SDS-PAGE (Figure 4.5B)] than

heat treatment (Lanes 2–5, Figures 4.4A and 4.5A), which generated a comparatively greater proportion of very high molecular weight aggregates (marked as X_5 – X_6 on native-PAGE and X_5 – X_6 on SDS-PAGE).

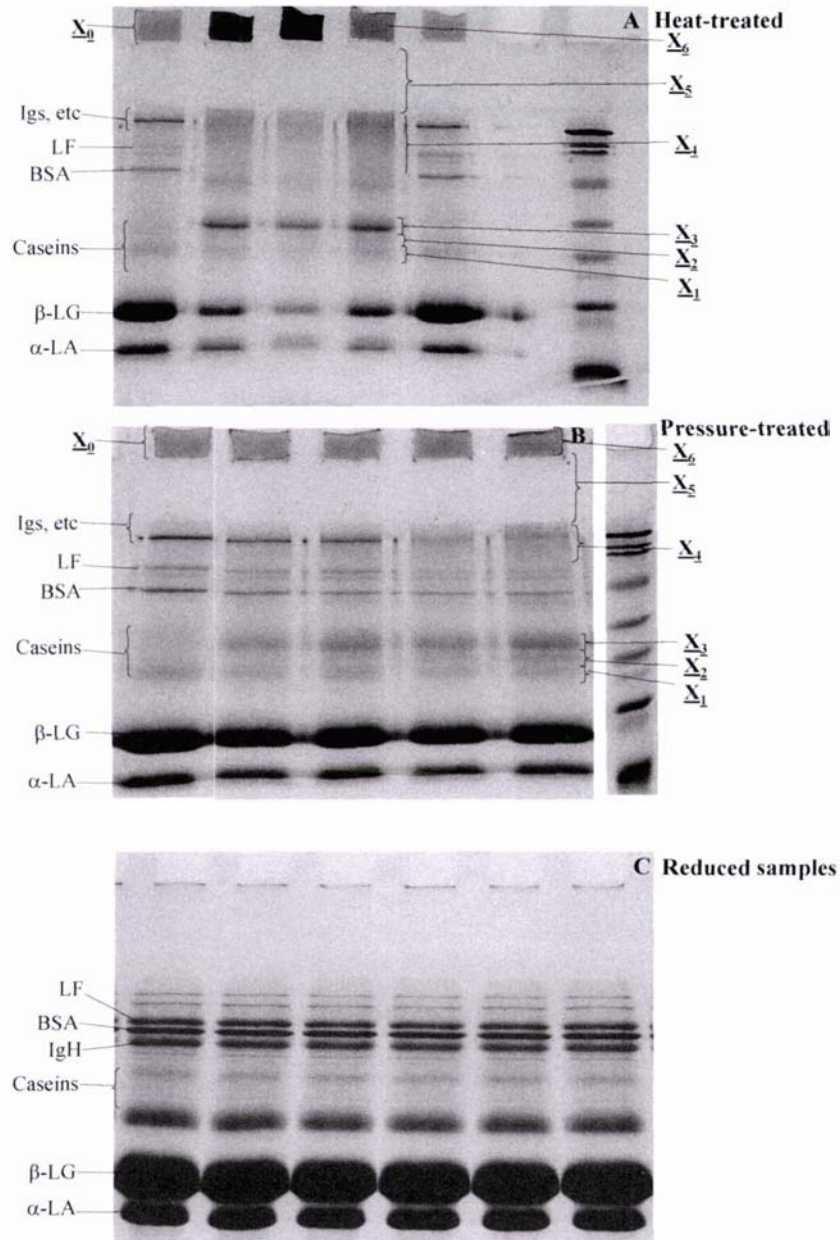


Figure 4.6. SDS-PAGE patterns (with set sample procedure) of (A) heat-treated and (B) pressure-treated WPC solutions (5% w/v). For the heat-treated samples (A), the PAGE pattern shows: samples heated at 72°C for 15 s (Lane 1), 100°C for 120 s (Lane 2), 120°C for 120 s (Lane 3) and 140°C for 5 s (Lane 4) and control sample (Lane 5). Similarly, for the pressure-treated samples (B), the PAGE pattern shows: control sample (Lane 1), and samples pressure treated at 200 MPa for 30 min (Lane 2), 400 MPa for 30 min (Lane 3), 600 MPa for 30 min (Lane 4) and 800 MPa for 30 min (Lane 5). Molecular weight markers (Mr) are also shown beside Figures 4.6A and 4.6B. (C) SDS^R-PAGE pattern of reduced samples corresponding to the samples shown in Figure 4.6B.

Reduction of the control and pressure-treated samples prior to SDS-PAGE (e.g. Figure 4.5C) resulted in the disappearance of the intermediate (X_1 – X_3) and high molecular weight (X_4 – X_6) protein aggregates and all samples gave patterns that were essentially the same, with only bands corresponding to reduced monomeric β -LG, α -LA, BSA, Igs and LF etc. This was due to the reduction of intra- and intermolecular disulphide bonds by the disulphide bond reducing agent (2-ME) and therefore different proteins present in these aggregates (X_1 – X_6) migrated as SDS complexes of reduced monomeric proteins. These proteins can be called reduced SDS-monomeric proteins or ‘total reducible proteins’ (see Figure 4.3). The SDS-PAGE patterns of the reduced samples suggested that almost all of the high molecular weight protein aggregates formed by heat and pressure treatments were disulphide linked and that covalent bonds other than disulphide bonds were not formed.

4.3.6 2D PAGE analysis

A combination of two PAGE techniques can be used to determine the composition of the protein aggregates and/or the types of bonds by which the protein aggregates are held together in the heat- or pressure-treated samples. However, 2D-PAGE does not provide direct evidence of specific interactions between individual protein species, e.g. aggregates caught-up in the resolving gel of non-reduced SDS gel could be resolved to give band corresponding to β -LG and α -LA in the reduced SDS (second) dimension. This strongly indicate that these proteins were aggregated via sulphhydryl-disulphide interchange reaction, but the exact disulphide bond directly involved into aggregation of two proteins can be identified in detail by further analysis such as MS-MS of their tryptic digests.

To characterise various intermediate species of protein aggregates and the high molecular weight aggregates formed as a consequence of heat or pressure treatment, the samples were further analysed using 2D PAGE (2D native:SDS-PAGE and 2D SDS:SDS^R-PAGE). A schematic diagram showing the basic principles and the separation of protein spots on 2D PAGE is given in Figure 3.5.

2D native:SDS-PAGE

In the 2D native:SDS-PAGE procedure, a sample containing protein complexes is analysed using 1D PAGE in a Tris HCl buffer at pH 8.7, called alkaline- or native-PAGE. The proteins or protein complexes separated on native-PAGE consist of all complexes including covalent (e.g. disulphide bonds) and non-covalent (e.g. hydrophobic)-bonded aggregates. The 1D native-PAGE gel strip with its separated protein bands is transferred to SDS-PAGE in the second dimension (i.e. transferred into a dissociating environment). Once the proteins in the strip are partially equilibrated with the SDS to form SDS-protein complexes, they are electrophoresed into a new (SDS) environment in a second dimension SDS-PAGE. SDS-PAGE in the second dimension dissociates non-covalent bonds (mainly hydrophobic aggregates) from the non-reduced native-PAGE gel strip, whereas the covalent bonds (disulphide bonds) remain unaffected.

The 2D native- and then SDS-PAGE pattern of the control WPC samples is shown in Figures 4.7A and 4.7F. The 2D native:SDS-PAGE gels presented in Figure 4.7 consist of two main components. The first component is the main body of the 2D gel, which includes the sample gel strip (a') that was used as the sample source to run on 2D PAGE. The second component is the stained gel strip (a''), which is the duplicate of the sample gel strip, but is stained separately and is aligned on the top of the sample gel strip once the main 2D gel has been completely stained and destained.

Identification of protein spots in the 2D PAGE patterns of the control samples – 2D native:SDS-PAGE

Comparison of the stained gel strip (a'') and the sample gel strip (a') obtained from the 1D PAGE pattern of the control sample showed that the monomeric protein bands corresponding to Igs, LF, BSA, β -LG, α -LA and α -LA*, which were present on the stained gel strip in the 1D PAGE pattern, were resolved to give corresponding protein spots on the 2D gels. These protein spots resolved on the 2D gels, corresponding to each protein, can be identified by comparison with the positions of bands on the stained gel strip (marked as a'' in the horizontal direction) and with the positions of protein bands on the PAGE pattern of the corresponding SDS sample (vertical PAGE pattern in the

left-hand channel, marked as SNR). The bands close to the top of the resolving gel of the 1D native-PAGE sample gel strip (a') (Figure 4.7F) were resolved into three spots in the second dimension. One spot was identified as IgG₁ (Marnila & Korhonen, 2003; Farrell et al., 2004), another spot corresponded to LF and the third, which had the mobility of a protein with an apparent molecular mass of about 25–45 kDa, was probably an aggregated casein, most likely κ -casein that had been hydrophobically bonded in the absence of SDS (in the first dimension native-PAGE).

2D SDS:SDS^R-PAGE

This type of 2D PAGE technique separates the initial mixture of proteins using SDS-PAGE with all the native and process-induced disulphide bonds intact. These separated proteins and protein aggregates are treated with a disulphide bond reducing agent, 2-mercaptoethanol (2-ME) while still in the gel strip. This gel strip is then used as the sample source for the second dimension PAGE analysis. SDS-PAGE in the second dimension separates the proteins as the reduced SDS–protein species. Thus the components of each of the various disulphide-bonded aggregates can be identified.

Identification of protein spots in the 2D PAGE patterns of the control samples – 2D SDS:SDS^R-PAGE

The 2D SDS:SDS^R-PAGE pattern of the control WPC samples is shown in Figures 4.9A and 4.9F. Comparison of the stained gel strip (a'') and the sample gel strip (a') showed that the bands corresponding to IgG, LF, BSA, β -LG and α -LA, identified on the stained 1D gel strip (a''), were resolved to give corresponding protein spots in the 2D SDS:SDS^R-PAGE gel, forming a diagonal line of spots. Most of the material shown in the 2D gel (e.g. Figure 4.9A) lay close to the diagonal, indicating that only small changes in mobility had occurred as a consequence of the reduction of the control sample. The major spots on the diagonal from the lower right-hand side were: α -LA, β -LG, residual caseins, IgG, BSA and LF. These protein spots resolved on the 2D gels, corresponding to each of these proteins, can be identified by comparison with the positions of bands on the stained gel strip (marked as a'' in the horizontal direction) and with the positions of protein bands on the PAGE pattern of the corresponding reduced SDS sample, which is the same as Figure 4.5C (vertical PAGE pattern in either the left-

hand or right-hand channel, marked as SR). There was a series of faint spots in the centre of the 2D gel (marked as C' in Figures 4.9A and 4.9F) that were tentatively identified as residual κ -caseins present in the WPC. A pair of spots to the left of the diagonal appeared to be reduction products that had been dissociated from the protein bands separated from the top of the resolving gel of the sample gel strip (a'). These spots were identified as IgL (light chain) and IgH (heavy chain) separated under reducing conditions. The other spot was identified as monomeric β -LG, which was dissociated from dimeric β -LG in the sample gel strip. A small quantity of disulphide-linked β -LG dimer was found in the control WPC sample. The presence of small quantities of dimeric β -LG in commercial WPCs is common (Havea et al., 1998).

4.3.7 Characterisation of heat- and pressure-induced protein aggregates using 2D PAGE

2D native: SDS-PAGE – heat treatment

Comparison of the 2D native:SDS-PAGE pattern of the control sample (Figure 4.7A) with those of the heat-treated samples (Figures 4.7B–4.7E) showed that the changes observed on the 1D native-PAGE patterns of different heat-treated samples were reflected clearly on the corresponding 2D native:SDS-PAGE patterns. For example, it was observed that the intensities of the protein spots corresponding to Igs, LF and BSA decreased on the 2D native:SDS-PAGE pattern of the sample heat treated at 72°C for 15 s (Figure 4.7B), compared with the PAGE pattern of the control sample (Figure 4.7A), whereas the intensities of the spots corresponding to β -LG and α -LA remained almost unchanged. These results confirmed that Igs, LF and BSA are comparatively more heat sensitive than β -LG and α -LA. The samples heat treated at 100 or 120°C for 120 s or 140°C for 5 s exhibited significantly different 2D PAGE patterns (Figures 4.7C–4.7E), showing that the spots corresponding to monomeric Igs, LF and BSA were completely absent, whereas the intensities of the spots corresponding to β -LG and α -LA had decreased significantly. The loss of these monomeric proteins was coupled with the formation of aggregates of various sizes (marked as X₁–X₆ or blurred regions observed on the stained gel)

2D native:SDS-PAGE – pressure treatment

Similarly, comparison of the 2D native:SDS-PAGE pattern of the control sample (Figure 4.7F) with those of the pressure-treated samples (Figures 4.7G–4.7J) indicated many changes in the 2D PAGE patterns of the pressure-treated samples, including a decreasing intensity of the spots corresponding to monomeric proteins and the simultaneous appearance of new spots corresponding to protein aggregates that were resolved from the region marked as X_1 – X_6 of the stained gel strip (a”).

It was found that the intensity of all native-like whey protein spots decreased with the severity of the pressure treatment. However, the faint spots corresponding to native-like monomeric BSA were still observable after pressure treatments of 400 and 600 MPa for 30 min. This observation confirmed earlier results (Figure 4.4B) that these whey proteins are resistant to pressure treatment. Unlike the 2D PAGE pattern of the sample heat treated at 72°C for 15 s (Figure 4.7B), many new spots appeared in the PAGE pattern of the sample pressure treated at 200 MPa for 30 min (Figure 4.7G). The spots corresponding to Igs, LF, BSA and α -LA remained almost unchanged in the pattern of the sample pressure treated at 200 MPa for 30 min (Figure 4.7G); however, there was a slight decrease in the intensity of the spot corresponding to β -LG and the formation of new distinct spots corresponding to hydrophobically linked β -LG dimer, trimer, tetramer etc. (resolved mostly from the X_1 – X_3 region of the sample gel strip (a') and marked as β -LG₂, β -LG₃, β -LG₄ in region Y' of Figure 4.7G). It was observed that, compared with the PAGE patterns of the heat-treated samples (Figures 4.7B–4.7E), there was more material in the region marked as Y' (which corresponded to the spots resolved from the bands marked as X_1 – X_4) in the PAGE pattern of the pressure-treated samples (Figures 4.7G–4.7J). This observation confirmed the results of the 1D PAGE (Lanes 3–5, Figure 4.4B), suggesting that pressure treatment generated comparatively smaller aggregates than heat treatment (Lanes 3–5, Figure 4.4A). Very large aggregates were caught up at the top of the gel in the sample loading well (the region marked as X_6 on the 1D gel strip) and were partly dissociated to show a series of spots that corresponded to various protein bands in the left-hand channel (i.e. spots resolved from X_6). These results essentially showed that the high molecular weight material (caught up at the top of the 1D native-PAGE sample gel strip, which was dissociated by SDS-PAGE in the second dimension) in the 2D PAGE patterns of the severely pressure-

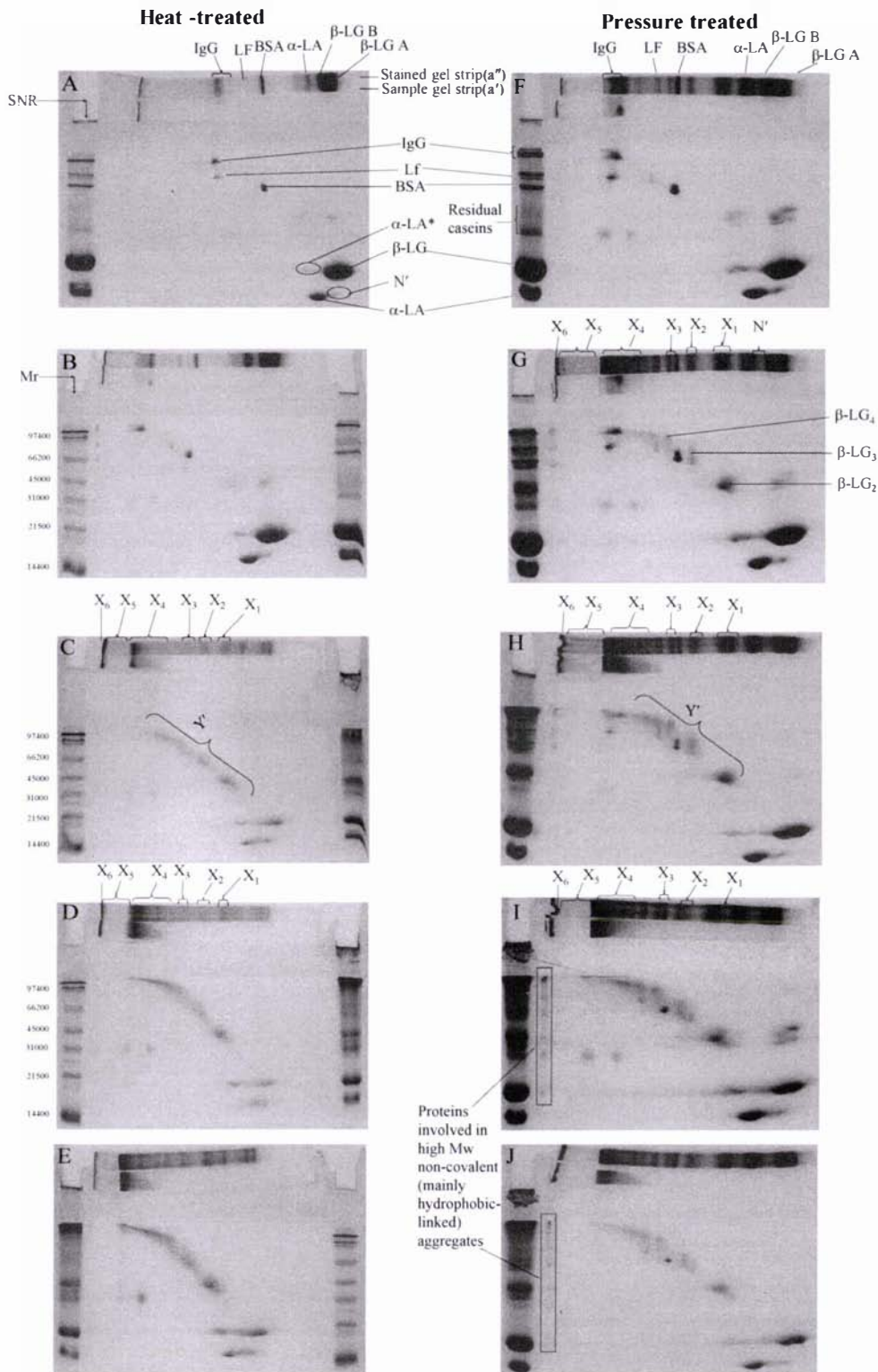


Figure 4.7. Native- and then non-reduced SDS-PAGE patterns of: (A) control sample, and samples heated at (B) 72°C for 15 s, (C) 100°C for 120 s, (D) 120°C for 120 s and (E) 140°C for 5 s. Similarly, native- and then non-reduced SDS-PAGE patterns of: (F) control sample, and samples pressure treated at (G) 200 MPa, (H) 400 MPa, (I) 600 MPa and (J) 800 MPa. The protein spots on the 2D gels were identified by comparison with the positions of bands on the stained gel strip or the positions of bands on the vertical PAGE pattern of the corresponding SDS sample in the left-hand or right-hand channel, marked as SNR.

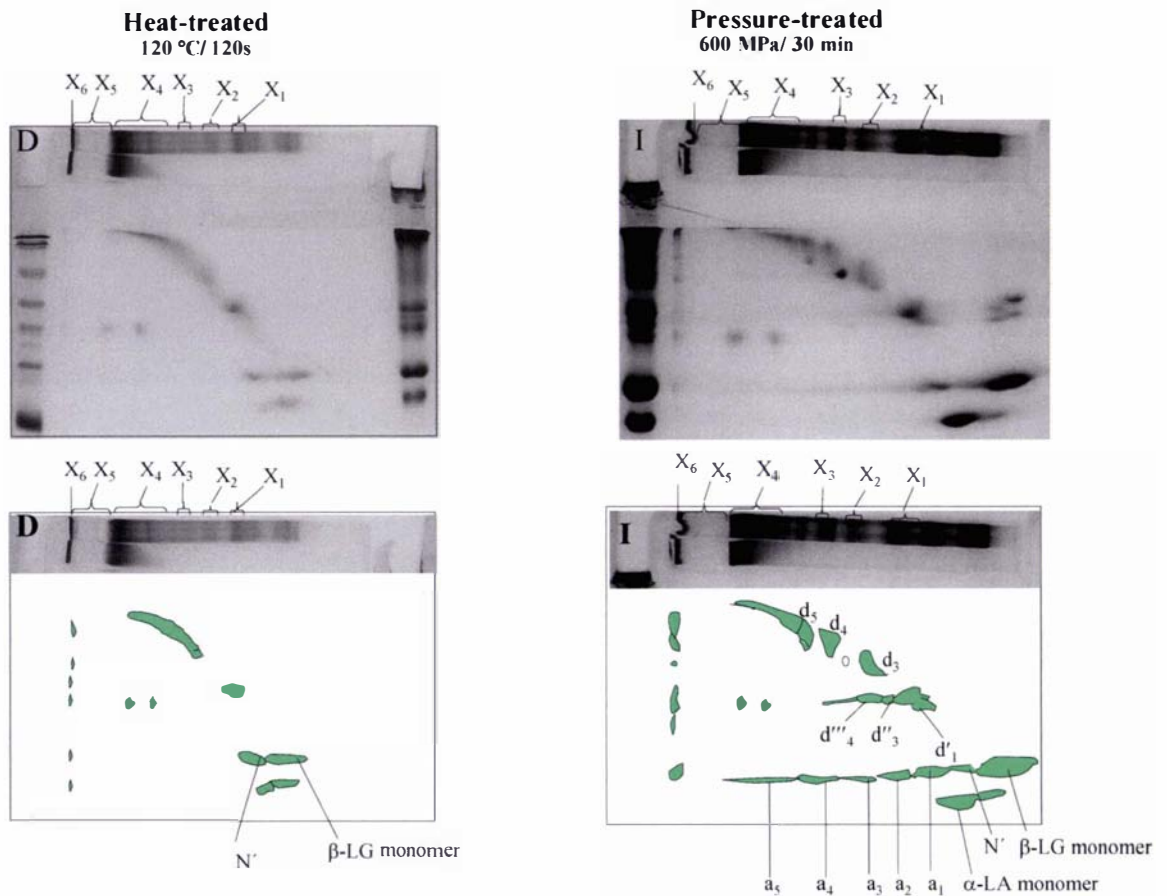


Figure 4.8. 2D native- and then non-reduced SDS-PAGE patterns of severely heat- and pressure-treated samples, showing the formation of new interaction products/whey protein complexes, shown in green colour. The new protein spots (corresponding to those shown in green and marked as a_1 , a_2 , a_3 , a_4 , a_5 , d_3 , d_4 , d_5 , d'_1 , d''_3 , d'''_4 , etc.) that appeared on the PAGE patterns were formed as a result of the heat or pressure treatment.

treated samples (Figures 4.7H–4.7J) corresponded to hydrophobically bonded aggregates consisting of Igs, LF, BSA, β -LG and α -LA.

Also a series of spots appeared in line with the SDS-monomeric β -LG spot in the second dimension in the form of a continuous horizontal line (in the region between the β -LG spot and the β -LG band in the PAGE pattern of the corresponding sample in the left-hand lane) up to the top of the gel (e.g. Figure 4.7I), which was resolved from the region marked as X_1 – X_6 of the 1D native-PAGE sample gel strip. The spots corresponding to such aggregates observed on the 2D native- and SDS-PAGE patterns of the pressure-treated samples were very faint and could be missed unless careful

observations are made. A schematic comparison of such aggregates present in the PAGE patterns of the severely heat-treated sample (Figure 4.7D) and the sample pressure treated at 600 MPa for 30 min (Figure 4.7I) is presented in Figure 4.8, giving a clear idea of the various types of aggregates formed in the heat- and pressure-treated samples. These results demonstrated that hydrophobically linked trimers, tetramers, pentamers, hexamers etc. of β -LG were formed by the effects of pressure treatment (e.g. marked as a₁, a₂, a₃, a₄, a₅, d₃, d₄, d₅ etc. in green colour spots in Figure 4.8I). When the 2D native:SDS-PAGE patterns of the heat-treated samples (e.g. Figure 4.7D) were compared with those of the pressure-treated samples (e.g. Figure 4.7I), it was clear that the range of intermediate aggregates observed on the 2D PAGE pattern of the pressure-treated samples was not present on the 2D PAGE pattern of the heat-treated samples. These results also suggested that pressure treatment generated a greater proportion of intermediate-sized hydrophobically linked aggregates than did heat treatment.

The results of 2D PAGE suggested that severe heat treatments generated hydrophobically linked protein aggregates involving all the whey proteins, which were resolved from region X₄ and X₆ of the sample gel strip and the aggregates resolved in region Y' may be disulphide-linked as they were not completely dissociated in the the SDS environment in the second dimension. In the severely heat-treated samples (e.g. 100 or 120°C for 120 s, 140°C for 5 s), the material in the region Y' was faint (Figures 4.7C–4.7E) because the heat-induced aggregates formed were so large that they could not enter the gel. Comparison of the stained and sample gel strips on 2D PAGE also suggested that the proportion of high molecular weight material that could not be dissociated by SDS increased as the severity of the heat treatment increased.

2D SDS:SDS^R-PAGE

In the 2D SDS:SDS^R-PAGE procedure, the protein components in the sample are separated on the 1D SDS-PAGE gel, the proteins are reduced with excess 2-ME while they are still in the gel strip and the components are identified after an SDS-PAGE analysis in the second dimension. This combination disrupts the disulphide-linked protein aggregates.

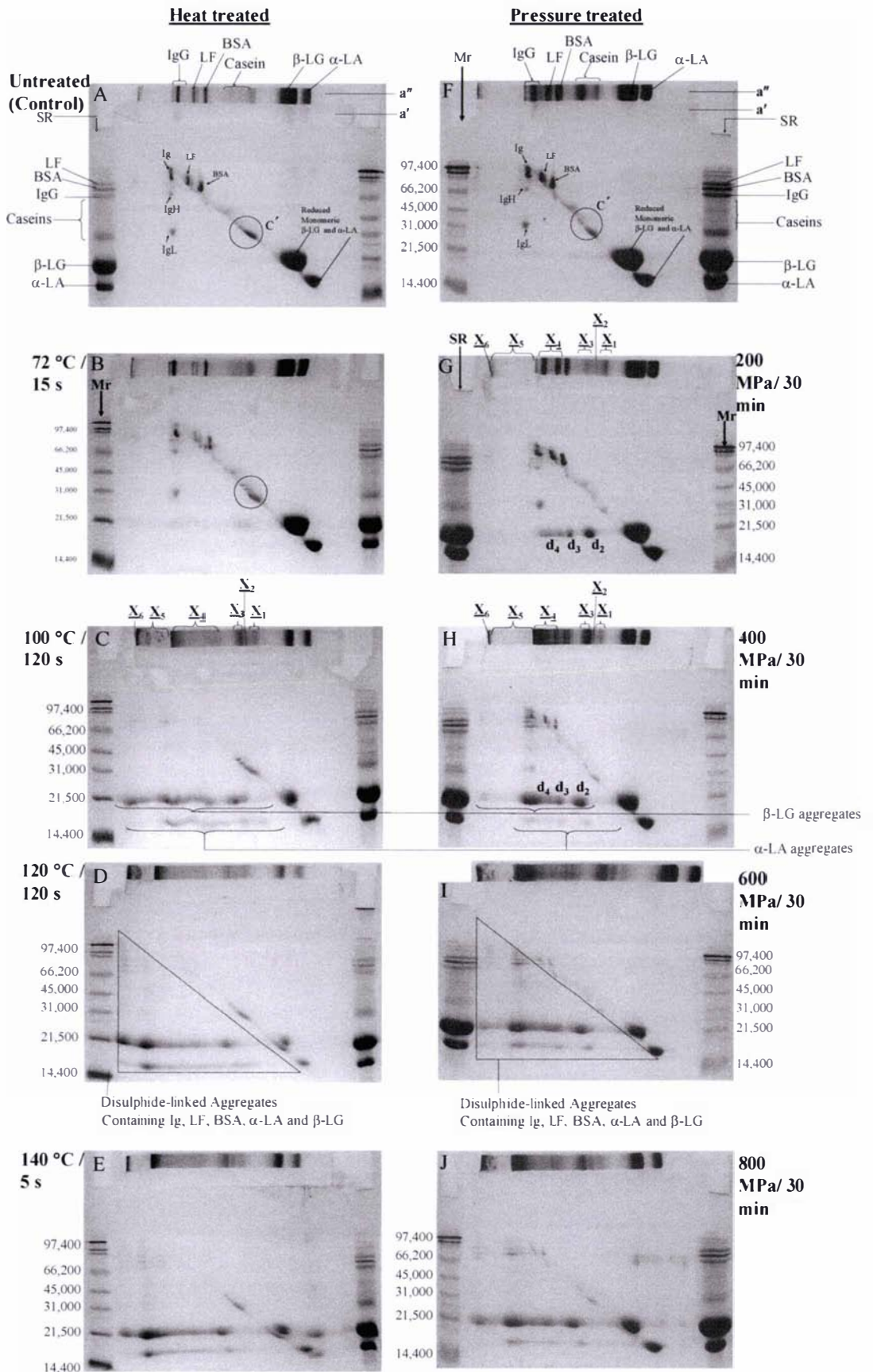


Figure 4.9. SDS- and then reduced SDS-PAGE patterns of: (A) control sample, and samples heated at (B) 72°C for 15 s, (C) 100°C for 120 s, (D) 120°C for 120 s and (E) 140°C for 5 s. Similarly, SDS- and then reduced SDS-PAGE patterns of: (F) control sample, and samples pressure treated at (G) 200 MPa, (H) 400 MPa, (I) 600 MPa and (J) 800 MPa for 30 min.

Comparison of the stained gel strip and the sample gel strip on the 2D SDS- and then reduced SDS-PAGE patterns of heat-treated (Figures 4.9B–4.9E) or pressure-treated (Figures 4.9G–4.9J) samples with their respective control samples (Figure 4.9A or 4.9F) indicated that all the protein bands and heat- or pressure-induced protein aggregates observed on 1D SDS-PAGE were resolved from the reduced sample gel strip and migrated into the second dimension SDS gel, giving corresponding protein spots.

2D SDS:SDS^R-PAGE – heat treatment

The changes observed in the 1D PAGE patterns of the heat-treated (Lanes 2–5, Figure 4.5A) and pressure-treated (Lanes 2–5, Figure 4.5B) samples were clearly reflected in the corresponding 2D PAGE patterns for the heat-treated samples (Figures 4.9B–4.9E) and the pressure-treated samples (Figures 4.9G–4.9J). In addition, it was also possible to observe some other changes that were not very clear from the 1D PAGE patterns (because of overlapping/blurring of the protein bands).

Comparison of the 2D SDS- and then reduced SDS-PAGE pattern of the control sample (Figure 4.9A) with those of the heat-treated samples (Figures 4.9B–4.9E) suggested that the intensities of the reduced SDS-monomeric protein spots (resolved from the 1D SDS sample gel strip of the heat-treated samples) decreased with the severity of the heat treatment, with the concomitant appearance of many new spots on the 2D PAGE patterns of the heat-treated samples. It was found that, after mild heat treatment (72°C for 15 s), the intensities of the spots corresponding to Igs, LF and BSA decreased but the spots corresponding to β -LG and α -LA remained almost unchanged. These changes in the intensity of bands or spots may not be obvious from the images of the PAGE patterns presented in this chapter. However, there was minor decrease in the intensity of these bands and this observation is clear when we see in the original PAGE gel after staining and destaining. These minor changes may not be very obvious in the photograph or scan and printed picture of the gels due to level of histogram and grey scale used in scanning gel images in order to produce sharp picture. Such minor changes observed on the original PAGE gels are particularly difficult to reproduce on the scanned and printed images. The 2D PAGE patterns of the samples heat treated at 100 or 120°C for 120 s and 140°C for 5 s (Figures 4.9C–4.9E) showed a significant decrease in the intensities of the spots corresponding to β -LG and α -LA, whereas the spots corresponding to reduced SDS-monomeric Igs, LF and BSA were completely absent in

the PAGE patterns of these samples. A series of new spots, resolved from the region marked as X₁–X₆ of the SDS sample gel strip, were also apparent on these PAGE patterns (Figures 4.9C–4.9E). These new spots corresponded to reduced monomeric LF, Igs, BSA, β -LG and α -LA in the left- or right-hand channel (Figures 4.9B–4.9E), indicating that all these proteins were involved in disulphide-linked aggregation as a consequence of heat treatment of the WPC solutions. There was an almost continuous horizontal line to the left of the reduced monomeric β -LG spot. This material seemed to be separated from the region marked as X₂–X₆ of the sample gel strip. A similar pattern of a horizontal line was also observed for α -LA, and lined up to the left of the spot corresponding to reduced α -LA. The dark-staining spots on these horizontal lines corresponding to reduced β -LG or α -LA were identified as reduced disulphide-bonded dimers of β -LG and α -LA and/or 1:1 complexes of α -LA: β -LG, β -LG trimer, tetramers etc. All these disulphide-linked aggregates observed on the 2D SDS- and then reduced SDS-PAGE pattern of a representative heat-treated sample (140°C for 5 s) are identified in detail in Figure 4.10A. Comparison of the 1D PAGE (Lanes 2–5, Figure 4.5A) and corresponding 2D PAGE (Figures 4.9B–4.9E) patterns also makes such changes and their explanation clearer.

2D SDS:SDS^R-PAGE – pressure treatment

Similar comparison of the 2D PAGE pattern of the control sample (Figure 4.9F) with those of the pressure-treated samples (Figures 4.9G–4.9J) indicated that new spots corresponding to dimer, trimer, tetramer etc. of β -LG (represented by spots marked as d₂, d₃, d₄) were observed on the PAGE pattern of the sample pressure treated at 200 MPa (Figure 4.9G), whereas the spots corresponding to Igs, LF, BSA and α -LA remained almost unaffected after this pressure treatment. More severe pressure treatments such as 400, 600 or 800 MPa for 30 min considerably decreased the intensities of the protein spots corresponding to Igs, LF and BSA. It appeared that α -LA was incorporated in disulphide-linked aggregates after a pressure treatment of 400 MPa for 30 min (Figure 4.9H), confirming the results of 1D PAGE (Lanes 3–5, Figure 4.5B). Also, new spots corresponding to reduced SDS-monomeric Igs, LF, BSA, β -LG and α -LA were resolved from region X₄ of the SDS sample gel strip (Figures 4.9H–4.9J), suggesting that a pressure treatment of 400 MPa generated disulphide-linked aggregates consisting of these proteins. Increasing proportions of these whey proteins (Igs, LF,

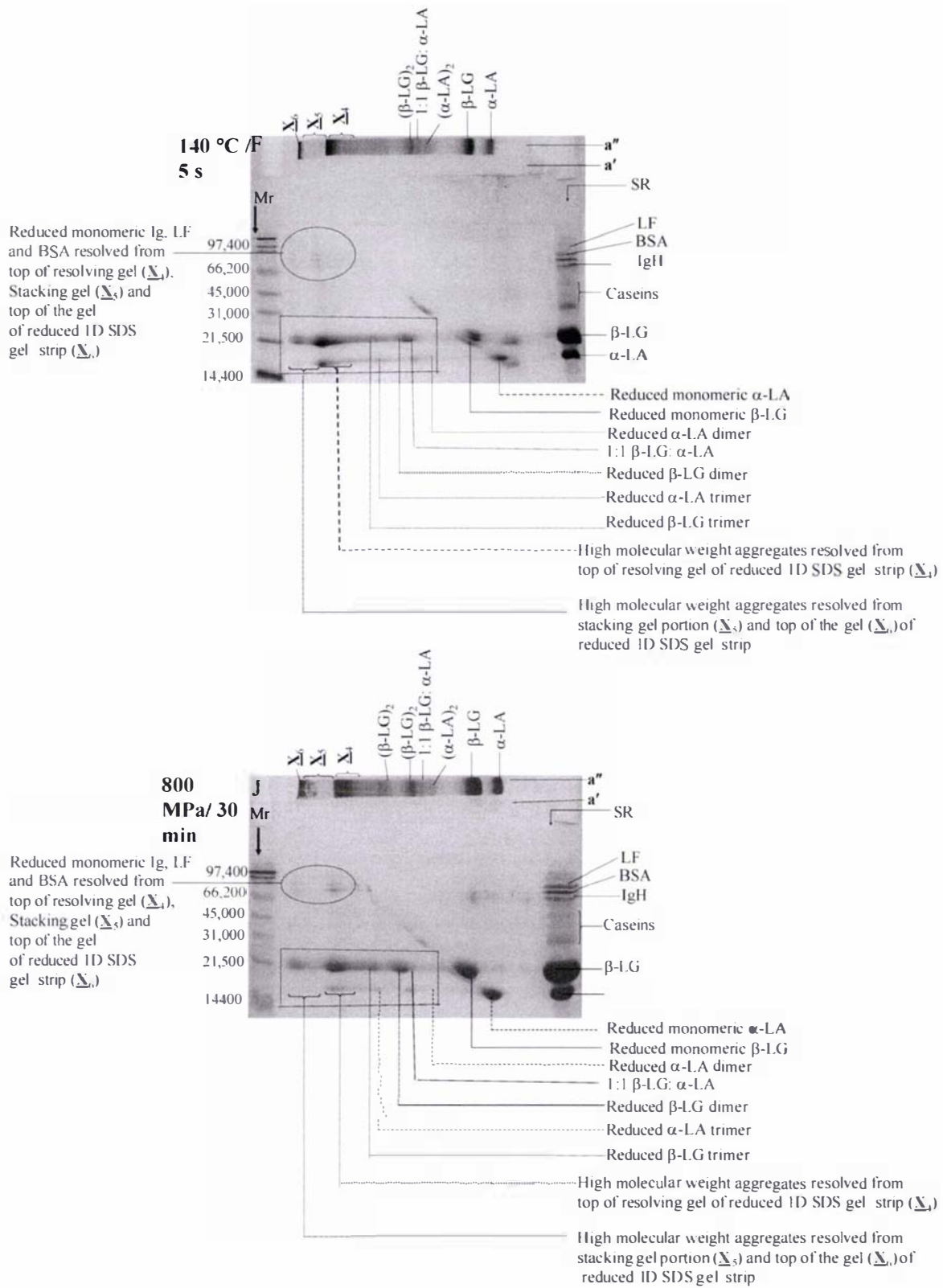


Figure 4.10. Detailed identification of the disulphide-linked aggregates and protein interactions on 2D SDS- and then reduced SDS-PAGE patterns of (A) heat-treated (140°C for 5 s) and (B) pressure-treated (800 MPa for 30 min) WPC solutions.

BSA, β -LG and α -LA) were involved in disulphide-linked aggregation after pressure treatment of WPC solutions at 600 MPa for 30 min and higher.

As observed on the 2D SDS- and then reduced SDS-PAGE patterns of the heat-treated samples (Figures 4.9C–4.9E), an almost continuous horizontal line (e.g. Figures 4.9I, 4.9J) consisting of aggregates of β -LG was observable (to the left of the reduced SDS-monomeric β -LG spot) in the 2D PAGE patterns of the samples pressure treated at 400 MPa for 30 min and higher (Figures 4.9H–4.9J). These patterns of the pressure-treated samples suggested that disulphide-linked dimers, trimers, tetramers and higher polymers of β -LG were induced by these pressure treatments. Similar patterns were also evident for α -LA, showing an almost continuous line to the left of the reduced monomeric α -LA spot and the α -LA band in the PAGE pattern of the reduced sample (Figures 4.9H–4.9J).

The faint spot (the region marked as X₂, Figures 4.9H–4.9J) observed between the α -LA dimer (approximate molecular weight 29 kDa) and the β -LG dimer (approximate molecular weight 37 kDa) bands on the 1D PAGE gels was resolved to give a disulphide-linked 1:1 α -LA: β -LG complex (approximate molecular weight 33 kDa). All these disulphide-linked aggregates observed on the 2D SDS- and then reduced SDS-PAGE pattern of a representative pressure-treated sample (800 MPa for 30 min) are identified in detail in Figure 4.10B.

It can be observed from the 2D SDS- and then reduced SDS-PAGE patterns of the pressure-treated samples (Figures 4.9H–4.9J) that disulphide-bonded aggregates (dimers, trimers and higher polymers) of β -LG and/or α -LA were formed after pressure treatments of 400 MPa or higher (Figures 4.9H–4.9J). It was also observed that the proportion of aggregates that were resolved from the region between the β -LG band and the top of the resolving gel (region marked as X₁–X₄) was comparatively greater in the 2D PAGE patterns of the pressure-treated samples (Figures 4.9G–4.9J) than in the 2D PAGE patterns of the heat-treated samples (Figures 4.9B–4.9E), which again confirmed the results of 1D PAGE (Figure 4.5B).

4.4 General discussion

In their native state, proteins are stabilised by covalent bonds (including disulphide bridges), electrostatic interactions (ion pairs, polar groups), hydrogen bonds and hydrophobic interactions. In contrast to heat treatment, where covalent as well as non-covalent bonds are affected, HP treatment at room and mild temperatures disrupts only relatively weak bonds (hydrogen bonds, hydrophobic bonds, ionic bonds). Therefore, it is expected that heat treatment and pressure treatment will have different effects on the structures of different proteins and therefore are likely to generate different aggregation patterns. However, little evidence demonstrating this has been shown in the literature, particularly in the situation where samples from the same aliquots are heat or pressure treated and analysed using common comparable methods.

As mentioned earlier, the main aim of this preliminary exploration was to determine the effects of heat and pressure treatment on the denaturation and aggregation of whey proteins and to determine possible differences and similarities in the heat- and pressure-induced aggregations of whey proteins, using identical methods. This was achieved by successful applications of various PAGE techniques, either singly or in combination and with or without reduction. Each of these PAGE techniques uses different principles to identify the type, size and composition of processing-induced protein aggregates. The 2D PAGE techniques in the present investigation were useful in determining which proteins formed reducible, most probably, disulphide bonds or non-covalent bonds (mainly hydrophobic bonds) with one another in a technically and conceptually straightforward way. The 2D PAGE techniques used in this study were the same as those used to study heat-induced interactions in WPC solutions (Havea et al., 1998) or in pure β -LG solutions (Manderson et al., 1998), in order to identify the composition of the aggregates.

Another purpose of the present investigation was to study the protein aggregation that precedes heat- or pressure-induced gel formation from WPC solutions. For this purpose, it was important that the concentrations of the samples (5 % w/v) were selected carefully in the present study. Also, it is known that WPC solutions of concentration 8% or greater are prone to heat-induced gel formation (Havea, 1998; Havea et al., 1998) under favourable conditions. If such WPC concentrations had been selected for some of

the heat treatments used in the present study, the samples would have gelled in the heat exchanger. Therefore the WPC concentrations (5 % w/v) were selected so that gels would not be formed, in order to be able to study pre-gelation aggregation in heat- or pressure-treated WPC solutions. The time and temperature combinations (i.e. 72°C for 15 s, 100 or 120°C for 120 s and 140°C for 5 s) used in the present study were selected from the regime of routine commercial heat treatments to give an idea of possible changes in the structure of proteins that may be induced by these heat treatments.

Sensitivities of whey proteins to heat and pressure

The results of the present study confirmed that the sensitivities of the different whey proteins to heat treatment and pressure treatment were different. Of the three major whey proteins (β -LG, α -LA and BSA), β -LG was the most sensitive to pressure treatment whereas BSA was the most sensitive to heat treatment. Also, it was found that Igs and LF were comparatively more resistant to pressure treatment than to heat treatment. However, the exact order of their sensitivity was difficult to determine accurately using PAGE results, partly because of overlapping bands of β -LG or α -LA aggregates generated by heat or pressure treatment of the WPC solutions. After the mild heat treatment (72°C for 15 s), α -LA and β -LG were largely in their monomeric form, but a proportion of the heat-sensitive whey proteins (e.g. BSA, Igs and LF) were aggregated and disulphide bonded to one another. Rather surprisingly, mild pressure treatment (200 MPa for 30 min) affected BSA less than β -LG. This observation was supported by López-Fandiño et al. (1996), who reported that BSA in raw milk is resistant to pressures up to 400 MPa, and Hinrichs et al. (1996), who reported that β -LG is more susceptible than α -LA and BSA to pressure denaturation. Huppertz et al. (2002) also reviewed several reports related to different pressure sensitivities of whey proteins. One explanation for the greater stability of BSA is that BSA has one SH group and 17 disulphide bonds and the energy received under pressure treatment is too small to disrupt all the disulphide bonds and to change the molecular structure of BSA. The present study also showed that, compared with β -LG, α -LA was much more resistant to denaturation under pressure, which is in agreement with the recent finding (Huppertz et al., 2004b), but was significantly different from the trend reported for heat-treated samples (e.g. Havea et al., 1998). Differences in the barostabilities of α -LA and β -LG may be linked to the more rigid molecular structure of α -LA (López-Fandiño et al.,

1996), caused partially by the different numbers of intramolecular disulphide bonds in the two proteins (Hinrichs et al., 1996; Gaucheron et al., 1997), or due to the lack of a free sulphhydryl group in α -LA (López-Fandiño et al., 1996).

Another possible explanation for the differences in the pressure sensitivities of β -LG, α -LA and BSA is the presence of a central hydrophobic cavity in the structure of β -LG and the absence of such a cavity in α -LA and BSA. This central hydrophobic cavity of β -LG (Hummer et al., 1998; Jameson et al., 2002) could be collapsed by moderate pressure treatment (Balci & Wilbey, 1999; Yang et al., 2003), by forcing water inside the cavity. Although the findings from this study need to be verified using a greater range of treatments, recent work (Considine et al., 2005a, 2005b, 2006a, 2006b) supports this contention; when a hydrophobic ligand, such as retinol, palmitic acid, SDS, myristic acid or conjugated linoleic acid, was bound to β -LG in its cavity, β -LG was more resistant to pressure-induced denaturation and aggregation (also see Appendix 2).

Size and type of heat- and pressure-induced aggregates

The present study showed that disulphide-linked and hydrophobically bonded dimers, trimers etc. were generated after a pressure treatment of 200 MPa, which was the lowest pressure used in the present study. However, it was observed that pressure treatment of WPC solutions generated comparatively greater proportions of hydrophobically linked aggregates than did heat treatment (see Figure 4.7). Both heat treatment and pressure treatment generated 1:1 disulphide-bonded dimers between α -LA and β -LG, similar to those reported by Havea et al. (1998) in a heated WPC system. Some of the larger aggregates from both heat- and pressure-treated WPC solutions appeared to contain all of the whey proteins that were linked through disulphide bonds. The pathway from native protein to aggregated disulphide-bonded particles will include intermediate conformations in which thiol groups or disulphide bonds become exposed to one another so that disulphide bond interchange can take place. Both pressure and temperature alter the energetics of the water–protein surface interactions, as well as the internal atomic packing, and modify the protein conformation in different ways.

Further, the results of the present study indicated that different proportions of aggregates of various sizes were generated by heat and pressure treatments. Pressure treatment of WPC solutions generated a greater proportion of comparatively smaller protein aggregates (such as dimers, trimers, tetramers etc.) than did heat treatment of WPC solutions, whereas heat treatment generated a greater proportion of very high molecular weight aggregates (which could not enter the gel or were caught up in the stacking gel) than did pressure treatment of WPC solutions. The differences in the aggregation behaviours of proteins generated by heat treatment and pressure treatment may well be related to the different effects of heat and pressure on protein structure. Consequently, there appeared to be some differences and similarities in the denaturation and aggregation of the proteins in the heat- and pressure-treated WPC samples. Therefore, it may be possible to modify the functional properties of whey proteins using pressure treatment or combinations of pressure treatment and heat treatment

It is expected that a proportion of heat-sensitive whey proteins (mostly Igs, LF and some BSA) would have been denatured during the manufacture of the WPC. Therefore, the results of the present study are applicable only to commercial WPC or to the whey from pasteurised milk, and are not applicable to the whey from raw milk.

4.5 Conclusions

The effects of heat treatment and pressure treatment on the proteins in WPC solutions were similar in general terms, with some proteins forming disulphide-bonded aggregates more readily than other proteins. Also, both these treatments generated hydrophobically as well as disulphide-linked protein aggregates. The most stable protein in both systems was α -LA. However, there were important differences, including the sensitivities of the different whey proteins in the heat- and pressure-treated WPC solutions. β -LG aggregated faster than BSA when WPC solutions were pressure treated but more slowly than BSA when WPC solutions were heat treated. It is likely that the presence of a hydrophobic cavity in β -LG but not in BSA or α -LA allows β -LG to be more sensitive to pressure treatment than expected. Another important difference was that HP treatment generated a greater proportion of smaller or intermediate-sized protein aggregates (such as dimers, trimers etc.) than did heat treatment. This was

probably a consequence of the different effects of heat and pressure on the structure of the native proteins and the disulphide-bonded dimers.

Apart from these findings, the effects of various WPC concentrations and pressurising temperatures on the pressure-induced denaturation, aggregation and gelation of WPC may be very important for its commercial application. However, it appears that only limited information is available on this subject. Based on the results of the present study, given that heat treatment and pressure treatment have different effects on the different whey proteins, it would be interesting to know how WPC solutions of different concentrations behave under pressure or when pressure treated at different pressurising temperatures. Thus, the present study was expanded (Chapters 5 and 6) by using a wider range of WPC concentrations and pressure treatments for various times at various pressurising temperatures to study in detail how a WPC solution behaves under these conditions and thus to define more fully the pressure-induced aggregation and protein–protein interactions that occur in these systems.

CHAPTER 5

Effects of Protein Concentration, Pressurising Temperature and holding time on the Aggregation of Whey Proteins¹

5.1 Introduction

When solutions of whey proteins are heated, the whey proteins modify their structure and interact with one another to form aggregates and gels, depending on the temperature, duration of the heat treatment, the concentration of the proteins, the pH and the ionic strength. Some of the earlier reports on heat-induced denaturation and aggregation (as summarised in Chapter 2) also suggested that the type, size and composition of heat-induced whey protein aggregates are dependent on the concentration of the protein solution and the severity of the heat treatment employed. It has been reported that significantly higher proportions of intermediate-sized aggregates (such as dimer, trimer, tetramer etc.) are formed when dilute (0.5–2%) whey protein solutions are heated, whereas much greater proportions of high molecular weight aggregates are generated (or whey protein gels are formed under appropriate conditions) when whey protein solutions at sufficiently higher concentration (e.g. 8–12%) are heated (e.g. Havea et al., 1998).

Some of the previous studies on the effects of high pressures on whey proteins (e.g. Wong & Heremans, 1988; Okamoto et al., 1990; Dumay et al., 1994, 1998; Funtenberger et al., 1995, 1997; Van Camp & Huyghebaert, 1995b; Van Camp et al., 1996, 1997a, 1997b; Iametti et al., 1997; Jegouic et al., 1996, 1997; Famelart et al., 1998; Havea, 1998; Kanno et al., 1998; Keim & Hinrichs, 2004) have indicated that the operating pressures and the protein concentrations appear to be important factors that influence the extent of whey protein denaturation, aggregation and gelation. However, comparatively little such information on the effects of protein concentration, holding time or pressurising temperature is available in the literature. Also, the limited ranges of pressure treatments, protein concentrations and pressurising temperatures used in the previous studies seemed insufficient to generate a clear trend of the effects of these

¹ Part of the content presented in this chapter has been presented as a poster at the ADAS-ASAS Joint Annual meeting, Minneapolis, Minnesota, July 9-13, 2006 and published in Dairy Foods: Chemistry and Microbiology section, *Journal of Dairy Science* (2006) 89, Suppl. 1, p 176.

parameters on denaturation and aggregation of whey proteins. Moreover, the cumulative or interactive effects of these parameters have not been evaluated.

In this context, the effects of a range of protein concentrations, and various pressure–time combinations, pressurising temperatures and combinations thereof on the denaturation and aggregation of whey proteins were evaluated in WPC solutions. The results are reported in this chapter.

5.2 Experimental

Whey protein concentrate (WPC) solutions of various concentrations (0.5, 2, 5, 8, and 12 % w/v) were prepared by dissolving the appropriate quantity of acid WPC (approximately 80% protein) in Milli-Q water and were pressure treated, and the control and pressure-treated samples were analysed using native- and sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described in Chapter 3.

For the samples pressure treated at different pressurising temperatures, the temperature of the pressurising fluid was adjusted as described in Chapter 3.

A summary of the overall experimental plan, including preparation and analysis of the samples, is presented in Figure 5.1A and 5.1B.

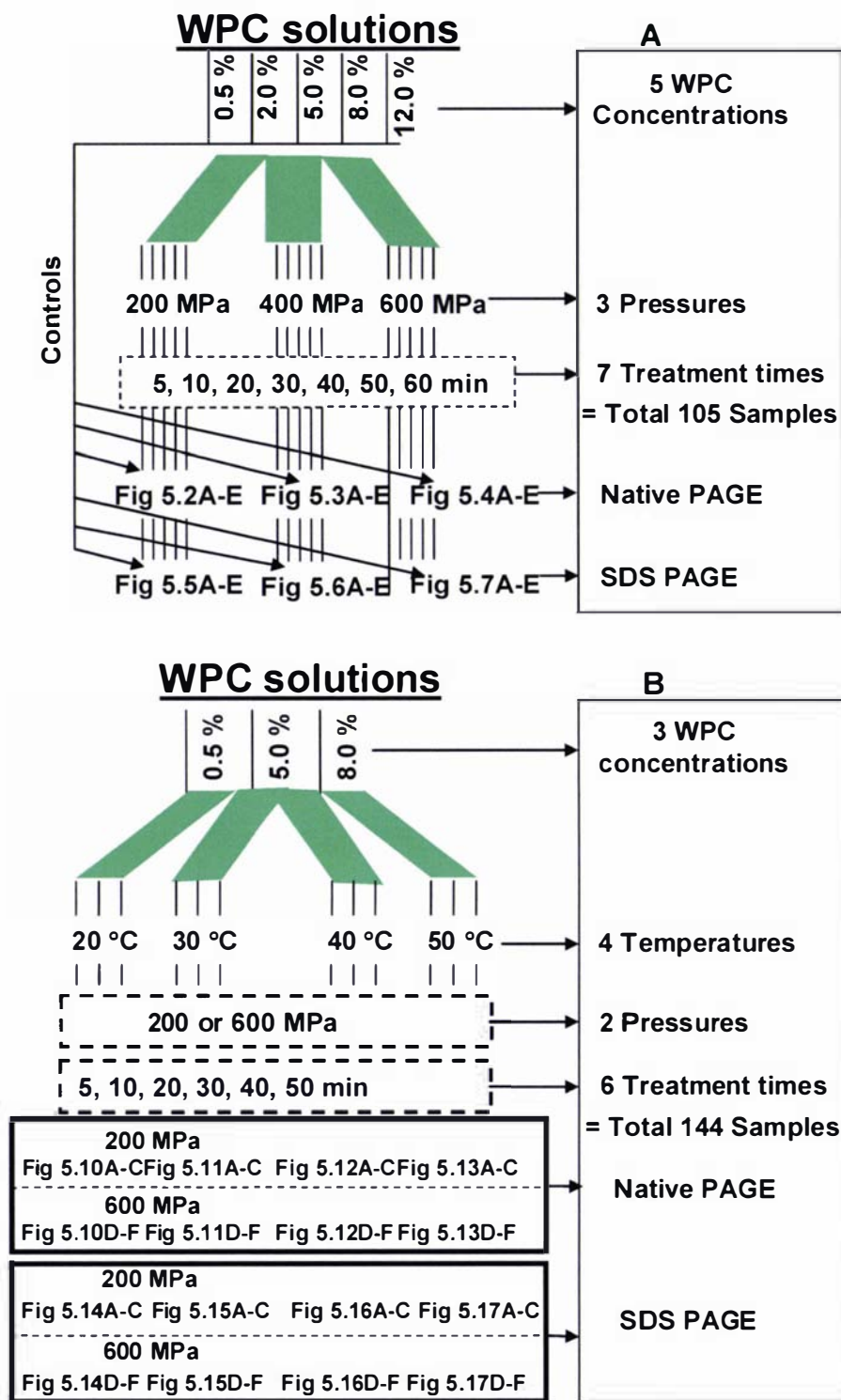


Figure 5.1. Outline of experimental plan for the study of the effects of a range of protein concentrations, various pressure–time combinations, various pressurising temperatures and combinations thereof on the denaturation and aggregation of whey proteins. Experimental plan (A) was designed to address the effect of different WPC concentrations, pressure-treatments and combination thereof. Plan (B) was designed to address the effects of different pressurising temperature, WPC concentrations, pressure treatments and combination thereof.

5.3 Results and discussion

5.3.1 Effects of whey protein concentration and pressure intensity on pressure-induced aggregation in WPC solutions pressure treated at 20°C

In the present study, one representative pressure in each of the regimes of mild, medium and severe pressure treatment was selected in order to generate an overall view of pressure-induced denaturation and aggregation over a wide pressure range. In this context, 200, 400 and 600 MPa were identified as mild, medium and severe pressure treatments respectively. In selecting these pressure regimes, some consideration was also given to previous reports on the pressure-induced denaturation of pure β -lactoglobulin (β -LG) solutions (Considine et al., 2005b, 2006a, 2006b). Similarly, WPC solutions of five different concentrations (0.5, 2, 5, 8 and 12% w/v) were selected to study the effects of whey protein concentration over a wide range, covering dilute solutions to concentrated solutions.

Identification of protein bands on native- and SDS-PAGE

The native-PAGE patterns of 0.5, 2, 5, 8 and 12% (w/v) WPC solutions pressure treated at 200, 400 and 600 MPa at 20°C for different times (from 5 to 60 min) are presented in Figures 5.2A–5.2E, 5.3A–5.3E and 5.4A–5.4E respectively. The corresponding SDS-PAGE patterns are presented in Figures 5.5A–5.5E, 5.6A–5.6E and 5.7A–5.7E respectively.

