STUDIES ON THE EFFECTS OF HEAT AND HIGH PRESSURE TREATMENTS ON FAT GLOBULE SURFACE LAYERS IN RECOMBINED MILK

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ABSTRACT

The present study examined the effects of heat treatment, high pressure (HP) treatment or combined heat and HP treatments, either before or after homogenization, on recombined milk systems. The main focus was to explore the changes induced by these treatments on the surface layers of recombined fat globules, milk proteins and rheological properties of acid gels.

Heat treatments caused denaturation of whey proteins; the degree of denaturation was dependent on temperature, holding time and to a lesser extent on the placement of heat treatment. Recombined milks that underwent heat treatment before or after homogenization had similar levels of whey protein denaturation. The amounts of caseins and denatured whey proteins adsorbed on the surface of fat globules of recombined milk heated before homogenization were significantly lower than those heated after homogenization, indicating different interaction mechanisms in these two systems.

Increases in treatment pressure used in HP treatment resulted in decreased amounts of caseins, while whey proteins adsorbed onto the surface layers of fat globules increased. This was probably due to the dissociation of casein micelles under HP treatment and the interactions between HP-induced denatured whey proteins and casein particles on the surface layers of fat globules.

Combined heat and HP treatments induced changes on adsorbed caseins and whey proteins on fat globule surface layers. HP treatment induced additional denaturation of whey proteins in heated milks, resulting in slightly increased amounts of denatured whey protein adsorbed onto the surface layers.

Gelation pH, final G' and yield stress values of acid gels prepared from recombined milks heated before or after homogenization were dependent on temperature, holding time and the placement of heat treatment. These changes were attributed to the extent of denaturation of the whey proteins and their interactions with casein particles adsorbed onto the fat globule surface and in the serum. Differences in acid gels prepared from recombined milks heated before and after homogenization were

attributed to the relative proportions of caseins and whey proteins at the surface layers of fat globules resulting in different interactions with protein strands in the gel network.

The acid gels prepared from recombined milks HP-treated either before or after homogenization had shorter gelation times, higher gelation pH, final G' and yield stress values compared with untreated recombined milk and the effects were dependent on treatment pressure, temperature, holding time and the placement of HP treatment. The denaturation of whey proteins and their interactions with casein particles were responsible for these changes. In HP-treated recombined milks the proportions of caseins and denatured whey proteins adsorbed onto the surface layers of fat globules had significant effects on the acid gel structure. When HP treatment was applied after homogenization, the proteins on the surface layer were present as a layer which might provide better sites for the interactions with the protein strands in the gel matrix.

The application of these processing treatments to recombined milk could provide new avenues to the dairy industry for manufacturing novel products with enhanced texture and nutritional properties.

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CHAPTER 1

INTRODUCTION

Recombined milks are widely used in countries where the milk production is insufficient as the compositions of these milks can be easily standardized. The preparation of recombined milk involves the reconstitution of skim milk powder (SMP), mixing with anhydrous milk fat (AMF) or vegetable fat followed by homogenisation. This process essentially creates an oil-in-water emulsion. The recombined milk can be used to manufacture all kinds of dairy products, such as recombined evaporated milk, cheese and yoghurt. During the manufacture of recombined milk, fat is dispersed via homogenisation into small fat droplets and fat globule surface layers are formed by the adsorption of skim milk proteins, i.e., caseins and whey proteins. The composition of these layers is markedly affected by processing treatments which ultimately influence the properties of the recombined dairy products. The protein-protein and protein-fat interactions involving fat globules have been shown to influence the heat stability (Sharma, Singh, & Taylor, 1996b) and acid gelation properties of recombined milk systems (Cho, Lucey, & Singh, 1999).

In the dairy industry, heat treatment is employed mainly to destroy microorganisms and to modify the functional properties of dairy products. Although the heat treatment is a very useful procedure, severe heat treatment often causes unwanted changes to the sensory and nutritional quality. Recently, non-thermal technology, such as high pressure (HP) treatment is gaining acceptance for food processing due to the increasing consumer demand for microbiologically safe, nutritious and fresh-like food products. In addition, HP treatment has the potential to produce new products with modified textures and functional properties (Balci & Wilbey, 1999; Buchheim, Schrader, Morr, Frede, & Schütt, 1996; Datta & Deeth, 1999; Huppertz, Fox, de Kruif, & Kelly, 2006).

Most previous research on the effects of HP treatment on dairy products has concentrated on denaturation of whey proteins, rheological and microstructural aspects of individual proteins, i.e., caseins and whey proteins (e.g. Anema (2008c); Huppertz and de Kruif (2007); Needs, Stenning, Gill, Ferragut, and Rich (2000)), using skim milk or whole milk. No specific study has been carried out on the effects of HP treatments on recombined milk systems, especially in relation to changes in the surface layers of

fat globules. These along with the interactions between milk proteins in recombined milk systems remain unclear.

Both heat and HP treatments have significant but different effects on milk proteins. Extensive studies have been carried on the interactions of milk proteins during heat treatments on milk systems (see review, Singh (1995); Singh and Ye (2010)), but studies on interactions involving fat globules in recombined milks are limited. The relationships between the changes in fat globule surface layers induced by heat or HP treatments and the functionality of dairy products have not been fully explored. In addition, combined utilization of heat and HP treatments might be beneficial to the dairy processing industry in terms of functionality, texture and cost of production process. Thus, the main objective of the present study is to determine the composition of the fat globule surface layers of heat- and HP-treated recombined milks and explore how acid gelation properties are affected by the modification of fat globule surface layers. This should provide a scientific background for the development of future technological methods of controlling the compositions of surface layers and enhancing the functional properties of recombined milks.

CHAPTER 2

LITERATURE REVIEW

2.1. Milk and milk composition

This study deals with recombined milk that contains several components derived from regular milk. Therefore, a brief review of the composition of normal milk is given. Milk is a complex fluid containing water, fat, lactose, proteins, and minerals in different states of dispersion. Lactose and a portion of mineral salts are found in solution; proteins and the remainder of mineral salts are found in a colloidal suspension; and fat is found as an emulsion stabilized by milk fat globule membrane (MFGM) materials. The general composition of bovine milk is shown in Table 2.1.

Table 2.1 *Typical milk composition, from Bylund (1995) and Fox (2003)*

Components	Diameter (nm)	State in milk	Sub-component	Amount (% w/w)
Water	-	-	-	86.00
Total solids	-	-	-	14.00
Fat	2000-6000	Oil-in-water emulsion	-	5.00
Lactose	0.5	True solution		4.70
Protein				
Casein micelles	50-300	Colloidal suspension	Total casein	2.80
			α_{s1}-Casein	1.10
			α_{s2}-Casein	0.30
			β-Casein	1.00
			κ-casein	0.40
Whey proteins	4-6	Molecular solution	Total whey proteins	0.70
			β-Lg	0.35
			α-La	0.14
			Others	0.21
Minerals	-		Total minerals	0.75
		True solution/colloidal suspension	Calcium	0.17
		True solution/colloidal suspension	Phosphate	0.23
		True solution	Potassium	0.19
		Mainly true solution	Others	0.16

2.2. General characteristics of milk proteins

Milk proteins are heterogeneous mixtures of caseins and whey proteins (Fox & Mulvihill, 1982). Caseins, the major protein group in milk (~80% of total), have been defined as phospho-proteins which can be precipitated from milk by acidification to pH 4.6 at 20°C. The residual proteins are referred to as whey proteins.

2.2.1. Caseins

Caseins in milk are present in colloidal aggregated forms of casein and mineral calcium phosphate, known as casein micelles. Each micelle composes of 20 - 150,000 casein molecules. Casein proteins can be distinguished into α_{s1}-, α_{s2}-, β-, and κ-caseins which are present in the approximate proportions of 4:1:4:1 (Davies & Law, 1980). The α_{s1}-, α_{s2}-, β-caseins are calcium-sensitive proteins, whereas κ-casein is calcium-insensitive protein. κ-Casein interacts with and stabilizes the calcium-sensitive proteins to form the stable colloidal state of casein micelles. The surface of the micelles consists primarily of κ-casein, with its hydrophilic C-terminal region protruding from the surface, as a polyelectrolyte brush. This brush provides electrostatic and steric repulsion, which prevents inter-micelle aggregation. The structure and properties of casein micelles have been reviewed recently (Dalgleish, 2011; de Kruif & Holt, 2003; Farrell, Malin, Brown, & Qi, 2006; Fox, 2003; Horne, 2009). Various models of casein micelle structure have been proposed and progress has been reviewed regularly. These models include the submicelle model (Schmidt, 1982; Walstra, 1999), the dual-binding model (Horne, 1998; 2006; 2009) and the nanocluster model (de Kruif & Holt, 2003; Holt, 1998).

According to submicelle model (Figure 2.1), the casein micelles are composed of smaller proteinaceous subunits, the submicelles, held together via colloidal calcium phosphate (CCP) linkages. Most of κ-casein is situated on the outside of the micelle, and the protruding chains of its C-terminal end give the micelle a ‘hairy’ surface, which provides stabilization to the casein micelles.

In the nanocluster model (Figure 2.2), casein micelle is formed by calcium phosphate nanoclusters, i.e., the complexes between calcium sensitive caseins and amorphorus calcium phosphate. The nanoclusters are distributed homogenously throughout the

casein micelle and are likely to be linked through hydrophobic bonding and electrostatic interactions. The polypeptide chains in the core (internal structure) are partly cross-linked by nanoclusters. The hairy layer with low density of polypeptide (external structure), provides steric and/or charge stability to native casein particles (Horne, 2009). According to this model, the casein micelles are more or less spherical, highly hydrated, and fairly open particles (Dalgleish, 2011).

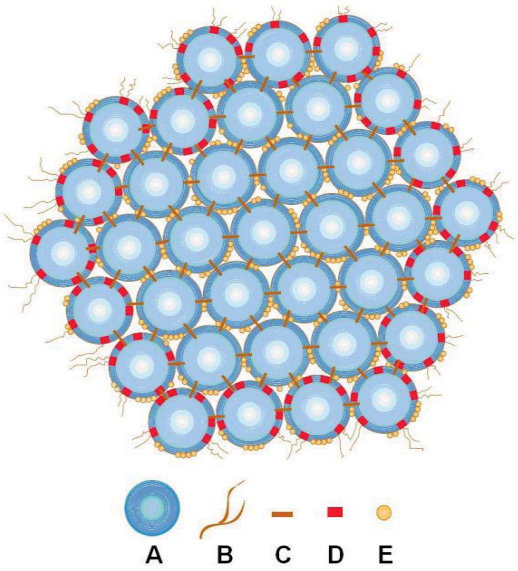


Figure 2.1. Submicelle model of casein micelle; A: a submicelle; B: protruding chain of κ-casein (and possibly β-casein); C: calcium phosphate; D: κ-casein; E: phosphate groups, from Walstra (1999).

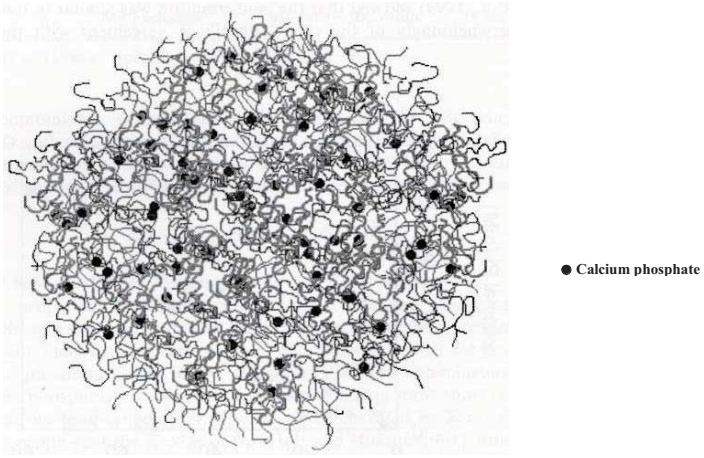


Figure 2.2. Nanocluster model of casein micelles, from de Kruif and Holt (2003).

The dual-bonding model (Figure 2.3) is based on hydrophobic and electrostatic interactions. In this model, the protein-protein interactions occur between hydrophobic regions (rectangular bars), whereas the protein hydrophilic regions (loops), containing phosphoserine cluster, bind the colloidal calcium phosphate clusters (triangles). κ–Casein, presents on the surface, limits further growth of casein micelle (Horne, 2006).

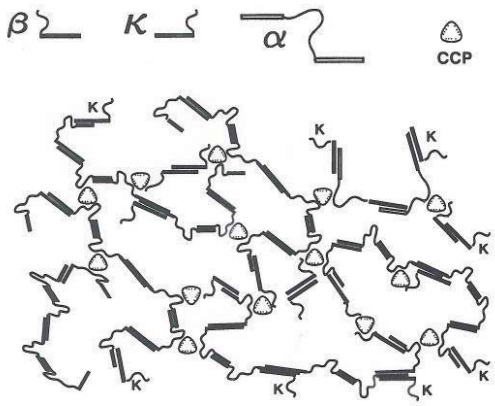


Figure 2.3. Dual bonding model of casein micelle, from Horne (1998).

Despite disagreement over the exact casein micelle structure, it can be pointed out that the well-defined casein micelles do not exist and the structure of casein micelles is more open and fluid. κ-Casein is located near the surface of casein micelles, where it is able to stabilize the calcium-sensitive caseins, to allow rapid and specific hydrolysis by proteases and to permit the complex formation with whey proteins upon heating milk (Horne, 2006). The α_{s1}-casein is in the core region and β-casein is in the core of the native micelle in a milk environment (~39°C, pH 6.6 - 6.7) but can move to the surface or into the serum when the micelle is in non-native environment (Considine, Patel, Anema, Singh, & Creamer, 2007). The CCP stabilizes the micelle and removal of CCP results in dissociation of casein micelles into smaller particles.

2.2.2. Whey Proteins

Whey proteins are the proteins that remain soluble after the precipitation of casein. The principal whey proteins are β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (Igs) (Singh & Havea, 2003). Whey proteins are mainly globular with a rather uniform distribution of hydrophobic/hydrophilic amino

acids along their polypeptides chains (Mulvihill & Donovan, 1987). β-Lg and α-La have a considerable native internal architecture, consisting of several different non-covalent bonds occurring within or between protein molecules or between protein groups and solvent molecules (Relkin, 1996). As whey proteins are globular proteins, they can be denatured by heat treatment. β-Lg, MW 18,400 Da, represents ~ 50% of the whey proteins (Figure 2.4). It exists as a monomer or as a dimer depending on the pH, ionic strength and temperature. Native β-Lg contains two disulphide bonds and a free thiol group, which is buried within the protein structures (Hambling, McAlpine, & Sawyer, 1992). The functionality of β-Lg is influenced greatly by the presence of the sulfhydryl group and by conformational changes because these determine the availability of the sulfhydryl group for reactions. Thus, under appropriate conditions, β-Lg readily participates in sulphydryl-disulphide interchange reactions with other proteins containing disulphide bonds which affect many of its characteristics, such as solubility (Hambling, et al., 1992).

α-La, with a molecular weight of 14,300 Da, has a very compact, nearly spherical overall shape (Figure 2.5). It account for approximately 25% of whey proteins. It is stabilized by four disulphide bonds and does not contain a free thiol group (Brew, 2003; Brew & Grobbler, 1992). α-La binds calcium very tightly, which may stabilize the molecule against irreversible denaturation (Hiraoka & Sugai, 1984). It is a small protein containing 123 amino acid residues, and has been well characterized.

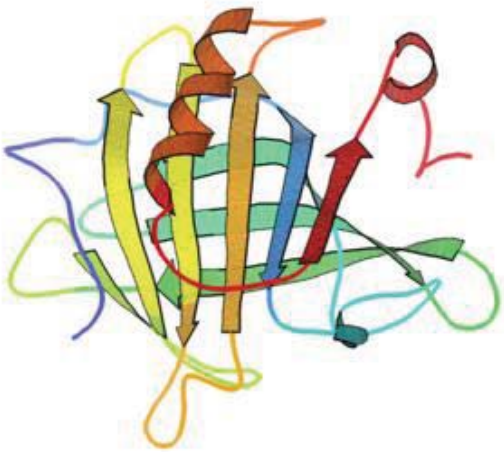


Figure 2.4. Structure of a β-Lg subunit. Ribbons denote the secondary structure with arrows for beta strands and spirals for alpha-helices, from Swaisgood (2004).

α-La does not polymerize by itself when heated above 70°C (Schokker, Singh, & Creamer, 2000). Heat treatment of α-La has very little effect on aggregation and disulphide-bond interchange, partly because no free thiol group is available (Calvo, Leaver, & Banks, 1993).

2.2.3. Effects of heat treatment on milk proteins

Protein structures can be readily destabilized from their native state by introducing relatively minor changes in environmental conditions (Mulvihill & Donovan, 1987). Application of heat or pressure or changes in other conditions causes proteins to lose their native structure and the compact molecule begins to unfold into a disordered, random structures. The denaturation or unfolding of proteins is thus characterised as the breaking of stabilizing forces in the native structure, and conversion to a less ordered conformation (Privalov & Gill, 1988).

Thermal 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 increased reactivity of such groups. Through sulphydryl-disulphide interchange and hydrophobic interactions, the unfolded proteins molecules may associate with each other to form aggregates, which will become insoluble as they grow in size.

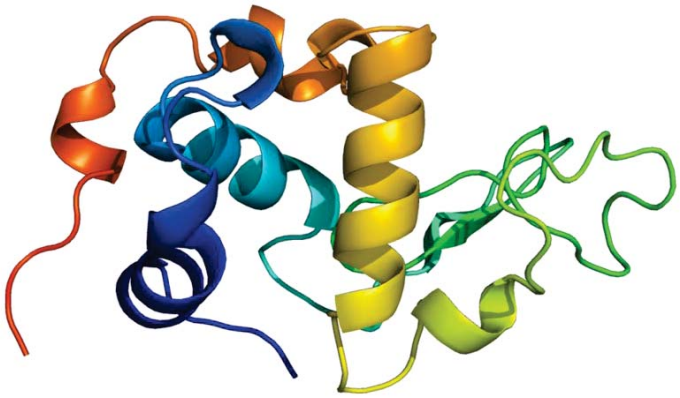


Figure 2.5. Structure of α-La, from Swaisgood (2004).

Severe heat treatments can lead to interactions with other protein molecules, which result in intermolecular association and aggregation and finally precipitation or gelation depending on the protein concentration, heating and cooling rates, pH and ionic strength (de Wit & Klarenbeek, 1984; Mulvihill & Donovan, 1987; Singh, 1995; Singh & Havea, 2003).

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 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).

As discussed by others (Mulvihill & Donovan, 1987; Roefs & de Kruif, 1994; Singh & Havea, 2003), the denaturation of whey proteins is generally assumed to be at least two-step process. Initially, the native protein unfolds co-operatively 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 (Griko & Kutyshenko, 1994). It is also important to note that the general term 'denaturation' may encompass a number of denatured forms of proteins, varying from slight changes in the tertiary structure, without changes to the secondary structure (e.g. non-native form), to major changes and unravelling of the secondary structure.

There are distinct differences in the thermal stability of individual whey proteins. The minor whey proteins (LF, BSA and Igs) begin to denature at 65°C, whereas, the major whey proteins (α-La and β-Lg) are more heat stable and denaturation occurs at temperature above 70°C (Fox, 1995). The kinetics of α-La and β-Lg irreversible denaturation in heated skim milk have been published (Anema & McKenna, 1996; Dannenberg & Kessler, 1988c; Oldfield, Singh, Taylor, & Pearce, 1998). There are

changes in temperature dependence of the rate constants at a temperature of 80°C and 90°C for α-La and β-Lg respectively, due to the different rate-determining steps. The denaturation or unfolding reactions being rate determining in the low temperature ranges, whereas the aggregation reaction being rate determining in the higher temperature ranges (Anema & McKenna, 1996; Dannenberg & Kessler, 1988c; Oldfield, et al., 1998).

When milk is heated at ~70°C and above, the whey proteins unfold, denature irreversibly (Havea, Singh, Creamer, & Campanella, 1998; Relkin, 1996) and eventually aggregate through hydrophobic bonding and thiol/disulphide exchanges with themselves and with, essentially κ-casein, leading to the so-called whey protein/ κ-casein complexes (Jang & Swaisgood, 1990; Singh & Creamer, 1991a). Lowe et al. (2004) reported that there were specific reactions between κ-casein on the casein micelle surface and denatured β-Lg which involved thiol-didulfide exchange. Two disulfide bridges and a free sulfhydryl group present in the native structure of β-Lg play an important role in its heat-induced interactions with κ-casein (Cho, Singh, & Creamer, 2003).

The caseins possess low levels of secondary and tertiary structure, a feature that contributes to their remarkable stability at high temperatures. However, when subjected to severe heat treatment, caseins undergo changes such as dephosphorylation and proteolysis (Fox & Mulvihill, 1982; Singh, 1995; Singh & Creamer, 1992). 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 & Mulvihill, 1982; Singh & Creamer, 1992). These changes have been reviewed in details elsewhere (de Kruif & Holt, 2003; Singh, 1995).

The change in casein micelle size during heating depends on heating temperature, holding time and pH of milk. Mild heat treatment (< 70°C/20 min) at natural pH causes small change in casein micelle size. However, the change in casein micelles size induced by heat treatment at temperature up to 100°C is dramatically dependent on pH of milk. This change can be related to the interaction with denatured whey proteins and also the aggregation of casein micelles (at severe heat treatment) (Anema & Li, 2003a, 2003b; Dalgleish, Pouliot, & Paquin, 1987).

Depending on heating temperature and holding time, heat treatment of milk can cause a pH-dependent dissociation of casein from the casein micelles (Anema & Klostermeyer, 1997; Anema & Li, 2000; Singh & Creamer, 1992). At pH ≤ 6.7, regardless of heating temperature and holding time, very little dissociation of casein micelles occurs. As pH > 6.7, the dissociation of casein micelles increases as pH increases. The composition of dissociated casein depends on heating temperature. At temperature up to 70°C, all the caseins are found in the proportion not different from that of natural milk. At temperature above 70°C, the level of κ-casein continue to increase whereas the level of α- and β-caseins decrease. The change in dissociation behaviour of casein micelle at temperature above 70°C appears to due to the denaturation of whey proteins and their interactions with κ-casein (Anema & Li, 2000).

2.3. General characteristics of milk fat globules

2.3.1. Size distribution of milk fat globules

Fat in milk is present predominantly in spherical droplets ranging from 0.2 - 15 μm in diameter; bovine milk typically contains > 10^{10} fat globules per mL. Milk fat globules are responsible for, or contribute to, some of the properties and phenomena observed in liquid dairy products and are integral to the manufacture and characteristics of many dairy products. The size distribution of milk fat globules depends greatly on the measurement methods. The average globule size can be expressed in many ways; however, the volume-surface average diameter, d_{32} or d_{VS} and volume-moment mean diameter, d_{43} or d_{VM} are most commonly used. These diameters can be calculated from:

$$\text{Volume-surface average diameter } d_{vs} = \sum \mathrm{d}V / \sum \mathrm{d}S = \sum d^3 \mathrm{d}N / \sum d^2 \mathrm{d}N$$

$$\text{Volume-moment mean diameter } d_{VM} = \sum \mathrm{d}M / \sum \mathrm{d}V = \sum d^4 \mathrm{d}N / \sum d^3 \mathrm{d}N$$

The specific surface area of the fat globule is given by $A = 6\phi/d_{vs}$ where ϕ is the volume fraction of fat.

2.3.2. Milk fat globule membrane (MFGM)

Milk fat globules are naturally surrounded and stabilized by a complex layer of surface active membrane materials, the milk fat globule membrane (MFGM), which accounts for approximately 2 - 6% of the globule mass or 0.3 to 0.4 g/L of whole milk (Keenan

& Mather, 2002). The membrane, approximately 10 nm thick, consists of a complex mixture of proteins, phospholipids, glycoproteins, triglycerides, cholesterol, enzymes and other minor components. The MFGM acts as a natural emulsifying agent, preventing flocculation and coalescence of fat globules in milk and protecting milk fat against enzyme action. The functional properties of MFGM may be attributed to its chemical composition and physical structure (Lee & Sherbon, 2002). However, the composition of MFGM is markedly affected by processing treatments, such as cooling, drying, heating and homogenization. The MFGM contains approximately 50% protein, which accounts for about 1% of the total milk proteins. The proteins of MFGM are of interest for their roles in the interactions with plasma proteins during dairy processing.

2.3.3. Isolation of MFGM materials

Isolation of MFGM materials involves four major steps: separation, removal of skim milk components, destabilization and recovery of the membrane. The separation of milk fat globules is easily completed by centrifugation using a cream separator. After separation, skim milk components are removed from the cream, usually by washing at a given temperature with physiological buffers such as milk salt buffer. There are a number of techniques which can be used to release membrane materials from the washed fat globules: (1) physical agitation in a blender or homogenization at a reduced temperature (<10°C); (2) freezing and thawing the washed globules; or (3) direct extraction with non-ionic detergents, bile salts or polar aprotic solvents. The released membrane is then collected by centrifugation (Keenan & Mather, 2002; Mather, 2000; McPherson, Dash, & Kitchen, 1984).

2.3.4. Effects of heat treatment on MFGM

It is known that industrial treatments have a major impact on the MFGM (van Boekel & Walstra, 1995). Among those treatments, heat is the most important factor affecting the MFGM. Aggregation of MFGM proteins, loss of MFGM proteins and phospholipids, and adsorption of caseins and whey proteins on the surface of the MFGM have been reported (Evers, 2004). Interactions between whey proteins and the MFGM proteins occur that are believed to involve sulphydryl-disulphide interactions (Kim & Jimenez-Flores, 1995; Lee & Sherbon, 2002). Houlihan, Goddard, Kitchen and Masters (1992) found both β-Lg and α-La bound to MFGM after heating whole milk at 80°C. On

heating whole milk at sufficiently high temperatures, both β-Lg and α-La interact with the milk fat globule membrane (MFGM) proteins, and the amounts increase with increasing temperature or duration of heat treatment up to a certain maximum level (Ye, Singh, Oldfield, & Anema, 2004). Thermal denaturation of both β-Lg and MFGM proteins results in disulphide linkage of MFGM aggregates and β-Lg complexes. Lee and Sherbon (2002) reported that MFGM in milk heated at 80°C for 3 min contains approximately 0.03 g of β-Lg and 0.008 g of α-La per 100 g of fat globules. The interactions between β-Lg and MFGM proteins are reported to be temperature-dependent (Ye, Anema, & Singh, 2004).

Heat treatment of whole milk at temperature even lower than pasteurization (72°C for 15 s) can considerably alter the composition of MFGM. Heat treatment causes changes in the MFGM by promoting interactions between proteins and native MFGM components (Dalgleish & Banks, 1991; Houlihan, Goddard, Nottingham, Kitchen, & Masters, 1992; Kim & Jimenez-Flores, 1995; Sharma & Dalgleish, 1993). A number of studies have shown that, upon heating milk, β-Lg associates with the MFGM, and the association commences at 60 - 65°C, which is lower than the denaturation temperature of β-Lg (~78°C) (Corredig & Dalgleish, 1996b; Dalgleish & Banks, 1991; Houlihan, Goddard, Nottingham, et al., 1992; Kim & Jimenez-Flores, 1995; Ye, Anema, & Singh, 2008). Although, the mechanism of heat-induced incorporation of whey proteins to the MFGM is not clearly understood, there appears to be a number of possible pathways. (1) β-Lg may associate with the MFGM via sulfydryl-disulfide interchange reactions (Dalgleish & Banks, 1991; Dalgleish & Sharma, 1993; Houlihan, Goddard, Kitchen, et al., 1992); (2) it may displace polypeptides in the MFGM, either by directly competing or because MFGM may breakdown during heating, leaving gaps through which the whey proteins (which may or may not be denatured at that stage) may adsorb to the newly exposed fat surface (Dalgleish & Banks, 1991), and (3) more complex interaction rather than direct SS bond formation alone (Kim & Jimenez-Flores, 1995). Ye, Anema, et al. (2004) suggested that association of β-Lg occurs after the native β-Lg dimer dissociates into monomers, but before the free thiol group is exposed. The thiol-disulfide interchange reactions are initiated by free thiol groups of MFGM proteins, which become available for interactions at much lower temperature. Hence, the association between MFGM proteins and β-Lg could occur at approximately 60°C.

The amount of protein associated with the fat globules increased on heating; the newly-bound protein was largely denatured whey proteins, particularly β-Lg (Corredig & Dalgleish, 1998; Dalgleish & Banks, 1991). Lee and Sherbon (2002) reported that after heating whole milk at 80°C for 18 min, whey protein comprised about one fifth of the total proteins in the membrane. Sharma and Dalgleish (1993) reported that if milk was heated prior to homogenization, less whey proteins were incorporated into the MFGM than if the order of these steps is reversed. The association of denatured whey proteins with the MFGM increases its protein content and reduces that of lipids proportionately. The amount of β-Lg associated with MFGM increases with heating time at 60 - 95°C and reaches a plateau level of approximately 1.0 $mg \cdot g^{-1}$ fat, which amounts to only approximately 1% of the total β-Lg in milk (Corredig & Dalgleish, 1996b). It is not known why the heat-induced associations between MFGM proteins and β-Lg reach this limiting value and what prevents further association between these proteins (Singh, 2006).

There have been conflicting reports with respect to the association of α-La with the MFGM proteins. This association probably occurs as a result of sulfydryl-disulfide exchange in a similar manner to that exhibited by β-Lg. α-La exhibited much lower binding than β-Lg. This was probably because the thermal denaturation of α-La is considerably slower than that of β-Lg and occur significantly only at high temperatures (Dalgleish & Banks, 1991), or as α-La has no SH group, there is little opportunity for SH-SS exchange reactions to occur (Singh, 1993), or because the proportion of α-La in milk is relatively much less than that of β-Lg. Corredig and Dalgleish (1996b) reported that the maximum amount for α-La in the MFGM of heated milk was 0.20 – 0.25 mg/g fat. However, Dalgleish and Banks (1991) did not detected α-La in MFGM preparations from milks heated at 80°C. Houlihan, Goddard, Nottingham et al. (1992) reported a low level of α-La in all heated samples.

Small amounts of κ-casein also appear to associate with MFGM upon heating whole milk above 80°C (Houlihan, Goddard, Kitchen, et al., 1992; Ye, Singh, Taylor, & Anema, 2004b). However, Lee and Sherbon (2002) did not observed the presence of κ-casein. Dalgleish and Banks (1991) proposed that the presence of caseins on the surfaces of milk fat globules may have resulted from partial homogenization during the handling of the milk and they did not support the suggestion that κ-casein either

associated with MFGM proteins or formed κ-casein/β-Lg complexes on the fat globule surface. Ye, Anema et al. (2004) reported that the casein micelles may be directly associated with the MFGM protein through κ-casein, and the association involves disulfide bonding between κ-casein and MFGM components. Alternatively, β-Lg already associated with the MFGM proteins could subsequently interact with the casein micelles through κ-casein (Singh, 2006).

Heat treatment of milk, either batch heating or indirect ultra-high temperature (UHT) heating at 90 - 150°C did not influence d_{vs} (Van Boekel and Folkerts, 1991) whereas direct UHT heating reduced d_{vs} progressively with increasing temperature. Lee and Sherbon (2002) found that heat treatment of whole milk at 80°C for 3 min had no significant influence on the size of milk fat globules.

2.4. Recombined milk

Recombined milk is an alternative product of liquid milk whose component ratio is always unstable due to milking interval, stage of lactation, intra-mammary infection, and so on. In the manufacture of recombined milk, skim milk, water and fat/oil are mixed and homogenized to yield a stable product. The composition and desired properties of recombined milk can be standardized and adjusted. The skim milk and fat/oil can be obtained from different sources. As a result, recombined milk is widely used in the production of dairy products.

2.4.1. Surface layers of recombined milk fat globules

During homogenization of recombined milk, the fat is subdivided into minute particles resulting in marked increases in fat surface area. As a consequence, the milk proteins migrate to the newly-created fat surface and form a surface stabilising layer (Mulder & Walstra, 1974; Walstra & Oortwijn, 1982). The fat/oil (e.g. anhydrous milk fat; AMF) generally used in the manufacture of recombined milk contains little, or no, MFGM materials. Therefore, the surface layer of recombined milk fat globules consists predominantly of milk proteins, i.e., caseins and whey proteins. The amounts and types of proteins adsorbed at the fat globules surface influence the properties of the recombined milk such as droplet coalescence, aggregation, oxidation creaming properties, and heat stability (Deeth, 1997; Sharma, et al., 1996b). Recombined milk properties are similar to those of fresh homogenized milk, but considerably different

from natural whole milk (Sharma, et al., 1996b). The fat globules in recombined milk are shown in Figure 2.6. Sharma et al. (1996a) reported that the average protein load in recombined milk prepared from skim milk powder and AMF was ~6 mg/m^2. However, Oortwijn and Walstra (1979, 1982) reported a protein load value in the range 8 - 10 mg/m^2 for recombined milk prepared from fresh skim milk and AMF. The difference may be attributed to the determination methods and milk materials.

Sharma et al. (1996a) used centrifugation of milk to recover the cream layer, washing to remove entrapped serum, and direct determination of protein composition of the fat globule surface layer. In contrast, Oortwijn and Walstra (1979, 1982) used "depletion method", which involved determination of protein composition of the continuous aqueous phase before and after homogenization, thereby estimating the composition of the fat globules surface layers by difference.

Protein load on the fat globule surface is influenced by protein and fat concentration (Oortwijn & Walstra, 1979; Sharma, et al., 1996b), fat globule size (McCrae & Muir, 1991; Sharma, et al., 1996a, 1996b; Singh, 1993), and homogenisation parameters (Oortwijn & Walstra, 1979; Sharma, et al., 1996b). The protein load of the fat globules in recombined milk is also influenced markedly by the form of protein present in continuous phase. Protein load is higher when casein micelles are present than when only whey proteins are present (Oortwijn & Walstra, 1979; Sharma & Singh, 1998, 1999).

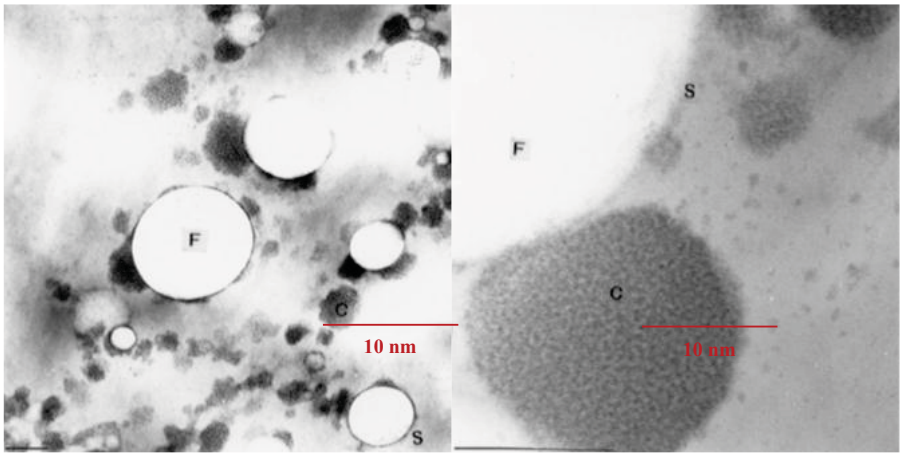


Figure 2.6. Fat globules in recombined milk. Fat globules (F), casein micelle (C) and surface layer (S), from Sharma (1993).

Further, the surface protein load decreases with increasing temperature during emulsification, with increasing homogenization pressure, and is increased by heat treatment of milk prior to emulsification (Oortwijn & Walstra, 1979; Sharma, et al., 1996b). In general, larger fat globules had a lower protein load than smaller fat globules.

Cho, Lucey & Singh (1999) reported that the protein load on recombined milk fat globules made with heated whey protein concentrate (WPC) was higher than those made with unheated WPC. This is probably due to the presence of denatured and aggregated whey proteins in the heated WPC, which subsequently adsorb on the fat globule surface during homogenization. Oortwijn and Walstra (1979) also reported that heat treatment of whey protein prior to homogenization with milk fat resulted in an increased protein load on milk fat globule surface.

Casein micelles on the fat globule surface of recombined milk often appear to be disrupted, which may be due either to homogenization or to the process of adsorption (Sharma, et al., 1996b). The extent of disruption of micelles in recombined milk is greater than that in homogenized fresh milk and increases with the temperature of homogenization from 40 - 70°C (Oortwijn & Walstra, 1982; Sharma, et al., 1996b). Destabilization of casein micelles by reducing the CCP content reduces the protein load on the fat globules in recombined milk and alters the proportions of individual caseins on the globule surface (Sharma, et al., 1996b). It is far more difficult to remove κ-casein than α_s- or β-caseins from the fat globule surface in recombined milk, suggesting that part of κ-casein is associated directly with the globule surface (Sharma, et al., 1996b; 2003). The concentration and type of proteins adsorbed at the fat globule surface influence the properties of the recombined milk.

The mechanism of disaggregation of casein is not clearly understood. One possibility is that (1) homogenization causes disruption of the casein micelle structure and subsequently the disrupted micelles are adsorbed onto the fat globule surface (Walstra & Oortwijn, 1982), or (2) the disruption of the casein micelles occurred after they are adsorbed onto the fat globule surface. Walstra and Oortwijn (1982) proposed that casein micelles if adsorbed onto a partially covered oil/water interface, may spread to cover the interface. Such spreading would be driven by thermodynamically favourable

changes in interfacial free energy which is sufficient to disrupt the micelles. Sharma et al. (1996b) found more disaggregated casein particles at the fat globule surface in recombined milk systems than in fresh homogenized milk systems. The researchers claimed that some structural change occurred in the casein micelles during manufacture of non-fat dry milk which made them more susceptible to disaggregation. They also claimed that the apparently "intact" micelles on the surface of fat globules were not likely to be in their native state since native micelles do not bind to hydrophobic (polystyrene lattices) surfaces (Dalgleish, 1989). Therefore, the micelle structure must re-arrange before the micelle interacts with the fat surface.

The adsorbed whey proteins on the surface of fat globule of recombined milk could possibly have come directly from the serum during homogenization or be adsorbed as part of a heat-denatured whey protein-casein micelles complex, which may have been formed during the manufacture of non-fat dry milk. However, Sharma et al. (1996b) reported that there was insignificant change in amount of adsorbed whey proteins when adsorbed casein micelles were dissociated by EDTA, indicating that whey proteins at the surface must have been adsorbed directly from the serum.

It has been reported that casein adsorbed in preference to whey proteins and α_{s1}-casein adsorbed in preference to β-casein (McCrae, Hirst, Law, & Muir, 1994; Oortwijn & Walstra, 1979; Sharma, et al., 1996a, 1996b). Caseins are the main proteins that made up the surface layer (~90% w/w), while whey proteins are present in small amounts (~10% w/w). However, whey proteins cover 25 - 33% of the total surface area in recombined milk (Sharma, et al., 1996a; Walstra & Oortwijn, 1982). The preferential adsorption of casein over whey protein may be partly attributed to a considerable proportion of casein adsorbing in the form of intact micelles, compared to whey proteins which adsorb in molecular form (Sharma, et al., 1996a). Walstra and Oortwijn (1982) proposed that, since protein adsorption during homogenization occurred via convection, large protein particles adsorbed in preference to small protein particles. The hypothesis also predicts that large protein particles would be preferentially adsorbed onto smaller fat globules. Sharma et al. (1996a) reported that smaller fat globules had larger attached casein micelles.

The greater adsorption of α_s-casein may be due to preferential adsorption of casein micelles that contain larger amount of α_s-casein (Walstra & Oortwijn, 1982). Previous studies reported that as the micelles size increased, the κ-casein content diminished (Donnelly, McNeill, Buchheim, & McGann, 1984; McGann, Donnelly, kearney, & Buchheim, 1980). Therefore, if large-sized casein micelles were adsorbed in preference to smaller ones, there should have been relatively less adsorption of κ-casein. However, Sharma et al. (1996b) found slightly more adsorbed κ-casein than in the original recombined milk. They proposed that mechanisms of adsorption were more complex than that predicted by Walstra and Oortwijn (1982) based on casein micelles differing in size. Such adsorption may involve rearrangement of casein components within micelles and changes in micelles size distribution.

Sharma et al. (1996b) reported that when washed cream was dispersed in EDTA, the extent of dissociation of caseins followed the order: β-casein > α_{s1}-casein >> κ-casein. This suggested that the micellar adsorption at the fat globule surface most likely occurred through interactions with κ-casein molecules, while α_{s1}- and β-caseins were less likely to be involved. The addition of 0.02% (v/v) mercaptoethanol to EDTA solution of washed cream had no effect on the extent of dissociation of α_{s1}- and β-caseins. However, the dissociation of κ-casein increased dramatically and there was a slight increase in dissociation of β-Lg. These suggested that a proportion of κ-casein was present as a disulfide-linked polymer at the fat globule interface and a proportion of β-Lg was present as a disulfide-linked polymer or as a κ-casein/β-Lg complex. And that these could be partially dissociated from the surface when S-S bonds were disrupted.

2.4.2. Effect of heat treatment on surface layer of recombined milk fat globules

Sharma et al. (1996b) investigated the effects of preheat treatment of skim milk in the temperature range 60 - 120°C for 5 min before homogenization on fat globule diameter (d_{vs}), protein load and proportion of individual proteins adsorbed at the fat globule of recombined milks. They found that the d_{vs} of recombined milks were unaffected by preheating temperature. The protein loads of recombined milks, prepared using skim milk preheated in the temperature range 90 - 120°C, were ~10% higher than those prepared using skim milk heated at lower temperature (6 vs. 5.2 mg/m^2). Preheating in

the temperature range 60 - 80°C caused an increase in the proportions of β-Lg and a small decrease in all caseins. Heating above 80°C caused no further appreciable change. Consequently, the casein/whey protein ratio decreased (from 8.8 to 5.4) as the preheating temperature was increased from 60 - 80°C. The increase in the proportion of β-Lg adsorbed at the fat surface in preheated milk could be attributed to the protein complexes associated with casein micelles during preheating (Singh & Fox, 1987) and subsequent adsorption of these complexes during homogenization. These changes may be responsible for the small observed increase in the protein load. This indicated that preheating of skim milk prior to homogenization at temperatures at which β-Lg denatures and binds to the casein micelles; i.e. above 70°C, results in an increase in the proportion of β-Lg adsorbed at the fat surface, which leads to a small increase in the protein load.

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 details over the last 6 decades and the subject has been reviewed several times (Jelen & Rattray, 1995; Mulvihill & Donovan, 1987; Singh, 1995). Most of the 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-Lg and its interaction with other proteins, including caseins. Some of the studies also reported that in addition to intermolecular covalent (disulphide) bonds, non-covalent interactions (such as hydrophobic and ionic interactions) are also involved in the heat-induced interactions of milk proteins.

2.5. High pressure processing of milk

High pressure (HP) is a novel method of food processing wherein the food is subjected to elevated pressures (pressures up to 900 MPa) with or without the addition of heat to achieve microbial inactivation or to alter the food attributes in order to achieve desired qualities. HP treatment has been described as high pressure processing (HPP), high hydrostatic pressure processing (HHP) or ultra-high pressure processing (UHP). This process can inactivate pathogenic and spoilage microorganisms with fewer changes to product “freshness” as compared to conventional food preservation processes. HP causes disruption of hydrophobic and ionic bonds but not covalent bonds, so many

small molecules in foods, including flavour compounds and vitamins, are left intact (Farr, 1990). Foods processed by HP may then be superior in both nutritional and functional quality as compared with their thermally processed counterparts. HP-processed foods are reported to have better flavour, colour, texture and nutrient retention compared to thermally-processed foods (Balasubramaniam, 2003; Balci & Wilbey, 1999; Knorr, 1993; Torres & Velazquez, 2005; Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002). HP treatment can also cause rheological changes in food, which result in beneficial sensory and structural effects. Therefore, HP treatment can be used in the preparation of food with different functional properties and in the development of new products (Balasubramaniam, 2003; Datta & Deeth, 1999; Datta & Deeth, 2003; Trujillo, et al., 2002). The major advantages of HP for the processing and preservation of foods are elimination or significant reduction of heating, thus avoiding thermal degradation of food components; high retention of flavour, colour and nutritional value; uniform and instant treatment of the product under pressure; reduced requirement for chemical additives; and potential for the design of new products due to the creation of new textures, tastes and functional properties (Datta & Deeth, 1999). Recent equipment advances, successful commercialization of HP products and a consumer demand for minimally processed, high quality and safe foods have led to considerable research interest in HP technology. Significant research interest in HP, also, arises from the differences in chemical and physical effects on the food products when compared with traditional heating processes (Anema, Lauber, Lee, Henle, & Klostermeyer, 2005). Some of the HP processed products commercially available in Japanese, European and the United States markets include jams, jellies, fish, meat products, salad dressing, rice cakes, juices, salsas, guacamole and oysters (Balasubramaniam, 2003; Trujillo, et al., 2002). Several other pressure-treated foods are now manufactured, but the total value is relatively small (Datta & Deeth, 2003).

Research into the application of HP treatment for milk preservation began when Hite (1899) demonstrated that the shelf life of milk and other food products could be extended by pressure treatment. HP treatment can cause substantial modification to almost all phases of the milk system. For example, HP treatment causes changes in milk fat crystallisation (Buchheim & El-Nour, 1992), whey protein denaturation (Hinrichs, Rademacher, & Kessler, 1996a; López-Fandiño, Carrascosa, & Olano, 1996; López-Fandiño & Olano, 1998a; Scollard, Beresford, Needs, Murphy, & Kelly, 2000),

increased exposure of hydrophobic groups in milk proteins (Guacheron et al., 1997) and shifts in the mineral equilibrium (Lee, Anema, Schrader, & Buchheim, 1996). Casein micelles can be disaggregated into smaller particles or aggregated into larger particles depending on the pressure treatment conditions applied (Anema, Lowe, & Stockmann, 2005; Huppertz, Fox, & Kelly, 2004c). Whey proteins are denatured and the denatured whey proteins interact with the remnants of the casein micelles (Hinrichs, et al., 1996a). These changes lead to modification of physio-chemical and functional properties of milk, such as acid gelation (Needs et al., 2000; Schwertfeger & Buchheim, 1998) or rennet coagulation (López-Fandiño, et al., 1996; López-Fandiño & Olano, 1998a; Needs, Stenning, et al., 2000). Of particular significance for milk are the effects of HP on milk proteins which are discussed further.

Even through, HP is unlikely to replace heat treatment for the safe production of liquid milk; there may be some niche products for which heat treatment is inappropriate, and for which microbiological quality could be improved by HP treatment. The main reason for on-going investigation in HP is that pressure brings about modifications to milk components (particular proteins), which can lead to altered functionality and the possibility of novel or improved dairy products. The technology and associated processing equipment have now been developed to the stage where it is feasible to establish processing plants (Datta & Deeth, 2003). There are emerge commercial pressure-treated dairy products available in the market such as longer life yoghurt with live and culture, probiotic drink, colostrum health drink and mozzarella cheese (Linton, Mackle, Upadhyay, Kelly, & Patterson, 2008).

2.5.1. Principle of high pressure processing

Two principles underlie the effect of high "hydrostatic" pressure on foods. Firstly, the principle of Le Chatelier-Braun according to which any phenomenon (phase transition, chemical reactivity, change in molecular configuration, chemical reaction) accompanied by a decrease in volume (negative ΔV) will be enhanced by increase in pressure and vice versa. Secondly, pressure is instantaneously and uniformly transmitted in all directions, independently of the size and the geometry of the food and the equipment size (based on Pascal's principle). This is known as isostatic pressure. Consequently, under HP treatment the food is treated evenly throughout and no particles escape the treatment, unlike thermal processing and other preservation technologies such as pulsed

electric field technology, in which different parts of the food can be treated at different rates. This ensures the absence of dead spots and localized over processing which are problems in other treatments (Datta & Deeth, 2003; Torres & Velazquez, 2005).

Pressure effects on the structure and texture of foods can be marked and variable and the mechanisms are not fully understood. During HP treatment, only the noncovalent bonds (hydrogen bonds, ionic bonds, and hydrophobic bonds) in the food component are broken or formed depending on the volume decrease of the system; covalent bonds are not affected during the pressurization. This unique characteristic suggests that reactions like Maillard reaction and production of cooking flavours (off flavours) that commonly occur in thermal treatments will not occur during HP treatment of foods. However, most biochemical reactions are influenced by HP since they often involve a change in volume. The pressure-induced effects are mostly concerned with secondary and tertiary structural changes in large molecules. Consequently, enzymes and polysaccharides in an aqueous environment undergo reversible or irreversible conformational changes resulting in denaturation, dissociation, aggregation or gelation. By contrast, the heat-induced breaking of covalent bonds in both small and large molecules causes changes to colour, flavour and other sensory.

Proteins show a HP-induced disordering in their structures that is similar, but not identical to that induced by heating (Balny, Masson, & Heremans, 2002). Proteins undergo HP-induced unfolding of the structures that may be largely attributed to penetration of water into the structure (Hummer, Garde, García, Paulaitis, & Pratt, 1998). As the pressure increases, the protein-water system may be packed more efficiently and have a lower volume when water molecules are incorporated into the structures, swelling the protein (Hummer, et al., 1998).

2.5.2. High pressure equipment and operation

A schematic diagram of basic equipment design used for HP processing is presented in Figure 2.7. A typical HP system usually consists of four main parts: a high pressure vessel and its closure (Figure 2.8), high hydrostatic pressure generating pumps or pressure intensifiers for generating desired target pressures (Figure 2.9), a temperature-control device and a material-handling system (Balasubramaniam, 2003; Datta & Deeth, 2003; Mertens & Deplace, 1993).

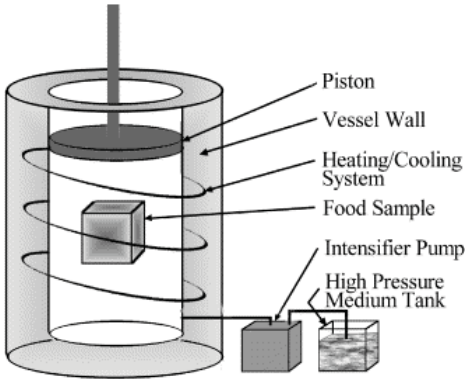


Figure 2.7. Schematic diagram of basic equipment design for HP treatment of foods, from Huppertz, Kelly and Fox (2002).

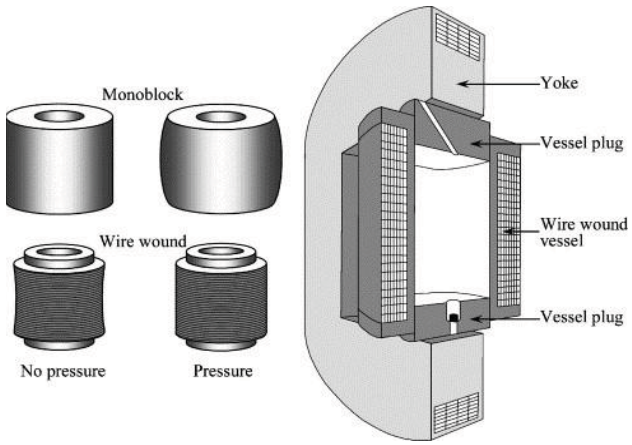


Figure 2.8. Monoblock casting technology for moderate pressure/size vessels, wire-winding technology for vessels and yoke used in larger size and higher pressure applications, from Torres & Velazquez (2005).

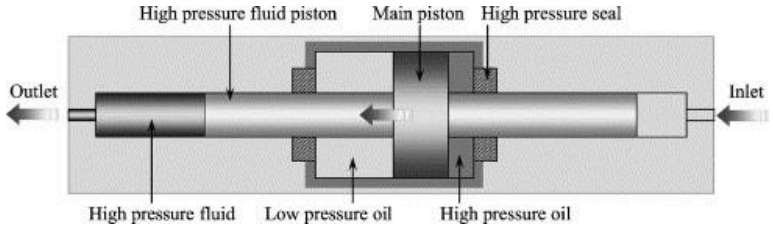


Figure 2.9. High hydrostatic pressure pump or pressure intensifier, from Torres & Velazquez, (2005).