The bands corresponding to monomeric whey proteins [immunoglobulins (Igs), lactoferrin (LF), bovine serum albumin (BSA), β -LG and α -lactalbumin (α -LA)] were resolved on native- and SDS-PAGE, giving clear PAGE patterns. Each band was identified by comparing its position with that of the respective band in the native- and SDS-PAGE patterns of pure proteins and WPC (see Chapter 4, Figures 4.2, 4.4 and 4.5) and was marked appropriately on the left-hand side of each PAGE pattern (Lane 1, Figures 5.2–5.7). Some material that did not enter the gel was also present in the untreated (control) samples, and was marked as X_0 on the native-PAGE patterns and as \underline{X}_0 on the SDS-PAGE patterns (Lane 1, Figures 5.2–5.7). Representative PAGE patterns of reduced samples are presented (i.e. in Figure 5.5F).

Effects of different protein concentrations and pressure treatments on aggregation of proteins

General comparison of the PAGE patterns of the control samples (Lane 1, Figures 5.2–5.7) with those of the pressure-treated samples (Lanes 2–8, Figures 5.2–5.7) indicated that the intensities of the monomeric bands corresponding to native-like and SDS-monomeric β -LG, α -LA, BSA, IgG and LF decreased in the PAGE patterns of the pressure-treated samples. However, the extent of the decrease in the intensities of the native-like and SDS-monomeric β -LG, α -LA, BSA, IgG and LF bands was found to be dependent on the concentration of WPC, the severity of the pressure treatment employed, the holding time of the samples at a particular pressure and combinations thereof. This general trend was in agreement with the trend reported by Huppertz et al (2004a) for β -LG and α -LA in the pressure treated bovine milk samples. Detailed aspects of the denaturation and aggregation as observed on the native-PAGE and SDS-PAGE patterns of samples of various concentrations pressure treated at 200, 400 and 600 MPa are described in the following sections.

Native-PAGE

WPC solutions of different concentrations pressure treated at 200 MPa

Comparison of the native-PAGE pattern of the control sample (Lane 1, Figure 5.2A) of the 0.5% w/v WPC solution with that of samples pressure treated at 200 MPa and 20°C for various times up to 60 min (Lanes 2–8, Figure 5.2A) indicated that the PAGE patterns of samples pressure treated at 200 MPa for 5, 10 and 20 min (Lanes 2–4, Figure 5.2A) were almost identical to the PAGE pattern of the control sample (Lane 1, Figure 5.2A) showing that there were minimal changes in the samples pressure treated at 200 MPa for up to 20 min. However, some changes were noticeable in the PAGE patterns of the samples pressure treated for 30 min and longer (Lanes 5–8, Figure 5.2A), as shown by the appearance of a new intense band corresponding to non-native monomers (N') and β -LG dimer (intense band just above the α -LA band). No bands corresponding to β -LG trimer, tetramer etc. appeared on this PAGE pattern, suggesting that the pressure-induced changes were limited mainly to the formation of β -LG dimers when dilute WPC solutions (0.5% w/v) were subjected to mild pressure treatment (200 MPa).

Comparison of the native-PAGE pattern of the untreated (control) sample of the 2% w/v WPC solution (Lane 1, Figure 5.2B) with those of the pressure-treated samples (Lanes 2–8, Figure 5.2B) showed almost similar changes in the pressure-treated samples. However, the bands corresponding to β -LG dimer (β -LG₂) were comparatively more intense (Lanes 5–8, Figure 5.2B) than those observed in the corresponding PAGE patterns of the 0.5% w/v WPC solutions (Lanes 5–8, Figure 5.2A).

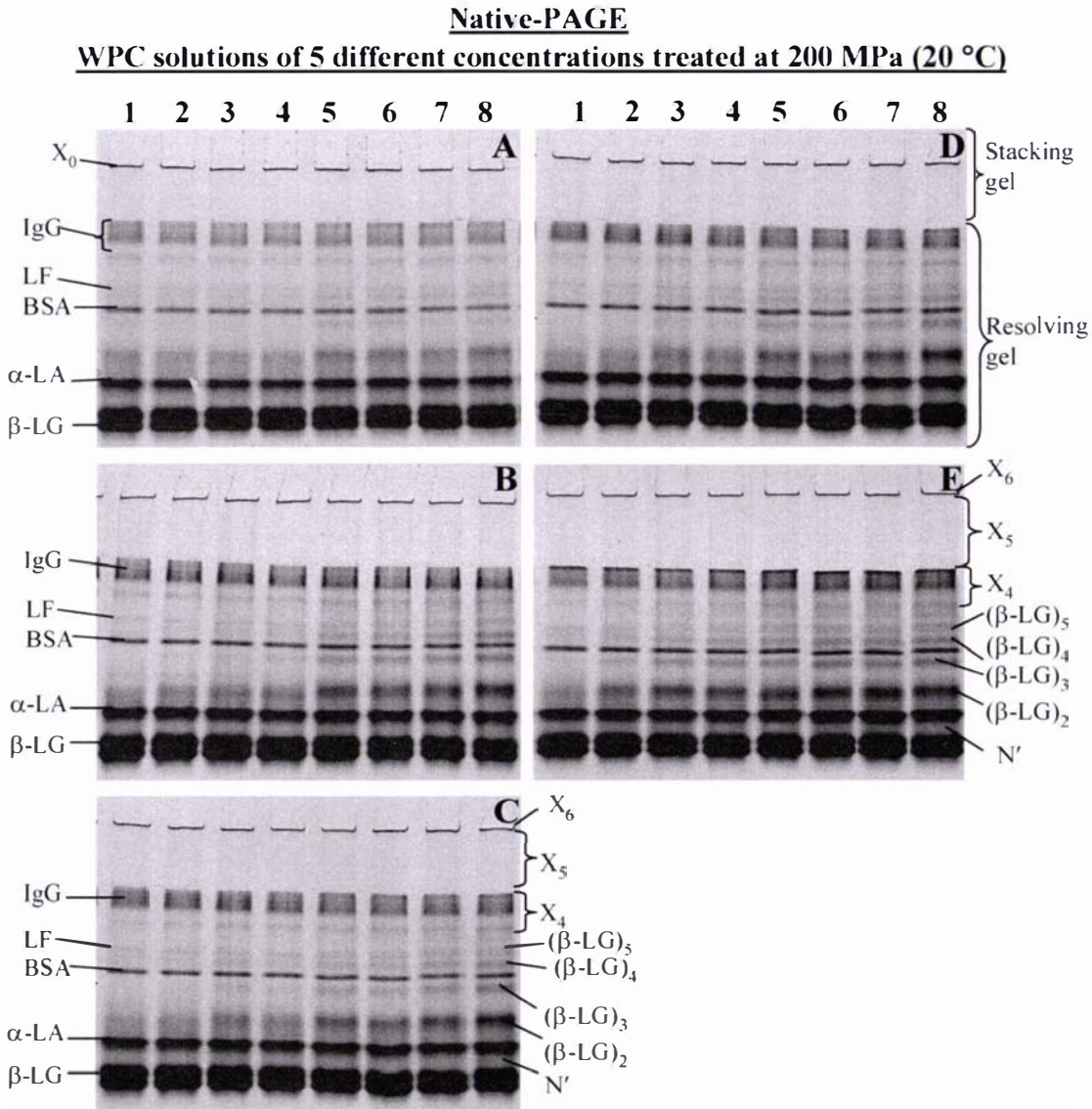


Figure 5.2. Native-PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 200 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

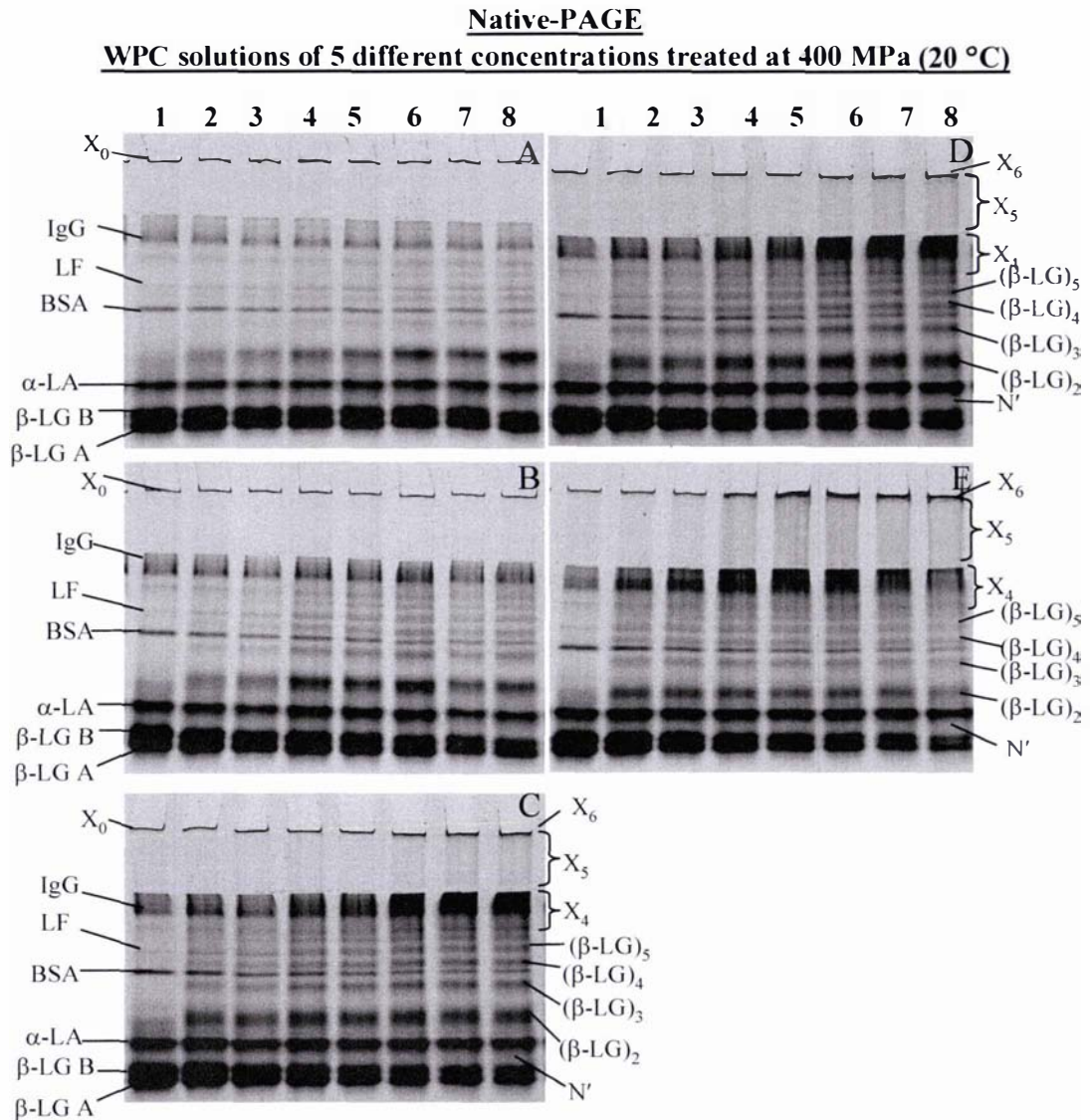


Figure 5.3. Native-PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 400 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

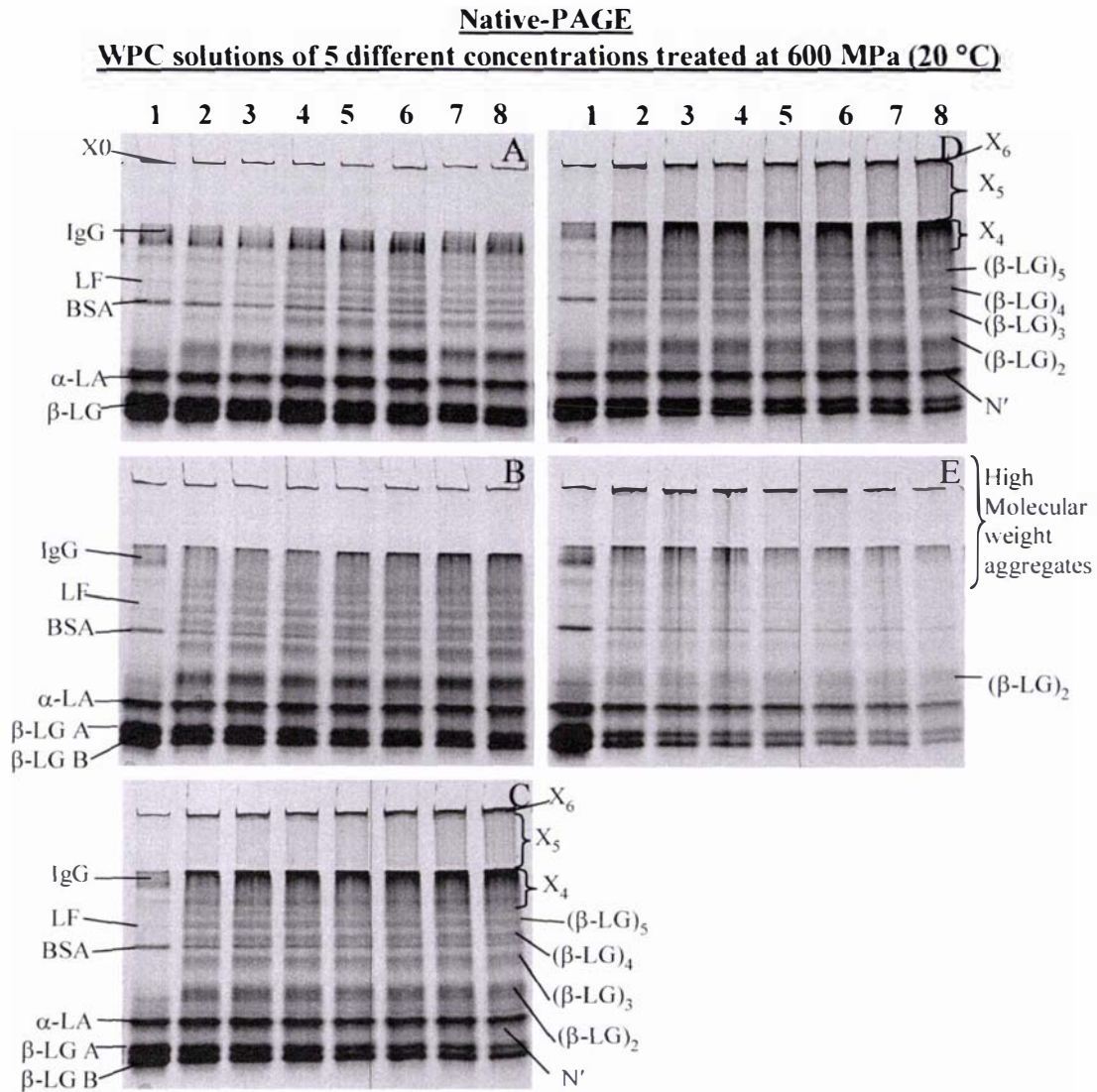


Figure 5.4. Native-PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

In addition, several new discrete bands (resolved in the region between the newly formed band corresponding to β -LG dimer and the top of the resolving gel) were visible in the native-PAGE patterns of the 2% w/v WPC solutions pressure treated at 200 MPa for 30 min and longer (Lanes 5–8, Figure 5.2B). These bands were identified as trimers, tetramers, pentamers etc. (β -LG₃, β -LG₄, β -LG₅ etc.) and high molecular weight polymers of β -LG. These results clearly showed that comparatively larger size pressure-induced aggregates were formed (i.e. the size of the aggregates increased from dimers to trimers, tetramers, pentamers etc.) when the WPC concentration was increased from 0.5 to 2% w/v.

Further comparison of the PAGE patterns of pressure-treated solutions of the 5, 8 and 12% w/v WPC solutions (Lanes 2–8, Figures 5.2C–5.2E respectively) with those of their respective controls (Lane 1, Figures 5.2C–5.2E respectively) showed that the onset of changes, such as the formation of β -LG dimer, trimer etc., occurred earlier than for the pressure-treated samples of the 0.5 and 2% WPC solutions. These changes became clearer as the concentration of the WPC solution was increased from 2 to 5, 8 and 12% w/v. For example, in the native-PAGE patterns of the 12% w/v WPC solutions (Figure 5.2E), the bands corresponding to β -LG₂, β -LG₃, β -LG₄, β -LG₅ etc. appeared after a pressure treatment of 5 min (Lane 2, Figure 5.2E). These polymers became larger after pressure treatment of the samples for extended holding times and a proportion of high molecular weight polymers, which were caught up at the top of the resolving gel (marked as X₄) or within the stacking gel (marked as X₅), were generated (Lanes 3–8, Figure 5.2E). These results suggested that the size of the pressure-induced aggregates increased with increasing concentration of the WPC solutions and with increasing holding time when samples were treated at a particular pressure.

WPC solutions of different concentrations pressure treated at 400 and 600 MPa

The native-PAGE patterns of the WPC solutions (0.5, 2, 5, 8 and 12% w/v) pressure treated at 400 and 600 MPa (20°C) for 5–60 min (Lanes 2–8, Figures 5.3A–5.3E and 5.4A–5.4E respectively) showed the combined effects of various concentrations and more severe pressure treatments (i.e. 400 and 600 MPa). It is apparent from these PAGE patterns that the intensities of the bands corresponding to native-like β -LG, α -LA and BSA decreased and the onset of aggregation occurred much earlier when samples from

the same aliquots were pressure treated at 400 MPa (Lanes 2–8, Figures 5.3A–5.3E) or 600 MPa (Lanes 2–8, Figures 5.4A–5.4E) than at 200 MPa (Lanes 2–8, Figures 5.2A–5.2E). Also, comparatively greater proportions of well-defined newly formed bands (corresponding to β -LG₂, β -LG₃, β -LG₄, β -LG₅ etc.) were observed in the region between the α -LA band and the top of the resolving gel in the PAGE patterns of the 0.5, 2, 5, 8 and 12% w/v WPC solutions pressure treated at 400 (Lanes 2–8, Figures 5.3A–5.3E) and 600 MPa (Lanes 2–8, Figures 5.4A–5.4E) than in the PAGE patterns of their counterparts pressure treated at 200 MPa (Lanes 2–8, Figures 5.2A–5.2E). Moreover, comparatively greater proportions of very high molecular weight aggregates, i.e. those caught up at the top of the resolving gel, those within the stacking gel or those that could not enter the gel (marked as X₄, X₅, X₆ respectively), were formed when 5, 8 or 12% w/v WPC solutions were pressure treated at 400 MPa (Figures 5.3C–5.3E) or 600 MPa (Figures 5.4C–5.4E) than when samples from the same aliquots were pressure treated at 200 MPa (Figures 5.2C–5.2E). In some instances, the aggregates became so large that they were immobilised in the sample well (marked as X₆) or did not enter the gel and were subsequently washed away during the staining and destaining procedure [for example, this was clear from the native-PAGE patterns of the 12% w/v WPC solutions pressure treated at 600 MPa (Figure 5.4E)].

These results suggested that, after mild pressure treatment (e.g. 200 MPa), small aggregates, such as dimers and trimers etc., were formed and that a comparatively very small proportion of aggregates of high molecular weight, such as those caught up at the top of the resolving gel, those within the stacking gel and those that could not enter the gel (marked as X₄, X₅, X₆ respectively), were formed. In contrast, when samples from the same aliquots were subjected to moderate or severe pressure treatment (e.g. 400 and 600 MPa), significant proportions of large or very large aggregates (marked as X₄, X₅, X₆ respectively) were formed.

SDS-PAGE

Comparison of the SDS-PAGE patterns of control WPC solutions (Lane 1, Figures 5.5A–5.5E, 5.6A–5.6E and 5.7A–5.7E) with those of samples pressure treated at 200, 400 and 600 MPa (at 20°C) for 5–60 min (Lanes 2–8, Figures 5.5A–5.5E, 5.6A–5.6E and 5.7A–5.7E) indicated that the trends observed on the SDS-PAGE patterns were

similar to those observed on the native-PAGE patterns (Figures 5.2–5.4). However, unlike native-PAGE, no well-defined bands corresponding to tetramers (β -LG₄), pentamers (β -LG₅) etc. were clearly observed on the SDS-PAGE patterns of the pressure-treated samples. Instead, only the bands corresponding to β -LG dimer (β -LG₂) and β -LG trimer (β -LG₃) were clearly resolved on the SDS-PAGE patterns of the pressure-treated samples. In addition, there was a dark-staining blurred region between the β -LG bands and the top of the resolving gel in the SDS-PAGE patterns of the pressure-treated samples.

Again, it can be observed from the SDS-PAGE patterns of the pressure-treated samples (Figures 5.5A–5.5E, 5.6A–5.6E and 5.7A–5.7E) that the size of the aggregates increased gradually (i.e. a progressive increase from α -LA and β -LG dimers to trimers, tetramers and high molecular weight aggregates) as the concentration of the WPC solutions, the severity of the pressure treatment and the holding time of the samples at a particular pressure increased. High molecular weight aggregates, i.e. those caught up at the top of the resolving gel, those within the stacking gel or those that could not enter the gel (marked as X₄, X₅ and X₆ respectively), were observed in the PAGE pattern of 2, 5, 8 and 12% w/v WPC solutions pressure treated at 400 MPa (Figures 5.6B–5.6E) and 600 MPa (Figures 5.7B–5.7E). Moreover, it was noticed that pressure-induced gels were formed when 12% w/v WPC solutions were pressure treated at 600 MPa at 20°C. It will be interesting to characterise the pathways of such pressure-induced gel formation in details by extending the present study (see Chapter 6).

These results confirmed that both the concentration of protein in the sample and the severity of the pressure treatment are important. The combination of a lower concentration and mild pressure treatment generated only small-sized aggregates, whereas the combination of pressure treatment and concentrated WPC solutions (e.g. 8 or 12% WPC solutions) led to the formation of very large aggregates or very high molecular weight protein aggregates (> 500 kDa) that could not enter the gel, which explains why cross-linking and gel formation accelerate when WPC solutions of higher protein concentrations are subjected to severe pressure treatments for longer holding time (also see Appendix 3).

The above results showed that the effects of pressure treatment on the proteins in WPC solutions followed an almost similar trend to those of heat treatment (Havea et al., 1998), which showed that the loss of native-like monomeric and SDS-monomeric β -LG in WPC solutions increased as the protein concentration and the holding time of heat treatment increased.

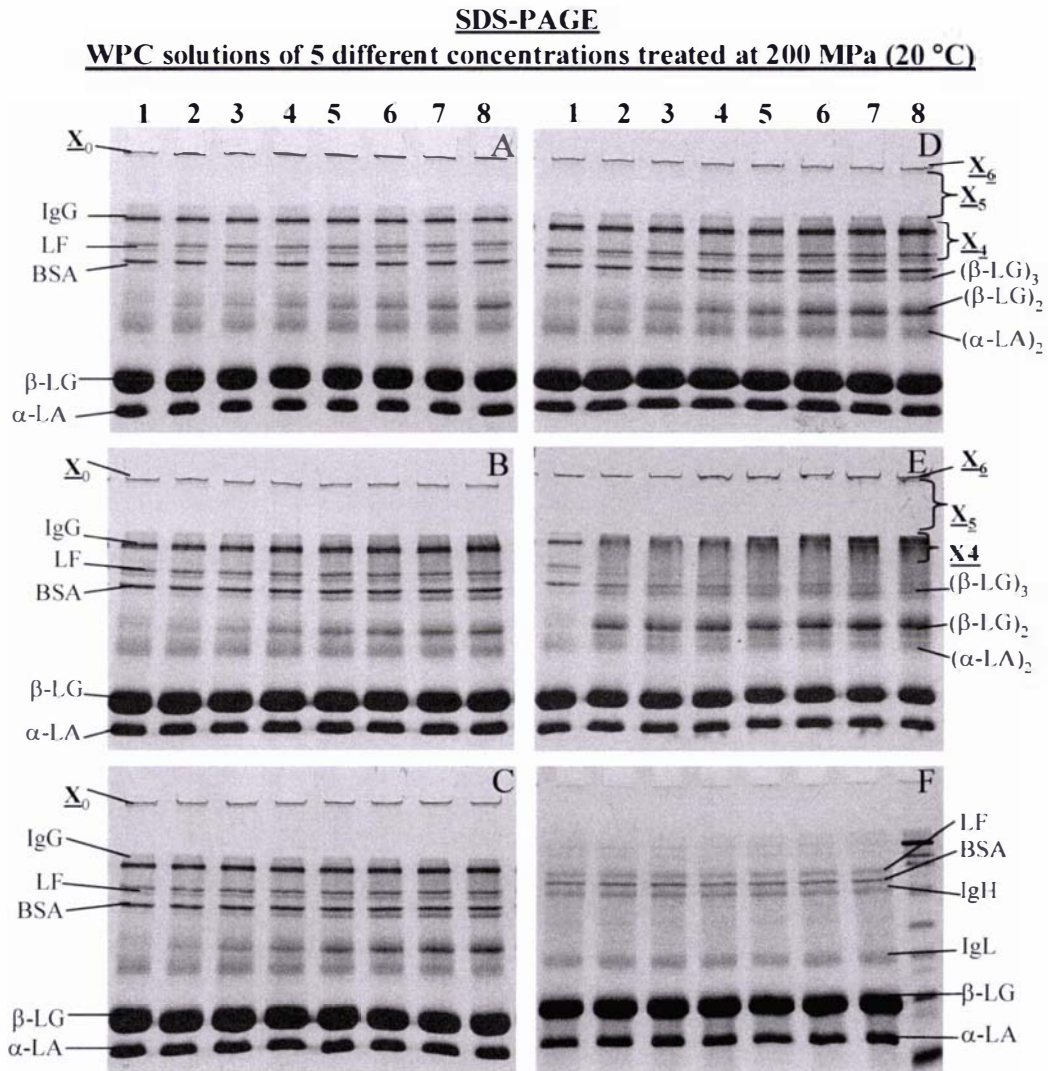


Figure 5.5: SDS PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 200 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8). (F) SDS-PAGE patterns of reduced samples corresponding to the samples shown in Figure 5.5B.

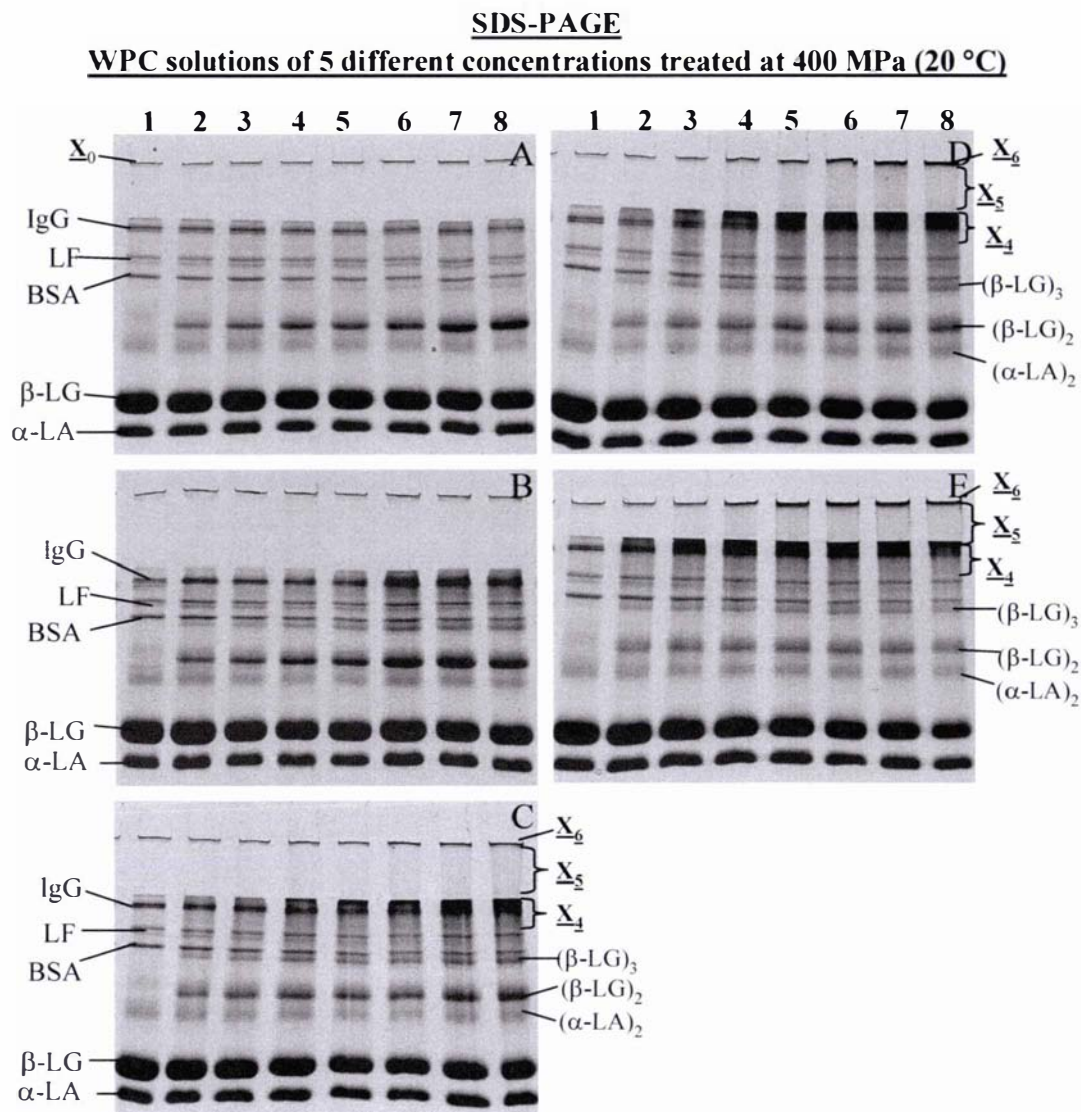


Figure 5.6. SDS-PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 400 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

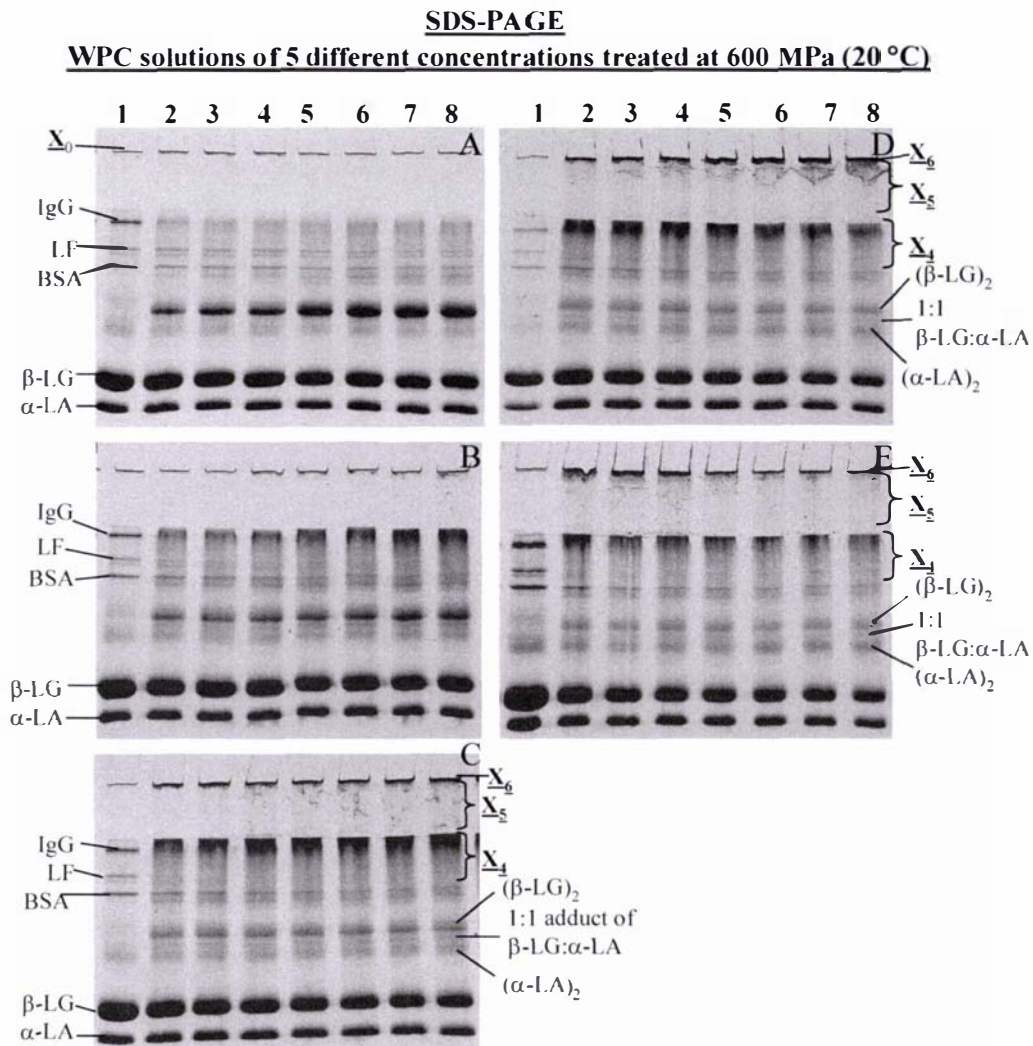


Figure 5.7. SDS-PAGE patterns of (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions: untreated (control) sample (Lane 1), and samples pressure treated (at 20°C) at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

Effects of protein concentration and pressure treatment on BSA and α -LA

It was observed from the native- (Figures 5.2A–5.2E) and SDS- (Figures 5.5A–5.5E) PAGE patterns that mild pressure treatment (200 MPa at 20°C) had little effects on the bands corresponding to BSA and α -LA, confirming some of the earlier reports and the results shown in Chapter 4. The native- (Figures 5.3A–5.3E) and SDS- (Figures 5.6A–5.6E) PAGE patterns of samples from the same aliquots that were pressure treated at 400 MPa at 20°C showed that the intensities of the bands corresponding to both β -LG and BSA were affected, but that the intensity of the band corresponding to α -LA was least affected. However, when samples from the same aliquots were pressure treated at 600 MPa at 20°C, the intensities of the bands corresponding to β -LG, BSA and α -LA were affected, as shown by the native- (Figure 5.4A–5.4E) and SDS- (Figure 5.7A–5.7E) PAGE patterns, particularly when 12% w/v WPC solutions were pressure treated at the severe pressure of 600 MPa at 20°C (see Figures 5.4E and 5.7E). These results indicated that α -LA was the most resistant of the whey proteins to pressure treatment up to 400 MPa at 20°C, confirming some of the earlier reports (López-Fandiño et al., 1996; Tanaka & Kunugi, 1996; Tanaka et al., 1996; Scollard et al., 2000; Huppertz et al., 2002, 2004b). Similarly, BSA seemed to be comparatively more resistant than β -LG, when pressure treated at up to 400 MPa at 20°C (Hayakawa et al., 1992; Lopez-Fandino et al., 1996), but, at higher pressures (e.g. 600 MPa at 20°C), the rate of decrease in the intensity of the band corresponding to BSA seemed to be comparatively faster than that of the band corresponding to β -LG.

Quantitative PAGE analysis

The changes in the relative intensities of the bands (relative to the band intensity of the control/untreated sample on each PAGE pattern) corresponding to native-like and SDS-monomeric β -LG (as measured using laser densitometry) on the PAGE patterns (Figures 5.2–5.7) of the WPC solutions of various concentrations pressure treated at 200, 400 and 600 MPa at a pressurising temperature of 20°C are presented in Figures 5.8 and 5.9. An attempt was also made to measure the intensities of the bands corresponding to α -LA and BSA, as affected by the pressure treatment of WPC solutions. However, some difficulties and errors were encountered, particularly for the native-PAGE patterns, because the bands corresponding to α -LA and BSA overlapped

with the new bands corresponding to the pressure-induced aggregates of β -LG (i.e. new pressure-induced bands corresponding to non-native monomers, dimers, trimers and tetramers). Moreover, it has been reported previously that β -LG, being the major whey protein in WPC, dominates the heat-induced (Mulvihill & Kinsella, 1987; Boye et al., 1995) and pressure-induced (Van Camp et al., 1996, 1997a, 1997b; Kanno et al., 1998; Belloque et al., 2000) aggregation and gelation of WPC solutions and therefore the overall behaviour of WPC is strongly affected by the behaviour of β -LG. Considering these reasons, only the integrated intensities of the bands corresponding to β -LG are plotted (Figure 5.8 and 5.9) and most results are discussed in relation to the pressure-induced behaviour of β -LG.

It is apparent from Figure 5.8 that the decrease in native-like and SDS-monomeric β -LG was comparatively greater and faster in the WPC solutions of 0.5, 2, 5, 8 and 12% w/v samples pressure treated at 400 MPa (Figure 5.8B) or 600 MPa (Figure 5.8C) than in the WPC solutions from the same aliquots pressure treated at 200 MPa (Figure 5.8A). When the samples were pressure treated at 200 MPa (Figure 5.8A), the decrease in native-like and SDS-monomeric β -LG was small (within 20–25 %), suggesting that there were no significant effects of protein concentration when the WPC solutions were subjected to mild pressure treatment. In contrast, when the WPC solutions were pressure treated at 400 MPa (Figure 5.8B) or 600 MPa concentration of monomeric β -LG decreases considerably (Figure 5.8C). For example, there was \approx 20% residual monomeric β -LG in concentrated (12% w/v) WPC solutions subjected to a severe pressure (600 MPa) for 40 min or longer, compared with \approx 70–80% residual monomeric β -LG when WPC solutions from the same aliquots were subjected to a mild pressure (200 MPa) for 40 min. From these plots, it can be seen that the effect was more pronounced when severe pressure treatment was coupled with an increased WPC concentration in the sample (Figure 5.8C; see also Figures 5.9A–5.9E).

Further, it was observed that the decrease in native-like and SDS-monomeric β -LG was much greater after a longer holding time (20 min or more) at 400 MPa (Figure 5.8B) and 600 MPa (Figure 5.8C) than after a longer holding time at 200 MPa (Figure 5.8A) (see also Figure 5.9). These results suggest that the rate and the extent of the denaturation of monomeric β -LG is dependent on the protein concentration, the severity of the pressure treatment, the holding time of the sample at the particular pressure and

combinations thereof. This behaviour of β -LG in pressure-treated WPC solutions was somewhat similar to that of β -LG in heat-treated WPC solutions (e.g. Havea et al., 1998; Singh & Havea, 2003).

Differences between SDS-monomeric and native-like β -LG

From the plotted values (Figures 5.8 and 5.9), it can be seen that there were some differences (≈ 5 –30%) between the residual native-like β -LG and the residual SDS-monomeric β -LG in the pressure-treated samples, suggesting that a proportion of the pressure-induced aggregates of β -LG were non-covalently linked (mainly hydrophobically linked), depending on the concentration of the WPC solutions (Figure 5.9) and the severity of the pressure treatment employed (Figure 5.8, also see Figure 5.9). These results were somewhat similar to those reported and discussed earlier (Gezimati et al., 1996, 1997; Dalgleish et al., 1997; Schokker et al., 2000; Havea et al., 2001; Hong & Creamer, 2002) for heat-treated solutions of whey proteins. It was found that the difference between residual SDS-monomeric β -LG and residual native-like β -LG increased as the concentration of the WPC solution increased (see Figure 5.9). For example, the greatest ($\approx 30\%$) difference between residual SDS-monomeric β -LG and residual native-like β -LG was found in the 12% WPC solutions pressure treated at 600 MPa (see Figure 5.9E), suggesting that comparatively higher proportions of hydrophobically linked aggregates were generated when the samples were pressure treated at higher concentrations and at higher pressures.

These results are also in partial agreement with the results of Yang et al. (2001), who reported the formation of hydrophobic molten globules when β -LG samples were pressure treated at 600 MPa at 50 °C, and are supported by the results of Aouzelleg et al. (2004), who suggested that pressure intensity is the most important parameter in bringing about molecular changes in the protein. Earlier reports on the pressure-induced aggregation of β -LG also indicated that factors such as the concentration of protein (Dumay et al., 1994), the buffer type and molarity used for preparation of the protein solutions (Funttenberger et al., 1995) and the pressure intensity, pressurising time and pressurising temperature (Funttenberger et al., 1995; Yang et al., 2001; Aouzelleg et al., 2004) affect pressure-induced changes in the protein structure, and the denaturation and aggregation of β -LG.

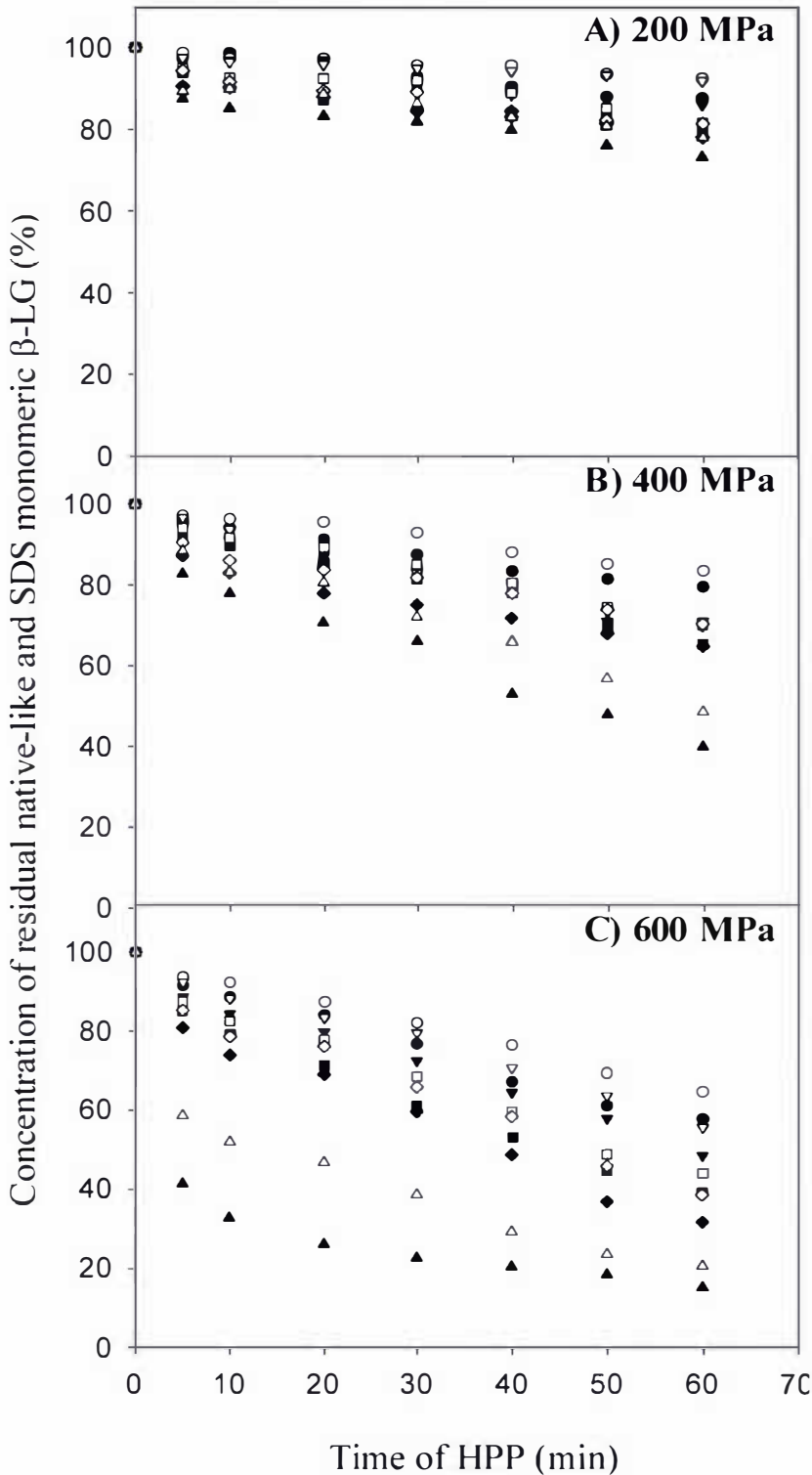


Figure 5.8. Residual native-like and SDS-monomeric β -LG in 0.5, 2, 5, 8 and 12% (w/v) WPC solutions pressure treated at (A) 200 MPa, (B) 400 MPa and (C) 600 MPa at 20°C for various times. Residual native-like β -LG present in ●: 0.5%; ▼: 2%; ■: 5%; ◆: 8%; ▲: 12% w/v WPC solutions; and residual SDS-monomeric β -LG present in ○: 0.5%; ▽: 2%; □: 5%; ◇: 8%; △: 12% w/v WPC solutions.

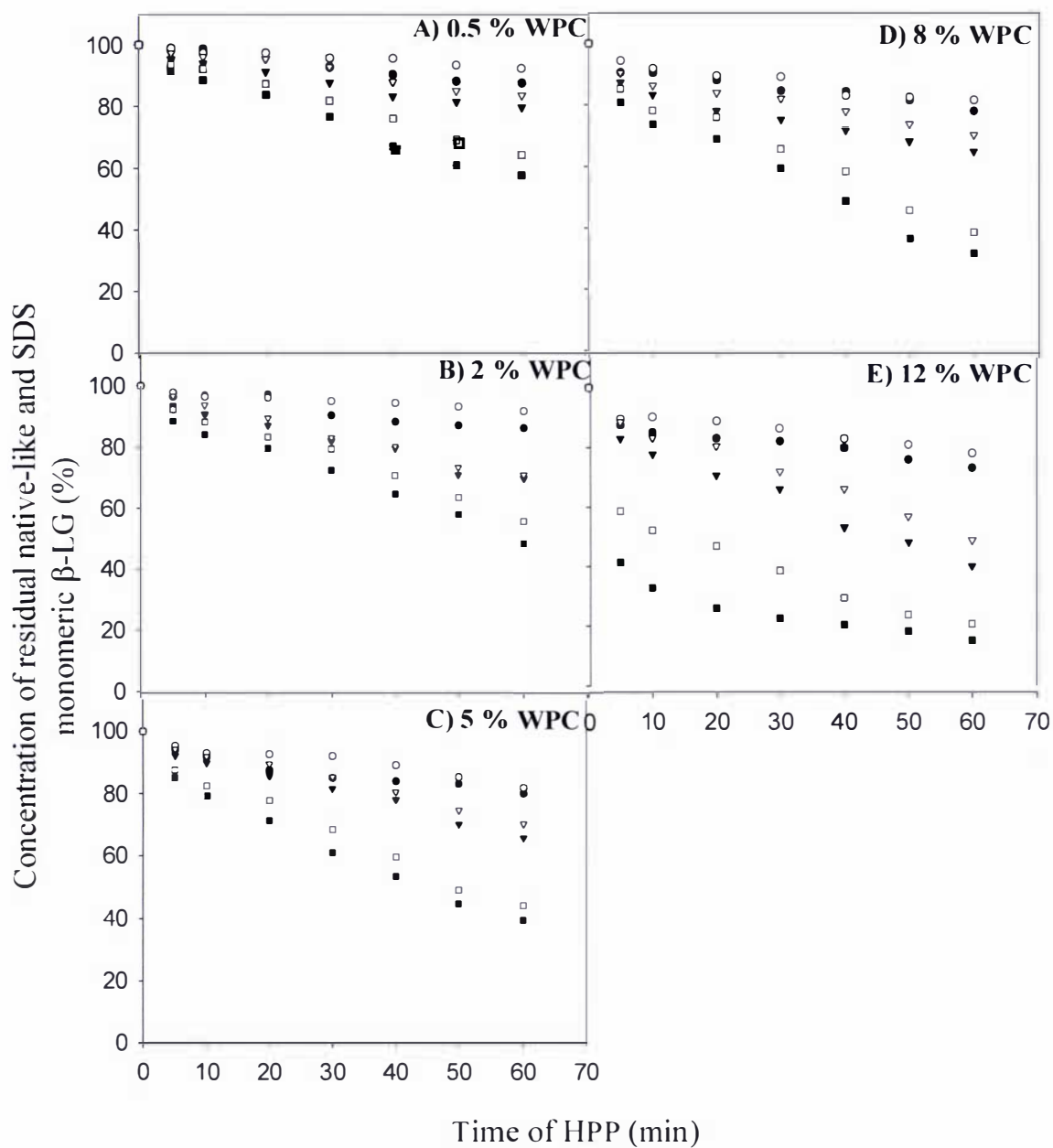


Figure 5.9. Residual native-like and SDS-monomeric β -LG in (A) 0.5% w/v, (B) 2% w/v, (C) 5% w/v, (D) 8% w/v and (E) 12% w/v WPC solutions pressure treated at 200, 400 and 600 MPa at 20°C for various times. Residual native-like β -LG in WPC solutions pressure treated at ●: 200 MPa; ▼: 400 MPa; ■: 600 MPa; and residual SDS-monomeric β -LG in WPC solutions pressure treated at ○: 200 MPa; ▽: 400 MPa; □: 600 MPa.

5.3.2 Effects of different pressurising temperatures on aggregation of whey proteins

It has been reported that pressure treatments employed at elevated temperature are more effective for the destruction of microorganisms (e.g. Farkas & Hoover, 2000). Therefore, it may be expected that different pressurising temperatures may have different effects on the denaturation and aggregation of proteins (Huppertz et al., 2004a). However, little has been reported in the literature on the effects of different pressurising temperatures on the denaturation and aggregation of proteins. Therefore, the study was extended to explore the combined effects of dilute (0.5% w/v), intermediate (5% w/v) and concentrated (8% w/v) WPC solutions after mild (200 MPa) and severe (600 MPa) pressure treatments at four different pressurising temperatures (20, 30, 40 and 50°C). The effects of these parameters were evaluated in order to determine the individual and combined effects of protein concentration, severity of pressure treatment and pressurising temperature. This experimental design (Figure 5.1B), in which samples from the same aliquots were subjected to various combinations of pressure treatments and pressurising temperatures, allowed simple comparison of the effects of different pressurising temperatures at selected protein concentrations and pressure treatments.

Native- and SDS-PAGE

The native- and SDS-PAGE patterns of WPC solutions (0.5, 5 and 8% w/v), subjected to mild (200 MPa) and severe (600 MPa) pressure treatment at 20, 30, 40 and 50°C, are presented in Figures 5.10–5.13 and 5.14–5.17 respectively.

The native-PAGE patterns of the 0.5, 5 and 8% w/v WPC solutions subjected to mild pressure treatment (200 MPa) at 20°C for 30 min and longer (Lanes 5–7, Figures 5.10A–5.10C) showed some minor changes (e.g. the appearance of a faint band corresponding to β -LG dimer), confirming earlier results (Figures 5.2A–5.2E, 5.8 and 5.9). In contrast, pressure treatment of samples from the same aliquots of the 0.5% w/v WPC solution pressure treated at 600 MPa at 20°C (Figure 5.10D) gave several changes in the native-PAGE pattern (including the formation of several new bands corresponding to dimers, trimers, tetramers and higher polymers etc.). In addition to these new bands, a proportion of high molecular weight polymers (marked as X₄, X₅ and X₆) were apparent in the native-PAGE patterns of the 5 and 8% w/v WPC solutions

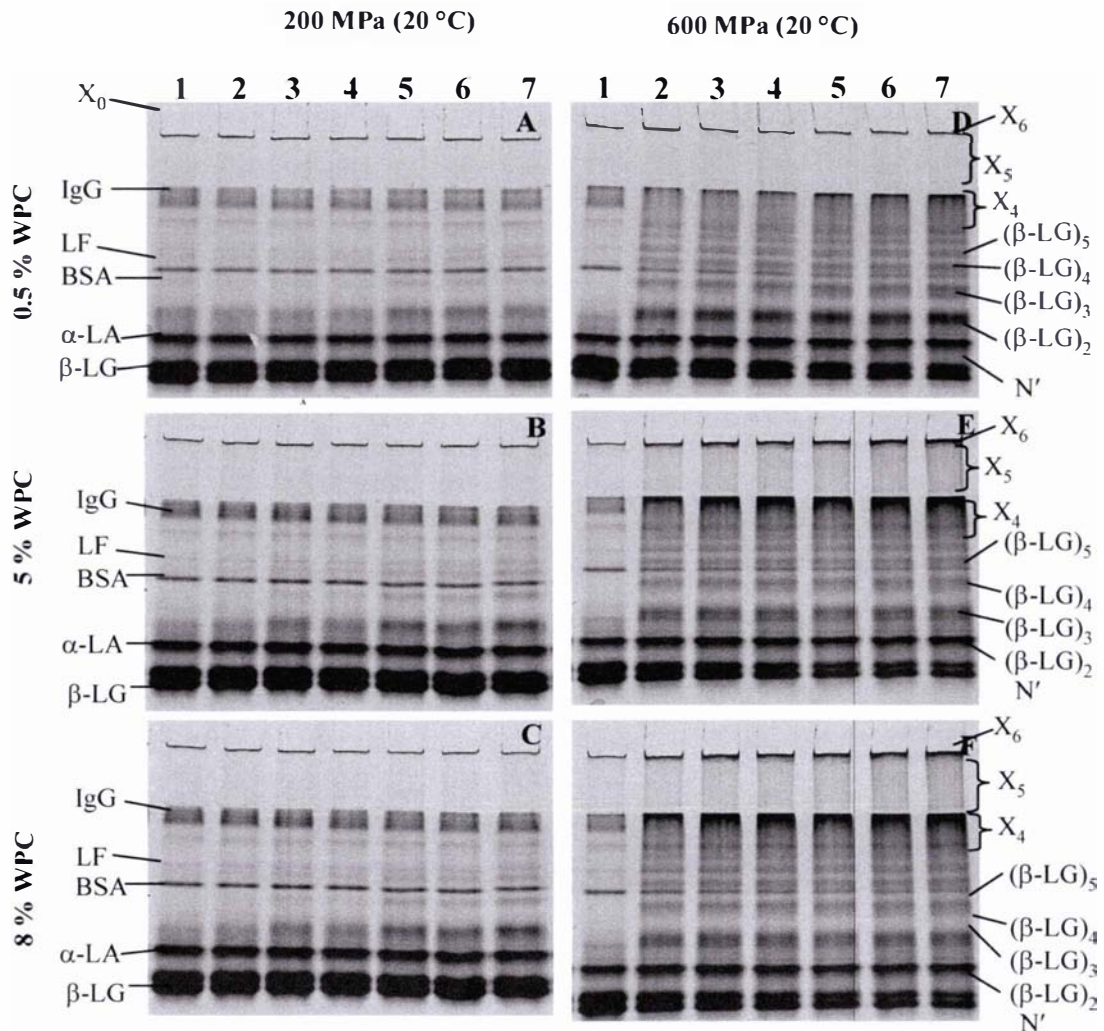


Figure 5.10. Native-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 20°C. Similarly, native-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 20°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

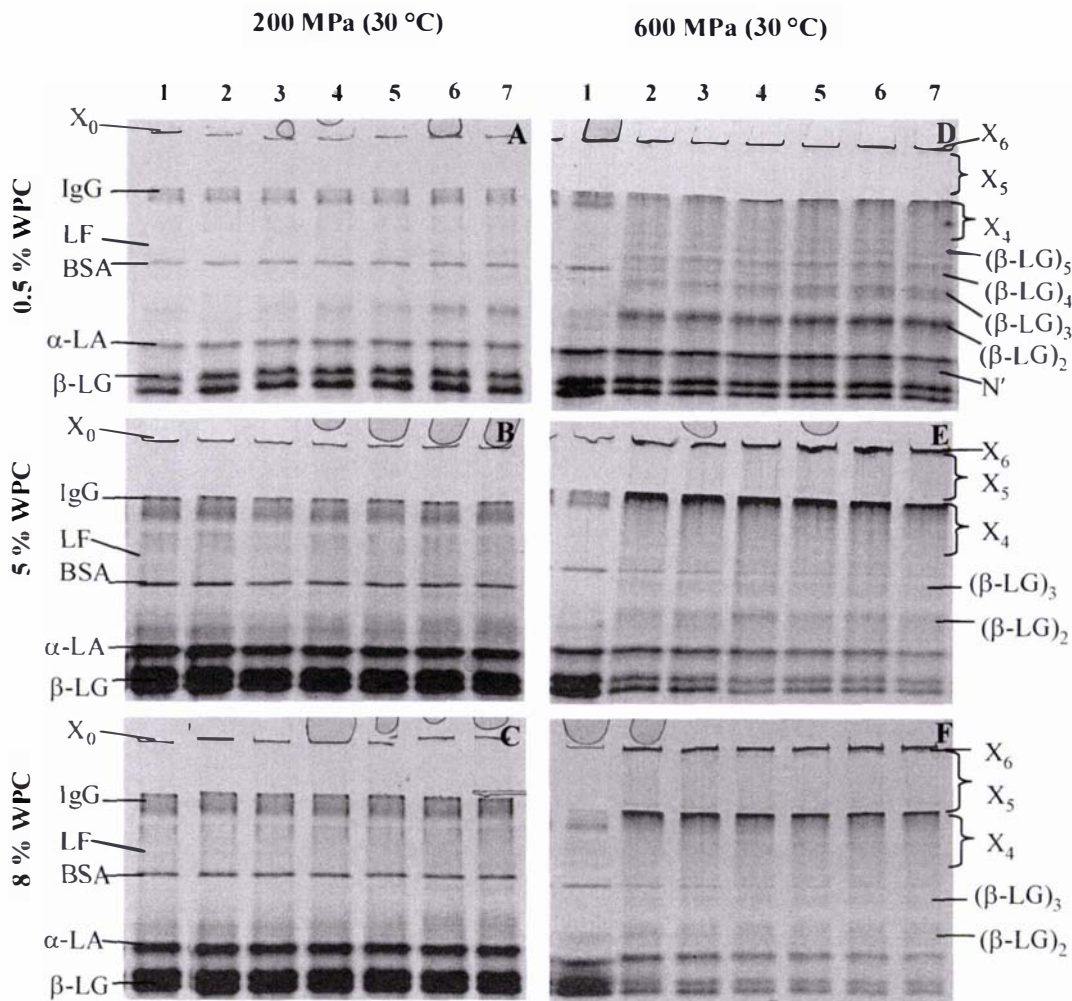


Figure 5.11. Native-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 30°C. Similarly, native-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 30°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

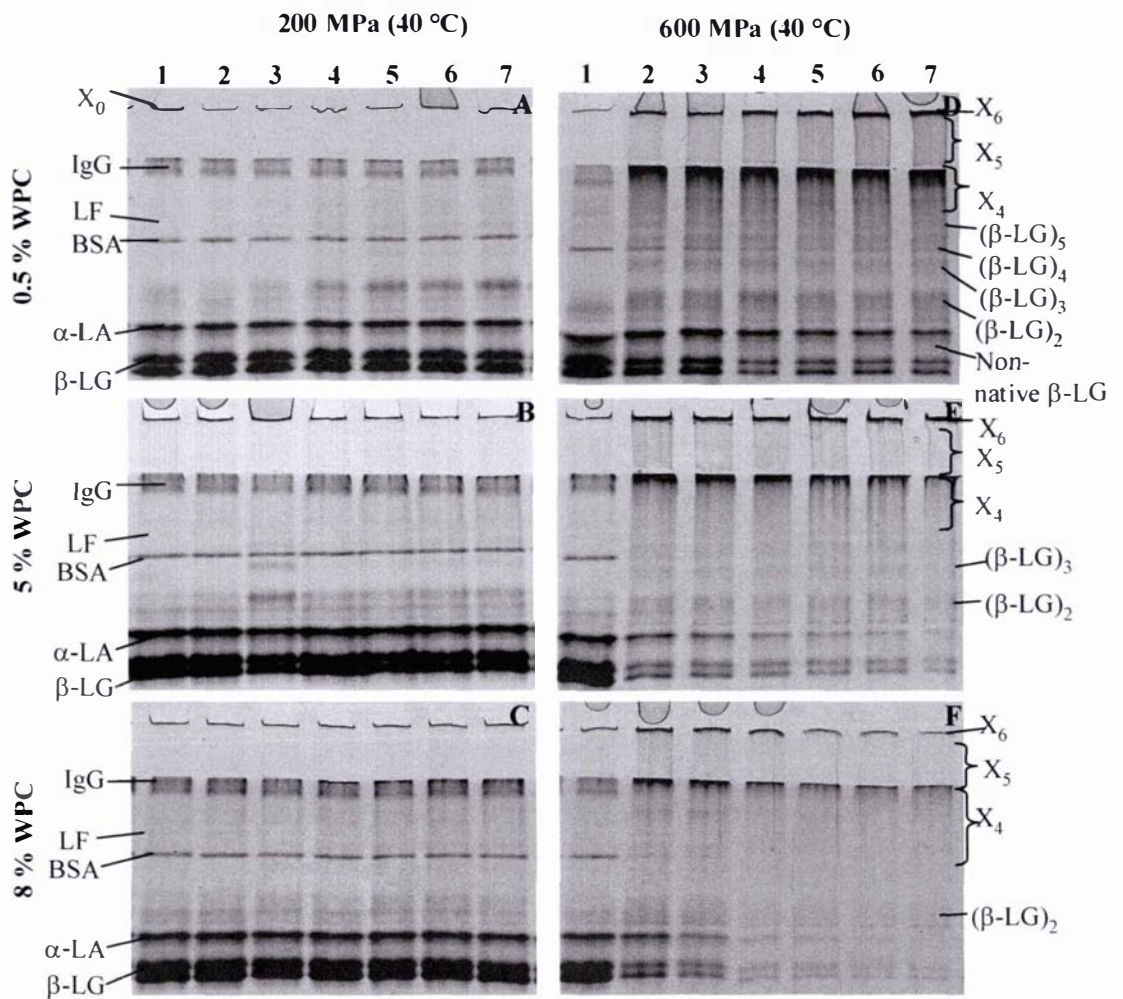


Figure 5.12. Native-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 40°C. Similarly, native-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 40°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7) in each PAGE patterns.

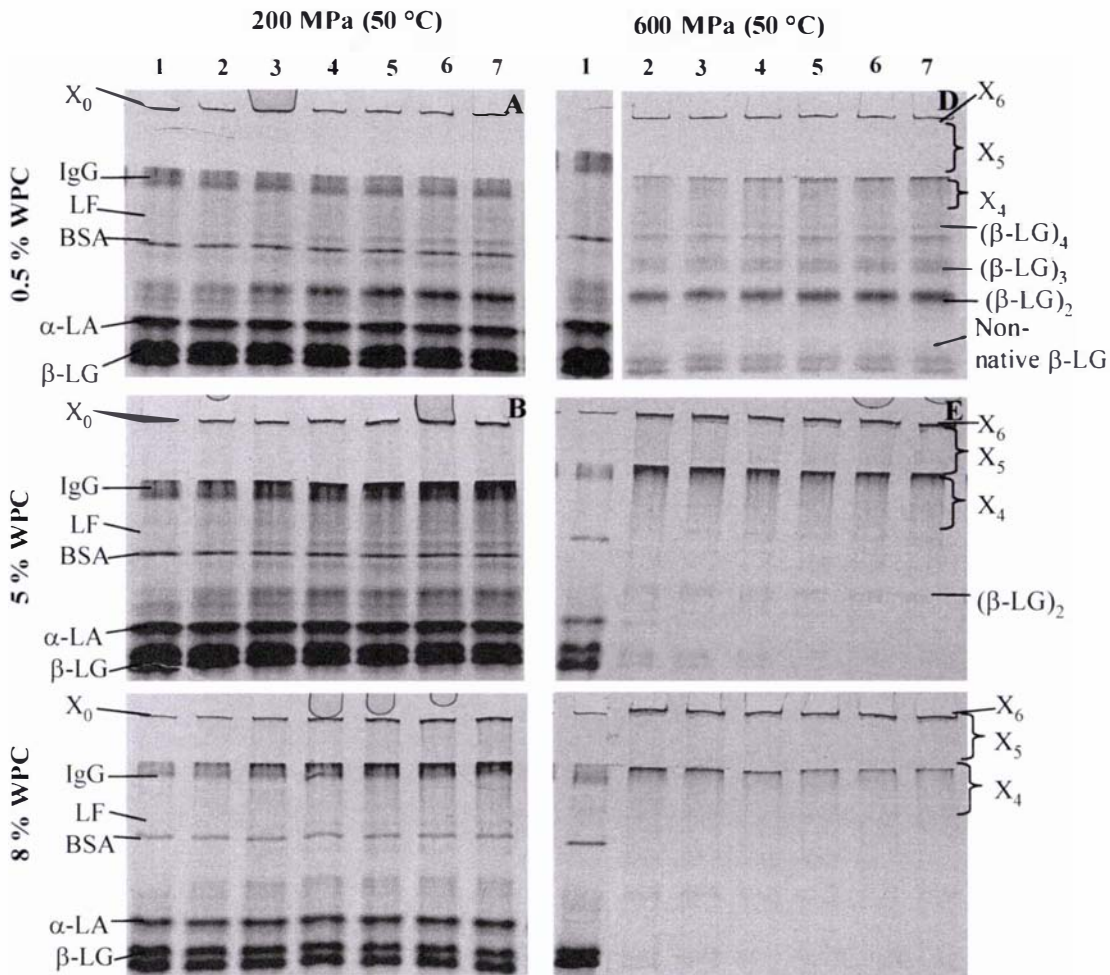


Figure 5.13. Native-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 50°C. Similarly, native-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 50°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

pressure treated at 600 MPa at 20°C (Figures 5.10E and 5.10F). These PAGE patterns clearly showed that, at a pressurising temperature of 20°C, the protein concentration appeared to have minor effects when used in combination with mild (200 MPa) pressure treatment (Figures 5.10A–5.10C), but had significant effects when used in combination with severe (600 MPa) pressure treatment (Figures 5.10D–5.10F).

Almost similar trends were apparent for the samples subjected to mild (200 MPa) pressure treatment at 30, 40 and 50°C, with the exception that comparatively greater proportions of larger protein aggregates were observed in the PAGE patterns as the pressurising temperature was increased from 20 (Figure 5.10A–5.10C) to 30, 40 and 50°C (Figures 5.11A–5.11C, 5.12A–5.12C and 5.13A–5.13C). The effects were comparatively more pronounced when 0.5, 5 and 8% w/v WPC solutions from the same aliquots were pressure treated at 600 MPa (instead of 200 MPa) and coupled with increasing pressurising temperature from 20 (Figure 5.10D–5.10E) to 30, 40 and 50°C (Figures 5.11D–5.11F, 5.12D–5.12F and 5.13D–5.13F).

The SDS-PAGE patterns of the 0.5, 5 and 8% w/v WPC solutions subjected to mild (200 MPa) and severe (600 MPa) pressure treatment at 20, 30, 40 and 50°C (Figures 5.14–5.17) showed almost similar trends to those observed on the native-PAGE patterns of the corresponding samples (Figures 5.10–5.13).

Comparison of the native-PAGE patterns of WPC solutions (0.5, 5 and 8% w/v) pressure treated at 200 and 600 MPa at 20, 30, 40 and 50°C showed that the major whey proteins (α -LA and BSA) were minimally affected when samples were pressure treated at 200 MPa. However, when the severe pressure treatment (600 MPa) was used in combination with elevated pressurising temperatures (30, 40 and 50°C), there were significant effects on the denaturation and aggregation of all the whey proteins, including β -LG, α -LA and BSA. It was observed that the concentration of native-like β -LG, α -LA and BSA decreased significantly for the samples pressure treated at 600 MPa at $\geq 40^\circ\text{C}$ (Figures 5.12D–5.12F and 5.13D–5.13F) with the concomitant formation of large and very large (molecular weight > 500 kDa) aggregates (marked as X₄, X₅ and X₆). It was also noticed that small proportions of intermediate-sized protein species (such as dimer, trimer etc.) were formed at such pressure–temperature combinations (Figures 5.12D–5.12F and 5.13D–5.13F), showing that the combination of higher

pressurising temperature and severe pressure treatment generated very large protein aggregates.

Again, it can be observed from the SDS-PAGE patterns of the pressure-treated samples (Figures 5.14A–5.14F, 5.15A–5.15F, 5.16A–5.16F and 5.17A–5.17F) that the size of the aggregates gradually increased (i.e. a progressive increase from α -LA and β -LG dimers to trimers, tetramers and high molecular weight aggregates) as the protein concentration in the WPC solutions, the severity of the pressure treatment and the holding time of the samples at a particular pressure increased. Also, it can be observed that the concentration of SDS-monomeric β -LG, α -LA and BSA was comparatively higher (as indicated by comparatively darker intensity of the bands corresponding to these proteins) in the SDS-PAGE patterns than in the corresponding native-PAGE patterns, which may be attributed to the dissociation of hydrophobically linked aggregates in the SDS-PAGE environment.

Effects on BSA and α -LA

These results also showed that the aggregation behaviours of β -LG, α -LA and BSA were quite different when WPC samples were pressure treated at different pressurising temperatures. It was particularly interesting to note from the native- and SDS-PAGE patterns that pressure-resistant whey proteins, such as α -LA and BSA, aggregated much faster as the pressurising temperature was increased from 30 to 40 and 50°C at 600 MPa. It was found that the intensities of the bands corresponding to α -LA and BSA decreased faster than the intensity of the band corresponding to β -LG when WPC samples were pressure treated at 600 MPa at pressurising temperatures of 40 and 50°C (5.12D–5.12F, 5.13D–5.13F, 5.16D–5.16F and 5.17D–5.17F).

These results suggested that α -LA and BSA became more reactive when the pressurising temperature was increased to $\geq 40^\circ\text{C}$ at 600 MPa. There are several possible reasons for such behaviour of these proteins. It has been reported that the denaturation of α -LA is partly reversible (Ruegg et al., 1977). It may be possible that α -LA unfolded reversibly when pressure treated at lower temperature (Tanaka & Kunugi, 1996; Tanaka et al., 1996). Another possibility is that α -LA was in a molten globule

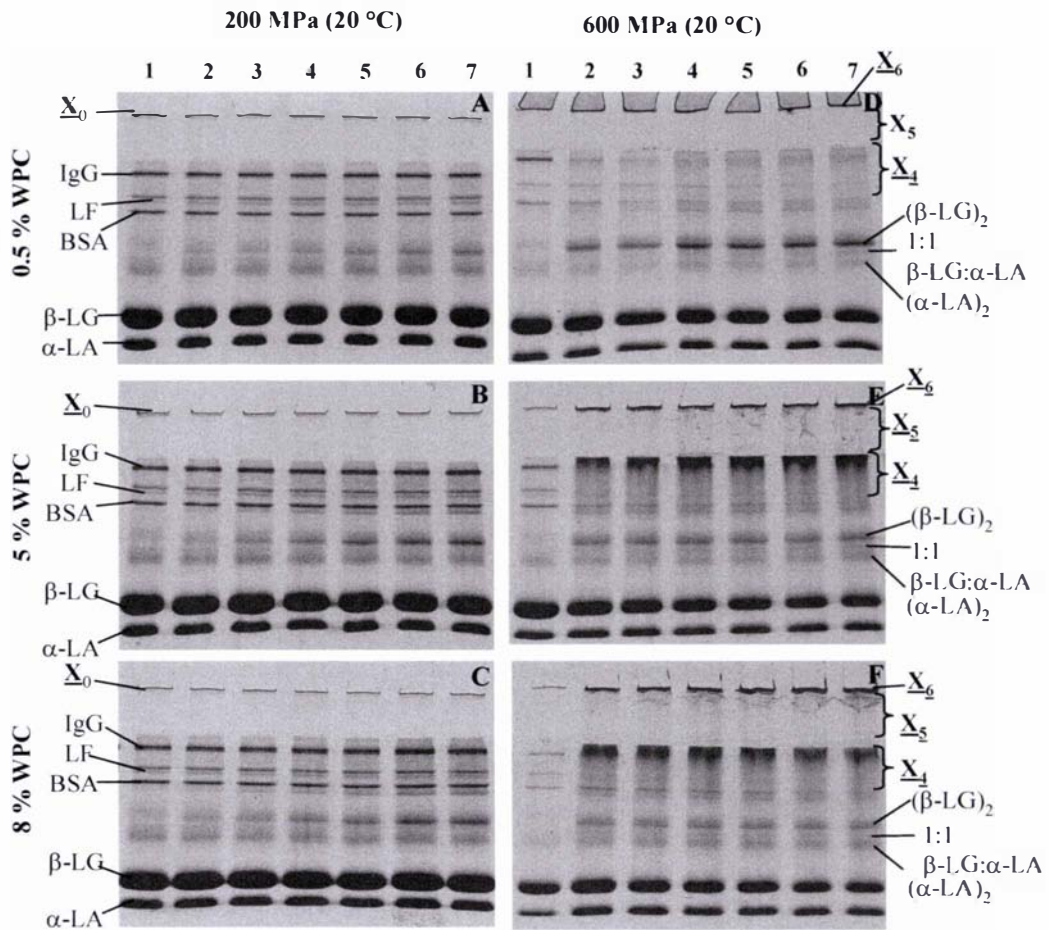


Figure 5.14. SDS-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 20°C. Similarly, SDS-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 20°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

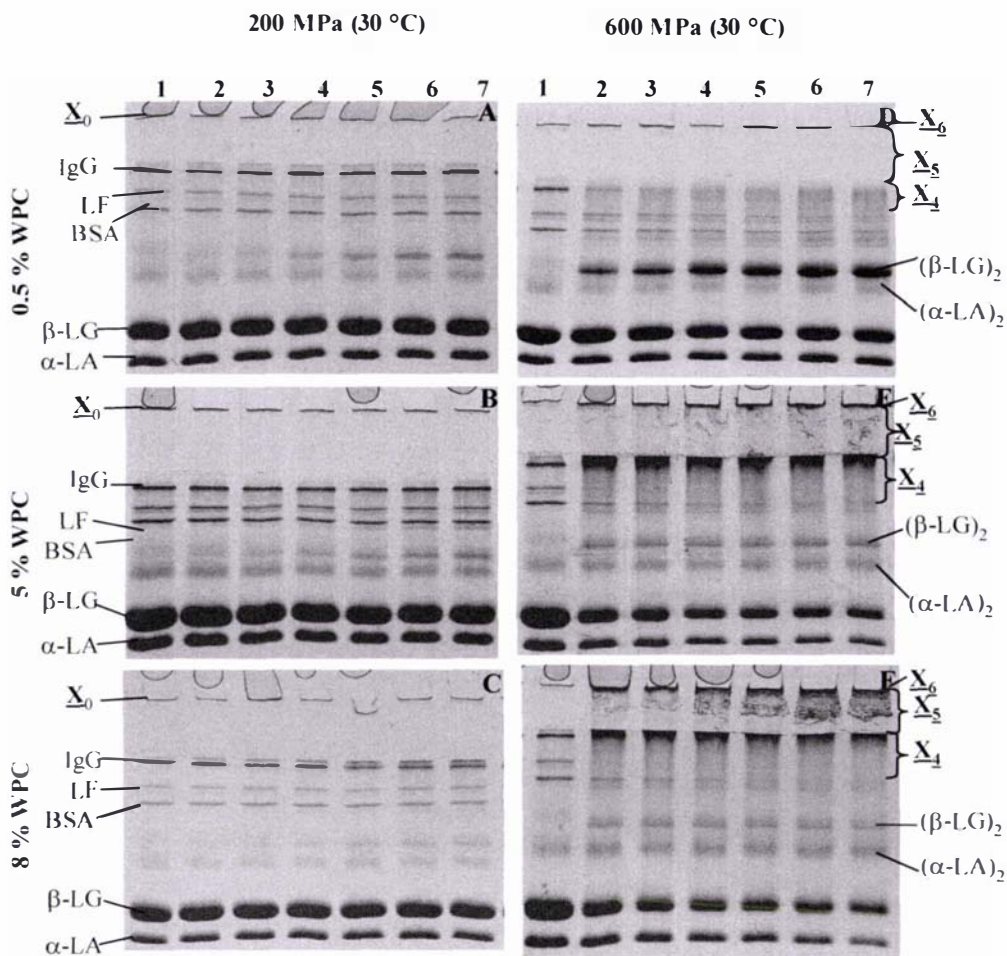


Figure 5.15. SDS-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 30°C. Similarly, SDS-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 30°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

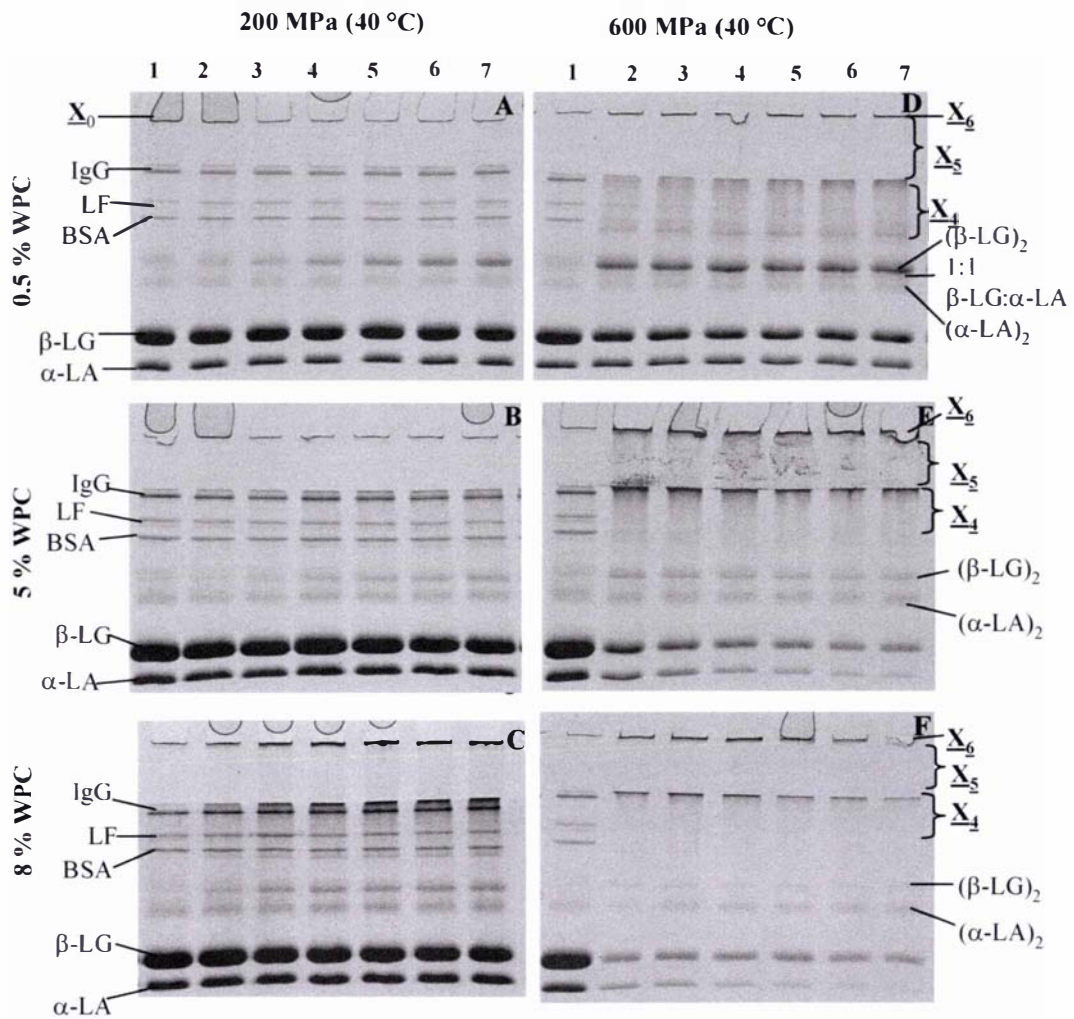


Figure 5.16. SDS-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 40°C. Similarly, SDS-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 40°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

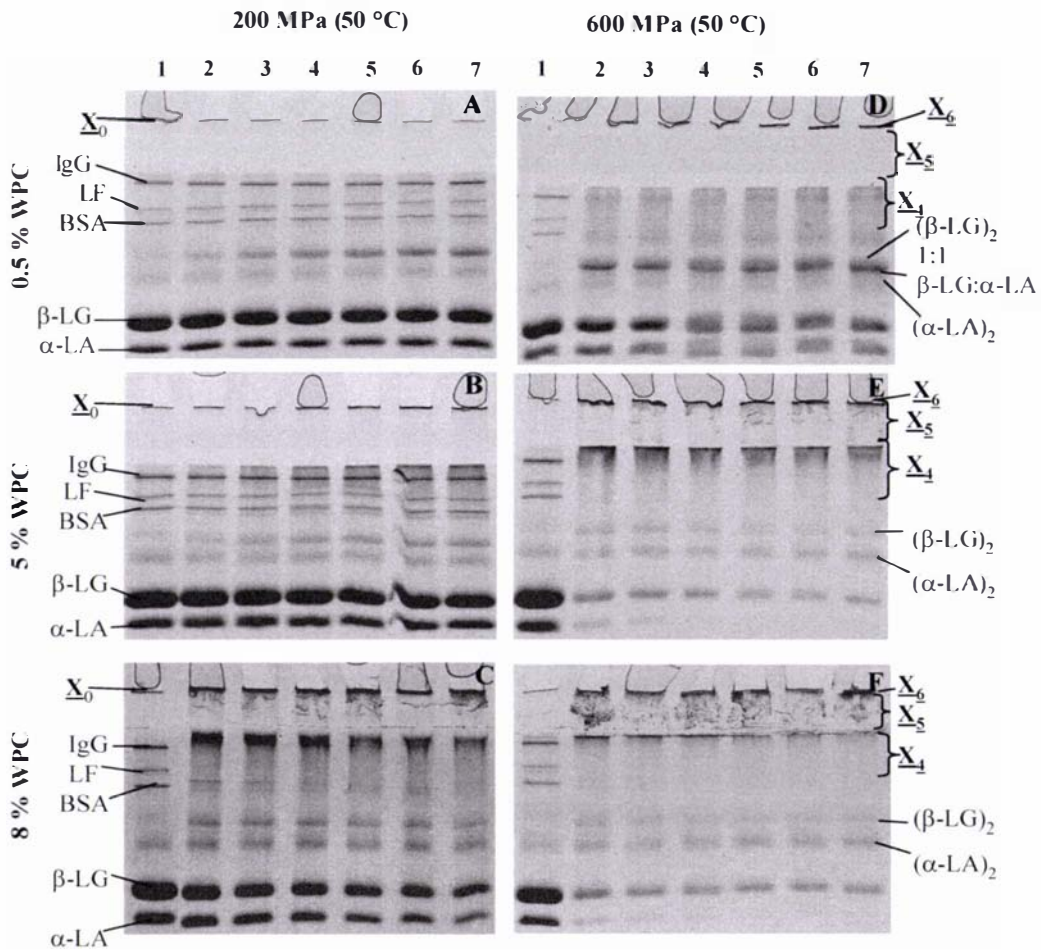


Figure 5.17. SDS-PAGE patterns of WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v pressure treated at 200 MPa at a pressurising temperature of 50°C. Similarly, SDS-PAGE patterns of WPC solutions of concentration (D) 0.5% w/v, (E) 5% w/v and (F) 8% w/v pressure treated at 600 MPa at a pressurising temperature of 50°C. In each of the PAGE patterns, the untreated (control) samples (Lane 1) and samples pressure treated for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

form and that the structural changes were partially or fully reversible (Lasselle et al., 2003) when the samples were subjected to mild pressure treatment and when the pressurising temperature was low ($\approx 20^\circ\text{C}$). However, it seems from the results of the present study that the higher pressurising temperatures appeared to have irreversible effects on disulphide bond interactions and that the formation of new disulphide bonds was enhanced by the higher pressurising temperatures. Therefore, it is possible that, at higher pressurising temperature, α -LA unfolded and exposed its disulphide bonds and

some irreversible changes in the structure of α -LA took place or the α -LA irreversibly reacted with β -LG and BSA and some new irreversible disulphide interactions occurred. However, it would be interesting to determine whether α -LA behaves in the same manner when solutions of pure α -LA are pressure treated at higher pressurising temperatures in the absence of any free-thiol-group-containing whey protein (such as β -LG). Yet again, the faster aggregation of α -LA and BSA in samples subjected to severe pressure treatment may be attributed to adiabatic heating due to the combined effects of higher temperatures and severe pressure treatment; when WPC solutions are pressure treated at higher pressure (600 MPa) and higher pressurising temperature (e.g. 50°C), the actual temperature of the sample reaches approximately 61°C (i.e. it is exposed to nearly the denaturation temperature of α -LA) due to adiabatic heating (for example see Figure 3.4 for adiabatic effects, Chapter 3) and the sample is actually pressurised at approximately 61°C for 5–7 min (the time required for the temperature of the sample to equilibrate back to the preset temperature), which may lead to partial denaturation of heat sensitive whey proteins (see Td data in table 2.6, chapter 2). These factors might have significant effects on the properties of proteins when protein-containing foods are pressure treated at different pressures and at different pressurising temperatures.

Similarly, as discussed earlier (see Chapter 4), BSA contains 17 disulphide bonds and has a more rigid structure than β -LG. Heat treatment unfolds BSA faster than β -LG, but BSA is quite resistant to pressure treatment when pressure treated at 200 MPa at 20 °C. This may be because when samples are pressure treated at low pressure or at a combination of ambient temperature ($\approx 20^\circ\text{C}$) and mild pressure (200 MPa), the structure of BSA is not much affected as the energy supplied may not be sufficient to disrupt its disulphide bonds or affect the thiol group. However, it seems that the thiol of BSA is exposed and becomes reactive when higher pressures (more than 400 MPa) are employed, particularly in combination with elevated pressurising temperatures ($\geq 30^\circ\text{C}$).

Quantitative PAGE analysis

The relative intensities of the bands corresponding to residual native-like and SDS-monomeric β -LG from the native- and SDS-PAGE patterns of 0.5, 5 and 8% w/v WPC

solutions, after pressure treatment of 200 MPa and 600 MPa at 20, 30, 40 and 50°C, are plotted in Figures 5.18 and 5.19 respectively.

From Figure 5.18, it is clear that the concentration of native-like and SDS-monomeric β -LG was not much affected in the WPC solutions pressure treated at 200 MPa. Little decrease in the native-like and SDS-monomeric β -LG was noticed in the 0.5% (Figure 5.18A), 5% (Figure 5.18B) and 8% (Figure 5.18C) WPC solutions pressure treated at 200 MPa at 20, 30, 40 and 50°C, suggesting that both the pressurising temperature and the protein concentration did not have significant effects when the WPC solutions were subjected to mild (200 MPa) pressure treatment.

In contrast, when samples from the same aliquots were subjected to severe (600 MPa) pressure treatment (Figure 5.19), a significant decrease in residual native-like and SDS-monomeric β -LG was observed. A comparatively greater decline in native-like and SDS-monomeric β -LG was observed even when dilute (0.5% w/v) WPC solutions were pressure treated at 600 MPa at 30°C and higher (Figure 5.19A). This effect was even more pronounced when WPC solutions of 5% (Figure 5.19B) and 8% w/v (Figure 5.19C) were pressure treated at 600 MPa.

Also, there appeared to be two distinct stages of aggregation of β -LG when WPC solutions of 5% (Figure 5.19B) and 8% w/v (Figure 5.19C) were pressure treated at 600 MPa, at a pressurising temperature of 30°C and higher compared with when the same solutions were pressure-treated at 600 MPa at a pressurising temperature of 20°C. However, such trend was not apparent when WPC solutions from the same aliquots were pressure-treated at 200 MPa at pressurising temperatures of 20, 30, 40 or 50°C. This trend of different stages of aggregation of β -LG may be explained as discussed in previous studies with pure β -LG solutions (Considine et al., 2005a, 2005b, 2006a). It may be possible that the kinetics of denaturation of β -LG when the WPC solutions were subjected to severe pressure treatment at higher temperatures (30°C or higher) may have been different from those when the WPC solutions were pressure-treated at lower temperature (20°C).

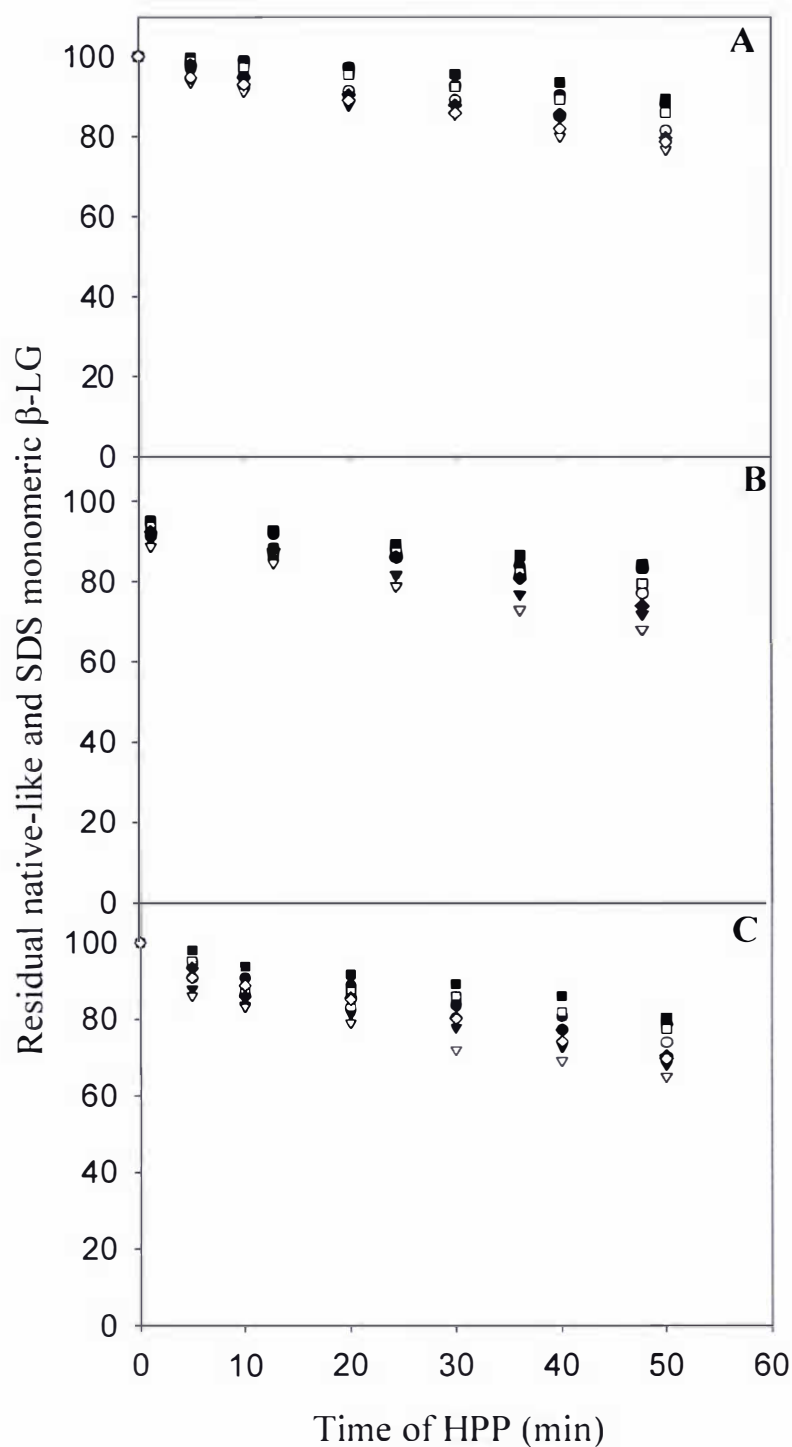


Figure 5.18. Residual native-like and SDS-monomeric β -LG in WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v after pressure treatment at 200 MPa at 20, 30, 40 and 50°C for various times. Residual native-like β -LG in WPC solutions pressure treated at ●: 20°C; ○: 30°C; ▼: 40°C; ▽: 50°C; and residual SDS-monomeric β -LG in WPC solutions pressure treated at ■: 20°C; □: 30°C; ◆: 40°C; ◇: 50°C.

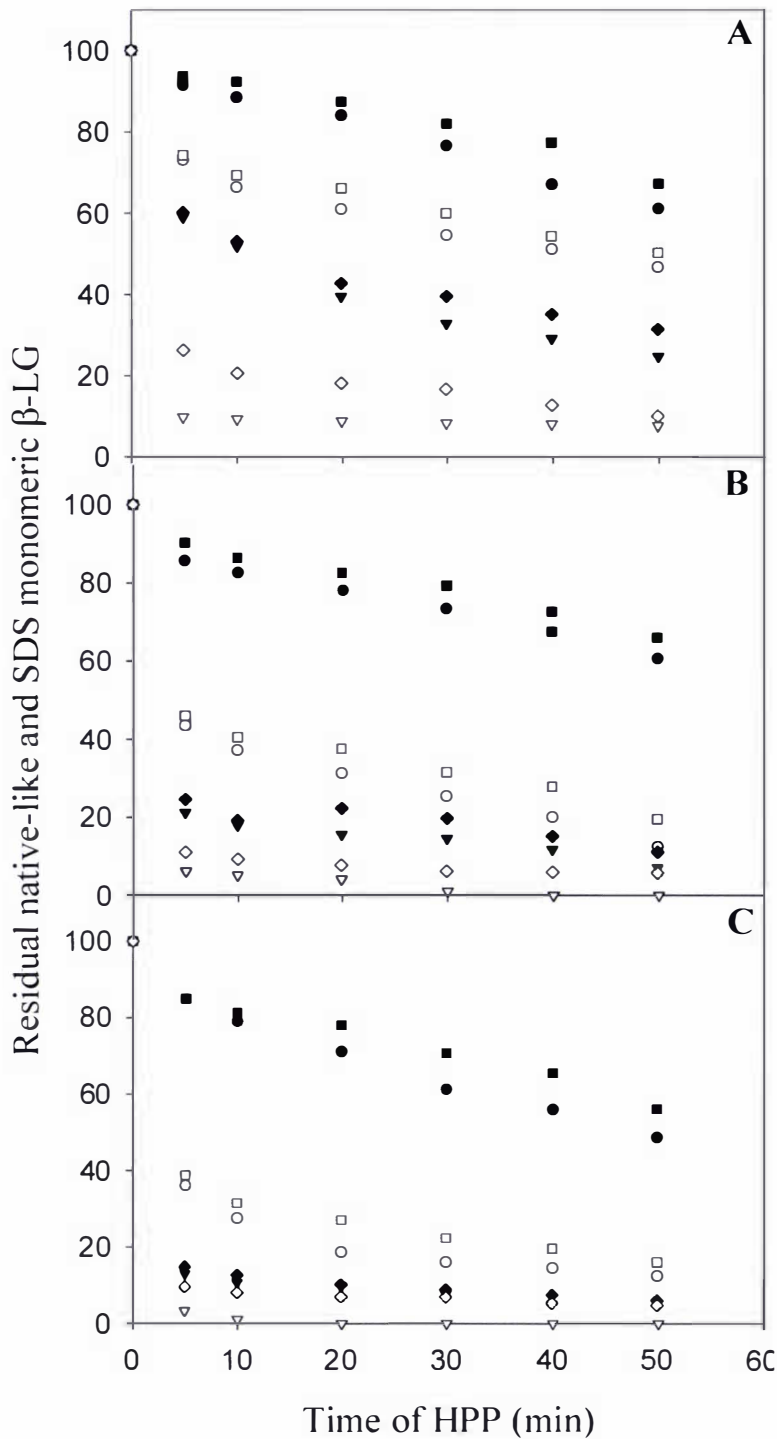


Figure 5.19. Residual native-like and SDS-monomeric β -LG in WPC solutions of concentration (A) 0.5% w/v, (B) 5% w/v and (C) 8% w/v after pressure treatment at 600 MPa at 20, 30, 40 and 50°C for various times. Residual native-like β -LG in WPC solutions pressure treated at \bullet : 20°C; \circ : 30°C; \blacktriangledown : 40°C; \triangledown : 50°C; and residual SDS-monomeric β -LG in WPC solutions pressure treated at \blacksquare : 20°C; \square : 30°C; \blacklozenge : 40°C; \diamond : 50°C.

Overall, comparison of these data showed that the effects were more pronounced when the combination of severe pressure treatment, higher pressurising temperature and higher WPC concentration was employed than when the combination of mild pressure treatment, higher pressurising temperature and higher WPC concentration was employed. Together, these results show that the factors such as pressurising temperature, severity of pressure treatment and concentration of WPC solution are very important and should be carefully considered when protein solutions are pressure treated. Also, it is likely that the denaturation trend of the major whey proteins such as β -LG will change considerably when these parameters are varied, either individually or in combination.

5.4 General Discussion

Compared with the thermal denaturation and aggregation of whey proteins, little attention has been paid to the effects of protein concentration, pressurising temperature and holding time on the pressure-induced aggregation of whey proteins. As there is ample literature on thermal denaturation and aggregation (see Chapter 2), the main focus of this discussion is on pressure-induced changes in the whey proteins. Some other factors (such as the effects of pH and the calcium content), which are important in thermal denaturation, aggregation and gelation of whey proteins, are not included in the present study. This is partly because of the size and complexity of the experiments. However, such parameters may form part of future studies.

Many aspects of the pressure-induced denaturation and aggregation of whey proteins, as affected by protein concentration, pressurising temperature and holding time have been discussed in the respective results and discussion sections. However, one of the important points to note [when studying the effects of elevated pressurising temperature (e.g. $\geq 40^\circ\text{C}$) in combination with the severe pressure treatments (e.g. 600 MPa) used in the present study] is that the adiabatic heating may accelerate the rate of denaturation and aggregation of the whey proteins in the early stages of each pressurisation cycle, depending on the preset pressurising temperature in a particular study. As shown in the results of the present study (see Chapter 3 and Section 5.3.2), the temperature of the sample increased during pressurisation due to adiabatic heating. The time taken for the temperature to equilibrate back to the preset temperature was about 5–7 min after the

desired pressure had been achieved. This suggested that there was a significant increase in temperature ($\approx 11^\circ\text{C}$) due to adiabatic heating and that the samples were pressure treated at the elevated temperature for 5–7 min (the time taken for the temperature to equilibrate back to the preset temperature). This is of concern, particularly when samples are pressure treated at higher pressure (e.g. 600 MPa) and higher pressurising temperature ($\geq 40^\circ\text{C}$) for a short holding time (e.g. for holding times of 5–10 min), because, in this case, the depressurisation cycle will begin before the temperature of the sample has equilibrated back to the preset temperature. This means that, when a sample is pressure treated at 600 MPa and at a pressurising temperature of 50°C , the actual temperature of the sample rises to about 61°C due to adiabatic heating and the sample is exposed to this temperature ($\approx 61^\circ\text{C}$) and severe pressure (600 MPa) for 5–10 min or until the temperature returns to the preset temperature. In such a case, the sample will be pressurised at a higher temperature than is desired, which may cause a greater extent of denaturation. Moreover, because the pressure increase is not instantaneous (i.e. the rate of pressure increase is approximately 300 MPa/min, and it takes some time to reach the desired pressure), and thus some denaturation of the proteins may occur during the pressurisation process. For example, when samples are pressure treated at 600–800 MPa, it will take 2–3 min to achieve the desired pressure. Therefore, this factor should be given consideration while planning the experiments or during the commercial trials.

Attempts were made to evaluate the kinetics of whey protein changes in samples pressure treated at different temperatures. We also tried to fit the data to different order reactions, but it was not possible to get a complete fit of the data to 1.0, 1.5, 2.0 or 2.5 order reactions in the majority of cases, which may be partly attributed to the faster denaturation of β -LG in the initial 5–10 min due to adiabatic heating. Once the majority of the β -LG had been denatured in the initial 5–10 min (particularly in the severely pressure-treated samples), the rate of loss of native-like and SDS-monomeric β -LG was slow and constant for an extended period of time. Such factors would make a kinetic evaluation of the data difficult. Ideally, it is important to compensate for such adiabatic heating and pressurisation effects. However, these effects have not been considered in most of the previous studies, including the present study. Therefore, the adiabatic heating for each pressurising temperature and each pressurisation cycle should be considered as an independent parameter for evaluation under high pressure processing and such effects need to be evaluated in detail in a separate study.

The results of the present investigation on the effects of various protein concentrations and pressurising temperatures on the aggregation of proteins generated useful information on how the aggregation of whey proteins precedes pressure-induced gel formation, when the solutions of WPC of various protein concentrations are subjected to different pressure treatments. The present study also showed that pressure-induced gels can be formed when WPC solutions of sufficiently high concentration (e.g. 12% w/v) are subjected to severe pressure treatment (600 MPa) at relatively low temperatures (20°C). It will be useful to carry out detailed characterisation of aggregation products and pathways involved in formation of such pressure-induced gels. Therefore, in Chapter 6, the mechanisms of pressure-induced gel formation from concentrated (i.e. 12% w/v) WPC solutions (800 MPa, 22°C) are studied in detail.

5.5 Conclusions

It was found that various parameters, such as protein concentration, intensity of pressure, holding time and pressurising temperature, have considerable effects on the pressure-induced aggregation and gelation of whey proteins. These parameters should be considered during process optimisation and during high pressure processing of protein-containing food systems, in new product development or for applications of high pressure processing as a novel process in the food industry, because many functional properties of dairy or food products are the consequence of the unique behaviour of proteins.

The present study also showed that the effects of pressure treatment on the proteins in WPC solutions followed an almost similar trend to the effects of heat treatment. However, pressure treatment generated a comparatively greater proportion of intermediate-sized aggregates (such as dimer, trimer etc.) at lower protein concentrations and lower pressures than did heat treatment.

It was also observed that the behaviour of different whey proteins (β -LG, α -LA and BSA) was quite different when WPC solutions were pressurised at different pressurising temperatures. In particular, α -LA and BSA became more reactive when the pressurising temperature was increased from 20 to 40°C and higher at severe (600 MPa) pressure, which may be partially explained by the structural changes in these proteins as the

pressurising temperature was increased. These preliminary results may suggest some potential use of different protein concentrations, holding time, intensity of pressure treatment and pressurising temperatures and combinations of these factors to manipulate food systems. However, further studies will be necessary to confirm these observations and to find mechanisms behind such behaviour of different whey proteins and their aggregation patterns.

CHAPTER 6

High-pressure-induced Aggregation and Gelation of Whey Proteins in Concentrated (12% w/v) WPC Solutions¹

6.1 Introduction

As the heat-induced gelation of whey proteins is considered to be one of the most important functional properties of whey protein ingredients, many studies to evaluate the mechanisms of the heat-induced aggregation and gelation of whey proteins have been conducted (e.g. Xiong & Kinsella, 1990; Matsudomi et al., 1991, 1992, 1993, 1994; Calvo et al., 1993; Doi, 1993; Hines & Foegeding, 1993; Tang et al., 1993, 1994; 1995; McSwiney et al., 1994a, 1994b; Aguilera, 1995; Gezimati et al., 1996, 1997; Havea et al., 1998, 2001, 2002, 2004), but little is known about the mechanism of the pressure-induced aggregation and gelation of the whey proteins in whey protein concentrate (WPC) solutions.

As with thermal processing, the pressure-induced denaturation of globular proteins in concentrated solutions can lead to extensive aggregation and the formation of a gel structure (Dumay et al., 1994; Van Camp & Huyghebaert, 1995a, 1995b; Kanno et al., 1998; Famelart et al., 1998; Keim & Hinrichs, 2004; also see Chapter 5). Several aspects of the aggregation and gelation of whey proteins by high pressure may be of interest in relation to the use of milk proteins as functional ingredients in many foods. Previous work on the high-pressure-induced denaturation and aggregation of β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) in a model system after pressure release showed that high pressure treatment of whey protein solutions promotes unfolding and aggregation of β -LG through sulphhydryl–disulphide interchange reactions (Dumay et al., 1994, 1998; Funtenberger et al., 1995, 1997; Jegouic et al., 1997; Van Camp et al., 1997a, 1997b). Intermolecular interactions and irreversible aggregations

¹ Part of the content presented in this chapter has been published as a peer-reviewed paper in *Journal of Agricultural and Food Chemistry* (2005) 53 9590–9601.

The content from this chapter was also presented as a poster at the 4th IDF Symposium on Recombined Milk and Milk Products, Cancun, Mexico, 9–12 May 2004.

are induced at high protein concentration (e.g. see results of Chapter 5; Wong & Heremans, 1988; Dumay et al., 1994; Iametti et al., 1997) and, as a result of aggregation and gel formation, may lead to textural changes in the final products. However, the detailed aggregation pathways and the interaction products that may be generated in the process of pressure-induced gel formation have not been characterised in detail. Moreover, comparatively little information is available on the effects of severe pressure treatment (800 MPa) for extended holding times on mixtures of the whey proteins. Detailed characterisation of the same samples using a range of different techniques may be useful for a detailed understanding of the pathways involved in pressure-induced whey protein gel formation.

In this context, in the present study, concentrated solutions of WPC (12% w/v) were pressure treated at 800 MPa at ambient temperature (22°C) for various times up to 120 min and the resultant samples were characterised, after pressure release at various stages of pressure treatment, using rheological measurements, size exclusion chromatography (SEC) and transmission electron microscopy (TEM). Also, the intermediate aggregates and interaction products formed by the pressure treatment of WPC solutions were characterised using various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques.

6.2 Experimental

The detailed procedure followed for sample preparation, pressure treatment and analysis of control and pressure-treated samples is described in Chapter 3. A summary of the experimental plan specific to the results presented in this chapter is outlined in Figure 6.1.

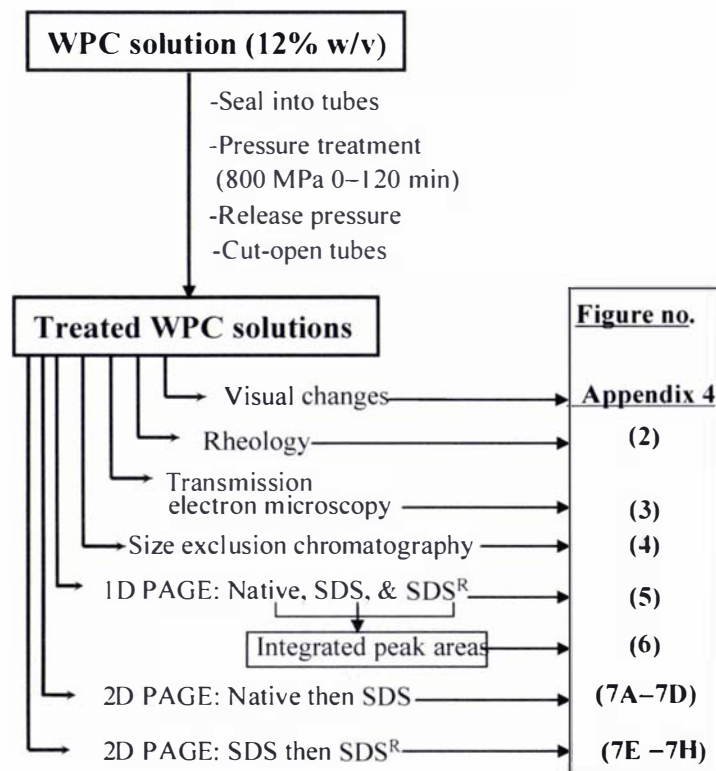


Figure 6.1. Summary of the experimental protocol used in this study of the pressure-induced aggregation and gelation of whey proteins. Note that the sample treated for 0 min is untreated (control) samples and it does not mean that pressure was released immediately once 800 MPa was reached.

6.3 Results and discussion

6.3.1 Visual changes

When the WPC solutions were pressure treated at 800 MPa at ambient temperature (22°C), the colour of the solutions changed from opalescent pale dun to pale yellow and finally to whitish yellow. The clarity of the samples decreased initially and then increased with longer pressure treatments. After a pressure treatment of 800 MPa for 20 min, the consistency of the samples was almost unchanged. The consistency increased with pressure treatment of the samples for longer holding times and the samples became viscous after a pressure treatment of 60 min at 800 MPa (see Appendix 4). The samples were quite viscous after 90 min of pressure treatment. The solutions were transformed

into stable, elastic, almost translucent gels after 120 min of pressure treatment (Appendix 4).

6.3.2 Rheology

The viscosity as a function of shear rate for the control sample and the sample pressure treated for 20 min remained independent of the shear rate, exhibiting Newtonian behaviour (Figure 6.2A). After longer pressurisation times (40 and 60 min), the pressurised samples deviated from Newtonian behaviour, to exhibit pseudo-plastic behaviour. Furthermore, the viscosity of these samples increased with an increase in the pressurisation time (see Figure 6.2A, inset a). To quantify the extent of pseudo-plasticity, the viscosity (η) as a function of the shear rate ($\dot{\gamma}$) was calculated using a power law equation of the form $\eta = k \dot{\gamma}^{n-1}$ where k is the consistency index and n is the flow behaviour index, which is related to the extent of pseudo-plasticity of the samples (note that, for a Newtonian liquid, $n = 1$). It was found that n was equal to 0.96, 0.70 and 0.32 for 20, 40 and 60 min of pressurisation respectively.

The frequency sweep measurement showed that, for pressure treatment times longer than 40 min, the elastic modulus G' was higher than the viscosity modulus G'' (Figure 6.2B). In addition, the elasticity of these gels (G^*) increased exponentially with the time of pressure treatment (Figure 6.2B, inset b). However, even after a longer pressure treatment time (90 min), these samples still behaved as weak gels. To further illustrate the effect of high pressure on the viscoelasticity of these samples, $\tan \delta (= G''/G')$ is reported in Figure 6.2C. It was observed that the sample pressurised for 40 min remained mainly liquid up to a frequency of 1 Hz, with a $\tan \delta$ value near to 1, and started to become elastic at higher frequencies. However, for the samples pressurised for 60 and 90 min, $\tan \delta$ was less than 0.45, which indicated the elastic nature of these samples, suggesting that the elastic gels were formed after pressure treatment of 90 min.

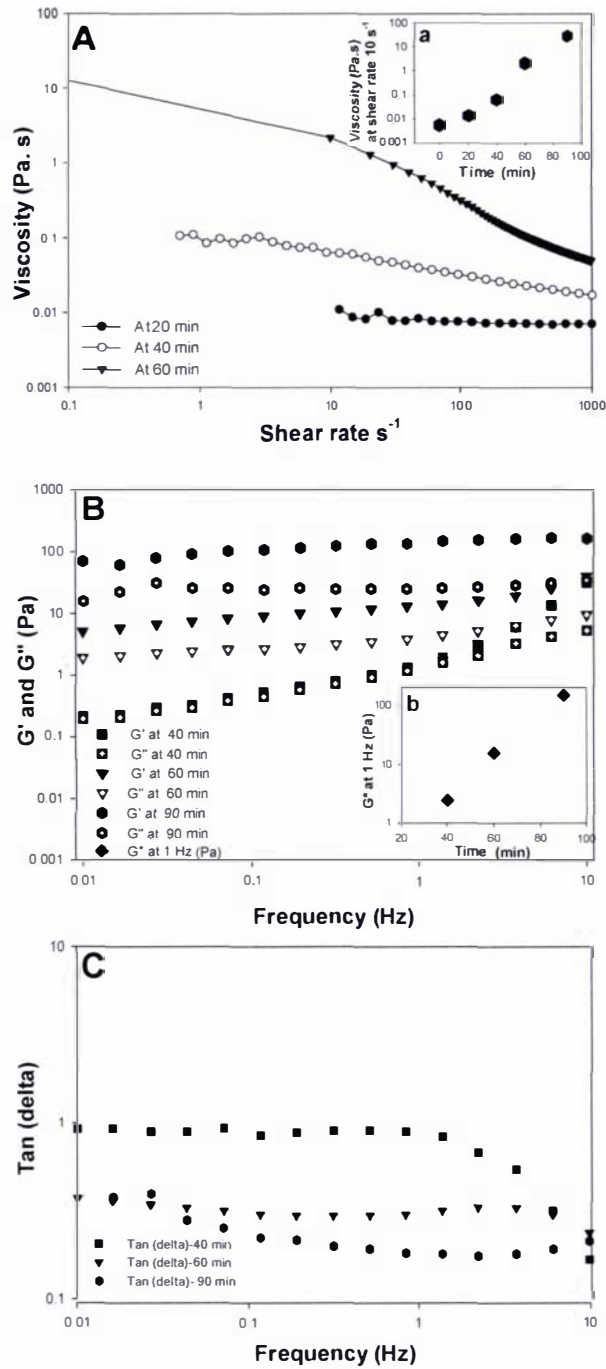


Figure 6.2. Effect of pressure treatment (800 MPa at 22°C) for (A) 20, 40 and 60 min on the shear rate dependence of the sample viscosity measured at 20°C, for (B) 40, 60 and 90 min on the frequency dependence of G' and G'' , and for (C) 40, 60 and 90 min on the frequency dependence of $\tan \delta$. Inset (a) shows the viscosity for the samples pressure treated for 20, 40, 60 and 90 min at a shear rate of $10 s^{-1}$. Inset (b) shows G^* for the samples pressure treated for 40, 60 and 90 min at 1 Hz.

6.3.3 Transmission electron microscopy

Two sets of pressure-treated samples were fixed, stained and examined using TEM, and a typical set of micrographs is shown in Figures 6.3A–6.3F. The micrograph of the untreated control sample (Figure 6.3A) did not show any sign of aggregated proteins, whereas the micrograph of the WPC sample that was pressure treated (800 MPa) for 20 min (Figure 6.3B) showed the formation of fine hair-like strands of almost uniform size. These fine strands were distributed randomly throughout the sample. The structure of these fine strands appeared to be comparable with the ‘fine-stranded’ structures of heat-induced β -LG gels (12% w/v) reported earlier (Stading & Hermansson, 1991; Langton & Hermansson, 1992). In addition, some clusters of strands (aggregates) were observed after a pressure treatment of 20 min.

The aggregates became larger after 40 min of pressure treatment (Figure 6.3C). There were more aggregates of almost similar size after 60 min of pressure treatment and some appeared to be linked to give even larger aggregates (Figure 6.3D). Longer pressure treatment times (Figures 6.3E and 6.3F) did not appear to increase the concentration and size of the aggregates, but there appeared to be more low density areas. This ‘particulate’ material looked similar to that observed in the heated whey protein system (Stading & Hermansson, 1991; Langton & Hermansson, 1996), and appeared to be consistent with scanning electron micrographs of pressure-treated WPC solutions (Van Camp et al., 1997a, 1997b; Kanno et al., 1998). These results also compared well with those of Walkenström & Hermansson (1997), who reported that, at pH 6.8 in a mixed gelatin and WPC system, the mixed gel formed a phase-separated system, composed of an aggregated network and a phase with fine strands; the aggregated network proved to be made up of both gelatin and WPC.

Overall, it appeared that the particulate aggregate structures were formed as a consequence of 40–60 min of pressure treatment at 800 MPa, but there appeared to be no further significant increase in particle size after longer pressure treatments (Figures 6.3D–6.3F) despite the changes in the rheological properties (Figure 6.2).

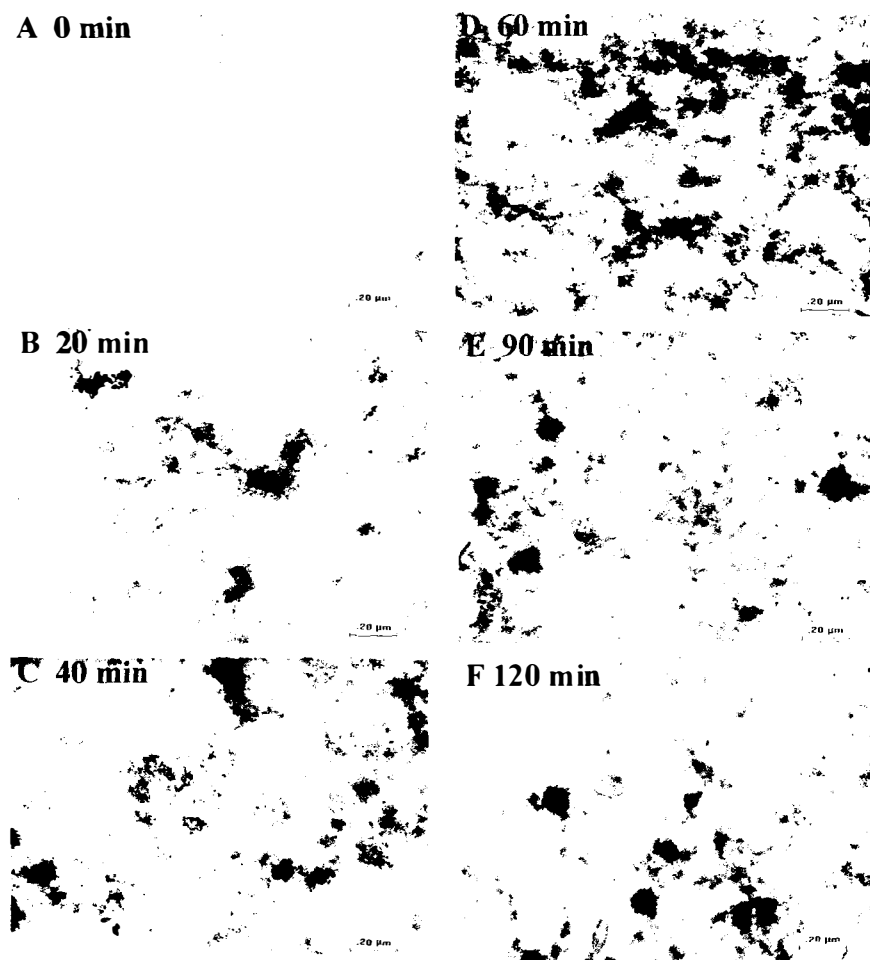


Figure 6.3. Transmission electron micrographs of WPC solutions (12% w/v) pressure treated (800 MPa at 22°C) for (A) untreated sample, (B) 20 min, (C) 40 min, (D) 60 min, (E) 90 min and (F) 120 min. After pressure treatment, each individual sample was removed, fixed, stained and photographed. Note that the sample treated for 0 min is untreated samples and it should not be confused or mean that pressure was released immediately once 800 MPa was reached.