The most important part is the pressure vessel, which is usually a forged monolithic cylindrical vessel constructed of forged steel or low-alloy steel of high tensile strength, or reinforced with tensioned wire windings. The wall thickness is determined by the maximum working pressure, the vessel diameter and the number of cycles the vessel is designed to perform. This thickness can be reduced by using multi-layer, wire-wound or other pre-stressed designs. For larger volumes at higher pressures, prestressed wire-wound vessels are needed for safe, durable and reliable operation. Typically, yoke (structure for restraining end closure) is used to hold the top and bottom seals (Figure 2.8). A typical batch process cycle consists of loading the vessel with the pre-packaged product, and then fills the reminder of the vessels with pressure-transmitting fluid. The vessel is closed and the desired process pressure is achieved through compression of pressure transmitting fluid. After holding the product for desired time at target pressure, the vessel is depressurized and the product is unloaded. Pressure holding times of 10 min or less may be required to develop a commercially viable process. Batch processes can be used for any kind of food in a flexible package, often the final consumer package.

Non-liquid products, such as cooked ham, chicken breasts and salami, are usually sealed in flexible packages before being placed in the HP vessels filled with a pressure-transmitting medium, which in most isostatic pressure applications is water mixed with a small amount of soluble oil for lubrication and anticorrosion purposes. Liquid food can be compressed directly in the HP vessels, eliminating the use of a pressure-transmitting medium (Datta & Deeth, 2003; Earnshaw, 1996). The former is referred to as indirect compression, the latter as direct compression. Pressure treatment can be performed in batch or semicontinuous operation. A variation of the batch method is the pulsed high-pressure process where the pressure is raised and lowered at repeated intervals of several minutes. Use of pulsed pressure or oscillatory pressure treatments has been shown to be generally more effective than an equivalent single pulse of equal time in term of micro-inactivation or sterilization. The difference in effectiveness varies, and the measure of improved inactivation by pulsed pressurization must be weighed against the design capabilities of the pressure unit, added wear on the pressure unit, possible detrimental effect to the sensory quality of the product, and possible additional time required for cycling (Balasubramaniam, 2003).

Semi-continuous systems for treating liquid foods use two or more pressure vessels containing a free-floating piston to compress liquid foods. A low-pressure transfer pump is used to fill the pressure vessels. As the vessel is filled, the free piston is displaced. When filled, the inlet valve is closed and pressure-transmitting fluid is introduced behind the free piston to compress the liquid food. After an appropriate holding time, releasing the pressure on the pressure transmitting fluid decompresses the system. A pump is used to move the free piston towards the discharge port. The treated liquid food can be filled aseptically into pre-sterilized containers. The batch vessels in a semi-continuous system are connected such that, when one vessel discharges the product, the second system pressurized, while the third system gets loaded. In this way, the output is maintained in a continuous fashion. This minimizes the operation time and allows a portion of the energy contained in the vessel under pressure to be used to pressurize another vessel, i.e. energy recuperation, thus reducing operation cost (Datta & Deeth, 2003).

The typical pressure-temperature curves for batch HP treatment are shown in Figure 2.10. The pressure is generated by direct compression, indirect compression, or by the heating medium. Pressure come-up time (AB) is the time required to increase the pressure of the sample from atmospheric pressure to the process pressure. The rate of compression is proportional to the power of the pump used. Pressure holding time (BC) is the time interval between the end of compression and the beginning of decompression. Once the pressure is released, expansion occurs.

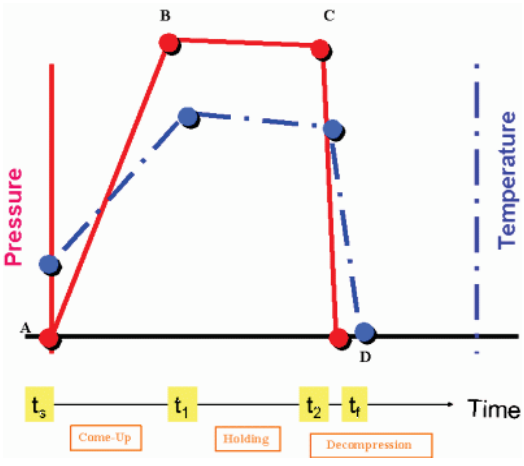


Figure 2.10. Typical pressure-temperature curves for HP treatment of foods, from Balasubramaniam, (2003)

Decompression time is the time required to bring a food sample from process pressure to atmospheric pressure (Balasubramaniam, 2003). During pressurization, an increase in temperature (up to 3°C for each 100 MPa for aqueous systems) occurs due to adiabatic heating (AB); a corresponding decrease in temperature occurs during depressurization (CD). These changes make a temperature control device necessary for some applications (Datta & Deeth, 2003; Mertens & Deplace, 1993). Sample temperature curves follow the pressure curve during pressure come-up time and exhibited slight time lag (up to 30 sec) to reach maximum temperature during pressure holding time. This could be attributed to material phase change under pressure and is likely to influence the microbial inactivation kinetics. The degree of temperature increase and time lag to reach maximum temperature under pressure depends upon the composition of product being processed. Upon pressure release, the temperature drops back to or below the initial temperature.

2.5.3. Effects of high pressure on milk proteins

Effects of HP on casein micelles and whey proteins have been studied in considerable detail in recent years. A number of studies have shown that HP treatment of milk or skim milk at pressure > 200 MPa causes the disruption of casein micelles and unfolding and aggregation of whey protein (Buchheim, Schrader, et al., 1996; Desobry-Banon, Richard, & Hardy, 1994; Guacheron, et al., 1997; Scollard, et al., 2000). HP-induced effects on milk proteins affect the properties of milk and may present opportunities for the creation of new or improved dairy products (Huppertz, Fox, et al., 2004c). In particular, the reaction of whey proteins with casein micelles is of technological importance; as an example, essential parameters of the rennet and acid induced coagulation may be influenced.

2.5.3.1. Effects of high pressure on casein micelles in milk

The effects of HP on casein micelles depend on the holding time, treatment temperature and pressure, and milk pH (Datta & Deeth, 2003). Under HP treatment, hydrophobic and electrostatic interactions between proteins are disrupted and CCP is solubilised (Buchheim, Schrader, et al., 1996; de la Fuente, Olano, Casal, & Juarez, 1999; López-Fandiño, Fuente, Ramos, & Olano, 1998); as a result, considerable changes in the size, structure and composition of the casein micelles occur (Huppertz, Fox, et al., 2004c).

Anema, Lowe et al. (2005) observed a small decrease in casein micelle size in reconstituted milk HP-treated at 100 MPa, with slightly greater effects at longer holding time (10 - 60 min) or higher treatment temperatures (10 - 40°C). At 150 - 200 MPa, there was no apparent effect of HP treatment on the average casein micelle size in raw (Huppertz, Fox, et al., 2004c) and reconstituted milk (Desobry-Banon, et al., 1994; Guacheron, et al., 1997). Above 200 MPa casein micelles are irreversibly disintegrated into smaller particles (Needs, Capellas, et al., 2000). However, there are some indications that an increase in particle size precedes the disintegration of the casein micelles, especially at pressures between 200 and 300 MPa and at elevated temperatures (Guacheron, et al., 1997). HP treatment at 250 MPa increases average micelle size by ~30% and that the effect was greater with increasing holding time, treatment temperature and milk pH (Huppertz, Fox, et al., 2004c; Huppertz, Fox, & Kelly, 2004d). At 300 MPa, there was an intermediate behaviour, with micelle aggregation and disintegration observed. Aggregation was observed at higher treatment temperatures and shorter holding times, whereas disintegration was observed at lower treatment temperatures or at the longer holding times at the higher treatment temperatures (Anema, Lowe, et al., 2005). Treatment at ≥300 MPa reduced micelle size by 40 - 50% in raw (Garcia-Risco, Villamiel, & López-Fandiño 2002; Huppertz, Fox, & Kelly, 2004a; Needs, Stenning, et al., 2000), or reconstituted milk (Anema, Lowe, et al., 2005; Desobry-Banon, et al., 1994; Guacheron, et al., 1997).

These conflicting results should be critically discussed.

At 200 MPa, partial disintegration of casein micelles occurred and at ≥ 400 MPa the disintegration was completed (Desobry-Banon, et al., 1994; Guacheron, et al., 1997; Needs, Stenning, et al., 2000). Anema, Lowe et al. (2005) reported that the effects of HP at pressure ≥ 400 MPa on casein micelles disintegration was more rapid at higher treatment temperatures although the final casein micelles size was similar in all samples regardless of the treatment temperature or pressure.

According to the casein micelles model suggested by Horne (1998), the disruption of the casein micelles through HP treatment may involve dissolution of the CCP, disruption of the hydrophobic interactions or a combination of these two effects. Above a critical value (~200 MPa), micelles are disrupted under HP treatment, probably as a

result of solubilisation of CPP and disruption of hydrophobic and electrostatic interactions (Needs, Stenning, et al., 2000). HP-induced changes in casein micelle size are generally irreversible on subsequent storage of the milk, except of the increase in micelles size at 250 MPa, which is partially reversible (Huppertz, Fox, et al., 2004c). However, it is difficult to establish the effect of HP treatment on CCP and hydrophobic interactions as in-situ measurements are difficult and the effects are likely to be reversible on pressure release. Considine et al. (2007) reviewed the effects of HP treatment on whey proteins including the in situ measurement. The changes in micelles size also depend on heat treatment both before and during HP treatment (Guacheron, et al., 1997; Huppertz, Fox, et al., 2004d). At 200 MPa at 10°C, the casein micelle size decreased slightly, whereas, at higher treatment temperatures, the size increased as a result of aggregation (Anema, Lowe, et al., 2005). HP treatment of reconstituted milk at 250 MPa at 20°C had an insignificant effect on micelle size; however, treatment at 40°C increased micelle size and treatment at 4°C reduced it (Guacheron, et al., 1997). The presence of large casein aggregates has been found in milk HP-treated at 250 – 400 MPa at 40 – 60°C (García-Risco, Olano, Ramos, & López-Fandiño 2000; Guacheron, et al., 1997; Huppertz, Fox, & Kelly, 2004e; Law et al., 1998). The increases in casein micelle size might be the result of a combination of the formation of casein aggregates (García-Risco, et al., 2000; Guacheron, et al., 1997; Law, et al., 1998) and complexation of denatured β-Lg with micelles.

The casein micelles in raw or pasteurized skim milk are more sensitive to pressure than heat-induced casein-whey protein complexes in UHT-treated milk. HP treatment (100 - 500 MPa) of UHT-treated skim milk reduced its turbidity, but to a lesser extent than in raw or pasteurised skim milk (Buchheim, Schütt, & Frede, 1996). The increase in turbidity observed on HP treatment of raw or pasteurised milk at 40°C was not observed for UHT milk, possibly because HP-induced interactions between casein micelle fragments and whey proteins are not possible in UHT milk as the whey proteins are already denatured and aggregated (Huppertz, et al., 2002). In contrast to UHT milk, HP-induced shifts in the turbidity of pasteurised skim milk are not reversible on storage after treatment, which may be linked to the fact that in systems pre-treated at relatively low temperature whey proteins interact with micelle fragments during HP treatment, thereby limiting the surface available for reaggregation of micelle fragments. However,

in UHT milk denatured whey proteins are covalently bound to micellar casein before HP-induced disintegration of casein micelles occurs, preventing such interactions.

The ionic calcium concentration was unaltered (de la Fuente, et al., 1999) or slightly increased by HP treatment (López-Fandiño & Olano, 1998b) while levels of total serum calcium and phosphorus increased to a similar extent by HP treatment of skim milk, reconstituted skim milk (Desobry-Banon, et al., 1994) and raw whole milk (López-Fandiño & Olano, 1998b). It is believed that this additional non-ionic calcium and phosphorus are released by the fragmentation of the micelles structure by pressure (Johnston, 1995). Lee, et al. (1996) observed that increasing the calcium concentration in a calcium caseinate suspension increased the resistance of micelles in the system to HP-induced disruption. This was suggested to be related to the fact that introduction of more calcium to the system shifts the calcium equilibrium from the soluble to the colloidal phase.

The dissociation of individual caseins was in the order $\beta>\kappa>\alpha_{s1}>\alpha_{s2}$, corresponding to the ester-phosphate content. This indicates that the caseins more tightly bound to the CCP dissociated to a lesser extent (López-Fandiño & Olano, 1998b). Also, HP possibly resulted in the formation of large fragments (possibly containing denaturing β-Lg) and/or some aggregation (Johnston, Austin, & Murphy, 1992). Indeed, β-Lg in skim milk was found to be denatured above 200 MPa and reaggregated to casein micelles or submicelles (López-Fandiño & Olano, 1998a). Hinrichs et al. (1996a) reported irreversible changes and reduction in size of casein micelles around 230 MPa.

Regardless of treatment temperature, HP treatment of skim milk at > 300 MPa at natural pH cause a dramatically decrease in casein micelle size from 200 to 100 nm and a significantly increase in the level of serum phase casein (Anema, Lowe, et al., 2005; Huppertz, Fox, et al., 2004c). At 200 - 300 MPa, an increase in casein micelles could be observed depending on pH and treatment temperature, pressure and time. The increase in casein micelles size is a consequence of the aggregation of casein micelle. This aggregation is increased as increased treatment temperature and time (Anema, Lowe, et al., 2005; Gaucheron et al., 1997; Huppertz, Fox, et al., 2004c).

2.5.3.2. Effects of high pressure on whey proteins

The amount of non-casein nitrogen in milk serum was shown to decrease with increasing pressure, suggesting denaturation and insolubilisation of whey proteins (Johnston, et al., 1992). Under suitable pressures (100 - 300 MPa), whey protein can undergo partial, but fully reversible, unfolding of their native molecular structures (Datta & Deeth, 2003). Denaturation of individual whey proteins in milk is commonly determined by measuring their level in the pH 4.6-soluble fraction of milk and expressing the level of denaturation relative to control samples (López-Fandiño, et al., 1996; Scollard, et al., 2000).

Denaturation of β-Lg begins to occur at a pressure > 100 MPa (Huppertz, Fox, et al., 2004c; López-Fandiño, et al., 1996; López-Fandiño & Olano, 1998b; Ye, Anema, et al., 2004). Application of higher pressures results in considerable denaturation of β-Lg, reaching 70 - 80% after treatment at 400 MPa (López-Fandiño, et al., 1996; López-Fandiño & Olano, 1998b; Scollard, et al., 2000; Ye, Anema, et al., 2004). Ye, Anema, et al. (2004) reported that the extent of denaturation increased with increasing pressure, with an abrupt and large increase between 300 and 400 MPa. Relatively little further denaturation of β-Lg occurred at 400 - 800 MPa (Scollard, et al., 2000). About 90% of β-Lg was denatured at 800 MPa (Ye, Anema, et al., 2004). Almost complete denaturation has been reported at 750 MPa at 30°C for 30 min, or at 450 MPa at 60°C for 15 min (Datta & Deeth, 2003). The denaturation reaction of β-Lg, which is generally considered as a two-step reaction of unfolding and aggregation, is limited by the rate of unfolding up to a pressure of 200 MPa. At higher pressures, β-Lg denaturation is limited by the aggregation rate (Hinrichs & Rademacher, 2005). The level of denaturation of β-Lg is influenced by heating the milk before pressurisation (Guacheron, et al., 1997; Needs, Capellas, et al., 2000), by the temperature during HP treatment (López-Fandiño & Olano, 1998b) and by the pH of milk during treatment (Arias, Lopez-Fandino, & Olano, 2000).

There are two recent studies on the kinetic of pressure-induced denaturation of whey proteins under HP treatment (Anema, Stockmann, & Lowe, 2005; Hinrichs & Rademacher, 2005). Hinrichs and Rademacher (2005) found that the pressures (up to 800 MPa) and temperatures (up to 70°C) act synergistically on denaturation of α-La and

β-Lg and the aggregation reactions are the rate-determining step. However, Anema, Stockmann et al. (2005) reported a change in pressure dependence at 300 MPa for β-Lg, and suggested that aggregation reactions are rate-determining at low pressures, whereas denaturation or unfolding reactions are rate determining as the pressure increased.

The level of denaturation caused by heat treatments before pressurisation may influence the amount of denaturation measured afterwards, with studies reporting very different extents of denaturation of β-Lg following HP treatment at 600 MPa of pasteurised milk (Needs, Capellas, et al., 2000) or reconstituted skim milk (Guacheron, et al., 1997). The reaction order of HP-induced denaturation of β-Lg is 2.5 (Hinrichs & Rademacher, 2005; Hinrichs, Rademacher, & Kessler, 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.

A synergistic effect of temperature and pressure on the denaturation of β-Lg has been reported (Guacheron, et al., 1997; Hinrichs & Rademacher, 2005; López-Fandiño & Olano, 1998b), with similar levels of denaturation (almost 100%) after treatment of raw milk at 300 MPa at 50-60°C or at 400 MPa at 40-60°C (López-Fandiño & Olano, 1998b). Denaturation was reduced by HP treatment at 4°C relative to 20°C (Guacheron, et al., 1997).

Compared to β-Lg, α-La is much more resistant to denaturation by HP. The reaction order of HP-induced denaturation of α-La is 2 (Hinrichs & Rademacher, 2005). Studies on raw milk (Huppertz, Fox, et al., 2004c; López-Fandiño, et al., 1996; López-Fandiño & Olano, 1998b; Ye, Anema, et al., 2004), reconstituted skim milk (Guacheron, et al., 1997) and pasteurised skim milk (Needs, Capellas, et al., 2000) have concluded that α-La is resistant to denaturation at pressures up to 500 MPa. Above this pressure, the denaturation of α-La increased with increasing pressure, treatment time and temperature, and milk pH (Huppertz, Fox, et al., 2004c). About 10% and 50% of α-La was denatured after 30 min at 600 and 800 MPa (20°C), respectively (Ye, Anema, et al., 2004). Huppertz, Fox, et al. (2004c) reported that denaturation of α-La reached ~72% after 30 min at 800 MPa. However, treatment at higher temperatures (50 - 60°C) greatly increased the extent of denaturation (López-Fandiño & Olano, 1998b).

Differences in the pressure-induced instability of α-La and β-Lg may be linked to the more rigid molecular structure of the former (Guacheron, et al., 1997; López-Fandiño, et al., 1996), caused partially by the numbers of intra-molecular disulphide bonds in the two proteins. α-La is stabilized by four intramolecular disulfide bonds compared to two stabilizing β-Lg (Guacheron, et al., 1997; Hinrichs, et al., 1996b; López-Fandiño, et al., 1996; Needs, Capellas, et al., 2000). There is also the lack of a free sulphydryl group in α-La (López-Fandiño, et al., 1996) and significant differences in the secondary structures of two proteins. It has been reported that the molecular structure of α-La is more stable than that of β-Lg, and that aggregation takes place only if, during unfolding, free SH groups are available from other molecules (Hinrichs, et al., 1996a). α-La heated at high temperature can form dimers and larger aggregates (Lyster, 1970), but in HP treated solution of pure α-La, no transformation of monomer α-La to larger disulphide-bonded aggregates is observed (Patel, Singh, Anema, & creamer, 2004), unless the pressure is very high, ~1000 MPa (Jegouic, Grinberg, Guingant, & Haertle, 1997). In analogy to thermal treatment, it can be assumed that α-La is only denatured if free SH-groups are available. Due to the fast pressure-induced aggregation of β-Lg among each other and with α_{s2}- and κ-caseins, not enough free SH-groups are available for the oligomerization with α-La, resulting in the observed low rate of aggregation (Hinrichs & Rademacher, 2005). β-Lg also has a large cavity in its structure which occupies volume. This is unfavourable under HP treatment, and therefore the protein will unfold rather than have an unfavourable cavity in its structure. Similar to α-La, BSA is also stable to pressure up to 500 MPa (Anema, Stockmann, et al., 2005; Hinrichs & Rademacher, 2005; Huppertz, Fox, et al., 2004c).

HP-treatment of milk induces interactions between whey protein and casein micelles. Huppertz, Fox, et al. (2004c) reported that the majority of denatured whey protein in HP treated milk was sedimentable, and presumably associated with the casein micelles under more severe centrifuging conditions. Both α_{s2}- and κ-caseins contain disulfide bonds, but α_{s2}-casein is more resistant to reduction than κ-casein polymers. It is uncommon to find α_{s2}-casein bonded to any of the whey proteins under heat (< 100°C) or pressure (< 150 MPa) treatments (Patel, Singh, Anema, & Creamer, 2006). However, Patel et al. (2006) observed whey proteins/α_{s2}-casein complexes in HP-treated milk. They suggested that pressures either disrupted the casein micelles structure

sufficiently to expose α_{s2}-casein or modified the structural arrangements of α_{s2}-casein within the micelle to the extent that the disulfide bonds become accessible to the -SH of β-Lg (or it aggregates) or the aggregates of other proteins.

Patel et al. (2006) reported that the size of whey protein/casein aggregates was smaller in HP-treated samples than in heat-treated samples. This difference could be attributed to different effects of heat and HP treatments on the protein structure as well as the sensitivity of different proteins to heat (Lf > Ig > BSA > β-Lg > α-La) and HP (Lf > β-Lg > Ig > BSA > α-La) treatments. The disulfide-linked complexes of β-Lg and κ-casein were formed at low pressure (200 MPa/30 min), whereas interaction between β-Lg and α_{s2}-casein became apparent only at higher pressures (400 MPa/30 min).

2.5.3.3. Effects of high pressure on milk fat globules

Relatively few studies have examined the effects of HP on milk fat globules. HP treatment of milk at a pressure up to 600 MPa has little effect on milk fat globule size (Cheftel & Dumay, 1996; Gervilla, Ferragut, & Guamis, 2001; Huppertz, Fox, & Kelly, 2003). Kanno, Uchimura, Hagiwara, Ametani and Azuma (1998) reported that pressures < 400 MPa did not affect the mean diameter or the size distribution of milk fat globules, but higher pressures (400 – 800 MPa) increased the former and broadened the latter. Huppertz et al. (2003) reported that HP treatment of raw whole milk in the range of 100 – 600 MPa for 30 min at 20°C had no significant effect on the size of milk fat globule ($d_{3,2}$), except for a slight increase in $d_{4,3}$ after treatment at 600 MPa for 30 min. Ye, Anema, et al. (2004) found that the average fat globule sizes ($d_{3,2}$), measured in SDS/EDTA buffer, did not change after raw milk was subjected to pressure treatment in the range of 100 to 800 MPa for 30 min. These results indicate that the milk fat globules were not disrupted during the HP treatment. However, the $d_{3,2}$ values of the milks dispersed in water before particle size measurement increased slightly with increasing pressure up to 700 MPa, but remained constant thereafter (Ye, Anema, et al., 2004). This slight increase was considered to be due to disruption of casein micelles during HP treatment, which reduces their contribution to light scattering (Needs, Stenning, et al., 2000), and therefore gives an apparent increase in the size of the other scattering particles. Treatment time also had little effect on milk fat globule size, with no significant effect being observed on treatment of milk at 200, 400 or 600 MPa for 0 - 60 min.

Microscopic analysis of milk immediately after HP treatment showed little difference between milk fat globules of untreated milk or milk treated at 200 or 600 MPa. HP treatment did not result in aggregation of globules. After 4 h of storage at 5°C, clusters of milk fat globules were apparent in untreated milk and in milk treated at 200 MPa, the aggregates in the latter milk being larger than those in the former. In milk treated at 600 MPa, only slight clustering of milk fat globules was observed after storage for up to 24 h at 5°C (Huppertz, et al., 2003).

Huppertz et al. (2003) reported that HP treatment of raw whole milk slightly increased the amount of cream centrifugally-separated from HP-treated milks. The protein content and the % of total protein in the centrifugally skimmed milk of whole milk treated at 200, 400 or 600 MPa was considerably lower than in that of untreated milk. The protein content and % of total protein were lowest in skimmed milk from whole milk treated at 200 MPa, with little difference between samples treated at 400 or 600 MPa (Huppertz, et al., 2003). The reduced amount of protein in the skim milk fraction of centrifugally-separated HP-treated whole milk, compared to that of untreated milk, when considered together with the slight increase in the amount of cream centrifugally separated from HP-treated milk, indicates association of milk proteins, either caseins or whey proteins, with MFGM on HP treatment (Huppertz, et al., 2003). HP-induced denaturation of α-La or β-Lg occur possibly facilitating HP-induced interactions of whey proteins with the MFGM. Furthermore, HP treatment of milk solubilises individual caseins, especially of β- and κ-caseins (Arias, et al., 2000; López-Fandiño, et al., 1998), which may adsorb onto the MFGM. Huppertz et al. (2003) reported that the amount of milk protein associated with the milk fat globules was increased by HP treatment, the extent of increase being maximal at 200 MPa.

Ye, Anema, et al. (2004) reported that β-Lg associated with MFGM proteins via disulfide bonds during the HP treatment (at 100 to 800 MPa for 30 min) of whole milk. The amount of β-Lg associated with the MFGM increased with an increase in pressure up to 800 MPa and with increasing pressure treatment time. The maximum value for β-Lg association with the MFGM was approximately 0.75 mg/g of fat. At very high pressure, α-La (≥ 700 MPa) and κ-casein (500 MPa) also interacted with the MFGM protein. The level of α-La association (< 0.2 mg/g of fat) was much lower than that for β-Lg. Association of β-Lg and α-La with the MFGM involved sulfydryl-disulfide

exchange reactions, with the thiol group being provided by β-Lg or MFGM proteins. κ-Casein also associated with the MFGM via disulfide bonding with MFGM proteins or β-Lg already associated with the MFGM. It was suggested that β-Lg/κ-casein complexes may have formed during HP treatment and became incorporated into the MFGM (Ye, Anema, et al., 2004). Heat treatment of milk at 90°C for 15 min after pressure treatment (at 400 and 800 MPa) resulted in a marked increase in β-Lg association with the MFGM, similar profiles to the milk that had been heat treated only. The amount of β-Lg associated with the MFGM in heated milk or milk first treated at high pressure and then heat treated (approximately 1.5 mg/g of fat) was about twice that in pressure-treated milk without heating (Ye, Anema, et al., 2004).

At this stage, no systematic studies have been reported on high pressure induced interactions between the recombined milk fat globules and serum protein, although these interactions are important for recombined dairy products.

2.6. Acid-induced milk gelation

Acid milk gels have been characterized as an open network of casein particle chains arranged as thick aggregates, leaving large cavities filled with liquid (Roefs, de Groot-Mostert, & van Vliet, 1990). The network is inhomogeneous, consisting of casein particle strands alternating with thicker nodes. These strands and nodes form large clusters which aggregate to form the gel network, while the liquid phase is immobilized inside the protein network. The formation of acid gel network is influenced by many factors such as heat treatment, total solids content and pH of milk. An understanding of mechanisms involved in structure formation is important in order to characterize the texture of acid gels (Kalab, Allan-Wojtas, & Phipps-Todd, 1983). The structural properties of gels are achieved through interactions between the major milk components, i.e., casein micelles, whey proteins and fat globules. van Vliet (1988) suggested that two types of interactions between the dispersed particles and the gel matrix could be distinguished: (1) no interaction between the gel matrix and the dispersed particles, resulting in a decrease in the gel modulus with increasing volume fraction of filler, and (2) a strong interaction between filler and matrix, resulting in an increase in the modulus of gel with increasing volume fraction of filler material.

The effect of fat globules in acid gel can be constructive or destructive, depending on the membrane composition (de Wit & Klarenbeek, 1984). A decrease in storage modulus (G') with increasing fat content is found in acid gels with non-interacting particles (van Vliet & Dentener-Kikkert, 1982). In this system, the dispersed emulsion droplets are situated in pores in the gel and not connected to the matrix. For the interacting fat particles (e.g. in recombined milk), G' increases with increasing fat content of acid gel (van Vliet & Dentener-Kikkert, 1982). In this case, the fat globules are integrated into the protein matrix (Harwalkar & Kalab, 1986; Tamime, Kalab, & Davies, 1984; Tamime, Kalab, Muir, & Barrantes, 1995) and act as "copolymers" forming links within and between strands and clusters of a casein-based particle network (Xiong, Auguilera, & Kinsella, 1991). The protein on the surface of fat globules adds extra bonding units to reinforce a protein-based matrix, leading to a higher gel strength (Aquilera & Kessler, 1988). Interactions are possible via cross-linking between fat globule membrane proteins and casein particles in the bulk phase. This indicates that the nature of interactions in gel systems, containing fat globules, can be changed by altering the compositions of fat globule membrane.

2.6.1. Rheological properties of acid milk gels

Rheology is defined as the study of the flow and deformation of materials. It is quantitatively concerned with the relationships between the variables, stress and strain, and their relationship with time. Stress is the force applied per unit area and strain is the formation or response to the force with time. In a viscoelastic substance that response is not instantaneous, but occurs over a period. A viscoelastic material is a substance that has some of the properties of a liquid (viscous) as well as those of a solid (elastic) (Ferry, 1980). Dynamic oscillatory techniques are useful in studying dispersions, emulsions and gelling materials. By applying small strain, the oscillating viscometer gives information on what is close to the condition of zero shear (Ferry, 1980). In dynamic rheology, using constant strain rheometer, a small sinusoidally varying strain is applied to the test sample and the response of the material to the varying strain is measured. The amplitude of the oscillation is kept sufficiently small so that the structure of the material is not damaged (i.e. within the linear viscoelastic range). For an elastic material, the stress response to an oscillatory strain shows no phase shift or phase angle (i.e., $\delta = 0°$). In a viscous material that is subjected to a

sinusoidally varying strain, the stress response shows a phase angle shift of 90°. Viscoelastic materials show intermediate phase angles between 0 and 90°. From the amplitude of the sinusoidal strain and the phase angle, the rheological properties of the gel can be characterised in terms of storage (or elastic) modulus (G') and loss or viscous modulus (G''). The storage modulus is the ratio of in-phase stress to strain and a measure of the energy stored per oscillation cycle. On the other hand, the loss modulus is a measure of the energy dissipated (released) per oscillation cycle. The phase angle (δ) is related to the storage and loss moduli through the equation:

$$\tan \delta = G''/G'$$

The larger the value of the tan δ, the more liquid-like the material is behaving and vice versa (Ferry, 1980). For a perfectly elastic material, all the energy is stored and the stress and strain will be in-phase and G'' will be zero. For a liquid possessing no elastic character, all the energy is dissipated as heat and G' will be zero and the stress and strain are completely out-of-phase. When milk gels are subjected to an applied (shear) stress, the energy of deformation is not completely conserved as in an ideal elastic solid nor is it dissipated as in a liquid. Stress applied for a short time results in the elastic character of the gel predominating but if applied for a long time, the viscous character of the gel predominates, and the stress is neither completely in-phase nor completely out-of-phase (Figure 2.11).

2.6.2. Effects of processing on rheological properties of acid milk gels

2.6.2.1. Effect of heat treatment on acid gelation

Heat treatment of milk prior to acidification is one of the most important factors affecting the rheological properties of acid gels. The treatment is often used to modify texture, consistency, and rheological properties of acid gels (Lucey, Munro, & Singh, 1999). Heat treatment of milk before acidification causes interactions between β-Lg and casein (mainly κ-casein) which affect the formation and structure of acid-induced gels (Lucey, Teo, Munro, & Singh, 1997).

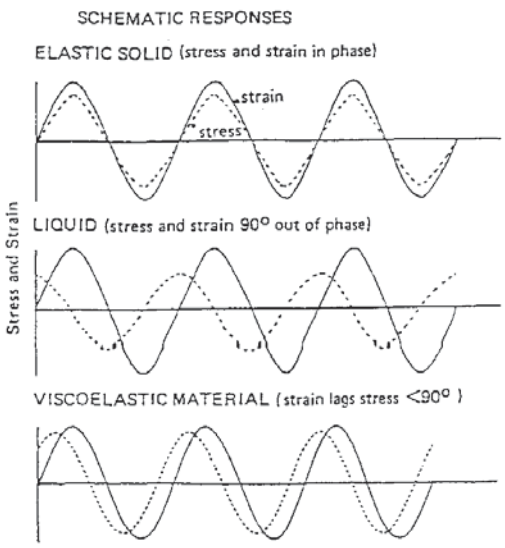


Figure 2.11. Dynamic experiment showing response of elastic solid, liquid and viscoelastic material, from Ferry (1980).

When milk is heated above 70°C, denatured whey proteins associate with casein micelles via the formation of intermolecular disulphide bonds (Singh, 1995) and participate in the gel matrix . Thus, heating milk above 70°C prior to acidification results in a decrease in gelation time and increases in the G' and gelation pH of the acid gel (Anema, Lee, Lowe, & Klostermeyer, 2004; Cho, et al., 1999; Lucey, Tamehana, Singh, & Munro, 1998a). During heating, the denatured whey proteins associate with the casein micelles as filamentous appendages that can provide sites for increased cross-linking during acidification and hence an increased firmness of the gel network (Lucey, Munro, et al., 1999). The level of whey protein denaturation, particularly β-Lg, is correlated with the firmness of acid milk gels (Dannenberg & Kessler, 1988b). The incorporation of denaturation of whey proteins in the acid milk gels produces a structure with a higher protein concentration and an increased number of cross-links (Anema, Lee, et al., 2004; Lucey & Singh, 1998; Lucey, Teo, et al., 1997).

Dannenberg and Kessler (1988b) reported that when the denaturation of β-Lg increased from 10 to 60%, there was a linear increase in firmness of acid gels. van Vliet and Keetals (1995) found that the dynamic moduli of acid gels made from low-heat (pasteurized at 72°C for 15 s) skim milk were much lower, and their fracture strain was higher than gels made from high-heat (heating at 85°C for 5 min) skim milk. Lucey,

Teo, et al. (1997) reported that G' of acid gels made from skim milks increased with an increase the severity of heat treatment. However, the strain at yield point decreased markedly with increasing heat treatment of reconstituted milk making these gels more brittle and easier to fracture. The large increase in the G' value of acid gels made from heated milks was explained as denatured whey proteins actively contributing to the rigidity of the gel, i.e., forming additional cross-linkages (Lucey, Munro, et al., 1999). Lucey, Tamehana, Singh and Munro (1998b) found that acid gels containing fat globules stabilized by heated WPC had very short gelation times and high gelation pH in both unheated and heated recombined milk systems, confirming that denatured whey proteins are responsible for the increased gelation pH of acidified milk. In unheated milk, native whey proteins remain soluble, do not interact with casein particles during acidification, therefore, gelation does not occur until the pH of unheated milk approaches isoelectric point of casein (about pH 4.6) (Lucey, Tamehana, et al., 1998a; Lucey, van Vliet, Grolle, Geurts, & Walstra, 1997). The main factor responsible for the formation of acid-induced gels from unheated milk is the reduction in the net negative charge on casein particles as the isoelectric point is approached; in these systems, the gel matrix consists exclusively of casein particles (Lucey, van Vliet, et al., 1997). The aggregated denatured whey proteins are insoluble at their isoelectric points. Therefore, as the pH of heated milk approaches pH 5.3, the whey proteins can aggregate and the gelation occurs at a pH considerably higher than that of unheated milk (Lucey, Tamehana, et al., 1998a; Lucey, Teo, et al., 1997; Lucey, van Vliet, et al., 1997).

Cho et al. (1999) reported that in unheated or heated (80°C for 30 min) reconstituted skim milk systems, gels containing fat globules stabilized by non-interacting materials such as unheated whey protein concentrate (WPC) had low G' compared with those containing interacting materials such as caseins or denatured whey proteins. Heated reconstituted skim milk systems had also higher G' values than unheated reconstituted skim milk systems. Lucey & Singh (1998) reported that the firmness of acid gels prepared from heated milk with the denatured whey proteins associated with the casein micelles was markedly higher than the firmness of acid gels in which the whey proteins were pre-denatured, and therefore not associated with the casein micelles. This indicated that the association of denatured whey proteins with the casein micelles is important for the marked increase in the G' of acid gels made from heated milk.

Several investigators have observed differences in microstructure of acid gels as a result of heat treatment of milk. Davis, Shankar, Brooker and Hobbs (1978) reported that heated milk (95°C for 10 min) had filamentous appendages attached to casein micelles, which appeared to consist of denatured whey proteins. Kelab et al. (1983) and Parnell-Clunies (1987) also observed the formation of 'spikes' on the micellar surfaces of heated milk. These appendages appear to inhibit micellar contact and fusion, which prevented the formation of very large aggregates (Davies, et al., 1978). In gel made from unheated milk, casein micelles form aggregates or clusters in which the protein is irregularly or unevenly distributed, resulting in the formation of a 'coarse network' (Harwalkar & Kalab, 1981). The systematic presentation of acid milk gels prepared from reconstituted skim milk with different fat globule membrane materials is shown in Figure 2.12.

2.6.2.2. Effect of high pressure treatment on acid gelation

There have been a few studies on the effects of HP treatment of milk on the properties of acid gels. Johnston, Austin and Murphy (1993) reported an increase in the gel strength (G') and yield stress of acid gels prepared from HP-treated milk samples. The final G' of the acid gels from the HP-treated samples was markedly higher than that from the heated samples although the HP-treated samples had a lower yield point than the heated samples. Pressurization at 600 MPa or higher was required to obtain a substantial effect on the rheology of acid-induced gels (Anema, Lauber, et al., 2005). Acid gels prepared from HP-treated milk had higher firmness and yield strength than those of acid milk gels made from untreated milk (Harte, Luedecke, Swanson, & Barbosa-Canovas, 2003).

The rheological properties of acid milk gels prepared from HP-treated milk were very similar to those of acid milk gels prepared from heated milks (Anema, Lauber, et al., 2005). The increase in gel strength for acid milk gels made from HP-treated milk may be related to the HP-induced denaturation of whey proteins, and may involve a similar mechanism to that observed when heated milk is acidified to form a gel (Anema, Lee, et al., 2004; Lucey & Singh, 1998; Lucey, Teo, et al., 1997).

An increase in temperature will always increase a reaction rate due to the reduction in the energy difference between the initial state (reactant) and the activated complex. An

increase in pressure may retard or increase the rate of a reaction depending on whether the volume of activated complex is increased (retarded rate) or decreased (increase rate) relative to the initial state. In addition, pressure and temperature can have different effects on the interactions that maintain protein and colloidal structure (such as hydrophobic and other non-covalent interactions, mineral and ion equilibrium etc.). As a consequence, heat and HP treatments are expected to have different effects on milk systems. It is clearly evident that the chemical and physical changes to the milk protein systems as induced by temperature and HP-treatment are different, as would be expected from a basic thermodynamic understanding.

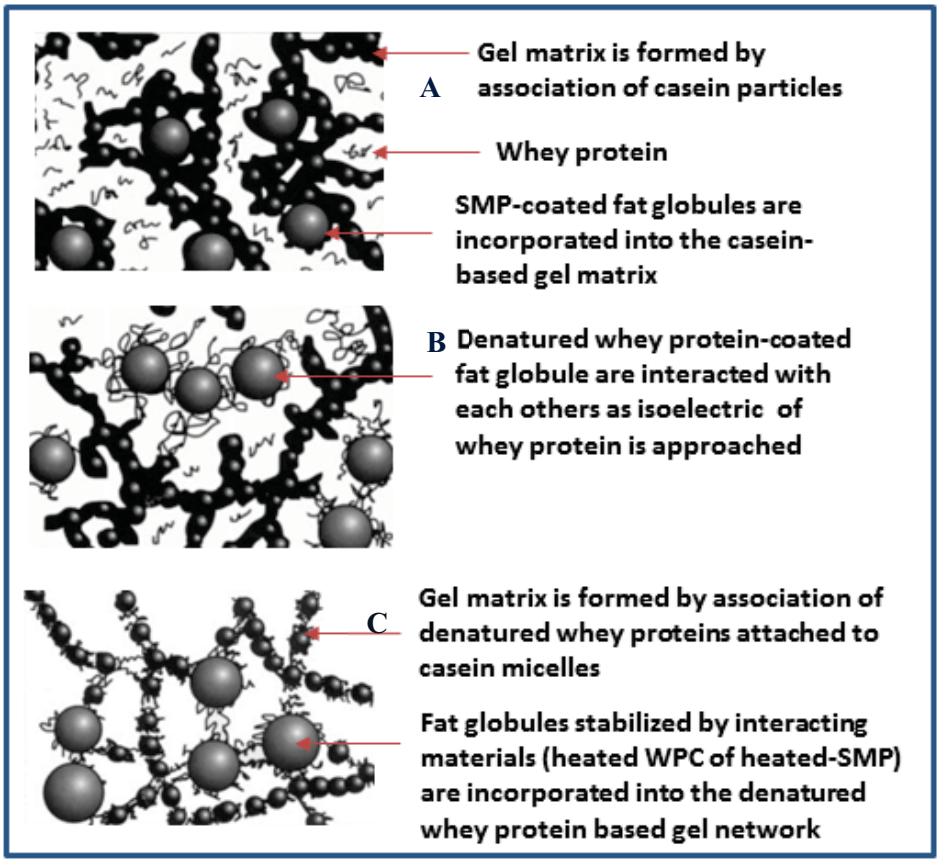


Figure 2.12 A systematic presentation of acid gels made from unheated (A, B) and heated (C) reconstituted skim milks with fat globules stabilized by different materials. In unheated system, the gel matrix is formed by association of casein particles. In heated system, the gel matrix is formed by association of denatured whey protein attached to casein micelles. Fat globules stabilized by interacting materials (SMP) are incorporated into the casein-based gel network. Fat globules stabilized by heated WPC interact with each other as the isoelectric point of whey protein is approached, from Cho et al. (1999).

2.7. Objectives

In the light of all the above, the main objective of the present investigation was to gain a better understanding of the characteristics of surface layers of milk fat globules in recombined milk and how these surface layer are modified by heat and HP treatments. The impact of these modifications on acid-induced gelation was also determined.

1. To investigate the effects of heat, HP and heat/HP treatments at different pressure-temperature-time combinations applied to milks before or after homogenization on whey protein denaturation, fat globule size and the surface layers of recombined milk fat globules.
2. To characterize the properties of acid gels prepared from recombined milks that underwent heat, HP and heat/HP treatments.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Low heat skim milk powder (LHSMP) (whey protein nitrogen index > 6.00) and anhydrous milk fat (AMF), which contained 99.95% milk fat and 0.05% moisture, were obtained from Fonterra Co-operative Group Ltd., New Zealand. The skim milk powder was composed of 33% protein, 54% lactose, 3.8% moisture, 0.8% fat and 8.4% ash.

All the chemicals used were of analytical grade obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA), unless specified otherwise.

The water used in all experiments was purified by reverse osmosis followed by carbon treatment and was deionised using Milli-Q apparatus (Millipore Corp., Bedford, MA, USA).

3.2. Methods

3.2.1. Recombined milk preparation

Reconstituted milks were prepared by adding low heat skim milk powder (LHSMP) to deionised water to a final concentration of 9% (w/v) at 40°C. Each mixture was stirred for 1 h using magnetic stirrer and allowed to equilibrate at ~20°C overnight. A small amount of sodium azide (0.02%) was added to all milk samples as a preservative. After 24 h, the reconstituted milk was warmed to 40°C, and melted AMF, at 50°C, was then added to the reconstituted milk to a concentration of 4% (w/v) AMF. The dispersion was stirred for 30 min, heated to 52°C, and mixed with a Silverson Laboratory mixer (model: L5M-A, Silverson, UK) for 1 min to produce an oil-in-water emulsion that was stable until homogenization. The mixture was then homogenized using a Rannie two-stage valve homogenizer (Model Lab, type 12.50H, DK-2620, Alberslund, Denmark) at 13.8 MPa and 3.5 MPa for the first and second stages respectively. The resulting recombined milk was cooled to room temperature (~20 - 23°C) and stored overnight at 5°C. The next day (after 18 hours), the recombined milk sample was warmed to 20°C in a water bath before further

analysis. The pH of milk sample was not standardized, but it was checked regularly to ensure that it was in the range of 6.6 – 6.7. The experimental protocols used to prepare untreated, heat-treated, HP-treated, and heat-/HP-treated recombined milk, before or after homogenization, are shown in Figures 3.1 and 3.2 respectively. When HP treatment was applied to reconstituted milk before homogenization, small batches of HP-treated reconstituted milks were homogenized in an APV homogenizer (APV1000, APV Homogenizers, Albertslund, Denmark) with a capacity lower than the Rannie Homogenizer.

3.2.2. Heat treatment

For the preparation of heat-treated milk, reconstituted milk or recombined milk was heated at pasteurization level (72°C for 15 s), ultra high temperature (UHT) level (140°C for 5 s), and at different combinations of time (0-30 min) and temperature (72-100°C) using a pilot-scale indirect UHT unit (spiral flow, indirect UHT Plant, Alfa Laval, Lund, Sweden) as shown in Figure 3.3. For all samples, the come up time to heat the milks to the required temperatures was < 2 s. The required holding time at pasteurization and UHT treatment were obtained by adjusting the flow of incoming milk. For the heat treatments at different time-temperature combinations, the milk samples were transferred after heating to the required temperature in the UHT unit to stainless steel beakers placed in a thermostatically controlled water bath set at the selected temperature and held for desired time. The beakers were covered with aluminium foil to minimize evaporation and stirred occasionally. After heat treatment, the milk samples were rapidly cooled to 20-23°C by immersion in ice water bath with agitation. The heat treatment at pasteurization, UHT, and high heat treatment (90°C for 30 min) conditions are used as control heat treatments as these time-temperature combinations are the commercial heat treatments used routinely in the dairy industry. The heat treatments were applied either before or after homogenization as shown in Figures 3.1 and 3.2.

3.2.3. High pressure treatment

HP treatments were applied to the milks, either before or after homogenization, as shown in Figures 3.1 and 3.2. The milk samples were transferred to polyethylene terephthalate bottles (330 mL) and tightly closed. Each bottle was then transferred to a polyethylene bag and vacuum-sealed. The samples in the bottles were HP-treated in a “Food Lab” high-pressure food processor (model: S-FL-065-200-9-W; Stansted Fluid Power Ltd., Stansted, Essex, UK) as shown in Figure 3.4.

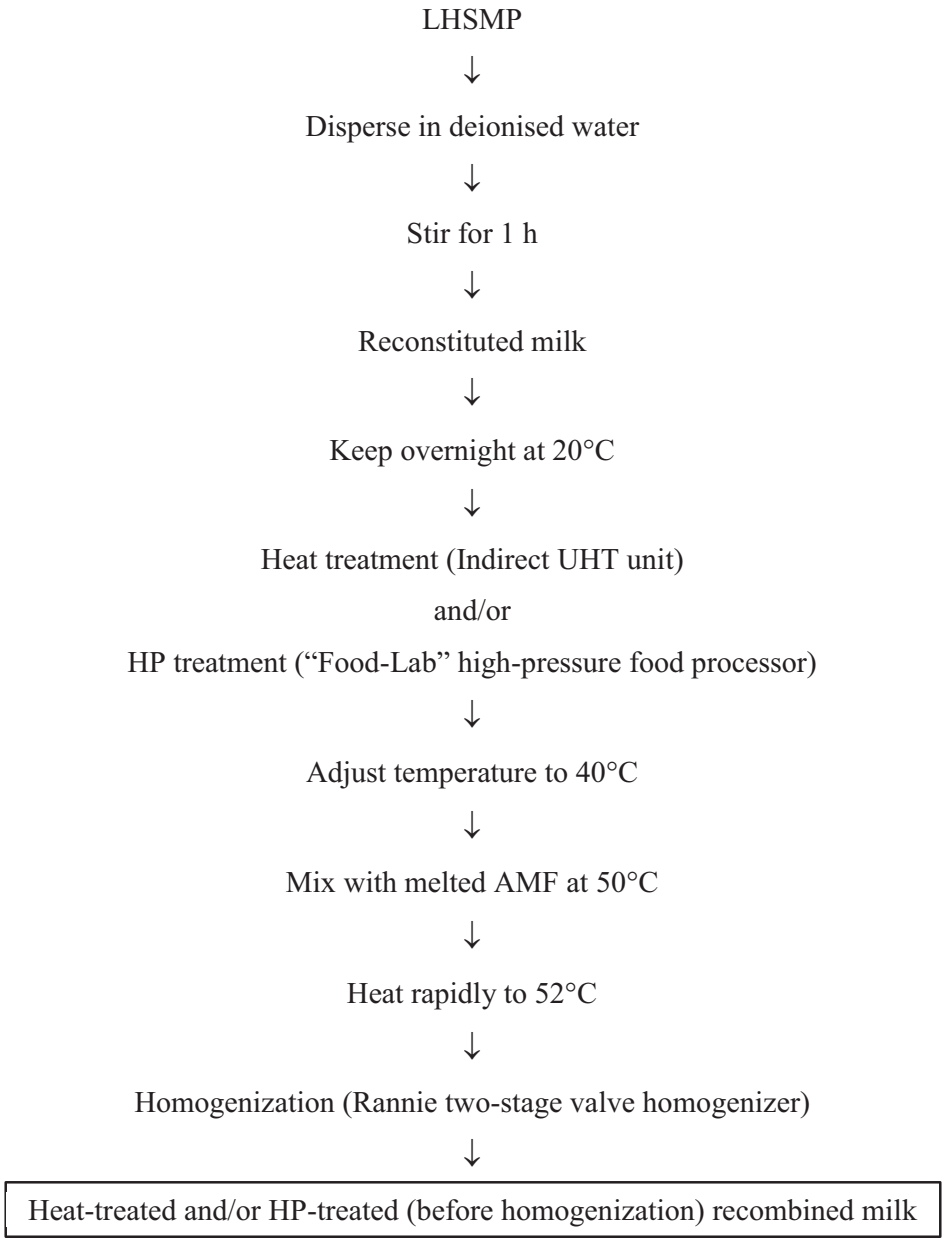


Figure 3.1. Experimental protocol for the preparation of recombined milk with heat and/or HP treatment before homogenization.

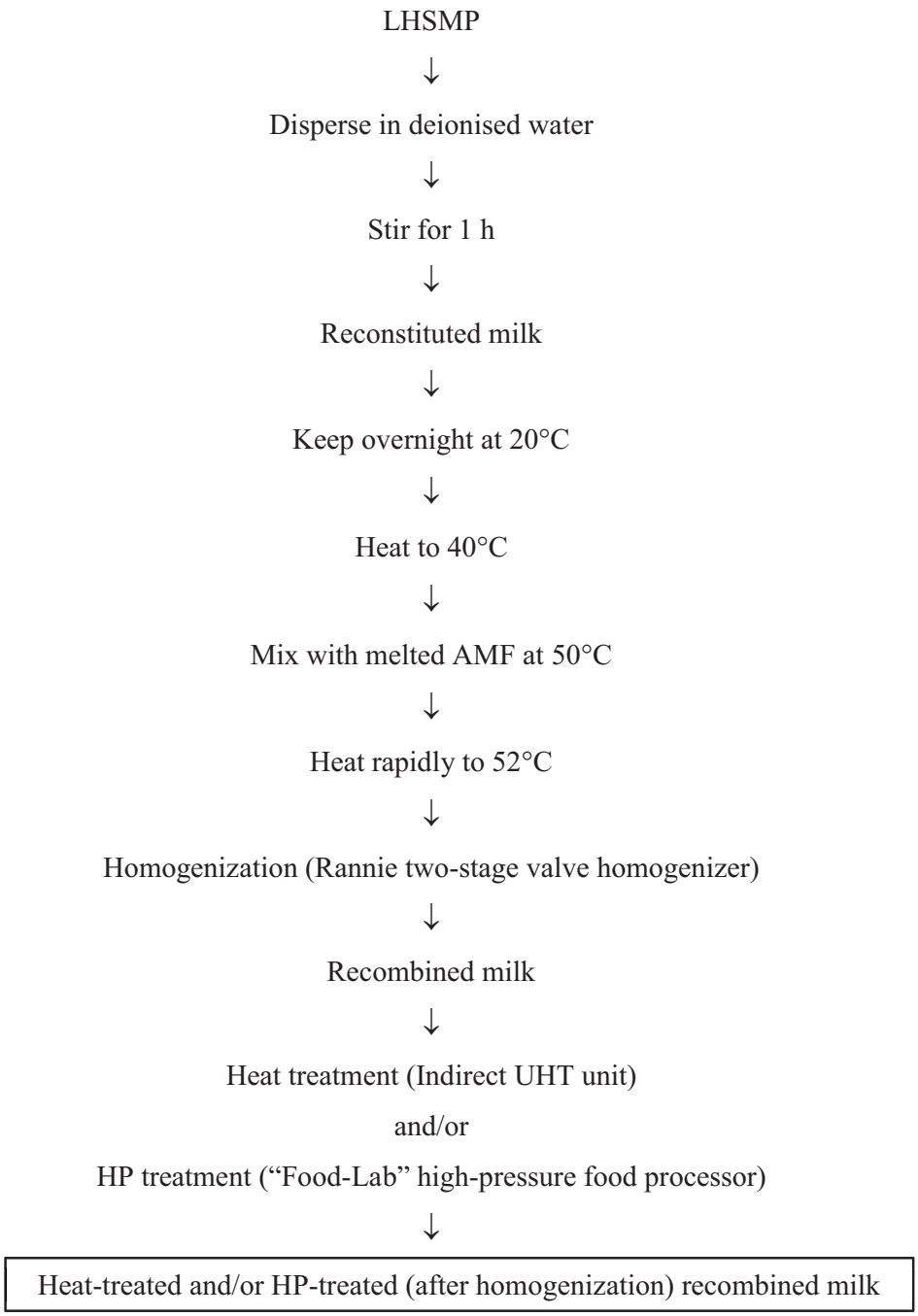


Figure 3.2. Experimental protocol for the preparation of recombined milk with heat and/or HP treatment after homogenization.