6.3.4 Size exclusion chromatography

The untreated sample gave a major peak eluting at about 29 min with a minor peak at 31 min (Figure 6.4A), using a TOSOH TSK-G4000_{PWXL} column (Supelco, Bellefonte, PA, USA). The major components of WPC (β -LG and α -LA) were the likely source of the major peak. The minor peak at 31 min was probably orotic acid because it showed a substantial absorbance at 320 nm (Dave Elgar, Fonterra Co-operative Group Ltd, Palmerston North, New Zealand, 1999, personal communication). After pressure treatment, the samples showed a marked and continuing decrease in the size of the major peaks corresponding to native whey proteins and increased absorbance between 20 and 28 min of elution time. For the sample pressure treated for 20 min (Figure 6.4B), there was a small broad peak at about 26 min, which corresponded to β -LG dimers (Schokker et al., 1999). There appeared to be a peak with an elution time of about 21 min in the sample pressure treated for 40 min (Figure 6.4C). This void volume 'peak' contained material that was too large to enter the gel pores and small enough to pass the filter (0.22 μ m) and therefore entered the column. In the region between 22 and 25 min of elution time, the absorbance corresponded to intermediate-sized aggregates, which probably corresponded to low mobility bands on PAGE patterns (e.g. Figure 6.5A, Lanes 2–5). It was observed that, as the pressure treatment time increased, the total quantity of material that entered the column decreased and the height of the peak that eluted between 21 and 27 min decreased (e.g. Figure 6.4F). Also, the size of peak corresponding to native protein was decreased to about 10% that of the control (Figure 6.4A), suggesting that about 90% of the original native protein had been denatured and polymerised to give high molecular weight aggregates of the size that would not pass a 0.22 μ m filter to enter the column. The resolution of the protein peaks in the present study was poor, compared with that obtained when a Superdex-75 column was used for similar studies on heated whey protein solutions (Schokker et al., 1999).

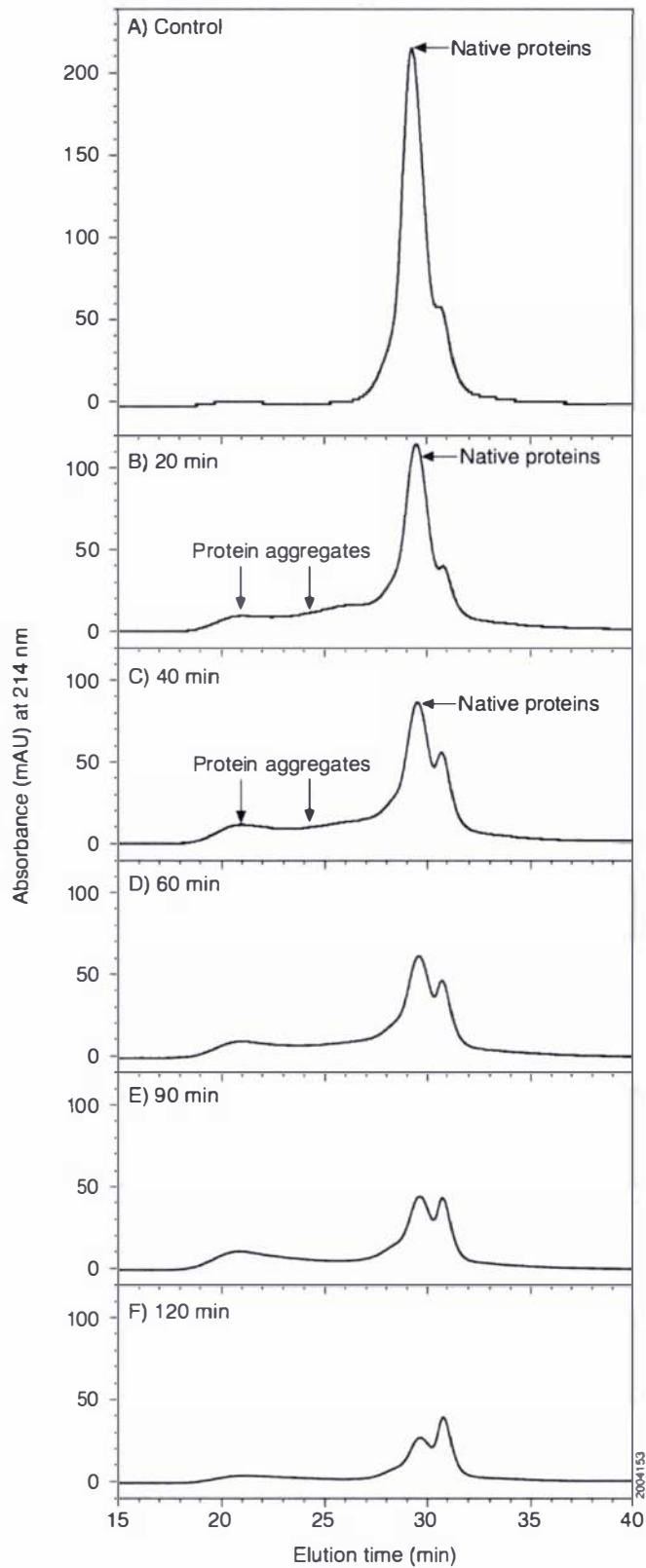


Figure 6.4. Size exclusion chromatograms of WPC solutions (12% w/v) for (A) untreated control samples and for samples pressure treated (800 MPa at 22°C) for (B) 20 min, (C) 40 min, (D) 60 min, (E) 90 min and (F) 120 min, monitored at 214 nm wavelength.

6.3.5 1D PAGE analysis

The PAGE patterns of control and pressure-treated WPC samples, analysed by 1D native-PAGE, sodium dodecyl sulphate (SDS)-PAGE and SDS-PAGE of reduced samples (SDS^R-PAGE), are presented in Figures 6.5A, 6.5B and 6.5C respectively. The identities of the protein bands in the control sample were determined as described earlier (Chapter 4) and the positions of the major whey proteins are noted on the left-hand side of the PAGE patterns of the control samples (Lane 1 in Figures 6.5A, 6.5B and 6.5C). Pressure-induced aggregates are denoted as X₁–X₆ and X₂–X₆ on the right-hand side of the native- and SDS-PAGE patterns respectively.

Native-PAGE

The native-PAGE patterns of the pressure-treated WPC solutions (Lanes 2–6, Figure 6.5A) showed that the intensities of the bands corresponding to native-like monomeric whey proteins decreased with increasing pressure treatment time. It was clear from the PAGE pattern that the WPC used in the present study contained a small proportion of glyco- α -LA (denoted as α -LA*), the details of which have been explained earlier (Chapter 4), and non-native β -LG species were also apparent in the 2D PAGE patterns of corresponding pressure-treated samples (Figures 6.7A–6.7D). Most of the changes observed in the pressure treated samples were similar to those described in Chapter 4.

The new bands X₂ and X₃ (Lanes 2–6, Figure 6.5A), which were not present in the control sample (Lane 1, Figure 6.5A), corresponded to β -LG dimers and trimers respectively. These species were also identified using 2D PAGE techniques (compare with corresponding 2D PAGE pattern in Figure 6.7). The bands X₂ and X₃ appeared to reach maximum concentrations in the samples that were pressure treated for 20 and 40 min (Lanes 2–3, Figure 6.5A) and thereafter their intensity decreased. Similarly, bands X₄ and X₅ reached maximum intensities in the sample that was pressure treated for 60 min (Lane 4, Figure 6.5). The lower intensity of region X₆ in the samples pressure treated for 90 min and longer (Lanes 5 and 6, Figure 6.5A) indicated that the pressure-induced aggregates present in these samples were too large to enter the stacking gel or to remain in the sample loading well of the stacking gel and therefore were lost during the staining and destaining procedures. This observation is consistent with the results of size exclusion chromatography (e.g. Figure 6.4F). These results suggest that the

pressure-induced interactions progressed towards the formation of larger and larger aggregates upon prolonged holding times. The largest aggregates on the top part of the stacking gel or caught in the sample well (X_6), within the stacking gel (X_5) and at or near the top of the resolving gel (X_4) probably consisted of both disulphide-linked and non-covalently associated proteins and contained protein aggregates of low net negative charge and/or high molecular weight.

SDS-PAGE

The major protein bands were also readily identified in the SDS-PAGE pattern of the control sample (Lane 1, Figure 6.5B). The bands corresponding to the A and B variants of β -LG were indistinguishable and migrated more slowly than α -LA, in contrast to their mobility in the native-PAGE pattern (Figure 6.5A).

The regions labeled X_2 – X_6 (Figure 6.5B) indicate the positions of new bands on SDS-PAGE that were formed as a consequence of the pressure treatment. The high molecular weight polymers were at the top of the stacking gel or in the sample loading well (X_6), within the stacking gel (X_5) and at or near the top of the resolving gel (X_4). These aggregates did not contain non-covalently associated proteins as they were not dissociated by SDS. The new bands X_2 and X_3 , which were not present in the control sample, corresponded to β -LG dimers and trimers and were identified as covalently linked dimers and trimers using 2D SDS:SDS^R-PAGE (compare with Figures 6.7E–6.7H). These bands appeared to attain maximum intensity after 40 min of pressure treatment (Lane 3, Figure 6.5B).

SDS^R-PAGE

Reduction of the control sample and the pressure-treated samples prior to analysis by SDS-PAGE gave almost identical patterns (Figure 6.5C), showing that there were no pressure-induced interactions leading to the formation of aggregates that could not be reduced using 2-mercaptoethanol. These results indicated that no covalent bonds other than disulphide bonds were formed.

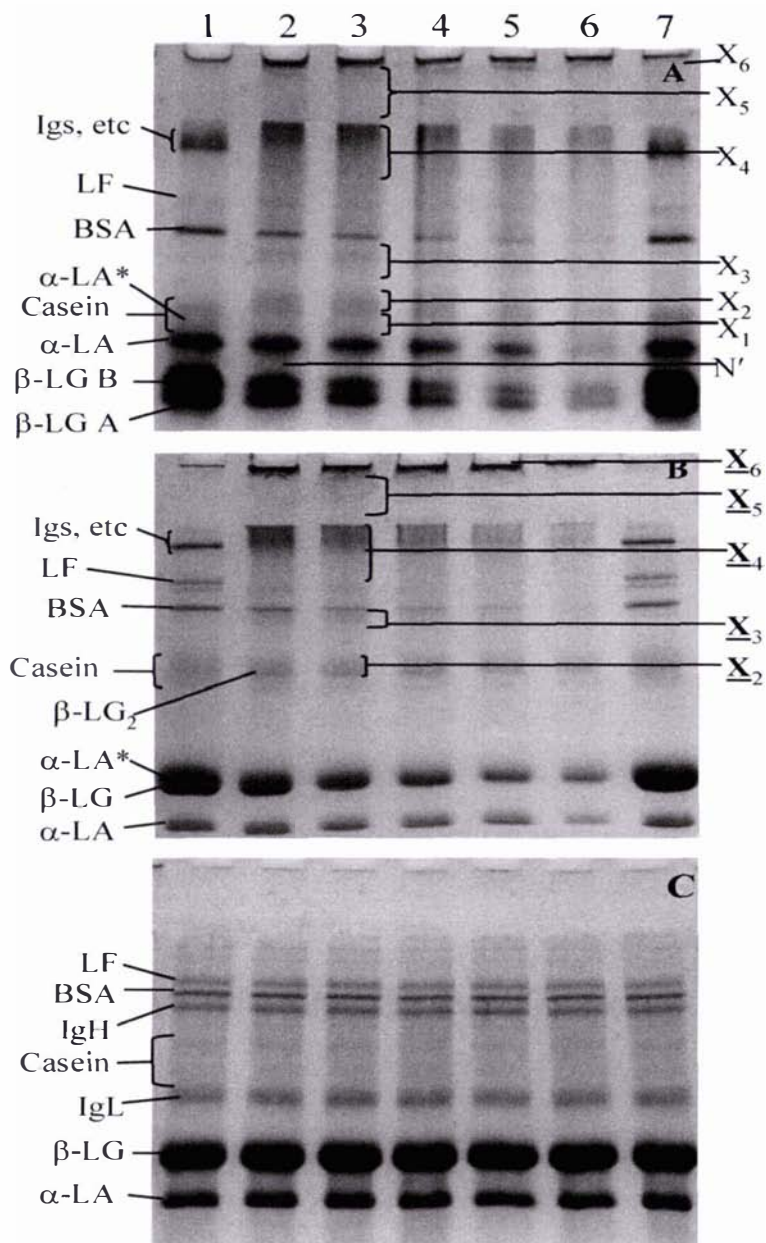


Figure 6.5. Typical 1D (A) native-, (B) SDS- and (C) SDS^R-PAGE patterns of pressure-treated (800 MPa for 0–120 min at 22°C) WPC solutions: control sample (Lane 1), samples pressure treated for 20 min (Lane 2), 40 min (Lane 3), 60 min (Lane 4), 90 min (Lane 5) and 120 min (Lane 6), and control sample (Lane 7). The identities of the major monomeric protein bands in the control samples are noted on the left-hand side and the new protein bands present as a consequence of the pressure treatment are denoted on the right-hand side as X₁–X₆ on native-PAGE (A) and X₂–X₆ on SDS-PAGE (B).

Formation of covalently linked and non-covalently linked protein aggregates

The integrated intensities (quantified data) of the bands corresponding to SDS-monomeric (Figure 6.6A) and native-like (Figure 6.6B) β -LG, α -LA and BSA were plotted against the pressure treatment time. The relative decrease in the intensities of the bands corresponding to SDS-monomeric and native-like β -LG, α -LA and BSA in the pressure-treated samples (compared with those in the PAGE pattern of the control sample) indicated that the concentration of β -LG decreased faster than that of α -LA or BSA (Figures 6.6A and 6.6B), supporting earlier findings as discussed in Chapter 4 as well as those reported earlier in milk samples (López-Fandiño et al., 1996; López-Fandiño & Olano, 1998a; Garcia-Risco et al., 2000; Needs et al., 2000a; Huppertz et al., 2004a; 2004b) or in whey (Huppertz et al., 2004b). These plots showed that the sensitivity of the pressure-induced aggregation of three major whey proteins was β -LG > BSA > α -LA, which was quite different from that obtained for the heat treatment of WPC solutions (BSA > β -LG \approx α -LA), using comparable analytical methods (Havea et al., 1998).

This quantitative analysis of the protein bands in the 1D native- and SDS-PAGE patterns of the control and pressure-treated samples (Figures 6.6A and 6.6B) showed that the loss of native-like proteins (Figure 6.6B) was comparatively greater than the loss of SDS-monomeric proteins (Figure 6.6A) for all major whey proteins, including β -LG, α -LA and BSA. Such a difference in the concentrations of the monomers, assessed by PAGE analysis, represents the 'non-native monomer' species (mainly non-covalently-linked (hydrophobic) aggregates) as discussed in chapter 4 and has been investigated and discussed previously (Havea et al., 1998; Manderson et al., 1998; Creamer et al., 2004; Considine et al., 2005a, 2005b).

Figure 6.6C shows the changes in non-native β -LG, α -LA and BSA with pressure treatment. Most of the monomeric β -LG was aggregated into disulphide-bonded dimers, trimers etc. (see Figure 6.7F) but some was converted into non-native monomeric β -LG (Figure 6.6C). These results indicated that approximately 5–25% of the aggregates formed during the pressure treatment of 12% WPC solutions for different holding times at 800 MPa were non-covalently linked (Figure 6.6C) and that the

majority (approximately 75–95%) of these aggregates were disulphide linked, confirming earlier results of Chapter 5.

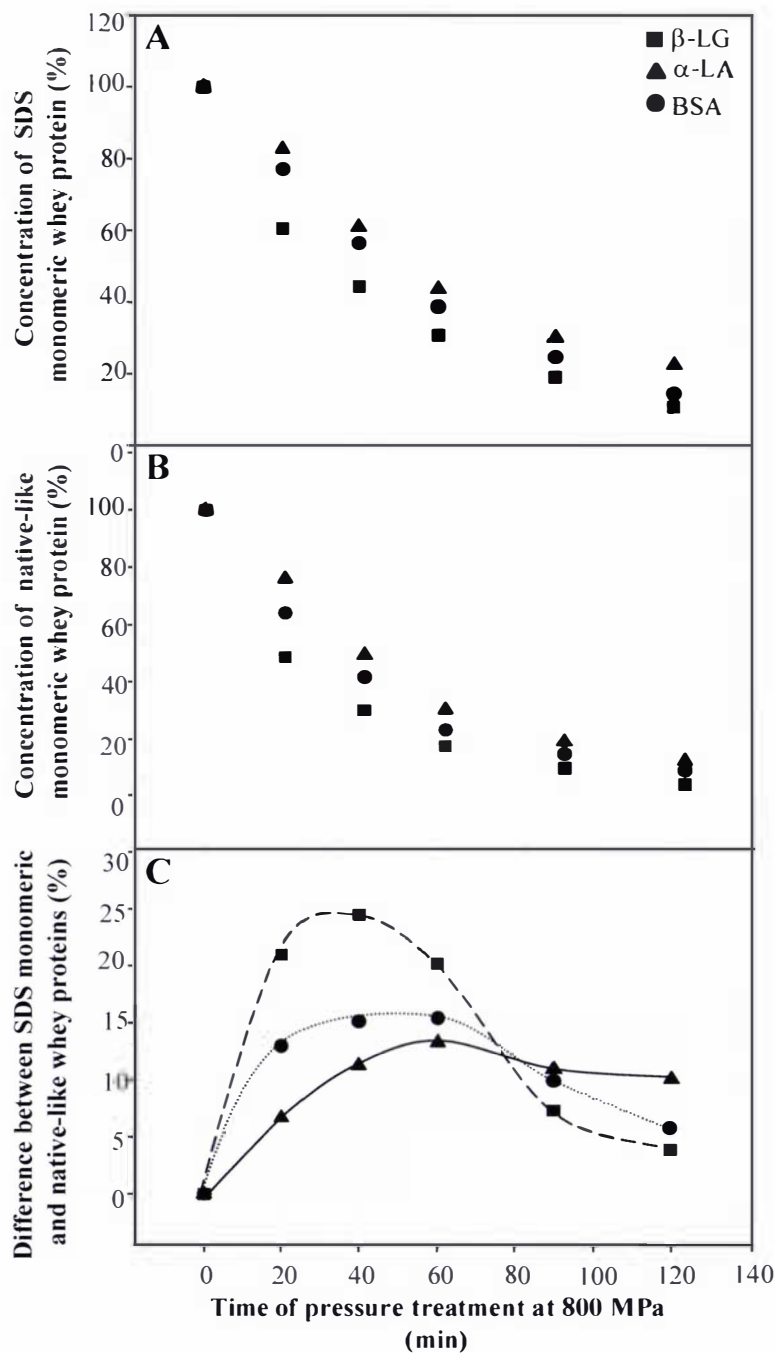


Figure 6.6. The decreases in the concentrations of (A) SDS-monomeric and (B) native-like monomeric β -LG, α -LA and BSA as a function of the pressure treatment time. (C) shows the differences in the concentrations of SDS-monomeric and native-like monomeric bands as a function of the pressure treatment time.

6.3.6 Characterisation of pressure-induced protein interactions using 2D PAGE

The WPC samples were further analysed using 2D PAGE techniques (native:SDS-PAGE and SDS:SDS^R-PAGE), in order to characterise the interaction products formed during pressure treatment. The general procedures that have been used previously to characterise pressure-induced aggregates (Chapter 4) in WPC solutions were followed to give the results shown in Figure 6.7.

2D native:SDS-PAGE of control and pressure-treated samples

The PAGE patterns of the control sample and the samples that were pressure treated for 20, 60 and 120 min are shown in Figures 6.7A–6.7D respectively. Each figure contains the horizontally mounted stained gel strip (marked as a'') and the sample gel strip (marked as a') from the 1D native-PAGE, the corresponding SDS-PAGE pattern of the non-reduced sample in the left-hand vertical lane (denoted as SNR) and a series of molecular weight standards (denoted as Mr) in the right-hand lane (Figure 6.7A).

The protein spots corresponding to β -LG, α -LA, α -LA*, BSA and lactoferrin (LF) monomers identified earlier in WPC samples (Chapter 4) were readily observed on the PAGE pattern of control samples (Figure 6.7A). The spot resolved adjoining to α -LA could be an α -LA dimer (marked as α -LA₂) (Chaplin & Lyster, 1986; Havea et al., 1998, 2000; Hong & Creamer, 2002), which may be present in commercial WPC samples as a consequence of processing.

Comparison of the 2D PAGE pattern of the control sample (Figure 6.7A) with those of the pressure-treated samples (Figures 6.7B–6.7D) showed that the intensities of all monomeric whey protein spots, including immunoglobulin G (IgG), LF, BSA, β -LG and α -LA, decreased with the time of pressure treatment, with the simultaneous appearance of new protein spots (as shown in the region Y', Figures 6.7B–6.7D). The protein spots corresponding to monomeric IgG and LF were absent from the samples that were pressure treated for 20 min or longer (Figures 6.7B–6.7D), but a faint spot of monomeric BSA was still observable even in the samples pressure treated for 60 min. Comparison of the sample gel strip (a') in the 2D gel with the stained gel strips (a'') of the control sample (Figure 6.7A) and the pressure-treated samples (Figures 6.7B–6.7D)

showed that the high molecular weight protein aggregates formed by pressure treatment (Figures 6.7B–6.7D, particularly marked as X₄–X₆ on the stained gel strip) were only partially dissociated by SDS PAGE in the second dimension. Some protein aggregates (marked as X₂, X₃, X₄, Figure 6.7B) dissociated from the region between the α -LA band and the top of the resolving gel of the sample gel strip to give many closely located protein spots on the 2D gels, forming an almost continuous faint inclined line (marked Y', Figure 6.7B) as described earlier in Chapter 4. The faint band with a mobility intermediate between monomer and dimer in the first dimension (Figure 6.5) had the same electrophoretic mobility in the second dimension as the monomer (marked as N', Figure 6.7A) and therefore was identified as non-native monomer, as described by Manderson et al. (1998). Also, as shown and described in Chapter 4, there was a series of closely located very faint spots in the region between the β -LG spot and the β -LG band in the left-hand lane in the PAGE pattern of the pressure-treated samples (Figure 6.7B). These spots corresponded to hydrophobically bonded non-native monomers of β -LG, β -LG dimer, trimer etc. generated as a result of the pressure treatment. As observed in Chapter 5, these results also confirmed that proportions of pressure-induced aggregates were non-covalently linked, when concentrated WPC solutions were subjected to severe pressure treatment.

2D SDS:SDS^R-PAGE of control and pressure-treated samples

The 2D SDS:SDS^R PAGE patterns of the control sample and the samples that were pressure treated for 20, 60 and 120 min are shown in Figures 6.7E–6.7H. Each PAGE pattern contains the stained gel strip (a'') from the 1D SDS-PAGE and the molecular weight markers (Mr). The reduced SDS sample loaded in the left-hand lane (denoted as SR) was the reduced sample of the corresponding sample used in the 1D SDS-PAGE (see Figure 6.5C).

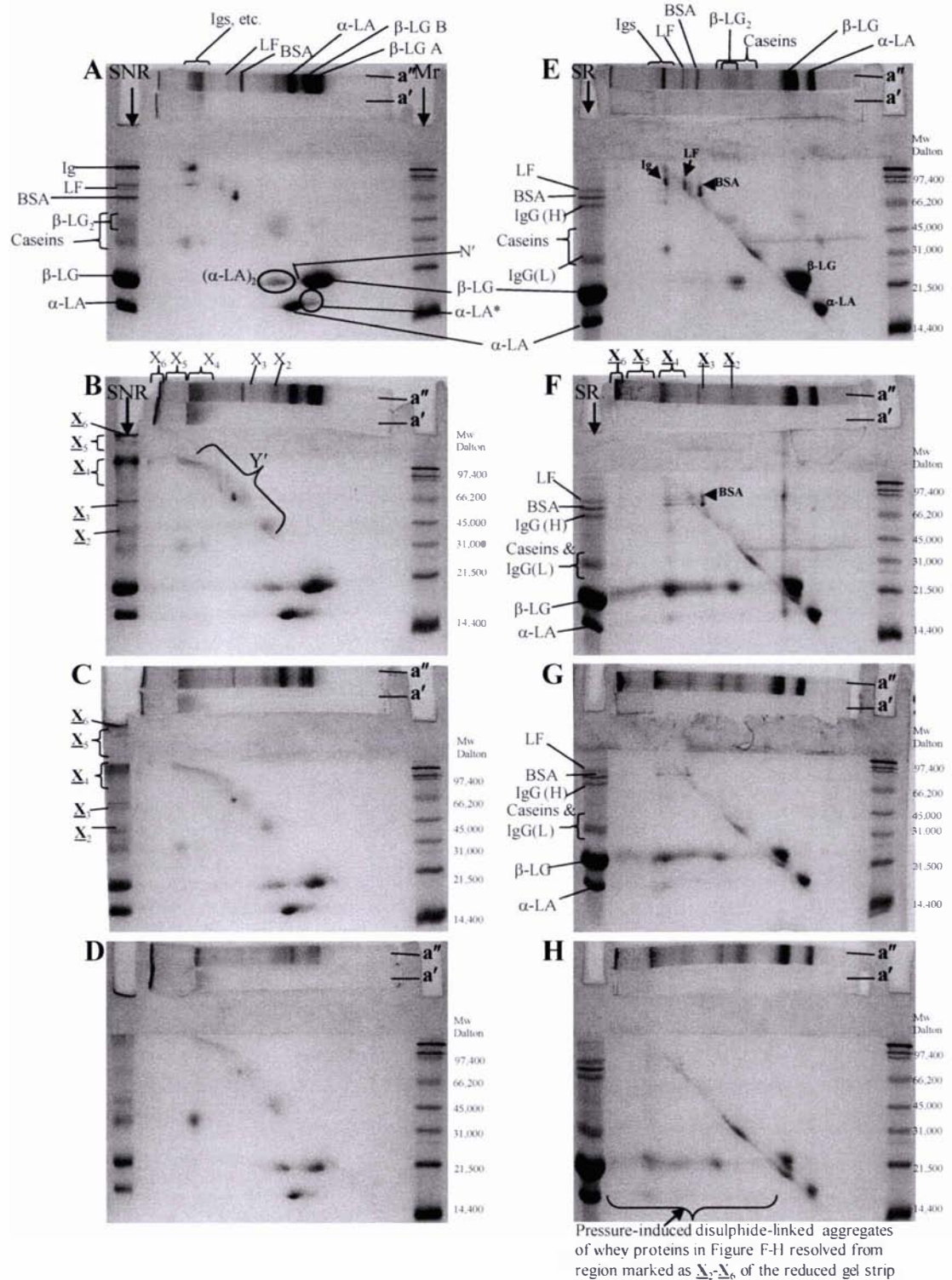


Figure 6.7. 2D PAGE patterns of control and pressure-treated WPC solutions (12% w/v). Native- and then non-reduced SDS-PAGE patterns of (A) the control, and samples pressure treated for (B) 20 min, (C) 60 min and (D) 120 min. Similarly, SDS- and then reduced SDS-PAGE patterns of (E) the control, and samples pressure treated for (F) 20 min, (G) 60 min and (H) 120 min.

The 2D SDS:SDS^R-PAGE pattern of the control sample is shown in Figure 6.7E. The spots corresponding to α -LA, β -LG, caseins, IgL, IgH, BSA and LF were clearly identified as described earlier (see Section 4.3.7 in Chapter 4).

Comparison of the PAGE pattern of the control sample (Figure 6.7E) with those of the pressure-treated samples (Figures 6.7F–6.7H) showed the presence of new spots corresponding to reduced SDS dimers, trimers and higher polymers of β -LG in the pressure-treated samples. The most significant spots were in a horizontal line that aligned with the large monomeric β -LG band in the left-hand lane (i.e. a series of spots between the spot corresponding to monomeric β -LG and the β -LG band on the left-hand side). From right to left in the 2D PAGE pattern, there was a large spot corresponding to reduced monomeric β -LG followed by a distinct dimer spot, then a distinguishable trimer spot and then a large spot resolved from the band corresponding to aggregated material resolved from the X₄ region of the reduced sample gel strip (Figure 6.5B). Further to the left was a faint β -LG streak that was resolved from the region that corresponded to X₅ and X₆ of the reduced sample gel strip (Figure 6.5B). These results suggested that pressure-induced disulphide-bonded dimers, trimers and high molecular weight polymers were generated.

A similar pattern of a horizontal line consisting of faint spots was also observed between the α -LA spot and the α -LA band on the left-hand side (Figures 6.7F–6.7H). Also, the spots corresponding to reduced BSA, IgG and LF were identified on the 2D PAGE pattern of the pressure-treated samples, suggesting that disulphide-bonded dimers, trimers etc. and disulphide-bonded complexes of β -LG with α -LA and BSA, IgG and LF were generated by the pressure treatment of WPC solutions. The low levels of α -LA polymers compared with β -LG polymers as well as the presence of a moderate level of α -LA monomer even after severe pressure treatment for 120 min at 800 MPa (Figure 6.6) reinforced the notion that α -LA was not as responsive as β -LG to high pressure treatment.

6.4 General discussion

The 2D native:SDS-PAGE data (Figures 6.7A–6.7D) show that medium- to large-sized polymers can be built up from smaller aggregates such as non-native monomers, dimers, trimers etc. These results are consistent with the TEM patterns (Figure 6.3), which show that there was an increase in the larger aggregates, which were apparent after various pressure treatments (Figure 6.3B). The SEC pattern (Figure 6.4B) also indicated that the native protein peak had diminished significantly after pressure treatment and the larger and larger polymers which could not enter 0.22 μ filter were formed. It has been postulated that β -LG, being a major whey protein, plays a major role in thermal (Mulvihill & Kinsella, 1987; Boye et al., 1995) or pressure-induced (Van Camp et al., 1997a, 1997b) aggregation and gel formation of WPC. Therefore, the major part of the discussion in this chapter is focused on effects of high pressure on β -LG. However, it is useful to begin with the knowledge that heated WPC solutions contained 1:1 disulphide-bonded adducts of α -LA and β -LG and that these were more obvious at lower WPC concentrations (Havea et al., 1998). This reaction was further explored by Hong & Creamer (2002) to show that β -LG could catalyse the formation of α -LA disulphide-bonded dimers and could form 1:1 adducts of α -LA and β -LG. However, analysis of the pressure-treated samples (Figures 6.7F–6.7H) showed that there was very little dimeric material containing α -LA, viz. α -LA dimer, α -LA:BSA or α -LA: β -LG, but that β -LG dimer was a major intermediate. More of such smaller adducts and intermediate aggregates were observed when WPC solutions were pressure treated at significantly lower concentration and lower pressures such as 200 MPa (as discussed in Chapter 5), as was the case for heat-treated samples (Havea et al., 1998). However, the examination of a model system helped to further clarify this situation (see Chapter 8).

The loss of native-like or SDS-monomeric α -LA (Figures 6.5 and 6.6) from the pressure-treated WPC solutions was moderate, but this may not be so if α -LA alone is pressure treated (Chapter 8). In milk (Huppertz et al., 2004a), α -LA is not affected at pressures up to 400 MPa. Of course, when a WPC solution is heated, α -LA is denatured almost at the similar rates to those for β -LG and BSA (Havea, 1998; Havea et al., 1998, 2002). The mechanism suggested involvement of thiols, notably CysH121 of β -LG in the first instance (Hong & Creamer, 2002), catalysing disulphide bond interchange within a suitable environment (suggested to be a molten globule) involving both α -LA

and β -LG (Livney et al., 2003). It is unlikely that the types of environments, such as hydrophobic/molten globule, that exist at high temperatures are comparable with those that will be available at high pressure, in which hydrophobic clusters are less stable (Lassalle et al., 2003). However, it was shown by Hong & Creamer (2002) that α -LA heat denatures faster in the presence of β -LG, probably because these proteins can form a stable adduct, which can then undergo thiol-catalysed disulphide bond interchange within the hydrophobic interior of the adduct. This mechanism could apply equally well to the pressure-induced denaturation and aggregation of α -LA in a mixture of whey proteins. As discussed in Chapter 4, the comparison of the present results with those in the literature also suggests that there are some similarities between the heat-induced aggregation and the high-pressure-induced aggregation of whey proteins. On the other hand, it has been reported that there are significant differences in protein denaturation and aggregation induced by heat compared with high pressure (e.g. Heremans et al., 1997) probably because the effects of high pressure on protein solutions are mainly related to the rupture of non-covalent interactions and subsequent re-formation of bonds within and between protein molecules, leading to the formation of oligomers and polymers, linked by hydrophobic interactions and disulphide bridges. Therefore, it is suggesting that the gels produced from whey proteins by high pressure may have different properties from those made by heat treatment.

As discussed in Chapter 2, gel formation within the neutral pH range is explained by the build-up of intermolecular disulphide bonds, which connect the whey proteins to form a network structure (Van Camp & Huyghebaert, 1995b; Tanaka & Kunugi, 1996; Van Camp et al., 1997a, 1997b; Kanno et al., 1998; Stapelfeldt & Skibsted, 1999), which is common for both heat- and high-pressure-induced gels. However, various studies have suggested that the structure and the properties of heat- and pressure-induced gels of β -LG are different (Van Camp & Huyghebaert, 1995a, 1995b; Van Camp et al., 1996; Dumay et al., 1998). In general, pressure-induced gels are weaker, less elastic and more exudative than heat-induced gels (Cheftel & Dumay, 1996; Dumay et al., 1998) and high pressure treatment generates gels with more porous structures and lower firmness, compared with heat-induced gels (Van Camp & Huyghebaert, 1995b; Zasytkin et al., 1996; Dumay et al., 1998). In this context, the results of the present study showed that the samples of 12% w/v WPC solutions that were pressure treated at 800 MPa at 22°C contained a range of disulphide-bonded and hydrophobically bonded protein aggregates

after pressure release (Figure 6.7) and gave visco-elastic gels (Figures 6.2). Samples that had been pressure treated for longer than 40 min contained particles (e.g. Figures 6.3C–6.3F) that appeared to be comparable with those found by others (Van Camp et al., 1997a, 1997b; Kanno et al., 1998) and were similar to the particulate structure induced by heat treatment (Stading & Hermansson, 1991; Havea et al., 2004).

As mentioned in the previous reports (Huppertz et al, 2006a; Considine et al., 2005b, 2006a, 2006b), and as discussed in Chapter 4, it appears that, at neutral pH, β -LG progressively unfolds as the pressure increases to 400 MPa. At this pressure, there are only transient structural elements (helices, strands and sheets) and almost no extensive structure. Disulphide bonds that originally stabilised the native structure of β -LG are strained and, in the presence of a free thiol, inter- and intramolecular disulphide bond interchange occurs readily (Creamer et al., 2004). At high pressures such as that used in the present study, where the hydrophobic effect, which helps to cluster the hydrophobic side chains, is significantly diminished (Heremans et al., 1997; Royer, 2002), the new disulphide bonds will be different from those in a heat-induced aggregation (Creamer et al., 2004; Patel et al., 2004; Considine et al., 2005a) and will vary with pressure. At 800 MPa and the time used in the present study, the proteins will have very little classical tertiary structure, but β -LG clearly associates progressively into disulphide-bonded dimers, trimers etc. (Figures 6.5–6.7) to give much larger aggregates.

In order to accommodate these two different viewpoints [(viz. extended holding time at high pressure gives more aggregation or gelation with particulate structure, which is observable after depressurisation (Figure 6.3), but nuclear magnetic resonance (NMR) and other data obtained after severe pressure treatment of whey proteins indicates that stable hydrophobic interactions are unlikely)], another process needs to be involved. It is possible that the particles seen in Figure 6.3 are not present at 800 MPa but are formed rapidly during (or after) pressure release and that the energy balance that favours hydrophobic interactions at lower pressures encourages the formation of these small particles. This explanation is supported by the larger proportion of protein in the particulate form that occurs between 60 and 90 min of pressure treatment, when the extent of inter-protein disulphide bonding is at an intermediate stage (Figures 6.5–6.7). In situ, pressure-induced gels are mainly stabilised by hydrogen bonds because this

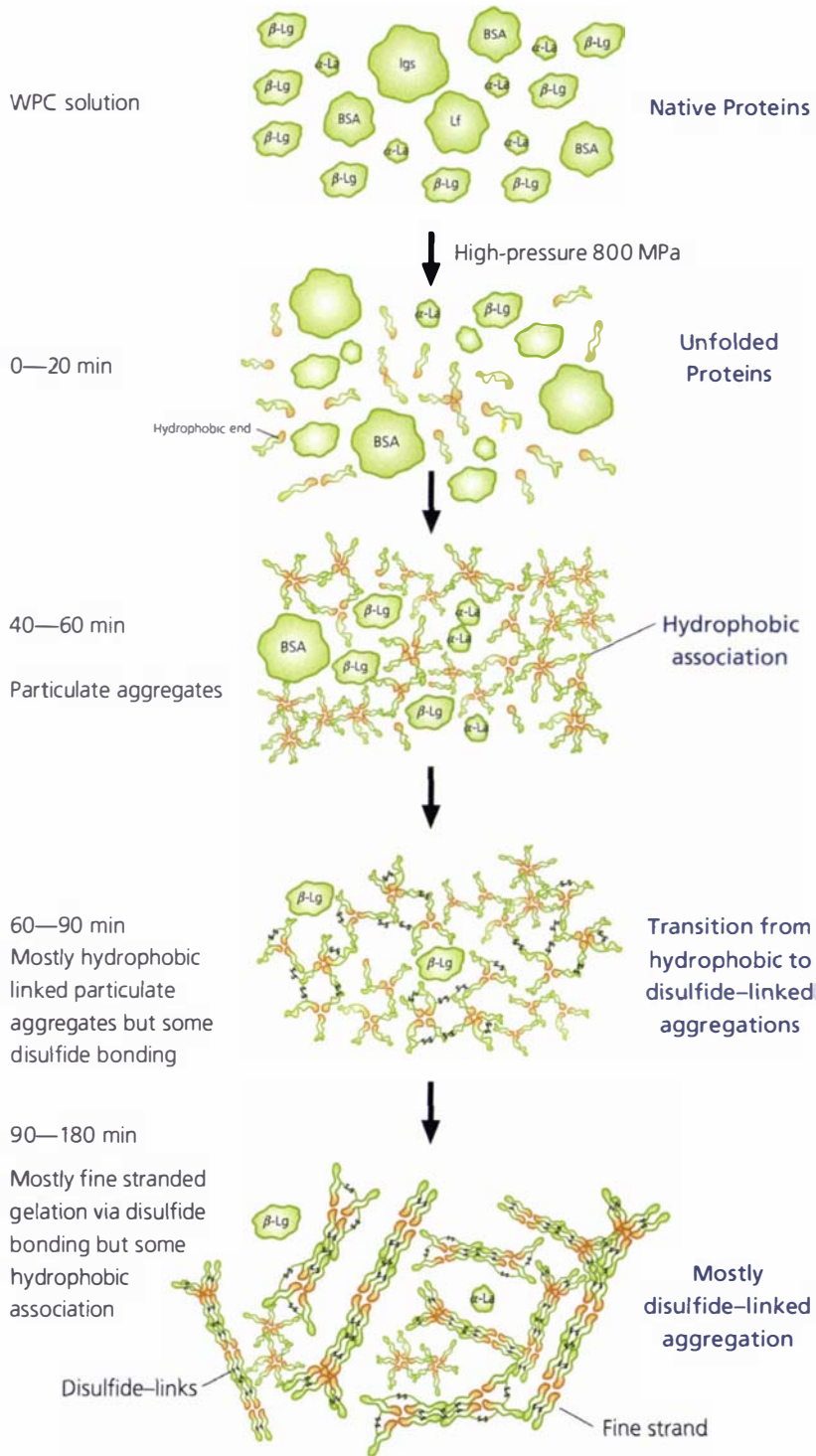


Figure 6.8. Schematic diagram of the possible changes in the whey proteins during pressure-induced gel formation.

reaction favours a negative reaction volume. Gel formation will be restrained as the pressure increases if considerable hydrophobic interaction is involved, because the reaction volume is positive (Masson, 1992). In such a case, it is assumed that the microstructure of the gel is built up during the pressure release phase because, as the pressure is reduced, hydrophobic interactions are again reinforced and the groups involved re-orient themselves (Suzuki & Taniguchi, 1972; Cheftel, 1992). With increasing pressure, protein molecules undergo a sequence of conformational changes due to the balance of stabilising interactions that are altered (Johnston et al., 1992a, 1992b) However, during the pressure release phase and after pressure treatment (as in the present study), new intermolecular interactions are formed and the proteins may become differently structured (Fertsch et al., 2003).

Another possible hypothesis is that during earlier stages (e.g. up to 60 min) of pressure treatment, large protein aggregates (particulate aggregates), held together by hydrophobic association were formed. During the later stages (90 and 120 min), these aggregates appear to disperse into fine stranded aggregates, which were held together by both hydrophobic and disulphide-linked associations (see TEM and PAGE results). Based on this explanation, it seems that the pressure-induced gel formation of WPC solutions followed novel pathways (see Figure 6.8). This hypothesis is speculation based on what might be possible, but comparative results of “in-situ” analysis and analysis of the same sample after pressure release will be required to confirm actual changes taking place during and after pressure-treatments.

As shown in the present study, the proteins in WPC solutions interact and gradually aggregate to form polymers of increasing size with increasing time of pressure treatment at 800 MPa. The identification of dimers, trimers etc. of β -LG and the other labile whey proteins using 2D SDS:SDS^R-PAGE suggests that disulphide bond interchange is involved, as previously suggested in Chapter 4 and in a number of other studies (Felipe et al., 1997; López-Fandiño et al., 1997; Kanno et al., 1998). The progress towards gel formation is slow relative to heat-induced gelation and this must be constrained either by the low rate of thiol-catalysed disulphide bond interchange or by the overall low energy gain as a result of gel formation.

Overall, the results of this study on a 12% w/v WPC solution at neutral pH are self-consistent and can be interpreted to give a coherent picture of the changes to the protein

components and their aggregation that occur during pressure treatment (800 MPa at 22°C for an extended period of time). It is likely that several β -LG molecules will become linked to one another by new disulphide bonds and that there will be a number of CysH residues that are capable of interacting with the disulphide bonds of other molecules. α -LA, as the second major protein, is a potential candidate for forming a disulphide bond with the newly formed β -LG strands and with available CysH residues. At this pressure, there will be negligible β -LG secondary structure, but α -LA will probably have some secondary structure, because of the stability of the molten globule state of this protein. Thus, α -LA will react slowly with the thiols of β -LG and will become incorporated into the growing protein strands and aggregates. It is envisaged that, after about 90 min of pressure treatment, most of the proteins will have become attached to one another via disulphide bonds and that there will be virtually no strong protein–protein interaction or association other than via disulphide bonds. This situation is very different from that in heat-treated WPC solutions, where hydrophobicity appears to be an important driving force. Once the pressure is reduced to atmospheric, the hydrophobic associations will be partly restored within the constraints of the new disulphide bonding structure.

6.5 Conclusions

In the current study to elucidate the pathway of pressure-induced gel formation, it was found that the pressure-treated samples showed a time-dependent loss of native whey proteins by SEC and 1D PAGE, and a corresponding increase in non-native proteins and protein aggregates of different sizes. These aggregates altered the viscosity and opacity of the samples and were shown to be cross-linked by intermolecular disulphide bonds and by non-covalent interactions using 1D PAGE (native, SDS and SDS^R) and 2D PAGE (native:SDS and SDS:SDS^R PAGE). As discussed in Chapter 4 and again confirmed by results of present study, the large internal hydrophobic cavity of β -LG may have been partially responsible for its sensitivity to high pressure treatments. Conversely, α -LA responds to pressure by modifying its structure to be more molten globule like and does not fully unfold at very high pressures. Various possible aspects and hypotheses in support of pressure-induced gel formation have been discussed in detail based on the results of the present study. It seemed likely that, at 800 MPa, the formation of a β -LG disulphide-bonded network preceded the formation of disulphide

bonds between α -LA or BSA and β -LG to form multi-protein aggregates, possibly because the disulphide bonds of α -LA and BSA are less exposed than those of β -LG either during or after pressure treatment. It may be possible that intermolecular disulphide bond formation occurred at high pressure and that hydrophobic association became important after the high pressure treatment.

CHAPTER 7

Effects of Heat and High Hydrostatic Pressure on Interactions of Proteins in Skim Milk¹

7.1 Introduction

The heat-induced interactions of caseins and whey proteins play an important role in the functionality of many dairy products (Haque & Kinsella, 1988; Jelen & Rattray, 1995), e.g. the heat stability of milk and the texture of yoghurt. Many studies have been reported on heat-induced interactions between β -lactoglobulin (β -LG) and κ -casein (κ -CN) in model systems or in milk, suggesting that the two disulphide bridges and the free sulphhydryl group present in the native structure of β -LG play an important role in its heat-induced interactions with κ -CN (Haque et al., 1987; Haque & Kinsella, 1988; Hill, 1989; Dalgleish, 1990; Jang & Swaisgood, 1990; Singh, 1995; Corredig & Dalgleish, 1999; Cho et al., 2003). Recently, high pressure (HP) processing has also been recognised as a tool for the modification of proteins (Cheftel, 1992; Hayashi, 1992; Balny & Masson, 1993), and thus is considered to be one of the most promising new processing technologies for modifying the functional and textural properties of dairy products (Johnston, 1995; Vardag & Körner, 1995; Messens et al., 1997; Datta & Deeth, 1999, 2003; Needs et al., 2000a, 2000b).

Most of the earlier work on the effects of HP on milk proteins has been on the denaturation behaviour (or kinetics) of individual proteins in milk (e.g. López-Fandiño et al., 1996, 1998; Felipe et al., 1997; García-Risco et al., 2000; Scollard et al., 2000; Huppertz et al., 2004a, 2004b), pasteurised milk (Needs et al., 2000a, 2000b) or reconstituted skim milk (Gaucheron et al., 1997). β -LG has been reported to interact with κ -CN, probably via disulphide bonds (Felipe et al., 1997; López-Fandiño et al., 1997, 1998; Needs et al., 2000b; Scollard et al., 2000) during HP treatments. Some of these studies also reported a decrease in the turbidity and the size of the casein micelles

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due to their disintegration as a consequence of pressure treatments between 250 and 600 MPa (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Needs et al., 2000b; Huppertz et al., 2004a, 2004c; Anema et al., 2005b). These studies explored the effects of HP on individual proteins, but little attention has been paid to characterizing the pressure-induced interactions of caseins and whey proteins, including interaction of α_{s2} -CN and β -LG.

Various one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE) techniques have been used to characterise aggregates and interaction products formed in heat-treated or pressure-treated (Chapters 4–6) WPC solutions. We have applied such techniques to characterise pressure-induced interactions of caseins and whey proteins in a more complex system, such as milk. It was necessary to modify the standard sodium dodecyl sulphate (SDS)-PAGE method and to develop another PAGE method (2D alkaline urea (AU):SDS-PAGE) in order to analyse the large process-induced aggregates formed in the presence of high casein concentrations.

7.2 Experimental

The detailed procedures for obtaining skim milk, for heat and pressure treatment of skim milk samples and for analysing various samples using appropriate 1D and 2D PAGE methods have been described in Chapter 3.

A summary of the experimental plan for generating and analysing the heat- and pressure-treated skim milk samples by PAGE methods is outlined in Figure 7.1.

The standard 2D SDS and then reduced SDS-PAGE method described in Chapter 3 and used in Chapters 4 and 6 was used with some modifications in order to trap very high molecular weight aggregates (the aggregates that were unable to enter the gel) present in the heat- and pressure-treated skim milk samples. In the modified method, the sample was mixed with the stacking gel mixture and was set within the sample loading well (Davis, 1964). See Figure 7.3 and results and discussion session for more detail. In addition, the combination of non-reduced AU- and then SDS-PAGE (as described in Chapter 3) was used for effective separation of the caseins from one another.

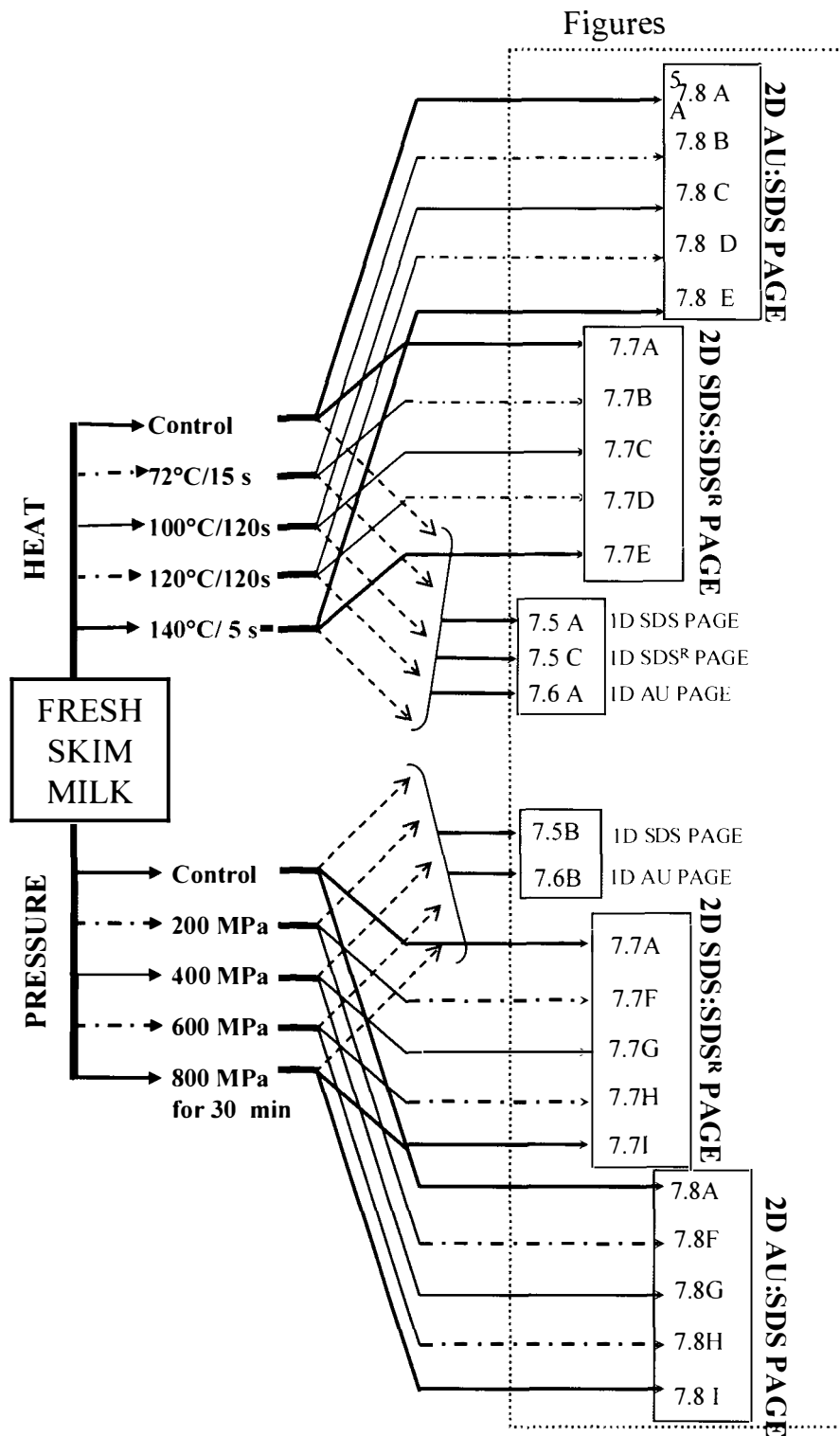


Figure 7.1. Outline of the steps taken from the raw skim milk, via a variety of heat and pressure treatments, to the PAGE analysis results. The heat treatments used corresponded to pasteurisation (72°C for 15 s), preheat treatments used for powder manufacture (100°C for 120 s or 120°C for 120 s) and UHT sterilisation (140°C for 5 s).

7.3 Results and discussion

7.3.1 Appearance of milk

Visual differences between heat- and pressure-treated skim milk samples were noticed directly after treatment (Figure 7.2). There was no distinct difference between the untreated control sample and the heat-treated skim milk sample (Figure 7.2A). However, the appearance of the pressure-treated sample changed to translucent or semi-transparent with a slightly yellow hue, which may be attributed to pressure-induced changes in the casein micelles (Figure 7.2B). When the milk was held at refrigerator temperature (about 5°C), it retained the semi-transparent appearance for long periods (several days). However, upon holding at room temperature, the milk became progressively more turbid, but did not return to the original appearance of the untreated milk. These observations are in agreement with the observations that have been reported previously (Needs et al., 2000a, 2000b; Gervilla et al., 2001).

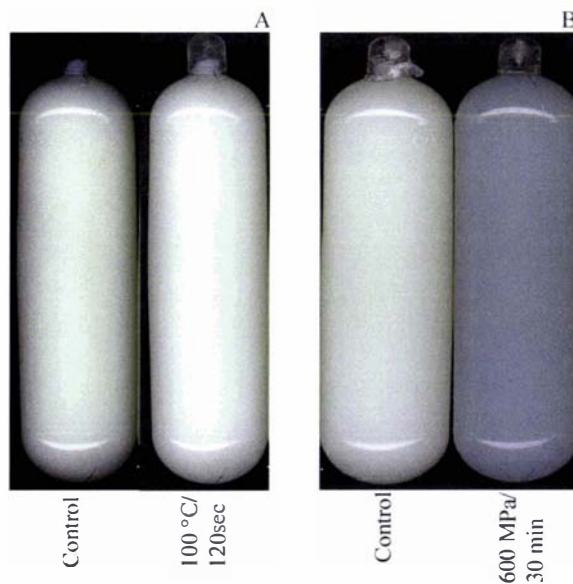


Figure 7.2. Changes in the appearance of skim milk after heat (A) and pressure treatment (B), as marked on each of the samples, compared with an untreated control.

7.3.2 Modification of standard SDS-PAGE

It was observed during preliminary experiments that some very large heat- or pressure-induced aggregates (molecular weight > 500 kDa) did not enter the stacking gel during electrophoresis; as a consequence, they were caught in the sample loading well and were subsequently lost during the staining and destaining procedures. While trying different approaches to resolve this problem, we found that mixing the heat- or pressure-treated samples with a polyacrylamide of similar composition to the stacking gel and setting them in the sample loading well using similar principles to those described by Davis (1964) trapped the protein aggregates with a molecular mass greater than about 500 kDa within the set sample gels (see Figure 7.3). This strategy prevented the loss of very large protein aggregates during electrophoresis and during the gel staining and destaining procedures. Consequently, a qualitative estimate of the composition of these very large aggregates could be made using the 2D SDS:SDS^R-PAGE procedure.

Another problem was that the standard native- and SDS-PAGE methods (Chapters 4–6) were not suitable for dispersing the samples (containing high concentrations of caseins) sufficiently to give clear patterns of each of the proteins. The caseins, which are naturally aggregated into micelles, are difficult to disperse at neutral pH. However, in SDS solutions, the casein components are readily dispersed as SDS complexes. All four caseins (α_{s1} -CN, α_{s2} -CN, β -CN and κ -CN) are readily separated by SDS^R-PAGE, but they have similar mobilities and therefore the bands corresponding to all four caseins overlap when higher sample loadings are used or when the sample contains high concentrations of caseins. It was found that the caseins are dispersible in 5 M urea solution and migrate satisfactorily at higher sample loadings in an AU-PAGE system. A preliminary study showed that a 2D PAGE system based on a urea separation in the first dimension followed by an SDS separation in the second dimension could effectively separate the caseins from one another.

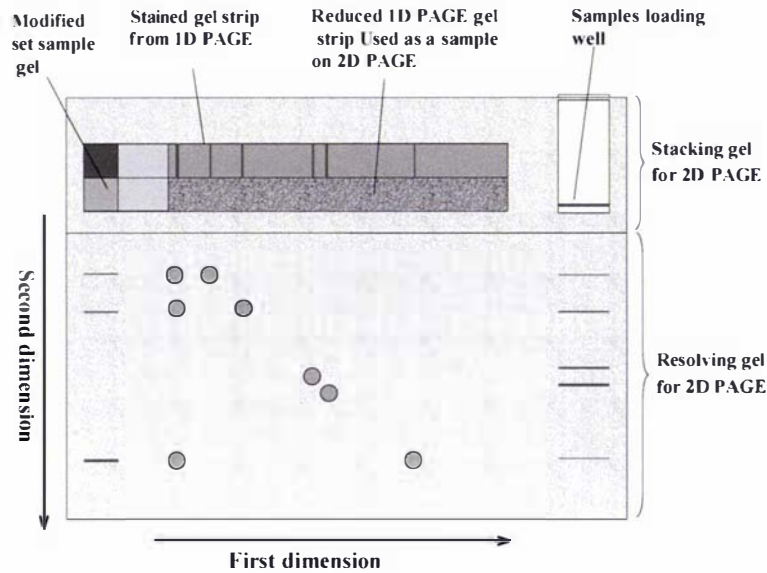


Figure 7.3. Diagram showing the preparation and running of a sample of heated milk in a modified 2D SDS:SDS^R-PAGE system. The milk sample is dispersed in SDS stacking buffer, mixed with an acrylamide gel solution and set in two sample wells of a standard SDS-PAGE gel. After electrophoresis of the samples, one of the gel strips is stained and the other is immersed in a SDS sample buffer containing 2-mercaptoethanol solution to reduce the covalent disulphide bonds. After washing, the unstained treated sample gel strip is clamped into another electrophoresis cell, the SDS resolving gel is set under it and then the SDS stacking gel is set around it. After the second dimension electrophoresis, staining and destaining, the previously stained gel strip is placed above and parallel to the strip that had been set into the gel. This set-up is then scanned and photographed (see Figure 7.7 for examples). This procedure allows the relationship between the stained spots/bands of the disulphide-bonded proteins and those of the reduced proteins to be established.

7.3.3 1D PAGE patterns of pure casein and whey proteins

The AU-PAGE and SDS-PAGE patterns of pure proteins, including skim milk (standard), lactoferrin (LF), bovine serum albumin (BSA), immunoglobulin G (IgG), α_{s1} -CN, α_{s2} -CN, β -CN, κ -CN are presented in Figures 7.4A and 7.4B respectively. The PAGE patterns of the pure proteins were useful for identifying individual caseins and whey proteins in the AU and SDS PAGE patterns of milk.

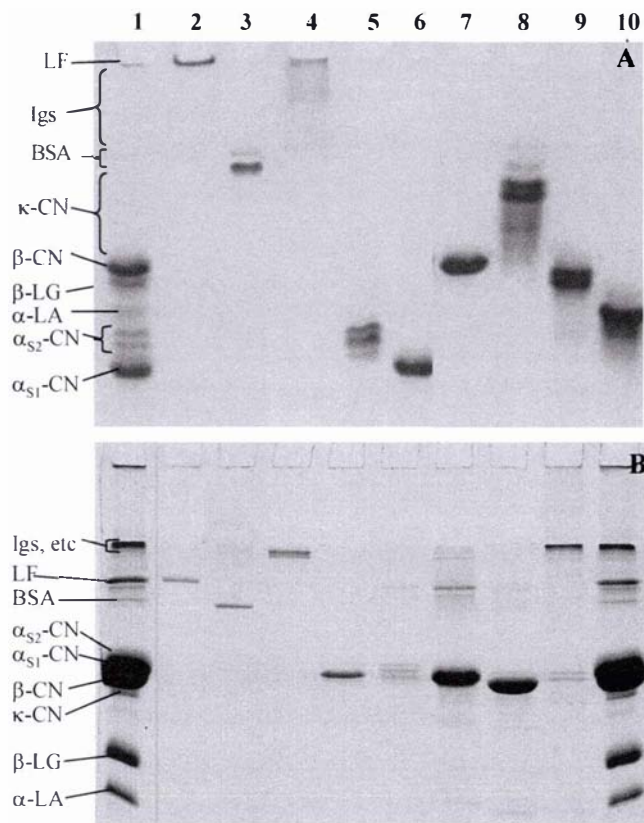


Figure 7.4. AU-PAGE (A) and SDS-PAGE (B) patterns of pure proteins.

The loading details on AU PAGE include, Skim milk standard (Lane 1), Lactoferrin (Lane 2), BSA (Lane 3), IgG (Lane 4), α s2-CN (Lane 5), α s1-CN (Lane 6), β -casein (Lane 7), κ -casein (Lane 8), β -lactoglobulin (Lane 9), α -lactalbumin (Lane 10).

The sample loading details on SDS PAGE pattern are: skim milk (Lane 1), Lactoferrin (Lane 2), BSA (Lane 3), IgG (Lane 4), α s1-CN (Lane 5), α s2-CN (Lane 6), α -CN (α s1- + α s2-CN) (Lane 7), β -CN (Lane 8), κ -CN (Lane 9) and skim milk standard (Lane 10).

7.3.4 Identification of protein bands on 1D PAGE patterns of control samples

1D SDS- and AU-PAGE

The bands corresponding to LF, BSA, IgG, α s1-CN, α s2-CN dimer, β -CN, κ -CN, β -LG and α -LA on the 1D SDS-PAGE patterns of the control samples (Lane 1, Figures 7.5A–7.5C) were clearly identified by comparison with the 1D SDS-PAGE patterns of the pure proteins (Figure 7.4B; Farrell et al., 2004) and their identities are noted on the left-hand side of each PAGE pattern. The bands corresponding to α s1-CN, α s2-CN, β -CN

and κ -CN were clearly identified on the AU-PAGE patterns of the control samples (Lane 1, Figures 7.6A and 7.6B), whereas the bands corresponding to whey proteins were not clearly resolved on the AU-PAGE patterns. In addition, for the purpose of identifying the bands corresponding to various caseins and whey proteins, the caseins and whey proteins were prepared by acid precipitation of skim milk ($\approx 50^\circ\text{C}$). The samples of skim milk and the whey and casein obtained from the same skim milk were analysed using comparable 1D (SDS- and AU-PAGE) and 2D (SDS:SDS^R-PAGE and AU:SDS-PAGE) PAGE (see Appendix 5). These PAGE patterns were useful in identifying the pattern and location of various protein bands in the PAGE patterns of milk. These protein bands are appropriately marked on the respective PAGE patterns (Figure 7.5A for SDS-PAGE and 7.6A for AU-PAGE). Some of the doubtful protein spots (e.g. corresponding to α_{s2} -CN dimer, κ -CN polymers and β -LG dimer) present in the 2D SDS:SDS^R-PAGE pattern of the untreated control sample were further analysed to confirm the identity of the protein components by mass spectrometry of their tryptic digests (see Appendix 6). Some large aggregates marked as \underline{X}_0 or X_0 were present in the SDS and AU PAGE patterns of the control samples respectively (Lane 1, Figures 7.5A, 7.5B, 7.6A and 7.6B).

1D SDS^R-PAGE

As described earlier (Chapter 4), when the control sample was reduced prior to analysis by SDS-PAGE (Figure 7.5C), the β -LG and α -LA bands were more intense. Also, the material that could not enter the gel or that migrated only a short distance in the non-reduced sample (marked as X_0 or \underline{X}_0 in Lane 1 of Figure 7.5A or 7.5B) was absent from the PAGE pattern of the reduced sample (Lane 1, Figure 7.5C).

7.3.5 1D PAGE of heat- and pressure-treated samples

The preparation of the heat- and pressure-treated milk samples is outlined in Figure 7.1 and the resultant 1D SDS- and AU-PAGE patterns are shown in Figures 7.5 and 7.6 respectively.

The large heat- and pressure-induced aggregates (Lanes 2–5, Figures 7.5A and 7.5B) were classified into three groups, i.e. those caught up at the beginning of the resolving

gel, those caught up in the stacking gel and those that could not enter the gel or were caught up in the sample loading well, and are marked as regions \underline{X}_4 , \underline{X}_5 and \underline{X}_6 respectively. In Figures 7.5A and 7.5B, \underline{X}_6 contained the material trapped in the set sample gels; in Figures 7.6A and 7.6B, X_6 contained the material that would normally be caught in the sample loading well at the top of the stacking gel.

SDS-PAGE of heat-treated samples

The nomenclature of the proteins in the control and heat- or pressure-treated samples has been described in detail in Chapter 4. The same nomenclature and terminology is used in this study of the heat- and pressure-induced denaturation, aggregation and interactions of proteins in the milk system.

The heat-treated samples were analysed by SDS-PAGE without disulphide bond reduction (Lanes 2–5, Figure 7.5A) and by SDS^R-PAGE (Lanes 2–5, Figure 7.5C) after disulphide bond reduction with 2-mercaptoethanol. In the PAGE pattern of the reduced samples (Figure 7.5C), Lanes 1–6 appeared to be similar to one another, suggesting the presence of disulphide-linked interactions in the heat-treated samples.

It was observed that the intensities of the bands corresponding to β -LG and α -LA decreased significantly in the severely heat-treated samples (Lanes 3–5, Figure 7.5A), whereas mild heat treatment, such as 72°C for 15 s (Lane 2, Figure 7.5A), did not affect the intensities of the bands corresponding to β -LG and α -LA. However, some decrease in the intensities of the bands corresponding to monomeric IgG, LF and BSA was observed in the PAGE pattern of the sample heated at 72°C for 15 s (Lane 2, Figure 7.5A). The bands corresponding to these heat-sensitive proteins (IgG, LF and BSA) were essentially absent in the PAGE patterns of the PAGE pattern of samples heat-treated at 100 and 120 °C for 120 s or at 140 °C for 5 s (Lanes 3–5, Figure 7.5A). The PAGE patterns of these severely heated samples also showed an increased density or dark-staining portion in the set sample gels (\underline{X}_6), indicating that significant proportions of large protein aggregates, which could not enter the gel, were present in the milks heated at 100°C for 120 s, 120°C for 120 s and 140°C for 5 s (Lanes 3–5 respectively, Figure 7.5A).

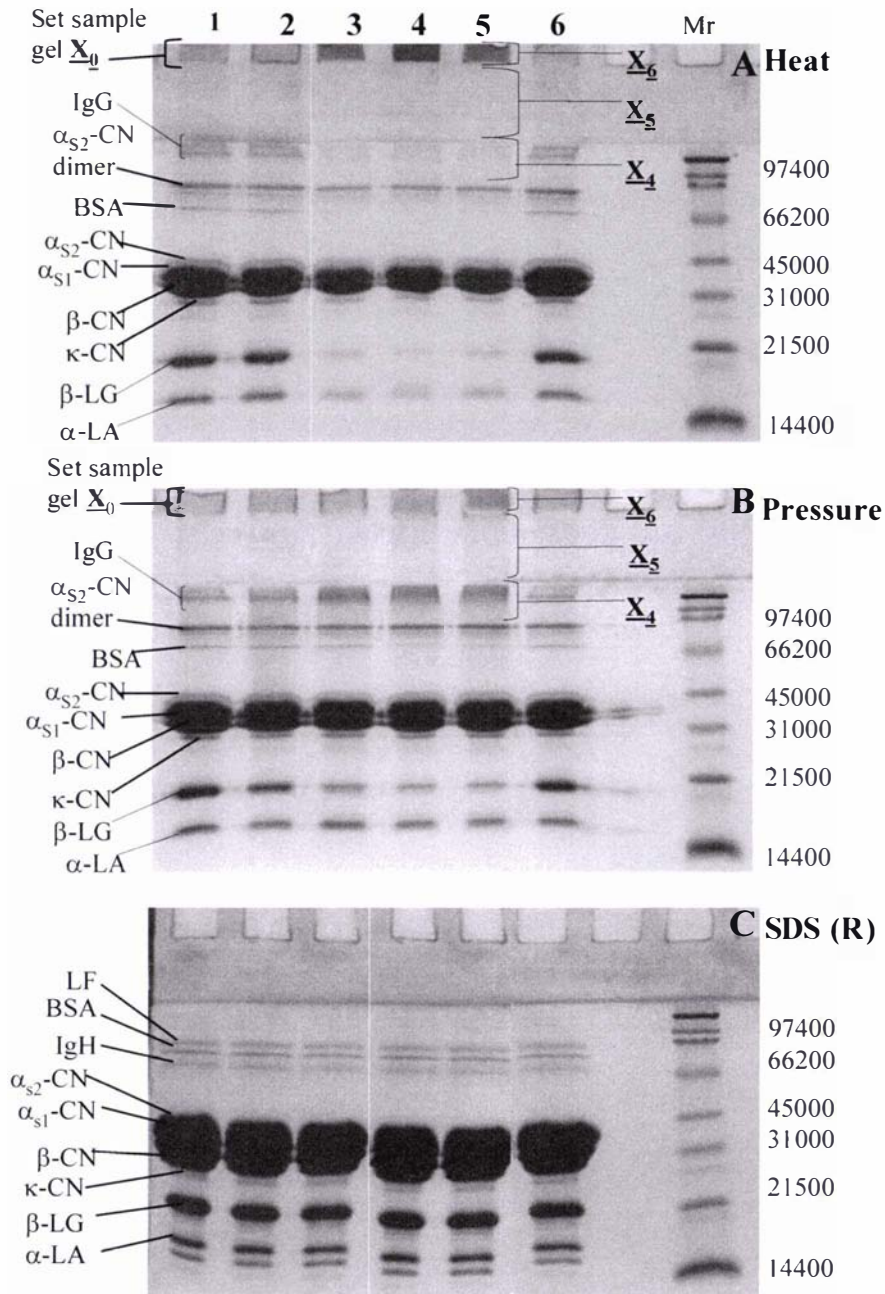


Figure 7.5. 1D SDS-PAGE patterns of non-reduced heat-treated (A) and pressure-treated (B) samples, and 1D SDS-PAGE pattern of reduced heat-treated samples (C). For the PAGE patterns of the heat-treated samples (A and C): control (Lane 1), heat treated at 72°C for 15 s (Lane 2), 100°C for 120 s (Lane 3), 120°C for 120 s (Lane 4) and 140°C for 5 s (Lane 5), and control (Lane 6). Similarly, for the PAGE patterns of the pressure-treated samples (B): control (Lane 1), samples pressure treated at 200 MPa for 30 min (Lane 2), 400 MPa for 30 min (Lane 3), 600 MPa for 30 min (Lane 4) and 800 MPa for 30 min (Lane 5), and control (Lane 6).

SDS-PAGE of pressure-treated samples

Similar analysis of the pressure-treated samples using SDS-PAGE (Figure 7.5B) showed steady changes with the extent of high pressure treatment from 200 to 800 MPa (Lanes 2–5). It was noticed that the intensities of the bands corresponding to β -LG, IgG and LF were the most affected. The changes in β -LG were apparent even in the sample pressure treated at 200 MPa for 30 min (Lane 2, Figure 7.5B). However, the intensity of the band corresponding to α -LA was not much affected until the sample pressure treated at 400 MPa (Lane 3, Figure 7.5B). Also, there were corresponding increases in the proportions of high molecular weight aggregates in the X₄, X₅ and X₆ regions in the severely pressure-treated samples (Lanes 3–5, Figure 7.5B). However, it was found that the proportion of aggregates that were caught at the start of the resolving gel (region marked as X₄) was greater in the PAGE patterns of the pressure-treated samples (Lanes 3–5, Figure 7.5B) than in the PAGE patterns of the heat-treated samples (Lanes 3–5, Figure 7.5A). In other words, the proportion of very large aggregates in the regions marked as X₅ and X₆ was less in the PAGE patterns of the samples pressure treated at 400–800 MPa (Lanes 3–5, Figure 7.5B) than in the PAGE patterns of the severely heat-treated samples (Lanes 3–5, Figure 7.5A).

AU-PAGE of control samples

Analysis of the control (untreated) milks using AU-PAGE (Lane 1, Figure 7.6A) showed that the caseins were well separated from one another. The bands corresponding to different proteins were appropriately marked on the left-hand side of the PAGE pattern (Lane 1, Figure 7.6A). Also, the effects of various genetic polymorphs of proteins, post-translational modifications, phosphorylation or glycosylation (Fox, 2003; Farrell et al., 2004) could be seen. β -LG gave rather blurred bands, probably as a consequence of thiol–disulphide interchange in the alkaline (pH \approx 8.7) chaotropic environment.

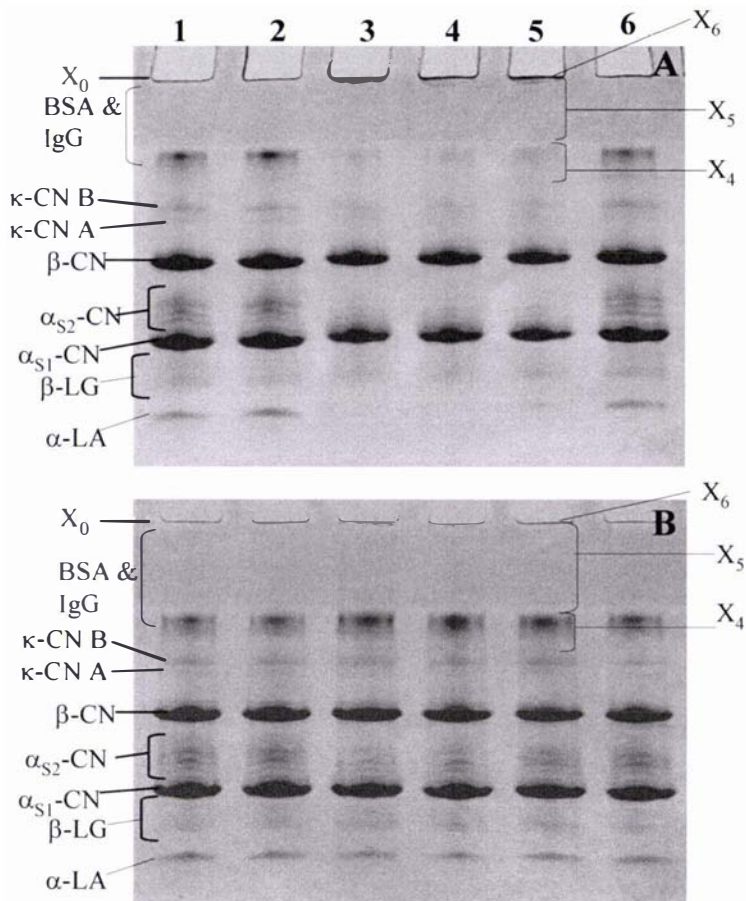


Figure 7.6. 1D AU-PAGE patterns of heat-treated (A) and pressure-treated (B) samples. For the PAGE patterns of the heat-treated samples (A): control (Lane 1), heat treated at 72°C for 15s (Lane 2), 100°C for 120 s (Lane 3), 120°C for 120 s (Lane 4) and 140°C for 5 s (Lane 5), and control (Lane 6). Similarly, for the PAGE patterns of the pressure-treated samples (B): control (Lane 1), samples pressure treated at 200 MPa for 30 min (Lane 2), 400 MPa for 30 min (Lane 3), 600 MPa for 30 min (Lane 4) and 800 MPa for 30 min (Lane 5), and control (Lane 6).

AU-PAGE of heat-treated samples

The AU-PAGE pattern of the sample heat treated at 72°C for 15 s (Lane 2, Figure 7.6A) was almost indistinguishable from that of the control sample (Lane 1, Figure 7.6A). The AU-PAGE patterns of the samples that had been heated more extensively (Lanes 3–5, Figure 7.6A) showed that the intensities of the bands corresponding to whey proteins and caseins (mainly κ -CN and α_{s2} -CN) decreased significantly. These results suggested that heat-induced interactions of whey proteins with κ -CN and possibly with α_{s2} -CN occurred. Also, it was evident from these AU-PAGE patterns that significant proportions of high molecular weight aggregates, marked as X₄, X₅ and X₆, were present in the AU-PAGE patterns of the samples heat-treated at 100°C for 120 s, 120°C for 120 s and 140°C for 5 s (Lanes 3–5, Figure 7.6A). These results are consistent with those shown in Figure 7.5A.

AU-PAGE of pressure-treated samples

Analysis of the pressure-treated samples using AU-PAGE showed similar trends (Figure 7.6B) to those observed in the SDS-PAGE patterns of the heat-treated samples (Figure 7.5B). The pressure treatment diminished the intensities of the bands corresponding to monomeric α_{s2} -CN and κ -CN in Lanes 3–5, with corresponding increases in the intensities of the regions marked as X₄, X₅ and X₆. However, the differences were not as clear-cut as shown in the SDS-PAGE results (Figure 7.5B).

When the regions marked as X₄, X₅ and X₆ in the PAGE patterns of the heat- and pressure-treated samples were compared, it was found that the proportion of aggregates caught at the top of the resolving gel was comparatively greater in the PAGE patterns of the pressure-treated samples (Lanes 3–5, Figure 7.6B), whereas the proportion of very large aggregates (that could not enter the gel or that were caught up in the sample loading well) was comparatively greater in the PAGE patterns of the heat-treated samples (Lanes 3–5, Figure 7.6A). These results are consistent with the results for heat- and pressure-treated WPC solutions (Chapter 4).

7.3.6 2D PAGE of heat- and pressure-treated samples

SDS:SDS^R-PAGE of heat-treated samples

The SDS:SDS^R-PAGE pattern of the untreated control sample of untreated skim milk (Figure 7.7A) had a series of spots that lay diagonally from the lower right of the gel to the upper left. As described in Chapter 4, the proteins that gave rise to these spots had comparable mobilities regardless of the action of 2-mercaptoethanol, i.e. they had not been part of a disulphide-bonded protein aggregate. The order of decreasing mobility on these PAGE pattern was: γ -CN, α -LA, β -LG, κ -CN monomer, β -CN, α_{s1} -CN, α_{s2} -CN monomer, α_{s2} -CN dimer and spots correspond to IgH, BSA and LF. To the left of the diagonal, there was a small spot of reduced α_{s2} -CN and two significant streaks that were reduced κ -CN, indicating that they had been disulphide bonded during the first dimension separation. The two κ -CN spots were resolved from the regions corresponding to X₄ and X₆ of the sample gel strip (a') shown in Figure 7.7A. This indicates that much of the κ -CN was present in polymerised form (disulphide-linked polymers of κ -CN) in the unheated control sample. The similar spots corresponding to polymeric κ -CN observed in the PAGE pattern of the sample heated at 72°C for 15 s suggested that this heat treatment did not affect the distribution of κ -CN significantly.

The samples that were subjected to more severe heat treatments (i.e. those heated at 100°C for 120 s, 120°C for 120 s and 140°C for 5 s) gave a significantly different pattern (Figures 7.7C–7.7E), compared with the control sample (Figure 7.7A), with the presence of all of the whey proteins, much of the κ -CN and a small proportion of the α_{s2} -CN in a series of new spots that were resolved by disulphide bond reduction of protein aggregates caught up in the set sample gel (region marked as X₆). These results suggested that significant proportions of very high molecular weight disulphide-linked casein and whey protein complexes consisting of α -LA, β -LG, BSA, LF, IgG, κ -CN and α_{s2} -CN were formed by severe heat treatment of the milk. The density of the reduced spot corresponding to α_{s2} -CN (resolved from the regions X₄–X₆ of the reduced 1D SDS gel strip) was comparatively greater in Figures 7.7D and 7.7E, showing that these heat treatments had significant effects in incorporating α_{s2} -CN into the heat-induced disulphide-bonded aggregates. However, the spot corresponding to α_{s2} -CN

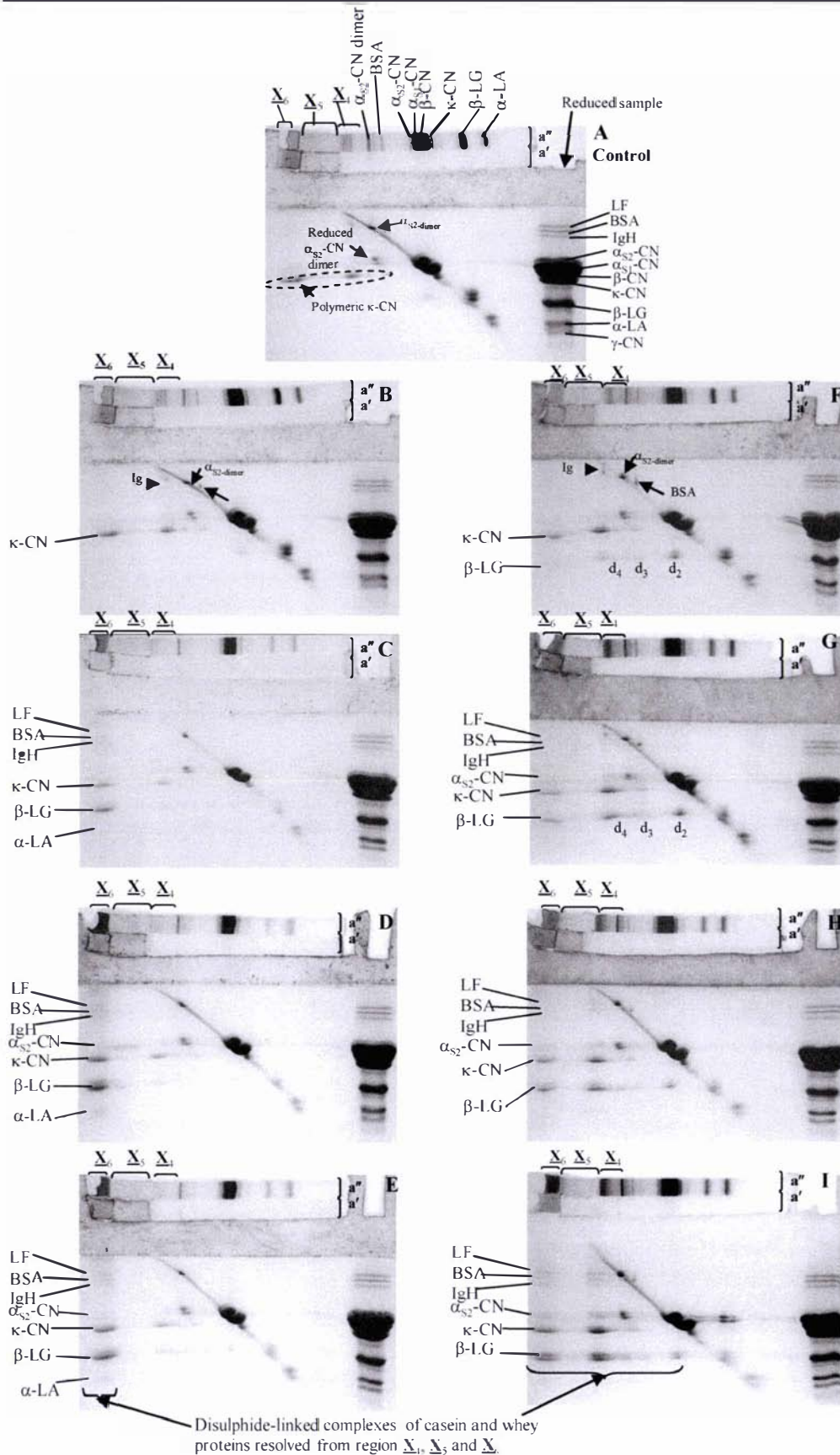


Figure 7.7. 2D SDS- and then reduced SDS-PAGE patterns of the control sample (A) and samples heat treated at 72°C for 15 s (B), 100°C for 120 s (C), 120°C for 120 s (D) and 140°C for 5 s (E). Similarly, 2D PAGE patterns of samples pressure treated at 200 MPa for 30 min (F), 400 MPa for 30 min (G), 600 MPa for 30 min (H) and 800 MPa for 30 min (I).

dimer remained almost unaffected after severe heat treatments, indicating that α_{s2} -CN dimer was much more stable to the severe heat treatments used in the present study. From these results, it can be concluded that a major proportion of the heat-induced interaction products formed were so large that they could not enter the gel, i.e. were caught up in the set sample gel portion (region marked as X_6), and that small and intermediate-sized aggregates (such as dimer, trimer, tetramer etc.) were not generated by the severe heat treatments used in the present study.

SDS:SDS^R-PAGE of pressure-treated samples

Comparison of the SDS- and then reduced SDS-PAGE pattern of the skim milk sample pressure treated at 200 MPa for 30 min (Figure 7.7F) with that of the untreated control (Figure 7.7A) showed that the intensity of the spot corresponding to monomeric β -LG decreased in the PAGE pattern of the sample pressure treated at 200 MPa for 30 min. This decrease in the intensity of the β -LG band was coupled with the appearance of new faint spots corresponding to β -LG dimer, trimer and tetramer (labeled as d_2 , d_3 and d_4 respectively on Figure 7.7F) and larger polymers of β -LG. Treatment at 400 MPa (Figure 7.7G) showed a further decrease in the intensity of the spot corresponding to monomer β -LG and the presence of several new spots that had been resolved from the X_4 and X_6 regions of the 1D SDS-PAGE sample gel strip, indicating that β -LG had been incorporated into high molecular weight disulphide-bonded aggregates that also included κ -CN and a minor quantity of monomer α_{s2} -CN.

As described in Chapter 4, an almost continuous horizontal series of spots was resolved to the left of the monomeric β -LG spot. The sample treated at 600 MPa gave a similar pattern (Figure 7.7H) with more β -LG, κ -CN and α_{s2} -CN as well as some α -LA resolved from regions X_6 and X_4 of the reduced gel strip and comparatively small proportions of spots corresponding to monomeric β -LG, α -LA, κ -CN and α_{s2} -CN. The same trend continued for the sample treated at 800 MPa (Figure 7.7I), with an even greater proportion of high molecular weight material trapped in the set sample gel (X_6) and region X_4 and proportionately more β -LG, α -LA, κ -CN, α_{s2} -CN, Ig, BSA and LF and possibly α_{s2} -CN dimer in the large aggregates from regions X_4 and X_6 . Once again these results suggested that greater proportions of intermediate-sized aggregates and those resolved from the top of the resolving gel (the region marked as X_4) were

generated by pressure treatment (Figures 7.7G–7.7I) than by heat treatment (Figures 7.7C–7.7E).

AU:SDS-PAGE of control sample

Almost all the spots corresponding to various caseins and whey proteins were identified by comparison with the 1D AU gel strip mounted horizontally on the top of the 2D PAGE pattern and by matching the mobility of each protein spot with the PAGE pattern of the non-reduced SDS sample in the vertical lane on the right-hand side.

The AU:SDS-PAGE pattern of the untreated control sample (Figure 7.8A) had two major spots near the centre of the 2D PAGE pattern, which were identified as α_{s1} - and β -CN. Another almost oval spot adjoining the α_{s1} -CN spot was identified as monomeric α_{s2} -CNs (α_{s2} -CN 10P to α_{s2} -CN 13P) (Farrell et al., 2004) and the small intensely stained spot was dimeric α_{s2} -CN, as marked on the 2D gel (Figure 7.8A). Monomeric κ -CN and products of post-translational modifications (Fox, 2003; Farrell et al., 2004) were resolved horizontally as a streak. β -LG was also resolved as a horizontal streak and α -LA, which also contained some glyco- α -LA, was resolved as a small spot under the β -LG streak. The high molecular weight whey proteins, including BSA, LF and Igs, gave a diagonal streak near the origin of the resolving gel region.

AU:SDS-PAGE of heat-treated samples

The 2D AU:SDS-PAGE pattern of the skim milk sample heat treated at 72°C for 15 s (Figure 7.8B) was almost similar to that of the control sample (Figure 7.8A), confirming earlier results from 1D AU-PAGE (Lane 2, Figure 7.6A) and 2D SDS and then reduced SDS-PAGE of the corresponding sample (Figure 7.7B). The spots corresponding to both monomeric β -LG and monomeric κ -CN were relatively unaffected in this PAGE pattern, supporting the 2D SDS-PAGE results shown in Figure 7.7B.

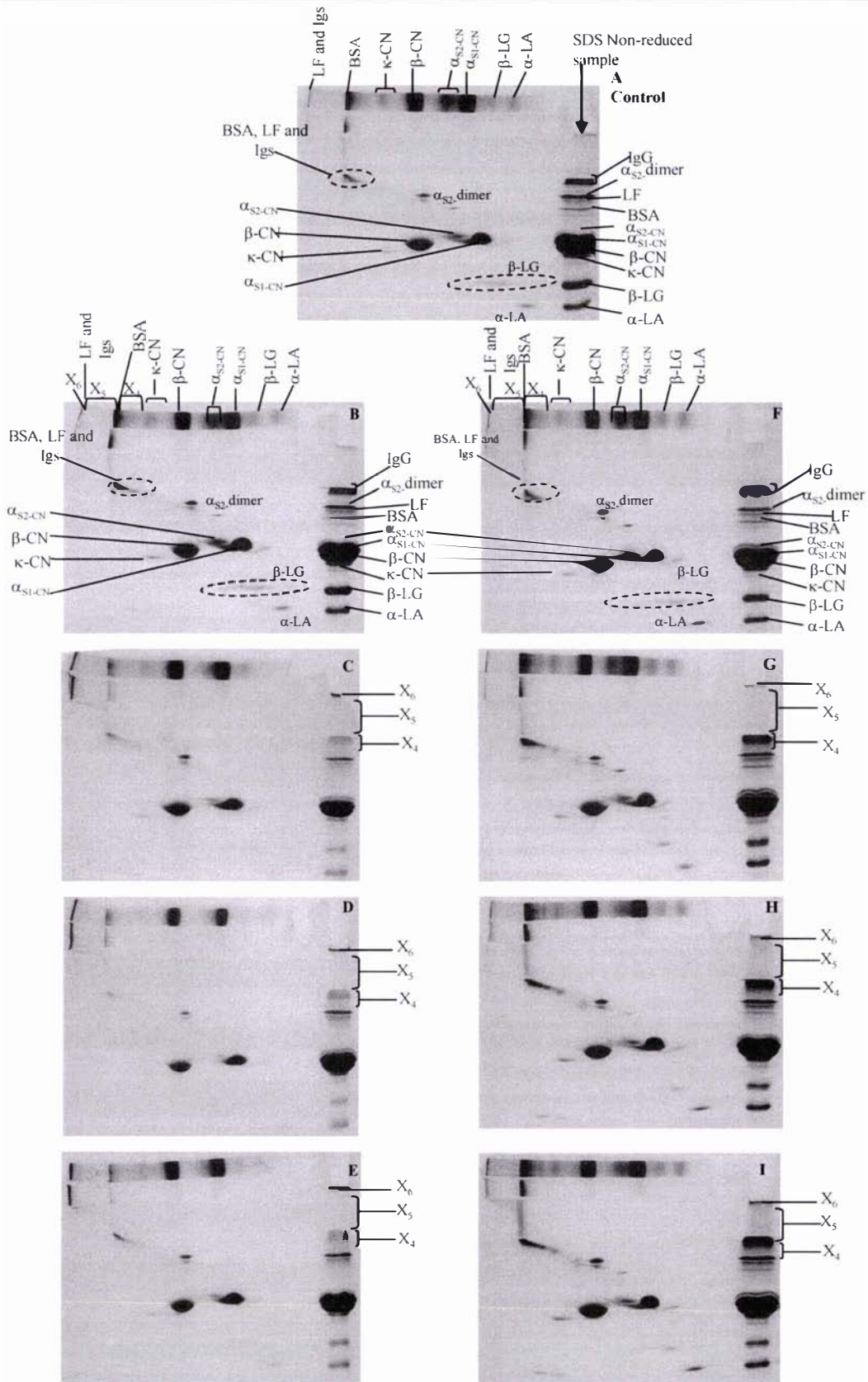


Figure 7.8. 2D AU- and then non-reduced SDS-PAGE patterns of the control sample (A) and samples heat treated at 72°C for 15 s (B), 100°C for 120 s (C), 120°C for 120 s (D) and 140°C for 5 s (E). Similarly, 2D PAGE patterns of samples pressure treated at 200 MPa for 30 min (F), 400 MPa for 30 min (G), 600 MPa for 30 min (H) and 800 MPa for 30 min (I).

The PAGE patterns of the samples heat treated at 100°C for 120 s, 120°C for 120 s and 140°C for 5 s (Figures 7.8C, 7.8D and 7.8E respectively) were similar to one another, although the intensities of the spots corresponding to both κ -CN and α_{s2} -CN diminished significantly after a heat treatment of 120°C for 120 s (Figure 7.8D), supporting the report that severely heat-treated milks (e.g. UHT-treated milk) contained β -LG: α_{s2} -CN complexes (Snoeren & Vander Spek, 1977).

AU:SDS-PAGE of pressure-treated samples

The 2D AU:SDS-PAGE pattern of skim milk pressure treated at 200 MPa for 30 min (Figure 7.8F) was similar to those of the control sample (Figure 7.5A) and the sample heat treated at 72°C for 15 s (Figure 7.8B). As the severity of the pressure treatment increased, the density of the low mobility band increased, with corresponding decreases in the spots corresponding to β -LG, κ -CN and monomer α_{s2} -CN (Figures 7.8F–7.8I). Because separation in a urea buffer smears some of the protein bands, this particular technique was found to be less useful for the whey proteins, but it was valuable for determining the roles/fates of the monomer, dimer and aggregates of α_{s2} -CN. After a pressure treatment of 400 MPa (Figure 7.8G), the spot corresponding to monomeric α_{s2} -CN was comparatively less dense. The intensity of the α_{s2} -CN spot further diminished after pressure treatments of 600 MPa (Figure 7.8H) and 800 MPa (Figure 7.8I), suggesting that α_{s2} -CN was involved in the pressure-induced interactions. The intensity of the spot corresponding to α_{s2} -CN monomer gradually decreased after higher pressure treatments, but that the intensity of the spot corresponding to α_{s2} -CN dimer did not change appreciably, suggesting that α_{s2} -CN dimer was comparatively more pressure resistant.

7.4 General discussion

The complexes of casein:whey protein complexes and the interaction pathways during heat treatment and pressure treatment of skim milk has not previously been studied in detail, partly because milk is a complex system in which all its components, including the caseins, whey proteins, lactose and milk salts, are present in their natural form. In particular, the caseins are present as casein micelles in which the caseins are held together by a combination of hydrophobic association and cross-linking via small calcium phosphate clusters (Holt & Horne, 1996; Horne, 1998).

Method development

In previous studies on whey protein systems, 2D (native and non-reduced SDS) PAGE has proven to be a suitable method for analysing heat-induced aggregates in model whey protein systems (Manderson et al., 1998; Havea et al., 2000, 2001; Schokker et al., 2000; Hong & Creamer, 2002) or in WPC solutions (Havea et al., 1998; Chapter 4). However, some initial experiments on the analysis of heat- and pressure-treated milk using 2D native and non-reduced SDS-PAGE showed that this 2D PAGE system was less than optimal for the separation of the milk proteins because the strong casein:casein association in the native-PAGE system prevented sufficient differentiation of the caseins, partly because the relatively high ratio of casein to whey protein in milk made it difficult to observe the whey proteins in the presence of the casein bands in the overloaded PAGE system. In addition, the SDS-PAGE system (e.g. Figure 7.5A, Lane 1), which worked well for whey protein samples (Havea et al., 1998, 2000, 2001; Chapter 4, Chapter 5), also poorly differentiated the SDS complexes of α_{s1} -CN, monomeric α_{s2} -CN, β -CN and monomeric κ -CN because they had similar mobilities. However, the 2D SDS and then reduced SDS-PAGE system (Figure 7.7) was suitable for showing that κ -CN was present in the large process-induced aggregates, which appeared as high molecular weight protein bands in the 1D SDS-PAGE in the first dimension.

Also, introduction of urea into the sample to be analysed and into the gel system (AU-PAGE) dispersed the caseins and separated them via their mobility at pH 8.5. The combination of AU-PAGE for the first dimension and SDS-PAGE for the second

dimension, e.g. Figure 7.8A, separated the various proteins and their heat- or pressure-induced aggregates effectively. This was particularly useful for analysing the various α_{s2} -CN and κ -CN polymers and aggregates, but less useful for analysing native β -LG, because disulphide bond interchange was facilitated in the AU-PAGE environment (pH 8.5, 5 M urea) (Figure 7.8A), thus giving rise to streaks of polymerising unfolded β -LG. Another modification was to set the sample in a large-pore polyacrylamide gel in the sample slot (Figure 7.5) [a procedure based on the concept of Davis (1964)], in order to retain the very high molecular weight aggregates that could not enter the gel, and therefore these aggregates could be analysed in detail during 2D electrophoretic analysis. When the set sample gel procedure was applied to the SDS:SDS^R-PAGE system (Figure 7.7), the protein aggregates that were trapped in the 1D sample gel strip (including those trapped in the set sample gel region) were reduced and the individual spots corresponding to reduced monomeric proteins were resolved in the second dimension. The components of these high molecular weight aggregates could then be determined; this showed the presence of both α_{s2} -CN and κ -CN, as well as β -LG and other whey proteins, in the pressure-induced aggregates (e.g. the series of new spots in the left-hand column resolved from region **X**₆ on the sample gel strip of Figure 7.7I), and showed the relative incorporation of these proteins in the aggregates induced by heat and pressure treatments.

Heat- and pressure-induced interactions of milk proteins

When β -LG and α -LA are present together at high temperature in model system or whey system, disulphide bond interchange can occur via a 'molten globule' state (e.g. Kuwajima, 1989; Griko, 2000; Lassalle et al., 2003), probably within a hydrophobic environment, as discussed in detail in Chapter 6 (also see Schokker et al., 2000; Hong & Creamer, 2002; Livney et al., 2003). However, in the milk environment, the situation is different; namely, there are two caseins, α_{s2} -CN and κ -CN, each of which contains two Cys residues: Cys 36 and 41 for α_{s2} -CN and Cys 11 and 88 for κ -CN (Farrell et al., 2004). α_{s2} -CN is naturally present as a monomer (with one intramolecular disulphide bond) and as a dimer (with two intermolecular disulphide bonds) (Hoagland et al., 1971; Rasmussen et al., 1992b, 1999). Although both forms are stable, the α_{s2} -CN dimer appears to be comparatively more stable in heat- or pressure-treated milks as shown in the results section (e.g. Figures 7.7D and 7.7H respectively). In contrast, κ -CN exists in

milk as a wide range of κ -CN polymeric species ranging from monomers to large polymers (with intermolecular disulphide bonds), with many in the 6–12 monomers per aggregate range (Rasmussen et al., 1992a; Farrell et al., 1998; Groves et al., 1998; Rasmussen et al., 1999).

The virtual absence of α_{s2} -CN from the heat-induced aggregates formed at 85–90°C in milk, as reported in the previous studies, might be because α_{s2} -CN is not a surface component of the micelle and therefore its disulphide bond(s) are inaccessible to the denaturing or denatured β -LG (Figure 7.9). On the other hand, κ -CN is on the surface of the micelles, and its disulphide bond(s) could be readily accessible to a thiol group of β -LG. Based on this fact, a diagrammatic representation of the consequences of the various inter-protein reactions that might take place during the heat treatment of milk at about 90°C is shown in Figure 7.9. α_{s2} -CN was apparent in the large aggregates (resolved from regions X_4 and X_6) in milk samples heated at temperatures above 100°C (Figures 7.7D and 7.7E), supporting an earlier report by Snoeren & Van der Spek (1977) and indicating that higher processing temperatures affect the proteins in a qualitatively different way.

In contrast, the effects of the pressure treatment of milk (Figure 7.10) were different from the effects of the heat treatment of milk (Figure 7.9). It can be postulated that both the casein micelles and the whey proteins are compressed at pressures up to about 150 MPa (Anema et al., 2005a, 2005b). Thereafter the micelles swell as the pressure increased up to 400 MPa, and hydrogen bonds (which are important in maintaining helix and sheet structures) and hydrophobic associations are diminished and colloidal calcium phosphate dissolves. As a consequence, the micelles absorb more water, swell and subsequently disperse (Anema et al., 2005a, 2005b).

At low pressure, β -LG loses some peripheral turns and the native dimer dissociates into native monomers. At higher pressures, it loses the major helix, and then some of the sheet structures, and finally the F, G and H motifs that are stable and contain the native Cys106–Cys119 disulphide bond, as discussed earlier in Chapter 6 and by Considine et al. (2006a, 2006b). A free thiol also becomes available to interact with previously inaccessible disulphide bonds and the intermediate species are probably very similar to

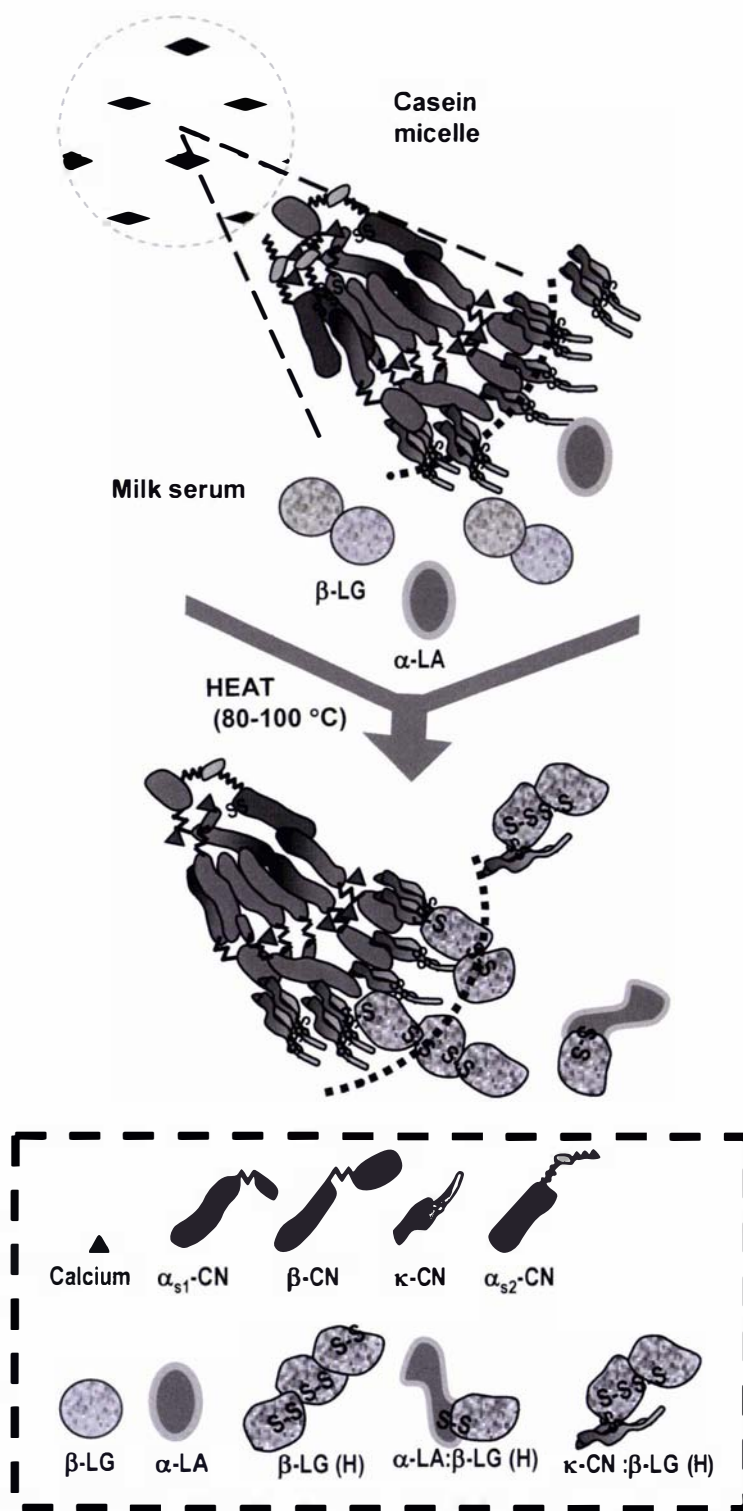


Figure 7.9. Pictorial representation of the likely effect of heating milk at 90°C. The native β-LG dimer dissociates and the monomer undergoes internal disulphide bond interchange to give reactive monomers that react with κ-CN at the surface (outer region) of the casein micelle. Native β-LG monomers can also form an adduct with α-LA, which then gives rise to α-LA dimers and α-LA:β-LG dimers. In the severely heat-treated samples, α_{s2}-CN also forms disulphide bonds with other proteins. (H) indicates possible heat-induced complexes.

the early heat-induced aggregates, but it is postulated that there is a stable disulphide-bonded dimer of β -LG (as well as a reactive dimer) that is not readily transformed into larger disulphide-bonded aggregates (Considine et al., 2005a, 2005b, 2006a, 2006b) as indicated in Figure 7.10, < 400 MPa. The present results show that, at 200 MPa, β -LG does not appear to become involved in interactions but is constrained to forming a stable, inactive, disulphide-bonded dimer (Figure 7.10) at < 400 MPa supporting the results of Considine et al. (2005b, 2006b). At higher pressures (> 400 MPa), the polymerisation of β -LG becomes the norm and the pressure-induced β -LG aggregation becomes similar to the heat-induced β -LG aggregation (Figure 7.9). This result shows that the β -LG in WPC (as discussed in Chapters 4 and 6) or in milk (present data) is not significantly modified by the other components, i.e. β -LG dominates the denaturation and aggregation pathway during pressure (> 400 MPa) treatment, as it has been shown to dominate the reaction at high temperature heat treatments.

Pressure treatment of milk at 200 MPa caused β -LG to form disulphide-bonded dimers and incorporated β -LG into aggregates, probably disulphide bonded to κ -CN. The other whey proteins appeared to be less affected at 200 MPa for 30 min. In contrast, pressure treatment at 800 MPa incorporated β -LG and most of the minor whey proteins, as well as κ -CN and much of the α_{s2} -CN, into large aggregates. The accessibility of α_{s2} -CN and the formation of complexes involving α_{s2} -CN, κ -CN and whey proteins in the pressure-treated milk is an important novel finding of the present study; this knowledge may explain the significant differences in the texture and functionalities of acid gel products made from heat and pressure treated milks. However, only a proportion of the α -LA was denatured or incorporated into the large aggregates. The relatively lower degree of α -LA reactivity at high pressures is probably related to the relative stability of this protein compared with β -LG, as discussed earlier in Chapters 4 and 6, and is based on the unusual pressure-dependent behaviour of α -LA (Kuwajima et al., 1990; Kobashigawa et al., 1999; Lasalle et al., 2003). These and other results show that the differences between the stabilities of the proteins and the accessibilities of the disulphide bonds of the proteins at high temperature or pressure affect the formation pathways that result in differences among the composition of resultant aggregation or interaction products (including their sizes) that ultimately may affect product functionalities.

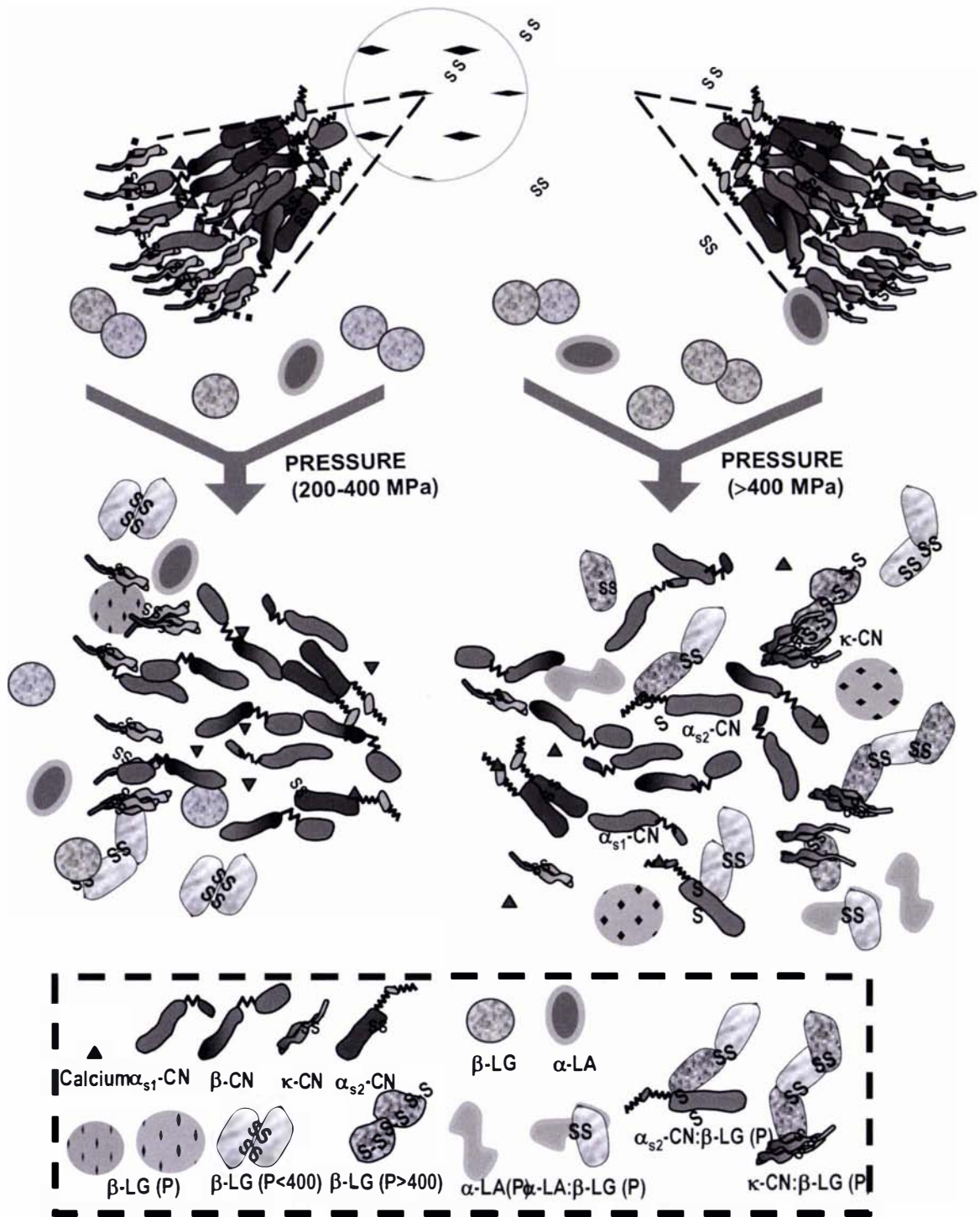


Figure 7.10. Pictorial representation of the likely effects of medium (≈ 250 – 400 MPa) and high (>400 MPa) pressure treatment at $\approx 22^\circ\text{C}$. The casein micelle swells at ≈ 250 MPa and β -LG unfolds and aggregates via disulphide bonds. β -LG forms disulphide-bonded dimers at lower pressure and probably aggregates with κ -CN, but does not form larger β -LG aggregate. The proportion of α -LA that is included in the aggregates is less than that of β -LG (Figure 7.5B) because it does not readily unfold. At pressures ≈ 400 – 600 MPa, α_{s2} -CN becomes available for thiol interchange reactions, assisted by the permeation of water into the micelle and the dissolution of the calcium phosphate. Also the β -LG molecules can polymerise into aggregates larger than dimers. (P) indicates possible pressure-induced complexes.

7.5 Conclusions

The modifications to the previously used PAGE methods were found to be useful in identifying the new disulphide-bonded species that were formed from the native proteins. Setting the samples in an open-pore gel (stacking gel mixture) prior to SDS-PAGE separation prevented the loss of very large aggregates during the PAGE analysis procedure so that the protein components of these aggregates could be determined after disulphide bond reduction. Consequently, it was possible to show that these large disulphide-bonded aggregates that were generated by high temperature ($> 100^{\circ}\text{C}$) or pressure ($> 400\text{ MPa}$) treatments of skim milk contained disulphide-linked complexes consisting of a high proportion of α_{s2} -CN as well as κ -CN, β -LG and other whey proteins.

The addition of urea to native-PAGE gels and to the treated milk samples dissociated the casein molecules (and the processing-induced protein aggregates) from natural, heat- or pressure-treated, micellar aggregates and the strong casein:casein hydrophobic associations, which allowed the α_{s2} -CN monomers and dimers to be identified and tracked.

The present study demonstrated that β -LG and other whey proteins interacted with α_{s2} -CN as well as κ -CN at high temperatures and at pressures greater than 400 MPa to form disulphide-linked high molecular weight complexes of casein and whey proteins. However, β -LG preferentially aggregated with κ -CN at 200 MPa. The accessibility of α_{s2} -CN and the formation of complexes involving α_{s2} -CN, κ -CN and whey proteins in the pressure-treated milk is an important novel finding of the present study; this finding may have significant implications for the functionality of product made from pressure treated milks. It was also found that dimer α_{s2} -CN was comparatively more stable than the monomer form after either high pressure treatment or high temperature treatment.

CHAPTER 8

Pressure-induced Interactions of Pure Proteins in a Model System

8.1 Introduction

Whey proteins are a heterogeneous group of proteins, with different amino acid compositions and molecular structures; it is therefore possible that the aggregation behaviour of the individual proteins is altered by the presence of other proteins. It has been demonstrated in many earlier studies that addition of bovine serum albumin (BSA) or α -lactalbumin (α -LA) to β -lactoglobulin (β -LG) (Matsudomi et al., 1991, 1993, 1994; Gezimati, 1995; Gezimati et al., 1996, 1997) increases the rigidity of the resultant gels because of the formation of disulphide-linked complexes between β -LG and α -LA or BSA. Some of the studies have also demonstrated that, in addition to disulphide-linked aggregation, non-covalently linked aggregates are formed in a heat-treated mixture of β -LG and BSA (e.g. Gezimati et al., 1995; 1996) or α -LA (Gezimati, 1995; Gezimati et al., 1997; Dalgleish et al., 1997; Schokker et al., 2000; Havea et al., 2001; Hong & Creamer, 2002). Havea et al. (2000), using binary and ternary mixtures of whey proteins, demonstrated the formation of new protein structures in heated mixtures of BSA and α -LA. Characterisation of heated mixtures of pure solutions of β -LG, α -LA or BSA, using one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), showed that homopolymers of each protein as well as heteropolymers of these proteins were observed (Havea et al., 2001). Extensive research has been conducted, using pure protein solutions in single, binary and ternary system to understand the synergistic effects of different milk proteins and their mixtures on heat-induced aggregation and gelation behaviour. However, there is comparatively little such information in the literature on the pressure-induced interactions of individual (pure solutions of β -LG, α -LA or BSA) whey proteins and how the pressure-induced denaturation and aggregation of one whey protein is affected by the presence of other whey proteins, when they are pressure treated in binary or ternary combinations.

Moreover, because of the complexity of a mixed whey protein system [whey protein concentrate (WPC) system], the role of β -LG, α -LA or BSA in the pressure-induced

aggregation and gelation of whey proteins when pressure treated in a WPC environment was not entirely clear from earlier results (Chapters 4–6). Therefore, some assumptions/speculations were made about the role of these proteins in the pressure-induced aggregation and gelation of WPC. Thus, it would be desirable to clarify the role of each protein in pressure-induced aggregation and interactions when a complex mixture of whey proteins (e.g. WPC) or caseins and whey proteins (e.g. milk) is pressure treated. This can be explored by studying the behaviour of the individual pure milk proteins alone and in binary and ternary mixtures after pressure treatment and analysing those using comparable methods. Therefore, the main aim of the present investigation was to use a model system to further explore the mechanisms of the pressure-induced interactions of different milk proteins.