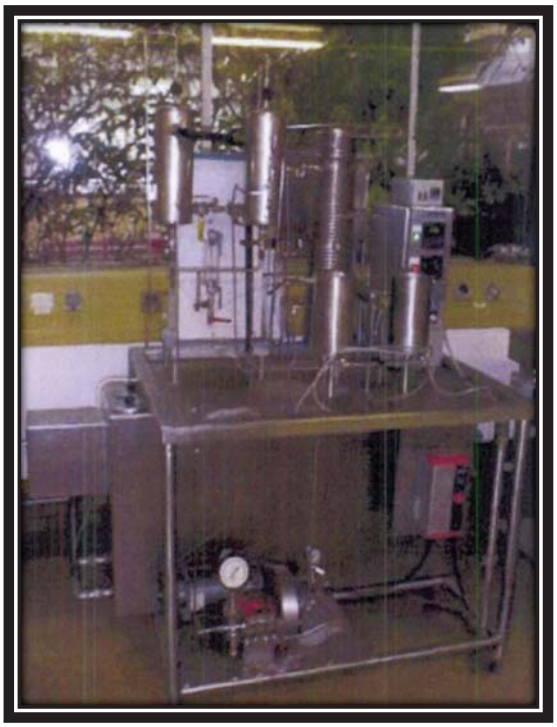


Figure 3.3. The photograph of a pilot-scale indirect UHT unit.

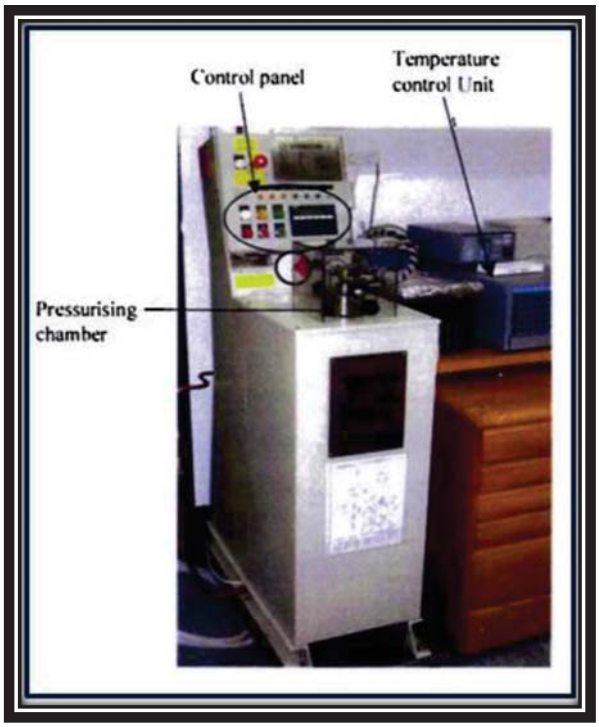


Figure 3.4. The photograph of "Food Lab" high-pressure food processor

The HP unit was equilibrated to the desired temperature by recirculating temperature-adjusted water through the water jacket associated with the unit. The HP unit and all samples were equilibrated to the desired temperature for at least 1 h before pressurization was commenced. The temperature change during pressurization/ depressurization cycles was monitored using the thermocouple associated with the unit and standard data logging equipment. An emulsion of 10% vegetable oil in water with surfactant and preservative was used as a pressure-transmitting fluid in the 65 mm × 220 mm cylindrical high-pressure chamber. The oil provides some lubrication to prevent mechanical wear in the unit. After HP treatment, the unit was automatically depressurized and the samples were processed immediately. The temperature of the pressurizing fluid and the sample increased (~2.0°C per 100 MPa) during adiabatic heating and decreased (~1.7°C per 100 MPa) as a result of adiabatic cooling. The pressurization rate and depressurization rate were ~ 6 and 27 MPa s^{-1}. All the HP-treated recombined milks were held at room temperature for 12 h before further analysis.

3.2.4. Chemical analysis

3.2.4.1. Protein analysis

Total protein was measured by determining total nitrogen using the macro-Kjeldahl method (AOAC, 2000) and multiplying it by a factor of 6.38. The samples were digested using a Kjeltec digester (Kjeltec1007 Digester, Tecator, Sweden) and distilled using a Kjeltec system (Kjeltec 1026 Distilling Unit, Tecator, Sweden).

3.2.4.2. Fat analysis

The total fat content of the recombined milk and cream were determined using the Mojonnier method for milk (IDF 16C, 1987) and cream (IDF 1C, 1987), respectively.

3.2.4.3. Milk fat globule diameter and size distribution

A Malvern MasterSizer MSE (Malvern Instruments Ltd, Worcestershire, UK) was used to determine the fat globule size distribution using the presentation code 2NAD provided by the instrument's manufacturer. The relative refractive index (N), i.e., the ratio of refractive index of fat globule (1.456) and that of the dispersion medium (1.330)

was 1.095. The milk sample was dispersed in water or in a 2% SDS/50 mM Ethylenediaminetetraacetic acid (ETDA) solution. The latter was used to dissociate the casein micelles, prior to fat globule size measurement (de Feijter, Benjamins, & Tamboer, 1987; Walstra, 1990). Two measurement results for fat globules are obtained by this technique: (1) volume-surface average mean diameters in μm, d_{32} (= $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of fat globules with diameter d_i) and (2) specific surface area (A) in square meters per gram fat globules. The measurements were performed in triplicate on freshly prepared samples at room temperature.

3.2.4.4. Calculation of protein load

The protein load, expressed as mg protein/m^2 of fat surface area, was calculated from the amount of protein adsorbed onto the washed cream layers and the specific surface area (A).

$$\text{Protein load (mg}\cdot\text{m}^2\text{)} = \frac{\text{Adsorbed protein (mg/g fat)}}{\text{A (m}^2\text{/g)}}$$

3.2.4.5. Isolation of surface layer of fat globules

The surface layer of fat globules was isolated from recombined milk, as described by Sharma et al. (1996a) and Ye, Anema, et al. (2004). Milk samples were centrifuged at 18,000 g for 20 min at 20°C in a temperature-controlled centrifuge (Sorvall RC5C, DuPont Co., Newtown, CT). Three fractions were obtained, i.e., top (cream) layer, middle layer and sediment. The top layer (cream) was carefully removed from the centrifuge tube using a spatula, and then washed with simulated milk ultrafiltrate (SMUF) (Jenness & Koops, 1962) to remove protein materials that were not bound to the fat globule membrane. The cream was dispersed in 10 volumes of either SMUF containing 6 M urea and 50 mM EDTA or SMUF alone and left at room temperature for 1 h. This mixture was recentrifuged at 18,000 g for 20 min at 20°C, and the top layer was collected. The cream was washed three times in order to remove any entrapped and loosely associated protein materials from the surface of fat globules. The proteins remaining in the cream layer after washing were considered to be adsorbed at the surface of fat globules. When the isolated surface layers of fat globules (cream) is washed in SMUF containing urea and EDTA, the casein micelles adsorbed at the fat

globule surface are dissociated and washed away. Only the protein molecules adsorbed directly at the interface of fat globules and the protein molecules bound to the interfacial protein layer via covalent bonds remain on the surface of the fat globules (Ye, Anema, et al., 2004).

3.2.4.6. Analysis of surface layer of fat globules

The individual proteins in the washed cream were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Singh and Creamer (1991b) and Havea et al. (1998). SDS-PAGE gels were run under dissociating (add SDS) and reducing (added β-marcaptoethanol) conditions. For all gel electrophoresis, the protein samples were analysed using the Mini-PROTEAN® 3 electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA).

Preparation of stock solutions

Acrylamide/Bis (30% T, 2.67% C)

Acrylamide/Bis mixture 37.5:1 (2.6% C) (30 g) was dissolved in approximately 60 mL of deionised water and made up to a final volume of 100 mL; it was filtered and stored in an amber bottle at 4°C.

1.5 M TRIS-HCl buffer, pH 8.8 (resolving gel buffer)

TRIS (tris hydroxymethyl aminoethane, 18.15 g) was dissolved in approximately 60 mL of deionised water and the pH adjusted to 8.8 with 6 M HCl. The volume was made to 100 mL with deionised water. This buffer was stored at 4°C.

0.5 M TRIS-HCl buffer, pH 6.8 (stacking gel buffer)

TRIS (6 g) was dissolved in approximately 60 mL of deionised water and the pH was adjusted to 6.8 with 6 M HCl. The volume was made to 100 mL with deionised water, and the buffer was stored at 4°C.

10% SDS

SDS (10 g) was dissolved with gentle stirring in deionised water and the volume made up to 100 mL. This solution was stored at room temperature.

10% (w/v) Ammonium persulphate (APS)

APS 100 mg was dissolved in 1.0 mL of deionised water. The solution was made up fresh each day.

0.4% (w/v) Bromophenol blue

Bromophenol blue (1.6 g) was dissolved in approximately 7 mL of 0.1 M NaoH and the volume made up to 400 mL in a measuring cylinder with deionised water.

SDS sample buffer, pH 6.8

The following solutions were added to 10 mL of deionised water: 0.5 M TRIS-HCl buffer (50 mL), glycerol (40 mL), 10% (w/v) SDS (80 mL) and 0.4% (w/v) bromophenol blue (10 mL). The sample buffer was stored at room temperature.

SDS electrode buffer, pH 8.3 (5X concentration)

Electrode buffer (5X) was made by dissolving TRIS (15 g), glycine (72 g) and SDS (5 g) in deionised water and the volume was made to 1 L. The pH of the buffer should be 8.6 (± 0.2) and the buffer was stored at 4°C. For each electrophoresis run, 80 mL of 5X electrode buffer was diluted to 400 mL with deionised water.

Preparation of resolving gel

To prepare 10 mL of resolving gel buffer, the following solutions were mixed: deionised water (2.0 mL), 1.5 M TRIS-HCl buffer (2.5 mL) and acrylamide/bis mixture (5.3 mL). The mixture was degassed for 15 minutes in a Buchner flask with rapid stirring. Immediately after that, 10% (w/v) SDS solution (100 μL), 50 μL of 10% (w/v) APS and 5 μL of TEMED (tetramethyl-ethylenediamine) were added. After gentle mixing, the contents were poured between electrophoresis casting plates (Mini-PROTEAN® 3 electrophoresis system, Bio-Rad Laboratories, Hercules, CA, USA).

Deionised water (200 μL) was added to form an upper layer and the resolving gel solution was allowed to polymerise at 20°C for 1 h. The water was poured off carefully and removed with pieces of filter paper before pouring the stacking gel.

Preparation of stacking gel

To prepare 5 mL of stacking gel, the following solutions were mixed: deionised water (3.05 mL), 0.5 M TRIS-HCl buffer (1.25 mL) and acrylamide/bris mixture (0.65 mL). The mixture was degassed for 15 min in a Buchner flask with rapid stirring. Immediately after that, 10% (w/v) SDS solution (50 μL), 25 μL of 10% (w/v) APS and 5 μL of TEMED were added by gentle swirling. The stacking gel was poured on the top of the set resolving gel. The slot former (10-slot plastic comb) was inserted between the plates to form appropriate slots for the samples. Polymerisation was carried out at room temperature for about 35 min, the combs were pulled out and the formed slots were rinsed with deionised water. The water was poured off carefully and removed using pieces of filter paper. The gel plates were then placed in the electrode chamber and samples were applied to the gel slots.

Sample preparation and electrophoresis

The composition of individual proteins in the washed cream were determined by SDS-PAGE, as described by Sharma et al. (1996b) and Ye (2003). The cream washed with SMUF (0.1 g) was dispersed in 3.0 g SDS sample buffer, whereas cream washed with SMUF containing 6 M urea and 50 mM EDTA (0.1 g) was dispersed in 0.5 g of SDS sample buffer. SDS displaced the proteins from the oil–water interface (de Feijter, et al., 1987) and stabilized the protein molecules in extended conformations. For reducing conditions, 2% β-mercaptoethanol was added to the samples which were then heated for 5 min in a boiling water bath. After heating in the SDS buffer, a further centrifugation at 800 g for 5 min was performed before PAGE analysis to remove the fat from the sample.

Two gels were placed in an electrode buffer chamber and SDS electrode buffer stock solution was used to fill the inner buffer chamber. The supernatants (10 μL) were injected to the slots of the gel. The gels were run in a Mini-PROTEAN® 3 electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) at 210 V, 70 mA

and 6.5 W using a Bio-Rad power supply unit (Model 1000/500, Bio-Rad, Richmond, CA, USA) until the tracking dye moved out of the gel. The approximate running time was 60 min, after which the gels were removed from the plates and transferred gently to staining solution.

Staining

After electrophoresis, the gels were removed from the casting assembly and placed in plastic containers containing 50 mL of staining solution (0.1% (w/v) amido black 10B dye dissolved in 10% acetic acid). The gel containers were put on a rocking table for 1 h for uniform staining.

Destaining

The staining solution was poured off and replaced with 100 mL destaining solution (10% glacial acetic acid). After rocking for 1 h, the destaining solution was replaced with 100 mL of fresh destaining solution and the container was rocked for a further 19 h. The destaining solution was changed three times during destaining.

Quantitation of the intensity of protein bands

The gel was scanned and photographed using a Molecular Dynamics Personal Densitometer (Model PD-SI computing densitometer, Molecular Dynamics, Sunnyvale, CA, USA). The stained proteins were scanned at 633 nm with a He/Ne laser with a spot size of 50 μm at a resolution of 100 μm. The integrated intensities of the caseins and whey proteins (β-Lg and α-La) bands were determined using ImageQuant software, version 5.0 (Molecular Dynamics, Sunnyvale, CA, USA), associated with the densitometer. The experiments were repeated and the variations in the integration results were found to be within 10%. The data plotted on the graphs are the averages of two integrations.

The total absorbance/integrated intensity of each protein band was determined and compared with the relative intensity of protein bands in untreated recombined milks (RM) run on each gel as a control. 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, so that the results could be compared.

The amount of individual proteins adsorbed on the surface layer of fat globules (i.e., cream washed using SMUF) was calculated as follows

$$\text{Protein adsorbed (mg}\cdot\text{m}^{-2}) = \frac{\text{Intensity of individual protein band (in each lane)}}{\text{Total intensity of all protein bands (in each lane)}} \times \text{Total protein (from nitrogen analysis)}$$

3.2.4.7. Denaturation of whey proteins

Untreated, heat-treated, HP-treated, and heat-/HP-treated milk samples were analysed for native β-Lg and α-La by native polyacrylamide gel electrophoresis (Native-PAGE) using a Mini-PROTEAN® 3 electrophoresis system (Bio-Rad Laboratories, Richmond, CA). Denaturation of β-Lg and α-La after those treatments was calculated by considering the initial contents of the untreated recombined milk.

Native resolving gel buffer

TRIS (72.6 g) was dissolved in approximately 180 mL deionised water. The pH of the solution was adjusted to 8.8 with 6 M HCl. The volume was made up to 200 mL with deionised water. The buffer was stored at 4°C.

Native stacking gel buffer

TRIS (30 g) was dissolved in approximately 400 mL of deionised water and the pH was adjusted to 6.8 with 6 M HCl. The volume was made to 500 mL with deionised water in a volumetric flask, and the buffer was stored at 4°C.

Native Gel sample buffer, pH 6.8

The following solutions were mixed together: native stacking gel buffer (200 mL), deionised water (600 mL), glycerol (80 mL), and 0.4% (w/v) bromophenol blue (20 mL). The pH of sample buffer should be 6.8. The native gel sample buffer was stored at 4°C.

Native electrode stock buffer, pH 8.3 (5X concentration)

Electrode buffer (5X) was made by dissolving TRIS (15 g) and glycine (72 g) in deionised water and the volume was made to 1 L. The buffer was stored at 4°C. For each electrophoresis run, 80 mL of 5X electrode buffer was diluted to 400 mL with deionised water.

Preparation of native resolving gel

To prepare 10 mL of resolving gel buffer, the following solutions were mixed: deionised water (3.75mL), resolving gel buffer (1.25 mL) and acrylamide/bis mixture (5.0 mL). The mixture was degassed for 15 minutes in a Buchner flask with rapid stirring. Immediately after that, 50 µL of 10% (w/v) APS and 5 µL of TEMED were added. After gentle mixing, the contents were poured between electrophoresis casting plates (Mini-PROTEAN® 3 electrophoresis system, Bio-Rad Laboratories, Hercules, CA, USA).

Preparation of native stacking gel

To prepare 5 mL of stacking gel, the following solutions were mixed: deionised water (3.15 mL), native stacking gel buffer (1.25 mL) and acrylamide/bris mixture (625 µL). The mixture was degassed for 15 min in a Buchner flask with rapid stirring. Immediately after that, 25 µL of 10% (w/v) APS and 5 µL of TEMED were added by gentle swirling. The stacking gel was poured on the top of the set resolving gel.

Native gel sample preparation

Milk (50 µL) was added to 950 µL of native sample buffer.

Native-PAGE preparation, electrophoresis, staining and destaining, and data analysis

Native-PAGE preparation, electrophoresis conditions, staining and destaining, and data analysis were carried out in the same manner as SDS-PAGE (see section 3.2.4.6). The approximate running time for Native-PAGE was 1.5 h.

3.2.4.8 Acid gelation of recombined milk

Recombined milk samples were acidified using glucono-δ-lactone (GDL) at the 1.8% (w/v) level at 30°C as described by Anema, Lauber, et al. (2005). The pH change over time was monitored using a combined electrode (model PHC2401; Radiometer, DK-2400 Copenhagen, Denmark) attached to a radiometer model PHM61 meter. The pH of the acid gels was ~4.04 after incubation for 6 h. The rheological properties of the recombined milk during acidification were monitored over time with low amplitude dynamic oscillation by measuring of storage modulus (G') and loss tangent (tan δ) using the techniques described by Lucey, Tamehana, Singh and Munro (2000) and Anema, Lauber, et al. (2005). A Physica rheometer MC301 (Anton Paar, Germany) was used. The measuring geometry (DG 26) consisted of two coaxial cylinders (diam. 24.68 and 26.68 mm). After the addition of GDL to the milk, the mixture was stirred for 2 min and then ~20 mL of the mixture was transferred to the rheometer. Vegetable oil was placed on the surface to prevent evaporation. Samples were oscillated at a frequency of 0.1 Hz, at a strain of 0.01; data were taken every 2 min for 6 h. The temperature of samples was maintained at 30°C. Gelation time was defined as the point when gels had a $G' \geq 1$ Pa. The effect of the time scale of the applied strain on the rheological properties of the set gels was determined at 30°C by frequency sweep ~6 h after the addition of GDL; frequency was varied from 0.01 to 10.0 Hz. After the frequency sweep, the temperature of the system was reduced to 5°C and the rheological properties of the set gel were monitored at 5°C for a period of 30 min to investigate the effects of gelation temperature on acid gel. A strain sweep was performed afterward by increasing the strain from 0.1% to 300% in order to measure the breaking strain and breaking stress of the acid gel. The maximum stress was considered to be that at which the gel broke, and the corresponding strain was defined as the breaking strain. All experiments were carried out in duplicate.

3.2.5. Statistical analysis

All the experiments were performed at least twice. Error bars on the data presented in the figures represent the standard deviations of the repeated measurements. The results from replicated experiments were analysed using an analysis of variance (ANOVA) and Post Hoc Multiple Comparisons (Tukey's honesty significant difference test Test) using

the SPSS 18 for Windows (SPSS Inc, Chicaco, IL). Differences were considered significant at $P \leq 0.05$.

CHAPTER 4

HEAT-TREATED RECOMBINED MILKS

4.1. Introduction

Heat treatment at temperatures higher than 70°C has profound effects on milk proteins. The whey proteins denature, and at the same time some caseins dissociate from casein micelles into the serum. The effects of heat treatment depend on heating temperature and holding time and also on the pH of the milk. The denatured whey proteins interact either with κ-casein or with other denatured whey proteins (Considine, et al., 2007; Sharma & Dalgleish, 1993; Singh, 1993). Heat treatment also affects fat globules; it induces the association of denatured whey proteins onto their surface layers (Sharma & Dalgleish, 1994; Ye, Singh, Taylor, & Anema, 2004a).

Recombined milk fat globules have no native milk fat globule membrane (MFGM) constituents in the protective layer surrounding the lipids (Oortwijn & Walstra, 1979, 1982; Walstra & Jenness, 1984). The surface layers of recombined milk fat globules are made up of primarily caseins (with the majority being α_s-caseins) as well as whey proteins (Darling & Butcher, 1978; McCrae, et al., 1994; Oortwijn & Walstra, 1979; Sharma, et al., 1996a). The interactions between the surface layers of recombined milk fat globules and serum proteins are similar to those found in heated skim milk (Dalgleish, 1990; Dannenberg & Kessler, 1988a; Dannenberg & Kessler, 1988c). These interactions occur via the formation of disulfide bonds between denatured whey proteins and caseins, especially κ-casein (Jang & Swaisgood, 1990; McKenzie, Norton, & Sawyer, 1971; Parris, White, & Farrell, 1990).

There are few studies on the effects of heat treatment on the surface layers of recombined milk fat globules (Oortwijn & Walstra, 1979; Sharma, et al., 1996b; Singh, Sharma, Taylor, & Creamer, 1996). Sharma et al. (1996b) reported that preheating reconstituted milk at temperatures above 70°C prior to homogenization increased the proportion of β-Lg absorbed on the surface layers of the fat globules, which led to a small increase in the protein load. However, whey proteins were adsorbed less on the surface layers of fat globules compared to those of milks heated after homogenization (Sharma, 1993; Sharma & Dalgleish, 1994). The composition of the surface layers of

fat globules directly influences the properties of recombined milk products, especially heat stability, creaming and acid gelation. The objective of this study is to investigate the effects of heat treatment either before or after homogenization on the surface layers of recombined milk fat globules.

4.2. Results and discussion

4.2.1. Untreated recombined milk

The fat and protein contents of recombined milks were approximately 4% and 3% respectively as shown in Table 4.1. The creams isolated from recombined milks contained about 58% fat and about 3% protein.

Table 4.1. *Some characteristics of recombined milk and cream washed with SMUF.*

Attribute	Recombined milks	Recombined milk cream washed with SMUF
Fat (%)	4.05 (0.28)	57.91 (1.30)
Protein (%)	3.14 (0.16)	3.20 (0.37)
Particle size ($d_{3,2}$, μm)		
Water	0.58 (0.01)	-
2% SDS + 50 m*M* EDTA	0.58 (0.01)	-
Specific surface area ($m^2 \cdot g^{-1}$)		
Water	10.52 (0.25)	-
SDS/EDTA	10.48 (0.17)	-
Protein load ($mg \cdot m^{-2}$)	-	5.10 (0.57)

Note. Standard deviations are in parentheses.

The specific surface area was found to be 10.5 $m^2 \cdot g^{-1}$, giving protein load value of ~5.1 $mg \cdot m^{-2}$. This value was consistent with that reported by Sharma et al. (1996a), i.e., ~5.7 $mg \cdot m^{-2}$, but differed from Oortwijn and Walstra (1979)'s findings (i.e., 8 – 10 $mg \cdot m^{-2}$). Oortwijn and Walstra (1979) used a 'depletion method' to estimate the protein load, whereas in this study the protein load in the cream layer was determined directly. In the depletion method, the difference between the amount of protein in the aqueous phase before and after homogenization was considered as the amount adsorbed. This depletion method presents several problems when used to

quantify the amount of protein adsorbed onto the surface layers of recombined milk fat globules (Hunt & Dalgleish, 1994; Sharma, et al., 1996a). Firstly, centrifugation of recombined milk at relatively high speed causes sedimentation of large casein micelles, which are difficult to re-disperse and hence the accurate measurement of protein in the aqueous phase is difficult. Secondly, the small fat globules are generally not separated by centrifugation and are therefore unavoidably included in the analysis of the aqueous phase. Finally, the amount of protein adsorbed on recombined milk fat globule is very small and thus there is very little difference between initial and final protein concentrations in the aqueous phase.

The SDS-PAGE patterns, under reducing conditions, of recombined milk and creams washed with SMUF or SMUF containing dissociating agents are shown in Figure 4.1. The surface layers of recombined fat globules (Figure 4.1B) contained higher proportions of caseins, but lower proportions of whey proteins than those of recombined milk (Figure 4.1A). The α_s (α_{s1} + α_{s2}) - and β-caseins were the major proteins adsorbed at the surfaces of fat globules, whereas κ-casein was present in smaller amounts and only faint bands of β-Lg and α-La were observed. When the cream was washed with SMUF containing dissociating agents, all the casein and whey protein bands were observed (Figure 4.1C), but in smaller amounts compared to those for cream washed with SMUF.

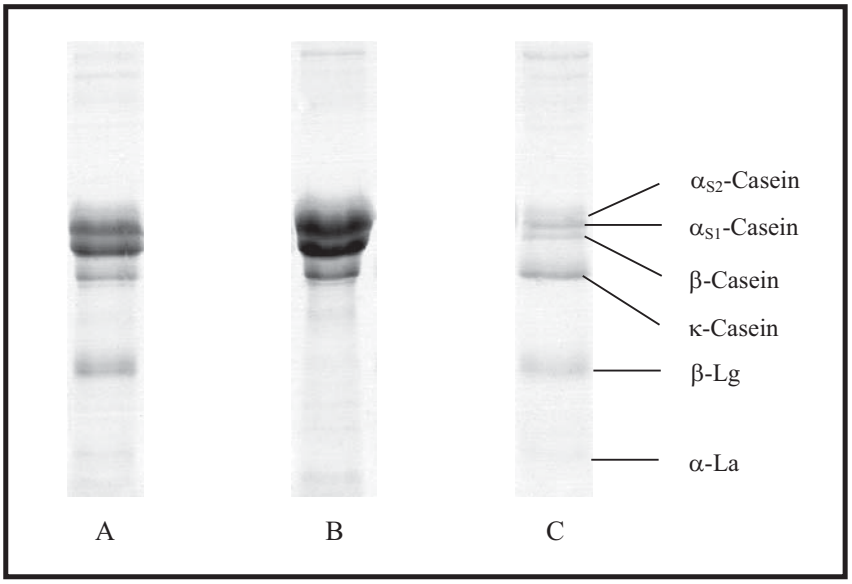


Figure 4.1 SDS-PAGE patterns under reducing conditions of recombined milk (A) and creams washed with SMUF (B) or SMUF containing dissociating agents (C). Sample dilution = 1:30.

The composition of individual proteins in recombined milk and creams washed with SMUF or SMUF containing dissociating agents are shown in Table 4.2. The surface layers of recombined milk fat globules consisted of caseins (~98%) and whey proteins (~2%). When the cream was washed with SMUF containing dissociating agents, the casein micelles adsorbed at the fat globule surface were dissociated and washed away. Only the protein molecules adsorbed directly at the interface of fat globules and the protein molecules bound to the interfacial protein layer via covalent bonds remain on the surface of the fat globules (Ye, Anema, et al., 2004). After dissociation, the surface layer of the recombined milk fat globule contained 83% caseins and 17% whey proteins. κ-Casein was the major protein; α_s- and β-caseins, β-Lg and α-La were also present, but in less proportions. Approximately 90% of α_s- and β-caseins dissociated from the surface layers of recombined milk fat globules when the cream was washed with SMUF containing dissociating agents, following by κ-casein and α-La (~ 60%) and β-Lg (~30%). These results indicate that κ-casein was the major casein adsorbed at the surface of the recombined milk fat globules. β-Lg was probably attached to κ-casein at the interface of recombined milk fat globules. These findings supported previous studies in recombined milks.

Table 4.2. *Individual proteins in recombined milk and creams washed with SMUF and SMUF containing dissociating agents.*

Proteins	Recombined milk		Washed Cream		
			SMUF		SMUF + Dissociating agents
	Amount[a] (mg)	(%)[b]	Amount[a] (mg)	(%)[b]	(%)[b]
α_s-Casein	1.07 (0.04)	34.00 (1.13)	2.03 (0.08)	39.76 (1.62)	26.62 (2.63)
β-Casein	1.24 (0.07)	39.39 (2.31)	2.22 (0.11)	43.57 (2.19)	33.36 (1.90)
κ-Casein	0.42 (0.01)	13.39 (0.40)	0.73 (0.06)	14.40 (1.09)	23.38 (1.40)
β-Lg	0.36 (0.02)	11.47 (0.75)	0.08 (0.05)	1.50 (0.99)	13.11 (0.88)
α-La	0.06 (0.06)	1.76 (1.85)	0.04 (0.01)	0.77 (0.22)	3.52 (0.27)
αs/β-Casein	0.86	-	0.91	-	0.80
Casein/Whey protein ratio	6.55	-	43.05	-	5.01

Note. Standard deviations are in parentheses. a = Amount of proteins calculated from the intensity of the protein band multiply by protein load. b = Percentage of individual protein calculated from total proteins bound to the fat globules

Sharma et al. (1996a) reported that when the surface layers of recombined milk fat globules were dispersed in EDTA, the extent of dissociation of caseins followed the order: β-caseins (77%) > α_S-caseins (58%) >> κ-casein (25%); whereas β-Lg and α-La did not appear to be affected. In this study, the ratio of caseins to whey proteins at the surface layers gave a mass fraction of whey proteins of ~0.02. As the protein load was ~5.1 $mg \cdot m^{-2}$, ~0.10 $mg \cdot m^{-2}$ of whey proteins must have been adsorbed. Oortwijn and Walstra (1979) reported that milk fat homogenized in an aqueous solution containing only whey proteins had a protein load of ~2%. Using the calculation of Oortwijn and Walstra (1979), the surface layers of recombined milk fat globules covered by whey proteins in this study would be 0.10/2 = 0.05. In other words, approximately 5% of the fat globule surface layers would be covered by whey proteins, while the rest would be covered by caseins. In a similar manner, Sharma et al. (1996a) also used Oortwijn and Walstra (1979)'s calculation and reported that the surface layers of recombined milk fat globules made from skim milk powder and AMF, the whey proteins occupied about 33% of the surface area. The proportion of whey proteins on the surface layers of recombined milk fat globules found in this study was different from the findings of Sharma et al. (1996a) probably due to the differences in skim milk types, the processing conditions, such as homogenization condition, as well as the analytical methods.

In this study, the caseins were preferentially adsorbed on the surface layers of fat globules compared to the whey proteins. This confirmed earlier observations on fresh homogenized milk (Darling & Butcher, 1978; McPherson, et al., 1984) and recombined milk (McCrae, et al., 1994; Oortwijn & Walstra, 1979; Sharma, et al., 1996a). The observed preferential adsorption of caseins over whey proteins was partly attributed to a considerable proportion of caseins adsorbed in the form of micelles, compared to whey proteins which were adsorbed in their molecular forms (Sharma, et al., 1996a). Walstra and Oortwijn (1982) proposed that the protein adsorption during homogenization occurred via convection, i.e., the casein micelles move faster under turbulent conditions than the whey proteins due to their larger size. As a consequence large protein particles were adsorbed in preference to small ones. In addition, whey proteins were incapable of displacing casein from the fat-water interface (Dalgleish & Banks, 1991), whereas, caseins are most likely to displace serum proteins (Walstra & Oortwijn, 1982). However, Sharma et al. (1996a) suggested that the adsorption mechanisms of the fat globule surface layer were more complex than those predicted by Walstra and Oortwijn

(1982) based on the casein micelles differing in size. Such adsorption may involve rearrangement of casein components within micelles and changes in micelles size distribution. In this study, β-casein was adsorbed slightly in preference to α_s-casein (Table 4.2).

Based on the above information, the structure of fat globules in recombined milk could be represented as shown in Figure 4.2. When reconstituted milk was recombined with milk fat, the caseins and whey proteins were adsorbed onto the surface layers of fat globules during homogenization, and the caseins were adsorbed in preference to the whey proteins. Some of caseins were primarily present in form of intact casein micelle and some as casein micelle fragments and the whey proteins were probably present in denatured forms, due to unfolding at the surface layer of fat globules.

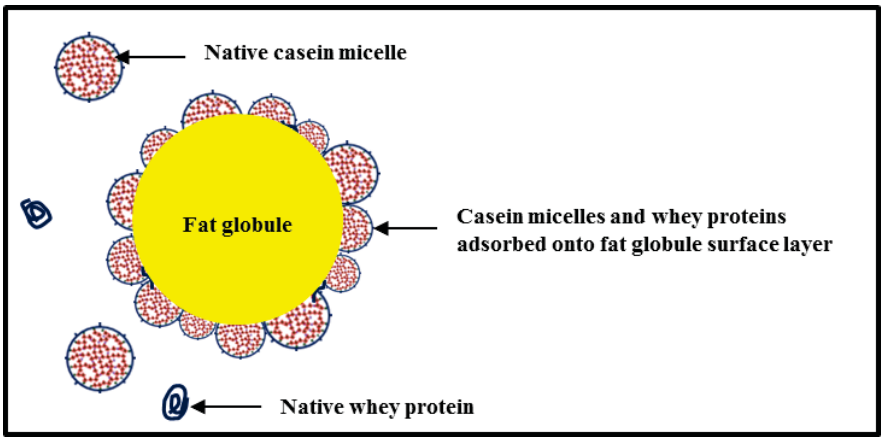


Figure 4.2. Schematic illustration of the state of protein and fat globules in recombined milk. The relative sizes of individual components are not to scale.

4.2.2. Heat-treated recombined milk

4.2.2.1. Whey protein denaturation

Figure 4.3 shows the native-PAGE patterns of recombined milks heated either before or after homogenization at different temperature-time profiles. The percentage of whey protein denaturation in heated recombined milks is shown in Appendix A.1. The relationships between denaturation levels of whey proteins and holding time and heating temperature are shown in Figures 4.4 and 4.5 respectively.

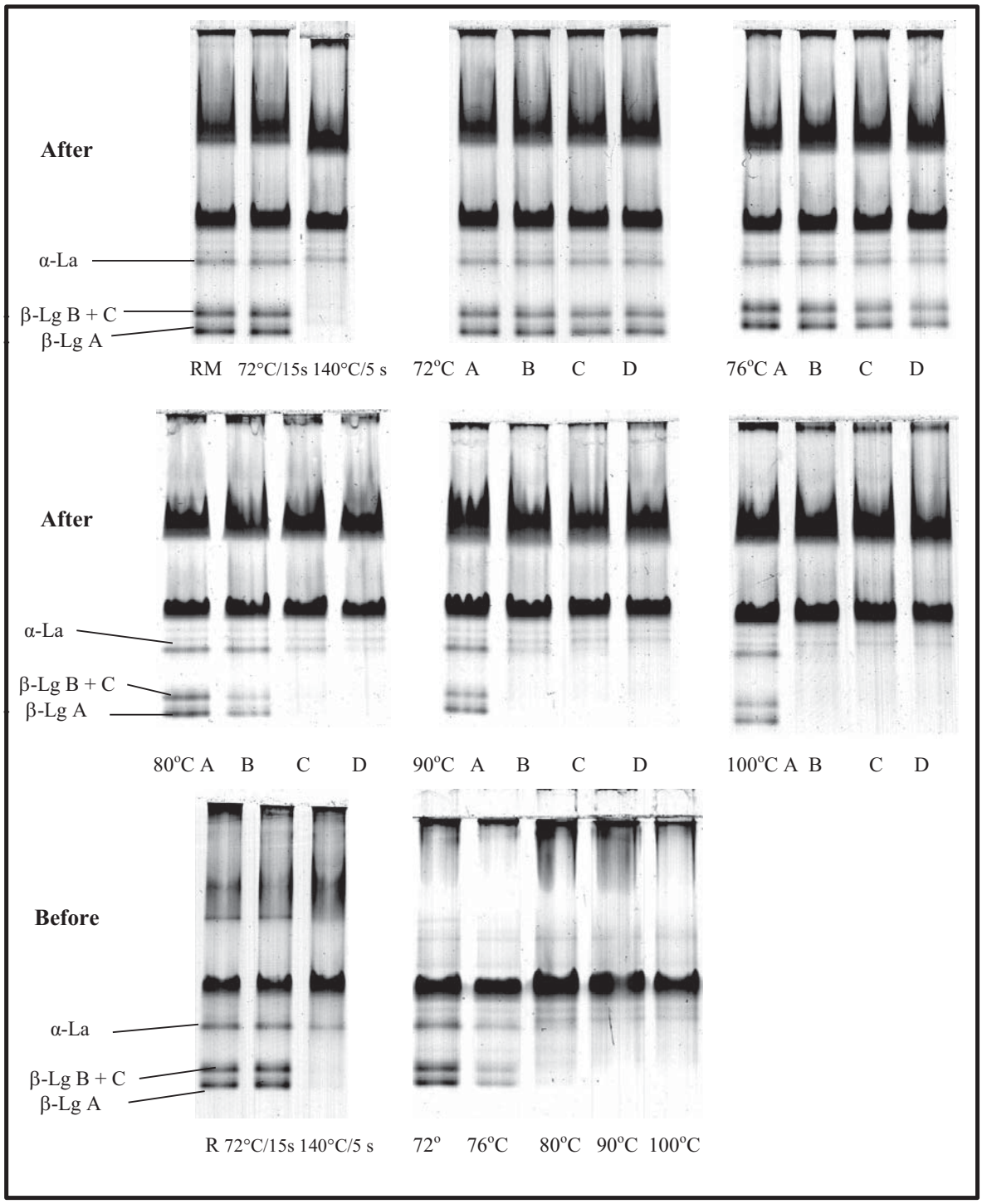


Figure 4.3. Native-PAGE patterns of recombined milks without heat treatment (RM) and with heat treatments after homogenization at 72°C for 15 s, 140°C for 5 s, and 72 - 100°C for 0 (A), 10 (B), 20 (C) and 30 (D) min. Heat treatments were also applied to milks before homogenization at 72°C for 15 s, 140°C for 5 s, and at 72 - 100°C for 30 min.

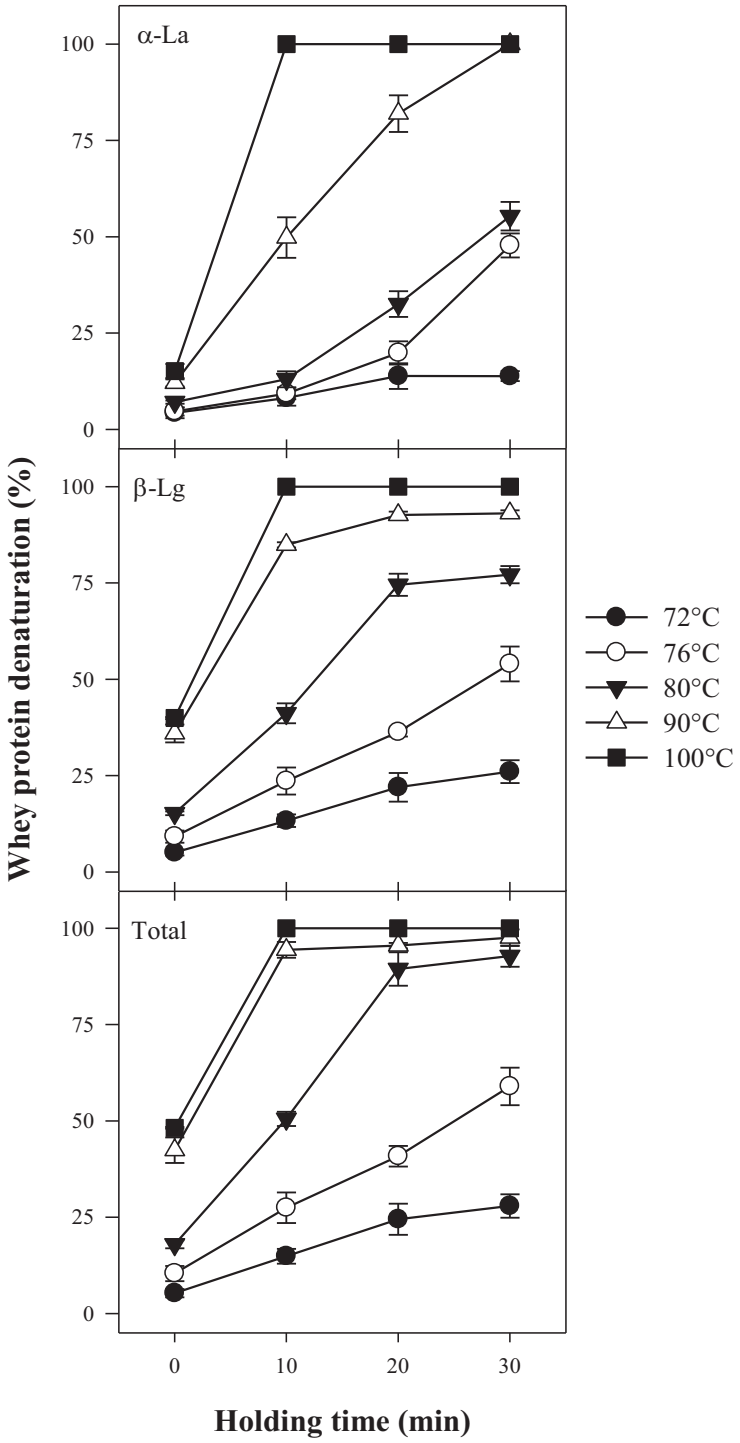


Figure 4.4. Denaturation of whey proteins as a function of holding time at different temperatures. Recombined milks were heated after homogenization.

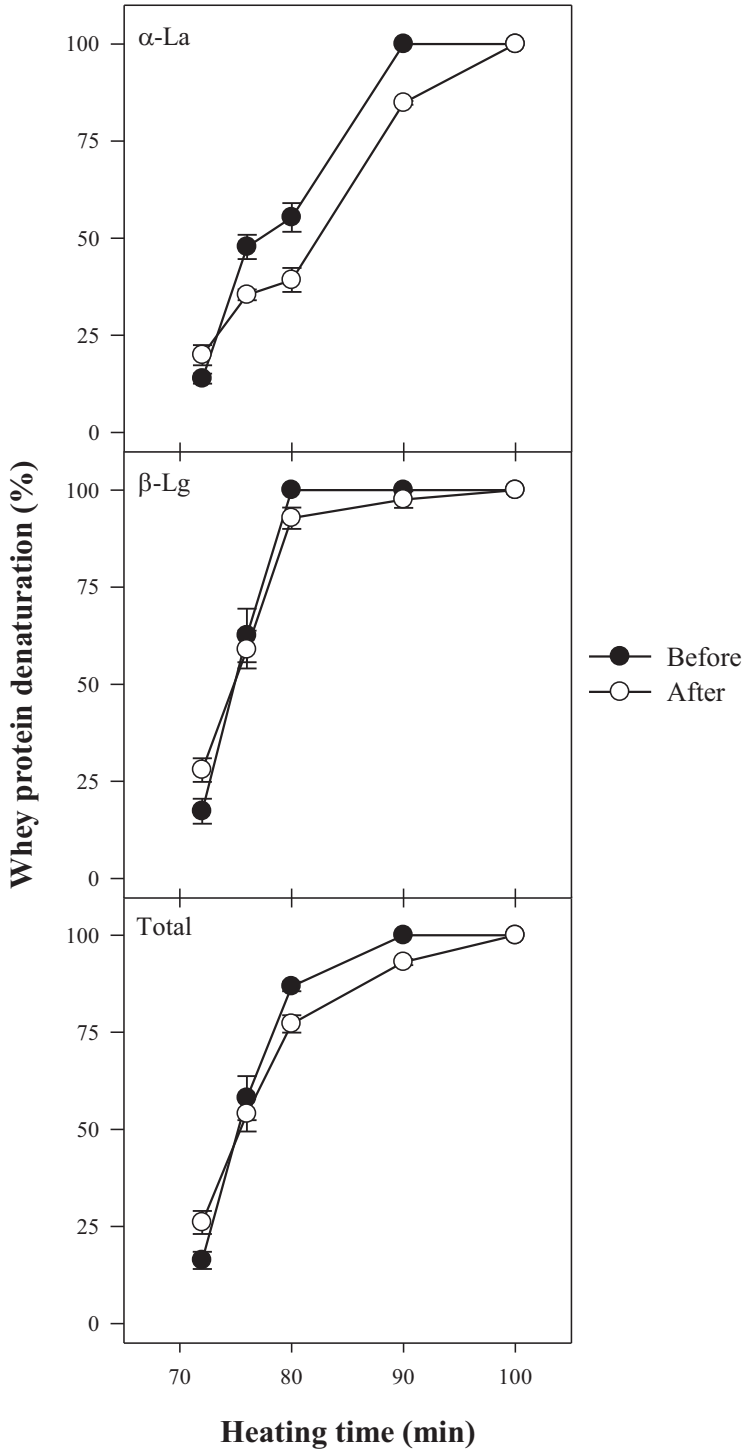


Figure 4.5 Denaturation of whey protein as a function of heating temperature for 30 min holding time either before or after homogenization.

Low heat treatment at the pasteurization level, i.e., 72°C for 15 s, resulted in low level of α-La and β-Lg denaturation, i.e., less than 5%. Heat treatment at ultra-high temperature (UHT) level, (i.e., 140°C for 5 s) denatured ~30% α-La and ~100% β-Lg. In the range 72 - 100°C, the extent of whey protein denaturation increased as the heating temperature and holding time increased. Heat treatment at 100°C for ≥ 10 min denatured all the α-La. High heat treatment at 80°C for ≥ 20 min and 90 - 100°C for ≥ 10 min resulted in denaturation of nearly all β-Lg. Recombined milks heated either before or after homogenization had similar levels of whey proteins denaturation (Figure 4.5); these results were expected as the denaturation depends on the concentration of native whey proteins in milks prior to heat treatment. These findings generally supported previous studies on skim milk. Anema, Stockmann, et al. (2005) reported that heat treatment of skim milk at 80°C for 2 min denatured about 20% of α-La and 35% of β-Lg, whereas heat treatment at 90°C for 15 min denatured most of β-Lg. The denaturation levels of α-La and β-Lg in heated skim milk increased as heating temperature increased, leading to almost complete denaturation for α-La and complete denaturation for β-Lg at 100°C (Anema, 2008b).

4.2.2.2. Fat globule diameter (d_{32}) and protein load

The fat globule diameter (d_{32}) and proteins load of heated recombined milk are shown in Appendix A.2. The d_{32} values of heated recombined milk fat globules dispersed in water and dissociating agents were approximately 0.55 and 0.54 μm respectively and the specific surface area were 10.80 and 11.04 $m^2 \cdot g^{-1}$ respectively. These values did not significantly change with the intensity of heat treatment applied to recombined milks.

The relationship between protein load and holding time is shown in Figure 4.6. The protein load slightly increase holding time up to 10 min. Extend holding time up to 30 min at did not always increase the protein load on the surface layer of fat globules. The surface layers of fat globules isolated from recombined milk heat at 72°C/15 s or 140°C/5 s had lower protein load than those of recombined milks heated at 72 - 100°C for 0 min. This was due to the adsorbtion of denatured whey protein that occurred during cooling time; i.e., it took approximately 2 min to cool heated milk samples to 20°C in ice bath. Whereas when the UHT plant was used for heating milks at 72°C/15 s or 140°C/5 s, the samples were immediately cooled to 20°C within 2 - 3 s.

Heated recombined milk fat globules had an average protein load of 7.78 ± 1.35 mg·m^{-2}, which was significantly higher than that of untreated recombined milk (~5.10 mg·m^{-2}). The increase in protein load was attributed to the association of denatured whey proteins onto the surface layers of the heated recombined milk fat globules which consist mainly of caseins.

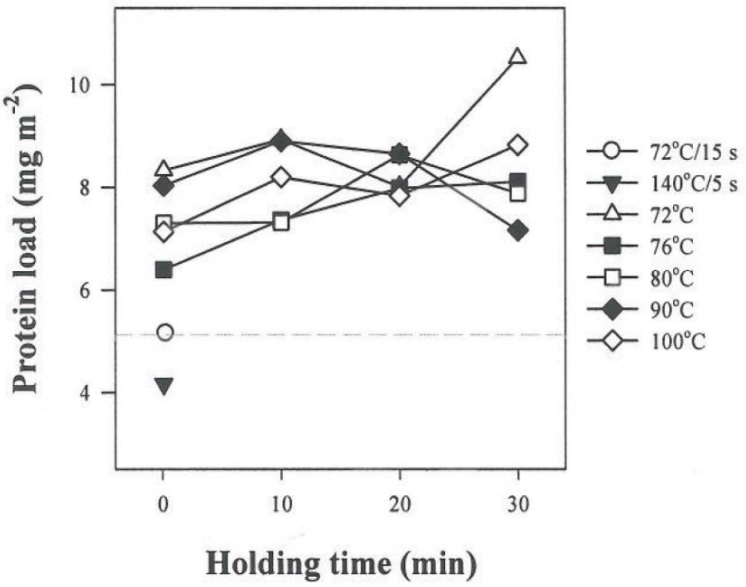


Figure 4.6. Protein load (mg·m^{-2}) on fat globules of recombined milks heated after homogenization.