8.2 Experimental

Details of the sources of the pure proteins, pressure treatment of the samples and analysis of the samples by 1D and 2D PAGE are given in Chapter 3. Specific details of the experimental plan followed in the present study are outlined in Figure 8.1.

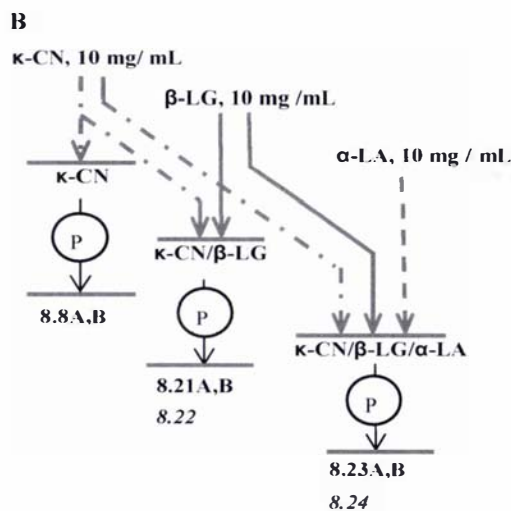
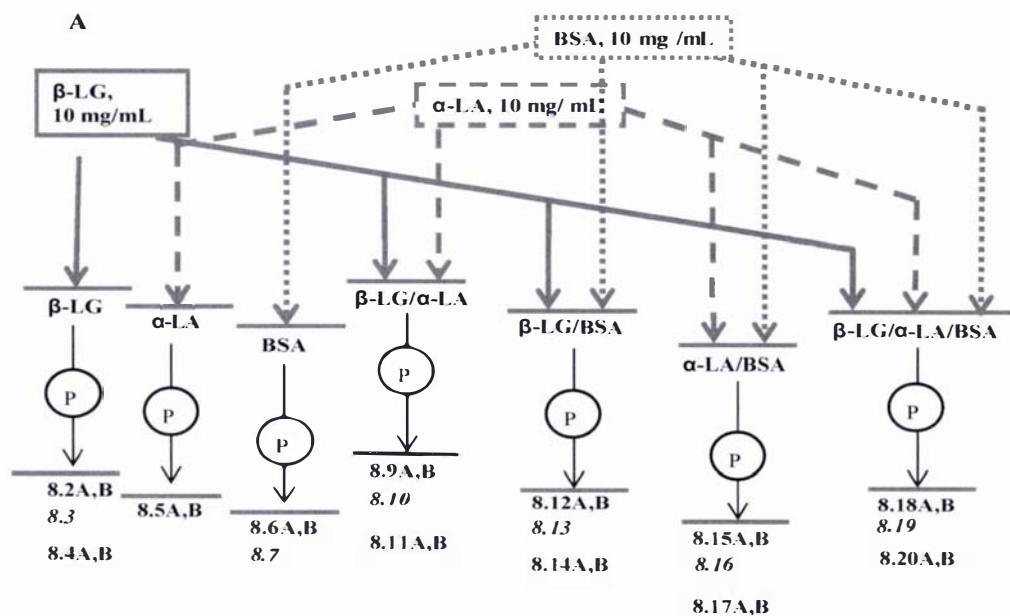


Figure 8.1. Outline of the experimental plan used in the present study. P indicates pressure treatment at 600 MPa of samples at 600 MPa for 5-60 min and the numbers given are respective figure number.

8.3 Results and discussion

8.3.1 Pressure treatment of pure protein solutions

β -Lactoglobulin

ID PAGE

The native- and SDS-PAGE patterns of control and pressure-treated β -LG solutions are presented in Figures 8.2A and 8.2B respectively. Lane 1 in each figure is the PAGE pattern of the untreated (control) sample and Lanes 2–8 are the PAGE patterns of samples held at 600 MPa for various times ranging from 5 to 60 min.

Comparison of the native-PAGE pattern of the control sample (Lane 1, Figure 8.2A) with that of the pressure-treated samples (Lanes 2–8, Figure 8.2A) showed that the intensity of the band corresponding to native-like β -LG decreased significantly after the initial pressure treatment of 5 min at 600 MPa (Lane 2, Figure 8.2A), with the simultaneous appearance of several new bands (corresponding to non-native monomers, β -LG dimers, trimers, tetramers etc.). This trend was similar to the results reported previously (Funtenerger et al., 1995). These newly formed bands in the pressure-treated samples are marked appropriately on the right-hand side of the PAGE pattern. The intensity of the band corresponding to native-like β -LG continued to decrease and simultaneously the new bands became more intense upon extended holding times at 600 MPa. Extended holding of the samples at 600 MPa also generated significant proportions of high molecular weight aggregates that were resolved at the top of the resolving gel (marked as X₄, Figure 8.2A) and some polymers that were caught up within the stacking gel (marked as X₅, Figure 8.2A). However, it appeared that very high molecular weight material (that could not enter the gel, X₆) was not formed in these pressure-treated samples, even after pressure treatment of dilute β -LG solutions at 600 MPa for 60 min. These results are different from that reported by Havea et al. (2001) for heat-treated β -LG samples; in this case, significant proportions of very large aggregates, including aggregates that could not enter the gel, were formed and there were comparatively fewer intermediate-sized species such as dimers, trimers and tetramers of β -LG.

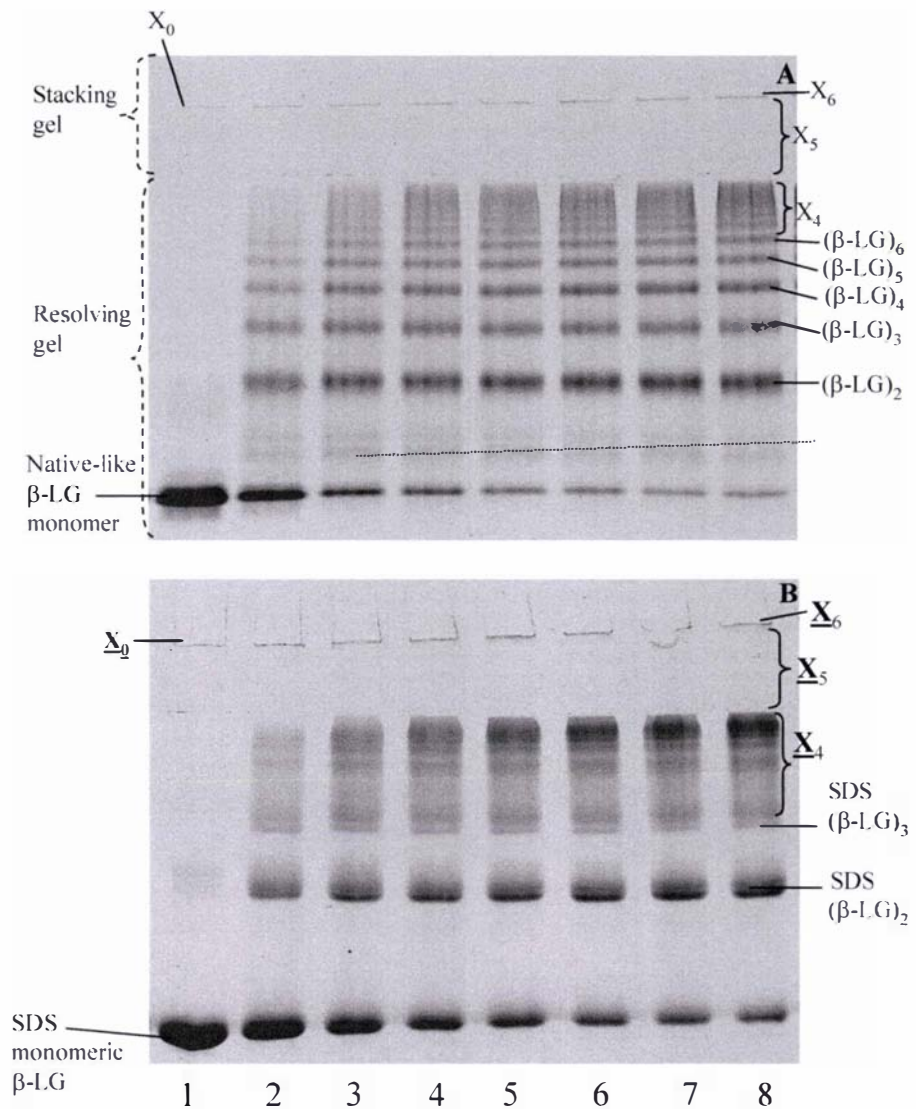


Figure 8.2. (A) Native-PAGE and (B) SDS-PAGE patterns of pure β -Lg AA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

Comparison of the SDS-PAGE pattern of the control sample (Lane 1, Figure 8.2B) with those of the pressure-treated samples (Lane 2–8, Figure 8.2B) indicated a similar trend to that observed for native-PAGE (Figure 8.2A), i.e. a decreased intensity of the band corresponding to SDS-monomeric β -Lg and the simultaneous appearance of new bands in the PAGE patterns of the pressure-treated samples. However, only bands corresponding to β -Lg dimer (molecular weight approximately 37 kDa) were

distinguishable in the SDS-PAGE patterns; bands corresponding to trimers, tetramers, pentamers and hexamers, as observed on the native-PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.2A), were not clearly distinguishable on the SDS-PAGE patterns of the corresponding samples (Lanes 2–8, Figure 8.2B). Instead, a dark-staining blurred region (marked as X₄) corresponding to aggregated protein species was observed between the band corresponding to SDS β -LG dimer and the top of the resolving gel, and the intensity of this region increased with extended holding time. However, small proportions of high molecular weight aggregates that were caught up within the stacking gel (marked as X₅) or that could not enter the gel (marked as X₆) were observed in the PAGE patterns of the severely pressure-treated samples (e.g. Lane 8, Figure 8.2B). This result for SDS-PAGE was also different from that reported by Havea et al. (2001) for heat-treated samples.

The intensities of the bands corresponding to residual native-like and SDS-monomeric β -LG were measured in the PAGE pattern of the control and pressure-treated samples using a laser densitometer (Figure 8.3). The plot suggested that there was a significant decrease in the intensities of the bands corresponding to residual native-like and SDS-monomeric β -LG (approximately 60% and 45% respectively) after initial holding (5 min) of the sample at 600 MPa. A gradual decrease was observed on further holding at 600 MPa for up to 40 min, with no further decrease after 40 min. The fast decrease in the native-like and SDS-monomeric β -LG during the initial period of pressure treatment may be attributed to adiabatic heating during pressurisation of the samples and the time taken to return to the preset temperature, as discussed in Chapter 5.

The higher concentration of residual SDS-monomeric β -LG compared with that of residual native-like β -LG suggested that a proportion of the aggregates were non-covalently linked. The difference between residual SDS-monomeric β -LG and residual native-like β -LG was greater (approximately 18%) in the initial \approx 10–20 min period of pressure treatment, and was gradually lowered to approximately 6% at extended holding times.

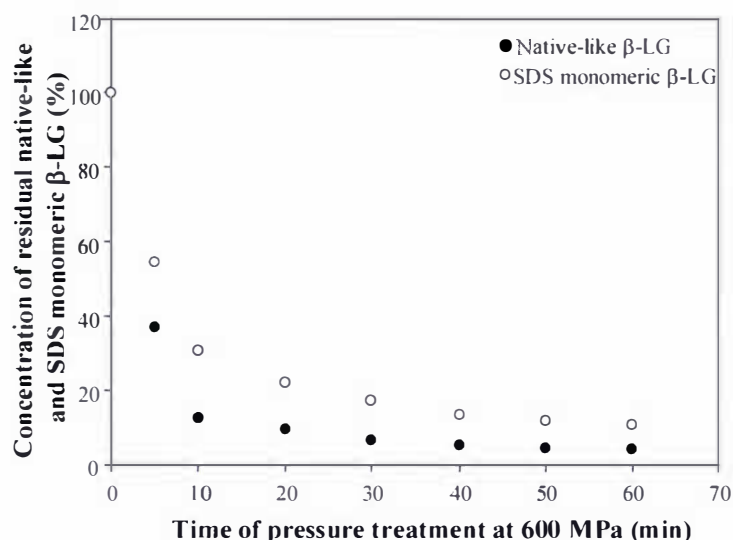


Figure 8.3. Concentrations of native-like (●) and SDS-monomeric (○) β -LG in solutions (10 mg/mL) of pure β -LG treated at 600 MPa for 0–60 min.

2D PAGE

The pressure-induced aggregates observed on 1D native- and SDS-PAGE were further characterised using 2D native:SDS-PAGE and 2D SDS:reduced SDS-PAGE (Figure 8.4). When 1D strips having lower sample loadings were used, it was difficult to observe the changes, particularly the minor changes in the proteins on the corresponding 2D PAGE. Therefore, in order to visualise majority of the changes clearly, the 1D PAGE strips (intended for use in subsequent 2D PAGE analysis) were overloaded. Thus, the stained gel strip (a'') used on each of the 2D PAGE patterns looks darker. The lower histogram settings used to generate scanned image in the ImageQuant program were also partly responsible for the darker look of the stained gel strip (a'').

The 2D native:SDS-PAGE pattern of the control sample of β -LG was that expected, with only a single large native-like β -LG spot. The 2D native:SDS-PAGE patterns of the pressure-treated sample (Figure 8.4A) showed that the intensity of the spot corresponding to monomeric β -LG was lowered considerably, with the simultaneous appearance of a series of new spots (on a diagonal). These spots were resolved from the bands corresponding to β -LG dimer, trimer, tetramer on the 1D native gel strip (marked as the sample gel strip a') under the dissociating environment (SDS-PAGE) in the second dimension.

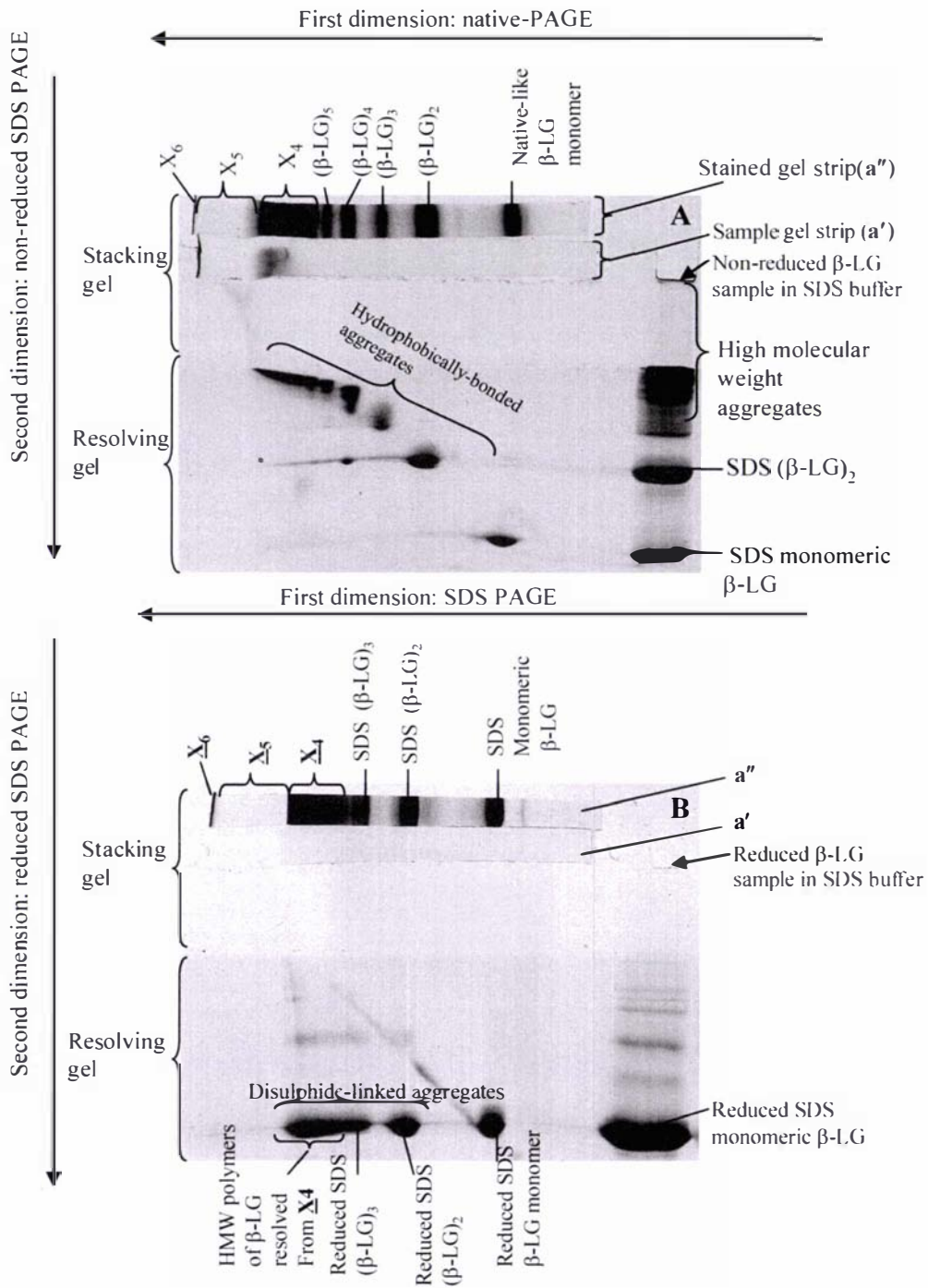


Figure 8.4. 2D (A) native:SDS-PAGE and (B) SDS:reduced SDS-PAGE patterns of a β -LG solution pressure treated at 600 MPa for 60 min.

Also, a dark streak corresponding to large polymers (resolved from the region marked as X₄ on the 1D native gel strip) was resolved at the top of the resolving gel of the 2D PAGE, indicating that pressure treatment of pure dilute β -LG solutions generated non-covalently linked (mainly hydrophobically bonded) dimers, trimers as well as high molecular weight polymers.

The 2D SDS:reduced SDS-PAGE patterns of the pressure-treated samples (Figure 8.4B) showed that the intensity of the spot corresponding to SDS-monomeric β -LG decreased significantly, with the simultaneous appearance of a horizontal streak containing a series of new spots that were resolved from the region between SDS-monomeric β -LG and the top of the resolving gel of the reduced sample gel strip (a'). These new spots were resolved from the bands that corresponded to dimers, trimers, tetramers and higher polymers of the stained gel strip (a'') after treatment of the 1D SDS gel strip with 2-mercaptoethanol, indicating that disulphide-linked dimers, trimers, tetramers and higher polymers of β -LG were induced by pressure treatment.

These results from the 2D native:SDS-PAGE and SDS:reduced SDS-PAGE patterns showed that pressure treatment of pure β -LG generated both hydrophobically bonded and disulphide-linked aggregates of β -LG. These results for pressure-treated samples were comparable with those reported for heat-treated pure β -LG solutions (Manderson et al., 1998; Havea et al., 2001).

α -Lactalbumin

1D PAGE

The native- and SDS-PAGE patterns of pure α -LA solutions pressure treated at 600 MPa for 0–60 min are presented in Figures 8.5A and 8.5B respectively. It was observed that the native-PAGE patterns of the untreated control (Lane 1, Figure 8.5A) and the pressure-treated (Lanes 2–8, Figure 8.5A) α -LA solutions were almost identical, suggesting that there were no effects when pure α -LA solutions were pressure treated.

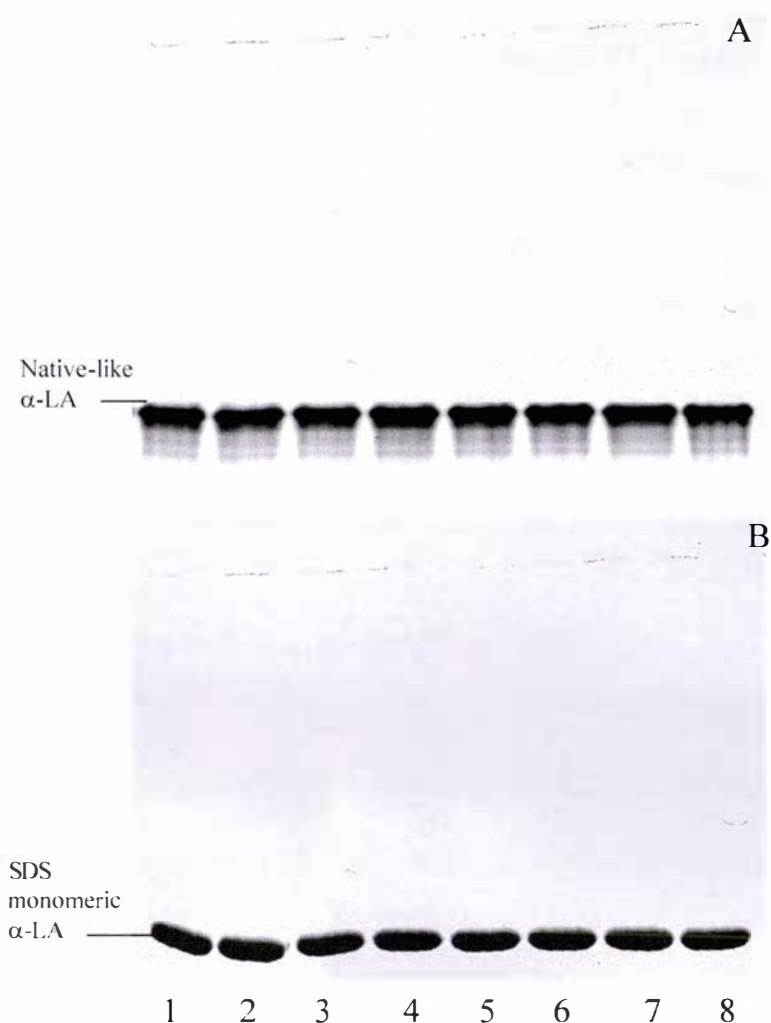


Figure 8.5. (A) Native-PAGE and (B) SDS-PAGE patterns of pure α -LA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

Similarly, no changes were observed on the SDS-PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.5B). Unlike β -LG, α -LA does not contain a free sulphhydryl group and therefore does not readily initiate the sulphhydryl–disulphide interchange reaction and considering this fact this result was as expected. These results for pressure-treated α -LA were different from those for heat-treated samples, in which the formation of hydrophobic and/or disulphide-linked aggregates of α -LA was reported (Chaplin & Lyster, 1986; Havea et al., 2000; Hong & Creamer, 2002; McGuffey et al., 2005).

As there were no apparent changes in the 1D PAGE patterns of pressure-treated α -LA samples, the intensities of the bands corresponding to residual native-like and SDS-monomeric α -LA were not measured and the pressure-treated samples were not further characterised using 2D PAGE.

Bovine Serum Albumin

1D PAGE

The native-PAGE pattern of the untreated control sample suggested that some of the BSA existed as a polymer (marked as p on the PAGE pattern) (Lane 1, Figure 8.6A).

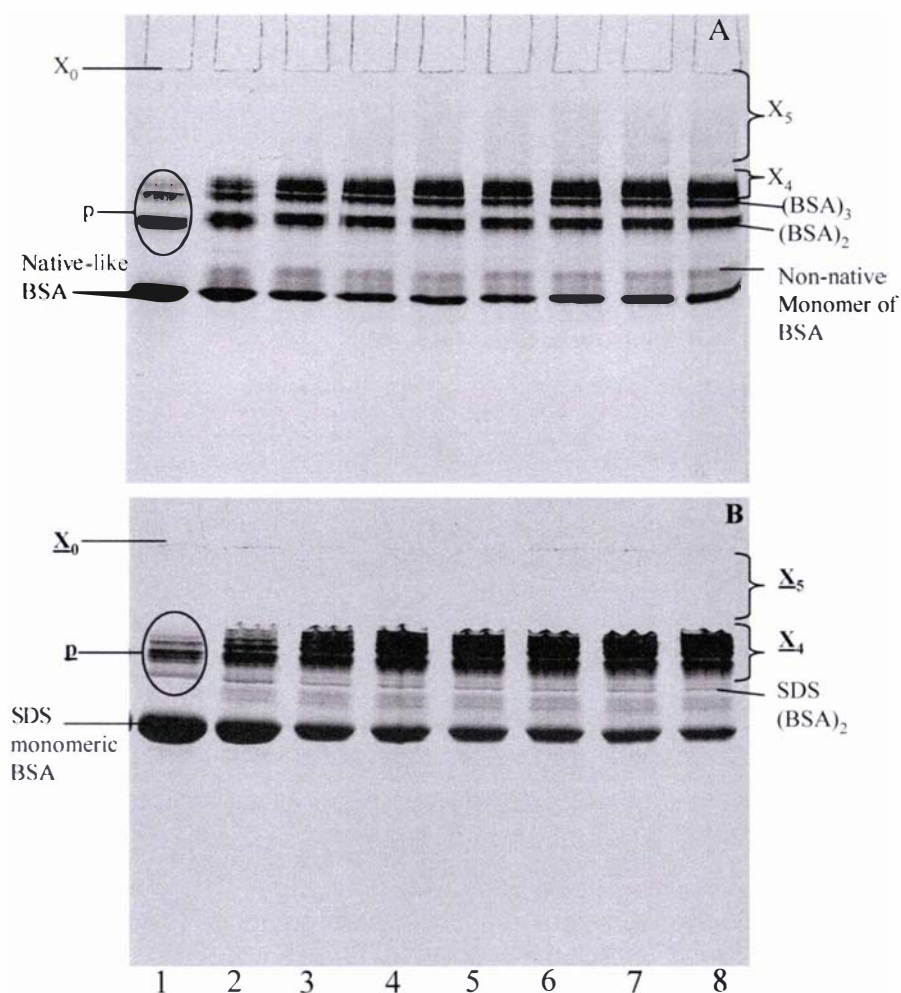


Figure 8.6. (A) Native-PAGE and (B) SDS-PAGE patterns of pure BSA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

It was observed that the intensity of the band corresponding to native-like BSA decreased significantly in the native PAGE pattern of pressure-treated samples (Lanes 2-8, Figure 8.6A) and that the new bands appeared between the band corresponding to native-like BSA and the top of the resolving gel. These results suggested that like β -LG, BSA also self-aggregated and formed non-native monomers, dimers, trimers and other high molecular weight polymers that were marked as X_4 and X_5 ; the proportion of such aggregates increased with longer holding times of the samples. The SDS-PAGE patterns of the pressure-treated samples (Lanes 2-8, Figure 8.6B) also indicated that the intensity of the band corresponding to SDS-monomeric BSA decreased and that some high molecular weight aggregates, which were mainly caught up as a dark-staining region (marked as X_4) near the top of the resolving gel, were observed. Unlike on the native-PAGE patterns of the pressure-treated samples (Lanes 2-8, Figure 8.6A), distinct bands corresponding to intermediate-sized protein aggregates were not observed clearly on corresponding SDS PAGE patterns (Lanes 2-8, Figure 8.6B).

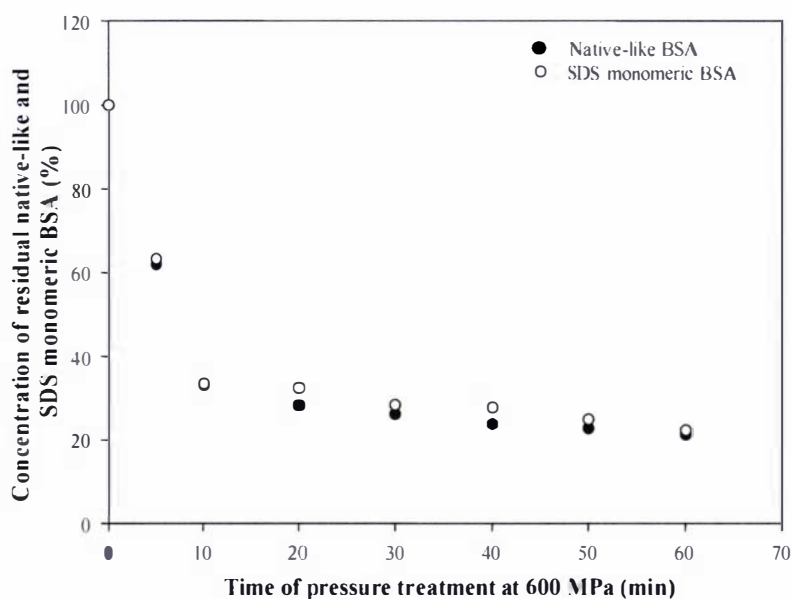


Figure 8.7. Concentrations of native-like (●) and SDS-monomeric (○) BSA in solutions (10 mg/mL) of pure BSA treated at 600 MPa for 0–60 min.

Plots (Figure 8.7) of the relative intensities of the bands corresponding to residual native-like BSA (Figure 8.6A) and residual SDS-monomeric BSA (Figure 8.6B) in the pressure-treated samples indicated that there was a sharp decline (approximately 65%) in the concentration of native-like and SDS-monomeric BSA during the initial 10 min holding period of the samples at 600 MPa. Further, it was observed that the concentrations of residual native-like and SDS-monomeric BSA in the pressure-treated samples were almost similar; suggesting that pressure treatment of pure BSA did not generate non-covalently bonded aggregates. This finding was different from the results for β -LG solutions, which showed that both non-covalently linked and covalently linked aggregates were present in the pressure-treated solutions (Figure 8.3).

κ -Casein

ID PAGE

κ -Casein (κ -CN) did not generate a satisfactory native-PAGE pattern, partly because significant proportions of κ -CN present in the control sample existed as κ -CN polymers. The band corresponding to monomeric κ -CN was not observed on the native PAGE patterns. The pressure treatment further polymerises the κ -CN and also generated some insoluble polymers of κ -CN (Figure 8.8A). The SDS-PAGE pattern of the control sample (Lane 1, Figure 8.8B) also showed that a small proportion of the κ -CN was monomeric κ -CN (molecular weight 24 000 Da), but that most of the κ -CN was in a polymeric form that was resolved in the regions marked as **X₄** and **X₅**. The SDS-PAGE pattern of pressure-treated samples (Lanes 2–7, Figure 8.8B) indicated that the proportion of monomeric κ -CN was not much affected by the pressure treatment, whereas the polymeric κ -CN aggregated further as a result of the pressure treatment to yield even higher polymers, as shown by the increasing proportion of polymeric material, marked as **X₅** and **X₆** (Lanes 2–7, Figure 8.8B). This result suggested that κ -CN self-aggregated to form very large polymers as a result of pressure treatment. It was not possible to carry out quantitative analysis of the monomeric κ -CN as most of the κ -CN already existed in a polymeric form in both the pressure-treated samples and the untreated control sample.

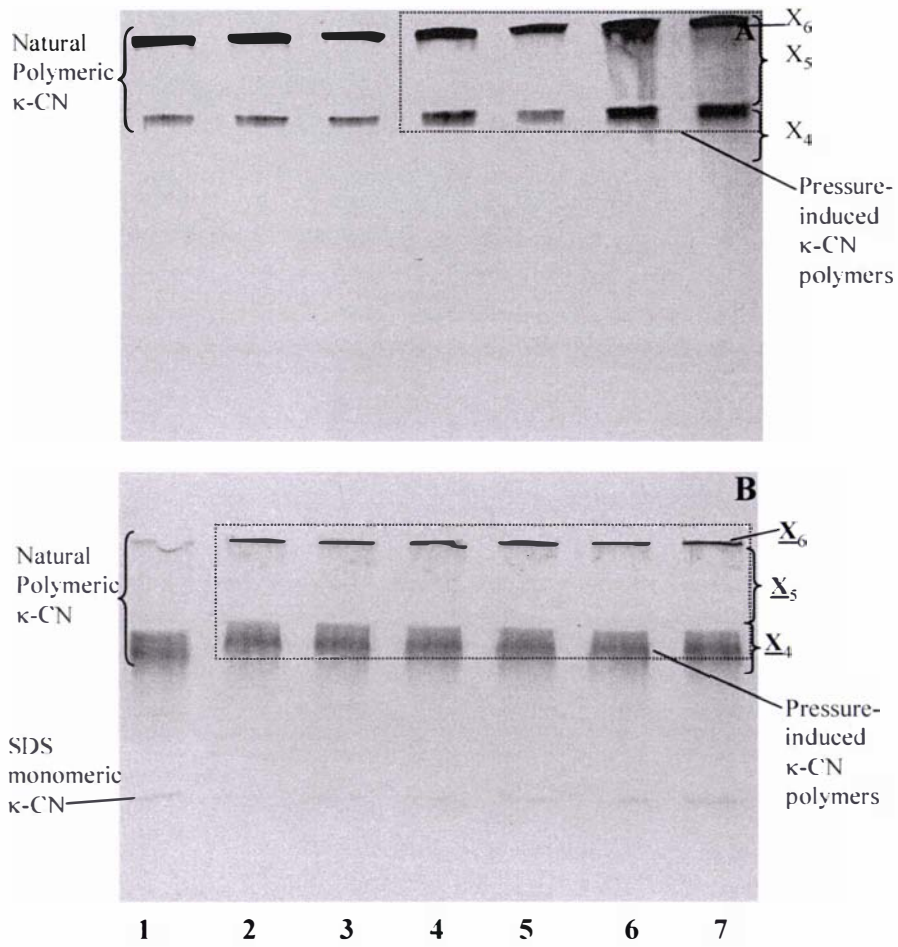


Figure 8.8. (A) Native-PAGE and (B) SDS-PAGE patterns of pure κ -CN solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6) and 50 min (Lane 7).

8.3.2 Pressure treatment of binary and ternary mixtures of pure proteins

Mixture of β -LG and α -LA

ID PAGE

Distinct bands corresponding to native-like β -LG and α -LA were clearly resolved on the native- (Lane 1, Figure 8.9A) and SDS- (Lane 1, Figure 8.9B) PAGE patterns of the control samples containing a binary mixture of pure β -LG and α -LA.

Upon pressure treatment, the intensities of the bands corresponding to native-like β -LG and α -LA decreased sharply (Lanes 2–8, Figure 8.9A). The extent of the decrease in the intensities of the bands corresponding to native-like β -LG and α -LA was comparatively greater and faster when a 1:1 binary mixture of β -LG and α -LA was pressure treated (Lanes 2–8, Figure 8.9A) than when solutions of pure β -LG (Lanes 2–8, Figure 8.2A) or pure α -LA (Lanes 2–8, Figure 8.5A) were pressure treated in isolation. This decrease in the intensities of the bands corresponding to native-like β -LG and α -LA in the pressure-treated samples (Lanes 2–8, Figure 8.9A) coincided with the appearance of several new bands, as labelled on the right-hand side of the native-PAGE pattern (Figure 8.9A). High molecular weight aggregates, marked as X_4 and X_5 , were also observed on the PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.9A).

The SDS-PAGE patterns of pressure-treated samples of a binary mixture of β -LG and α -LA (Lanes 2–8, Figure 8.9B) were very similar to those of a pressure-treated β -LG solution (Lanes 2–8, Figure 8.2B), except that the decrease in the intensity of the band corresponding to SDS-monomeric β -LG was comparatively faster when a 1:1 binary mixture of β -LG and α -LA was pressure treated than when a pure β -LG solution was pressure treated in isolation (Lanes 2–8, Figure 8.2B). Similarly, the intensity of the band corresponding to SDS-monomeric α -LA decreased faster when a 1:1 binary mixture of β -LG and α -LA was pressure treated (Lane 2–8, Figure 8.9B) than when pure α -LA was pressure treated alone in which α -LA was not affected (Lane 2–8, Figure 8.5B). Also, comparatively greater proportions of high molecular weight aggregates, marked as X_4 , X_5 and X_6 , were observed on the PAGE pattern when a

binary mixture of β -LG and α -LA was pressure treated than when β -LG (Figure 8.2B) or α -LA (Figure 8.5B) alone was pressure treated.

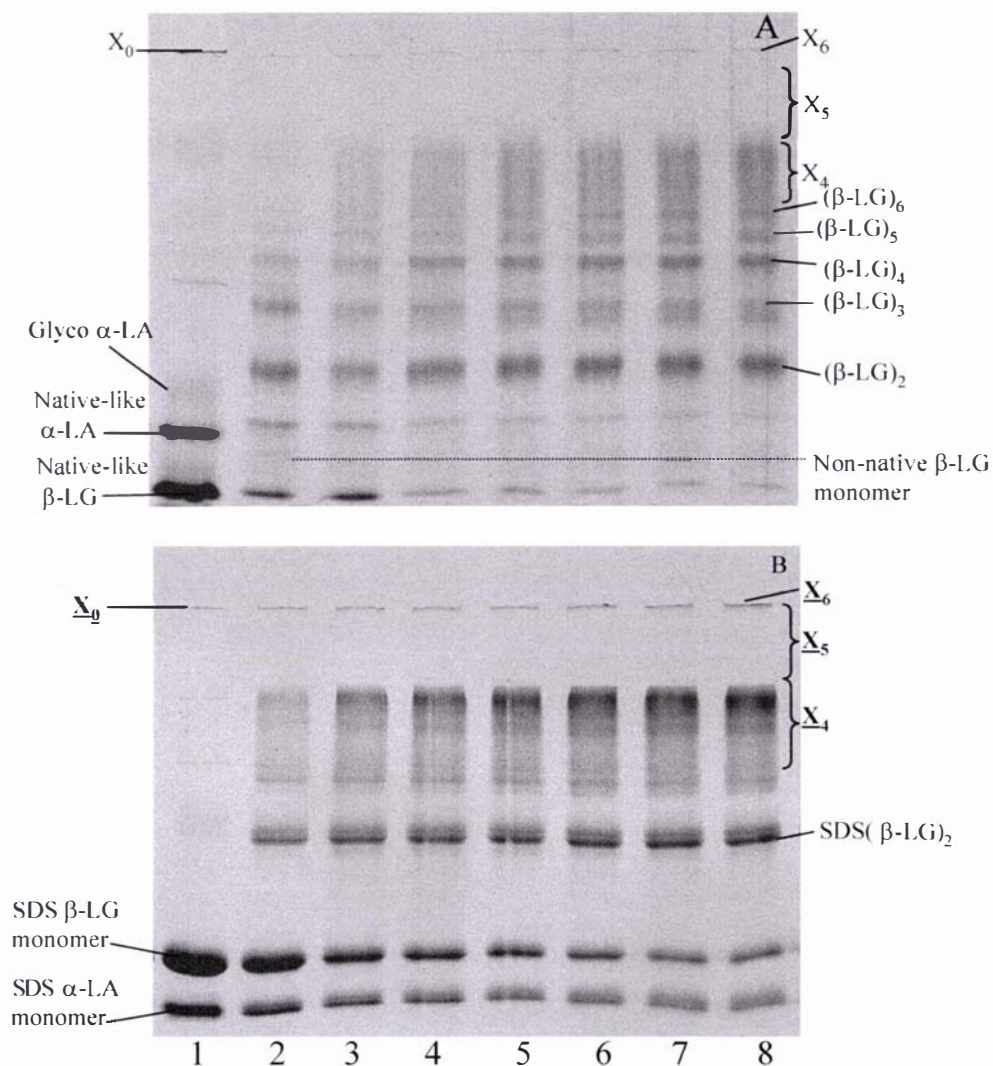


Figure 8.9. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1 binary mixture of pure β -LG and α -LA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

The plotted intensities (Figure 8.10) of the bands corresponding to native-like (Figure 8.9A) and SDS-monomeric (Figure 8.9B) β -LG and α -LA in the control and pressure-treated solutions indicated a sharp decline in the concentration of native-like β -LG and α -LA after a pressure treatment of 5–10 min. It was observed that the concentrations of native-like β -LG and α -LA decreased at almost similar rates throughout the pressure treatment. This behaviour of α -LA when a binary mixture of β -LG and α -LA was pressure treated was quite different from that when pure α -LA alone was pressure treated. On the other hand, the concentration of SDS-monomeric β -LG and, in particular, SDS monomeric α -LA decreased comparatively slowly, indicating that pressure treatment of a binary mixture of β -LG and α -LA generated a significant proportion of hydrophobically linked aggregates of β -LG and α -LA, and the greater proportion of such aggregates was contributed by α -LA rather than by β -LG.

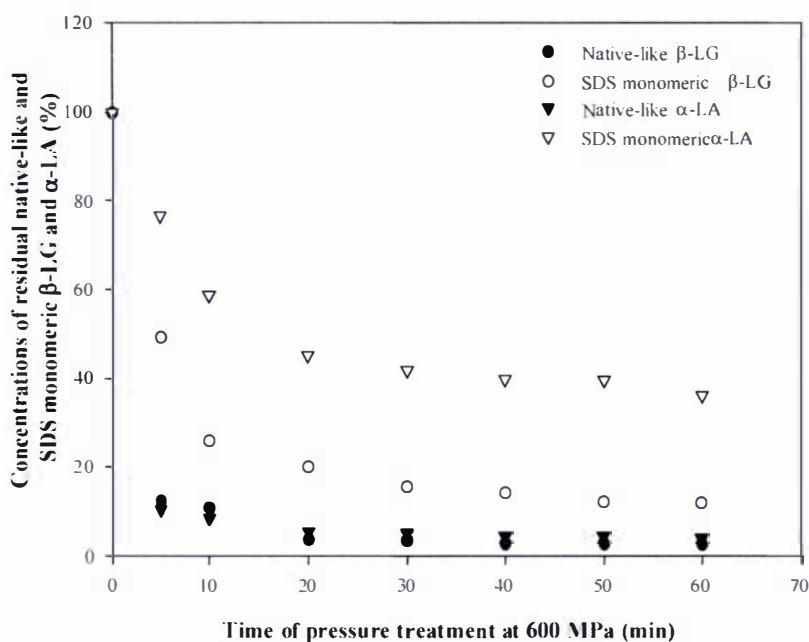


Figure 8.10. Concentrations of native-like β -LG (\bullet), SDS-monomeric β -LG (\circ), native-like α -LA (\blacktriangledown) and SDS-monomeric α -LA (\triangledown) in solutions (10 mg/mL) of a 1:1 binary mixture of pure β -LG and α -LA treated at 600 MPa for 0–60 min.

2D PAGE

The 2D PAGE patterns of pressure-treated samples of a 1:1 binary mixture of β -LG and α -LA are presented in Figure 8.11.

The 2D native:SDS-PAGE pattern (Figure 8.11A) showed the presence of a series of new spots (a diagonal series), resolved from the pressure-induced aggregates that were present in the region (marked as X_4 on the 1D native stained gel strip, a'') between the native-like α -LA band and the top of the resolving gel of the 1D native sample gel strip (a'). These new spots were resolved when the 1D native sample gel strip (a') was run on SDS-PAGE in the second dimension (i.e. in a dissociating environment), suggesting that significant proportions of hydrophobically linked aggregates were generated by pressure treatment of a 1:1 binary mixture of β -LG and α -LA. This observation confirmed the results of 1D PAGE presented in Figure 8.10. There was some material still present at the top of the resolving gel (that was not dissociated by SDS). Similarly, the 2D SDS:reduced SDS-PAGE pattern (Figure 8.11B) showed a series of new spots (as an almost continuous horizontal streak) that were resolved from the corresponding bands present in the region between the β -LG band and the top of the resolving gel of the reduced 1D SDS sample gel strip (a'). These new spots were identified as reduced SDS β -LG dimer, α -LA dimer and high molecular weight disulphide-bonded copolymers of β -LG and α -LA generated by the pressure treatment. These results were comparable with those reported earlier (Gezimati, 1995; Gezimati et al., 1997; Havea et al., 2001) in a heated binary mixture of β -LG and α -LA. The results of 2D PAGE confirmed the results of 1D PAGE (Figure 8.10) showing that the pressure treatment of a binary mixture of β -LG and α -LA generated both hydrophobically linked and disulphide-linked polymers of both β -LG and α -LA.

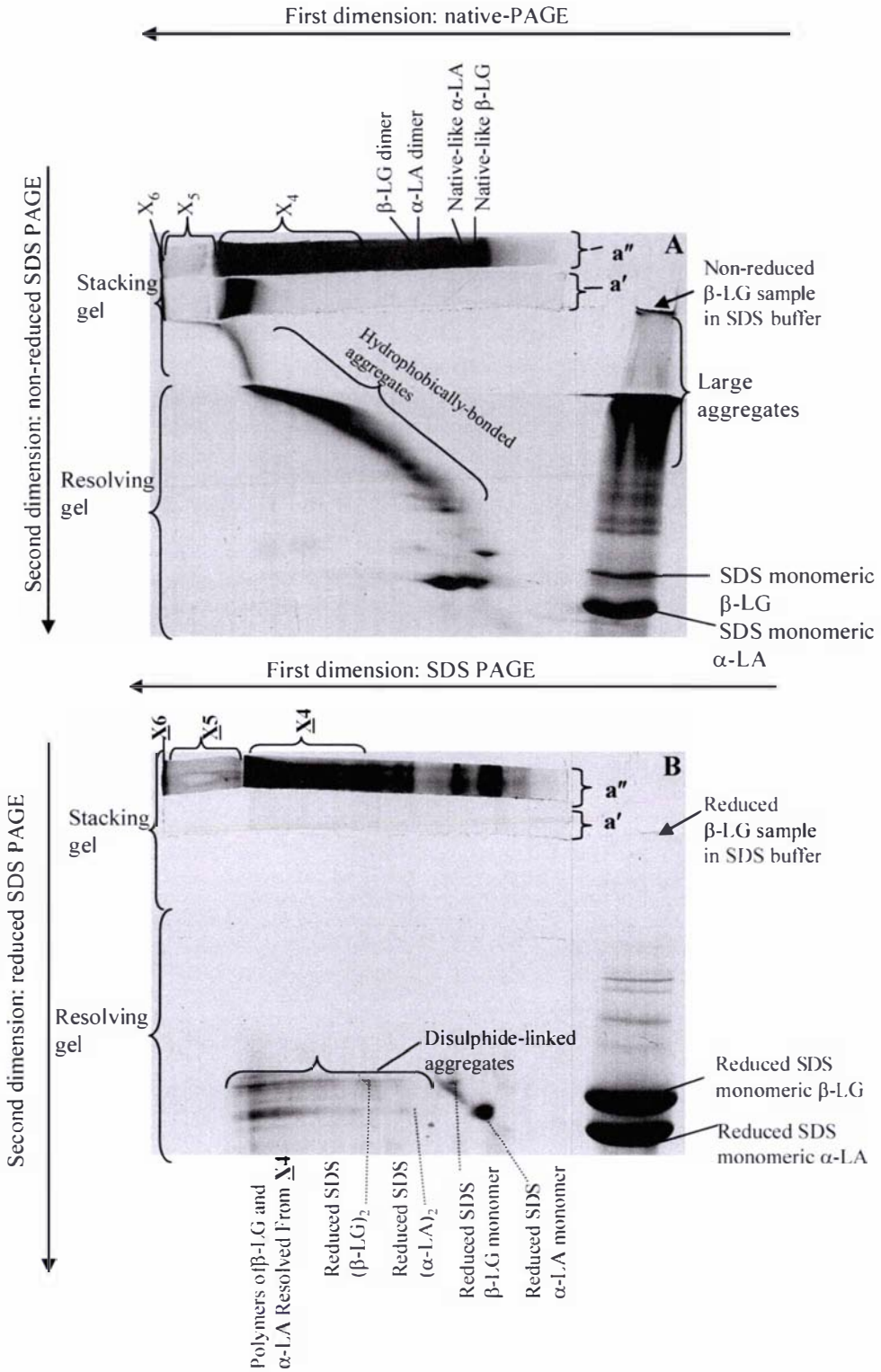


Figure 8.11. 2D (A) native:SDS-PAGE and (B) SDS:reduced SDS-PAGE patterns of a solution of a 1:1 binary mixture of β -LG and α -LA pressure treated at 600 MPa for 60 min.

Mixture of β -LG and BSA

1D PAGE

The native-PAGE patterns of control and pressure-treated samples of a 1:1 binary mixture of β -LG and BSA (Figure 8.12A) showed that the intensities of the bands corresponding to native-like β -LG and BSA decreased sharply in the PAGE pattern of the pressure-treated samples (Lanes 2–8, Figure 8.12A). The band corresponding to BSA almost diminished slowly in the PAGE pattern of the sample pressure treated for 40 min or longer at 600 MPa (Lanes 5-8, Figure 8.12A), whereas a faint band corresponding to β -LG was still observable in the PAGE pattern of the sample pressure treated for 60 min at 600 MPa. These results suggested that, when a binary mixture of BSA and β -LG was pressure treated at 600 MPa, the loss of BSA was greater and faster than that of β -LG. It appeared that almost all the BSA interacted with β -LG during the early stages of pressure treatment. This behaviour exhibited by BSA after severe pressure treatment was almost similar to the behaviour of BSA after heat treatment (Havea et al., 2001). This trend of the results confirmed that the behaviour of BSA was quite different when a mixture of whey proteins (WPC solution) was subjected to different pressure treatments (Chapters 4–6) than when a pure BSA solution alone was pressure treated (i.e. in the absence of β -LG; see Figures 8.6A and 8.6B), confirming that the presence of other whey proteins (particularly β -LG) in the WPC system catalyses the aggregation and interactions of BSA with other proteins. Several new discrete bands corresponding to a range of aggregates, including dimers, trimers, tetramers of β -LG, co-polymers of β -LG and BSA as well as high molecular weight polymers (marked as X₄ and X₅ respectively), were observed on the PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.12A).

A similar trend was apparent on the corresponding SDS-PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.12B). However, in contrast to the native-PAGE patterns (Figure 8.12A), only a distinct band corresponding to β -LG dimer was clearly observable. The native and SDS PAGE patterns of a pressure treated mixture of 1:1 binary mixture of β -LG and BSA were similar to those of native and SDS PAGE patterns of pressure treated pure β -LG solutions, indicating that β -LG dominated the aggregation behaviour of the mixed system.

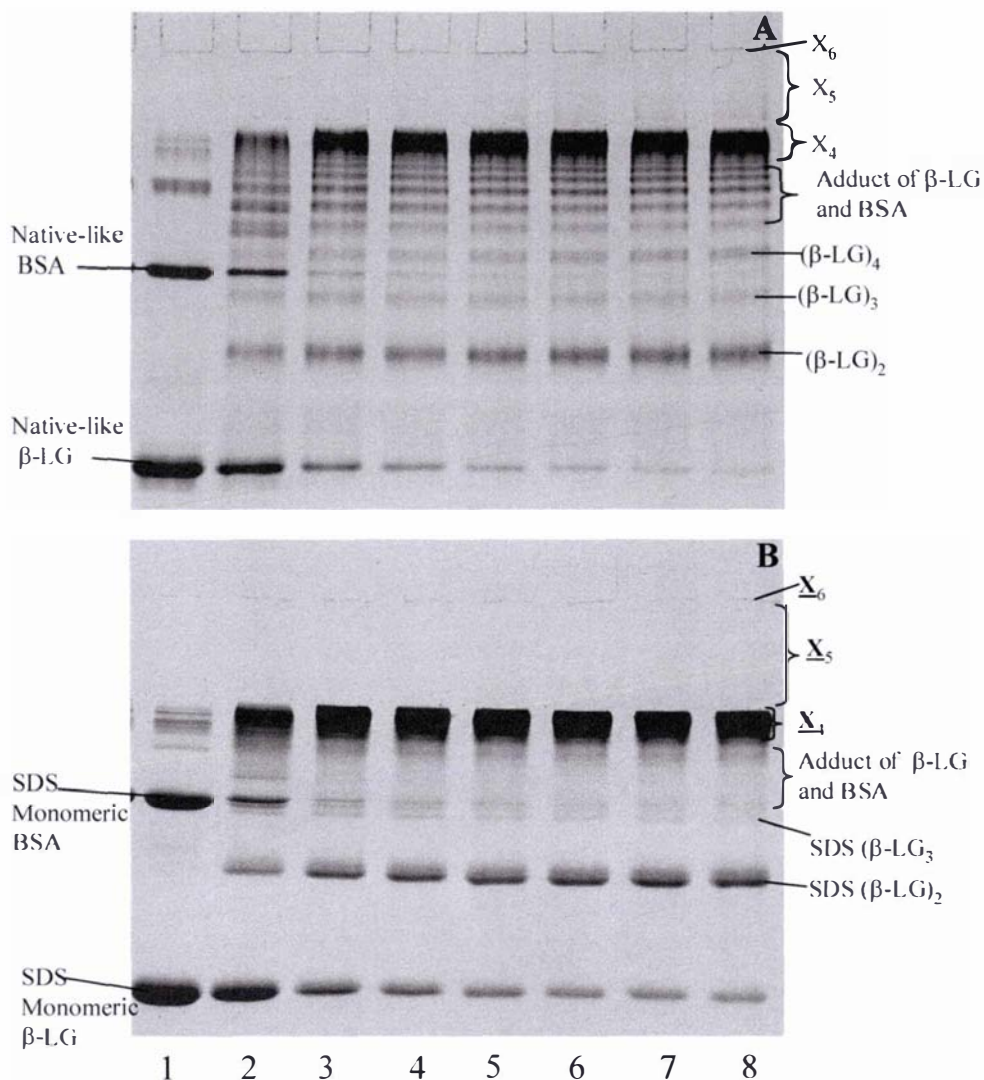


Figure 8.12. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1 binary mixture of pure β -LG and BSA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

The proportion of high molecular weight aggregates (marked as X_4) was comparatively greater, whereas high molecular weight aggregates (marked as X_5 or X_6) was comparatively less in the SDS-PAGE patterns as compared with the corresponding native-PAGE patterns (Lanes 2–8, Figure 8.12A). This probably suggests that the material caught up within the stacking gel (marked as X_5) in the native-PAGE patterns consisted of very large aggregates, a proportion of which were subsequently dissociated to comparatively smaller aggregates in the SDS environment, which is in general

agreement with earlier studies (McSwiney et al., 1994a; Havea et al., 2001) of a heat-treated mixture of the pure proteins.

The integrated data on the intensities of the bands corresponding to native-like and SDS-monomeric β -LG and BSA (Figure 8.13) showed that the concentrations of residual native-like and SDS-monomeric BSA were almost similar in all pressure-treated samples, suggesting that a small proportion of non-covalently bonded aggregates of BSA were generated in this case. A similar observation was made when BSA alone was pressure treated (see Figure 8.7). However, there was some difference between the concentrations of SDS-monomeric β -LG and native-like β -LG in this case, suggesting that presence of β -LG contributed to the formation of a proportion of non-covalently bonded aggregates. However, it can be seen that the difference between the concentrations of SDS-monomeric β -LG and native-like β -LG (approximately 10%) was small compared with that (approximately 30–40%) when a 1:1 binary mixture of β -LG and α -LA was pressure treated (see Figure 8.10). This observation suggested that comparatively greater proportions of hydrophobically linked aggregates were generated by pressure treatment of a mixture of β -LG and α -LA than by pressure treatment of a mixture of β -LG and BSA.

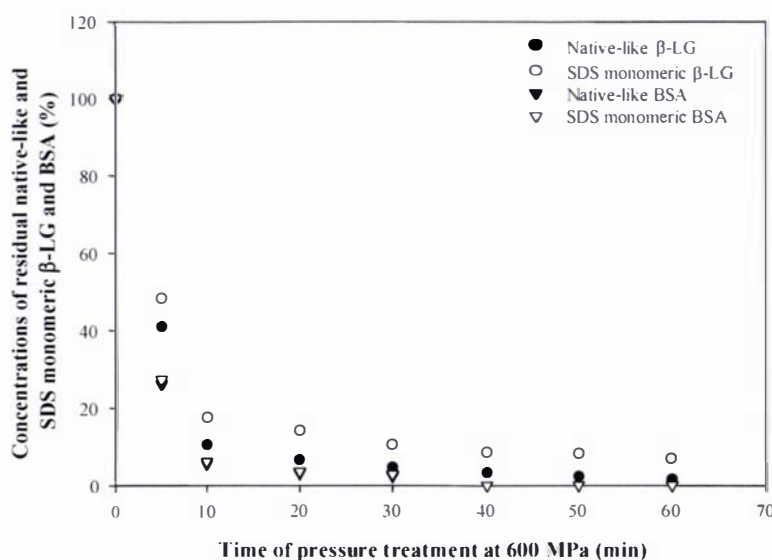


Figure 8.13. Concentrations of native-like β -LG (●), SDS-monomeric β -LG (○), native-like BSA (▼) and SDS-monomeric BSA (▽) in solutions (10 mg/mL) of a 1:1 binary mixture of pure β -LG and BSA treated at 600 MPa for 0–60 min.

Further, it was observed that the concentrations of both native-like and SDS-monomeric β -LG and BSA decreased comparatively faster when a binary mixture of β -LG and BSA was pressure treated than when pure β -LG (see Figure 8.3) or BSA (see Figure 8.7) was pressure treated alone.

2D PAGE

The 2D native:SDS-PAGE and 2D SDS:reduced SDS-PAGE patterns of a pressure-treated 1:1 binary mixture of β -LG and BSA are presented in Figures 8.14A and 8.14B respectively.

The 2D native:SDS-PAGE pattern (Figure 8.14A) showed that the intensities of the spots corresponding to monomeric β -LG and BSA decreased as a result of pressure treatment, with the simultaneous formation of high molecular weight aggregates. These aggregates were so large that they did not travel further in the 2D gel; some of these aggregates were resolved in the stacking gel or near the top of the resolving gel. Some newly formed spots resolved in the second dimension on SDS-PAGE, from the protein aggregates present in the region between the monomeric β -LG band and the top of the resolving gel of the 1D native sample gel strip (a'), corresponded to polymers of β -LG and BSA. A proportion of the material caught up at the top of the resolving gel and caught up within the stacking gel was not dissociated in the second dimension in the SDS-PAGE environment, suggesting that a proportion of the aggregates were other than hydrophobically linked.

On the 2D SDS:reduced SDS-PAGE pattern (Figure 8.14B), several new spots were resolved (as almost continuous horizontal streaks), in line with the spots corresponding to SDS-monomeric β -LG and BSA, to the left of the diagonal. These new spots were resolved from the region between the SDS-monomeric β -LG band and the top of the gel of the reduced 1D SDS sample gel strip (a') (i.e. after disulphide bond reduction of the proteins within the sample gel strip), which coincided with the bands corresponding to reduced SDS dimers of β -LG and high molecular weight co-polymers of β -LG and BSA on the stained gel strip (a''), suggesting that these aggregates were disulphide linked.