4.2.2.3. Composition of fat globule surface layers in heated recombined milk

The SDS-PAGE patterns under reducing conditions of surface materials isolated from fat globules of recombined milks heated in the range 72 - 100°C for 0 - 30 min are shown in Figures 4.7 and 4.8. The PAGE patterns of creams washed using SMUF (Figure 4.7) showed that pasteurization at 72°C for 15 s did not significantly change the protein composition of fat globule surface, but ultra-high temperature treatment, i.e., UHT at 140°C for 5 s, showed higher amounts of whey proteins compared to those of untreated samples (Figure 4.7). Increase in heating time from 0 to 30 min at 72 to 100°C did not apparently change casein bands, but increases in the intensity of β-Lg band were observed

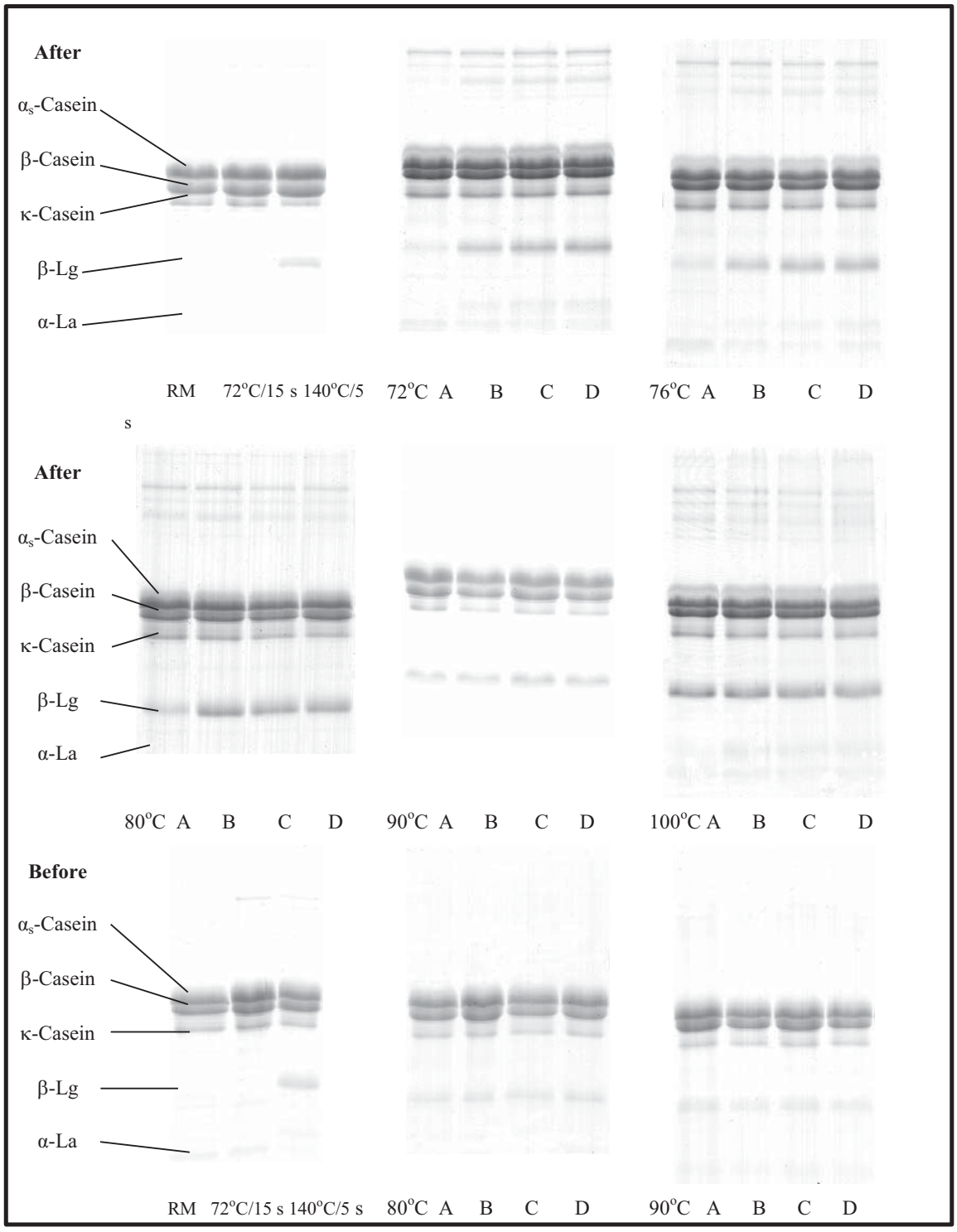


Figure 4.7. SDS-PAGE patterns under reducing conditions of membrane materials isolated from recombined milks: untreated (RM); heated either before or after homogenization at 72°C for 15 s, 140°C for 5 s, and 72 - 100°C for 0 (A), 10 (B), 20 (C) and 30 min (D). Creams were washed with SMUF.

SDS-PAGE gels of cream isolated from recombined milks heated at 80 and 90° C before or after homogenization and dispersed in SMUF containing dissociating agents are shown in Figure 4.8. In the samples heated after homogenization the casein bands, except κ-casein, became very faint but the β-Lg band was still prominent. The samples heated before homogenization showed more intense casein and whey protein bands. This suggests that the adsorbed casein material was less susceptible to dissociation in the EDTA/urea buffer in samples heated before homogenization.

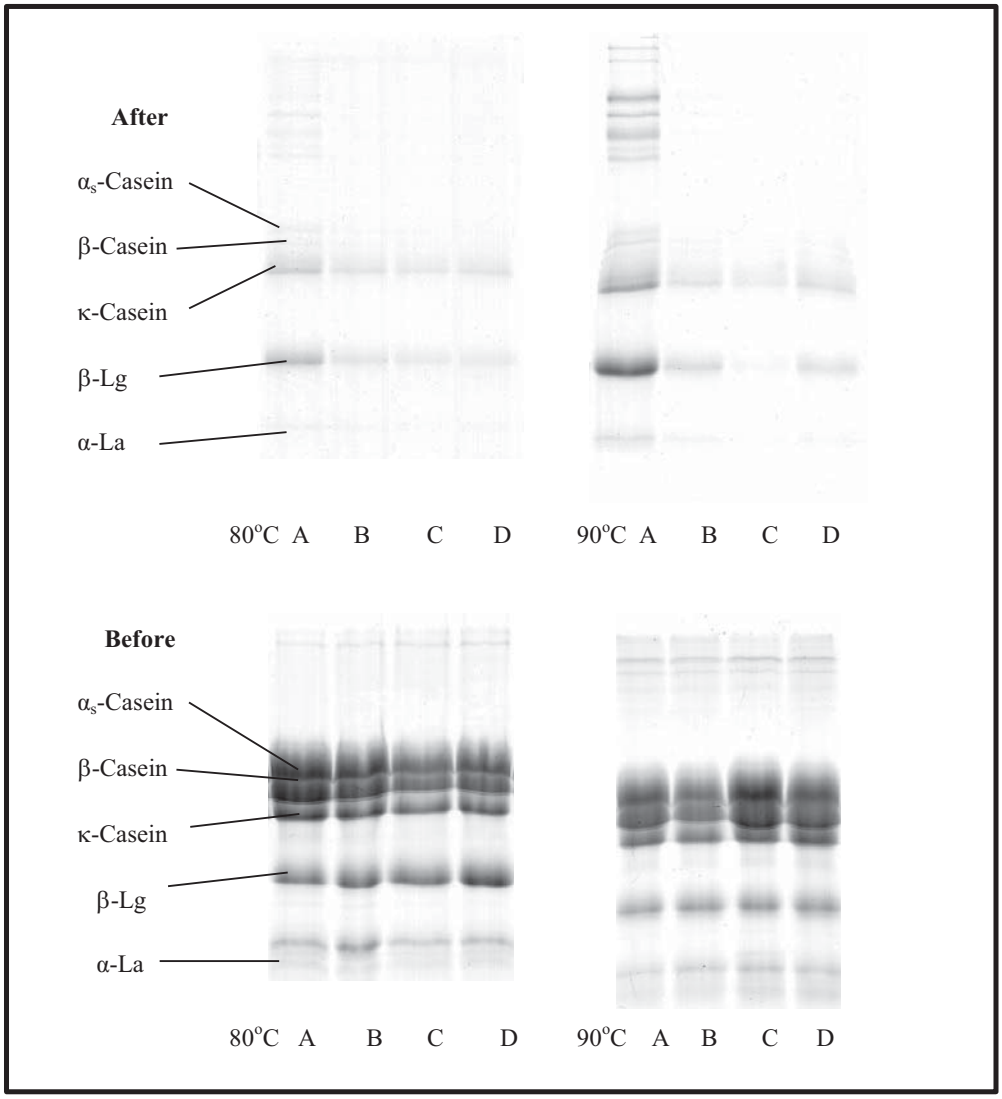


Figure 4.8. SDS-PAGE patterns under reducing condition of membrane materials isolated from recombined milks heated either before or after homogenization at 80 and 90°C for 0 (A), 10 (B), 20 (C) and 30 min (D). Creams were washed with SMUF containing dissociating agents.

Quantitative analysis of PAGE gels (Figure 4. 9) showed that the amounts of caseins at the globule surface ($mg \cdot m^{-2}$) in heated samples varied between 6.5 to 7.5 $mg \cdot m^{-2}$. However, the casein loads of heated samples were significantly higher than that of unheated sample (~5.1 $mg \cdot m^{-2}$). The amount of β-Lg increased significantly with increased heating temperature and time; the amount of β-Lg at the surface appeared to reach the maximum value of about 1.2 $mg \cdot m^{-2}$.

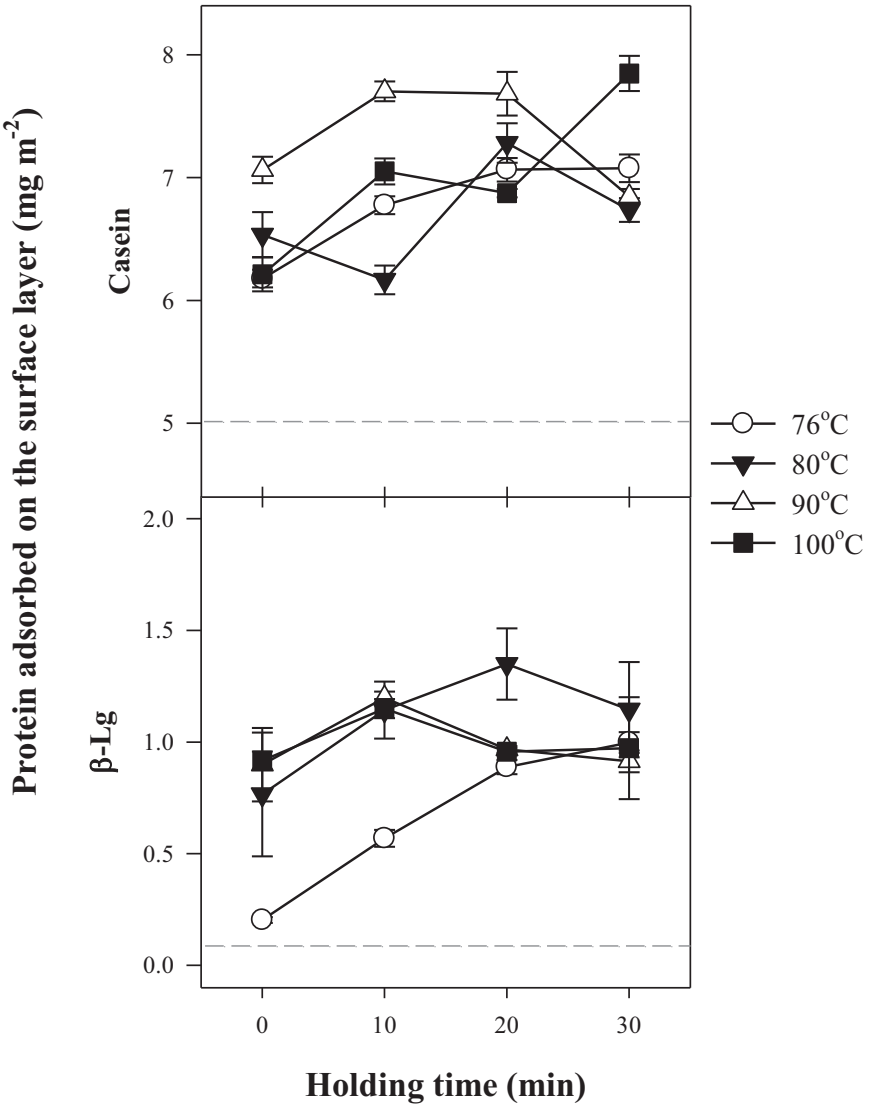


Figure 4.9. Changes in the amounts of caseins and whey proteins on the surface layers of fat globules isolated from recombined milks heated at 76 - 100°C for 0 - 30 min after homogenization. Dash lines represent those of untreated recombined milk.

These findings are in agreement with the studies on whole milks. Ye, Singh, Taylor, et al. (2004a) reported that on heating whole milk at temperatures lower than 80°C, the amount of β-Lg associated with the MFGM increased with increasing heating time and temperature. At temperatures greater than 80°C, the amount of β-Lg associated with the MFGM increased rapidly to a maximum value after which the level remained constant. The maximum amount of β-Lg associated with the MFGM after heat treatment of whole milk was ~1 $mg \cdot g^{-1}$ fat, which was about 10 - 15% of the total MFGM protein (6.5 - 8.5 $mg \cdot g^{-1}$ fat) and only ~1% of total β-Lg in milk (0.4% β-Lg in 4% fat whole milk) on weight per weight basis. Corredig and Dalgleish (1996b) and Sharma and Dalgleish (1993) also reported that the maximum amount of β-Lg associated with the MFGM of heated milk was ~0.7 $mg \cdot g^{-1}$ fat.

To investigate the effects of applied heat treatments before or after homogenization on the composition of caseins and whey proteins on the surface layers of fat globules, heat treatments at 72°C for 15 s, 140°C for 5 s and 72 - 100°C for 30 min and heat treatment at 80 - 90°C for 0 - 30 min were applied to milk samples either before or after homogenization (Appendix A3). Quantitative PAGE results for milks heated at 72 - 100°C for 30 min or heat treatment at 80 - 90°C for 0 - 30 min are shown in Figures 4.10 and 4.11.

In general, under all conditions used it was found that the amounts of caseins and β-Lg on the surface layers were higher, when recombined milks were heated after homogenization as compared to heat treatment of skim milk before recombining with milk fat and homogenisation. The trend was more obvious for β-Lg. When homogenized recombined milk was heated, the denatured whey proteins interacted with the fat globules, and with casein micelles. Since the fat globule surfaces in recombined milk are covered by disrupted casein micelles, it is expected that β-Lg and α-La will bind to them during heating. In addition, casein micelles with attached whey proteins in the serum could also bind to the fat globule surface layers, resulting in increase in both caseins and whey proteins at the surface.

When heat treatment was applied to milks before homogenization, the denatured whey proteins interacted with casein micelles, and then the casein micelles with the attached whey proteins were adsorbed to the fat globule surfaces during homogenization. In

addition, some denatured/aggregated whey proteins could adsorb directly at the fat globule surface. The differences in the amounts of caseins and whey proteins in the recombined systems heated before or after homogenization could be due to differences in the reactivity of whey proteins with the globule surfaces. Whey protein appeared to be more easily adsorbed when heat treatment was performed after the homogenization step, perhaps due to greater spreading of casein micelles at the surface, providing more sites for interactions.

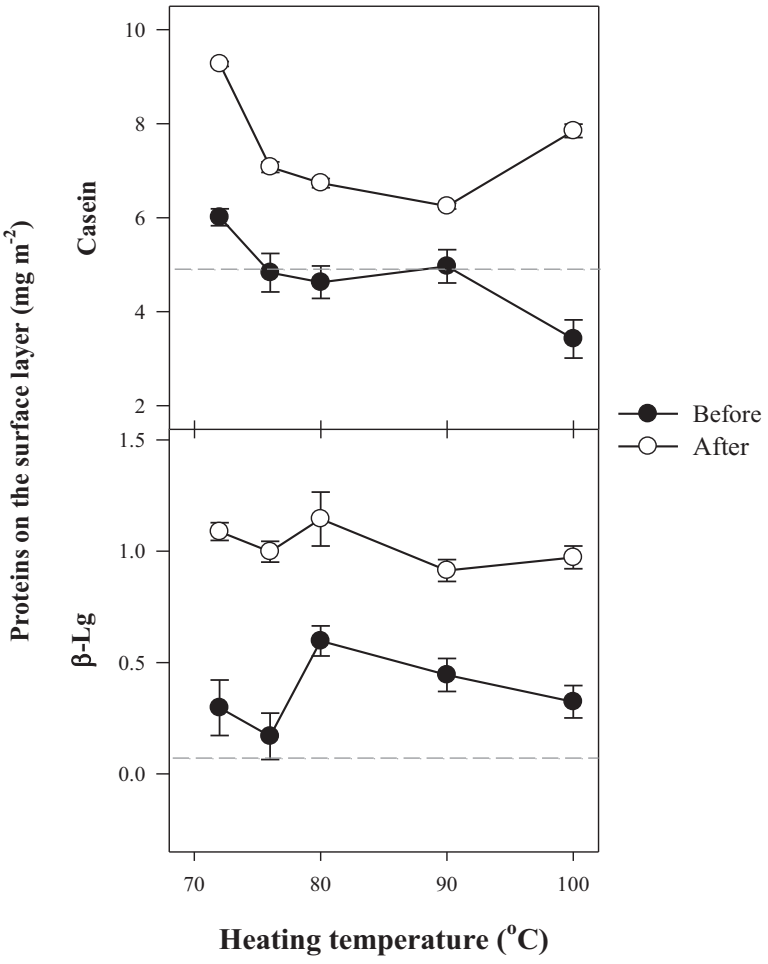


Figure 4.10. Changes in the amount of caseins and whey proteins on the surface layers of fat globules isolated from recombined milk heated at 72-100°C for 30 min either before or after homogenization. Dash lines represent those of untreated recombined milk.

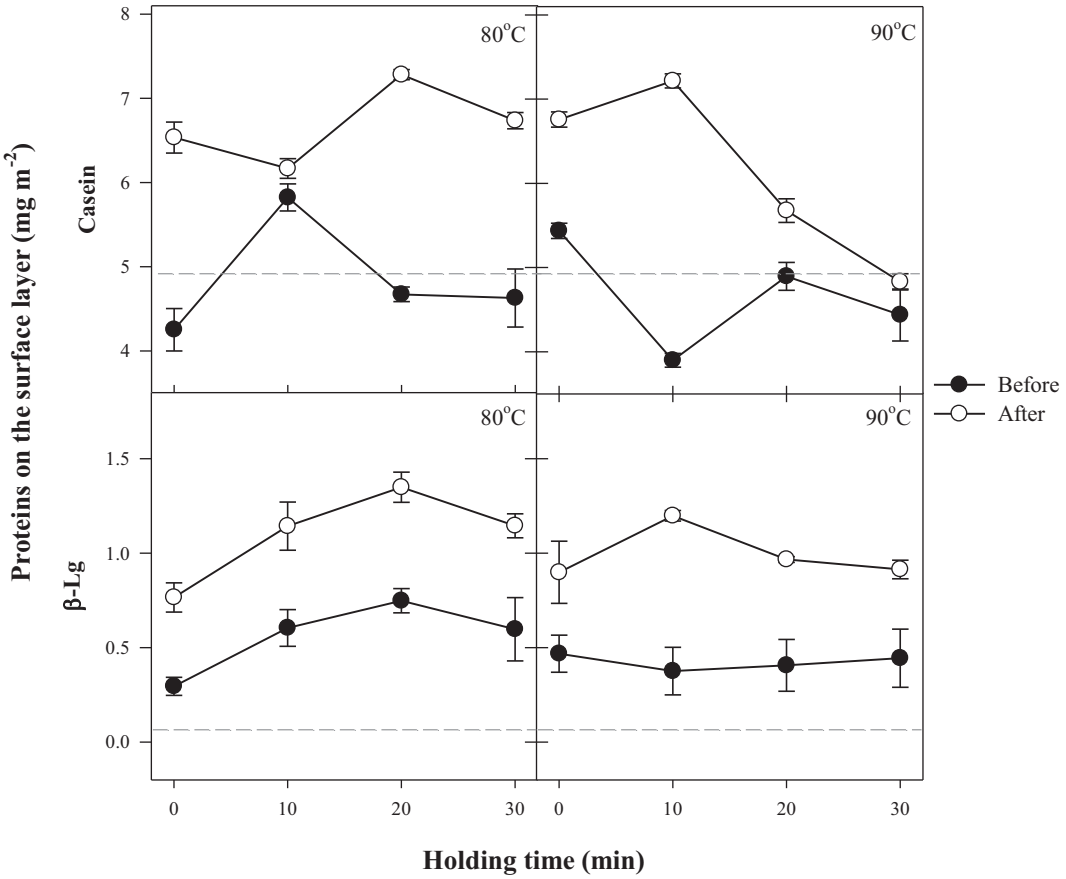


Figure 4.11. Changes in caseins and whey proteins on the surface layers of fat globules isolated from recombined milk heated at 80 - 90°C for 0-30 min either before or after homogenization. Dash lines represent those of untreated recombined milk.

4.2.2.4. Possible structures of proteins and fat globules in heated recombined milk

(1) Reconstituted skim milk heated before recombining/homogenization

Based on the studies on skim milk systems, when reconstituted milk was heated at temperatures ≥ 70°C, the casein micelles did not significantly change in size and internal structure, even though some caseins, especially κ-casein, dissociate from the casein micelles (Anema, 2007; Considine, et al., 2007; Singh & Fox, 1985). However, at those temperatures, the whey proteins denatured (as shown in Figures 4.3 and 4.4) and interacted with κ-caseins (present at the surface of the casein micelles or in the

serum) or interacted with each other, resulting in denatured whey protein aggregates (Anema, 2007; Considine, et al., 2007; Donato & Guyomarc'h, 2009; Vasbinder & de Kruif, 2003). The effects of heat treatment depend on heating conditions (i.e., temperature and holding time) and milk pH. Anema (2010b) observed the casein micelles with a range sizes (average ~150 nm) in skim milk heated at 90°C for 30 min as shown in Figure 4.12.

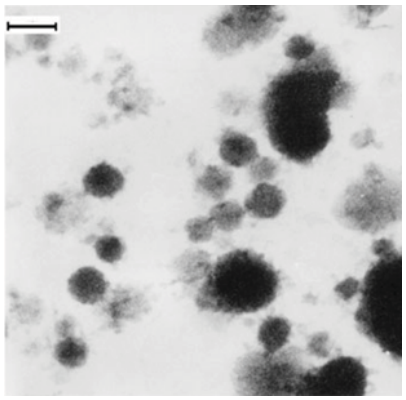


Figure 4.12. Transmission electron micrograph images of skim milk heated at 90°C for 30 min. The scale bar on the figure represents 100 nm, from Anema (2010b).

From the above information, the caseins and whey proteins present in heated reconstituted milk are illustrated in Figure 4.13. When heated reconstituted milk was recombined with milk fat, the casein micelles (with associated denatured whey proteins) and denatured whey proteins are adsorbed onto the surface layers of recombined milk fat globules during homogenization. At this processing procedure the fat globules were disrupted into smaller particles, as a result the casein micelles, with attached whey proteins, and denatured/aggregated whey proteins were adsorbed onto the newly formed fat globules. The proportions of caseins and whey proteins adsorbed onto the surface layer of fat globules were dependent on heating temperature and holding time. In general, the amount of caseins adsorbed onto the surface layer was somewhat variable, but clearly the amount of whey proteins adsorbed onto the surface layer increased as the severity of heat treatment increased. The possible surface layer of recombined milk heated before homogenization is shown in Figure 4.14.

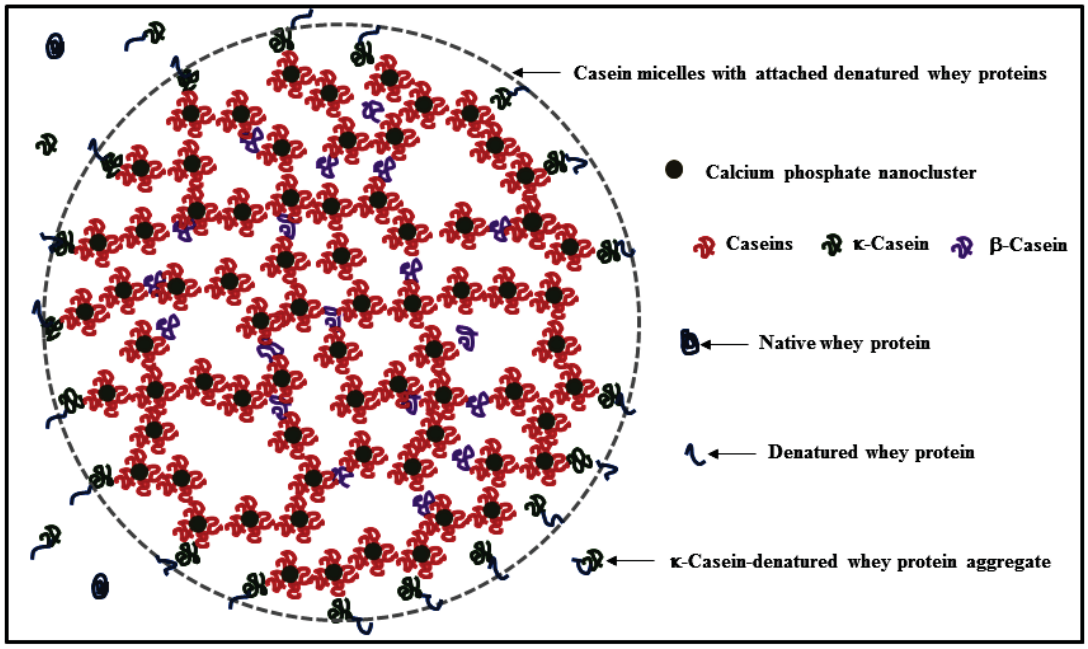


Figure 4.13. Schematic illustration of casein micelles and whey proteins in heated reconstituted milk. The relative sizes of individual components are not to scale.

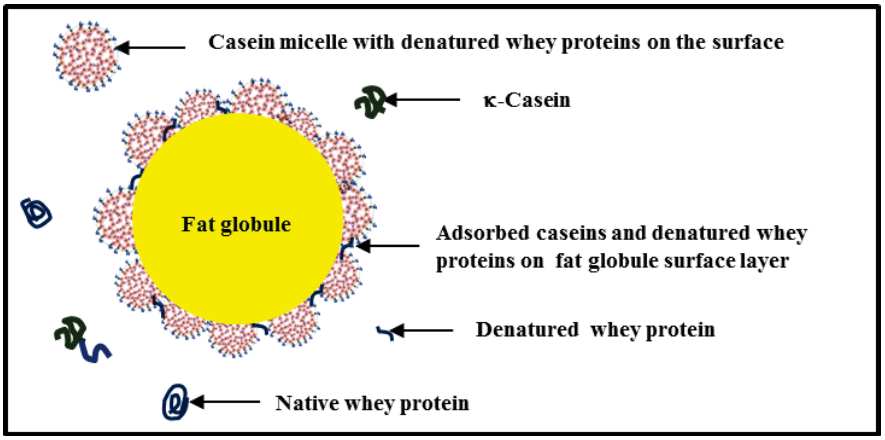


Figure 4.14. Schematic illustration of caseins, whey proteins and fat globules of recombined milk heated before homogenization. The relative sizes of individual components are not to scale.

(2) Recombined milk heated after homogenization

Different from the recombined milk heated before homogenization, in recombined milk heated after homogenization, the surface layers of fat globules were already formed before heat treatment was applied. In this system the surface layer was consisted

mainly of caseins (~98%) and small amount of whey proteins (~2%) as discussed in Section 4.2.2.3. Therefore, during heat treatment the denatured whey proteins induced by heat treatment can interact with the proteins on the surface layers of fat globules similar to what happens when skim milk is heated. During heat treatment denatured whey proteins would also interact with the casein micelles in the serum. Some of these casein micelles with attached whey proteins could interact with denatured whey proteins or casein micelles at the fat globule surface layer. As a result, the composition of surface layers of recombined milk fat globules changes significantly after heat treatments. The amount of denatured whey proteins adsorbed onto the surface layers depends on heating conditions, i.e., heating temperature and time. The caseins, whey proteins and fat globules of heated recombined milk are illustrated in Figure 4.15. This study found that the amounts of caseins and whey proteins adsorbed at the interface of fat globules were higher when heat treatment was applied to recombined milks after homogenization compared to when heating was applied before homogenization.

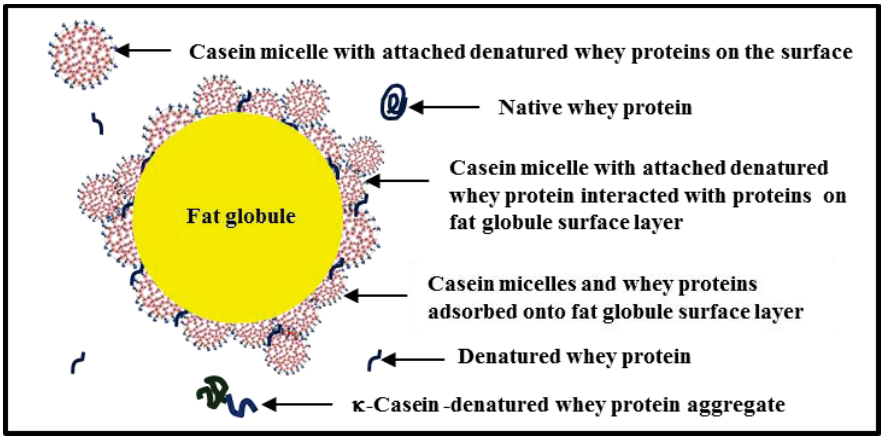


Figure 4.15. Schematic illustration of caseins, whey proteins and fat globules of recombined milk heated after homogenization

4.3. Conclusions

The surface layers of recombined milk fat globule are composed of caseins (98%) and whey proteins (2%). This study found that the κ-casein and β-Lg were the most prominent proteins at the surface of fat globules and are responsible for the interactions with other proteins. Heat treatment applied to recombined milks either before or after homogenization changes the proportion of caseins and whey proteins on the surface

layers of the recombined milk fat globules and the effects depend on heating temperature, holding time as well as the placement of heat treatment. It would be worth investigating further the contribution factors that resulted in higher amount of caseins and whey proteins adsorbed at the interface of fat globules of recombined milks heated after homogenization compared with those of recombined milk heated before homogenization. These findings indicate that the placement of heat treatment (whether it was performed before or after homogenization) has significant effect on the proteins adsorbed at the interface of fat globule, which need to be considered when designing new processes for recombined dairy products.

CHAPTER 5

ACID-GELATION OF HEAT-TREATED RECOMBINED MILKS

5.1. Introduction

Heat treatment of milk above the denaturation temperature of whey proteins (~70°C) prior to acidification has significant effects on the acid gelation properties of milk. Heat treatment decreases the gelation time, but increases gelation pH and gel strength (Anema, Lauber, et al., 2005; Bikker, Anema, Li, & Hill, 2000; Cho, et al., 1999; Lucey, Munro, & Singh, 1998). These effects have been attributed to the denaturation of whey proteins and their interactions with casein proteins.

Fat globules also have significant effects on the acid gelation properties of milk. The effects depend on whether the surface layers contain interactive or non-interactive materials. Non-interactive materials, such as native whey proteins, do not interact with the casein matrix. In contrast, interactive materials, such as caseins and denatured whey proteins, interact positively with the protein network and integrate into the casein matrix (Anema & Li, 2003a; Cho, et al., 1999; de Wit & Klarenbeek, 1984; Tamime, et al., 1995; van Vliet, 1988; van Vliet & Dentener-Kikkert, 1982).

Limited studies have been carried out on acid-induced gelation of recombined milk systems. Cho et al. (1999) investigated the effects of fat globules, stabilized with different surface layer materials, on acid gelation of recombined milks. They reported that acid gels containing fat globules stabilized by non-interacting materials, such as unheated whey protein concentrate (WPC) had lower gelation pH and final G' values, but longer gelation times compared to those stabilized by interacting materials, i.e., skim milk powder and heated whey protein concentrate.

This study is designed to investigate the effects of heat treatment carried out either before or after homogenization on acid gelation properties of recombined milk, which has never been done before. Major emphasis is placed on the how modifications of fat globule surface layers by heating influences acid gelation properties.

5.2. Results and discussion

5.2.1. Gelation pH and gelation time

The acid gelation properties of heated recombined milk are shown in Table 5.1. The initial pH of the recombined milk sample was approximately 6.67. After acidification with 1.9% GDL at 30°C for 6 h, the pH dropped to about 4.04 (Figure 5.1). In this discussion, the gelation pH and gelation time refer to those at which G' became ≥ 1 Pa, and the final G' value refers to that measured after 6 h of acidification. In untreated recombined milk, the gelation occurred at ~pH 4.73, which is in the range of that reported for skim milk (Oh, Wong, Pinder, Hemar, & Anema, 2007) and recombined milk (Cho, et al., 1999). In untreated milk, as the pH approached the isoelectric point of the caseins (~pH 4.6), the solubility of caseins diminished and these proteins aggregated to form a weak gel. In this system, the native whey proteins remained soluble in the serum, and did not interact with casein particles.

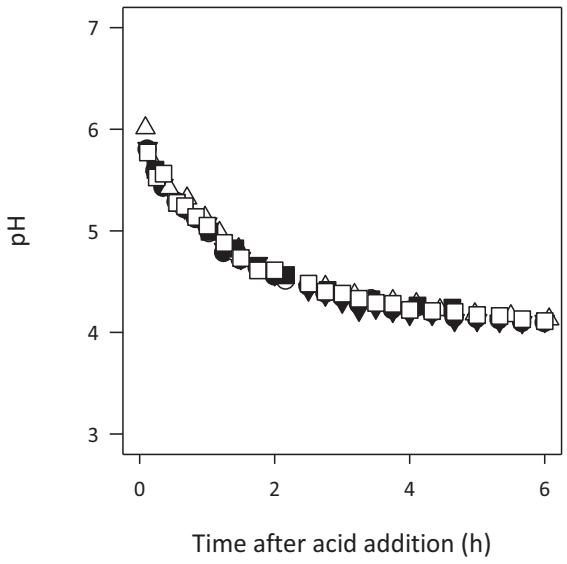


Figure 5.1. Changes in pH with time after GDL addition of recombined milks: (●) untreated; heated before homogenization at (○) 72°C for 15 s and (▼) 140°C for 5 s, or heated after homogenization for 30 min at (△) 76°C, (■) 90°C and (□) 100°C.

Table 0.1 *The acid gelation properties of heated recombined milks*

Milk	Gelation		*G'* (Pa)		Yield	
treatment	pH	Time (h)	30°C	5°C	Strain (%)	Stress (Pa)
Unheated	4.73 (0.00)	1.45 (0.00)	14.20[a] (0.20)	56.07[a] (2.18)	81.80[f] (0.00)	107.67[a,b] (4.73)
Heated before homogenization						
72°C/15 s	4.84[a] (0.02)	1.15[e] (0.03)	23.55[a] (1.45)	111.50 (6.50)	65.15 (4.65)	107.00[a,b] (10.00)
140°C/5 s	4.89[b] (0.01)	1.03[c] (0.02)	117.00[b] (2.00)	418.00[b] (3.00)	44.80[d,e] (0.00)	174.00[e,f,g] (4.00)
72°C/30 min	4.86[a,b] (0.01)	1.10[d,e] (0.02)	62.95 (0.75)	242.00 (1.00)	54.20[e] (2.50)	148.00[c,d,e] (2.00)
76°C/30 min	5.01 (0.00)	0.82 (0.00)	197.67 (2.08)	668.00[c] (9.00)	38.20[c,d](0.00)	204.67[g] (2.89)
80°C/30 min	5.09[c] (0.00)	0.68[b] (0.00)	234.67[c] (4.93)	721.00[d] (12.12)	21.17[a,b] (3.21)	125.00[b,c,d] (19.70)
90°C/30 min	5.14[d] (0.00)	0.62[a,b] (0.00)	238.67[c] (4.62)	685.33[c,d] (22.59)	15.67[a] (2.35)	85.50[a] (15.28)
100°C/30 min	5.12[c,d] (0.00)	0.65[a,b] (0.00)	243.50[c] (0.50)	707.00[d] (1.00)	28.30[b,c] (0.00)	149.50[c,d,e] (1.50)
Heated after homogenization						
72°C/15 s	4.78 (0.02)	1.31 (0.06)	17.13[a] (0.51)	73.60[a] (2.54)	83.50[f] (0.00)	108.33[a,b] (2.08)
140°C/5 s	4.89[b] (0.01)	1.04[c,d] (0.02)	106.67[b] (4.51)	394.33[b] (14.05)	35.97[c,d] (2.83)	141.00[b,c,d,e] (15.39)
72°C/30 min	4.86[a] (0.00)	1.12[e] (0.00)	42.87 (0.47)	195.00 (7.00)	51.70[e] (0.00)	116.67[a,b,c] (3.79)
76°C/30 min	4.94 (0.00)	0.95 (0.00)	138.00 (3.61)	505.00 (9.85)	37.57[c,d] (5.00)	187.67[f,g] (16.17)
80°C/30 min	5.12[c,d] (0.00)	0.65[a,b] (0.00)	288.00[d] (9.64)	960.33[e] (25.01)	38.23[c,d] (9.33)	249.33 (17.24)
90°C/30 min	5.14[d] (0.03)	0.62[a] (0.04)	325.33[d] (4.62)	950.67[e] (18.50)	17.03[a] (3.37)	132.00[b,c,d] (21.93)
100°C/30 min	5.12[c, d] (0.00)	0.65[a,b] (0.00)	300.50[d] (6.50)	929.00[e] (16.00)	20.60[a,b] (1.40)	158.50[d,e,f] (1.50)

Note. Standard deviations are in parentheses. [a] Samples with the same superscript letters within the same column are not significantly ($P \leq 0.05$) different from each other.

When recombined milks were heated either before or after homogenization at temperatures in the range of 72 - 80°C for 30 min, the gelation pH increased and gelation time decreased with increased heating temperature (Table 5.1). Further increase in heating temperature to 100°C after homogenization resulted in no statistical change in gelation pH and gelation time. Heat treatment applied either before or after homogenization did not cause a significant difference in gelation pH and gelation time of the recombined milks. These findings support previous studies on skim milk (Anema, Lauber, et al., 2005; Anema, Lowe, & Lee, 2004; Bikker, et al., 2000; Lucey, Tamehana, et al., 1998b; Oh, et al., 2007; Vasbinder, 2002), whole milk (Lucey, Teo, et al., 1997) and recombined milk (Cho, et al., 1999; Lucey, Munro, et al., 1998). Oh et al. (2007) reported that the gelation occurred at pH 5.23 for skim milk heated at 80°C for 30 min. Vasbinder (2003) found that heat treatment of skim milk at 80°C for 10 min or at higher temperature had higher gelation pH values compared to unheated skim milk. Cho et al. (1999) reported that the gelation pH of acid gel made from recombined milk heated at 80°C for 30 min before homogenization was 5.07.

When recombined milks were heated at temperatures above 70°C, the whey proteins denatured and interacted either with κ-caseins on the surface of casein micelles or interacted with each other forming whey protein aggregates (Corredig & Dalgleish, 1996a; Mulvihill & Donovan, 1987; Singh, 1995). The interactions between milk proteins induced by heat treatment involve non-covalent interactions and disulfide bonds (Lowe, et al., 2004). Denatured whey proteins cause casein micelles to aggregate at higher pH due to the higher isoelectric pH (~ 5.2) of the main whey proteins (β-Lg) than those of caseins. (Guyomarc'h, Queguiner, Law, Horne, & Dalgleish, 2003; Lucey, Teo, et al., 1997). In addition, the denatured whey proteins associated with the micelles alter the hydrophobic interactions between heated micelles, facilitating the gelation at higher pH values (Jean, Renan, Famelart, & Guyomarc'h, 2006).

The changes in gelation pH and time of heated recombined milks were related to the denaturation levels of whey protein; severe heat treatments increased whey protein denaturation levels (Appendix A.1) which in turn resulted in increased gelation pH, and decreased gelation time. However, when heat treatments applied to recombined milks were beyond those required to denature all the whey proteins, the gelation pH slightly decreased and gelation time slightly increased. Cho et al. (1999) and Lucey et al. , Tamehana, (1998a) reported that acid gels of recombined milks, made from unheated and heated (80°C for 30 min) reconstituted milks, containing fat globules stabilized by denatured WPC had a higher gelation pH than those stabilized by undenatured WPC. Their results suggested that denatured whey proteins present on the surface layers of fat globules are responsible for the increased gelation pH of acidified recombined milks.

5.2.2. Viscoelastic properties during acidification

Sections 5.2.2 and 5.2.3 will discuss the viscoelastic properties that arise during and after acidification of heated recombined milks respectively. The large deformation rheology after acidification will be discussed in Section 5.2.4.

The changes in G' with time after GDL addition for unheated and heated recombined milks are shown in Figure 5.2. Initially the G' was very low; it rapidly increased as the milk gelled and finally it reached a plateau value. The final G' value used in the discussion refers to that measured after 6 h of acidification. Acid gel made from

unheated recombined milk had very low final G' value (~ 15 Pa). Applied heat treatments at pasteurization level (72°C for 15 s) either before or after homogenization did not have significant effects on the final G' values. Heat treatments at this level induced ≤ 10% denaturation of whey proteins (Appendix A.1) which in turn resulted in little association of denatured whey proteins on the surfaces of casein micelles. Higher heat treatments (72 - 80°C for 30 min) either before or after homogenization resulted in significant increase in the final G' values, and the effects were more intense with increased heating temperatures. Further increase in heating temperatures to 100°C did not significantly change the final G' values. These results are in agreement with previous studies on skim milks (Graveland-Bikker & Anema, 2003; Oh, et al., 2007; Vasbinder, 2002) and recombined milks (Cho, et al., 1999; Lucey, Tamehana, et al., 1998a).

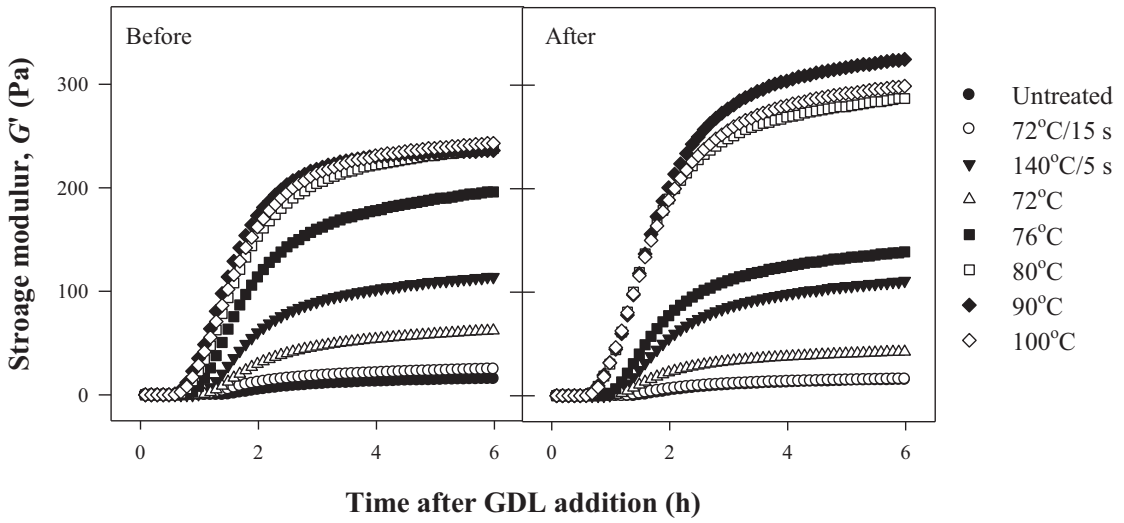


Figure 5.2. Changes in storage modulus as a function of time after GDL addition. Recombined milks were heated either before or after homogenization at different heating profiles.

Anema (2008b) reported that the final G' values of acid gels made from skim milks heated at temperatures below 70°C were similar to those of unheated milks, whereas heat treatments at 70-85°C increased and beyond 85°C decreased the final G' values. van Vliet and Keetals (1995) reported that the final G' values of acid gels made from

low heated (72°C for 15 s) skim milks were significantly lower than those made from high heated (85°C for 5 min) skim milks. Cho et al. (1999) found that acid gels made from unheated recombined milks had lower final G' values compared to those of heated (80°C for 30 min) recombined milks.

The changes of β-Lg denaturation (findings from Chapter 4), gelation pH, final G' and yield stress values as a function of heating temperature are shown in Figure 5.3. At heating temperatures up to 80°C, the increase in final G' values (Figure 5.3C) was related to the increase in denaturation levels of whey protein (Figure 5.3A). These finding supported previous studies on skim milks. Dannenberg and Kessler (1988b) reported that when the denaturation of β-Lg increased from 10 to 60%, there was a linear increase in the firmness of acid gel. Anema (2008b) found that when 50% of the β-Lg was denatured, the final G' value of acid gel increased almost 10-fold compared with that of skim milk without heat treatment. The increase in final G' value of acid gel made from heated recombined milk was attributed to additional cross-linkages between denatured whey proteins with the casein gel matrix (Lucey, Teo, Munro, & Singh, 1999). Denatured whey proteins attached to the surface of casein micelles during heating (i.e., bound denatured whey protein) are a critical factor in the increased gel strength of acid gels made from heated milk, i.e., more cross-linking of gels by bound denatured whey protein increased the gel strength (Lucey, Tamehana, et al., 1998b). However, further increase in heating temperatures applied to recombined milks up to 100°C either before or after homogenization did not significantly change the final G' values, even though the denaturation of whey proteins slightly increased to nearly 100%. Studies in skim milk systems have also reported that the final G' values of acid gels decreased when skim milks were heated beyond the levels that are required to denature all the whey proteins (Anema, 2008a; Anema & McKenna, 1996; Dannenberg & Kessler, 1988c; Lucey, Teo, et al., 1997). The placement of heat treatments at 72-100°C for 30 min either before or after homogenization had significant effects on final G' values, indicating that the protein gel structures were different. Acid gels made from recombined milks heated before homogenization at 72 - 76°C had higher final G' values, whereas those heated at 80-100°C had lower final G' values compared to acid gels made from recombined milks heated after homogenization (Figure 5.3C).

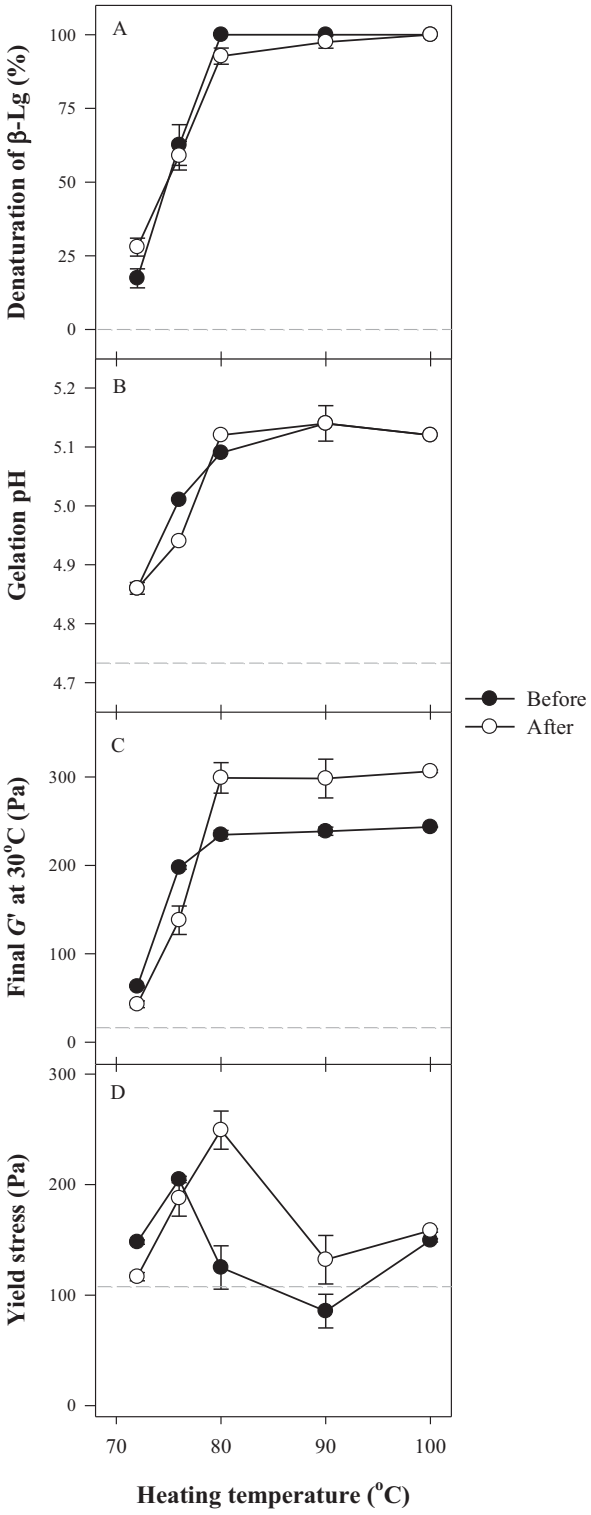


Figure 5.3. Denaturation of β-Lg and characteristics of acid gels as a function of heating temperature. Recombined milks were heated for 30 min either before or after homogenization. Dash lines represent those characteristic of untreated recombined milk.

However, there were no significant differences in gelation time of recombined milks heated before or after homogenization. The application of heat treatment after homogenization resulted in higher amount of caseins and whey proteins adsorbed on the surface of fat globules compared to those of milk heated before homogenization (Chapter 4). This might induce different interactions among milk proteins (discussed in Section 5.2.5). These findings support recent studies on heated skim milks which reported that even though the irreversible denaturation of the whey proteins is primarily responsible for the increased firmness of acid gels made from heated skim milks, the interaction behavior of the denatured whey proteins during heating is also important (Anema, Lauber, et al., 2005; Anema, Lowe, et al., 2004; del Angel & Dalgleish, 2006).

5.2.3. Viscoelastic properties after acidification

In section 5.2.2, the viscoelastic properties during acidification were discussed. In this section, the properties after acidification will be investigated. Figure 5.4 shows the frequency dependence of the storage modulus, G', and the loss modulus, G'', of acid gels made from recombined milks that underwent heat treatment either before or after homogenization. For all the acid gels, regardless of heat treatments applied, the plots of G' and G'' against frequency of the applied deformation were straight lines with a slope of approximately 0.14 ± 0.01 on logarithmic scale and G' was greater than G'', indicating that the gels were elastic. The slope of the log G' versus log frequency curves was not greatly influenced by heat treatment suggesting that the overall nature of the bonds in the gel did not vary greatly. The slope observed in this study is consistent with those reported for skim milks (Anema, Lauber, et al., 2005; Lucey, Teo, et al., 1997; Oh, et al., 2007; Roefs & Van Vliet, 1990) and recombined milks (Cho, et al., 1999).

After 6 h of acidification, the temperature of acid gel was reduced from 30°C to 5°C and the rheological properties were monitored. The final G' values at 5°C were approximately 3.60 ± 0.59 times of those at 30°C (Figure 5.5). The increase in G' values at lower temperature was attributed to the lower hydrophobic interactions resulting in less compact casein micelles. As a consequence, the casein particle size was increased within the acid gel network.

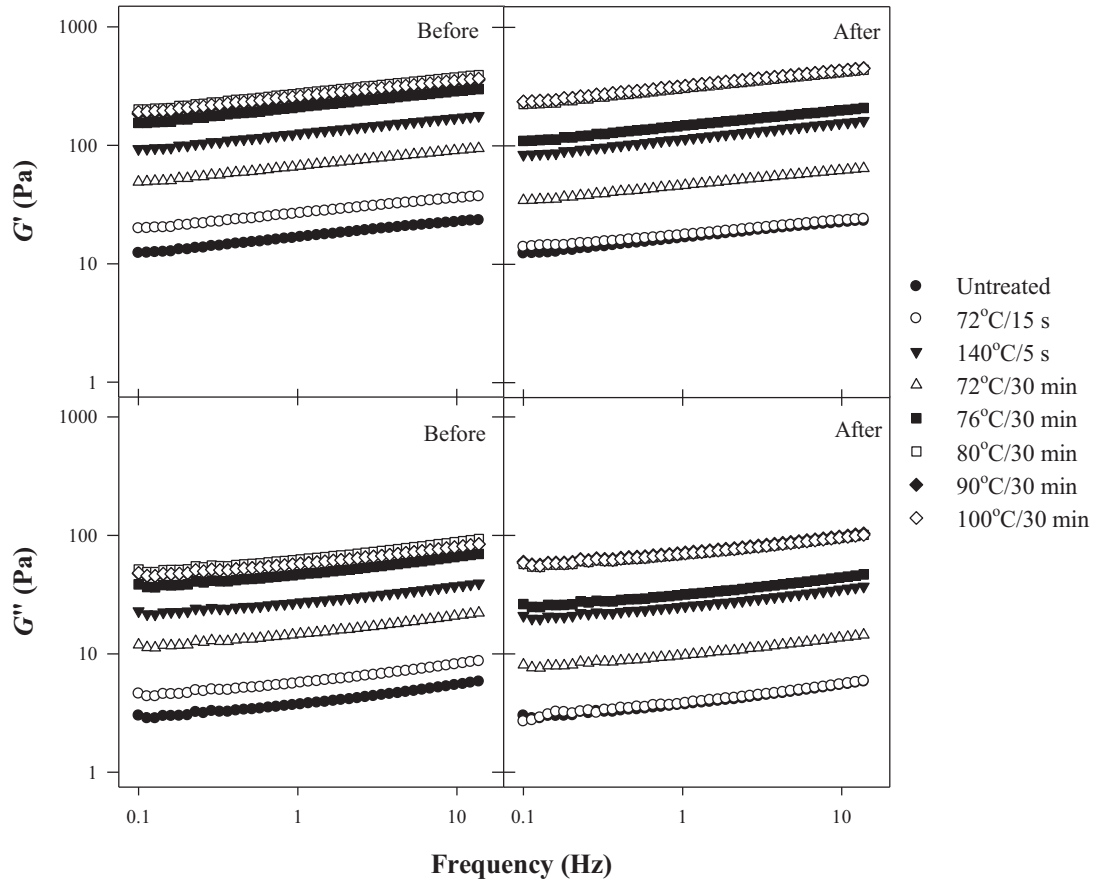


Figure 5.4. Storage modulus (*G′*) and loss modulus (*G″*) of acid gels at 30°C as a function of frequency. Recombined milks were heated either before or after homogenization.