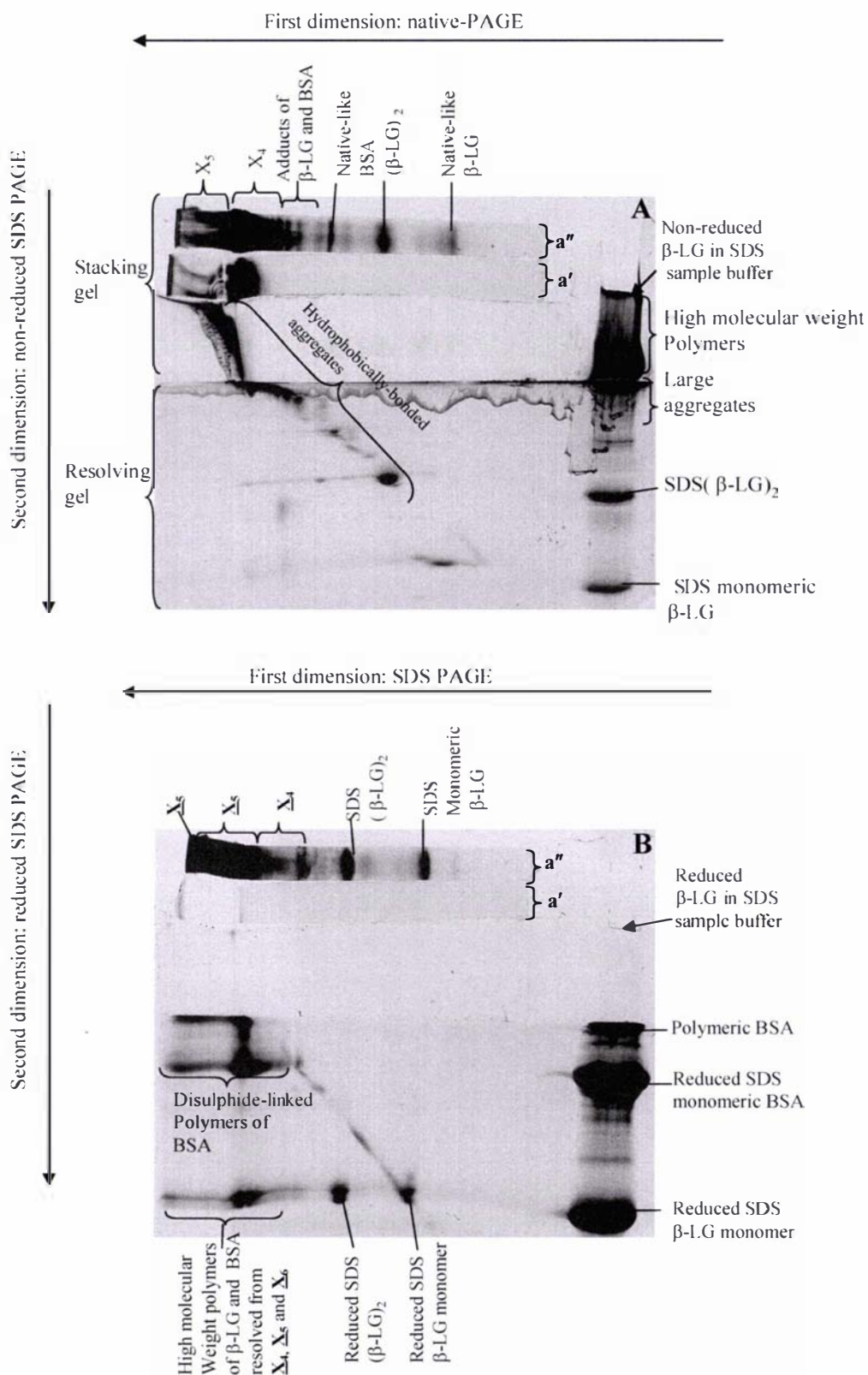


Figure 8.14. 2D (A) native:SDS-PAGE and (B) SDS:reduced SDS-PAGE patterns of a solution of a 1:1 binary mixture of β -LG and BSA pressure treated at 600 MPa for 60 min.

Mixture of α -LA and BSA

ID PAGE

Comparison of the native-PAGE patterns of a binary mixture of control α -LA and BSA (Lane 1, Figure 8.15A) and pressure-treated samples (Lanes 2–8, Figure 8.15A) indicated that the intensity of the band corresponding to native-like BSA decreased gradually in the pressure-treated samples. However, the decrease in the intensity of the band corresponding to native-like BSA was much slower than that observed when a binary mixture of β -LG and BSA (Figure 8.12A) was pressure treated. The band corresponding to native-like BSA was still clearly observable in the native-PAGE pattern of the sample pressure treated at 600 MPa for 60 min (Lane 8, Figure 8.15A); this behaviour of BSA was clearly different from that when a binary mixture of β -LG and BSA was pressure treated (Lane 8, Figure 8.12A). Similarly, the intensity of the band corresponding to native-like α -LA also decreased much more slowly (Figure 8.15A) compared with when a binary mixture of β -LG and α -LA was pressure treated (Figure 8.9A).

There were several new bands and a dark-staining region corresponding to aggregated material between the band corresponding to native-like BSA and the top of the resolving gel in the native-PAGE patterns of the pressure-treated samples (Lanes 2-8, Figure 8.15A).. Also, in this case, comparatively small proportions of high molecular weight aggregates (regions marked as X₄ and X₅) were generated.

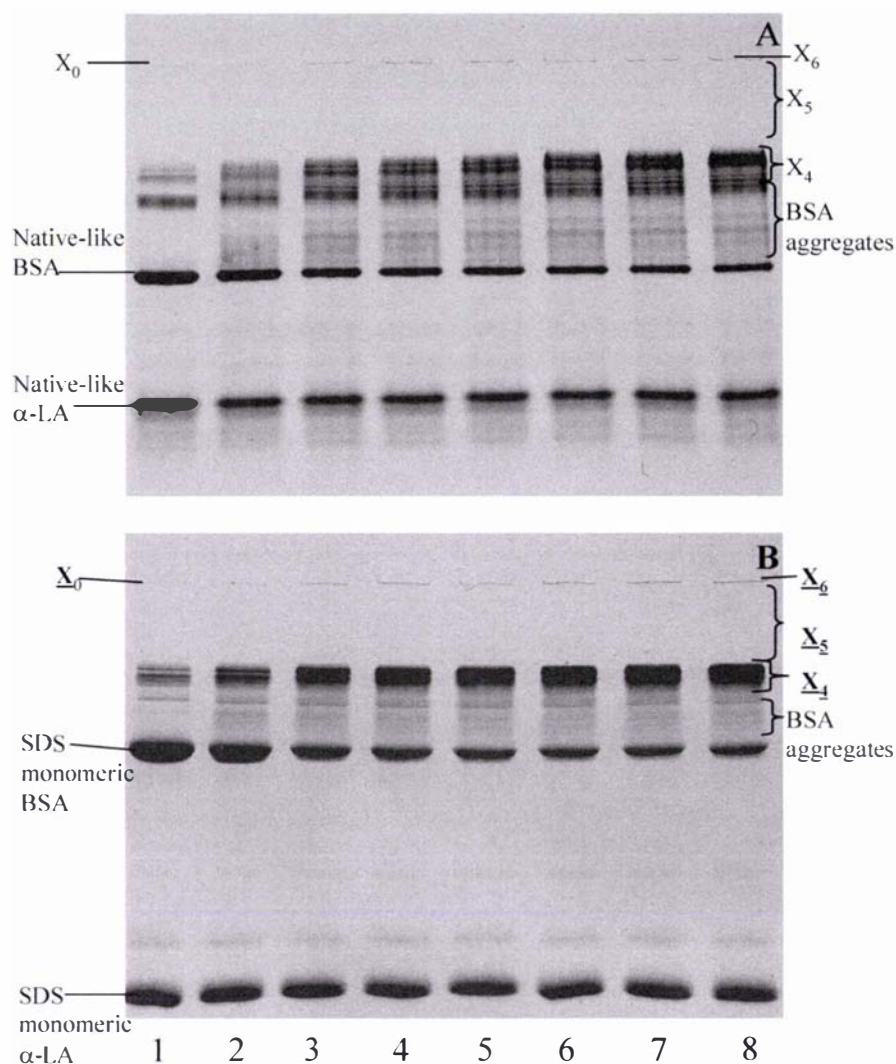


Figure 8.15. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1 binary mixture of pure α -LA and BSA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

The SDS-PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.15B) also indicated almost the same trend as for the native-PAGE patterns (Lanes 2–8, Figure 8.15A). There was little change in the intensity of the band corresponding to SDS-monomeric α -LA. A number of new discrete bands and a dark-staining region corresponding to aggregated material were observed between the band corresponding to SDS-monomeric BSA and the top of the resolving gel in the SDS-PAGE pattern of the pressure-treated samples (Lanes 2–8, Figure 8.15B). This aggregated material consisted of polymers of BSA alone, as well as co-polymers of α -LA and BSA (Figure 8.17B).

However, intermediate-sized aggregates consisting only of α -LA (such as α -LA dimers) appeared to be absent from both the native-PAGE (Figure 8.15A) and SDS-PAGE (Figure 8.15B) patterns.

These results clearly indicated that both α -LA and BSA behaved differently when pressure treated in the presence (Figure 8.9A and 8.12A respectively) versus in the absence of β -LG (Figure 8.15), suggesting that the reactivity of these seemed to be different in each system, depending on the presence or absence of β -LG in the system.

The relative intensities of the bands corresponding to residual native-like and SDS-monomeric α -LA and BSA in a pressure-treated mixture of α -LA and BSA (Figure 8.16) showed that the concentrations of native-like and SDS-monomeric BSA decreased faster than those of native-like and SDS-monomeric α -LA. It appears from these results that most of the BSA was self-aggregated (confirming earlier results; see Figures 8.6 and 8.7) and that α -LA played little role in the formation of co-polymers of α -LA and BSA when a mixture of α -LA and BSA was pressure treated. Also, the differences between the concentrations of SDS-monomeric and native-like α -LA and between the concentrations of SDS-monomeric and native-like BSA were very small in all pressure-treated samples. Thus, these results again confirm that comparatively small proportions of hydrophobically linked aggregates of BSA were generated when BSA was pressure treated either alone (e.g. see also Figure 8.7) or in combination with other whey proteins in binary systems (Figure 8.16; see also Figure 8.13). These results indirectly suggest that pressure-induced aggregation of BSA either alone or in combination with other whey proteins generated mainly disulphide-linked aggregates. This behaviour of BSA is in contrast to the behaviours of β -LG and α -LA, which generated both disulphide-linked and hydrophobically linked aggregates.

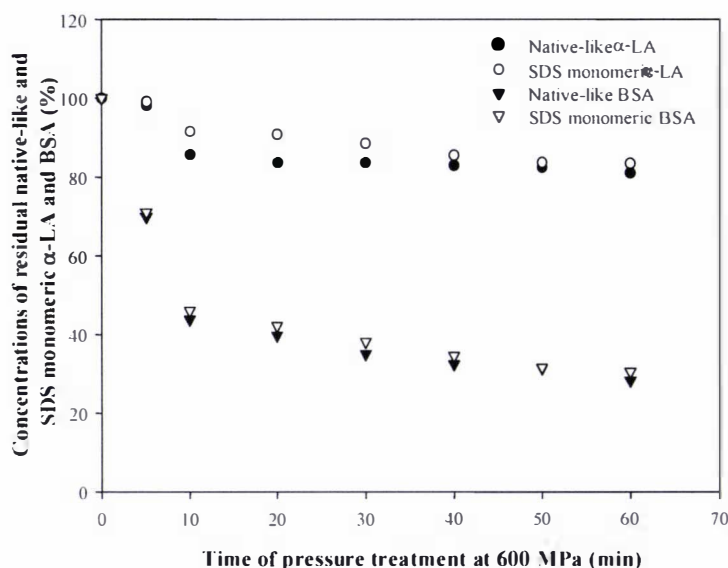


Figure 8.16. Concentrations of residual native-like α -LA (●), SDS-monomeric α -LA (○), native-like BSA (▼) and SDS-monomeric BSA (▽) in solutions (10 mg/mL) of a binary mixture of pure α -LA and BSA treated at 600 MPa for 0–60 min.

2D PAGE

Significant proportions of high molecular weight aggregates were resolved as a diagonal streak near the top of the resolving gel, and were not further dissociated by SDS in the 2D native:SDS-PAGE pattern (Figure 8.17A). Also, it seems that there was little effect on α -LA.

In contrast, the 2D SDS:reduced SDS-PAGE pattern (Figure 8.17B) clearly showed that spots or streaks corresponding to reduced monomeric BSA and α -LA were resolved from the high molecular weight aggregates present in the region between the SDS-monomeric BSA band and the region marked as **X**₄ of the reduced 1D SDS sample gel strip (a'). These results suggested that pressure treatment of a binary mixture of BSA and α -LA generated high molecular weight disulphide-linked co-polymers consisting of BSA and α -LA.

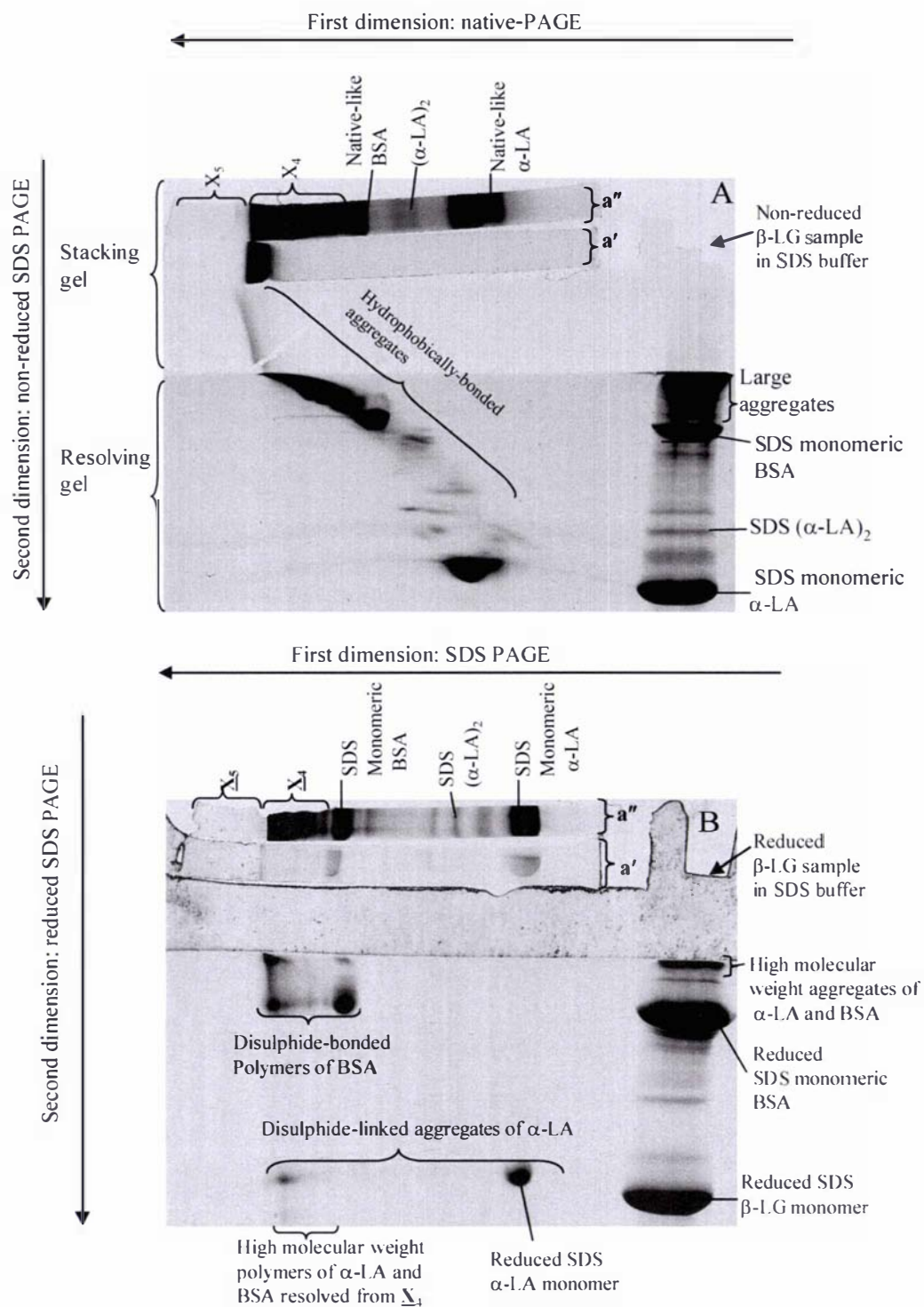


Figure 8.17. 2D (A) native:SDS-PAGE and (B) SDS:reduced SDS-PAGE patterns of a solution of a 1:1 binary mixture of α -LA and BSA pressure treated at 600 MPa for 60 min.

Mixture of β -LG, α -LA and BSA

1D PAGE

The native-PAGE patterns of control and pressure-treated samples of a 1:1:1 ternary mixture of β -LG, α -LA and BSA are presented in Figure 8.18A. Upon pressure treatment, sharp decreases in the intensities of the bands corresponding to native-like β -LG, α -LA and BSA were observed (Lanes 2–8, Figure 8.18A), with the simultaneous appearance of several new discrete bands corresponding to protein aggregates of β -LG, α -LA and BSA (including high molecular weight aggregates X_4 , X_5 , and X_6) as marked on the right-hand side of the native-PAGE patterns (Figure 8.18A).

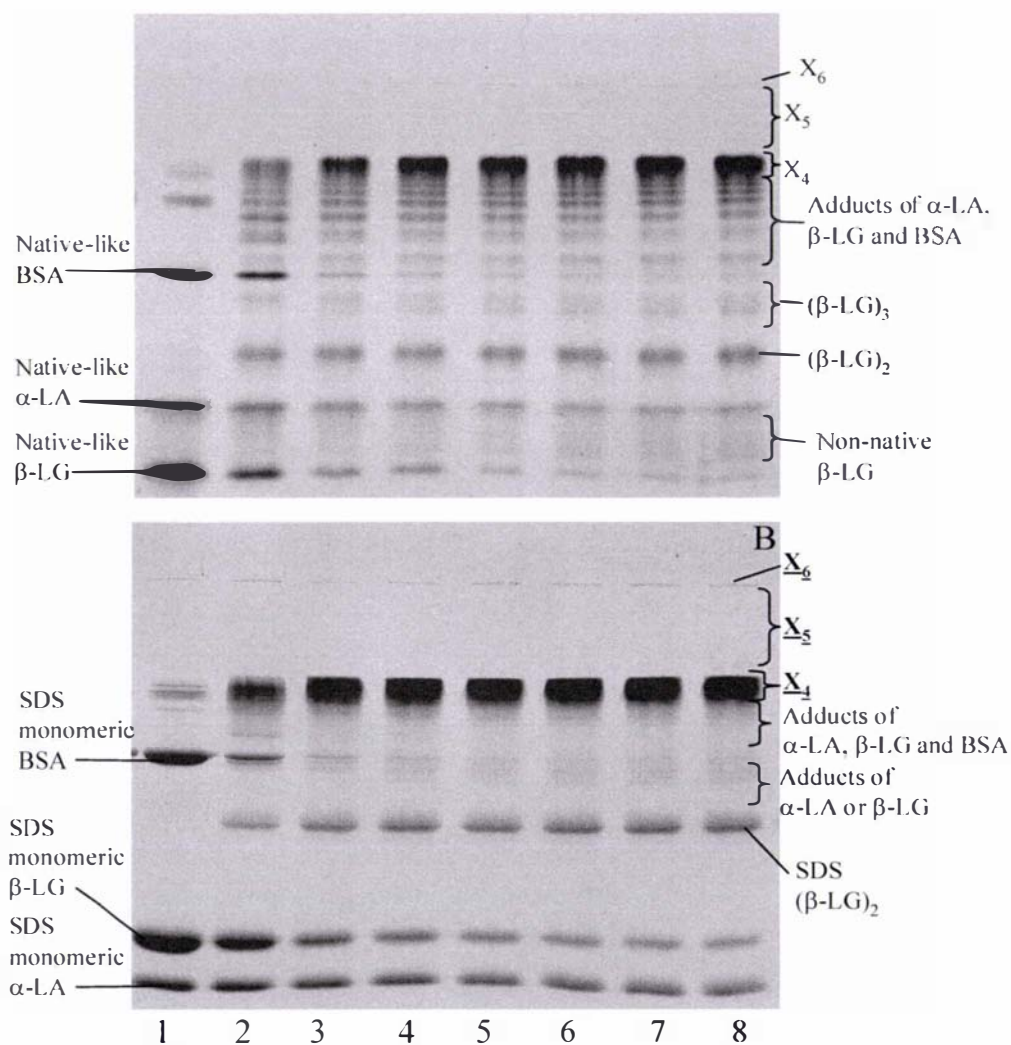


Figure 8.18. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1:1 ternary mixture of pure β -LG, α -LA and BSA solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

The SDS-PAGE patterns of the pressure-treated (Lanes 2–8, Figure 8.18B) samples suggested a similar trend to that of the corresponding native-PAGE patterns (Lanes 2–8, Figure 8.18A), showing significant decreases in the intensities of the bands corresponding to SDS-monomeric β -LG, α -LA and BSA and the concurrent appearance of new discrete bands corresponding to SDS β -LG dimer and a dark-staining region corresponding to high molecular weight aggregates between the band corresponding to the SDS β -LG dimer and the top of the resolving gel. As observed on the native-PAGE patterns (Figure 8.18A), the band corresponding to SDS-monomeric BSA was absent in the PAGE patterns of samples pressure treated for longer than 20 min at 600 MPa (Lane 5–8, Figure 8.18B), whereas the bands corresponding to monomeric β -LG and α -LA were still observable after severe pressure treatment (Lane 8, Figure 8.18B), suggesting that BSA was found to be more pressure sensitive when a ternary mixture of β -LG, α -LA and BSA was pressure treated than when pure BSA was pressure treated in isolation.

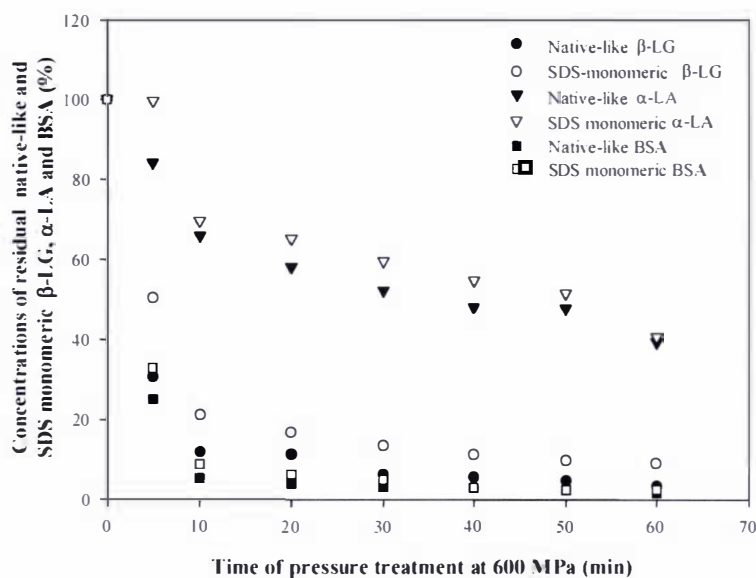


Figure 8.19. Concentrations of residual native-like β -LG (\bullet), SDS-monomeric β -LG (\circ), native-like α -LA (\blacktriangledown), SDS-monomeric α -LA (\triangledown), native-like BSA (\blacksquare) and SDS-monomeric BSA (\square) in solutions (10 mg/mL) of a ternary mixture of pure β -LG, α -LA and BSA treated at 600 MPa for 0–60 min.

From the plotted intensities of the bands corresponding to the concentration of residual native-like and SDS-monomeric β -LG, α -LA and BSA (Figure 8.19), it can be observed that the concentrations of native-like and SDS-monomeric BSA decreased fastest, followed by β -LG and α -LA. Also, it can be seen that there were some differences between the concentrations of residual SDS-monomeric and native-like β -LG and α -LA (but not BSA), suggesting that a proportion of the aggregates of β -LG and α -LA were hydrophobically linked.

2D PAGE

The 2D native:SDS-PAGE and 2D SDS:reduced SDS-PAGE patterns of pressure-treated samples of a ternary mixture of β -LG, α -LA and BSA are presented in Figures 8.20A and 8.20B respectively.

A series of new spots were resolved on 2D native:SDS-PAGE as a diagonal streak from the protein aggregates present in the region between the band corresponding to β -LG and the top of the resolving gel of the 1D native sample gel strip (a'). These new spots were dissociated in the SDS environment, suggesting that they were hydrophobically linked.

Similarly, the 2D SDS:reduced SDS-PAGE pattern of the pressure-treated samples (Figure 8.20B) showed that a series of new spots were resolved from the reduced 1D SDS sample gel strip (a'). The new spots were resolved as a continuous horizontal line to the left of the reduced monomeric β -LG, α -LA and BSA spots on the 2D PAGE pattern (i.e. to the left of the diagonal). These spots were resolved from the bands corresponding to intermediate-sized aggregates (e.g. β -LG and α -LA dimers) and from the high molecular weight aggregates accumulated in the region marked as X₄ and X₅ of the reduced sample gel strip (a'), suggesting that disulphide-linked co-polymers consisting of BSA, β -LG and α -LA were generated by pressure treatment.

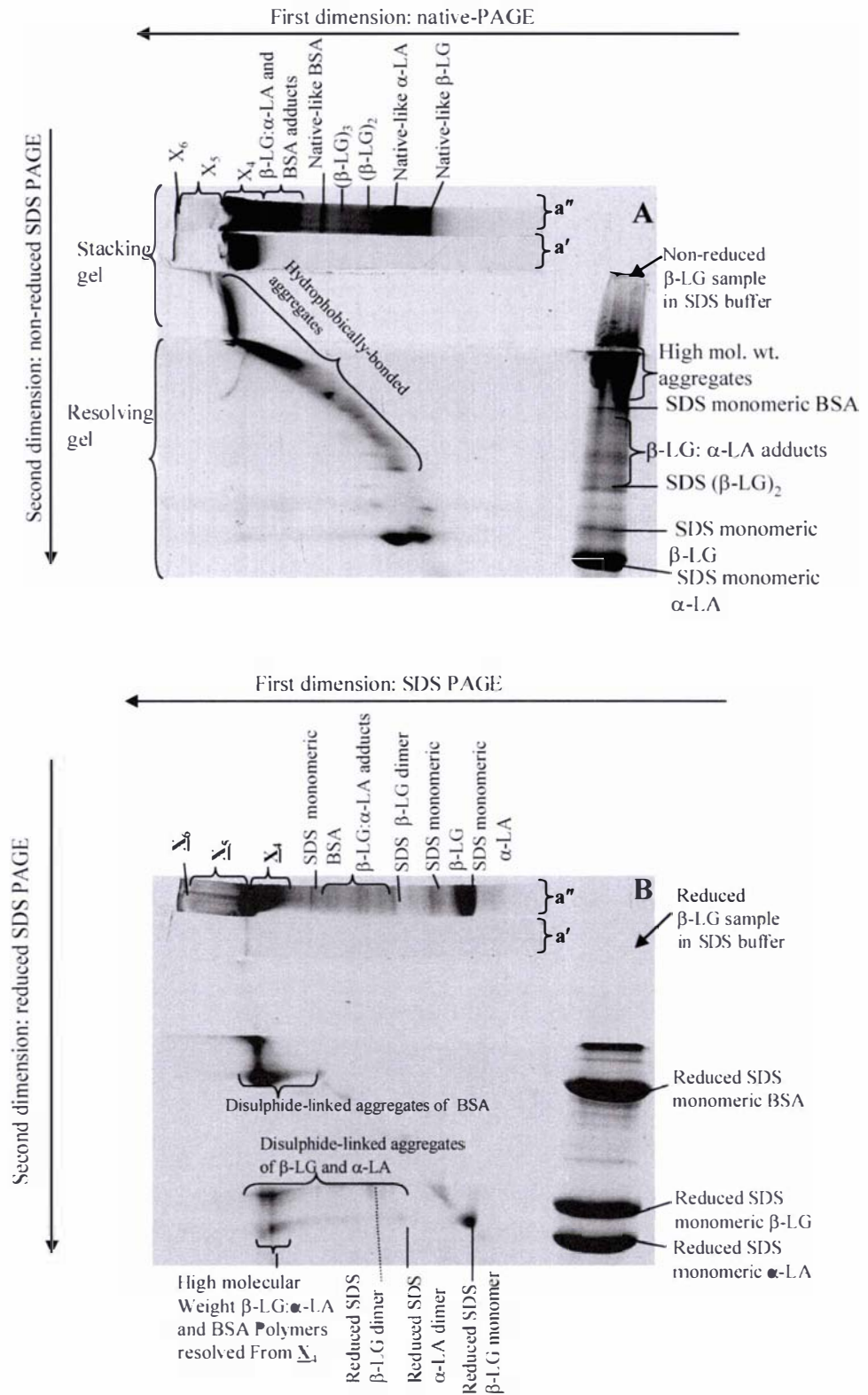


Figure 8.20. 2D (A) native:SDS-PAGE and (B) SDS:reduced SDS-PAGE patterns of a solution of a 1:1:1 ternary mixture of β-LG, α-LA and BSA pressure treated at 600 MPa for 60 min.

Mixture of β -LG and κ -CN

ID PAGE

The native-PAGE patterns of the control and pressure-treated samples (Figure 8.21A) showed that the band corresponding to native-like β -LG was resolved in the control samples (Lane 1, Figure 8.21A), giving a quite distinct clear monomeric band with almost no impurity or aggregated protein, and that most of the κ -CN existed in a polymeric form (which was resolved near the top of the resolving gel, was caught up in the stacking gel or could not enter the gel). It was found that the intensity of the band corresponding to native-like monomeric β -LG decreased sharply in the pressure-treated samples (Lanes 2–8, Figure 8.21A), with the concurrent formation of several new bands in the region between the native-like β -LG band and the top of the resolving gel, as marked on the right-hand side of the gel. High molecular weight polymers (marked as X_4 , X_5 and X_6) were also present in the PAGE pattern of the pressure-treated samples (Lanes 2–8, Figure 8.21A).

The SDS-PAGE patterns also showed that the intensity of the band corresponding to SDS-monomeric β -LG decreased in the PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.21B), with the simultaneous formation of aggregates (mainly β -LG dimers) and high molecular weight aggregates of β -LG and κ -CN (marked as X_4 , X_5 and X_6). Some very large, insoluble aggregates that did not penetrate the gel pore or that caught up within the stacking gel or could not enter the gel were also generated by pressure treatment. This result shows that a comparatively much higher proportion of very large aggregates was formed when a binary mixture of β -LG and κ -CN was pressure treated compared with when a binary or ternary mixture of β -LG in combination with α -LA and BSA was pressure treated (see Figures 8.9, 8.12 and 8.18).

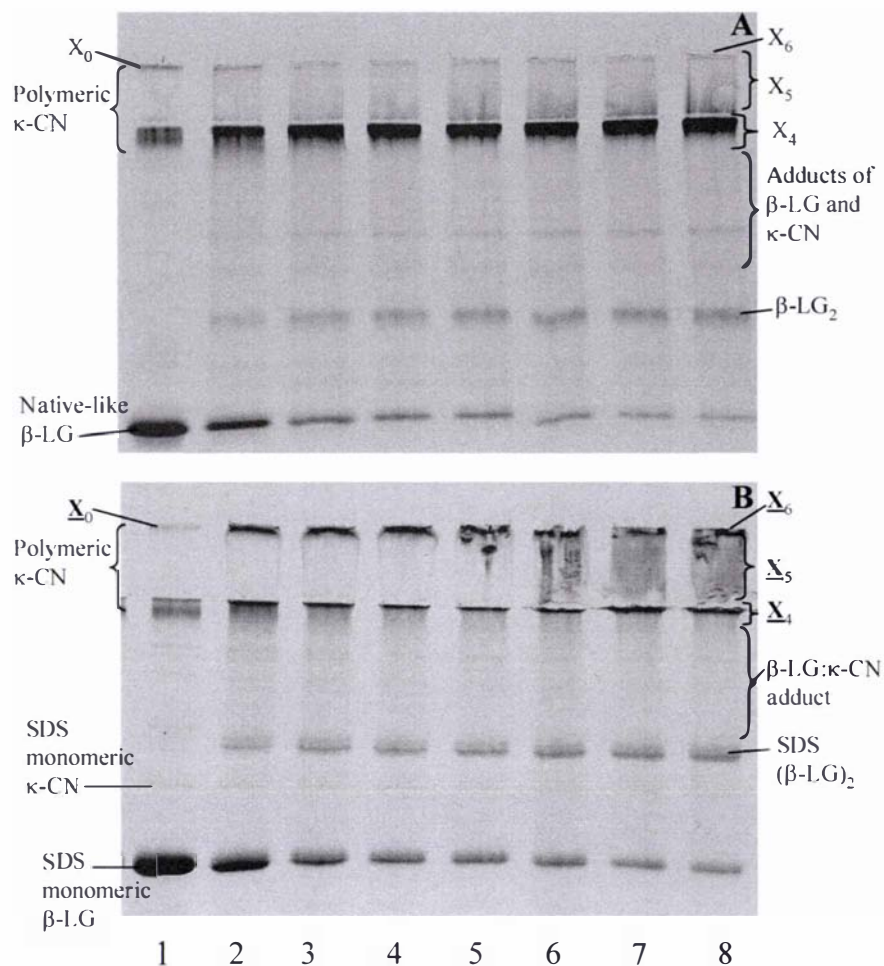


Figure 8.21. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1 binary mixture of pure β -LG and κ -CN solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

It was not possible to quantify the residual native-like or SDS-monomeric κ -CN as almost all the κ -CN was present in a polymeric form in all the samples, including the control samples (Lane 1, Figures 8.21A and 8.21B). The quantitative data on the residual native-like and SDS-monomeric β -LG were plotted (Figure 8.22). These results suggested that the concentration of native-like and SDS-monomeric β -LG decreased at a faster rate when a 1:1 binary mixture of β -LG and κ -CN was pressure treated than when a pure β -LG solution alone was pressure treated (Figure 8.3). Also, the difference between SDS-monomeric and native-like β -LG was comparatively smaller, compared with when a 1:1 binary mixture of β -LG and α -LA was pressure treated (Figure 8.10),

which suggested that the majority of the aggregates induced by pressure treatment of a binary mixture of β -LG and κ -CN were covalently linked.

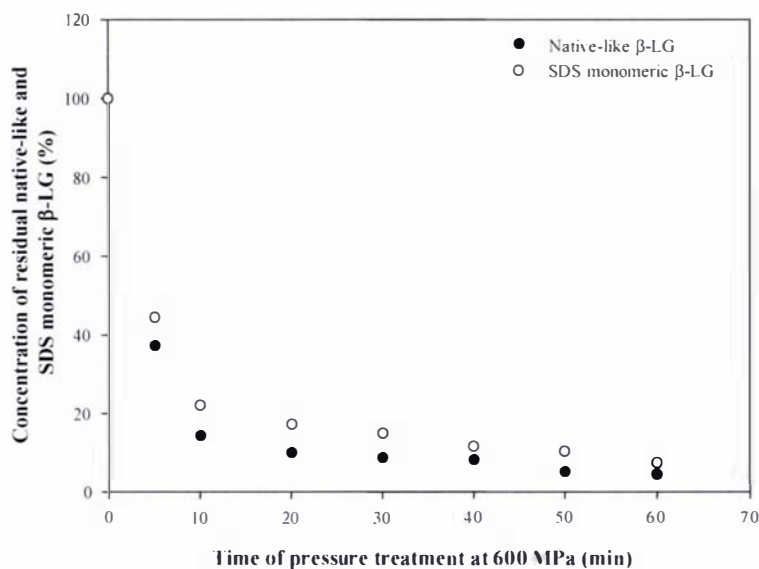


Figure 8.22. Concentrations of residual native-like β -LG (●) and SDS-monomeric β -LG (○) in solutions (10 mg/mL) of a binary mixture of pure β -LG and κ -CN treated at 600 MPa for 0–60 min.

Mixture of β -LG, α -LA and κ -CN

1D PAGE

The native PAGE patterns of the pressure-treated samples (Lanes 2–8, Figure 8.23A) showed a rapid decline in the intensity of the band corresponding to native-like β -LG after pressure treatment of the samples for a short time (Lane 2, Figure 8.23A). This band corresponding to native-like β -LG then almost disappeared upon longer holding (Lanes 3–8, Figure 8.23). The decrease in the intensity of the band corresponding to native-like proteins was coupled with the formation of polymers of various sizes, ranging from dimers, trimers and tetramers to high molecular weight polymers of β -LG and κ -CN that were caught up at in the region marked as X_4 , X_5 and X_6). Interestingly, the intensity of the band corresponding to native-like α -LA remained almost unchanged throughout the pressure treatment.

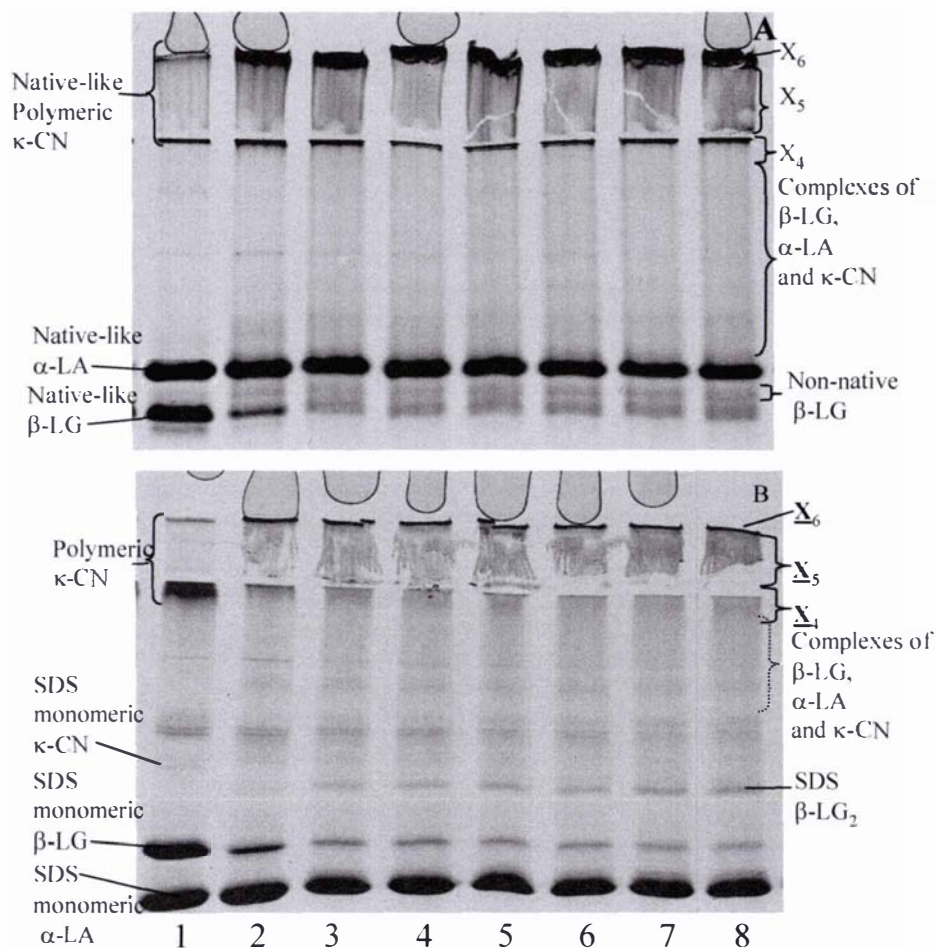


Figure 8.23. (A) Native-PAGE and (B) SDS-PAGE patterns of a 1:1:1 ternary mixture of pure β -LG, α -LA and κ -CN solutions (10 mg/mL): untreated control sample (Lane 1) and samples pressure treated at 600 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5), 40 min (Lane 6), 50 min (Lane 7) and 60 min (Lane 8).

An almost similar trend as for the native-PAGE patterns (Figure 8.23A) was shown by the SDS-PAGE patterns (Figure 8.23B). However, in contrast to the corresponding native-PAGE pattern (Lane 8, Figure 8.23A), faint bands corresponding to SDS-monomeric β -LG were still observable after a long pressure treatment. It was found that there was further polymerisation of κ -CN in the pressure-treated samples (see Lanes 2–8, Figure 8.23B). No major changes in the intensity of the band corresponding to SDS-monomeric α -LA were observed. New bands corresponding to SDS β -LG dimer appeared in the SDS-PAGE patterns of the pressure-treated samples (Lanes 3–8, Figure 8.23B). In addition, high molecular weight aggregates (marked as X_5 and X_6) were also observed.

From these results, it seems that there were preferential interactions of β -LG with κ -CN with little involvement of α -LA. Also, in contrast to the binary system containing β -LG and α -LA (Figure 8.9), comparatively fewer changes in α -LA were observed (Figure 8.23). This behaviour is similar to the response of these proteins to heat treatment (Cho et al., 2003).

The quantitative data on the concentrations of residual native-like and SDS-monomeric β -LG and α -LA (Figure 8.24) indicated that the concentration of native-like and SDS-monomeric β -LG decreased much faster than that of α -LA, indicating that α -LA reacted slowly when two highly reactive proteins such as κ -CN and β -LG were present in the system. It was not possible to obtain quantitative data on the decrease in the intensity of the band corresponding to monomeric κ -CN, as most of the κ -CN was in a polymeric form.

There were some differences between the concentrations of residual SDS-monomeric and native-like β -LG and between the concentrations of residual SDS-monomeric and native-like α -LA, suggesting that a proportion of the aggregates was non-covalently linked.

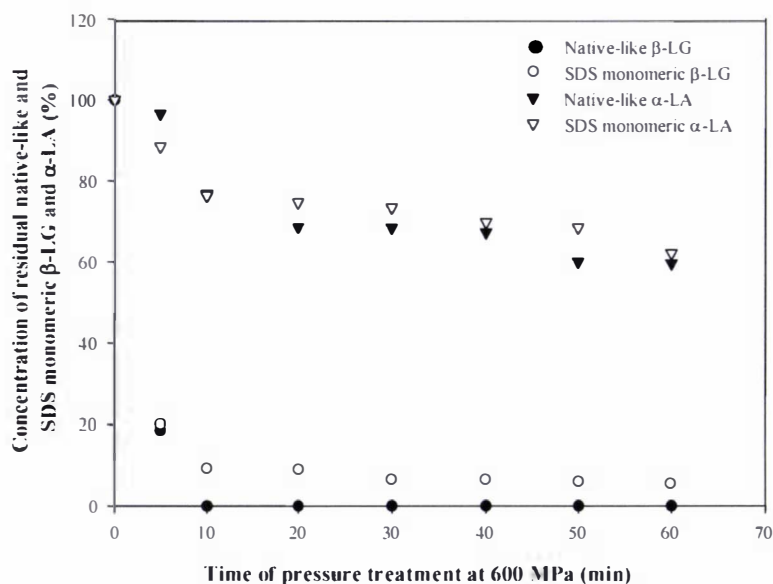


Figure 8.24. Concentrations of residual native-like β -LG (●), SDS-monomeric β -LG (○), native-like α -LA (▼) and SDS-monomeric α -LA (▽) in solutions (10 mg/mL) of a ternary mixture of β -LG, α -LA and κ -CN treated at 600 MPa for 0–60 min.

8.4 General discussion

Several studies have been reported on the effects of heat treatments on pure β -LG, α -LA or BSA alone or when heat treated in binary or ternary combinations in model systems (e.g. Chaplin & Lyster, 1986; Calvo et al., 1993; Gezimati et al., 1996, 1997; Dalglish et al., 1997; Manderson et al., 1998, 1999; Schokker et al., 1999, 2000; Havea et al., 2000, 2001; Hong & Creamer, 2002; Livney et al., 2003; Murayama & Tomida, 2004; McGuffey et al., 2005). Also there are reports concentrating on the effects of heat treatments on β -LG, α -LA or BSA in a heat-treated natural whey protein (e.g. WPC) system (e.g. Donovan & Mulvihill, 1987; Havea et al., 1998, 2002, 2004) or a milk system (e.g. Dannenberg & Kessler, 1988a, 1988b; Noh et al., 1989; Anema & McKenna, 1996; Anema, 2000, 2003a, 2003b). Considering that ample literature is already available on the above subjects, only the effects of pressure treatments on β -LG, α -LA, BSA or κ -CN alone or when pressure treated in binary or ternary combinations were evaluated in the present study. The present discussion is therefore mainly based on the effects of the pressure treatments observed in the present study and these results are discussed in comparison with the reported literature on the effects of heat treatments. Further, it should be considered that severe pressure treatments (e.g. 600 MPa) will have different effects on proteins, as compared with mild pressure treatments (e.g. 200 MPa) or medium pressure treatments (e.g. 400 MPa) and may have different mechanisms of denaturation and aggregation (Considine et al., 2005a, 2005b). However, it should be noted that the results of the present study and the explanations are based only on the interactions that occur with severe pressure treatments.

Further, it was obvious from the results of earlier chapters that it was challenging to study the heat- or pressure-induced interactions or denaturation, aggregation and gelation of whey proteins in a complex system (e.g. in WPC, Chapters 4–6) or the interactions of caseins and whey proteins in a milk system (e.g. in Chapter 7), in which all components (including whey proteins, caseins and other milk components such as lactose and minerals) are present in their natural environment, which might affect the behaviour of each of the milk proteins. Also, the role of some of the proteins when pressure treated in a complex system was not very clear.

Many previous studies on the effect of heat on β -LG (e.g. Manderson et al., 1998 1999; Croguennec et al., 2003; Creamer et al., 2004) and pressure on β -LG (e.g. Dumay et al., 1994; Funtenberger et al., 1997; Iametti et al., 1997; Moller et al., 1998; Considine et al., 2005a, 2005b; also see Chapters 5 and 6) indicated that the general reaction pathways for heat- and pressure-induced denaturation and aggregation of β -LG are similar but not identical. As discussed earlier (Chapters 4–6), there are some similarities and differences between heat- and pressure-induced changes in the proteins. Some of these differences in the denaturation and aggregation of proteins may be attributed to the differences in the rupture of non-covalent bonds and the subsequent re-formation of intra- and intermolecular bonds by heat compared with high pressure (Heremans et al., 1997). The effects of pressure treatments on individual proteins are discussed in the subsequent sections.

β -LG

As β -LG is the major whey protein and is the most sensitive whey protein, it dominates the reactions in a mixed protein system such as WPC or milk. Therefore, the effects of high pressure on β -LG are discussed in comparatively greater detail. As for heat-treated samples of β -LG, when β -LG is in an unfolding environment such as high pressure, the free SH group of Cys121 (CysH121) has the ability to interact irreversibly with disulphide bonds (see Figure 8.25; Considine et al., 2006a, 2006b).

Pressure also seems to dissociate native dimers into monomers, which then partially unfold (as shown in Figures 8.2 and 8.4) so that the thiol moiety of CysH121 can access the adjacent Cys106–Cys119 disulphide bond and generate a CysH119 with a free thiol. Either this CysH119 or the original CysH121 then interacts with a Cys66–Cys160 disulphide bond of the same or another β -LG molecule to transfer the thiol to CysH66 or CysH160. The thiol on CysH160 is likely to be able to access almost any available Cys residue because CysH160 is within two amino acids from the C terminus of β -LG. It was observed that comparatively small proportions of high molecular weight polymers were formed in these pressure-treated samples, which is a different trend from that for heat-treated samples (Havea et al., 2001). Such behaviour of whey proteins under pressure may well be related to enthalpy/entropy relationships or different effects of heat and pressure on the structure of whey proteins.

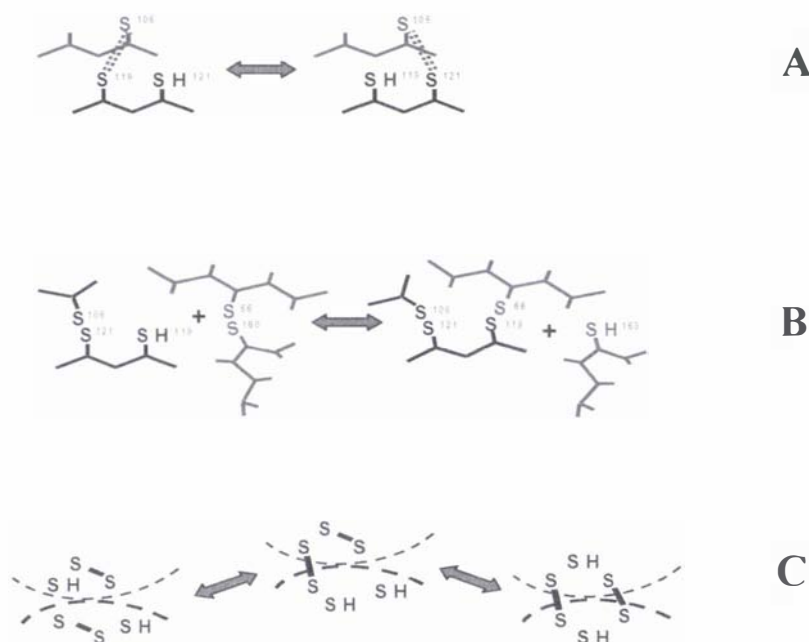


Figure 8.25. Thiol–disulphide interchange in β -LG. (A) The intramolecular interchange that precedes all other interchange reactions (Croguennec et al., 2003; Creamer et al., 2004). (B) The possible second interchange that involves the Cys66–Cys160 β -LG disulphide bond in the same molecule or in another β -LG molecule, which is spatially distant in native β -LG. (C) A plausible interchange involving two β -LG molecules to form a disulphide-bonded dimer without involving the interchange shown in (B).

α -LA

In contrast to β -LG, no changes in α -LA were observed when pure solutions of α -LA were subjected to severe pressure treatment in isolation, showing that α -LA does not readily aggregate when pressure treated alone. This behaviour is different from the earlier reports (Chaplin & Lyster, 1986; Havea et al., 2000, 2001; Hong & Creamer, 2002; McGuffey et al., 2005), reporting hydrophobic or disulphide-bonded aggregates of α -LA after severe heat treatment. Such differences might be attributed to the absence of a free sulphhydryl group in the structure of α -LA, as discussed in Chapters 4–7. In some studies (Kuwajima, 1989; Hirose, 1993; Lassalle et al., 2003), α -LA has been shown to form disulphide-linked polymers as well as modified monomers when heated under severe conditions (100°C for 30 min) and these are probably in the molten globule state. One of the reports suggested that α -LA changes its conformation from molten globule to the unfolded state without volume changes (Kobashigawa et al., 1999).

Mixture of α -LA and β -LG

It was found that α -LA alone is relatively non-reactive upon pressure treatment. However, the results of the present study (Figures 8.9 and 8.11) suggested that aggregation of α -LA occurs when it is pressure treated in the presence of β -LG, which is in agreement with earlier reports on pure protein systems (Tanaka & Kunugi, 1996; Jegouic et al., 1996; 1997). This was also in agreement with several reports on the effects of heat treatment on model systems (e.g. Noh et al., 1989; Matsudomi et al., 1992; Calvo et al., 1993; Hines & Foegeding, 1993; Qi et al., 1995; Dalgleish et al., 1997; Gezimati et al., 1997; Schokker et al., 2000; Havea et al., 2001; Hong & Creamer, 2002), showing that, in the presence of α -LA, a series of disulphide-linked (Matsudomi et al., 1992; Calvo et al., 1993; Gezimati et al., 1997) and hydrophobic (Calvo et al., 1993; Dalgleish et al., 1997; Gezimati et al., 1997; Havea et al., 1998, 2001; Oldfield et al., 1998, 2000; Schokker et al., 2000; Hong & Creamer, 2002) interactions between α -LA and β -LG occur. These PAGE patterns of heat-treated binary mixtures of α -LA and β -LG are similar to those when β -LG solution is heated on its own, suggesting that the mechanism of aggregation is governed by β -LG when a binary mixture of α -LA and β -LG is heated (Schokker et al., 2000; Hong & Creamer, 2002). An almost similar trend was observed in the present study (Figures 8.9A and 8.9B) when a mixture of α -LA and β -LG was pressure treated, suggesting that the pressure-induced mechanism is also governed by β -LG.

BSA

BSA has been reported as the most heat sensitive of the major whey proteins when heated alone (Clark et al., 1981; Shimada & Matsushita, 1981; de Wit & Klarenbeek, 1984; Takeda et al., 1989; Yamasaki et al., 1991). However, comparatively little information is available on the behaviour of pure BSA when pressure treated alone.

The results of the present study showed that, compared with β -LG, BSA was quite resistant to pressure treatment when solutions of BSA were pressure treated alone, which may be explained by the structural differences between these proteins and the number of disulphide bonds (as discussed in Chapters 4–6). Pressure resistance (100–400 MPa) has also been demonstrated by Hayakawa et al. (1992), probably because

pressure does not affect the α -helix and does not impart sufficient energy to disrupt disulphide bonds, thus maintaining the molecular structure of BSA. Like β -LG, BSA also self-aggregated and formed pressure-induced aggregates.

Mixture of β -LG and BSA

In comparison with pressure treatment of pure BSA (Figure 8.6) or β -LG (Figure 8.2) alone, when a 1:1 mixture of β -LG and BSA (Figure 8.12A) was pressure treated, the loss of both native-like and SDS-monomeric β -LG and BSA was faster and greater proportions of disulphide-bonded aggregates were formed.

When a 1:1 binary mixture of β -LG and BSA was pressure treated in the present study, BSA denatured faster than β -LG and it appeared that almost all the BSA interacted with β -LG during the early stages of pressure treatment. This behaviour of BSA was almost the same as that reported for heat treatment (Havea et al., 2001), which may be attributed to the availability of the free thiol from β -LG, which accelerates the reactivity of BSA. At lower pressure, 200–300 MPa, β -LG seems to be sensitive to high pressure and starts unfolding earlier than α -LA or BSA (results of Chapters 4–5), but, at higher pressure (e.g. 600 MPa), BSA also becomes more sensitive and starts to denature and aggregate faster. This may be because the lower pressure may not be providing sufficient energy to affect the large number of disulphide bonds (17 disulphide bonds) at 200–300 MPa, but the lower pressure (200–300 MPa) may be sufficient to affect the structure of β -LG or to collapse the central hydrophobic cavity of β -LG (as discussed in Chapter 4-6).

Mixture of α -LA and BSA

In a heat-treated mixture (Havea et al., 2000), it was found that BSA forms disulphide-bonded aggregates and catalyses the formation of differently structured α -LA monomers, dimers and adducts of α -LA with BSA. However, this did not occur when a binary mixture of α -LA and BSA was pressure treated in the present study, which may be attributed to pressure resistance of α -LA.

Mixture of β -LG, α -LA and BSA

When mixtures of β -LG, α -LA and BSA were heat treated, BSA formed polymers prior to the unfolding of either α -LA or β -LG (Gezimati et al., 1996, 1997; Havea et al., 2001). Moreover, it has been shown that heating α -LA in the presence of BSA or β -LG in WPC permeate (Havea et al., 2001) can induce the formation of α -LA dimers and copolymers of α -LA with β -LG and possibly with BSA. A somewhat similar trend was also observed in the pressure-treated mixture of α -LA, β -LG and BSA in the present study. However, in the pressure-treated samples, it appeared that β -LG, being the most pressure-sensitive whey protein, formed early aggregates, prior to the unfolding of either α -LA or BSA.

It was generally observed that, when pure BSA solutions or combinations/mixtures of BSA and other whey proteins (e.g. a binary mixture of BSA and α -LA) were pressure treated, almost all the aggregates were disulphide linked and only a small proportion of the aggregates were hydrophobically linked. However, pressure treatment of β -LG, a binary mixture of β -LG and α -LA or a ternary mixture of BSA, β -LG and α -LA generated aggregates comprising a mixture of hydrophobically linked and disulphide-linked aggregates. This may be attributed to structural differences in each of these proteins, and/or the effects of high pressure treatments on the structure of each of these proteins may be responsible for such differences.

Mixture of β -LG and κ -CN

When binary mixtures of β -LG and κ -CN were pressure treated, β -LG dimers and large polymers consisting of β -LG and κ -CN were formed as a result of interaction between β -LG and κ -CN. The majority of these aggregates were not dissociated by SDS, suggesting that they were mainly disulphide bonded. These results are in agreement with the available reports showing that pressure-induced interactions occur between β -LG and κ -CN (Felipe et al., 1997; López-Fandiño et al., 1997; López-Fandiño & Olano, 1998a, 1998b; Needs et al., 2000a, 2000b; Scollard et al., 2000), possibly via intermolecular disulphide bonds (López-Fandiño et al., 1997; Needs et al., 2000b).

Mixture of β -LG, α -LA and κ -CN

Pressure treatment of a mixture of β -LG, α -LA and κ -CN indicated that there was a rapid decline in the intensity of the band corresponding to monomeric β -LG and, interestingly, the intensity of the band corresponding to α -LA decreased very slowly (Figure 8.23). These results showed that, when all three proteins (β -LG, κ -CN and α -LA) were present in the system, reactions occurred preferentially between β -LG and κ -CN, with little involvement of α -LA. The pressure stability of α -LA and the lack of a free thiol in α -LA may be responsible for such behaviour of α -LA in the pressure-treated system. This behaviour was similar to the response of these proteins to heat treatment (Cho et al., 2003). In such system, only a small proportion of the aggregates were non-covalently linked and the majority of the aggregates were covalently linked (mainly disulphide linked) probably because of preferential reaction between β -LG and κ -CN.

8.5 Conclusions

This study on the effects of pressure treatments on pure proteins in isolated (i.e. individual) isolated systems, or in binary or ternary systems, generated useful information and clarified the role of each protein in pressure-induced aggregation and interactions of milk proteins. This study was a major step towards clarifying many doubts about the role of different proteins, when a complex mixture of proteins (such as WPC or milk) is pressure treated. However, more detailed studies using different experimental conditions (such as pH, ionic strength, and the natural WPC or milk systems with particular protein depleted or enriched etc) would be useful to further clarify the role of different proteins in the pressure-treated systems.