The changes in size and interactions altered the balance between inter- and intra-particle bonds, resulting in higher number density of inter-particle bonds between casein particles at the lower temperature. Therefore the *G′*and *G″* at lower temperature were higher (van Vliet, Roefs, Zoon, & Walstra, 1989). The increase in *G′* value at lower temperature was also attributed to other factors such as the swelling of casein particles, the greater rigidity of the protein network, and the increased viscosity of the continuous phase at the lower temperature (Anema, Lauber, et al., 2005; Bikker, et al., 2000; Lucey, Teo, et al., 1997; Oh, et al., 2007).

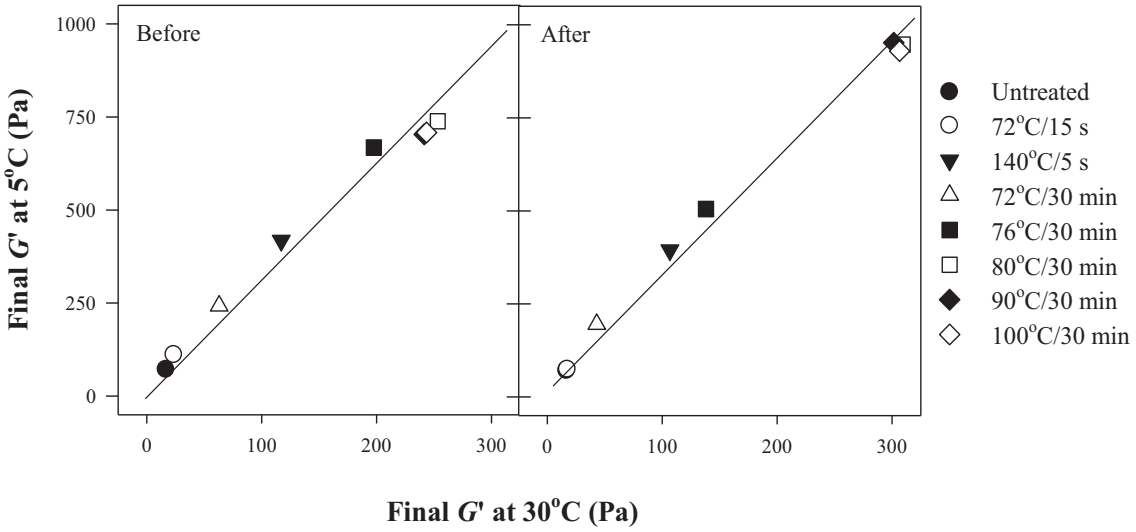


Figure 5.5. Comparison between the final storage modulus (G') at 30°C and 5°C. Recombined milks were heated either before or after homogenization.

However, studies on skim milk systems found that the final G' values at 5°C were approximately two times higher than those observed at 30°C (Anema, Lauber, et al., 2005; Bikker, et al., 2000; Lucey, van Vliet, et al., 1997; Oh, et al., 2007), indicating that the fat globules in recombined milk play an important role in the increased firmness at low temperature. At 5°C, the fat globules are less liquid-like compared to at 30°C. This might be responsible for the higher final G' value at lower temperatures.

Tan δ is a measure of the ratio of the deformation stored by the sample to the deformation energy liberated as heat, i.e., tan $\delta = G''/G'$. Figure 5.6 shows a plot of the final G' against the final G'' for all samples at 5°C and 30°C. It is evident from this plot that the final G' and G'' were linearly correlated and tan δ had a value of approximately 0.25 ± 0.01. The result indicates that the proportions of the deformation energy lost and stored remain constant regardless of the heat treatment applied, the final firmness of the sample, and the measuring temperature. Oh et al. (2007) reported that the relationship between final G' and final G'' was linear and tan δ was about 0.25 regardless of the temperature of the acid gel. Anema et al. (2005) and Lucey et al. (1997) have also reported a relatively constant tan δ value despite different treatment histories of the milk prior to acidification.

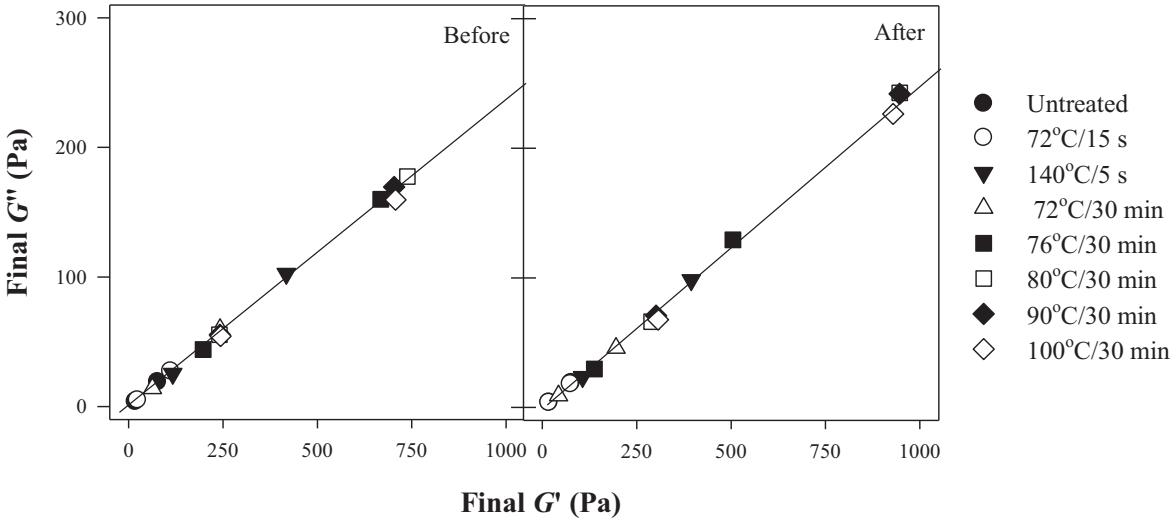


Figure 5.6. Comparison between the final storage modulus (G') and final loss modulus (G'') at 30°C and 5°C. Recombined milks were heated either before or after homogenization.

5.2.4. Large deformation rheology of acid gels

After 6 h of acidification, the acid gels were subjected to a strain sweep at 5°C and the shear stress was measured. The stress increased with increasing strain to a maximum, and then decreased markedly, indicating that the gel structure had been destroyed (Figure 5.7). The maximum stress was considered to be that at which the gel broke, and the corresponding strain was defined as the breaking strain. Both the breaking strain and stress were affected by heat treatments of recombined milk either before or after homogenization (Table 5.1). The changes in yield stress with heating temperatures are shown in Figure 5.3D. The breaking stresses increased as the levels of whey protein denaturation increased to about 50% and 75% for recombined milks heated before and after homogenization respectively; after those levels the breaking stress decreased as the levels of whey protein denaturation increased. However, further increase in heating temperature from 90°C to 100°C resulted in slightly increased yield stress. Recombined milks heated at 80 - 90°C for 30 min before homogenization had significant lower yield stress values compared to those of milks heated after homogenization.

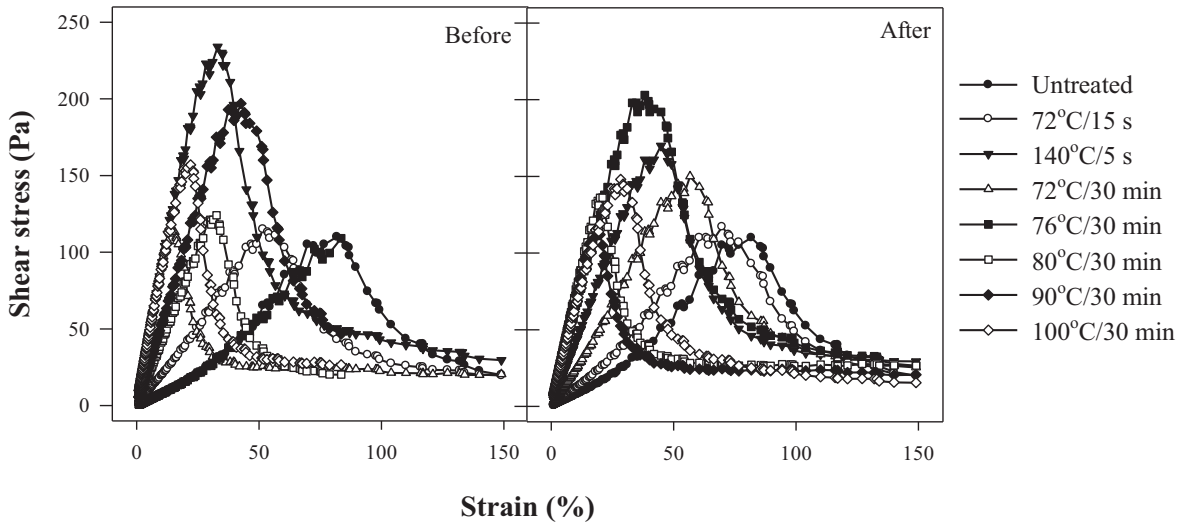


Figure 5.7. Shear stress as a function of strain for acid gels made from recombined milks heated either before or after homogenization.

The yield strain of acid gels made from recombined milks decreased as the heating temperature increased from 72°C to 90°C (Table 5.1), indicating that the gels became more brittle and easier to fracture as the heating temperature increased. Further increase in heating temperature to 100°C slightly increased the yield strains of acid gels. Recombined milks heated before or after homogenization at different heating profiles had similar yield strains, except for those heated at 72°C for 15 s and 80°C for 30 min.

These findings are consistent with that reported in skim milk systems (Anema, 2008b; Lucey, Teo, et al., 1997; van Vliet & Keetals, 1995). Anema (2008b) reported that acid gels made from skim milks heated at low treatment temperatures (≤ 70°C) had higher yield strains (~50%) and lower yield stress values (~25 Pa). However, at higher treatment temperatures, the yield strain decreased to about 30% and the yield stress increased to about 100 Pa. van Vliet and Keetals (1995) found that the yield strain of acid gel made from low-heated (72°C for 15 s) skim milk was higher than that of high-heated (85°C for 5 min) skim milk.

5.2.5. Factors contributing to the differences in acid gelation properties of recombined milks heated before or after homogenization

Recombined milks heated either before or after homogenization had similar levels of whey protein denaturation, but had significantly different final G' and yield stress values. These findings indicated that other factors in addition to denatured whey protein contributed to the firmness and the structure of acid gels. As discussed in Chapter 4, recombined milk heated after homogenization contained higher amount of caseins and whey proteins adsorbed on the surface layer of fat globules. It is possible that the interactions of milk proteins and the proteins on the surface layers of fat globules for these two recombined milk systems were different during acidification.

5.2.5.1. Gel network of acid gel made from recombined milk

In recombined milk, the casein micelles are present as colloidal particles and the whey proteins are in native forms. During homogenization, these proteins are adsorbed on to the surface layers of fat globules. In this study, the surface layers were composed of 98% caseins and 2% whey proteins (see Figure 4.2, Chapter 4). According to Cho et al. (1999) when recombined milk is acidified with GDL, the protein gel network is formed by the aggregation of casein micelles; the native whey proteins do not interact with the network, but fill the space between protein strands. Recombined milk fat globules are incorporated into the casein gel network through the interactions between adsorbed caseins and denatured whey proteins on the surface layers with the casein-based network. The illustration of acid gel made from recombined milk is shown in Figure 5.8.

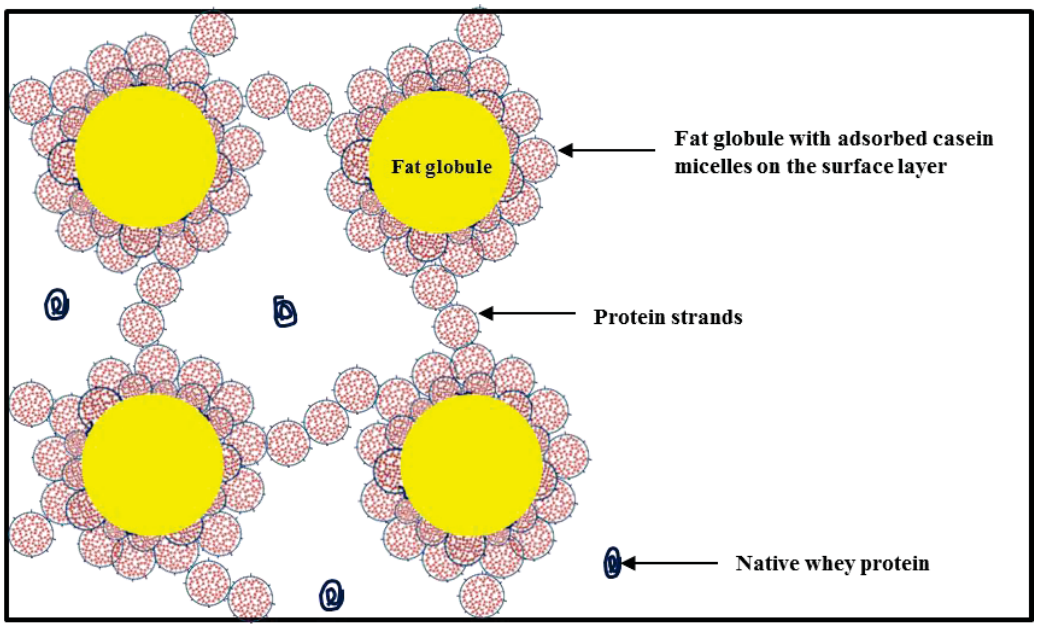


Figure 5.8. Schematic illustration of acid gels made from untreated recombined milk. The relative size of individual components are not to scale.

5.2.5.2. Gel network of acid milk gel made from recombined milk heated before homogenization

When heat treatment at temperatures higher than the denaturation temperature of whey proteins (~70°C) was applied to the reconstituted milk before homogenization, the whey protein denatured and interacted with κ-casein on the surfaces of casein micelles or with other denatured whey proteins, resulting in protein aggregates (Lowe, et al., 2004; Singh, 1995). During the recombination process with milk fat, the protein aggregates are adsorbed onto the surface layers of fat globules (as shown in Figure 4.14, Chapter 4). The whey protein aggregates facilitated the acid-induced gelation process as they change the isoelectric point from ~ 4.6 (for caseins) to ~ 5.2 (for whey proteins) resulting in increased gelation pH and reduced gelation time. The whey protein aggregates also contributed to the increase in final G' values as they strengthen the caseins strands via the interactions with casein matrix. As a consequence, the gel firmness of acid gel is increased in relation to the denaturation levels of whey proteins. The illustration of acid gel made from recombined milk heated before homogenization is shown in Figure 5.9.

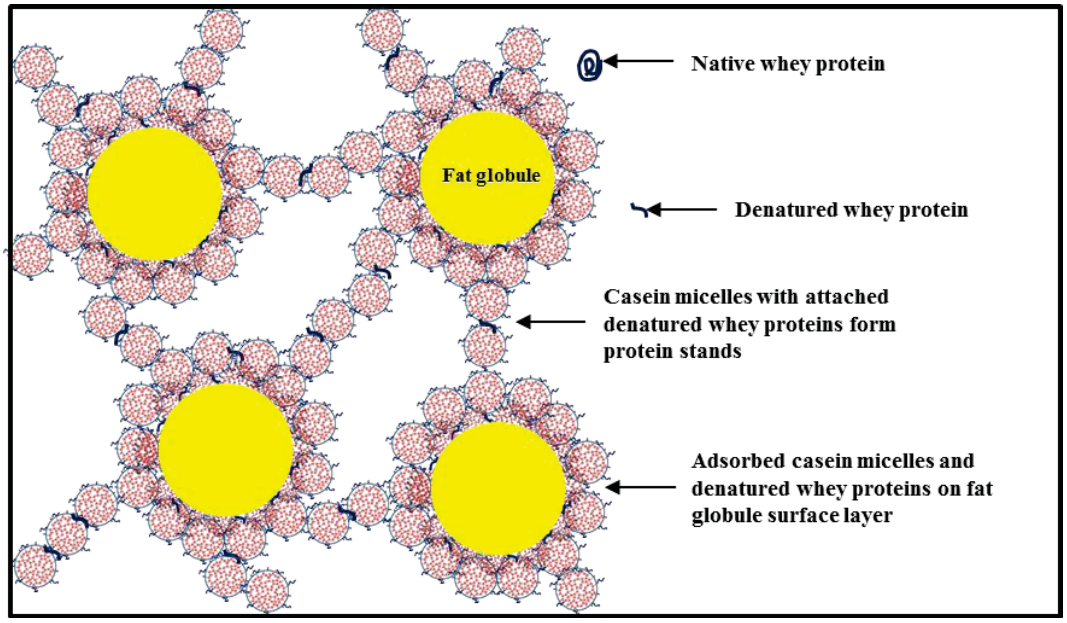


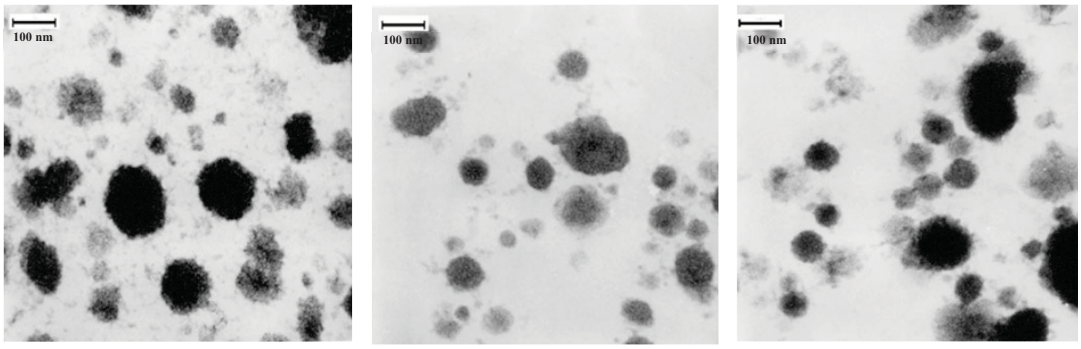
Figure 5.9. Schematic illustration of acid gel made from recombined milk heated before homogenization. The relative sizes of individual components are not to scale.

5.2.5.3. Gel network of acid gel made from recombined milk heated after homogenization

The discussion on characteristics and the illustration of recombined milk heated after homogenization are in Section 4.2.2.4 (Chapter 4). In general, the surface layers of heated recombined milk fat globules were composed of caseins and denatured whey proteins. The amount of adsorbed proteins on the surface layers depends on the severity of heat treatment. The whey proteins of heated recombined milks in the serum present in both native and denatured forms. The denatured whey proteins interacted with κ-casein on the surface of casein micelles present in the serum and on the surface layers of fat globules and also interacted with dissociated κ-casein in the serum. The denatured whey proteins also interacted with other denatured whey proteins in the serum.

According to Cho et al. (1999) when GDL was added to heated recombined milk, the protein networks were formed by the caseins with adsorbed denatured whey proteins on the surface, i.e., casein bound complexes The denatured whey proteins bound to the casein micelles are responsible for the increase in firmness of acid gels made from heated recombined milks. Anema (2010b) reported a size range (with the average of

150 nm) of casein micelles for heated (90°C/30 min) skim milk before acidification (pH 6.6) as shown in Figure 5.10 (A). During acidification, the size of casein micelles in heated (90°C/30 min) skim milk did not markedly change until the pH was below the gelation point (pH ~5.2) as shown in Figure 5.10 (B). In acid gel made from heated skim milk, the casein micelles appeared to be aggregated or linked, however the discrete micelles could still be observed as shown in Figure 5.10 (C). The illustration of acid gel prepared from recombined milk heated after homogenization is shown in Figure 5.11.



(A) pH 6.60 (B) pH 5.45 (C) pH 5.20

Figure 5.10. Transmission electron micrograph images of heated (90°C/30 min) skim milk taken before and during acidification, from Anema (2010b).

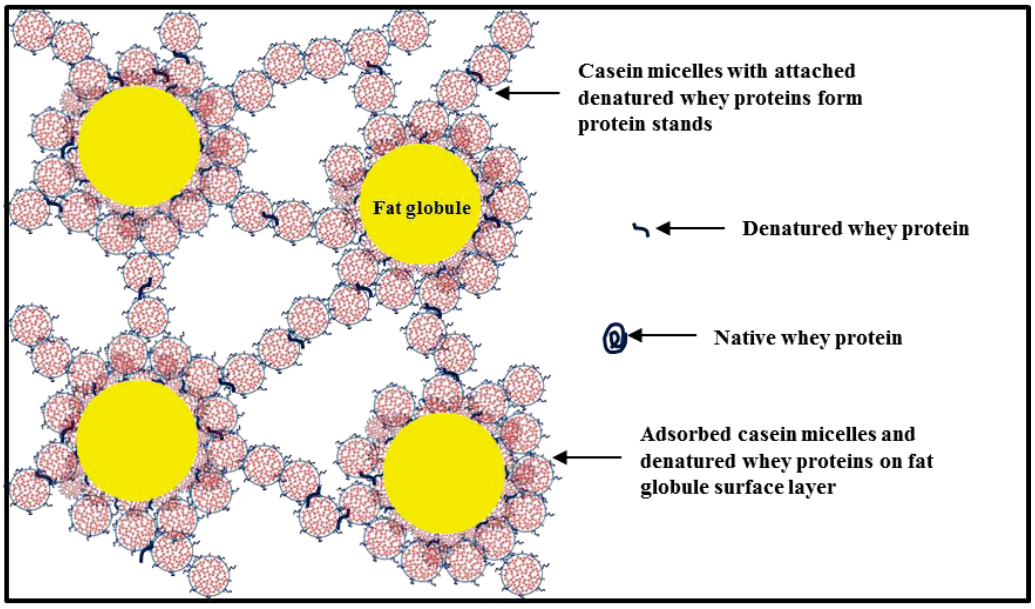


Figure 5.11. Schematic illustration of protein gel network of acid gel made from recombined milk heated after homogenization.

Acid gels made from recombined milks heated after homogenization have higher G' values than acid gels made from recombined milks heated before homogenization, although they have similar levels of whey protein denaturation. It is likely that the differences in the composition and reactivity of fat globule surfaces layer between two types of recombined milk systems played a role in affecting gel strength. The surface layer of fat globules in recombined system heated after homogenisation had higher levels of adsorbed caseins and denatured whey proteins which may have provided more sites for cross-linking fat globules with the casein network. Further studies are required to understand the details of possible interactions involved in these systems.

5.3. Conclusions

Heat treatment applied to recombined milk, either before or after homogenization, prior to acidification has profound effects on the acid-induced gelation properties. Increased heating temperatures up to 80°C resulted in decreased gelation time, but increased gelation pH and final G' values of the acid gels. However, further increase in heating temperature to 100°C did not significantly change the gelation pH and final G' values of acid gels. The placement of heat treatment at 72 - 100°C for 30 min either before or after homogenization had a significant effect on final G' and yield stress values, indicating that the protein gel structures were different. The recombined fat globules can interact with the casein matrix by cross-linking between surface layers of fat globules and caseins and denatured whey proteins in the protein gel matrix. The type of adsorbed layer around the fat globule possibly influences the acid gelation properties of recombined milk.

CHAPTER 6

HIGH PRESSURE-TREATED RECOMBINED MILKS

6.1. Introduction

HP treatment has significant effects on milk proteins. Casein micelles may aggregate or dissociate and whey proteins can denature, depending on the conditions of the HP treatment, i.e., pressure, temperature and time (Anema, Lowe, et al., 2005; Scollard, et al., 2000). Casein micelle size and internal structure induced by HP treatment are different from those of untreated and heated milks (Anema, 2010b; Huppertz, Kelly, & de Kruif, 2006). In addition, the size and internal structure of denatured whey protein aggregates induced by HP treatment and the subsequent interactions with caseins are different from those induced by heat treatments (Considine, et al., 2007). In whole milk systems, HP treatment at pressures up to 600 MPa does not have a significant effect on the fat globule size (Huppertz, et al., 2003; Ye, Anema, et al., 2004). While the size does not change, HP treatment at ≥ 100 MPa causes the association of β-Lg with MFGM proteins, and this increases with the treatment pressure and time. At very high treatment pressures, κ-casein (~500 MPa) and α-La (≥ 700 MPa) interact with the MFGM proteins (Ye, Anema, et al., 2004).

No information appears to be available on the effects of HP treatment on recombined milk systems, especially on HP-induced interactions between the surface layers of recombined milk fat globules and serum proteins. This study was conducted to investigate the effects of HP treatment at different pressure, temperature and time combinations on whey protein denaturation, particle size (d_{32}), protein load, and the surface layers of fat globules. Also investigated were the effects of HP treatment carried out before or after homogenization.

6.2. Results and discussion

In these experiments, milk samples were homogenized and subsequently HP-treated at 200, 400, and 600 MPa for 5, 15, 30, and 60 min at 10, 20 and 40°C. HP treatments were also applied to milk samples at 200, 400 and 600 MPa for 30 min at 20°C before homogenization. The results on whey protein denaturation, particle size (d_{32}), protein

load, surface layer and interface of recombined milk fat globules are shown in Appendix B.1 – B.4.

6.2.1. Whey protein denaturation

The native-PAGE patterns of HP-treated recombined milks and the extent of whey protein denaturation at different HP conditions are shown in Figures 6.1 and 6.2 respectively. At 200 MPa, the denaturation of β-Lg slightly increased as the pressurizing time increased. However, increasing treatment temperature at this pressure did not have a significant effect on the denaturation level of β-Lg (Figure 6.2, Appendix B.1). The denaturation of β-Lg at this pressure was ≤ 30%. At higher treatment pressures (400 and 600 MPa) and temperatures of 10°C and 20°C, the denaturation of β-Lg increased rapidly as the pressuring time increased. Increasing the pressurizing time from 5 min to 60 min at 400 MPa resulted in increased denaturation of β-Lg in recombined milk from ~20% to ~70% and from ~50% to ~90% at 10°C and 20°C respectively. At 600 MPa, increasing the pressurizing time from 5 min to 60 min resulted in increased denaturation of β-Lg from ~40% to ~80% and from ~75% to ~100% at 10°C and 20°C respectively. At 40°C, regardless of the pressurizing time, HP treatment at 400 and 600 MPa resulted in denaturation of nearly all β-Lg. The denaturation of β-Lg induced by HP treatment found in this study is consistent with previous studies on skim milk systems (Anema, Stockmann, et al., 2005; Hinrichs & Rademacher, 2005; Huppertz, Fox, & Kelly, 2004b; Scollard, et al., 2000; Ye, Anema, et al., 2004) which reported that the levels of β-Lg denaturation is increased at treatment pressure ≥ 200 MPa. About 90% of the β-Lg is denatured after HP-treated at 800 MPa for 30 min (Anema, 2008b). The denaturation increases with treatment pressure, but difference in rate with at pressure < 300 MPa and pressure > 300 MPa (Anema, 2008b). At any pressures, the denaturation of β-Lg increases with holding time (Hinrichs & Rademacher, 2005; Huppertz, Fox, et al., 2004c; Scollard, et al., 2000) and temperature, except at 200 MPa, i.e., at this low pressure, there is no significant effect of treatment temperature (Anema, Stockmann, et al., 2005; García-Risco, et al., 2000; Gaucheron, et al., 1997; Huppertz, Fox, et al., 2004c; López-Fandiño & Olano, 1998b).

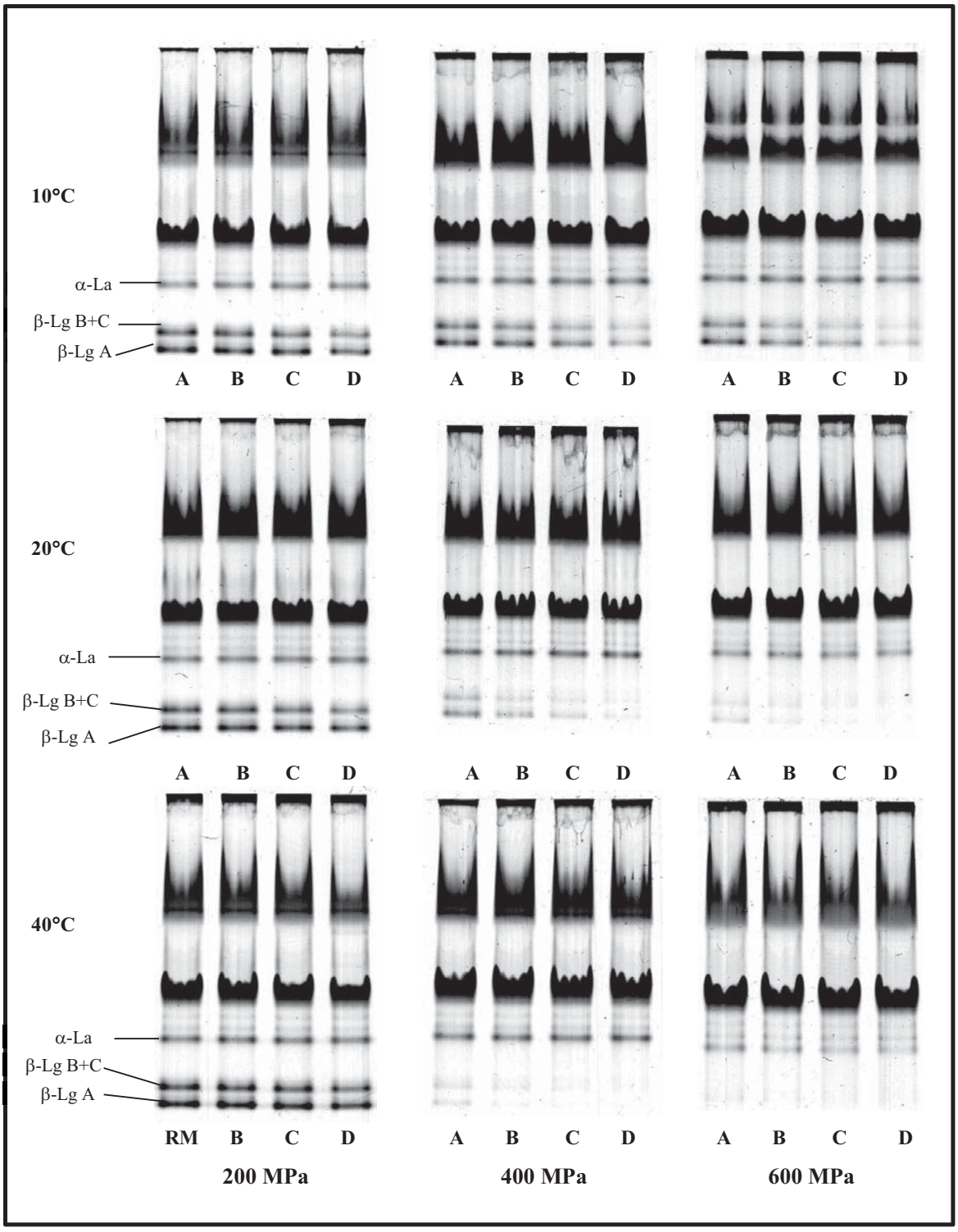


Figure 6.1. Native-PAGE patterns of HP-treated recombined milks. HP treatments were carried out after homogenization at 10, 20 and 40°C for (A) 5, (B) 15, (C) 30 and (D) 60 min. RM represents native-PAGE of untreated recombined milk.

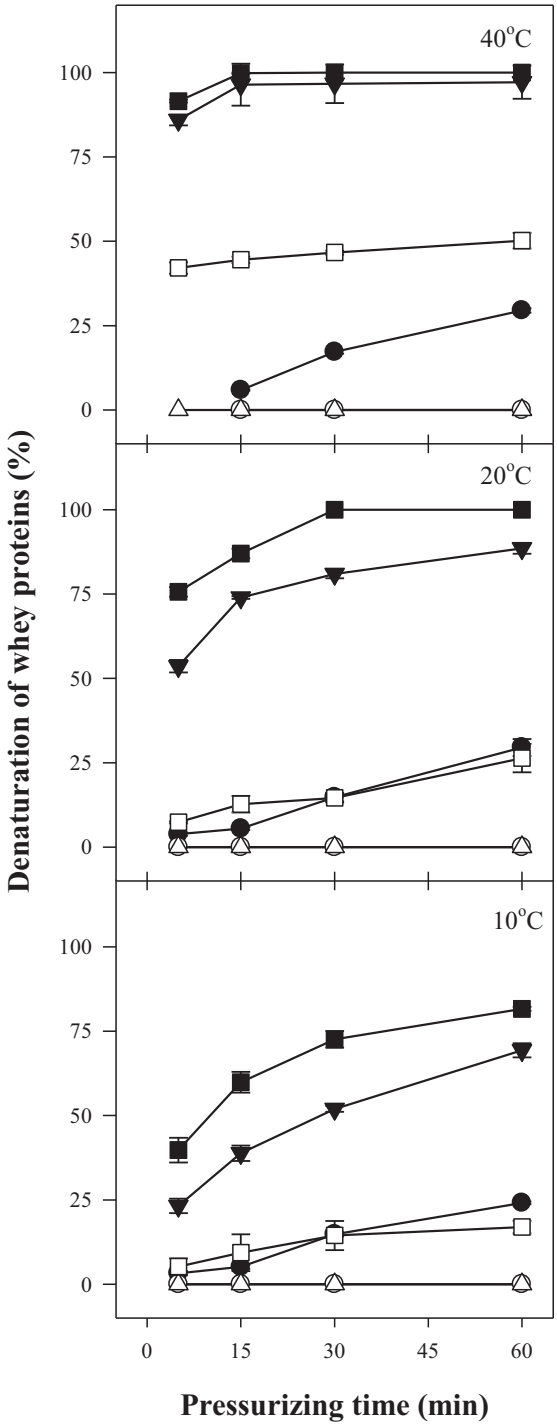


Figure 6.2. Denaturation of β-Lg (filled symbols) and α-La (open symbols) as a function of pressurizing time. HP treatments were carried out on recombined milks after homogenization at 200 (●,○), 400 (▼,△), and 600 (■,□) MPa.

A level of β-Lg denaturation of ~85% was achieved in a number of treatment pressure, temperature and time combinations: 600 MPa for 60 min at 10°C, 400 MPa for 30 min at 20°C, 600 MPa for 15 min at 20°C, 400 MPa for 5 min at 40°C as shown in Appendix B.1. These synergistic effects of treatment pressure, temperature and time on β-Lg denaturation have been reported previously. García-Risco et al. (2000) found that HP treatment at 300 MPa for 15 min at 50 - 60°C or at 400 MPa for 15 min at 40 - 60°C resulted in almost 100% denaturation of β-Lg.

Compared with β-Lg, α-La was more resistant to HP treatment and the denaturation of this protein did not occur until recombined milks had undergone HP treatment at 600 MPa (Figure 6.2). At this pressure, the denaturation increased as treatment temperature and time increased. Increased pressurizing time from 5 min to 60 min resulted in increased denaturation of α-La from ~5% to ~20%, from ~10% to ~25% and from 40% to 50% at 10°C, 20°C and 40°C respectively. These findings support previous studies on skim milk systems (Anema, 2008b, 2010a; García-Risco, et al., 2000; Hinrichs & Rademacher, 2005; Huppertz, Fox, et al., 2004b; Needs, Stenning, et al., 2000), which have reported that α-La is resistant to denaturation at pressure up to 500 MPa and beyond this pressure, the denaturation increases with increasing treatment pressure, temperature and time (García-Risco, et al., 2000; Gaucheron, et al., 1997; Hinrichs & Rademacher, 2005; Huppertz, Fox, et al., 2004c; López-Fandiño & Olano, 1998b; Scollard, et al., 2000). About 30% of α-La is denatured after HP-treated at 800 MPa for 30 min (Anema, 2008c).

According to the principle of Le Chatelier-Braun, any phenomenon (such as change in molecular configuration) that is accompanied by a decreased in volume (negative ΔV) will be enhanced by increase in pressure (Datta & Deeth, 2003; Torres & Velazquez, 2005), i.e., pressure favours a state with reduced volume. Hence, the native structure with a cavity is less favourable than a denatured state without cavity. β-Lg has a cavity in the structure, making it easier to denature under HP treatment, as a result β-Lg denatures faster than α-La. The greater resistance of α-La to HP treatment than β-Lg may also be attributed to the more rigid molecular structure, caused partially by the higher number of intra-molecular disulphide bonds, i.e., 4 compared to 2 (Gaucheron, et al., 1997; Hinrichs, et al., 1996b) and the absence of a free sulphydryl group in α-La (López-Fandiño, et al., 1996). Calcium may also play a part in α-La stability. β-Lg

contains a free sulphydryl-group which can participate in sulphydryl-oxidation or sulphydryl-disulphide interchange reaction (Hinrichs, et al., 1996b; López-Fandiño, et al., 1996). The native-PAGE patterns of recombined milks HP-treated before or after homogenization at 200 to 600 MPa for 30 min at 20°C and the relationships between denaturation of whey proteins and treatment pressure are shown in Figures 6.3 and 6.4 respectively.

Figure 6.4 shows that recombined milks that underwent HP treatment either before or after homogenization did not have different denaturation level of whey proteins. This result was expected since the denaturation of whey proteins depends on the concentration of native whey proteins and the environment that the whey proteins are in (milk serum), which were similar in milk samples HP-treated before or after homogenization.

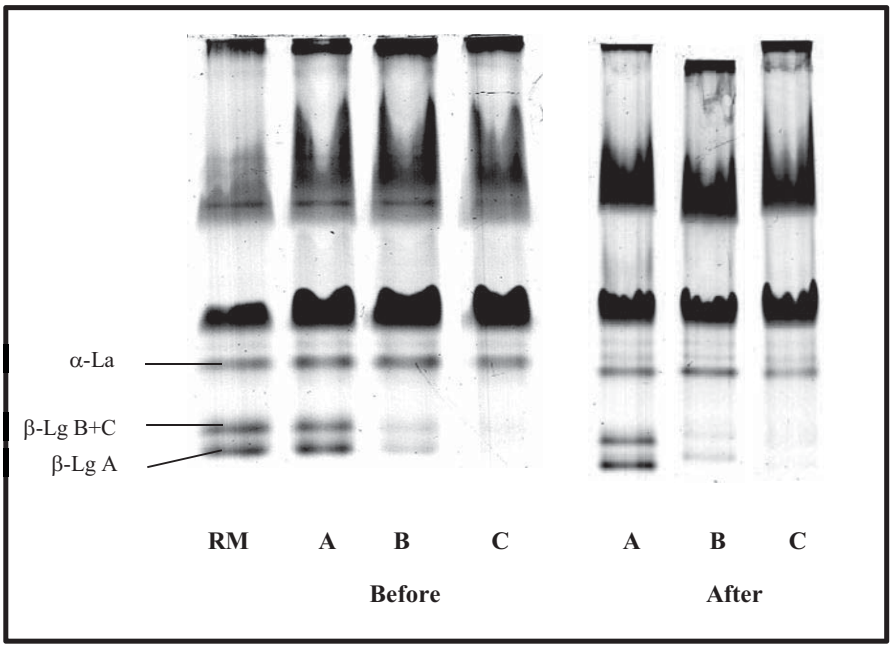


Figure 6.3. Native-PAGE patterns of HP-treated recombined milks. HP treatments were carried out at 20°C for 30 min before or after homogenization at (A) 200, (B) 400, and (C) 600 MPa. RM represents native-PAGE of untreated recombined milk.

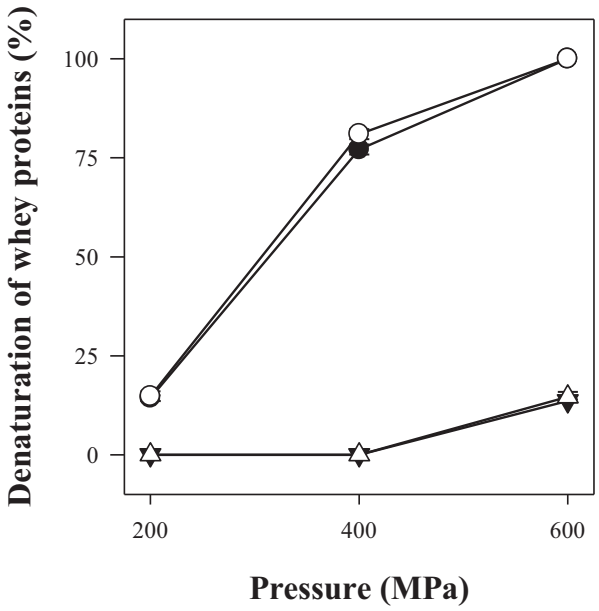


Figure 6.4. Denaturation of β-Lg (●,○) and α-La (▼,△) as a function of treatment pressures at 20°C for 30 min. HP treatments were applied to milks either before (filled symbols) or after (open symbols) homogenization.

The possible mechanism for HP-induced denaturation of whey protein in milk, as suggested by Huppertz, Fox et al. (2004b), is as follows; under HP treatment, β-Lg unfolds (Lakemond & van Vliet, 2008), resulting in the exposure of the free sulphydryl group in β-Lg (Vasbinder, van de Velde, & de Kruif, 2004); this free sulphydryl group interacts with κ-casein, β-Lg or α-La, and possibly α_{s2}-casein through sulphydryl-disulphide interchange reactions, resulting in aggregated protein complexes. During depressurization, unfolded β-Lg and α-La molecules that have not interacted with another protein may refold to a state closely related to their original native state.

In general, it can be concluded that β-Lg and α-La in recombined milk denatured at 200 and 600 MPa respectively and the denaturation levels increased as the treatment pressure, temperature and holding time increased. In addition, the effect of HP treatment on denaturation of whey proteins was pronounced at high treatment temperature, except those at 200 MPa. The lower barostability of β-Lg was attributed mainly to the cavity in its structure, which was unfavourable under HP treatment. These findings support previous studies on skim milk systems (Anema, Stockmann, et al., 2005; Huppertz, Fox, et al., 2006).

6.2.2. Diameter (d_{32}) and protein load of HP-treated recombined milk fat globules

The average diameter (d_{32}) and protein load of HP-treated recombined milk fat globules are shown in Appendix B.2. The d_{32} values of HP-treated recombined milks dispersed with dissociating agents before particle size measurement did not change significantly (d_{32} = 0.58 ± 0.02 μm), indicating that the fat globules did not disrupt under HP treatment. This finding supports previous studies on HP-treated whole milks (Huppertz, et al., 2003; Ye, Anema, et al., 2004) which reported that HP treatment up to 600 MPa did not have a significant effect on fat globule size. However, in this study when water was used as the dispersing medium, the d_{32} values of HP-treated recombined milk increased markedly from 0.56 ± 0.00 μm to 1.03 ± 0.09 μm as the intensity of HP treatment increased, indicating the aggregation of fat globules. This contrast with the finding on whole milk systems (Needs, Capellas, et al., 2000; Ye, Anema, et al., 2004), which reported a slight increase in d_{32} value of whole milk HP-treated at pressures up to 700 MPa. They claimed that the increase in d_{32} value was attributed to the disruption of casein micelles under HP treatment, which reduces their contribution to light scattering and therefore, give an apparent increase in other scattering particles. This was also apparent in recombined milk system. However, as the surface layers of recombined milk fat globules contained mainly casein (98%) and small amount of whey protein (2%), without MFGM materials, the interactions between milk proteins and the surface layer materials of fat globules induced by HP treatment are expected to be different from those of MFGM of whole milk. At severe HP treatment more whey proteins are denatured (discussed in Section 6.2.1). As most denatured whey proteins (especially β-Lg) in HP-treated milk associate with the casein micelles (Huppertz, Fox, et al., 2004c; Zobrist, Huppertz, Uniacke, Fox, & Kelly, 2005), it was expected that they were also associated with the caseins on the surface layers of recombined milk fat globules, forming the denatured whey protein-casein complexes. Those complexes might form the bridges, facilitating the aggregation of fat globules, which in turn increase the d_{32} values. The protein loads on fat globules of HP-treated recombined milks are shown in Figure 6.5. All the fat globules of HP-treated recombined milks had a lower protein load than those of untreated recombined milks, probably due to the dissociation of casein micelles induced by HP treatment. When HP treatment is applied to milk, the casein micelles dissociate and whey protein denatures (Considine, et al., 2007).

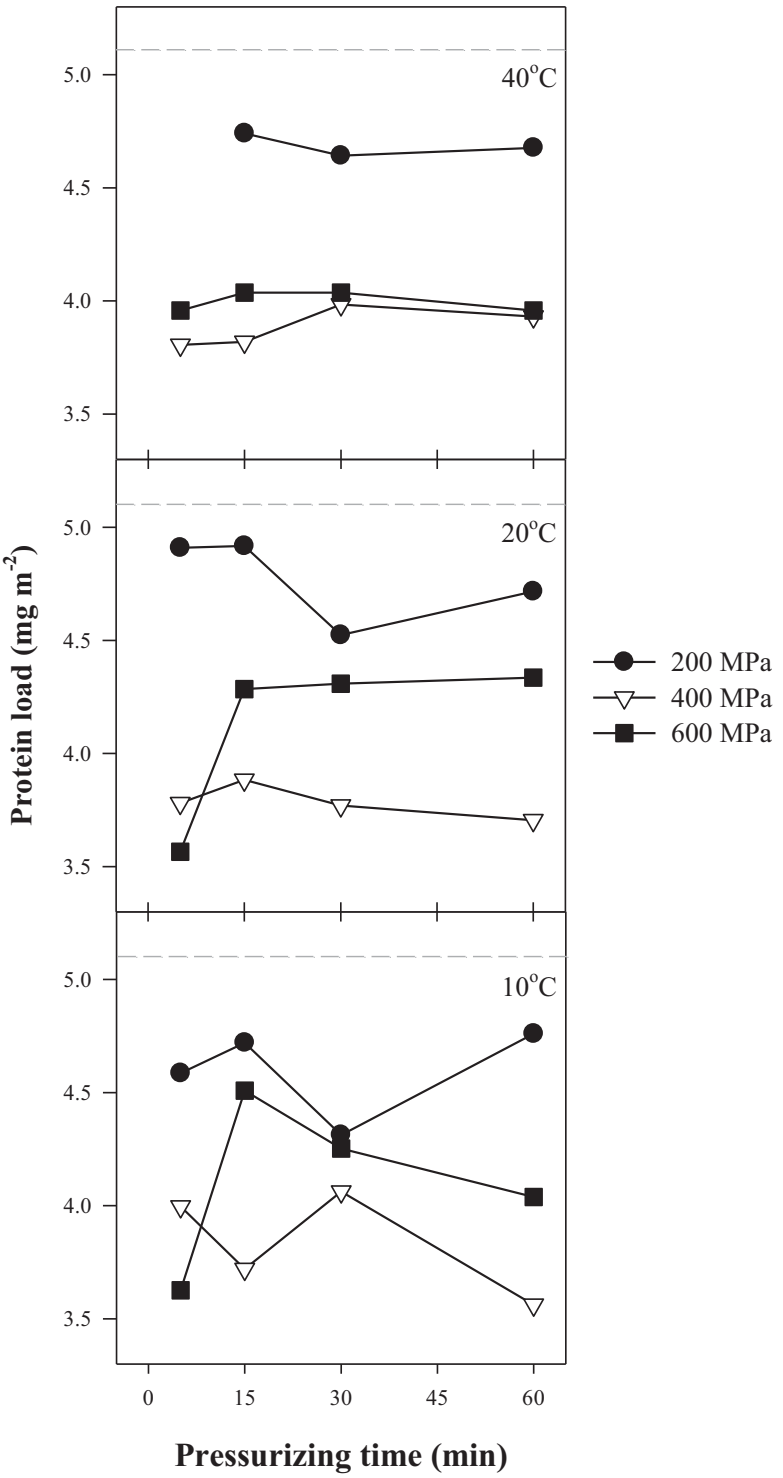


Figure 6.5 Protein load (mg·m^{-2}) on HP-treated recombined milk fat globules as a function of treatment pressures. HP treatments were carried out after homogenization at 10 - 40°C. Dash lines represent the protein load of untreated recombined milks.

As the surface layers of untreated recombined milk fat globules contained mainly casein (discussed in Chapter 4), they were expected to dissociate under HP treatment. As a consequence, the surface layers of HP-treated recombined milk fat globules contained less casein than those of the untreated counterparts. The denatured whey proteins induced by HP treatment can interact with the casein (Huppertz, Fox, et al., 2006; Patel, et al., 2006), and therefore will interact with those on the surface layers of the recombined milk fat globules, resulting in an increased amount of whey proteins on the surface layers of the fat globules. However, the proportion of whey proteins on the surface layers of recombined milk fat globule was very small (~2%), suggesting that the corresponding protein load depends mainly on caseins.

For all treatment temperatures, the fat globules of recombined milks HP-treated at 200 MPa had a higher protein load than those of recombined milks HP-treated at 400 and 600 MPa. Based on the information on skim milk systems (Anema, 2008b), the size of the casein micelles of skim milk HP-treated at 200 MPa is similar to the original size of the native casein micelles in untreated skim milk, whereas the casein micelle size markedly decreases to about half of the original when skim milk is HP-treated at pressures ≥ 300 MPa. This suggests that at pressures 400 - 600 MPa more casein was dissociated from the casein micelles that had been adsorbed on the surface layers of recombined milk fat globules, resulting in less caseins on the surface layers compared with those of recombined milks HP-treated at 200 MPa.

Since the casein micelle sizes of HP-treated skim milks at 400 and 600 MPa are similar (Anema, 2008b), the level of casein dissociation and the protein load of recombined milk fat globule would also expected to be the same, however, the whey proteins were denatured more at higher pressure and temperature. Therefore, more denatured whey proteins may associate with surface layers of fat globules. This was apparent for the proteins load in recombined milk HP-treated at 10 - 40°C for 15 – 60 min. However, recombined milks HP-treated at those pressures and at lower temperatures (10°C and 20°C) the protein load was higher at treatment pressure of 400 MPa. These finding were unexpected and might involve different interactions between milk proteins, which are still unknown and need further investigation. Increasing the pressurizing time, at constant pressure or temperature, tends to decrease the protein load on the surface layers

of recombined milk fat globules probably due to the higher dissociation of casein micelles under those HP treatments.

The protein load on fat globules of recombined milks HP-treated either before or after homogenization is shown in Figure 6.6. Recombined milks HP-treated at 200 and 600 MPa before homogenization had a higher protein load, whereas those HP-treated at 400 MPa had a lower protein load than recombined milks HP-treated after homogenization. In addition, recombined milks HP-treated at 400 MPa, either before or after homogenization, had a lower protein load compared with those HP-treated at 200 MPa and 600 MPa. All these findings imply that not only the treatment pressure, but also the placement of HP treatment (before or after homogenization) had an influence on the adsorbed proteins on the surface layers of HP treated recombined milks fat globules. Ye et al. (2004) reported that in a whole milk system, increased treatment pressures and times resulted in higher protein load on the MFGM as a result of the greater interaction between denatured whey proteins and the MFGM materials. However, this result was not apparent in recombined milk systems due to the different materials on the surface layers of fat globules.

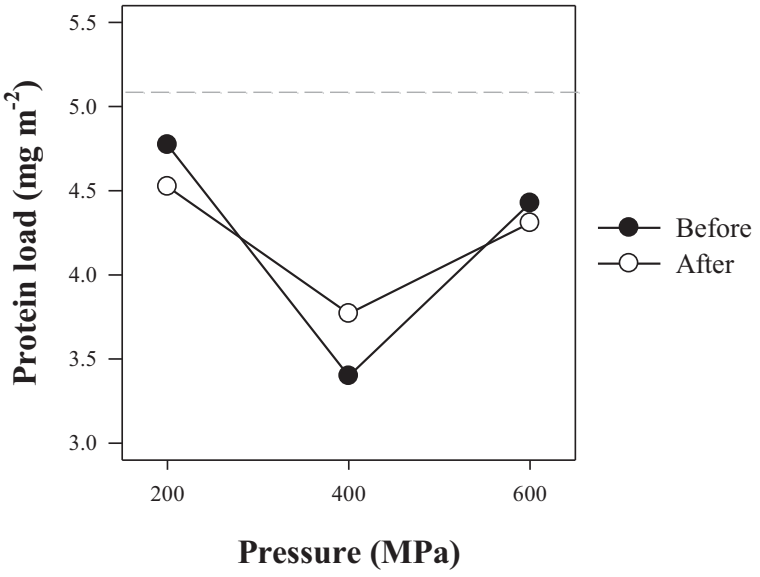


Figure 6.6. Protein load on HP (for 30 min/20°C)-treated (before or after homogenization) recombined milk fat globule as a function of treatment pressures. Dash line represents protein load of untreated recombined milk.

6.2.3. Composition of fat globule surface layers in HP-treated recombined milk

The amounts of caseins and whey proteins on the surface layers of HP-treated recombined milk fat globules are shown in Appendix B.3. The creams were washed with SMUF and SDS-PAGEs were run under reducing conditions. The SDS-PAGE patterns of surface layers isolated from HP-treated recombined milk fat globules are shown in Figure 6.7. The relationships between HP treatment and the amounts of proteins on the surface layers of recombined milk fat globules are shown in Figures 6.8 - 6.10. The amounts of individual proteins adsorbed onto the surface layer of fat globules were calculated from as described in Section 3.2.4.6.

Adsorbed α_s-casein on surface layer of fat globule

The α_s-casein was the main protein on the surface layers of recombined milk fat globules and accounted for approximately 45% of the total adsorbed proteins (Figure 6.7, Appendix B.3). Recombined milks HP-treated at 200 MPa had higher levels of α_s-casein on the surface layers of fat globules compared with those of untreated and HP-treated at 400 and 600 MPa recombined milks. However, recombined milks HP-treated at 400 MPa, at all treatment temperatures, had lower levels of α_s-casein compared with those HP-treated at 600 MPa, excepted for the milks HP-treated for 5 min at 10 and 20°C. Increase pressurizing time at 40°C did not have a significant effect on the amount of α_s-casein adsorbed on the surface layers of fat globules. However, increasing pressurizing times at treatment temperatures of 10°C and 20°C slightly decrease the amount of this protein adsorbed on the surface layers of fat globules.

Adsorbed β-casein on surface layer of fat globule

All HP-treated recombined milks have lower levels of β-casein adsorbed on the surface layers of fat globules compared to those of untreated recombined milks. Recombined milks HP-treated at 400 and 600 MPa at different treatment temperatures and times have lower levels of β-casein on the surface layers of fat globules compared to those HP-treated at 200 MPa. HP treatment at 400 MPa at 10°C and 20°C resulted in lower levels of β-casein on the surface layers of fat globules compared with those HP-treated at 600 MPa, except for the milk HP-treated for 5 min. Increased pressurizing time at all treatment pressures and temperatures resulted in only slight decrease in the amount of β-casein adsorbed on the surface layers of fat globules.

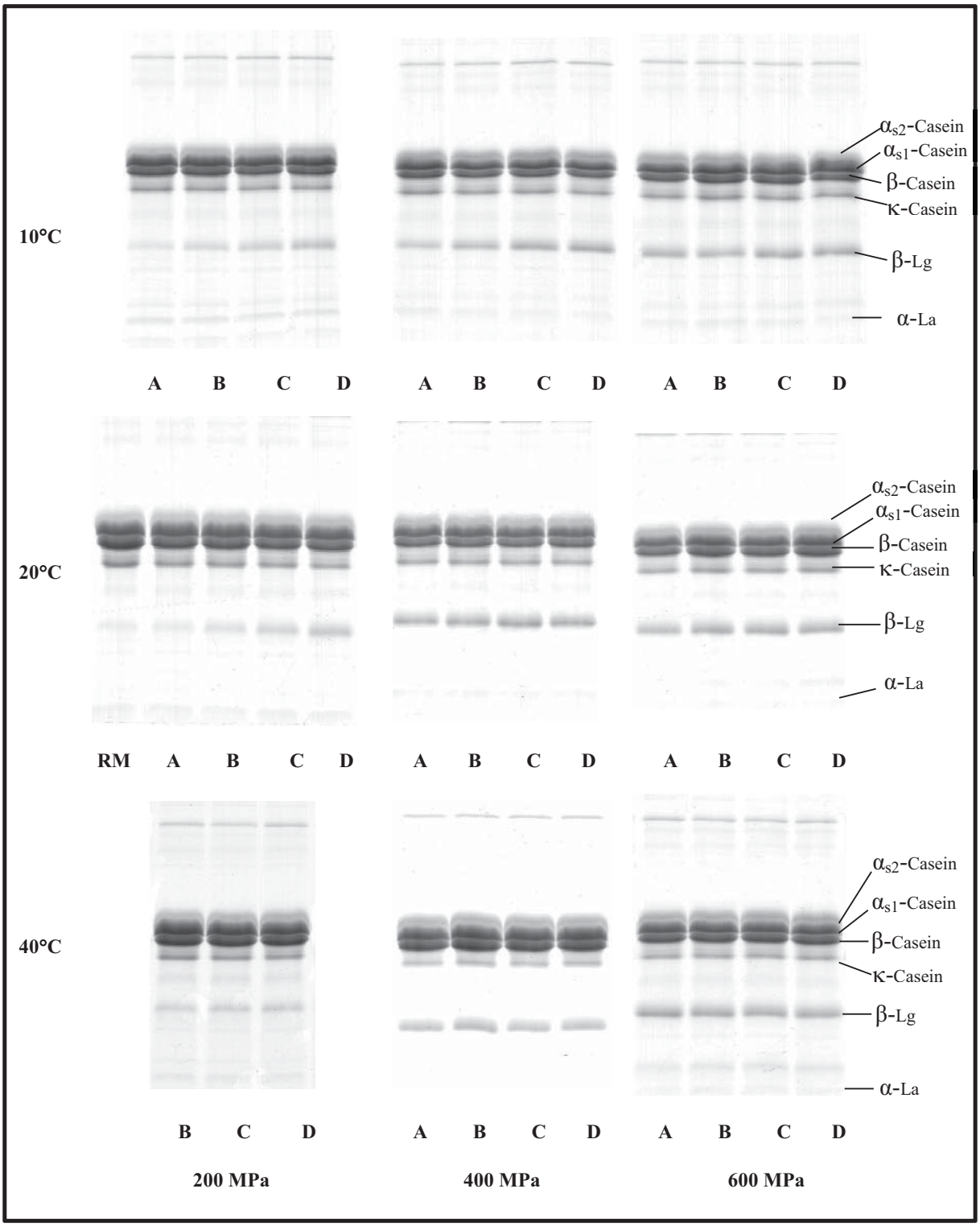


Figure 6.7. SDS-PAGE patterns of surface layers isolated from HP-treated recombined milk fat globules. HP-treatments were applied to recombined milks after homogenization at 10, 20 and 40°C for (A) 5, (B) 15, (C) 30, and (D) 60 min. RM represents the surface layers of fat globules of untreated recombined milk.

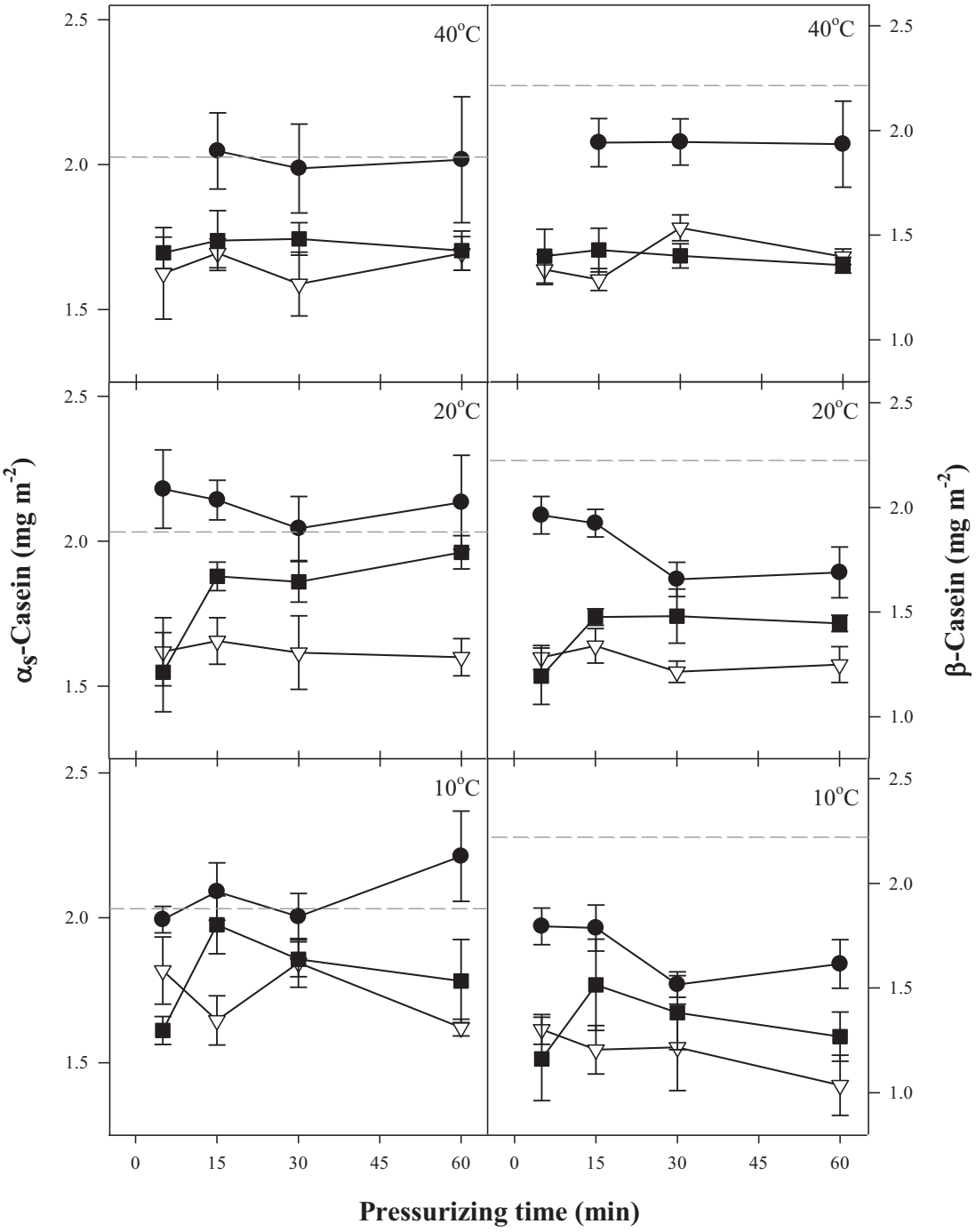


Figure 6.8. The amounts of α_s- and β-caseins on the surface layers of HP-treated recombined milk fat globules as a function of pressurizing times. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa. Dash lines represent the amounts of α_s- and β-caseins on the surface layers of fat globules isolated from untreated recombined milk.

Adsorbed κ-casein on surface layer of fat globule

All fat globules isolated from HP-treated recombined milks contained lower amount of κ-casein on the surface layers compared to those of untreated recombined milks. Recombined milk HP-treated at 200 MPa for all pressurizing times and temperatures had higher levels of κ-casein on the surface layers of fat globules compared to those HP-treated at 400 and 600 MPa. Recombined milks HP-treated at 400 and 600 MPa at 40°C had similar levels of κ-casein on the surface layers which were the lowest amount of κ-casein (~7%) on the surface layers of fat globules (Appendix B.3). This might be due to the dissociation of κ-casein from the micelles under HP treatment. κ-Casein locates on the surface of casein micelles which makes it easier to dissociate from the micelles under HP treatment (Considine, et al., 2007; Horne, 2006). The change in the adsorbed amount of κ-casein on the surface layers induced by HP treatment shows similar trend as those of α_s- and β-caseins.

Adsorbed β-Lg on surface layer of fat globule

All the fat globules isolated from HP-treated recombined milks had higher amount of adsorbed β-Lg compared with those of untreated recombined milks. The amount of this protein tended to increase as pressurizing time increased, except those HP-treated at 400 and 600 MPa. Recombined milks that underwent HP-treated at 200 MPa had lower amount of β-Lg on the surface layers of fat globules than those underwent HP-treated at 400 and 600 MPa. The higher amount of β-Lg was related to the higher denaturation level at a higher pressure, which resulted in greater interactions with κ-casein on the surface layers of HP-treated recombined fat globules. However, recombined milk HP-treated at 400 MPa at 20°C and 40°C had higher amount of this protein on the surface layer than those HP-treated at 600 MPa.

Adsorbed α-La on surface layer of fat globule

The amounts of α-La on the surface layers of fat globules isolated from recombined milks with and without HP treatments were very low. Recombined milks HP-treated at 200 - 600 MPa at 10 and 40°C for all pressurizing times had slightly higher levels of this protein compared to those of untreated recombined milks.

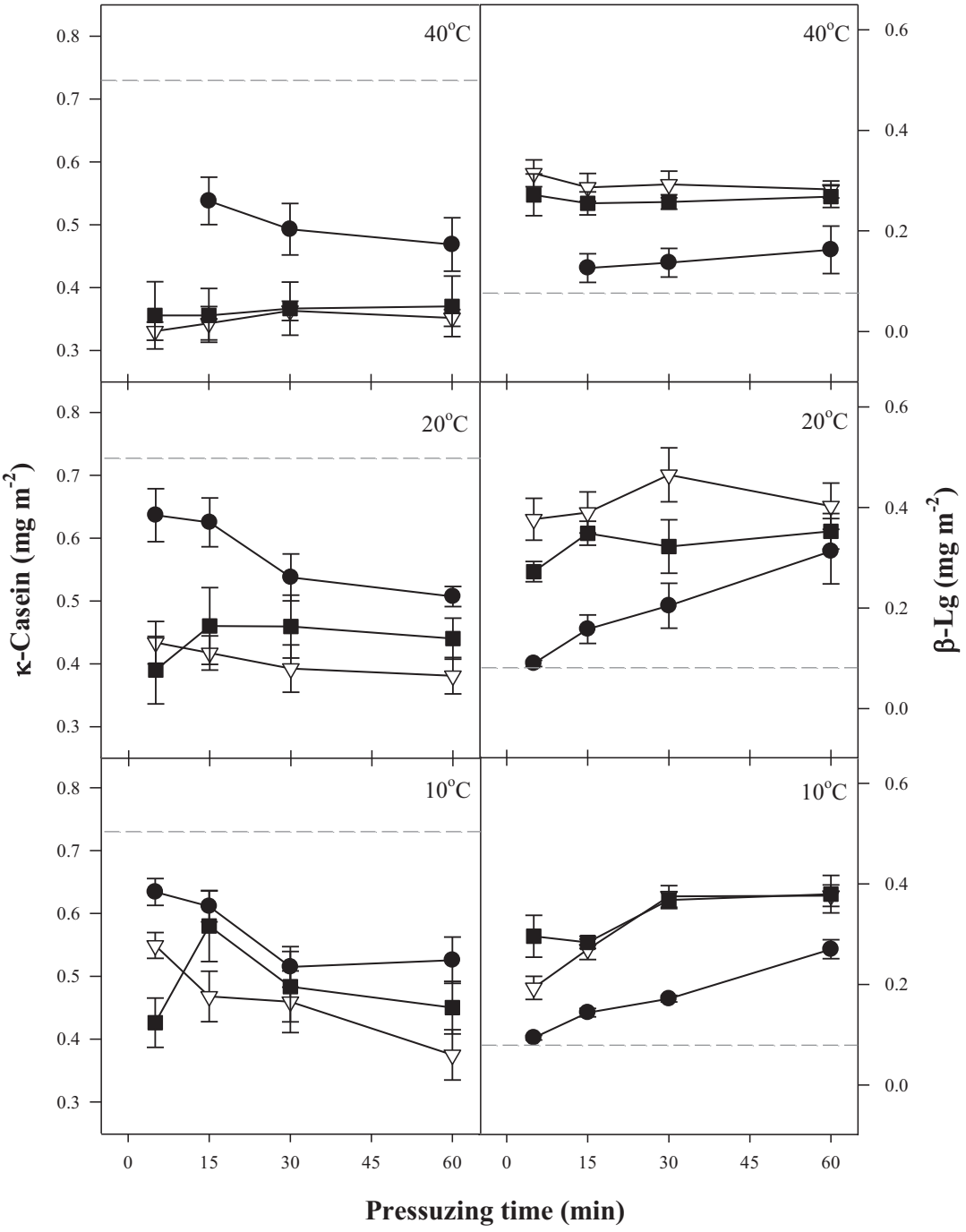


Figure 6.9. The amounts of κ-casein and β-Lg on the surface layers of fat globules isolated from HP-treated recombined milks as a function of pressurizing times. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa. Dash lines represent the amounts of κ-casein and β-Lg on the surface layers of fat globules isolated from untreated recombined milks.

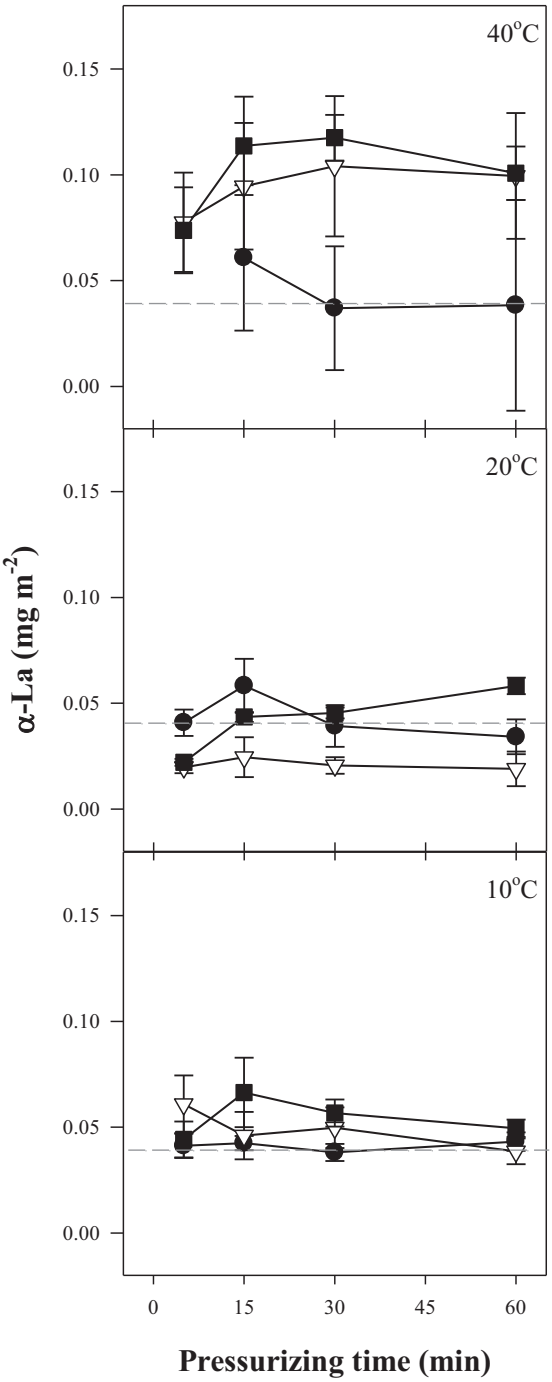


Figure 6.10. The amount of α-La on the surface layers of HP-treated recombined milk fat globules. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa. Dash lines represent the amount of α-La on the surface layers of fat globules isolated from untreated recombined milks.

HP treatment at 40°C and at 400 and 600 MPa resulted in more adsorbed α-La on the surface layers of fat globules. This was attributed to the denaturation of α-La at 600 MPa and the subsequent interactions with denatured β-Lg, forming aggregates which interacted with the adsorbed non-native casein particles via sulfhydryl groups on the surface of fat globules.

Figures 6.8 - 6.10 show that the surface layers of fat globules isolated from HP-treated recombined milks contained lower amount of β and κ-caseins, but higher amounts of β-Lg and α-La compared with those isolated from untreated recombined milk. Most of the fat globules isolated from HP-treated recombined milks contained lower amount of α_s-casein, except for those that underwent HP-treated at 200 MPa. In general, the fat globules of HP-treated recombined milks contained a slightly lower amount of caseins, but a higher amount of whey proteins. These findings suggest that the adsorbed caseins on the surface layers of recombined milk fat globules are dissociated under HP treatment and that some of the denatured whey proteins, induced by HP treatment, interacted with the non-native casein particles on the surface of fat globules.

To investigate the effects of HP placement on adsorbed proteins on the surface layers of fat globules, HP treatments at 200 - 600 MPa at 20°C for 30 min were applied to the milks before or after homogenization. The SDS-PAGE patterns under reducing conditions of surface layers isolated from fat globules of HP-treated recombined milks and the amount of proteins on the surface layers of fat globules as a function of treatment pressures are shown in Figures 6.11 and 6.12 respectively. Recombined milks HP-treated before or after homogenization composed of different amounts and proportions of caseins and whey proteins on the surface layers of fat globules (Figure 6.12, Appendix B.3). The amount of α_s-casein was significantly lower when HP treatments were applied before homogenization. At 200 and 600 MPa, recombined milks HP-treated before homogenization contained slightly higher β-casein than those of recombined milks HP-treated after homogenization. The levels of β-Lg in recombined milks HP-treated before homogenization were higher at 200 MPa, but lower at 400 - 600 MPa compared with those of recombined milks HP-treated after homogenization. At 400 MPa, recombined milk HP-treated before homogenization contained lower levels of β-casein and β-Lg, but similar level of α-La compared with recombined milk HP-treated after homogenization.

In reconstituted milk, the casein micelles and whey proteins are in native forms (discussed in Section 4.2.1, Chapter 4). Analogous to the effects of HP treatment on skim milk systems reported by Huppertz, Fox et al. (2006), applied HP treatment to reconstituted milk resulted in dissociation of casein micelles and denaturation of whey proteins. The extent of these effects depends on treatment pressure, temperature and time As a consequence, the HP-treated reconstituted milks contained non-native casein micelles (with the sizes smaller than those of untreated reconstituted milk), fragments of casein micelles (as some caseins dissociated from the micelles) and whey proteins (in both native and denatured aggregated forms). During homogenization, these proteins are adsorbed onto the surface layers of fat globules. The surface layers of fat globules isolated from recombined milk HP-treated before homogenization contained α_s-casein : β-casein : κ-casein : β-Lg in the ratio of 6 : 5 : 2 : 1 and 2.5 : 2.3 : 1 : 1 for milks HP-treated at 200 and 600 MPa respectively (Appendix B.3).

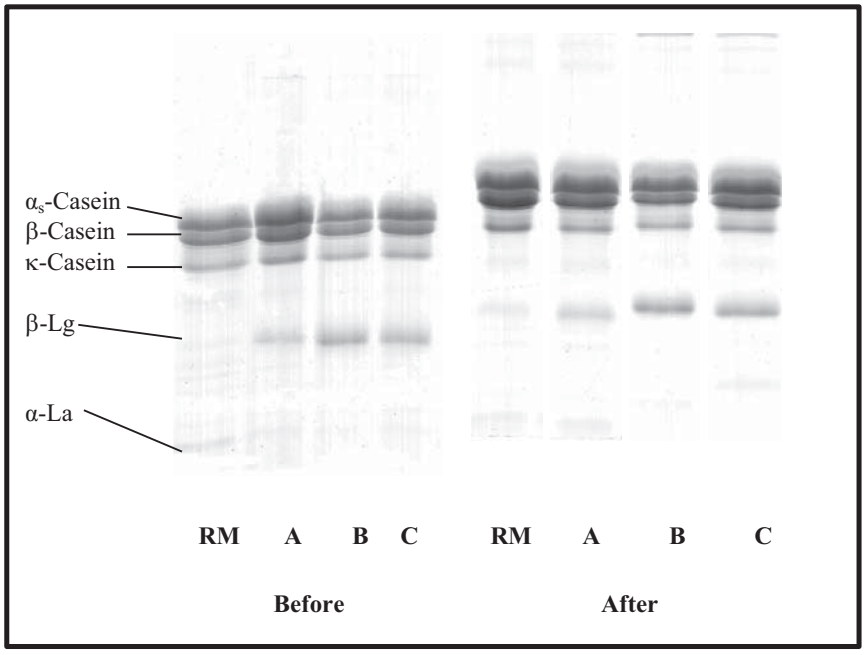


Figure 6.11. SDS-PAGE patterns of surface layers isolated from fat globules of HP-treated recombined milks. HP treatments were carried out before or after homogenization at 20°C for 30 min at (A) 200, (B) 400, and (C) 600 MPa. RM represents the surface layers of fat globules of untreated recombined milk.

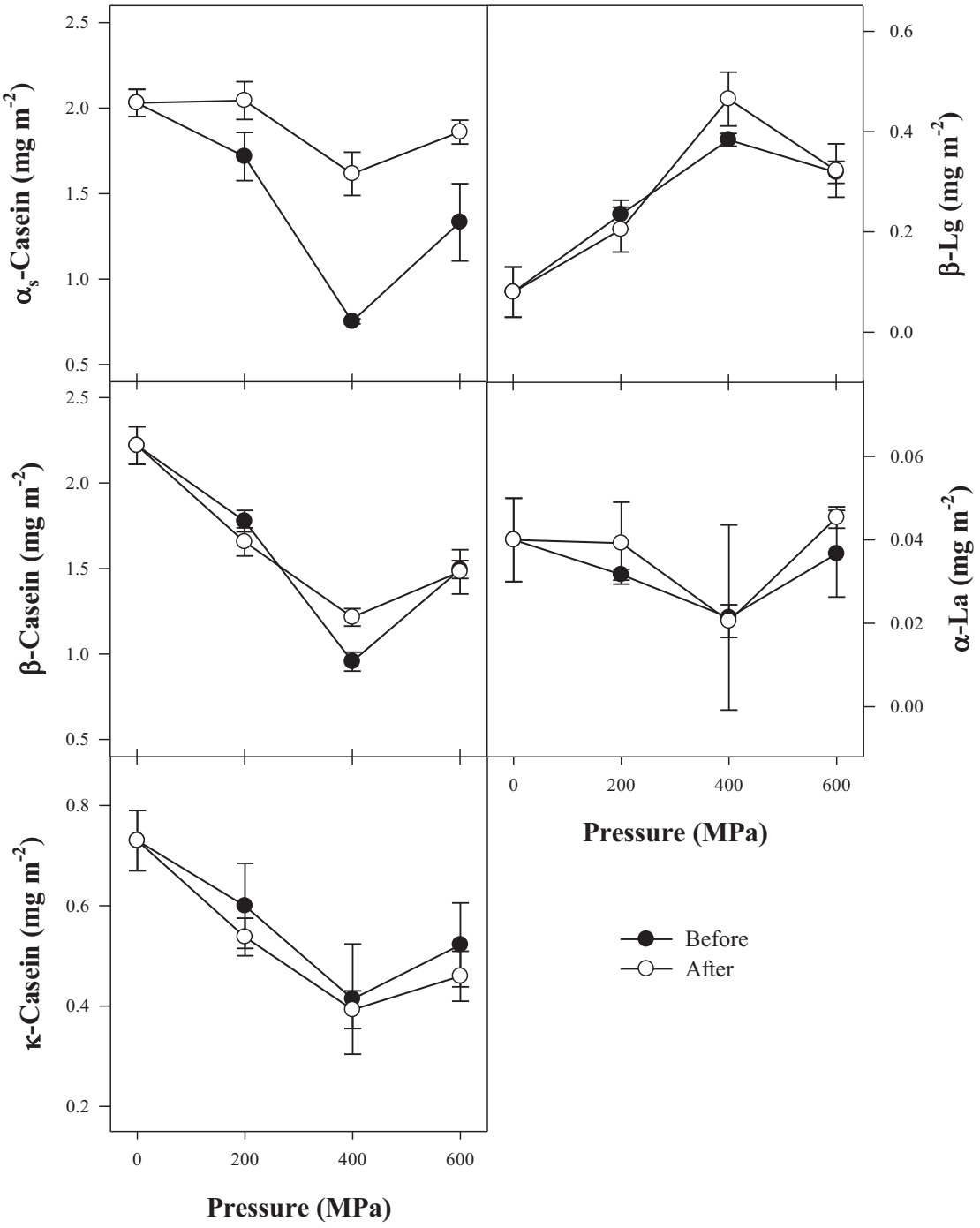


Figure 6.12. The amount of proteins on the surface layers of fat globules isolated from HP-treated recombined milks as a function of treatment pressures. HP treatments were applied to milks before or after homogenization at 200 - 600 MPa for 30 min at 20°C.

The amount of adsorbed α-La on the surface layers was very small. The proportion of β-Lg adsorbed onto the surface layers markedly increased as treatment pressure increased, suggesting the preferential adsorption of this protein, or adsorption of aggregates containing this protein on the surface layers of fat globules.

6.2.4. Proteins adsorbed directly at the interface of fat globules

To investigate the effects of HP treatment on the proteins that are adsorbed directly at the interface of recombined milk fat globules and the protein molecules that are bound to the interfacial protein layer via covalent bonds, the creams were washed with SMUF containing urea and EDTA. The SDS-PAGEs were run under reducing conditions. The SDS-PAGE patterns of protein at the interface of HP-treated recombined milk fat globules are shown in Figure 6.13. It is very interesting to find that applied HP treatment at 40°C to recombined milks after homogenization resulted in significantly lower level of proteins at the interface of fat globules compared to those underwent HP treatment at 20 and 10°C. At this temperature, the amounts of caseins (especially α_s- and β-caseins) at the interface were markedly decreased, whereas the amount of β-Lg was increased and distinct at this HP treatment. In order to compare the proteins adsorbed directly at the interface of fat globule, each individual protein was compared with that of untreated recombined milk as described in Section 3.2.4.6. The ratios of individual protein at the interface of fat globules as a function of pressurizing times are shown in Figures 6.14 - 6.16.

Adsorbed α_s-casein at the interface of fat globule

As shown in Figures 6.13 and 6.14, the amounts of α_s-casein (relative to the untreated recombined milk) at the interface of fat globules were higher in recombined milks HP-treated after homogenization at 200 MPa compared to those HP-treated at 400 and 600 MPa, except for that HP-treated at 200 MPa for 60 min at 20°C. Recombined milks HP-treated at 400 and 600 MPa did not have significant differences in the amounts of α_s-casein at the interface of fat globules. HP treatment at 40°C applied to recombined milks after homogenization markedly decreased the amount of α_s-casein at the interface of fat globule (Figure 6.13). Recombined milk HP-treated at 40°C at 400 and 600 MPa had very faint bands of α_s-casein.

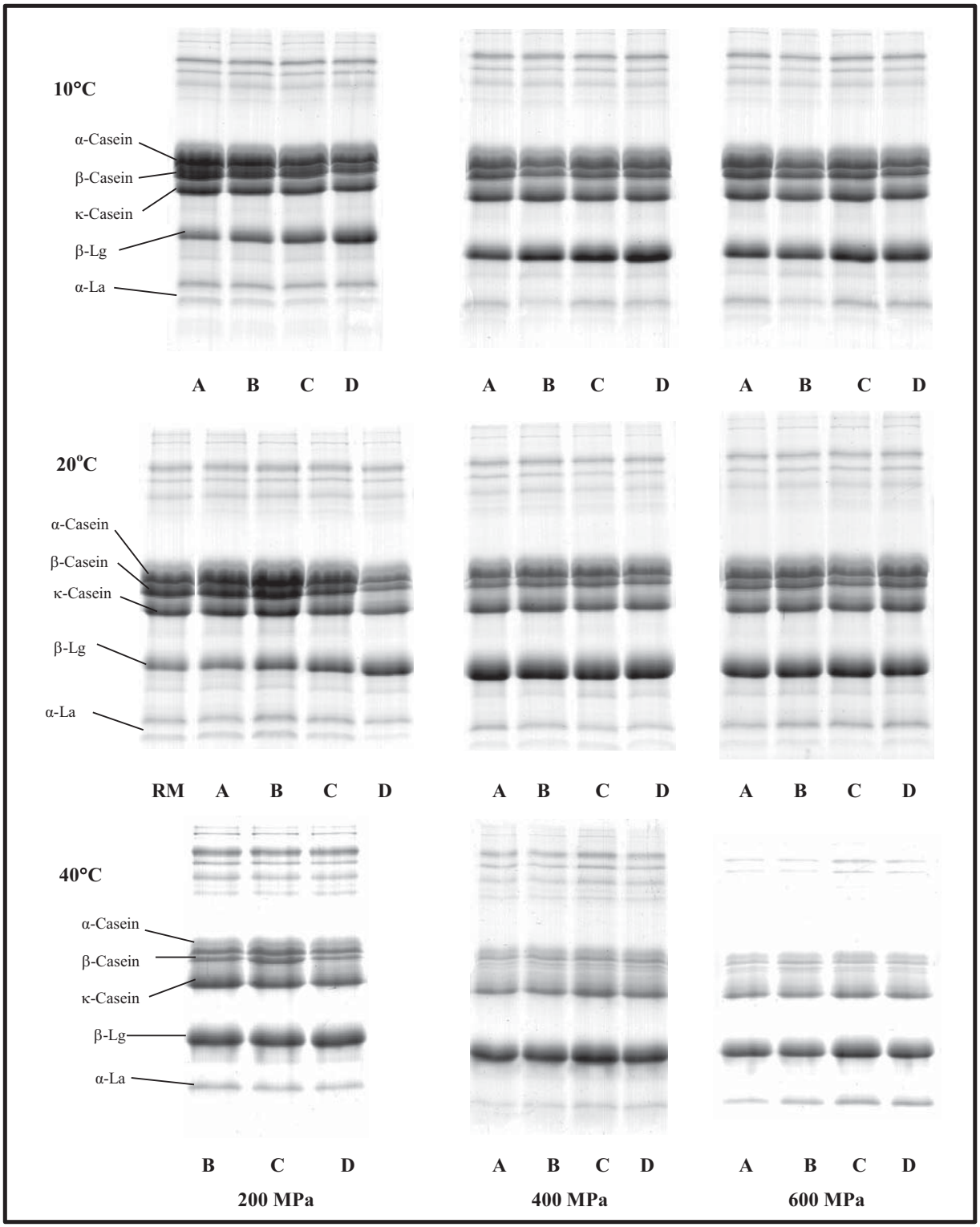


Figure 6.13. SDS-PAGE patterns under reducing conditions of proteins at the interface of fat globules isolated from HP-treated recombined milks. HP-treatments were carried out after homogenization for (A) 5, (B) 15, (C) 30, and (D) 60 min. RM represents the interface of fat globules isolated from untreated recombined milk.

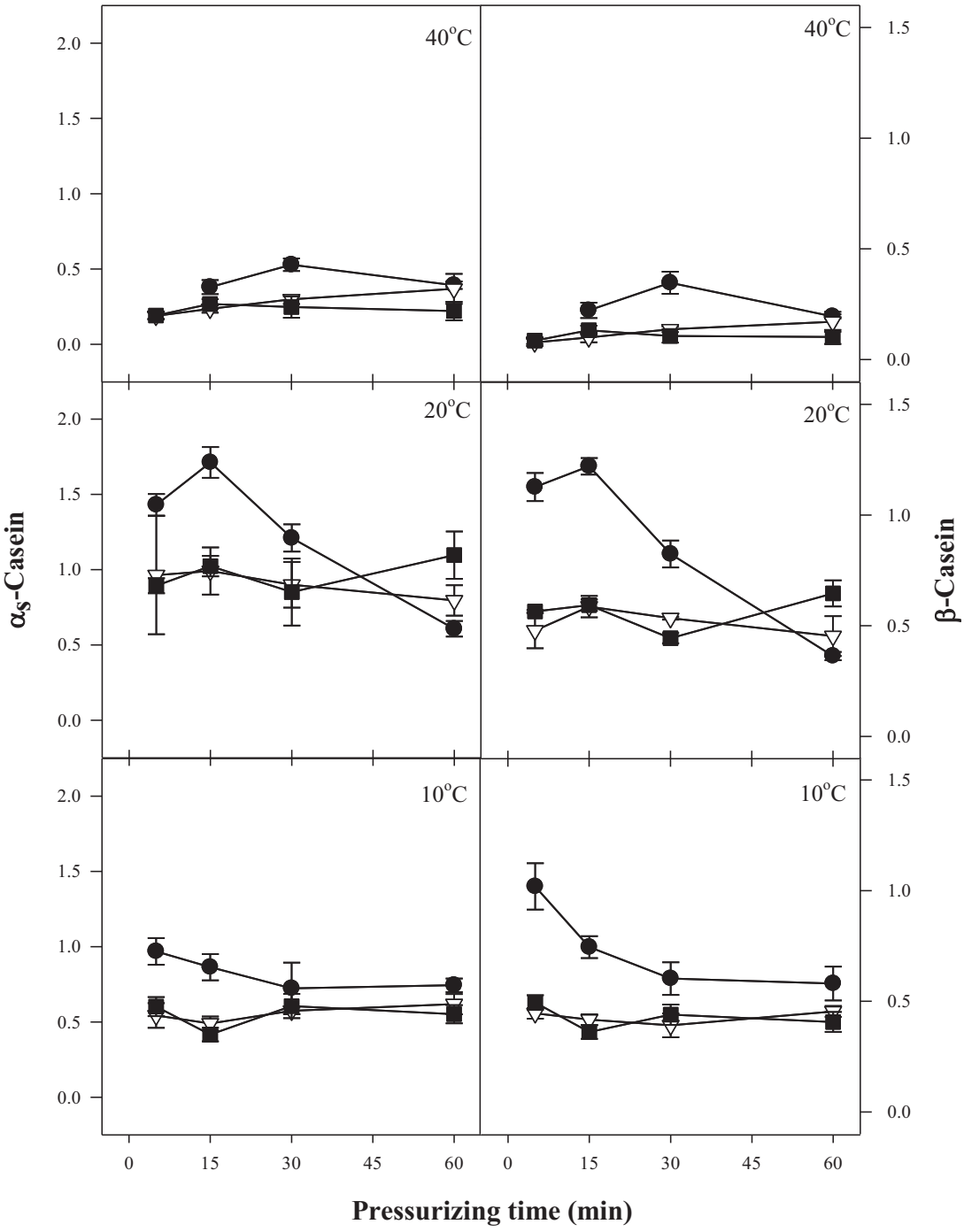


Figure 6.14. The ratio of α_s- and β-caseins at the interface of HP-treated recombined milk fat globules to those of untreated recombined milk as a function of pressurizing time. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa.

Adsorbed β-casein at the interface of fat globule

HP-treated recombined milks have lower amount of β-casein at the interface of fat globules than that of untreated recombined milk, excepted for those HP-treated at 200 MPa at 20°C for 5 and 15 min and those HP-treated at 200 MPa at 10°C for 5 min. HP treatment at 200 MPa resulted in higher amount of β-casein at the interface of fat globules compared to those HP-treated at 400 and 600 MPa. Recombined milk HP-treated at 400 MPa and 600 MPa did not have significantly different amounts of β-casein at the interface of fat globules.

Adsorbed κ-casein at the interface of fat globule

HP-treated recombined milks had lower amounts of κ-casein than those of untreated recombined milks, except for the milks HP-treated at 200 MPa for ≤ 15 min at 20°C. Figure 6.15 shows that treatment temperature had a significant effect on the amount of this protein at the interface of fat globules. Recombined milks HP-treated at 40°C had lower amount of κ-casein at the interface of fat globules compared to other HP-treated recombined milks. At 10°C, treatment pressure did not have a significant effect on the amount of κ-casein at the interface...

Adsorbed β-Lg at the interface of fat globule

All HP-treated recombined milks had higher levels of β-Lg at the interface of fat globules compared with that of untreated recombined milk (figure 6.13). Increased pressurizing time at all treatment pressures resulted in increased levels of β-Lg at the interface of the fat globules. At 10°C and 20°C, HP treatment at 400 and 600 MPa resulted in similar levels of β-Lg at the interface of fat globules, which were higher than those HP-treated at 200 MPa. However, at 40°C HP treatments at 400 MPa resulted in higher levels of β-Lg at the interface of fat globules than those of milk HP-treated at 200 and 600 MPa. The higher level of β-Lg at the interface of fat globules was attributed to the denaturation of β-Lg induced by HP treatment and the interactions with the non-native casein micelles at the interface of fat globules.

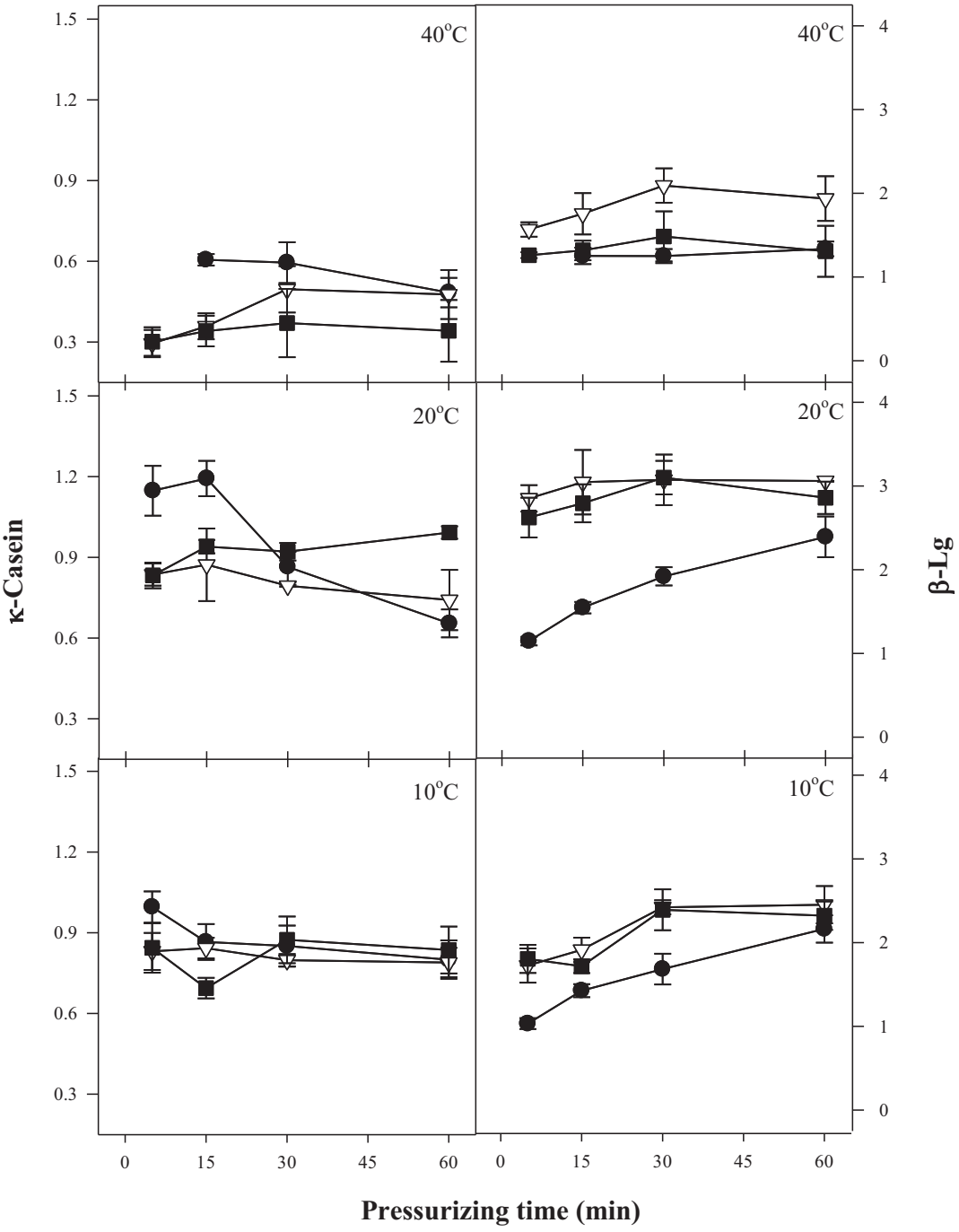


Figure 6.15. The ratio of κ-casein and β-Lg (compared with those of untreated recombined milk fat globules) at the interface of HP-treated recombined milk fat globules. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa.

Adsorbed α-La at the interface of fat globule

The amounts of α-La at the interface of fat globules of recombined milks HP-treated at 10°C were slightly lower than those of untreated recombined milk. At 20°C, the amount of this protein was higher at HP treatment of 200 MPa for 5 - 30 min and at 600 MPa for 30 min compared to that of untreated recombined milk. At 400 MPa the amount of α-La was lower than those of untreated recombined milks. Recombined milk HP-treated at 40°C had lower levels of α-La at the interface, except for those HP-treated for ≥ 30 min. The denaturation of α-La occurred at HP treatment of 600 MPa and the denaturation level was higher at higher treatment temperature (Figure 6.2). The denatured α-La interacted with β-Lg and probably included in the β-Lg-casein aggregates at the interface of HP-treated fat globules. As the levels of α-La was very low, so small changes may be reflected as large difference in the ratio of this protein adsorbed at the interface of fat globules compared to that of untreated recombined milk.

The SDS-PAGE patterns of individual proteins at the interface of fat globules isolated from recombined milks HP-treated before or after homogenization at 20°C for 30 min are shown in Figure 6.17. The relationship between individual proteins adsorbed at the interface of fat globules is shown in Figure 6.18. The proteins were measured as a ratio relative to the same protein in the untreated recombined milk (control). There were slight differences in the proportion of individual proteins at the interface of HP-treated recombined milk fat globules. Recombined milks HP-treated before homogenization at all treatment pressures resulted in lower levels of α_s- and κ-caseins and α-La, but higher level of β-Lg at the interface of fat globules than those HP-treated after homogenization. The amount of β-casein at the interface of fat globules was lower in recombined milks HP-treated before homogenization at 200 - 400 MPa, but higher at 600 MPa compared with those HP-treated after homogenization. These results showed that the placement of HP treatment had an influence on proteins adsorbed at the interface of fat globules and on the interactions between milk proteins.

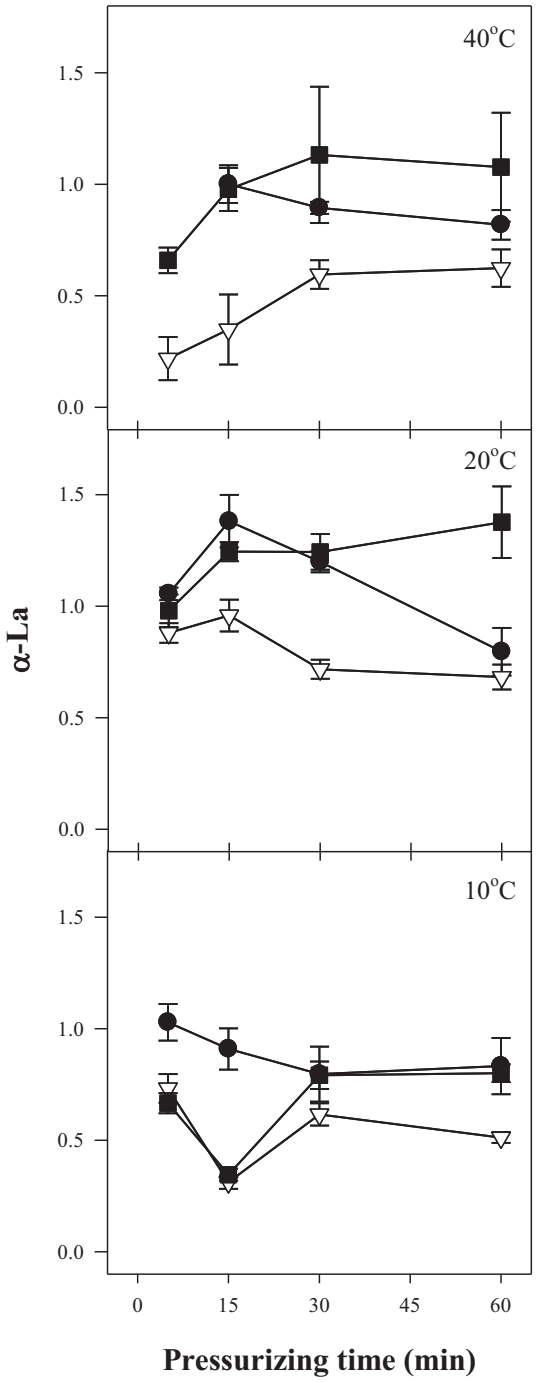


Figure 6.16. The ratio of α-La (compared to that of untreated recombined milk fat globules) at the interface of HP-treated recombined milk fat globules. HP treatments were carried out after homogenization at (●) 200, (▽) 400, and (■) 600 MPa.

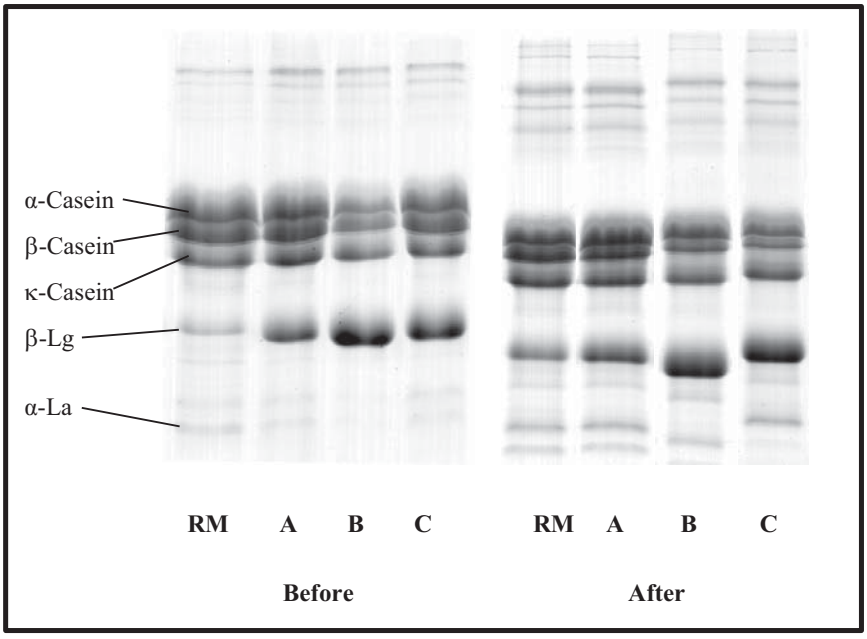


Figure 6.17. SDS-PAGE patterns under reducing conditions of proteins at the interface of fat globules isolated from HP-treated recombined milks. Creams were washed with SMUF containing dissociating agent. HP-treatments were carried out either before or after homogenization at 20°C for 30 min at (A) 200, (B) 400, and (C) 600 MPa. RM represents proteins at the interface of fat globules isolated from untreated recombined milk.