CHAPTER 9

Overall Conclusions and Recommendations

9.1 Introduction

Although the potential of high pressure (HP) processing of foods was first demonstrated in 1899 by Hite, considerable research interest in HP processing has been generated only over the last two decades, partly because of the previous unavailability of suitable commercial-scale equipment and because of high initial capital costs. Most of the previous studies on pressure-induced denaturation, aggregation and gelation have concentrated mainly on β -lactoglobulin (β -LG) in model systems, i.e. as an academic exercise. A number of studies evaluating the microstructural and rheological properties of pressure-induced whey protein gels or the effects of HP processing on the casein micelles in milk have also been reported. However, there is limited understanding of the mechanisms involved in the pressure-induced aggregation and gelation of whey proteins or the casein–whey protein interactions in complex natural systems such as whey protein concentrate (WPC) or milk. Such aspects are important because, in natural systems such as WPC or milk, the presence of other proteins, lactose and minerals will influence the behaviour of the proteins. In addition, the effects of heat treatment and pressure treatment have been studied on different samples, in different systems and using different experimental protocols or analytical methods. Therefore, in many instances, it is difficult to make comparisons and draw conclusions about the behaviour of milk and whey from these results.

In this context, the effects of HP processing on the denaturation, aggregation and gelation of whey proteins in WPC, and the interactions of caseins and whey proteins in milk and in model systems using pure proteins, were explored. Changes in the milk proteins, as induced by selected pressure and heat treatments, were compared, where possible, using a common approach, common methodology and samples from the same aliquot to compare the effects of heat and HP on milk proteins. However, the purpose of the comparison was to provide an indication of the changes in proteins induced by particular heat or pressure treatments (i.e. generally accepted treatments within mild, moderate and severe heat and pressure treatment regimes were considered); therefore, it was not a direct comparison. Any heat treatment (such as pasteurisation, sterilisation or

preheat treatment) should not be directly correlated with a particular pressure treatment, in terms of their functionalities or their abilities to inactivate microorganisms.

The pathways and the products of denaturation, aggregation and gelation in whey proteins, and the casein–whey protein complexes formed in heat- and pressure-treated milk systems, were elucidated. In most cases, the findings have been discussed in terms of key similarities and differences generated as a result of these two treatments, as observed in the present study or reported in the literature. Possible pathways/hypotheses have been proposed based on the results from the present study and relevant discussion has been included in the appropriate chapters. However, to support the conclusions, some of the important aspects of the discussion are reiterated in this chapter.

9.2 Comments and conclusions

Although several possible principal methods [such as size exclusion chromatography (SEC), light scattering, microscopy, rheology, circular dichroism etc.] could have been used, suitable polyacrylamide gel electrophoresis (PAGE) techniques (with appropriate modifications, where necessary) were found to be valuable for the detailed characterisation of the aggregation pathways of whey proteins and the covalent interaction products of caseins and whey proteins in heat- and pressure-treated samples. Each of the PAGE techniques uses different principles to separate and identify the proteins and protein complexes. The two-dimensional (2D) PAGE techniques in the present investigation were useful in determining which proteins formed reducible bonds (mainly disulphide bonds) or non-covalent bonds (mainly hydrophobic bonds) with one another. Application of a common approach and a common methodology to heat- and pressure-treated samples from the same aliquots generated useful information, including the type and size of aggregates, the proportions of covalent and non-covalent aggregates, the forces involved in protein–protein interactions, and the molecular mass ranges of aggregates of individual proteins or mixtures of proteins. Furthermore, modification of the PAGE methods to set the sample in the stacking gel portion within the sample loading well allowed better characterisation of the very large aggregates (> 500 kDa), which could not enter the gel. In some instances, combination of PAGE techniques with other available techniques (such as SEC, microscopy, rheology) provided greater understanding of the behaviour of the various milk proteins and their

interactions as affected by HP treatments. The major findings of the present study are listed below.

1) There were some differences and similarities in the denaturation and aggregation of the proteins in heat-treated and pressure-treated WPC samples. It was found that each of the whey proteins had different sensitivities to heat treatment and pressure treatment, confirming the findings of some of the earlier studies (e.g. Hinrichs et al., 1996; López-Fandiño et al., 1996; Gaucheron et al., 1997; Huppertz et al., 2004a). The sensitivity of the major whey proteins to pressure was in the order β -LG B > β -LG A > immunoglobulin (Ig) > lactoferrin (LF) > bovine serum albumin (BSA) > α -lactalbumin (α -LA) compared with Ig > LF > BSA > β -LG B > β -LG A > α -LA for heat. It has been proposed that the presence of a large internal hydrophobic cavity in β -LG, but not in BSA or α -LA, allows β -LG to be more sensitive to pressure treatment (Jameson et al., 2002); α -LA responds to pressure by modifying its structure to be more molten globule like and does not fully unfold at very high pressures. The relatively lower degree of reactivity of α -LA at high pressures is probably related to the relatively greater stability of this protein compared with β -LG (as discussed in Chapters 4 and 6) and is based on the unusual pressure-dependent behaviour of α -LA as reported previously (e.g. Kuwajima et al., 1990; Kobashigawa et al., 1999; Lasalle et al., 2003). Also, the heat-sensitive proteins, such as Igs and LF, were comparatively more resistant to pressure treatment, which may be attributed to various other structural differences; this has been discussed in Chapters 4–6.

The present study also indicated that different proportions of aggregates of various sizes were generated by heat treatment and pressure treatment. Pressure treatment of WPC solutions generated a greater proportion of comparatively intermediate-sized protein aggregates (such as dimers, trimers, tetramers and larger polymers) than did heat treatment (Chapter 4), which was probably a consequence of the different effects of heat and pressure on the structure of the native proteins and the disulphide-bonded dimers.

Both heat treatment and pressure treatment generated disulphide-bonded and hydrophobically bonded aggregates of whey proteins, consisting of Igs, LF, BSA, β -LG and α -LA (Chapters 4–6), as well as 1:1 disulphide-bonded dimers between α -LA and β -LG, similar to those reported by Havea et al. (1998) in a heated WPC system.

Quantitative data (Chapter 5) suggested that up to 30% of the β -LG was involved in hydrophobic aggregation and that approximately 70% of the β -LG was involved in disulphide-linked aggregation. Consequently, there appeared to be considerable similarity between the products of heat treatment and the products of pressure treatment of WPC.

2) It was found that various experimental parameters, such as protein concentration, intensity of pressure treatment, holding time and pressurising temperature, have considerable effects on the pressure-induced aggregation and gelation of whey proteins (Chapter 5). The combination of low protein concentration and mild pressure treatment led to minimal loss of native-like and SDS-monomeric β -LG, whereas the combination of high protein concentration and severe pressure treatment led to significant loss of both native-like and SDS-monomeric β -LG. Once again, this indicated that the effects of pressure treatment on the proteins in WPC solutions followed a similar trend to the effects of heat treatment, which demonstrated that the loss of native-like and SDS-monomeric β -LG in WPC solutions increased as the protein concentration and the holding time of heat treatment increased (Havea et al., 1998).

Moreover, the combination of severe pressure (e.g. 600 MPa) and elevated pressurising temperature had significant effects on the denaturation and aggregation of all whey proteins. The pressure-resistant whey proteins such as α -LA and BSA became more reactive and involved in the aggregation as the pressurising temperature was increased, particularly at pressurising temperatures of 40°C and higher. The rate of loss of native-like and SDS-monomeric α -LA was greater than that of native-like and SDS-monomeric β -LG and BSA when the pressurising temperature was increased to 40°C and higher. As discussed in Chapter 5, pressure treatment generated comparatively greater proportions of intermediate-sized protein aggregates at lower protein concentrations and lower pressures, or that were present only in the samples that were pressure treated at the lower pressurising temperatures, than did pressure treatment of concentrated protein solutions. However, comparatively small proportions of intermediate-sized protein aggregates were formed when the combination of severe pressure treatment and the higher pressurising temperatures was used [i.e. even when dilute WPC solutions were subjected to severe (600 MPa) pressure treatment at the higher pressurising temperatures of 40 and 50°C].

3) The pressure-treated samples of concentrated WPC solutions (12% w/v) showed a time-dependent loss of native whey proteins and a corresponding increase in non-native proteins and protein aggregates of different sizes (Chapter 6). These aggregates altered the viscosity and the samples almost gelled and were shown to be cross-linked by intermolecular disulphide bonds and by non-covalent interactions. Based on the results, it was proposed that the formation of a β -LG disulphide-bonded network preceded the formation of disulphide bonds between α -LA or BSA and β -LG to form multi-protein aggregates, possibly because the disulphide bonds of α -LA and BSA are less exposed than those of β -LG either during or after pressure treatment. It was envisaged that, after about 90 min of pressure treatment, most of the proteins would have become attached to one another via disulphide bonds and that there would be virtually no strong protein–protein interaction or association other than via disulphide bonds. This situation is very different from that in heat-treated WPC solutions, where hydrophobicity appears to be an important driving force. Once the pressure was reduced to atmospheric, the hydrophobic associations would be partly restored within the constraints of the new disulphide-bonding structure. This led to the possible explanation that intermolecular disulphide bond formation occurred at high pressure and that hydrophobic association became important after the high pressure treatment. Various possible hypotheses for pressure-induced gel formation are discussed in Chapter 6.

4) The compositions of the casein–whey protein complexes formed during heat and pressure treatment of skim milk have not previously been studied in detail. Modifications to the previously used PAGE methods, such as setting the samples in an open-pore gel prior to sodium dodecyl sulphate (SDS)-PAGE separation, prevented the loss of the larger aggregates during the PAGE analysis so that the protein components of these aggregates could be determined after disulphide bond reduction using a 2D PAGE analysis (Chapter 7). One-dimensional (1D) alkaline urea (AU)-PAGE and 2D AU:SDS-PAGE dissociated the strong casein–casein hydrophobic associations, and the processing-induced disulphide-bonded protein aggregates of caseins and whey proteins in the control or processed samples, which allowed the α_{s2} -casein (α_{s2} -CN) monomers and dimers to be identified and tracked. Consequently, it was possible to show that these large disulphide-bonded aggregates that formed at high temperatures ($> 100^{\circ}\text{C}$) or pressures ($> 400\text{ MPa}$) contained complexes consisting of a high proportion of α_{s2} -CN as well as κ -CN, β -LG and other whey proteins. The effects of heat treatment and

pressure treatment on the interactions of caseins and whey proteins in milk were significantly different. α_{s2} -CN as well as κ -CN aggregated with β -LG at high temperatures and at pressures greater than 400 MPa. However, β -LG preferentially aggregated with κ -CN at 200 MPa. The accessibility of α_{s2} -CN and the formation of complexes involving α_{s2} -CN, κ -CN and whey proteins in the pressure-treated milk, as demonstrated using the modified 2D PAGE technique and as explained by possible proposed reactions of the caseins and whey proteins in pressure-treated milk (Figures 7.7, 7.9 and 7.10, Chapter 7), was an important novel finding of the present study. It was also found that dimeric α_{s2} -CN was comparatively more stable than the monomer form after either HP treatment or high temperature treatment. These findings also partly demonstrated that the differences between the stabilities of the proteins and the accessibilities of the disulphide bonds of the proteins at high temperature or pressure affect the pathways that give the differences among the resultant aggregates, the sizes of the aggregates and the product functionalities.

5) Finally, a study on the effects of the pressure treatment of pure proteins alone and in binary and ternary systems generated useful information and confirmed the role of each protein in pressure-induced denaturation, aggregation and interactions. This study was a major step in clarifying some doubts about the role of different proteins in complex systems (e.g. in aggregation and gelation of WPC or interactions of caseins and whey proteins in milk).

α -LA and BSA exhibited different behaviour when pressure treated in the presence, compared with in the absence, of β -LG. The presence of β -LG in the system was found to catalyse the reactions of other proteins such as α -LA or BSA but the reactions of β -LG were not significantly affected by α -LA or BSA, as discussed in Chapter 8.

9.3 Recommendations for future study

Effects of adiabatic heating

The temperature of the pressurising fluid and the sample increases during pressurisation as a result of adiabatic heating and decreases as a result of adiabatic cooling (Chapter 3). Adiabatic heating may accelerate the rate of denaturation and aggregation of proteins in the early stages of each pressurisation cycle, depending on the desired (preset) pressurising temperature in a particular study. The temperature equilibrates back to the preset temperature within about 5 min of achieving the desired pressure. This is of concern, particularly when samples are pressure treated at higher pressure and higher pressurising temperature and for a short time (e.g. for holding times of 5–10 min), because, in this case, the depressurisation cycle will begin before the temperature of the sample equilibrates back to the preset temperature, and therefore the sample will be pressurised at a higher temperature than is desired, which may give an incorrect prediction of denaturation. Moreover, because the pressurisation and depressurisation are not instantaneous (e.g. a pressure increase of approximately 300 MPa/min), some denaturation of the proteins may occur during the pressurisation process (e.g. when samples are pressure treated at 600–800 MPa, it would take 2–3 min to achieve the desired pressure). Ideally, it is important to compensate for such adiabatic heating and pressurisation effects. However, the effects of this important parameter have not been considered in most of the previous studies, including the present study. Therefore, the adiabatic heating for each pressurising temperature and each pressurisation cycle should be considered as an independent parameter for evaluation under HP processing and such effects should be evaluated in detail in a separate study.

Effects of pressurising fluid

Different pressurising fluids (e.g. water, mineral oil, emulsions etc) have different temperature responses and different compressibility. This may result into varied extent of adiabatic heating and therefore may affect the denaturation and aggregation of proteins, particularly when the combination of pressure treatment and heat is applied to inactivate pressure-resistant spores. Available literature suggests that different pressurising fluids have considerable effects on microbial inactivation. However, comparatively little information is available on the effects of the pressurising fluid on

proteins. Therefore, a detailed investigation of this subject will be useful both from a scientific viewpoint and from a commercial viewpoint.

Effects of pH and ionic strength on the pressure-induced denaturation of whey proteins

It is known that the pH values and ionic strengths of samples have marked effects on the heat-induced denaturation, aggregation and interactions of proteins. However, comparatively little attention has been given to study the effects of these factors on pressure-induced aggregation and interactions of proteins. A detailed study of such aspects would be helpful in providing greater understanding of the subject, which might be useful in relating the laboratory and the processing data.

Comparison of the behaviours of proteins under pressure 'in situ' and after pressure release

Many reports have indicated that proteins may behave differently when analysed under pressure 'in situ' and after pressure treatment. Therefore, it may be important to study and compare these behaviours in detail, using suitable common techniques. The lack of suitable equipment, such as high pressure cells for circular dichroism and nuclear magnetic resonance, limits such investigations. Some of the recent reports have exploited "in-situ" light scattering properties of milk (Huppertz, Kelly and de Kruif, 2006) or changes in the pH changes of protein solutions (Orlien et al., 2007) under pressure using pressure chamber with special window

Effects of ligands on pressure-induced denaturation and aggregation

Previous work has shown that the binding of ligands or small molecules to β -LG at near neutral pH delays its denaturation and aggregation (Considine et al., 2005a, 2005b, 2006). Similar effects may be possible in WPC systems (see Appendix 2) or with other whey proteins such as α -LA and BSA. This may open up the possibility for a new HP-treated ingredient with bound ligands, with modified functional properties, such as gel formation. Therefore, a comparison of the properties of gels made from proteins with and without added ligands or solutes would be another area of interest for future study.

Effects of the addition/depletion of particular caseins or whey proteins on the functionality of pressure-treated systems

α_{s2} -CN, which is buried inside the casein micelles, is generally not accessible for interactions with β -LG or other whey proteins. However, the results and the proposed pathways of the present study suggest that solubilisation of the casein micelles under pressure treatment makes α_{s2} -CN easily accessible to whey proteins. This will have significant effects on the functionality of milk products made from pressure-treated milk, as suggested by the results of an initial investigation that was carried out in the present study. However, it will be helpful to evaluate the full mechanisms by studying the effects of the addition/depletion of particular caseins or whey proteins on the functionality of pressure-treated systems.

Effects of a combination of heat and pressure

A combination of heat treatment and pressure treatment has been reported to have significant effects on microbial inactivation as well as on the functionality of milk proteins. HP processing alone, HP processing in combination with heat treatment and the effects of HP processing in combination with low temperature (e.g. 4°C) may be subject of interest for investigations using different systems such as milk, WPC/whey protein isolate (WPI) and model system using pure proteins.

Behaviour of WPI vs WPC

WPI has recently become very popular in the beverage and food industry because of its high protein content and excellent functional properties. Although it is generally expected that WPI will behave in the same way as WPC under pressure treatment, it would be useful to confirm this by carrying out extensive studies using WPI in place of the WPC used in the present study. Also, WPIs from different sources, such as mineral acid casein whey, rennet casein whey or cheese whey, and made using different manufacturing processes, such as microfiltration, ultrafiltration or ion exchange, will give different properties and may behave differently under pressure treatment because of their different mineral contents, different amounts of β -LG, different proportions of glycomacropeptide etc.

9.4 Concluding remarks

In conclusion, research on the HP processing of dairy proteins is comparatively new area (compared with various aspects of heat treatment) and there is still a long way to go. It is important to ensure that research on the various areas of HP processing is addressed appropriately to realise its full commercial potential. As significant progress has already been made on the engineering aspects in the development of suitable equipment for processing, wider research to address various important aspects would help the food industry in the widespread use and acceptance of this technology. Extensive research to fully understand the behaviour of milk proteins is certainly an area of interest from the viewpoint of texture improvement/modification or for the creation of new products/ingredients with novel textures and functionalities using HP technology.

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Appendix 1

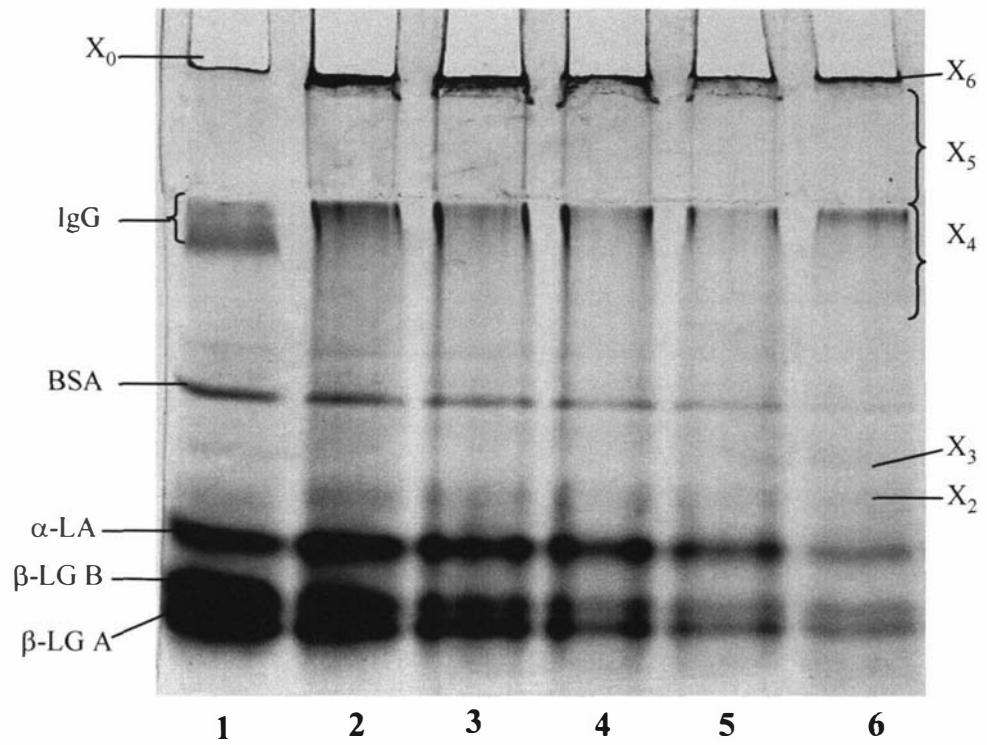


Figure 1.1. Native-PAGE of WPC solutions (2% w/v): control (Lane 1), and pressure treated at 400 MPa for 5 min (Lane 2), 10 min (Lane 3), 20 min (Lane 4), 30 min (Lane 5) and 40 min (Lane 6).

Appendix 2

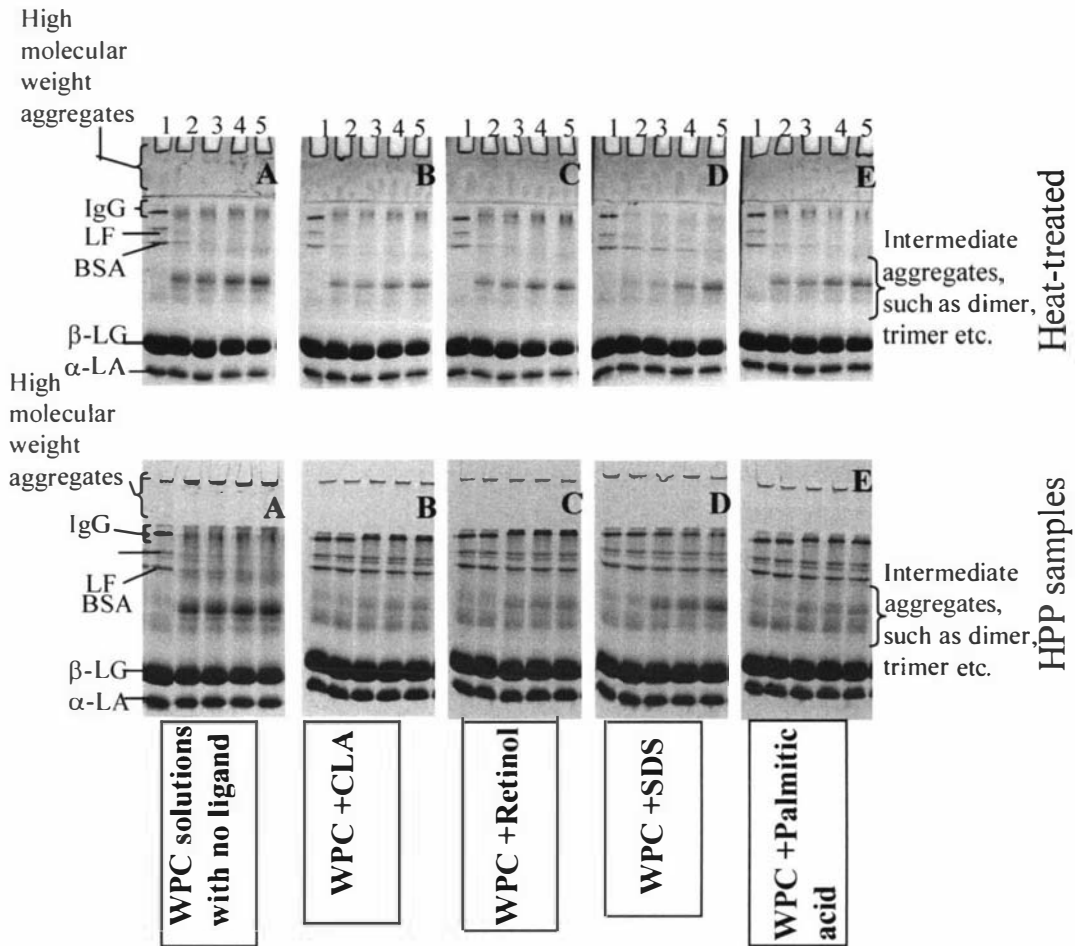


Figure 2.1. SDS-PAGE patterns of 2% WPC solutions, with and without ligands, that were untreated (Lane 1), heat treated at 80°C (top) and pressure treated at 600 MPa (bottom) for 1 min (Lane 2), 2 min (Lane 3), 3 min (Lane 4) and 4 min (Lane 5).

Appendix 3

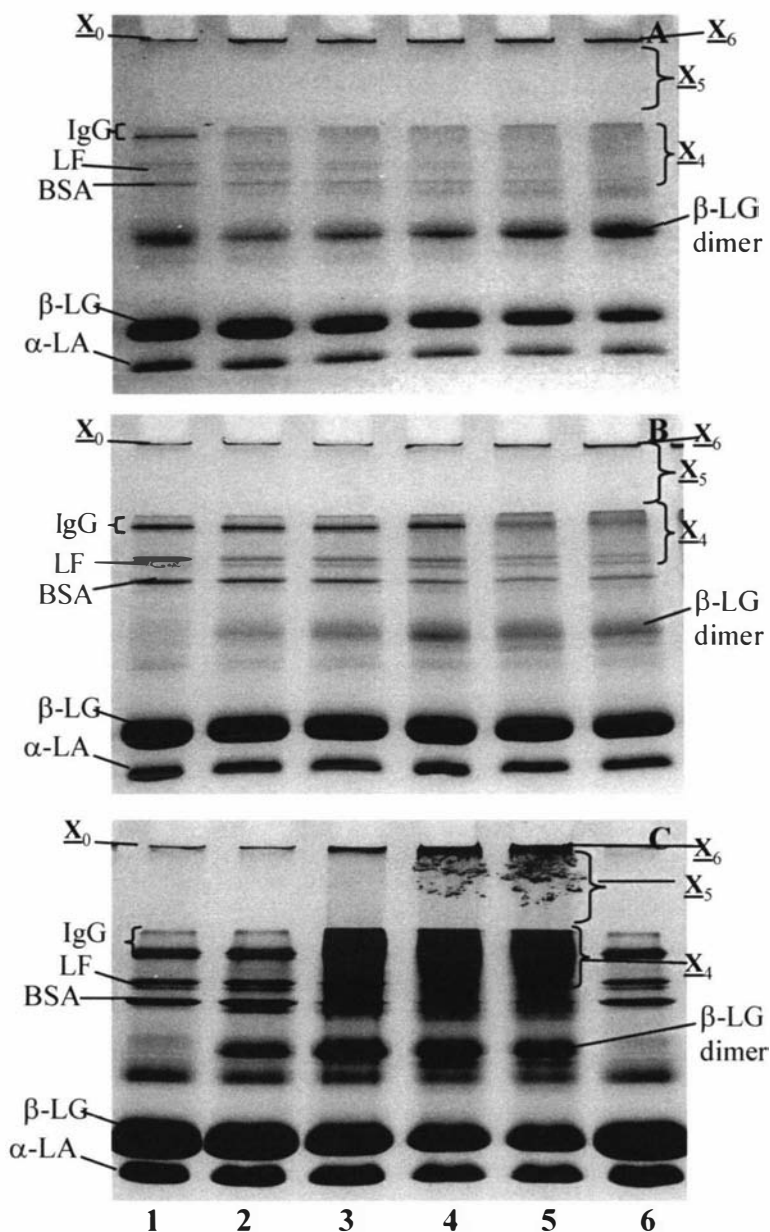


Figure 3.1. SDS-PAGE patterns of (A) 0.5%, (B) 5% and (C) 12% WPC solutions that were untreated (Lane 1) and pressure treated at 800 MPa for 20 min (Lane 2), 40 min (Lane 3), 60 min (Lane 4), 90 min (Lane 5) and 120 min (Lane 6).

Appendix 4

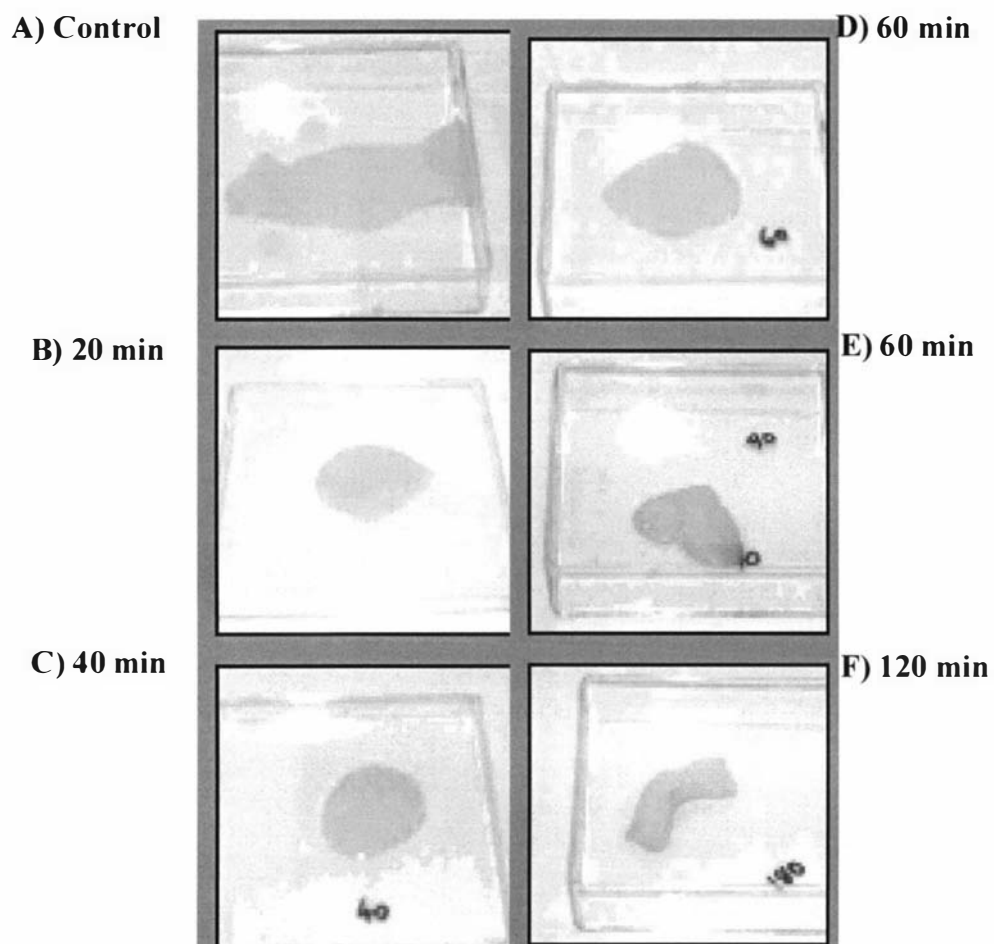


Figure 4.1. Physical appearance of the WPC solutions (12% w/v): untreated control (A), and samples pressure treated at 600 MPa (22 °C) and held for 20 min (B), 40 min (C), 60 min (D), 90 min (E) and 120 min (F). The colour and the physical appearance of the samples changed from liquid to semi-solid soft gel to self-supporting elastic gel structures.

Appendix 5

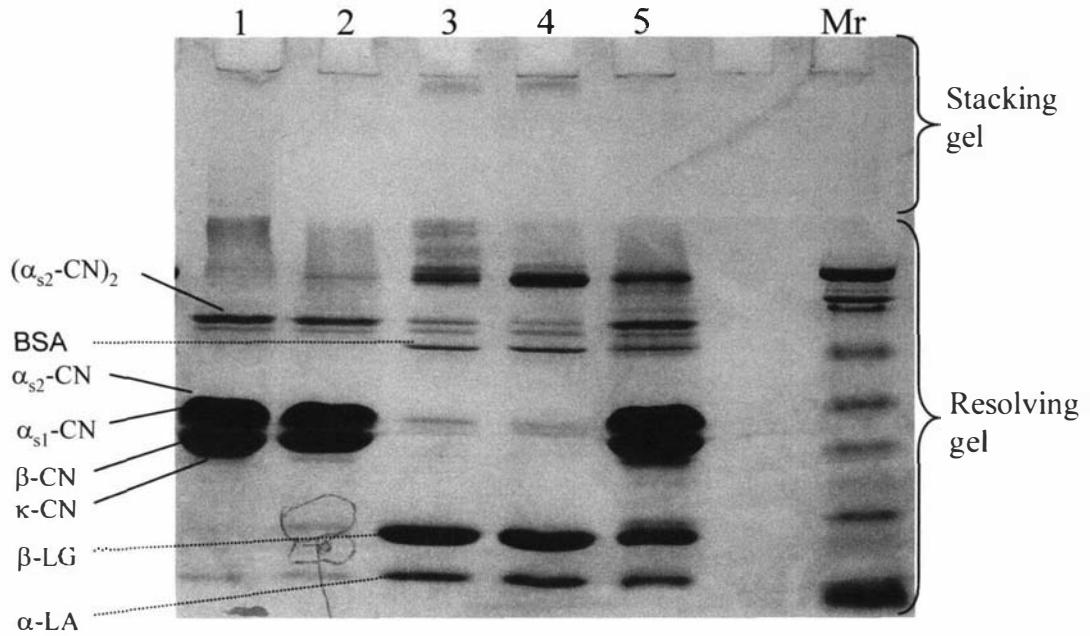


Figure 5.1. SDS-PAGE pattern of acid casein (Lane 1), rennet casein (Lane 2), acid casein whey (Lane 3), rennet casein whey (Lane 4) and skim milk (Lane 5). The acid casein, rennet casein, acid casein whey and rennet casein whey were produced from the same skim milk sample, and the SDS-PAGE patterns show the distribution of the casein and whey protein fractions. Lane Mr shows the molecular weight markers, which also helped to identify the bands corresponding to the various caseins and whey proteins.

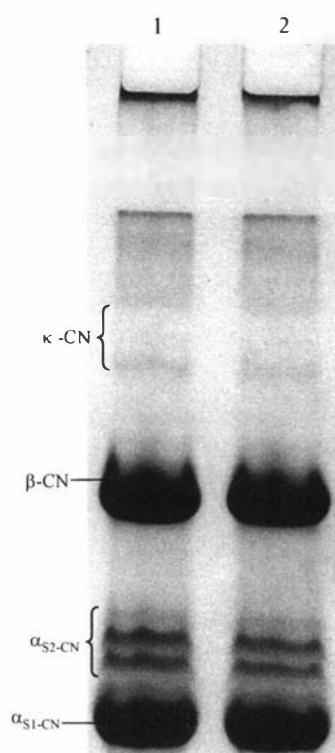
Appendix 5 (continued)

Figure 5.2. AU-PAGE patterns of acid casein (Lane 1) and rennet casein (Lane 2).

Appendix 5 (continued)

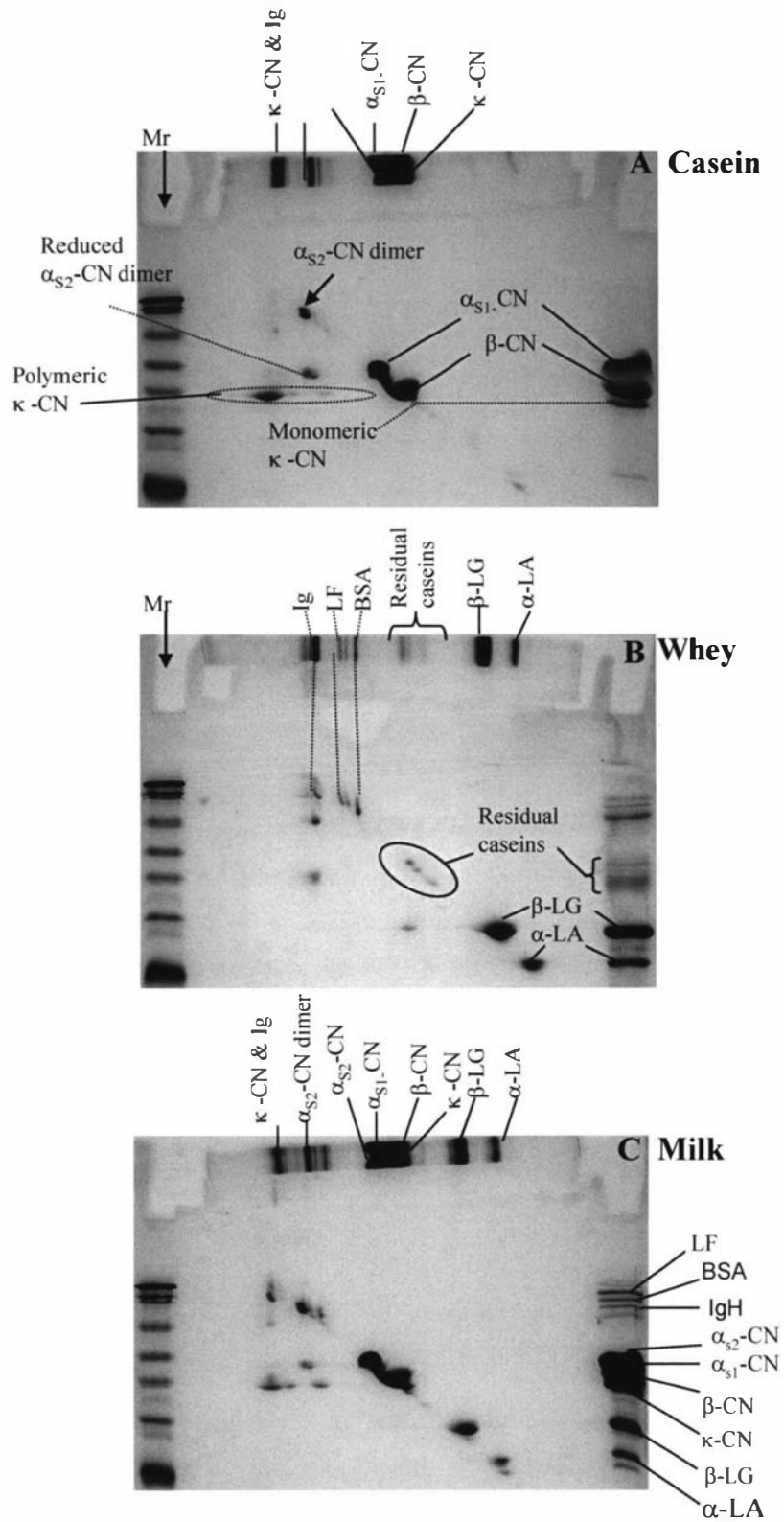


Figure 5.3. 2D SDS- and then reduced SDS-PAGE patterns of (A) acid casein fraction (Lane 1) and (B) whey fraction isolated from skim milk (pattern C). The acid casein and the acid casein whey were produced from the same skim milk sample, and patterns A and B show the distribution of the casein and whey protein fractions.

Appendix 5 (continued)

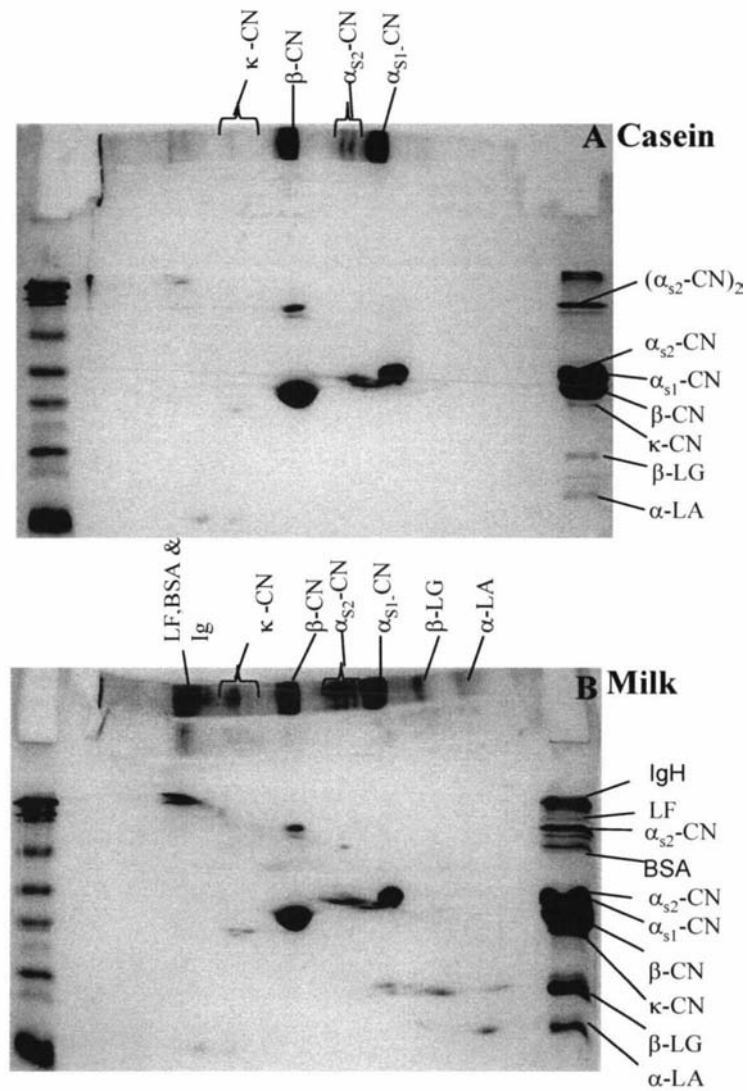


Figure 5.4. 2D AU- and then non-reduced SDS-PAGE pattern of (A) acid casein fraction (Lane 1) and (B) skim milk. The acid casein was produced from the same skim milk sample.

Appendix 6

Mascot Search Results

User : AVEENASH
Email : AVEENASH.BALA@FONTERRA.COM
Search title : D:\PE Sciex Data\Projects\API Instrument\Data\Datacsn200903.wiff : 1063 HP
MS data file : C:\TEMP\mas17.tmp
Database : Sprot 040303 (122210 sequences; 44861009 residues)
Taxonomy : Other mammalia (5169 sequences)
Timestamp : 24 Sep 2003 at 20:52:40 GMT
Significant hits: TRFL BOVIN (P24627) Lactotransferrin precursor (Lactoferrin)
CAS2 BOVIN (P02663) Alpha-S2 casein precursor [Contains: Ca
LACB BOVIN (P02754) Beta-lactoglobulin precursor (Beta-LG)
LACB SHEEP (P02757) Beta-lactoglobulin 1/B, 2/A, and 3/C pr
TRFL CAPHI (Q29477) Lactotransferrin precursor (Lactoferrin)
CAS2 CAPHI (P33049) Alpha-S2 casein precursor (Alpha-S2-CN)
TRYP PIG (P00761) Trypsin precursor (EC 3.4.21.4)
K1CJ BOVIN (P06394) Keratin, type I cytoskeletal VIB (Cytok
TRFL CAMDR (Q9TUM0) Lactotransferrin precursor (Lactoferrin)
TRFE BOVIN (Q29443) Serotransferrin precursor (Transferrin)

Probability Based Mowse Score

Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event.

Individual ions scores > 25 indicate identity or extensive homology ($p < 0.05$).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

| | | | | |
|-----------|--|--|---------------------|---|
| Format As | Peptide Summary | | | Help |
| | Significance threshold p< | 0.05 | Max. number of hits | 10 |
| | Standard scoring <input checked="" type="checkbox"/> | MudPIT scoring <input checked="" type="checkbox"/> | Ions score cut-off | 0 |
| | Show pop-ups <input checked="" type="checkbox"/> | Suppress pop-ups <input checked="" type="checkbox"/> | Sort unassigned | Decreasing Score |
| | | | | Show sub-sets <input type="checkbox"/> |
| | | | | Require bold red <input type="checkbox"/> |

 Error tolerant

1. TRFL BOVIN **Mass:** 80002 **Score:** 460 **Queries matched:** 13

(P24627) Lactotransferrin precursor (Lactoferrin)

Check to include this hit in error tolerant search or archive report

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|--|----------|----------|----------|-------|------|-------|----------|------|-------------|
| <input checked="" type="checkbox"/> <u>68</u> | 402.20 | 802.38 | 802.42 | -0.04 | 0 | 21 | 0.14 | 1 | EDLIWK |
| <input checked="" type="checkbox"/> <u>84</u> | 439.72 | 877.43 | 877.47 | -0.04 | 0 | 48 | 0.00031 | 1 | DSALGFLR |
| <input checked="" type="checkbox"/> <u>130</u> | 540.76 | 1079.51 | 1079.56 | -0.05 | 0 | 66 | 4.3e-006 | 1 | VDSALYLGSR |
| <input checked="" type="checkbox"/> <u>133</u> | 549.23 | 1096.44 | 1096.50 | -0.05 | 0 | 35 | 0.0046 | 1 | YYGYTGAFR |
| <input checked="" type="checkbox"/> <u>173</u> | 617.29 | 1232.56 | 1231.62 | 0.94 | 0 | 13 | 0.97 | 1 | ANEGLTWNSLK |

| | | | | | | | | | | |
|-------------------------------------|------------|--------|---------|---------|-------|---|----|----------|---|----------------|
| <input type="checkbox"/> | <u>190</u> | 633.26 | 1264.50 | 1264.58 | -0.08 | 0 | 14 | 0.71 | 2 | EPYFGYSGAFK |
| <input checked="" type="checkbox"/> | <u>199</u> | 430.54 | 1288.61 | 1288.67 | -0.06 | 1 | 29 | 0.018 | 1 | SVDGKEDLIWK |
| <input checked="" type="checkbox"/> | <u>202</u> | 653.80 | 1305.59 | 1305.65 | -0.06 | 0 | 55 | 5.2e-005 | 1 | ETTVFENLPEK |
| <input checked="" type="checkbox"/> | <u>205</u> | 439.90 | 1316.69 | 1316.75 | -0.06 | 0 | 42 | 0.0012 | 1 | LRPVAAEIYGTK |
| <input checked="" type="checkbox"/> | <u>206</u> | 660.82 | 1319.62 | 1319.66 | -0.04 | 0 | 12 | 1.1 | 1 | SFQLFGSPPGQR |
| <input checked="" type="checkbox"/> | <u>217</u> | 681.81 | 1361.60 | 1361.67 | -0.07 | 0 | 57 | 3.5e-005 | 1 | GSNFQLDQLQGR |
| <input checked="" type="checkbox"/> | <u>243</u> | 497.57 | 1489.70 | 1489.76 | -0.07 | 1 | 33 | 0.0079 | 1 | KGSNFQLDQLQGR |
| <input checked="" type="checkbox"/> | <u>262</u> | 531.25 | 1590.73 | 1590.80 | -0.07 | 0 | 37 | 0.0026 | 1 | ESPQTHYYAVAVVK |

2. CAS2 BOVIN **Mass:** 26173 **Score:** 344 **Queries matched:** 31

(P02663) Alpha-S2 casein precursor [Contains: Ca

Check to include this hit in error tolerant search or archive report

| | Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|-------------------------------------|-----------|----------|----------|----------|-------|------|-------|--------|------|---------|
| <input checked="" type="checkbox"/> | <u>48</u> | 634.36 | 633.36 | 633.38 | -0.03 | 0 | 35 | 0.011 | 1 | LNFLK |
| | <u>49</u> | 635.35 | 634.34 | 633.38 | 0.95 | 0 | (15) | 0.71 | 6 | LNFLK |
| <input checked="" type="checkbox"/> | <u>56</u> | 746.41 | 745.41 | 745.45 | -0.04 | 0 | 27 | 0.051 | 1 | VIPYVR |
| <input checked="" type="checkbox"/> | <u>57</u> | 762.45 | 761.44 | 761.48 | -0.04 | 1 | 16 | 0.81 | 1 | LNFLKK |

| | | | | | | | | | | |
|---|------------|---------|---------|---------|-------|---|------|---------|---|---------------------------|
| ☒ | <u>102</u> | 490.26 | 978.50 | 978.55 | -0.05 | 0 | (14) | 0.61 | 1 | FALPQYLK |
| ☒ | <u>103</u> | 490.26 | 978.50 | 978.55 | -0.05 | 0 | 52 | 0.00011 | 1 | FALPQYLK |
| ☒ | <u>104</u> | 979.51 | 978.50 | 978.55 | -0.05 | 0 | (19) | 0.21 | 1 | FALPQYLK |
| ☒ | <u>105</u> | 979.51 | 978.50 | 978.55 | -0.05 | 0 | (14) | 0.66 | 1 | FALPQYLK |
| ☒ | <u>106</u> | 490.26 | 978.51 | 978.55 | -0.04 | 0 | (29) | 0.02 | 1 | FALPQYLK |
| ☒ | <u>107</u> | 490.26 | 978.51 | 978.55 | -0.04 | 0 | (50) | 0.00019 | 1 | FALPQYLK |
| ☒ | <u>136</u> | 557.78 | 1113.55 | 1113.60 | -0.05 | 0 | (33) | 0.0093 | 1 | AMKPWIQPK + Oxidation (M) |
| ☒ | <u>137</u> | 557.79 | 1113.56 | 1113.60 | -0.04 | 0 | (14) | 0.78 | 1 | AMKPWIQPK + Oxidation (M) |
| ☒ | <u>138</u> | 558.00 | 1113.99 | 1113.60 | 0.38 | 0 | 34 | 0.0087 | 1 | AMKPWIQPK + Oxidation (M) |
| ☒ | <u>158</u> | 598.30 | 1194.59 | 1194.67 | -0.09 | 0 | (13) | 0.78 | 1 | NAVPIPTLNR |
| ☒ | <u>159</u> | 1195.62 | 1194.62 | 1194.67 | -0.06 | 0 | (25) | 0.047 | 1 | NAVPIPTLNR |
| ☒ | <u>160</u> | 598.32 | 1194.62 | 1194.67 | -0.05 | 0 | (4) | 5.7 | 1 | NAVPIPTLNR |
| ☒ | <u>161</u> | 598.60 | 1195.19 | 1194.67 | 0.51 | 0 | 46 | 0.00038 | 1 | NAVPIPTLNR |
| ☒ | <u>162</u> | 598.81 | 1195.60 | 1194.67 | 0.93 | 0 | (13) | 0.65 | 1 | NAVPIPTLNR |
| ☒ | <u>163</u> | 598.81 | 1195.60 | 1194.67 | 0.93 | 0 | (32) | 0.009 | 1 | NAVPIPTLNR |
| ☒ | <u>179</u> | 623.79 | 1245.57 | 1245.64 | -0.06 | 1 | (38) | 0.0025 | 1 | ITVDDKHYQK |
| ☒ | <u>180</u> | 416.20 | 1245.57 | 1245.64 | -0.06 | 1 | (45) | 0.00053 | 1 | ITVDDKHYQK |
| ☒ | <u>181</u> | 623.80 | 1245.58 | 1245.64 | -0.06 | 1 | 48 | 0.00029 | 1 | ITVDDKHYQK |

Appendices

| | | | | | | | | | | |
|---|------------|--------|---------|---------|-------|---|------|-------|---|-------------------------------|
| ✓ | <u>183</u> | 416.54 | 1246.60 | 1245.64 | 0.96 | 1 | (29) | 0.024 | 1 | ITVDDKHYQK |
| ✓ | <u>213</u> | 676.36 | 1350.70 | 1350.77 | -0.07 | 1 | (29) | 0.022 | 1 | RNAVPIPTLNR |
| ✓ | <u>214</u> | 451.24 | 1350.71 | 1350.77 | -0.07 | 1 | 38 | 0.003 | 1 | RNAVPIPTLNR |
| ✓ | <u>221</u> | 684.32 | 1366.63 | 1366.69 | -0.06 | 0 | (18) | 0.3 | 1 | ALNEINQFYQK |
| | <u>222</u> | 684.32 | 1366.63 | 1366.69 | -0.06 | 0 | (12) | 1.3 | 2 | ALNEINQFYQK |
| ✓ | <u>223</u> | 684.81 | 1367.60 | 1366.69 | 0.91 | 0 | (23) | 0.076 | 1 | ALNEINQFYQK |
| ✓ | <u>224</u> | 684.81 | 1367.60 | 1366.69 | 0.92 | 0 | 26 | 0.042 | 1 | ALNEINQFYQK |
| ✓ | <u>233</u> | 701.79 | 1401.57 | 1401.63 | -0.06 | 0 | 15 | 0.5 | 1 | TVDMESTEVFTK + Oxidation (M) |
| ✓ | <u>252</u> | 510.89 | 1529.66 | 1529.73 | -0.07 | 1 | 14 | 0.6 | 1 | TVDMESTEVFTKK + Oxidation (M) |

3. LACB BOVIN **Mass:** 20269 **Score:** 269 **Queries matched:** 7
(P02754) Beta-lactoglobulin precursor (Beta-LG)

Check to include this hit in error tolerant search or archive report

| Query | Observed | Mr (expt) | Mr (calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|--|----------|-----------|-----------|-------|------|-------|----------|------|-------------------------|
| <u>78</u> | 427.22 | 852.42 | 852.46 | -0.04 | 0 | 14 | 0.62 | 2 | ALPMHIR + Oxidation (M) |
| <input checked="" type="checkbox"/> <u>92</u> | 458.72 | 915.42 | 915.47 | -0.05 | 0 | 49 | 0.00025 | 1 | IDALNENK |
| <input checked="" type="checkbox"/> <u>126</u> | 533.27 | 1064.53 | 1064.58 | -0.05 | 0 | 20 | 0.2 | 1 | VLVLDTDYK |
| <input checked="" type="checkbox"/> <u>157</u> | 597.31 | 1192.61 | 1192.67 | -0.06 | 1 | 62 | 1.2e-005 | 1 | VLVLDTDYKK |
| <input checked="" type="checkbox"/> <u>178</u> | 623.26 | 1244.51 | 1244.58 | -0.06 | 0 | 64 | 6.3e-006 | 1 | TPEVDDEALEK |
| <input checked="" type="checkbox"/> <u>272</u> | 818.35 | 1634.68 | 1634.77 | -0.08 | 1 | (39) | 0.0021 | 1 | TPEVDDEALEKFDK |
| <input checked="" type="checkbox"/> <u>273</u> | 545.90 | 1634.69 | 1634.77 | -0.08 | 1 | 61 | 1.4e-005 | 1 | TPEVDDEALEKFDK |

Proteins matching the same set of peptides:

LACB BUBBU **Mass:** 20410 **Score:** 269 **Queries matched:** 7
(P02755) Beta-lactoglobulin precursor (Beta-LG)

4. LACB SHEEP **Mass:** 20308 **Score:** 223 **Queries matched:** 7
 (P02757) Beta-lactoglobulin 1/B, 2/A, and 3/C pr

Check to include this hit in error tolerant search or archive report

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|------------|----------|----------|----------|-------|------|-------|----------|------|-------------------------|
| <u>78</u> | 427.22 | 852.42 | 852.46 | -0.04 | 0 | 14 | 0.62 | 2 | ALPMHIR + Oxidation (M) |
| <u>92</u> | 458.72 | 915.42 | 915.47 | -0.05 | 0 | 49 | 0.00025 | 1 | IDALNENK |
| <u>126</u> | 533.27 | 1064.53 | 1064.58 | -0.05 | 0 | 20 | 0.2 | 1 | VLVLDTDYK |
| <u>157</u> | 597.31 | 1192.61 | 1192.67 | -0.06 | 1 | 62 | 1.2e-005 | 1 | VLVLDTDYKK |
| <u>178</u> | 623.26 | 1244.51 | 1243.59 | 0.92 | 0 | 19 | 0.2 | 3 | TPEVDNEALEK |
| <u>272</u> | 818.35 | 1634.68 | 1633.78 | 0.90 | 1 | (32) | 0.01 | 2 | TPEVDNEALEKFDK |
| <u>273</u> | 545.90 | 1634.69 | 1633.78 | 0.90 | 1 | 60 | 1.8e-005 | 2 | TPEVDNEALEKFDK |

5. TRFL CAPHI **Mass:** 79361 **Score:** 168 **Queries matched:** 5
 (Q29477) Lactotransferrin precursor (Lactoferrin

Check to include this hit in error tolerant search or archive report

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|------------|---------------|----------------|----------------|--------------|----------|----------|------------|----------|----------------------|
| <u>130</u> | 540.76 | 1079.51 | 1079.56 | -0.05 | 0 | 66 | 4.3e-006 | 1 | VDSALYLGSR |
| <u>133</u> | 549.23 | 1096.44 | 1096.50 | -0.05 | 0 | 35 | 0.0046 | 1 | YGYTGAFR |
| <u>173</u> | 617.29 | 1232.56 | 1231.62 | 0.94 | 0 | 13 | 0.97 | 1 | ANEGLTWNSLK |
| <u>202</u> | 653.80 | 1305.59 | 1305.65 | -0.06 | 0 | 55 | 5.2e-005 | 1 | ETTVFENLPEK |
| <u>228</u> | 695.30 | 1388.59 | 1388.74 | -0.15 | 1 | 4 | 6.9 | 9 | TSALECIRAIAGK |

6. CAS2 CAPHI **Mass:** 26543 **Score:** 127 **Queries matched:** 13
(P33049) Alpha-S2 casein precursor (Alpha-S2-CN)

Check to include this hit in error tolerant search or archive report

| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|------------|---------------|----------------|----------------|--------------|----------|----------|-----------|----------|-------------------------------|
| <u>48</u> | 634.36 | 633.36 | 633.38 | -0.03 | 0 | 35 | 0.011 | 1 | LNFLK |
| <u>49</u> | 635.35 | 634.34 | 633.38 | 0.95 | 0 | (15) | 0.71 | 6 | LNFLK |
| <u>57</u> | 762.45 | 761.44 | 761.48 | -0.04 | 1 | 16 | 0.81 | 1 | LNFLKK |
| <u>179</u> | 623.79 | 1245.57 | 1245.64 | -0.06 | 1 | (38) | 0.0025 | 1 | ITVDDKHYQK |
| <u>180</u> | 416.20 | 1245.57 | 1245.64 | -0.06 | 1 | (45) | 0.00053 | 1 | ITVDDKHYQK |
| <u>181</u> | 623.80 | 1245.58 | 1245.64 | -0.06 | 1 | 48 | 0.00029 | 1 | ITVDDKHYQK |
| <u>183</u> | 416.54 | 1246.60 | 1245.64 | 0.96 | 1 | (29) | 0.024 | 1 | ITVDDKHYQK |
| <u>221</u> | 684.32 | 1366.63 | 1366.69 | -0.06 | 0 | (18) | 0.3 | 1 | ALNEINQFYQK |
| <u>222</u> | 684.32 | 1366.63 | 1366.69 | -0.06 | 0 | (12) | 1.3 | 2 | ALNEINQFYQK |
| <u>223</u> | 684.81 | 1367.60 | 1366.69 | 0.91 | 0 | (23) | 0.076 | 1 | ALNEINQFYQK |
| <u>224</u> | 684.81 | 1367.60 | 1366.69 | 0.92 | 0 | 26 | 0.042 | 1 | ALNEINQFYQK |
| <u>277</u> | 558.00 | 1670.98 | 1671.93 | -0.96 | 2 | 1 | 11 | 6 | LNFLKKISQYYQK |
| <u>312</u> | 939.24 | 2814.71 | 2815.28 | -0.56 | 2 | 5 | 3 | 4 | EKLCTTSCEEVVRNANEEYSIR |