6.2.5. Possible structures of proteins and fat globules in HP-treated recombined milk

6.2.5.1 Recombined milk HP-treated before homogenization

In reconstituted milk, the casein micelles and the whey proteins are in native forms (as discussed in Section 4.2.1, Chapter 4). Their change in size and structure induced by HP treatment is different from those induced by heat treatment (Anema, 2008b; Patel & Creamer, 2009). Under HP treatment, the colloidal casein micelles disrupt and rearrange, resulting in the formation of non-native colloidal particles with the size dramatically smaller than those of native casein micelles in untreated milk (Considine, et al., 2007). The extent of the disruption depends on the treatment pressure and to a lesser extent, on the holding time (Anema, Lowe, et al., 2005) and the extent has been reported to be β-casein > κ-casein > α_{s1}-casein > α_{s2}-casein (López-Fandiño, et al., 1998).

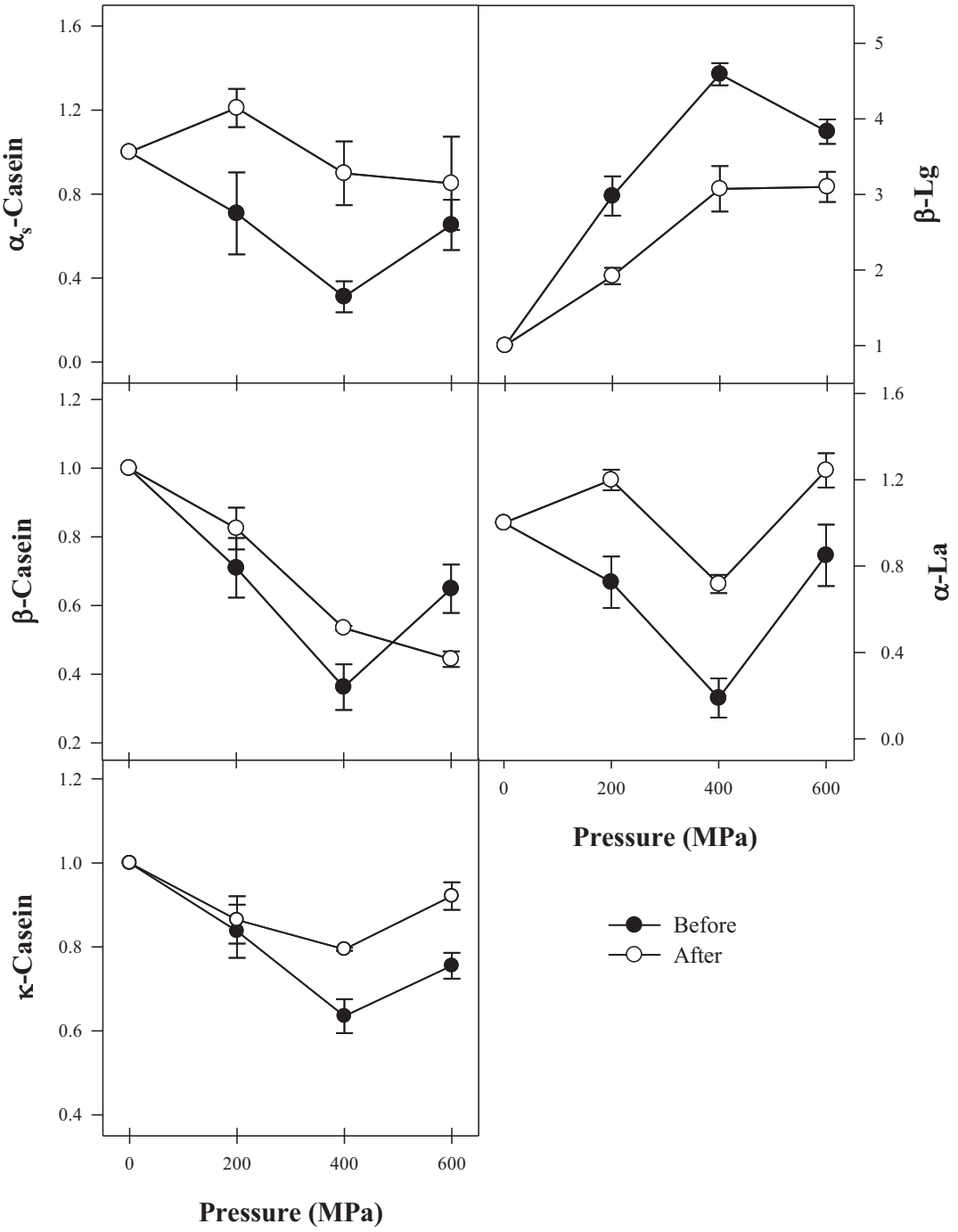


Figure 6.18. The ratio of individual proteins (compared to those of untreated recombined milk) at the interface of HP-treated recombined milk fat globules. HP treatments were carried out before or after homogenization at 200 - 600 MPa for 30 min at 20°C.

The disruption of the casein micelles is due to the dissolution of colloidal calcium phosphate (CCP) and the reduction of a hydrophobic effect under HP conditions (Huppertz, Fox, et al., 2004c; Huppertz, Fox, et al., 2004d; Needs, Capellas, et al., 2000; Needs, Stenning, et al., 2000; Regnault, Thiebaud, Dumay, & Cheftel, 2004; Schrader & Buchheim, 1998). Most of the non-native colloidal particles are formed during depressurization when the hydrophobic effects and mineral equilibrium state are rapidly returned to those of milk under ambient pressure (Hubbard, Caswell, Lüdemann, & Arnold, 2002; Huppertz, Kelly, et al., 2006). The casein micelles that have been dissociated under HP treatment do not reform to their original sizes, but remain as fragments or as partially re-aggregated particles (Anema, Lowe, et al., 2005; Dalgleish, 2011). Anema (2010b) observed very small particles in HP (400 MPa/30 min)-treated skim milks, and these particles were markedly smaller (< 50 nm) than the micelles observed in the untreated and heated (90°C/30 min) skim milks.

Limited information is available on the structure of colloidal particles formed during HP treatment of milk. It is possible that the CCP-like materials link the caseins through their serine phosphate groups and hydrophobic interactions between the hydrophobic regions of the casein proteins occur (Anema, 2010b) in other words, the "dual-binding model". The κ-casein is located on the surface and stabilizes the colloidal particles formed in HP-treated skim milks (Hubbard, et al., 2002; Huppertz & de Kruif, 2007). As the casein micelles in HP-treated skim milk are redistributed into a large number of small particles (Gaucheron, et al., 1997; Needs, Stenning, et al., 2000; Regnault, et al., 2004), the total surface area of the colloidal particles will be markedly increased (Anema, 2010b). As a consequence, the surface characteristics of the particles will be different and the density of κ-casein at the surface should be considerably lower than those of native casein micelles. Under HP treatment, in which hydrophobic interactions are reduced (Hummer, et al., 1998; Huppertz, Smiddy, Upadhyay, & Kelly, 2006) and the solubility of calcium and phosphate is increased (Huppertz, Fox, et al., 2006; Pouliot, Boulet, & Paquin, 1989), the whey proteins are denatured. The protein unfolding and the subsequent disulfide-bond interchanges induced by HP treatment are significantly different from those of heat treatment, resulting in different composition and structure and smaller size of aggregated whey proteins (Anema, Lauber, et al., 2005; Considine, et al., 2007; Huppertz, Fox, et al., 2004c; Patel, et al., 2006). In this study, β-Lg and α-La are denatured at 200 and 600 MPa respectively (as shown in

Figure 6.2). Most denatured β-Lg in HP-treated milk is associated with the casein micelles (Huppertz, Fox, et al., 2004c; López-Fandiño, et al., 1996; Zobrist, et al., 2005), probably through disulphide-sulphydryl-interchange reactions (Huppertz, Fox, et al., 2004b). Due to micellar fragmentation, in HP-treated milk β-Lg formed adducts with either κ- or α_{s2}-caseins, whereas in heated milk, β-Lg reacted with κ-casein (Considine, et al., 2007). Denatured β-Lg in HP-treated milk is found in various forms that are equivalent to those found in heated milk. The majority of denatured β-Lg in HP-treated skim milk are associated with the casein micelles, with a small proportion remaining non-sedimentable, either in the form of whey protein aggregates or associated with casein particles too small to be sediment (Huppertz, Fox, et al., 2004c).

Studies on skim milk systems have reported that HP treatment at 200 MPa at ambient temperature has little effect on the casein micelles size (Anema, Lowe, et al., 2005; Desobry-Banon, et al., 1994; Huppertz, Fox, et al., 2004c; Needs, Stenning, et al., 2000; Regnault, et al., 2004). Therefore, according to the size of native casein micelles in skim milk reported by Dalgleish (2011), the size of non-native casein particles in reconstituted milk HP-treated at 200 MPa at 20°C were in the ranges of 80 - 400 nm, with the average of 200 nm. However, the internal structure and the surface of non-native casein particles were expected to be different from those of untreated casein micelles as the caseins were dissociated and reassociated during HP treatment. At this pressure, approximately 10% whey proteins (only β-Lg) in reconstituted milk were denatured (Figure 6.2, Appendix B.1). The denatured β-Lg unfolded and formed disulphide-bonded dimers and probably aggregated with κ-casein, without forming larger β-Lg aggregates. At this pressure, α-La was not denatured and was not included in the aggregates. The illustration of caseins and whey proteins in reconstituted milk HP-treated at 200 MPa is shown in Figure 6.19. According to studies on skim milk systems, HP treatment at pressure higher than 300 MPa at ambient temperature reduces casein micelles size by ~50% (Anema, Lowe, et al., 2005; Desobry-Banon, et al., 1994; Huppertz, Grosman, Fox, & Kelly, 2004; Needs, Stenning, et al., 2000; Regnault, et al., 2004), therefore the non-native casein particles in reconstituted milk HP-treated at 400 - 600 MPa had average size of 100 nm. As shown in Figure 6.2, at these pressures, 50 - 70% β-Lg denatured. Under severe HP treatment (400 - 600 MPa), the denatured β-Lg polymerized into larger aggregates and α-La were incorporated into the aggregates as

this protein denatured at 600 MPa (Patel, 2007). Thus, the reconstituted milk HP-treated at 400 - 600 MPa at 20°C contained non-native casein particles with the size ~100 nm, fragment of caseins (i.e., dissociated caseins), denatured whey protein (mostly β-Lg)-casein aggregates and large size of denatured whey protein aggregates, which are illustrated in Figure 6.20.

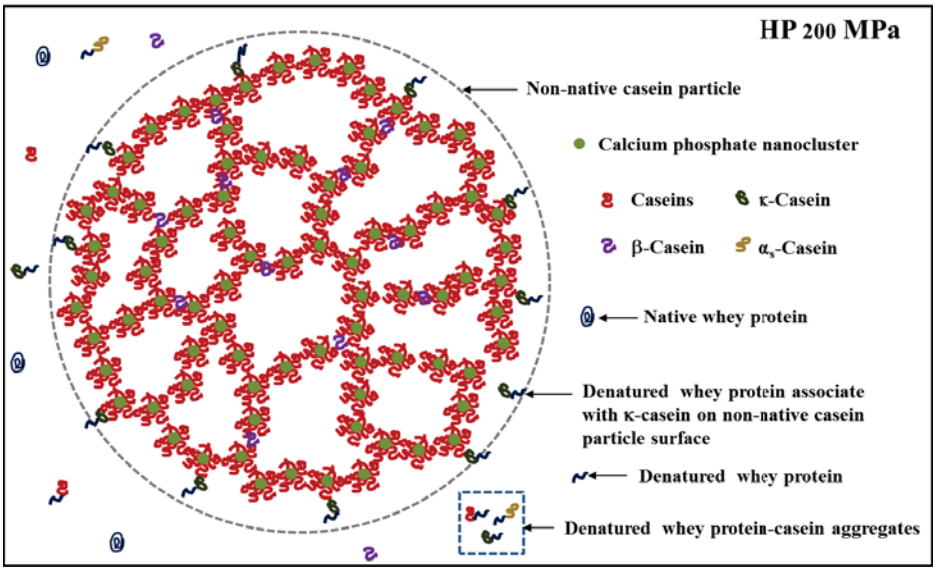


Figure 6.19. Illustration of caseins and whey proteins in reconstituted milk HP-treated at 200 MPa. The relative sizes of the individual components are not to scale.

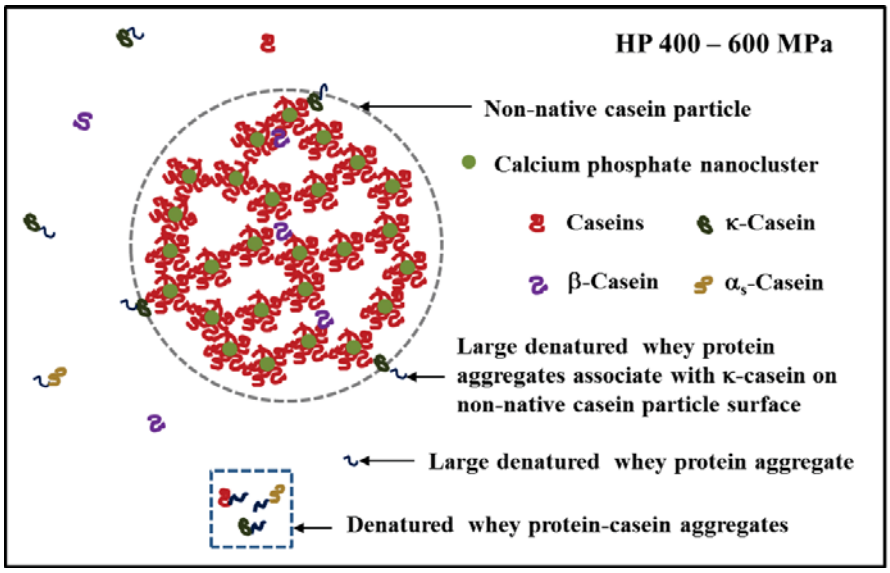


Figure 6.20. Illustration of casein particles and whey proteins in reconstituted milk HP-treated at 400 - 600 MPa. The relative sizes of the individual components are not to scale.

When HP-treated reconstituted milk was recombined with milk fat, the non-native casein particles, fragment of caseins (dissociated caseins) and denatured whey proteins aggregates were adsorbed onto the surface layers of fat globules. The amounts of caseins and whey proteins adsorbed depended on treatment pressure, temperature and time (as discussed in Section 6.2.3). Recombined milk HP-treated at 200 MPa for 30 min at 20°C before homogenization had approximately 92% caseins and 8% whey proteins on the surface layers of fat globules (Appendix B.3). The interface of fat globules contained caseins and whey proteins ~77% and ~23% respectively (Appendix B.4). At this pressure, the caseins presented as non-native casein particles with the average size of native casein micelles (~200 nm), based on the findings on skim milk systems reported by Dalgleish, (2011) and approximately 10% of whey proteins (only β-Lg) are denatured (Figure 6.2). Most of the denatured whey proteins were expected to associate with κ-casein on the surface of non-native casein particles, only small amounts were associated with κ-casein that had been dissociated from native casein micelles into the serum or were interacted with each other. The caseins, whey proteins and fat globules in recombined milk HP-treated at 200 MPa at 20°C before homogenization are illustrated in Figure 6.21.

In similar manner, when reconstituted milks HP treated at 400 - 600 MPa were recombined with milk fat, the non-native casein particles, the fragment of caseins (i.e., dissociated caseins) and denatured whey proteins induced by HP treatment were adsorbed onto the surface layers of fat globules during homogenization. As shown in Figures 6.8 - 6.9, the amount of caseins adsorbed at the surface layer decreased, whereas the amount of adsorbed whey proteins increased as treatment pressure increased, suggesting that the smaller size of caseins were adsorbed and more denatured whey proteins were associated with caseins on the surface layers of fat globules. Base of the reported in skim milk systems (Patel, 2007), denatured whey proteins in reconstituted milk HP-treated at 400 – 600 MPa before homogenization were expected to polymerize into the larger aggregates and subsequently adsorbed onto the surface layers of fat globules, as a consequence the amount of whey proteins on the surface layer increased. The amount of caseins and whey proteins adsorbed on the surface layer of the fat globule of recombined milk HP-treated before homogenization at 600 MPa for 30 min at 20°C were 85% and 15% respectively. The caseins and whey proteins occupied about 70% and 30% of the interface area of fat globules respectively (Appendix B.4).

The possible mechanism of recombined milk HP-treated at 600 MPa before homogenization is shown in Figure 6.22.

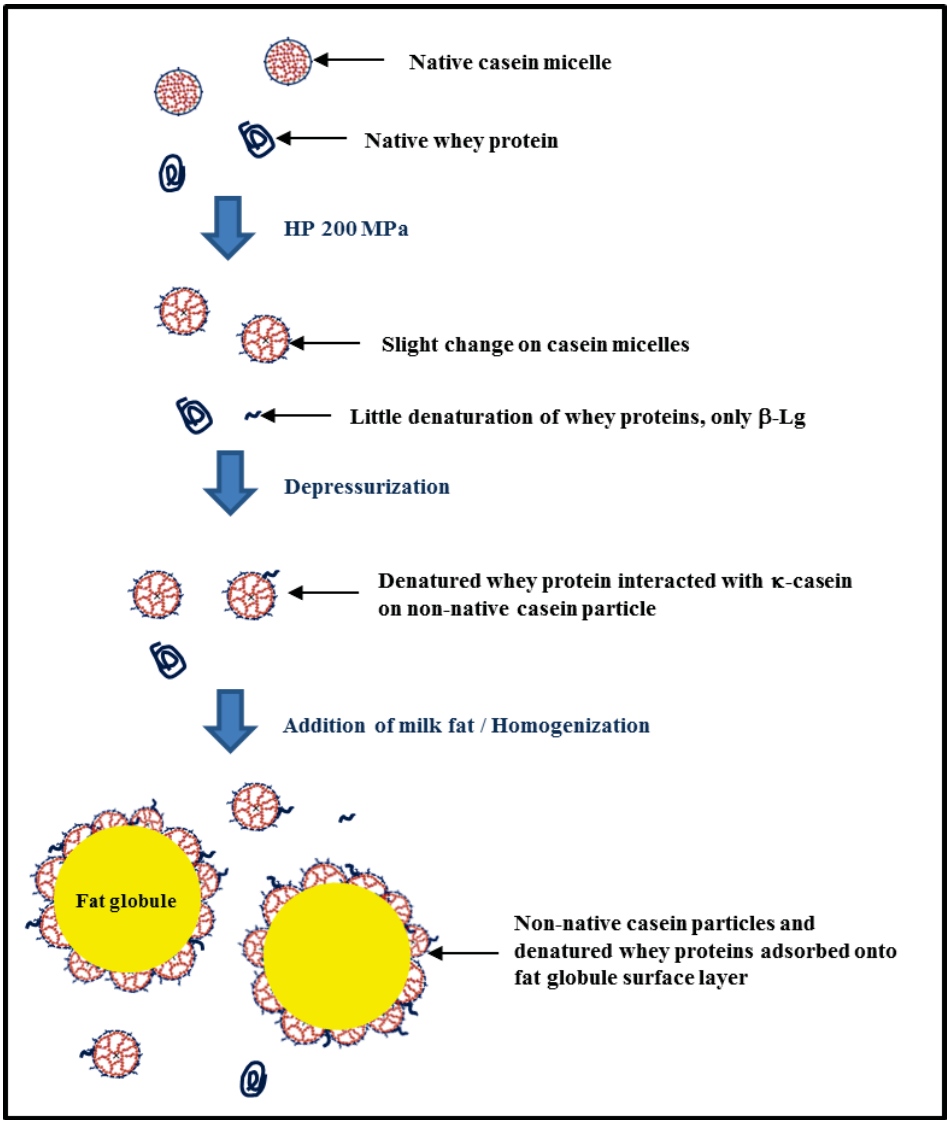


Figure 6.21. Schematic illustration of recombined milk undergoing HP treatment at 200 MPa before homogenization. The relative sizes of the individual components are not to scale.

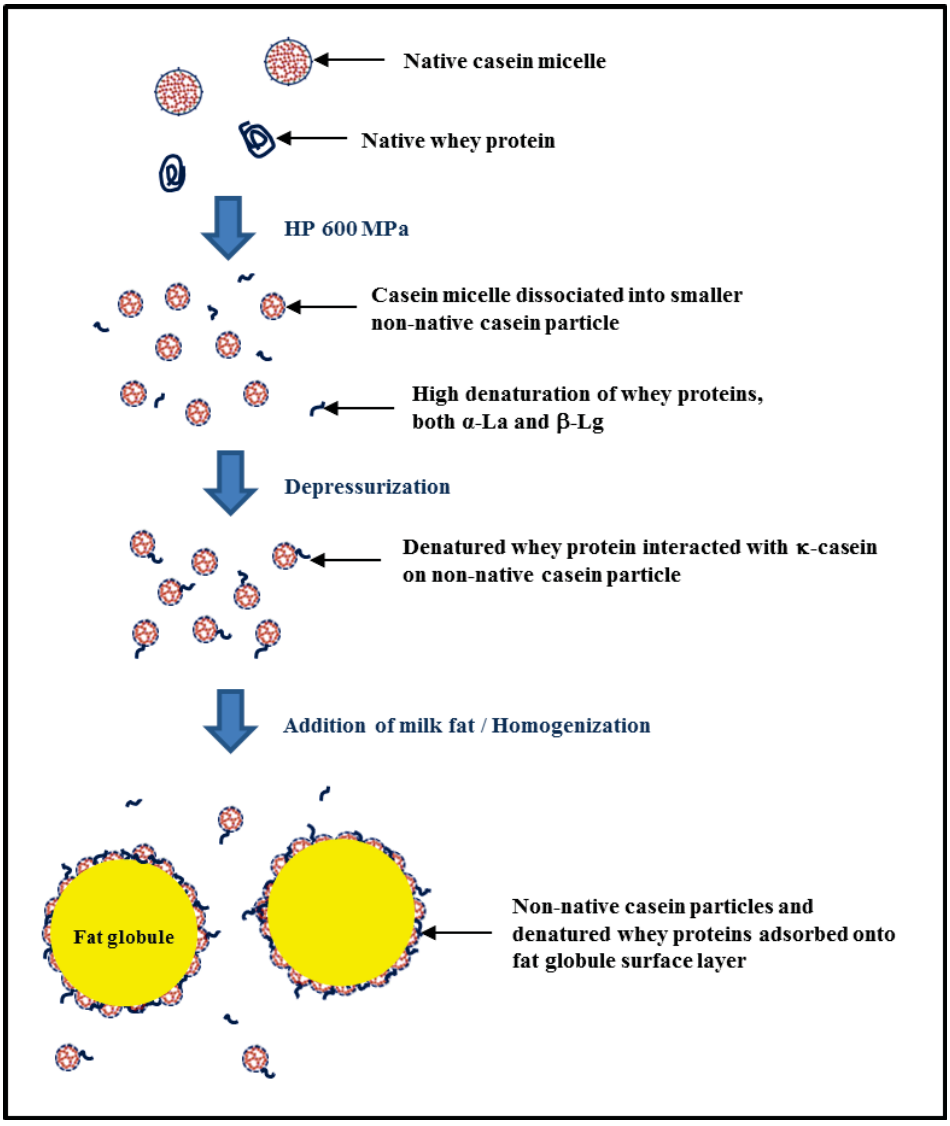


Figure 6.22. Schematic illustration of recombined milk undergoing HP treatment at 600 MPa before homogenization. The relative sizes of the individual components are not to scale.

6.2.5.2 Recombined milk HP-treated after homogenization

In untreated recombined milk, mainly caseins (~98%) and small amount of whey proteins (~2%) are adsorbed onto the surface layers of fat globules (discussed in Chapter 4, Figure 4.3). Most of whey proteins were present in the serum in native forms. When recombined milk was HP-treated, the caseins both in the serum and on the surface layers of fat globules was expected to dissociate and associate into non-native casein particles and the whey proteins denatured. The extent to which these occurred depended on the treatment pressure, temperature and holding time. As a result, the

caseins on the surface layers, i.e., non-native casein particles, were smaller in size and their internal structures different from those of untreated recombined milk. The denatured whey proteins formed associations with non-native casein particles, dissociated caseins and with other denatured whey proteins (Patel, et al., 2006). As a consequence, the amount of adsorbed whey proteins on the surface layers of the fat globules increased as the severity of HP treatment increased. According to Section 6.2.3 (Chapter 6), recombined milk HP-treated at 200 MPa for 30 min at 20°C after homogenization contained ~94% caseins and ~6% whey proteins on the surface layers of fat globules, and approximately 74% caseins and 26% whey proteins at the interface of fat globule (Appendix B.3 and B.4). As a result of HP treatment, the non-native casein particles were present as mono-layer on the fat globule surface layers and it was likely that the fat globules act as large casein micelles. Recombined milk HP-treated before homogenization had similar amounts of proteins (except α_s-casein) adsorbed onto the fat globule surface layers as recombined milk HP-treated after homogenization (Figure 6.12), but the proteins adsorbed at the fat globule interfaces were significantly lower (Figure 6.18). The illustration of the possible changes on recombined milk HP-treated after homogenization at 200 MPa is shown in Figure 6.23.

At 400 – 600 MPa, the non-native casein particles size decreased to about 50% of the native casein micelles; more caseins were dissociated from the micelles and the denatured whey proteins were polymerized into larger whey protein aggregates. The dissociation of caseins happened both in the serum and on the fat globules surface layers. Before depressurization the protein layer at the fat globule surface was very thin, however, after depressurization the protein layer became much thicker as a result of reassociation of caseins and also aggregation of denatured whey proteins on the fat globule surface layer. The illustration of caseins, whey proteins and fat globules in recombined milk HP-treated at 400 - 600 MPa after homogenization is shown in Figure 6.24.The surface layers of fat globules isolated from recombined milk HP-treated at 600 MPa for 30 min contained 88% casein and 12% whey proteins respectively (Appendix B.3). The interface of fat globules was composed of 56% caseins and 44% whey proteins respectively (Appendix B.4). These results suggested that more whey proteins are preferably adsorbed at the interface of fat globules as the severity of HP treatment increased.

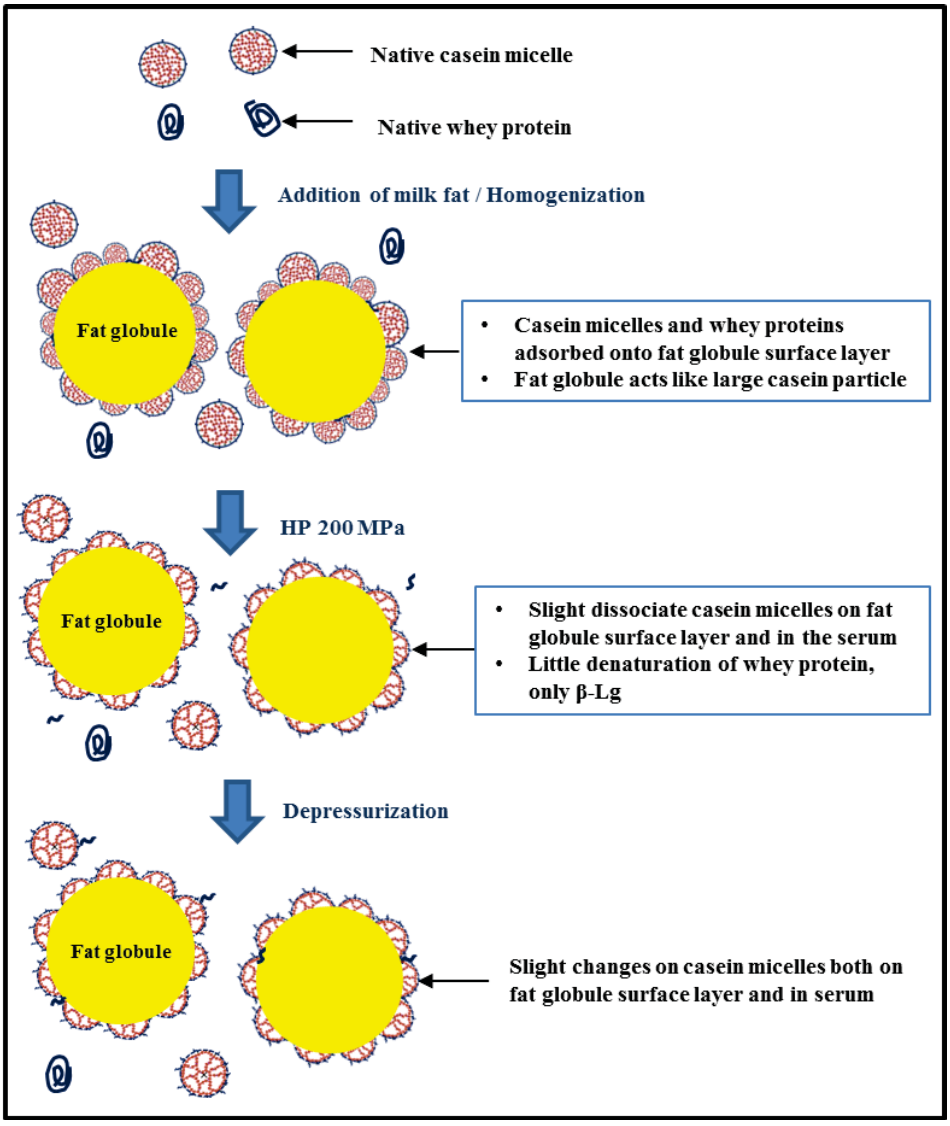


Figure 6.23. Schematic illustration of recombined milk undergoing HP treatment at 200 MPa after homogenization. The relative sizes of the individual components are not to scale.

6.3. Conclusions

Similar to skim milk systems, under HP treatment the casein micelles (both in the serum and on the surface layer of fat globules) dissociated and reassociated into non-native casein particles and the whey protein denatured. Increasing treatment pressure, temperature and time caused increases in whey protein denaturation, and whey proteins adsorbed onto the surface layers of fat globules of recombined milks. HP treatments applied to the milk either before or after homogenization produced different surface layers of fat globules, which in turn affect the characteristics of recombined milk.

However, the denaturation levels of whey proteins were similar in both milks. The interactions between surface layers of fat globules and milk proteins were different in recombined milks HP-treated before or after homogenization. Particle size of fat globules was not affected by HP treatment, indicating no coalescence, but increase in size was observed when dispersed in water, suggesting aggregation or bridging between proteins coated fat globules. The particle sizes were similar when HP treatments were applied to milk samples before or after homogenization.

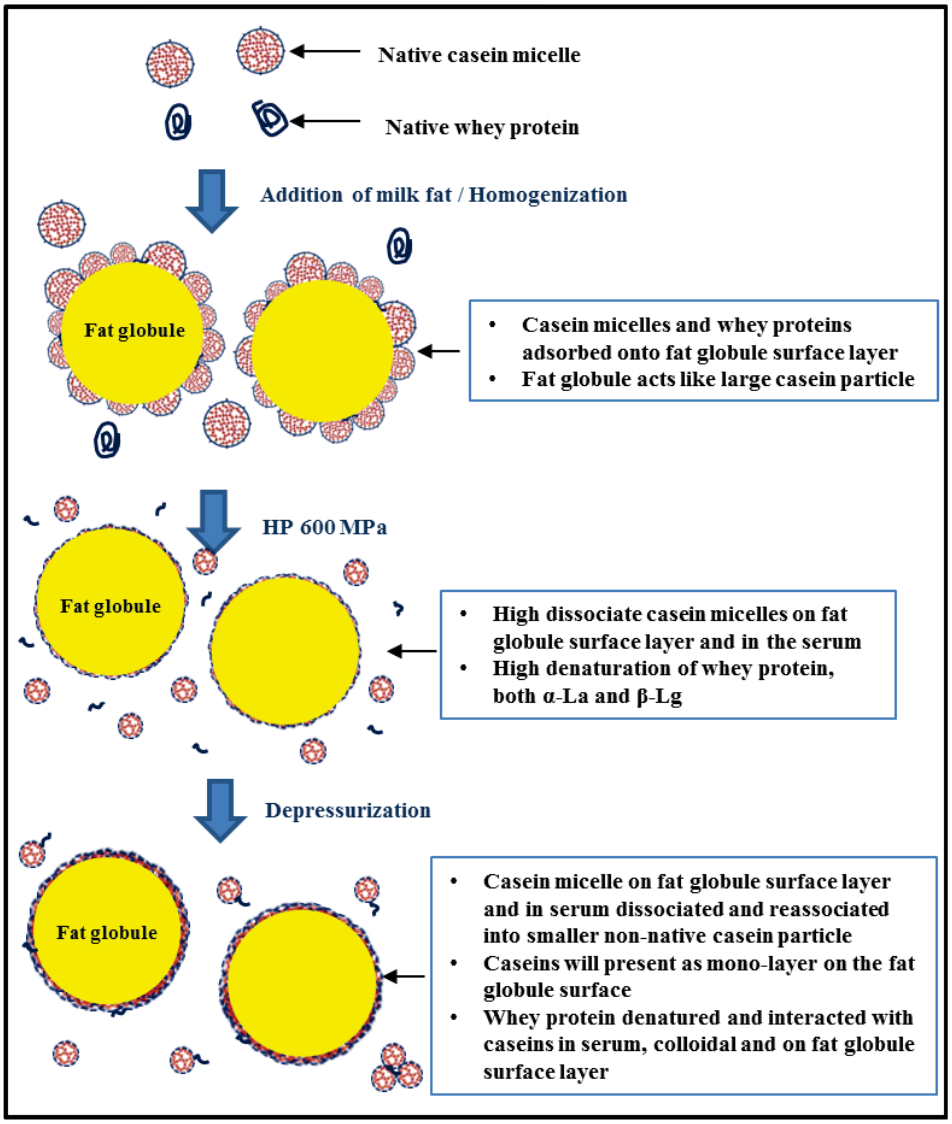


Figure 6.24. Schematic illustration of recombined milk undergoing HP treatment at 600 MPa after homogenization. The relative sizes of the individual components are not to scale.

The protein load changed with treatment pressure, i.e., decreased from 200 to 400 MPa and then increased again at 600 MPa. The similarity in behaviour for milks HP-treated before or after homogenization as shown in Figure 6.6., indicating similar levels of whey proteins on the surface layers. However, there were marked differences between proteins adsorbed at the interface of fat globules when recombined milk underwent HP treatment before and after homogenization

CHAPTER 7

ACID-GELATION OF HIGH PRESSURE-TREATED RECOMBINED MILKS

7.1 Introduction

Compared with the effects of heat treatment, there have been relatively few studies on the effects of HP treatment of milk on acid gelation properties. Anema (2010a) reported that the acid gels made from HP-treated skim milks had higher gelation pH, final $G'$and yield stress values and shorter gelation time compared with untreated skim milks. The effects of HP treatment have been attributed to the change in casein micelle size and structure, the denaturation of whey proteins and the interactions between milk proteins (Anema, 2010b; Anema, Lauber, et al., 2005; Hinrichs, et al., 1996b; Huppertz, Fox, et al., 2004c; Rademacher & Hinrichs, 2002). The mechanisms involved in the gel formation of acid gels made from HP-treated milks have been reported to be similar to those made from heated milks (Huppertz, Fox, et al., 2006).

This study reports on the investigation of the effects of HP treatment applied to milks either before or after homogenization on acid gelation properties (i.e., gelation pH, final G' and yield stress values), which has not previously been done.

7.2 Results and discussion

Recombined milks were subjected to HP treatments at 200 and 600 MPa for 15, 30, and 60 min at 20°C. In addition, to compare the effects of HP placement, milks were subjected to HP treatments at 200, 400, and 600 MPa for 30 min at 20°C either before or after homogenization with milk fat to prepare recombined milks. The acid gelation properties of HP-treated recombined milks are shown in Table 7.1.

7.2.1. Gelation pH and gelation time

The acid gels made from HP-treated recombined milks had higher gelation pH and shorter gelation time compared with those made from untreated recombined milk. The gelation pH slightly increased (from 4.73 to 4.92), whereas gelation time decreased (from 1.45 to 0.98 h) with increased treatment pressure and time.

Table 7.1 *Acid gelation properties of HP-treated recombined milks.*

Milk treatment	Gelation		*G'* (Pa)		Yield	
	pH	Time (h)	30°C	5°C	Strain (%)	Stress (Pa)
Untreated	4.73[a] (0.00)	1.45[e] (0.00)	14.20[a] (0.20)	56.07[a] (2.18)	81.80[c] (0.00)	107.67[b] (4.73)
HP treatment for different times at 200 MPa after homogenization						
15 min	4.73[a] (0.01)	1.45[e] (0.03)	14.20[a] (0.30)	59.77[a] (1.59)	81.80[c] (0.00)	97.10[a,b] (0.61)
60 min	4.79[b] (0.03)	1.27[d] (0.07)	29.80[b] (2.25)	119.33[b] (9.24)	57.57[b] (5.08)	100.83[a,b] (1.89)
HP treatment for different times at 600 MPa after homogenization						
15 min	4.85[c] (0.01)	1.14[c] (0.02)	64.53[e] (4.46)	240.00[e] (15.87)	43.10[a] (1.51)	180.33[c] (9.07)
60 min	4.92[e] (0.00)	0.98[a] (0.00)	217.00[g] (12.12)	675.00[i] (32.51)	38.20[d] (0.00)	302.33[g] (5.86)
HP treatment at different pressures for 30 min after homogenization						
200 MPa	4.77[b] (0.01)	1.33[d] (0.04)	21.77[a,b] (0.31)	102.00[b] (1.00)	60.50[b] (0.00)	92.90[a] (0.66)
400 MPa	4.87[c,d] (0.00)	1.08[b,c] (0.00)	82.70[c] (3.02)	302.00[f] (10.82)	46.90[a] (0.00)	184.33[c] (4.04)
600 MPa	4.92[e] (0.00)	0.98[a] (0.00)	155.33 (4.04)	561.33 (14.98)	51.70[e] (0.00)	259.33[f] (2.08)
HP treatment at different pressures for 30 min before homogenization						
200 MPa	4.79[b] (0.01)	1.28[d] (0.03)	25.27[a,b] (1.26)	111.67[b] (5.51)	60.50[b] (0.00)	110.00[b] (3.61)
400 MPa	4.84[c] (0.00)	1.15[c] (0.00)	48.37[d] (0.55)	175.00[d] (1.73)	60.50[b] (0.00)	167.00[d] (6.00)
600 MPa	4.90[d,e] (0.01)	1.03[a,b] (0.02)	94.63[c] (0.40)	350.67[g] (2.08)	45.50[a] (1.21)	207.00[e] (1.73)

Note. Standard deviations are in parentheses. [a] Samples with the same superscript letters within the same column are not significantly ($P \leq 0.05$) different from each other.

These changes were related to the denaturation levels of whey proteins induced by HP treatment, i.e., increased denaturation levels of whey protein resulted in increased gelation pH and decreased gelation time. The gelation pH and gelation time for acid gels made from recombined milks HP-treated before or after homogenization did not significantly differ because both milks had similar levels of whey protein denaturation (Appendix B.1).

The only exception to the above finding was that the gelation pH and gelation time of acid gel made from recombined milk HP-treated after homogenization at 200 MPa for 15 min did not significantly differ from those of untreated recombined milk. This was probably due to the whey protein denaturation being less than the level (~30%) required to induce the effects of HP treatment. In recombined milk, the whey proteins are in a native form and remain soluble (as discussed in Section 4.2.1). Therefore, gelation did not occur until the pH of acidified milk approached the isoelectric point of caseins (approximately pH 4.6). However, in HP-treated recombined milk, the whey proteins are denatured and are insoluble at their isoelectric point. Thus, as the pH of acidified milk approached pH 5.2 (the isoelectric point of the major whey protein, β-Lg), aggregation and gelation occurred. These findings support previous studies on acid gels

prepared from skim milks (Desobry-Banon, et al., 1994; Harte, Amonte, Luedecke, Swanson, & Barbosa-Canovas, 2002; Needs, Capellas, et al., 2000) which reported that HP-treated skim milk coagulates at higher pH compared with those made from untreated skim milks.

7.2.2. Viscoelastic properties during acidification

This section will discuss the viscoelastic properties that arise during the acidification of HP-treated recombined milk. The viscoelastic properties and the large deformation rheology after acidification will be discussed in Sections 7.2.3 and 7.2.4 respectively. The changes in G' with time after GDL addition of recombined milks HP-treated at different pressurizing times and treatment pressures are shown in Figures 7.1 and 7.2 respectively. Initially the G' was very low, followed by a rapid increase as the milk gelled until it reached a high value.

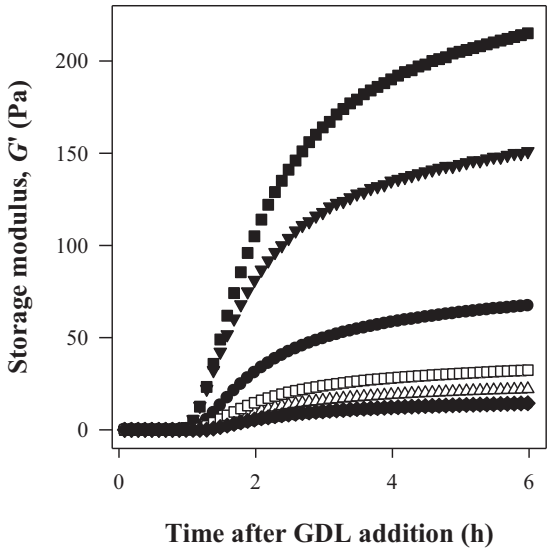


Figure 7.1. Changes in storage modulus (G') as a function of time after GDL addition. The acid gels were made from recombined milks HP-treated after homogenization at 20°C at 200 MPa (open symbols) and 600 MPa (filled symbols) for (○, ●) 15, (△,▼) 30 and (□,■) 60 min. The closed diamonds (◆) represent G' of untreated recombined milk. The change in G' of recombined milk HP-treated at 200 MPa for 15 min at 20°C (○) was on the same curve as that of untreated recombined milk (bottom curve).

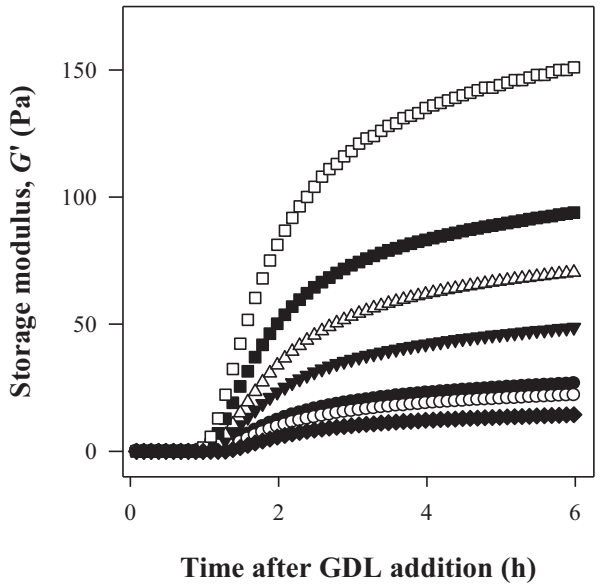


Figure 7.2. Changes in storage modulus (G') as a function of time after GDL addition. The acid gels were made from recombined milks HP-treated before (filled symbols) or after (open symbols) homogenization at 20°C for 30 min at (●,○) 200, (▼,△) 400 and (■,□) 600 MPa. The closed diamonds (◆) represent G' of untreated recombined milk.

The curves between G' and time after GDL addition of acid gels made from recombined milks without HP treatment and with HP treatment after homogenization at 200 MPa for 15 min at 20°C were identical, confirming that HP treatment at this combination did not have significant effects on the acid gelation properties (Figure 7.1). Increased treatment pressure and holding time resulted in increased final G' values of acid gels. It is also very interesting to find out that increased treatment pressure applied to milks resulted in significant differences in the final G'values between acid gels made from milk HP-treated before and after homogenization (Figure 7.2), which indicates the differences in protein gel structure between those two systems.

The effects of treatment pressure and time applied to recombined milk on β-Lg denaturation, gelation pH and final G' values are shown in Figures 7.3 and 7.4. The acid gels made from milk samples without HP treatment and with HP treatment at 200 MPa either before or after homogenization had low final G' values, i.e., ≤ 30 Pa (Figure 7.4). At this treatment pressure, the final G' values slightly increased (from 14 to 30 Pa) with increased pressurizing times (from 15 to 60 min).

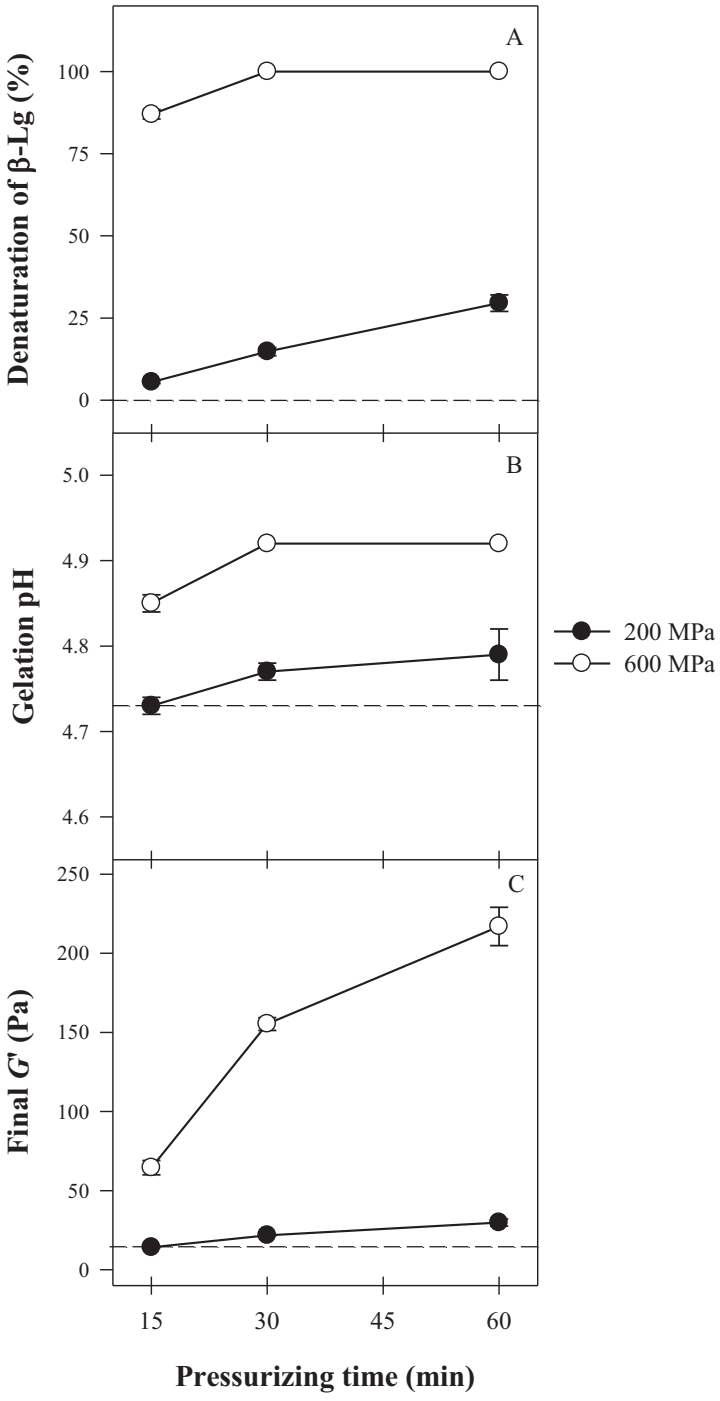


Figure 7.3. Denaturation of β-Lg and characteristics of acid gels as a function of pressurizing time. Recombined milks were HP-treated at 200 and 600 MPa after homogenization. Dash lines represent the denaturation and characteristics of untreated recombined milks.

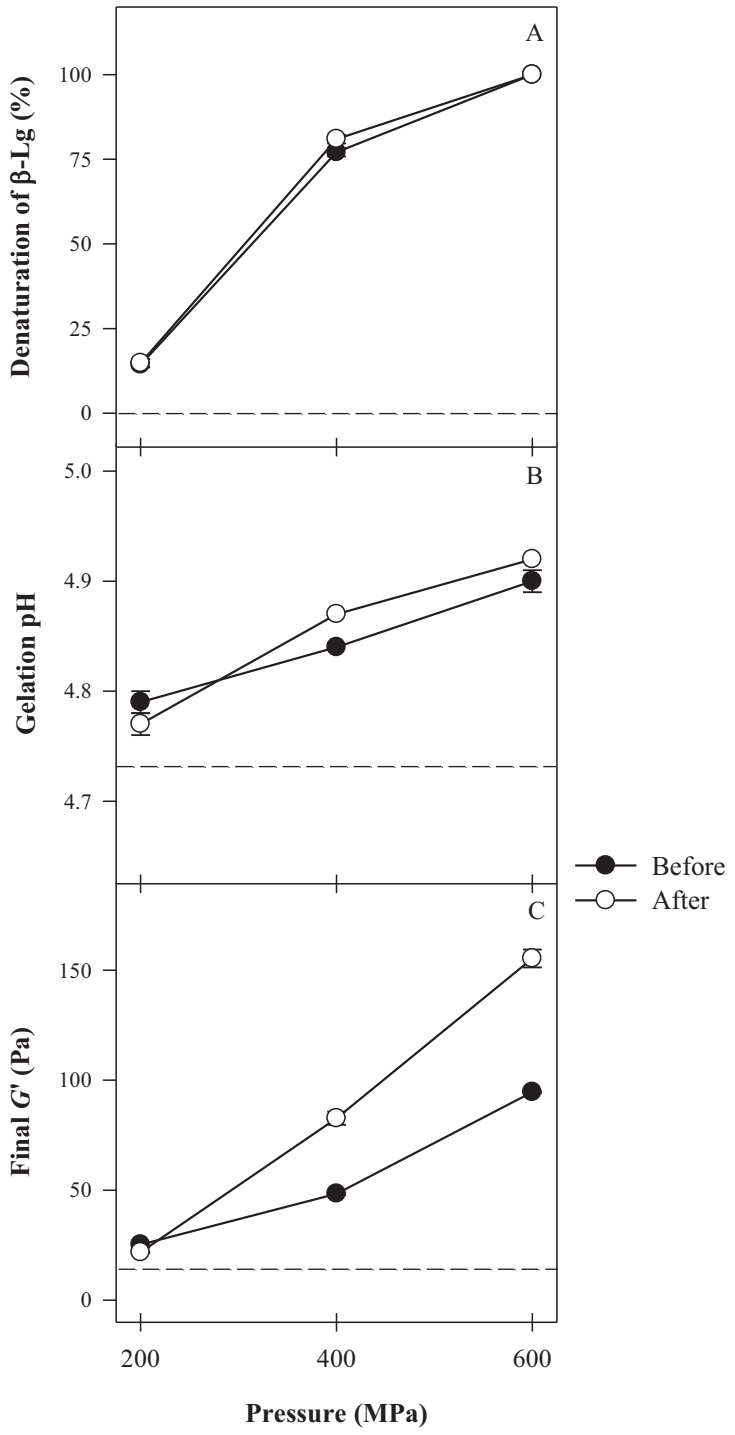


Figure 7.4. Denaturation of β-Lg and characteristics of acid gels as a function of treatment pressures. Recombined milks were HP-treated either before or after homogenization at 20°C for 30 min. Dash lines represent the denaturation and characteristics of untreated recombined milks.

At higher treatment pressures (400 and 600 MPa) the final G' values increased markedly with treatment pressure and time. At 600 MPa, the final G' values abruptly increased (from 65 to 217 Pa) as pressurizing times increased (from 15 to 60 min). The increase in final G' value was related to the increase in whey protein denaturation levels, i.e., a higher denaturation level resulted in an increase in final G' value as shown in Figures 7.3 and 7.4. However, there must be some other factors in addition to the whey protein denaturation level that gave rise to differences in the properties of gels prepared from milks heated before or after homogenization. The mechanism involved in the gel formation between those two systems need to be studied further.

This study found that the denaturation of β-Lg in HP-treated recombined milk had to be greater than 30% to induce a change in final G' value, which was consistent with previous studies on skim milk systems. Anema (2008c) reported that the final G' of HP-treated skim milk dramatically increases when treatment pressure is higher than 100 MPa. When the denaturation of β-Lg is ≤ 50%, the final G' values of acid gels make from HP-treated skim milks are not different from those of untreated skim milks (Anema, 2010a). The lower level of whey protein denaturation to induce changes in final G' value of HP-treated recombined milk might be attributed to the interactions between the adsorbed proteins on the surface layers of fat globules and proteins gel network.

The final G' values are affected by protein content (van Vliet & Keetals, 1995), strand curvature (Mellema, van Opheusden, & Van Vliet, 2002), bond type, i.e., the S-S interactions (Vasbinder, et al., 2004; Walstra, 2003) and micelle junction density. Figure 7.4C shows that the final G' values of acid gels made from milks HP-treated at 400 and 600 MPa before homogenization were significantly lower than those of recombined milks HP-treated after homogenization. At 600 MPa, the final G' value of acid gel made from milk sample HP-treated before homogenization was approximately 40% lower than that of milk sample HP-treated after homogenization. The lower final G' value indicated lower number of bonds connecting network structures, i.e., less bonds in key structure of acid gels made from milk HP-treated before homogenization compared with those made from recombined milks HP-treated after homogenization.

7.2.3. Viscoelastic properties after acidification

In Section 7.2.2, the viscoelastic properties during acidification were discussed. In this section, the properties of the gels after acidification will be examined. Figure 7.5 shows the frequency dependence of the storage modulus, G', and the loss modulus, G'', of the acid gels made from untreated milk and milks HP-treated at 200 MPa for 30 min at 20°C either before or after homogenization. For all the acid gels made from HP-treated milks, G' was higher than G'' (Table 7.1), indicating that the acid gels were elastic. In addition, for all the acid gels the plots of G' and G'' against frequency of the applied deformation were linear with a slope of 0.14 ± 0.01 on a logarithmic scale. These results are in agreement with those reported for acid gels made from skim and recombined milks (Cho, et al., 1999; Oh, et al., 2007; Roefs & Van Vliet, 1990).

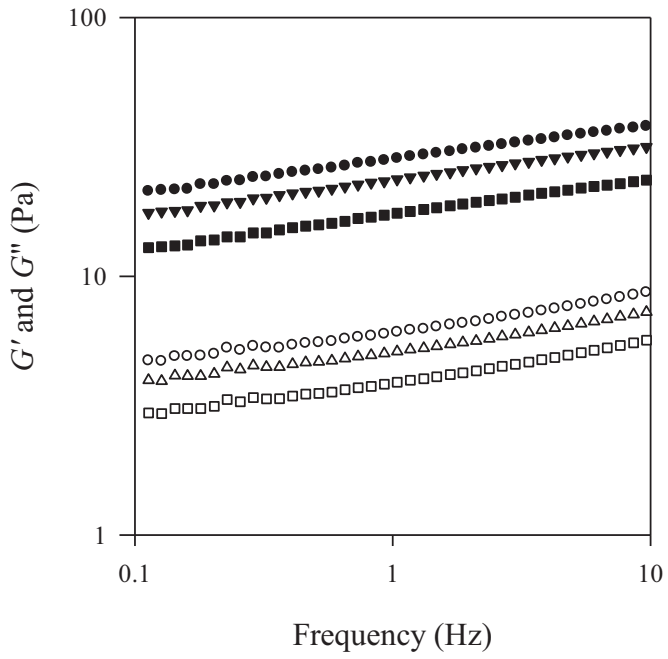


Figure 7.5. Storage modulus (G', filled symbols) and loss modulus (G'', open symbols) of acid gels at 30°C as a function of frequency. Acid gels were made from milks HP-treated either before (●,○) or after (▼,△) homogenization at 200 MPa for 30 min at 20°C. The squares (■,□) represent G' and G'' of acid gel made from untreated recombined milk.

After 6 h of acidification, the temperature of the acid gels was reduced from 30°C to 5°C and the rheological properties were monitored. For all acid gels made from HP-treated recombined milks, the final G' values at 5°C were 3.88 ± 0.42 times higher than those at 30°C (Figure 7.6). The effect of temperature on G' has been observed previously for acid gels made from heated skim milks (Anema, Lauber, et al., 2005; Lucey, Teo, et al., 1997; Oh, et al., 2007) and heated recombined milks (discussed in Chapter 5). The increase in G' value at lower temperature was attributed to the temperature dependent hydrophobic interactions, which play an important role in casein micelle assembly and acid-induced casein gel structures. At lower temperatures, the hydrophobic interactions were weak resulting in less compact casein micelles. Therefore, the casein particle size increased within acid gel networks. The increase in G' value at lower temperatures was also attributed to other factors such as the swelling of casein particles, the greater rigidity of the protein network, and the increased viscosity of the continuous phase at the lower temperatures (Anema, Lauber, et al., 2005; Bikker, et al., 2000; Lucey, Teo, et al., 1997; Oh, et al., 2007).

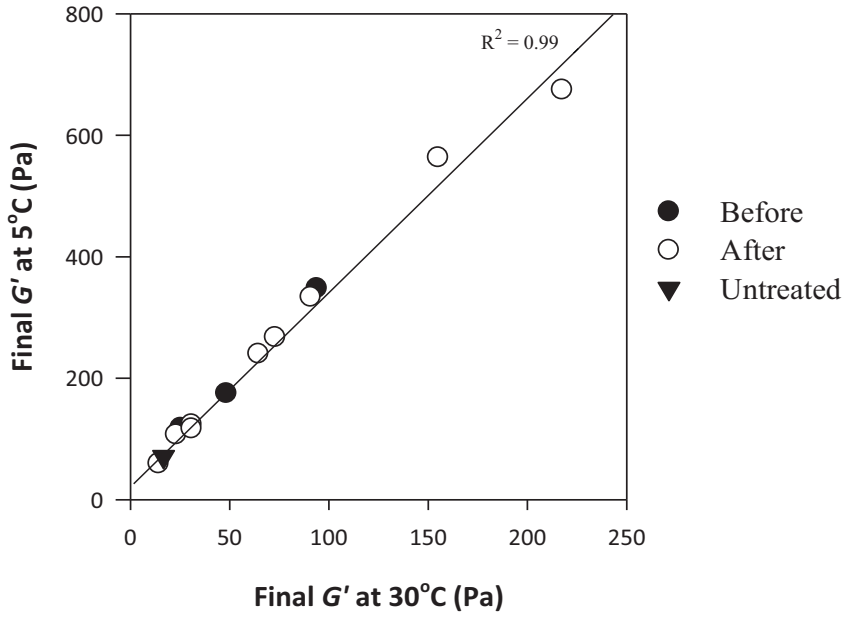


Figure 7.6. Comparison between the final storage modulus (G') at 30°C and 5°C. Acid gels were made from recombined milks HP-treated either before or after homogenization at different treatment pressures and times at 20°C.

However, most studies on acid gels made from skim milk reported that the final G' at 5°C are 2-3 times higher than that at 30°C (Anema, Lauber, et al., 2005; Oh, et al., 2007). The greater rigidity of the milk fat globules at 5°C contributed to the observed higher final G' value at 5°C compared to that at 30°C in recombined milk system. At 5°C the milk fat was less liquid-like than at 30°C, which may account for the differences in effect of temperature between skim and recombined milk systems.

Tan δ (G''/G') is the ratio of the deformation energy stored by the sample to the deformation energy liberated as heat. A change in tan δ indicates the changes in type and/or strength of the bonds in a gel network (van Vliet & Walstra, 1999). The plot of the final G' against the final G'' for all acid gels made from HP-treated recombined milks at the temperatures investigated (30°C and 5°C) is shown in Figure 7.7.

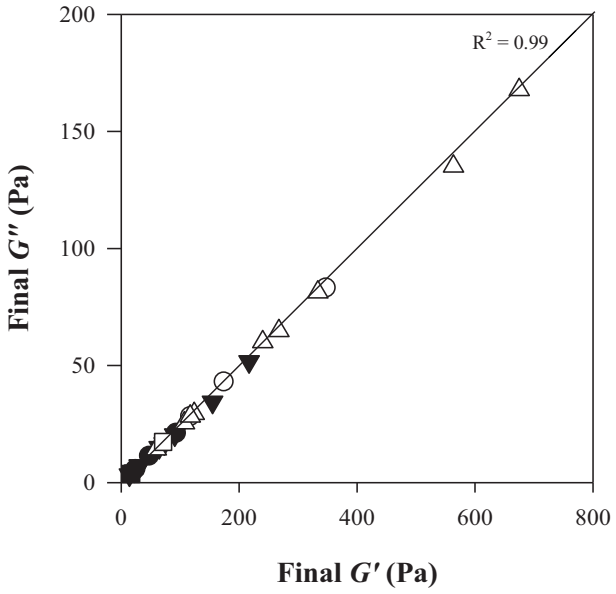


Figure 7.7. Comparison between the final storage modulus (G') and final loss modulus (G'') at 30°C (filled symbols) and 5°C (open symbols). HP treatments were carried out either before (●,○) or after (△,▼) homogenization at different treatment pressures and times at 20°C. The squares (■,□) represent the G' and G'' of acid gel made from untreated recombined milk.

The final G' and G'' values were linearly correlated and the tan δ was constant with a value of 0.25 ± 0.01 indicating that the proportion of the deformation energy stored and the deformation energy lost remained constant regardless of the HP treatment applied. In addition, the frequency dependence of G' and G'', the temperature dependence of G', and the tan δ were similar for all the acid gels made from HP-treated recombined milks, indicating that the changes in the elastic properties are accompanied by proportionate changes to the viscous properties. These results were in agreement with those reported previously in for acid gels made form skim and recombined milks (Anema, Lauber, et al., 2005; Lucey, Munro, et al., 1998; Lucey, Tamehana, et al., 1998b; Lucey, Teo, et al., 1997; Oh, et al., 2007).

7.2.4. Large deformation rheology of acid gels

After 6 h of acidification, the set gels were subjected to a strain sweep. As the strain increased, the measured stress also increased to a maximum and then decreased as the strain was increased further as shown in Figures 7.8 and 7.9.

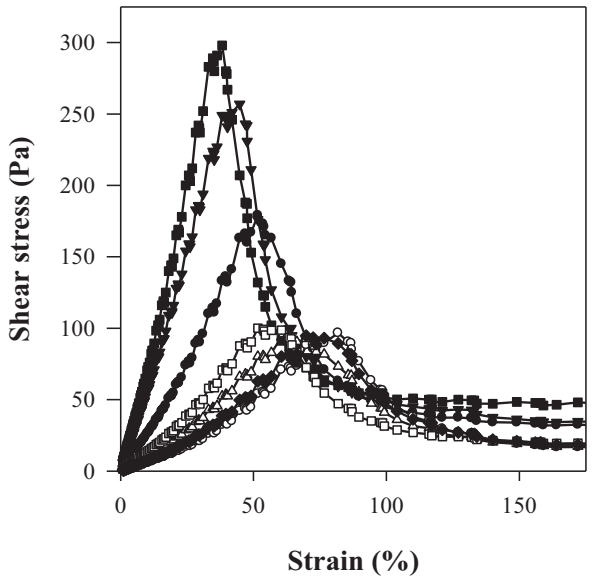


Figure 7.8. Shear stress as a function of strain for acid gels made from recombined milks HP-treated after homogenization at 20°C at 200 MPa (open symbols) and 600 MPa (filled symbols) for (○, ●) 15 min, (△,▼) 30 min, and (□, ■) 60 min respectively. The closed diamonds (◆) represent shear stress of acid gel made from untreated recombined milk

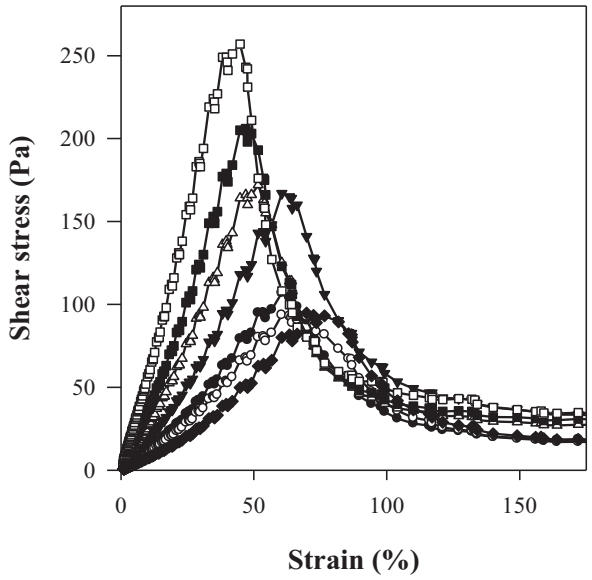


Figure 7.9. Shear stress as a function of strain for acid gels made from milk HP-treated before (filled symbols) or after (open symbols) homogenization at 20°C for 30 min at (●,○) 200 MPa, (▼,△) 400 MPa, and (■,□) 600 MPa respectively. The closed diamonds (◆) represent shear stress of acid gel made from untreated recombined milk.

The maximum stress represents the point at which the gel structure yields. The stress and strain at this point are regarded as the yield stress and yield strain respectively. The change in yield stress as a function of pressurizing time and treatment pressure is shown in Figure 7.10. For recombined milks HP-treated after homogenization at 200 MPa, the yield stress values were similar at different pressurizing times (Figure 7.10A). However, the yield stress values of acid gels made from HP-treated recombined milk increased with increases pressurizing time at higher pressure, i.e., 600 MPa (Figure 7.10A) and with increased treatment pressure (Figure 7.10B). These results and Figure 7.4A indicate that to induce the change in yield stress values, the denaturation of whey proteins in HP-treated recombined milk must be greater than 30%. Figure 7.10B also shows that the placement of HP treatment has a significant effect on the yield stress values of acid gels.

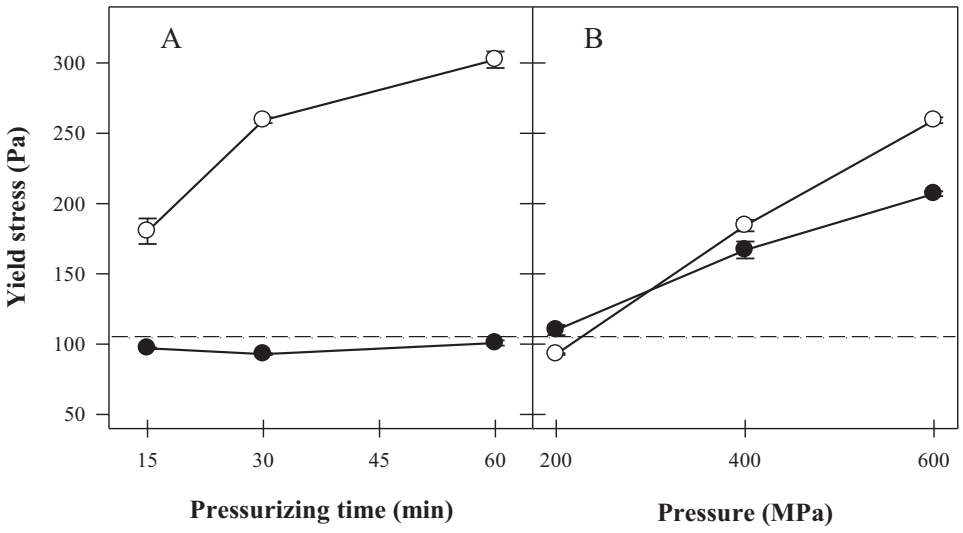


Figure 7.10. Change in yield stress as a function of pressurizing time and treatment pressure. (A) Recombined milks were HP-treated at 200 MPa (●) and 600 MPa (○) after homogenization. (B) Milk samples were HP-treated at different pressure for 30 min before (●) and after (○) homogenization. Dash line represents the yield stress of untreated recombined milks.

At lower treatment pressure (200 MPa), acid gels made from milk sample that underwent HP treatment before homogenization had higher yield stress value compared with milk sample HP-treated after homogenization. However, at higher pressure (400 and 600 MPa), milk samples that underwent HP treatment before homogenization had significantly lower yield stress values than recombined milk HP-treated after homogenization. Both HP-treated recombined milks had similar levels of whey protein denaturation (as shown in Figure 7.4A). These results imply that other factors apart from whey protein denaturation contributed to the yield stress value of acid gels.

The yield stress value depends on the number of bonds in the structure, but are unaffected by the strand curvature, i.e., the curve of the strand (Bremer, Bijsterbosch, Schrijvers, van Vliet, & Walstra, 1990; Mellema, et al., 2002) as during large deformation, curved strands have to be straightened before they are stretched and fractured (Mellema, et al., 2002; Renkema, 2004). This may be indicate that the location of denatured β-Lg is different between the two systems, with more β-Lg in the key structures of the gel network in the recombined milk HP-treated after homogenization than that of the milk HP-treated before homogenization.

These findings were in line with those reported previously for acid gels prepared from skim milks. Johnston et al. (1993)reported that the yield stress values of acid gels increased as treatment pressure and time applied to skim milk increased. Anema (2008c) found that the yield stress values increased markedly from ~25 Pa to ~250 MPa for acid gels made from skim milks without HP treatment and with HP-treated at 800 MPa respectively. Anema (2010a) reported that the yield stresses of the acid gels made from HP-treated skim milk were almost unchanged from that of untreated skim milk when the denaturation of β-Lg in HP-treated skim milks were ≤ 50%. Fat globules in recombined milk and the interactions between the caseins and whey proteins on the surface layers of fat globules with the protein gel matrix were responsible for breaking of the gels at a lower level of whey protein denaturation.

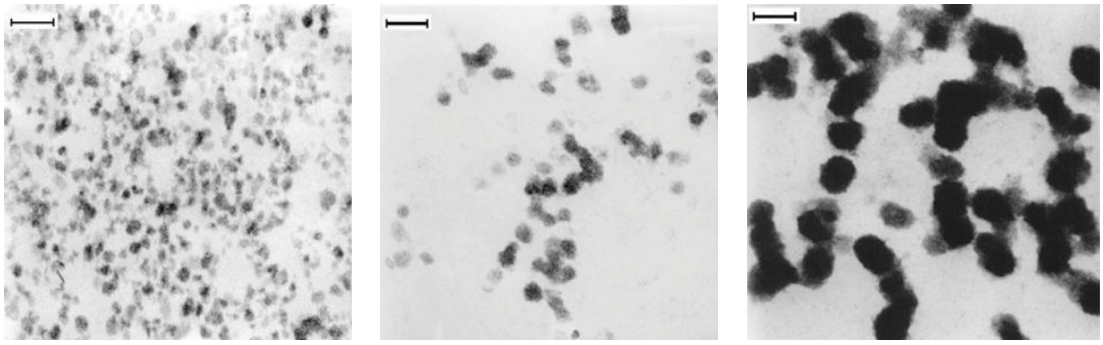
Yield strain depends on strand curvature (Lakemond & van Vliet, 2008; van Vliet & Keetals, 1995) and is affected by network structure and to a lesser extent the bond type between particles. In other words, high yield strains denote that relatively more strands are curved and/or more strongly curved (Bremer, et al., 1990; Mellema, et al., 2002; Renkema, 2004).

7.2.5. Factors contributing to the differences in acid gelation properties of recombined milks HP-treated before or after homogenization

In recombined milk, the casein micelles and the whey proteins are in native forms. These proteins are absorbed onto the surface layers of fat globules during homogenization as discussed in Chapter 4 (Figure 4.3). On acidification with GDL, the negative charge of κ-casein on the micelle surface is neutralized, resulting in a partial collapse of its extended conformation (de Kruif & Zhulina, 1996). As a consequence, a simultaneous loss of both electrostatic and steric stabilization occurs, allowing the micelles to come together and eventually to aggregate and gel (Lucey & Singh, 1998). According to the findings of Cho et al. (1999), the protein gel network of acid gel made from recombined milk is formed by the aggregation of casein micelles. The native whey proteins do not interact with the protein gel network, but fill the space between the protein strands (Vasbinder & de Kruif, 2003). The recombined milk fat globules are incorporated into the protein gel matrix through the interactions between adsorbed

caseins on the surface layers with casein-based network as discussed in Chapter 5 (Figure 5.8).

The change in casein micelle structure induced by HP treatment is the major factor responsible for the gelation properties of acid gels made from HP-treated skim milk (Anema, 2010a). Anema (2010b) reported that during acidification of HP (400 MPa/30 min)-treated skim milk, the size of colloidal casein particles progressively increases as the pH is reduced from its natural pH (pH ~6.60, Figure 7.11A).



A (pH 6.60) B (pH 5.45) C (pH 5.00)

Figure 7.11. Transmission electron micrograph (TEM) images of HP (400 MPa/30 min)-treated skim milk before and during acidification. The scale bar on each figure represents 100 nm, from Anema (2010b).

At pH ~5.45, the particle size increases from ~100 nm and there is evidence of the fusion of particles into chains and clusters (Figure 7.11B). With acidification to pH 5.00 (i.e., below gelation pH) this trend continue until the size reaching that of original native casein micelles and these particles are fused into chains and clusters (Figure 7.11C). On acidification with GDL, HP (400 MPa/30 min)-treated skim milk forms a weak gel at a relatively high pH (~ 6.0 - 5.5), indicating the inherent instability of the colloidal particles of HP-treated skim milk to acidification. Oh, Wong, Pinder and Anema (2011) reported that the protein strands are thinner and shorter in acid gels made from HP-treated (500 MPa, 20°C, 30 min) skim milks compared to those of heated (80°C, 30 min) skim milk as a result of the smaller protein aggregates in HP-treated milk (Patel, et al., 2006).

The application of HP treatment before or after homogenization on milk samples resulted in different compositions of the milk proteins on the surface layers of fat

globules (discussed in Chapter 6), which in turn had significant effects on the interactions between milk proteins as well as the structure of casein gel matrices. The surface layers of fat globules of recombined milks HP-treated before homogenization contained higher levels of κ-casein, lower level of α_s-casein, and similar levels of β-casein, β-Lg and α-La compared to those of recombined milks HP treated after homogenization.

HP treatment applied to milks either before or after homogenization affects the structure of acid gels. Those made from milks HP-treated before or after homogenization probably had differences in gel coarseness, strand curvature and type of interactions, as indicated by the differences in the final G', yield stress and yield strain values. As discussed in Sections 7.2.2 and 7.2.4, at low HP treatment, i.e., 200 MPa, the acid gels made from milk samples HP-treated before or after homogenization did not have significant differences in acid gelation properties. However, at higher pressures (400 and 600 MPa), the acid gels made from recombined milks HP-treated before homogenization have significantly lower final G' (Figure 7.4C) and yield stress values (Figure 7.10B) compared with those made from recombined milks HP-treated after homogenization. These milks had similar levels of whey protein denaturation (Figure 7.4A), indicating that other important factors contributed to the difference in acid gel structure.

The final G', yield stress and yield strain values are related to the number of bonds, the amount of covalent bonds in structure and the strands curvature respectively (Mellema, et al., 2002). Therefore, the acid gels made from recombined milk HP-treated before homogenization contained lower number of bonds in the gel structure and lower covalent bonds compared with acid gels made from milks HP-treated after homogenization. The possible gel network of acid gel made from HP-treated recombined milk is discussed in the following section. The lower final G' value indicated lower number of bonds connecting network structures, i.e., less bonds in the key structure of acid gels made from milk HP-treated before homogenization compared with those made from recombined milks HP-treated after homogenization.

7.2.5.1. Gel network of acid gels made from recombined milk undergoing HP treatment before homogenization

With increasing treatment pressure and time, under HP treatment, casein micelles increasingly dissociated into smaller particles and whey proteins denatured (Chapter 6). At a pressure of 200 MPa, the casein micelles only partially dissociated and reassociated into non-native casein particles with a size similar to those of native casein micelles (~200 nm) as discussed in Chapter 6. At this pressure, only low levels of β-Lg unfolded and formed disulphide-bonded dimers and probably aggregated with κ-casein, but did not form larger β-Lg aggregates. α-La did not denature at this pressure (see Figure 6.2, Chapter 6) and therefore was less apparent in the aggregates. At pressures of 400 - 600 MPa, the casein micelles dissociated into smaller non-native casein particles with the average size ~50% of native casein micelles, i.e., average size ~100 nm. At these pressures, β-Lg denatured (Figure 6.2, Chapter 6) and aggregated with caseins. α-La was not denatured at 400 MPa, but denatured at 600 MPa. The illustrations of reconstituted milks HP-treated at 200 – 600 MPa before homogenization are shown in Figures 6.19 and 6.20 (Chapter 6) respectively. When HP-treated reconstituted milks are recombined with milk fat, non-native casein particles, fragment of caseins, and denatured whey proteins aggregates were adsorbed onto the surface layers of fat globules during homogenization. The amounts of caseins and whey proteins adsorbed depend on treatment pressure and time as illustrated in Figures 6.21 and 6.22 (Chapter 6). Based on the information on acid gelation of HP-treated skim milks (Anema, 2010b), the behaviour of casein micelles in HP-treated recombined milk was expected to be similar. The fat globules of recombined milk behave like large casein particles as a result of casein micelles being adsorbed onto the surface layer of fat globules.

On subsequent acidification with GDL, the non-native casein particles in HP-treated recombined milks fused to form the aggregated particles with sizes similar to the original casein micelles size. The protein strands formed by dissociated casein particles are expected to be thinner and shorter than those of untreated recombined milk. The proteins on the surface layers of fat globules interacted with protein gel matrix, resulting in the incorporation of fat globules into the protein gel network. In acid gel prepared from milk HP-treated at 200 MPa before homogenization, the final G' value was low.

There was little denatured whey protein (~10%) involved in the gel structure and the non-native casein micelles were only slightly different from the native casein micelles. The structure of acid gel was not so different from that prepared from untreated recombined milk. The possible structure of acid gel made from milk HP-treated at 200 MPa before homogenization is illustrated in Figure 7.12.

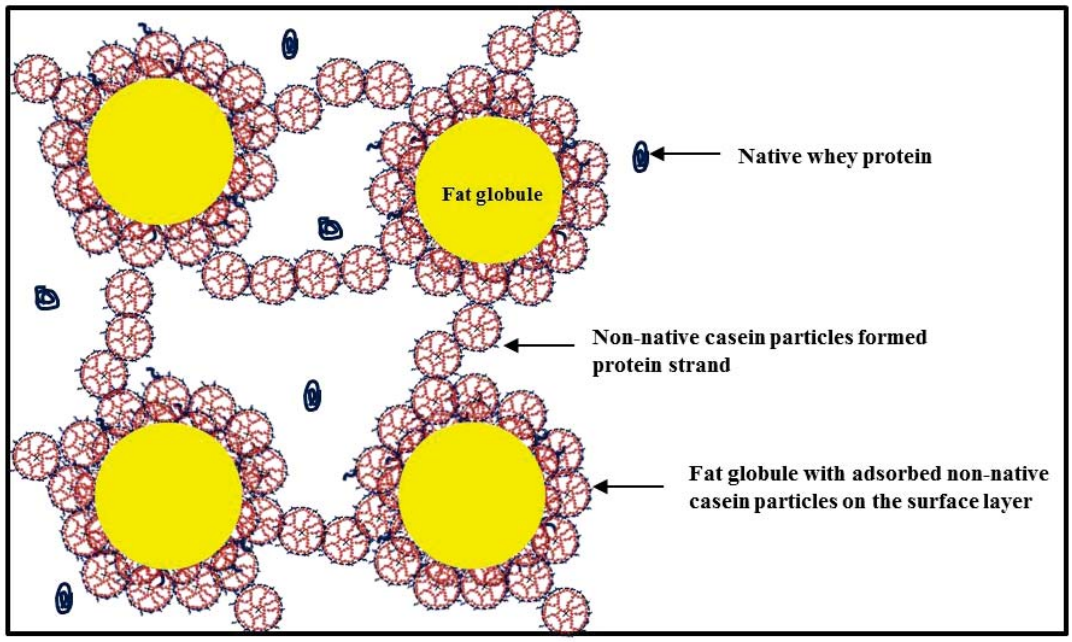


Figure 7.12. Possible acid gel formation in milk HP-treated at 200 MPa before homogenization, showing the interactions between casein micelles and other milk proteins. The relative sizes of individual components are not to scale

The possible gel formation of recombined milk that was HP-treated at 600 MPa before homogenization is illustrated in Figure 7.13. In this system, the final G' and yield stress values were higher as a result of the association of denatured whey proteins on the casein matrix. The non-native casein micelles were markedly changed both in structure and size during increase HP treatment. These caseins were then adsorbed onto the surface layers of fat globules as discrete non-native casein particles. The acid gel was firmer than those prepared from recombined milk HP-treated at 200 MPa due to the denaturation of whey protein as well as the changes in casein micelles structure. The casein micelles were smaller in size. The denatured whey proteins also helped to

strengthen these bonds as they interacted with the protein strands, resulting in higher final *G′* value.

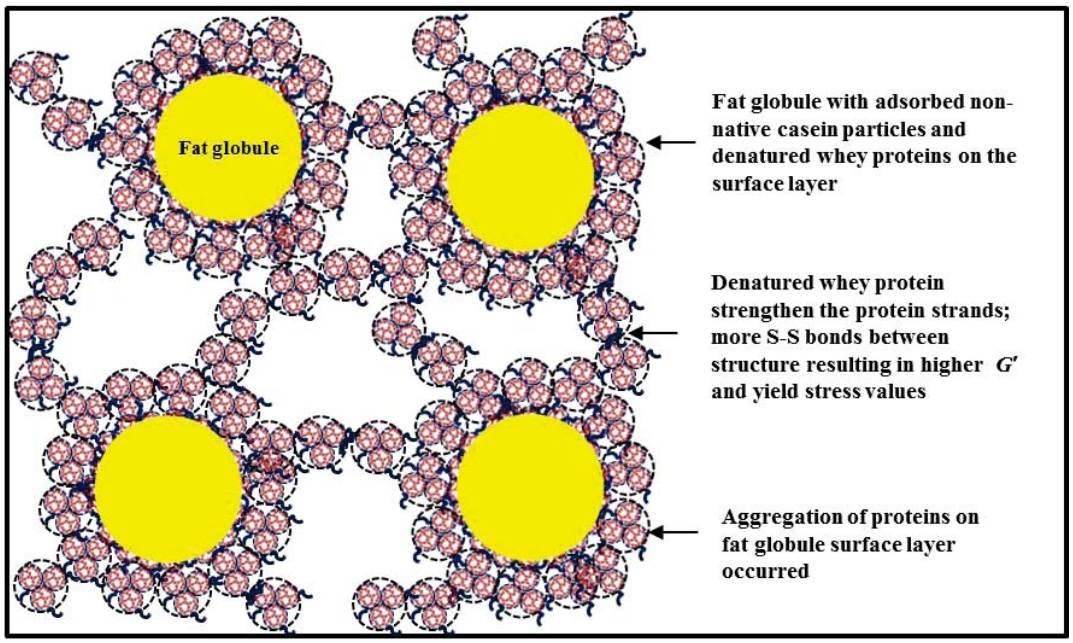


Figure 7.13. Schematic illustration of structure of acid gels made from milk HP-treated before homogenization at 600 MPa for 30 min at 20°C. The relative sizes of individual components are not to scale.

7.2.5.2. Gel network of acid milk gels made from recombined milk with HP treatment after homogenization

When HP treatments are applied to recombined milks after homogenization, the surface layers of fat globules have already been formed. Under HP treatment, casein micelles on the surface layers and in the serum are dissociated and reassociated into smaller particles and the whey proteins are denatured. The extents of these changes depend on the treatment pressure and time. As a result, the surface layers contain lower amounts of caseins and whey proteins compared to those of recombined milks without HP treatment and with HP treatment before homogenization as shown in Figures 6.23 and 6.24 (Chapter 6). In addition, more dissociated caseins are available in the serum to form the protein gel network. When HP-treated (after homogenization) recombined milks were subsequently acidified with GDL, the non-native casein particles fused to form the aggregated particles with size similar to the native casein micelles. The fat

globules interacted with the protein strands and were incorporated into the protein gels matrix. In untreated recombined milk, only a small amount of whey proteins are adsorbed onto the surface layer of fat globules with most of them being in native form in the serum. The casein micelles are the major milk proteins adsorbed onto the surface layer of fat globules. At 200 MPa, only slight changes occurred in the casein micelles both in the serum and on the surface layers of fat globules. Most of the whey proteins were not denatured. The acid gels made from these milks were similar to those of untreated recombined milk (Figure 5.8) and recombined milk HP-treated at 200 MPa before homogenization (Figure 7.12). Therefore, the acid gel had a low final G' and yield stress values similar to those of acid gel prepared from untreated and HP-treated (200 MPa) before homogenization recombined milks. The acid gel network of recombined milk HP-treated after homogenization at 200 MPa is shown in Figure 7.14.

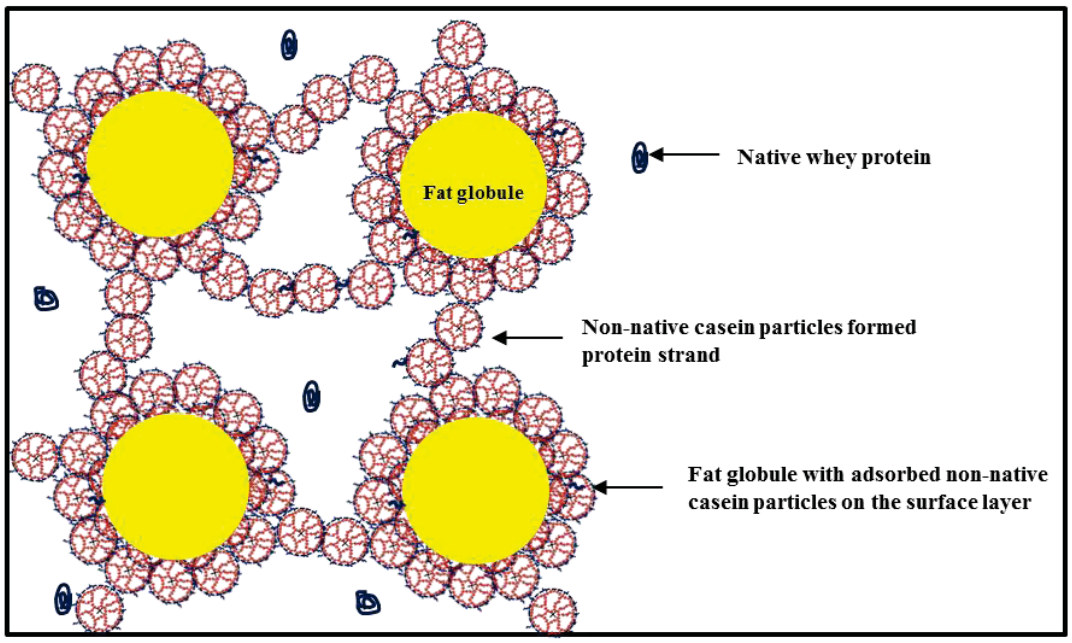


Figure 7.14. Schematic illustration of structure of acid milk gel made from recombined milks HP-treated at 200 MPa for 30 min at 20°C after homogenization. The relative of the individual components are not to be scale.

In recombined milk HP-treated at 600 MPa, the caseins on the surface layers of the fat globules and in the serum dissociated and reassociated into non-native particles with the size ~50% of native casein micelle. This meant that there were a large number of protein species available to form a stronger gel. The whey protein denatured and

interacted with the serum, colloidal particles and fat surface. When GDL was added to recombined milk HP-treated at 600 MPa, lots of interactive particles were available. The protein strands formed by small non-native casein micelles were finer and stronger. The denatured whey proteins interacted via their disulphide bonds, which produced acid gel with higher final G' and yield stress values. The acid gel prepared from recombined milk HP-treated after homogenizaion at 600 MPa had higher final G' value compared to those prepared from milk HP-treated at 600 MPa before homogenization due to the proteins on the surface layer were present as a layer which provided better sites for the interactions with the protein strands. The acid gel network of recombined milk HP-treated after homogenization at 600 MPa is shown in Figure 7.15.

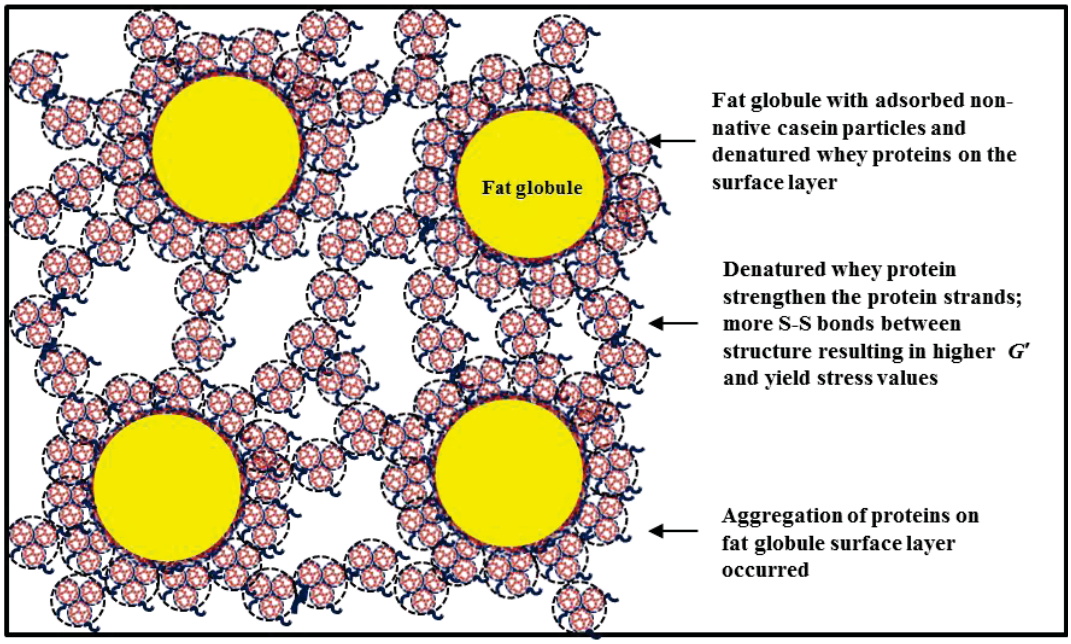


Figure 7.15. Schematic illustration of structure of acid milk gel made from recombined milks HP-treated at 600 MPa for 30 min at 20°C after homogenization. The relative of the individual components are not to scale.

The acid gels prepared from heated and HP-treated recombined milk were different from each other as follows:

- Acid gel made from heated recombined milk required less denaturation of whey protein to increase final G' value.

- 100% denaturation of β-Lg induced by heat treatment gave higher final G' value (Figure 5.3) than those recombined milks which had 100% denaturation induced by HP treatment (Figure 7.3).
- 100% denaturation of β-Lg induced by heat treatment always resulted in a maximum final G' value, whereas this behaviour was not observed with 100% denaturation of β-Lg induced by HP treatment.

7.3 Conclusions

HP treatment of recombined milk, either before or after homogenization, had significant effects on acid gelation properties. Increasing the severity of HP treatment, i.e., increased treatment pressure and time, resulted in decreased gelation time and increased gelation pH, final G' and yield stress values. The effects of HP treatment were attributed to the changes in casein micelles structure, the denaturation level of whey proteins, the composition of proteins on the surface layers of the fat globules as well as the interactions between milk proteins. The application of HP treatments before or after homogenization resulted in different protein compositions of the recombined milk fat globule surface layers, which in turn had significant effect on the structure of the acid gels. Applying HP treatment to recombined milk after homogenization resulted in acid gels with higher final G' and yield stress values compared to those of milks undergoing HP treatment before homogenization. The interactions between milk proteins in the serum and on the surface layer of recombined milk fat globules are thought to be responsible for the higher final G' and yield stress values.

CHAPTER 8

HEAT AND HIGH PRESSURE-TREATED RECOMBINED MILKS

8.1. Introduction

As discussed in Chapters 4-7, both heat and HP treatments had significant effects on casein micelles, whey protein denaturation, fat globule surface layers and acid gelation properties of recombined milks. The changes in fat globules and milk proteins induced by heat treatment were different from those induced by HP treatments. This study was designed to investigate the effects of combined heat and HP treatments applied to recombined milks on whey protein denaturation, surface layers of fat globules and acid gelation properties.

8.2. Results and discussion

The milk samples were heated at 80°C or 90°C for 30 min and then HP-treated at 200 - 600 MPa for 30 min at 20°C in a series of steps as shown in Figure 8.1.

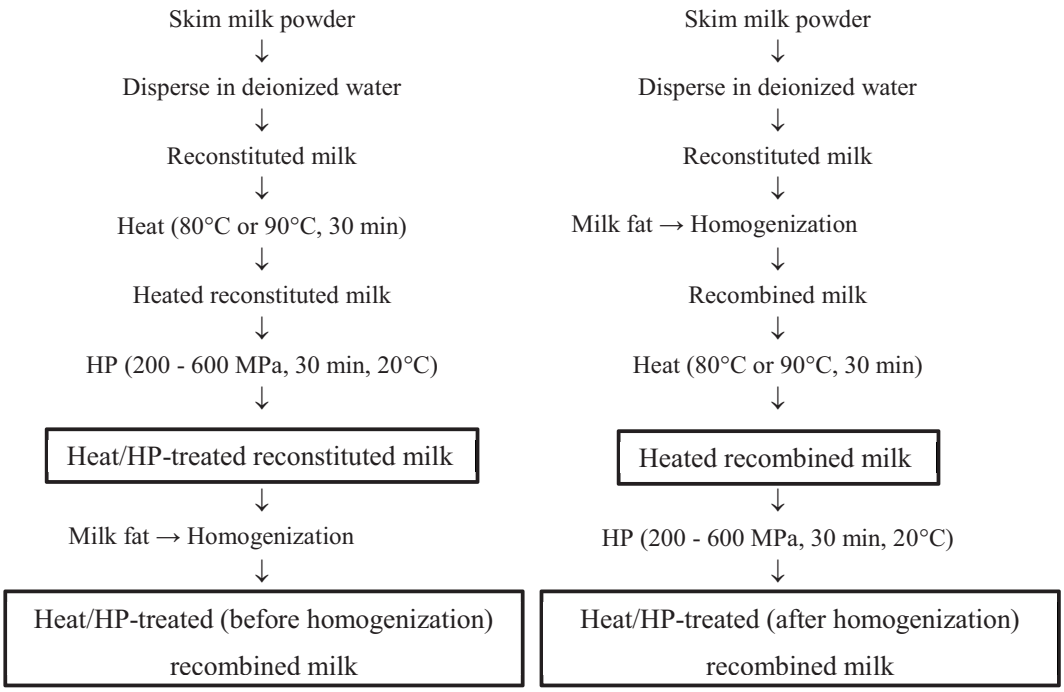


Figure 8.1. Experimental protocols to prepare recombined milks with heat/HP treatments before or after homogenization.

8.2.1. Whey protein denaturation

The extent of whey protein denaturation induced by heat/HP treatments is shown in Appendix C.1. The native-PAGE patterns of heat/HP-treated recombined milks are shown in Figure 8.2. The relationships between whey protein denaturation and heat/HP treatments are shown in Figure 8.3. Heat treatments at 80°C and 90°C for 30 min resulted in approximately 45% and 90% denaturation of α-La respectively. There was no difference in α-La denaturation between milk samples heated either before or after homogenization (Figure 8.3). Increased treatment pressure at 600 MPa resulted in slightly increased level of α-La denaturation.

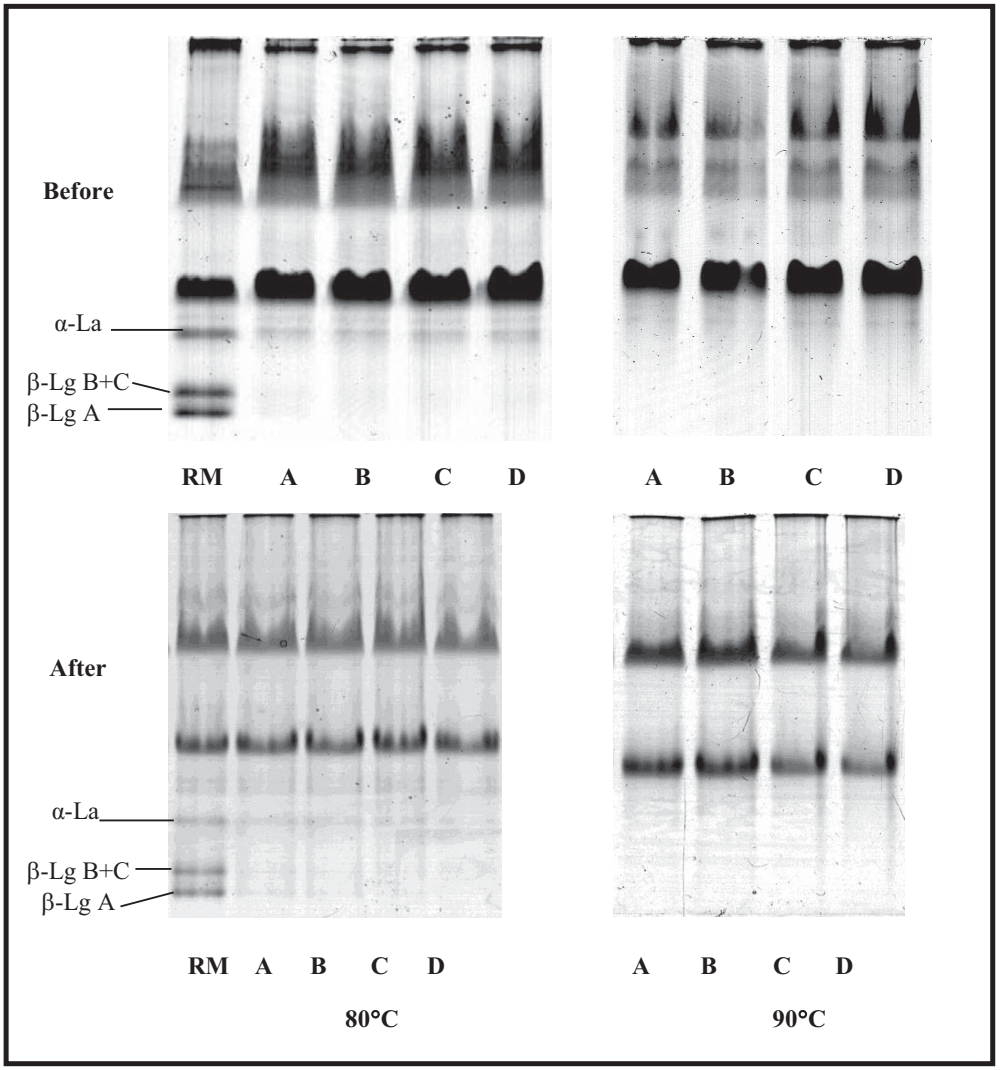


Figure 8.2. Native-PAGE patterns of heat/HP-treated recombined milks. HP treatments were carried out either before or after homogenization at (A) 0, (B) 200, (C) 400, and (D) 600 MPa. RM represents native-PAGE of untreated recombined milk.

Anema (2008b) also reported that about 15% of α-La was denatured at on heating skim milk 70°C for 30 min and further denaturation was observed at heating temperatures ≥ 70°C. No further denaturation of α-La was found when heated (90°C/15 min) skim milk was HP-treated at 400 MPa at 20°C for up to 120 min. In addition, the order of treatments (heated 80°C/HP or HP/heated 80°C) applied to skim milks had little effect on the denaturation level of α-La (Anema, 2008b).

Figure 8.3 shows that heat treatment of recombined milks at 80°C or 90°C for 30 min resulted in > 90% denaturation of β-Lg; only small amounts of native β-Lg were available for further denaturation under the subsequent HP treatment. Increasing treatment pressure applied to milk heated at 80°C for 30 min slightly increased the denaturation levels of β-Lg.

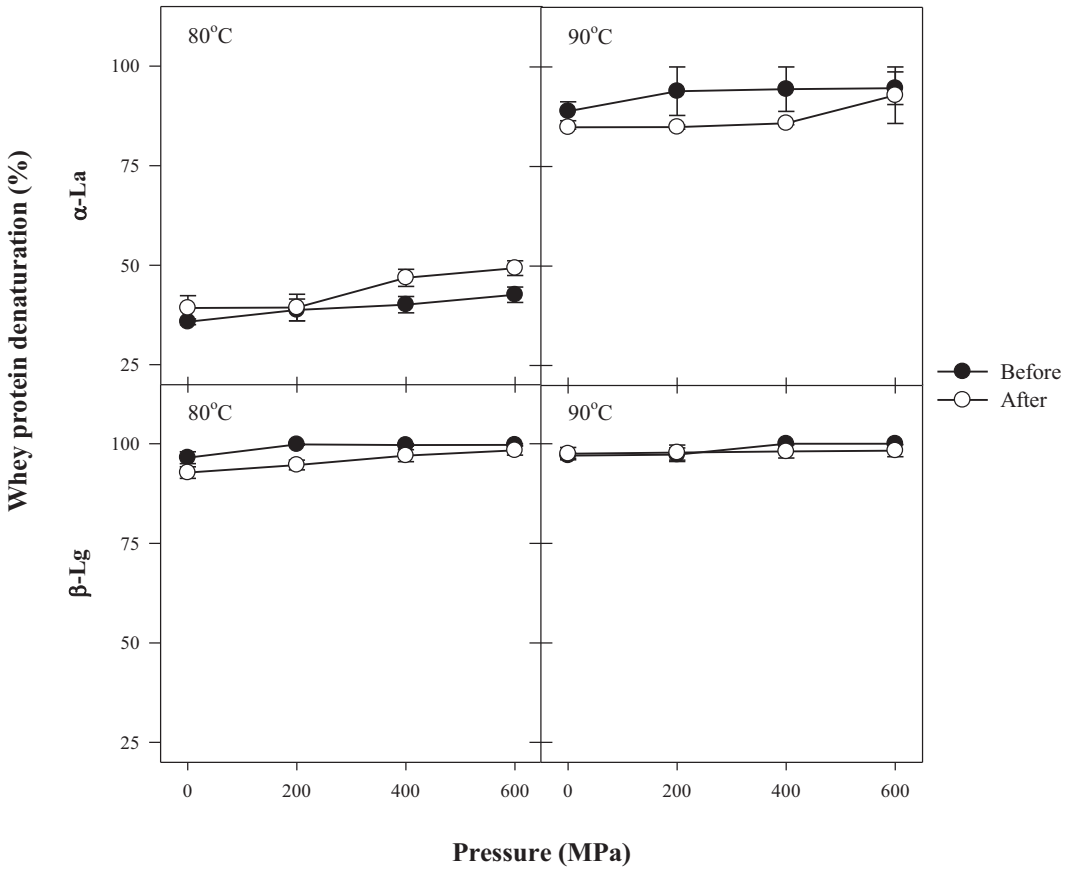


Figure 8.3. Denaturation of whey proteins as a function of treatment pressures (for 30 min at 20°C).

Heat treatments at 90°C for 30 min, either before or after homogenization, resulted in denaturation of nearly all β-Lg. Increasing the magnitude of HP treatment, either before or after homogenization, at this heating profile did not have a significant effect on denaturation level of β-Lg.

These results are in agreement with findings in Chapter 6 and previous studies on skim milk systems. Anema, Stockmann and Lowe (2005) reported that β-Lg was denatured when skim milk was heated at temperatures ≥ 70°C and the denaturation level increased as the temperature increased. They found that HP treatment (400 MPa at 20°C) applied to heated (90°C/15 min) skim milk without holding time results in an additional 10% denaturation of β-Lg and prolonged holding time for 60 and 120 min resulted in complete denaturation of this protein. Later, Anema (2008c) observed slight difference in denaturation levels of β-Lg between the heat/HP (500 MPa)-treated and the HP (500 MPa)/heat-treated skim milks.

Based on these studies, it can concluded that heat treatment was the dominant factor in whey protein denaturation, whereas HP treatment only had a significant effect at lower heating temperatures.

8.2.2. Composition of fat globule surface layers in heat/HP-treated recombined milk

The SDS-PAGE patterns of surface materials under reducing conditions isolated from heat/HP-treated recombined milks are shown in Figure 8.4 and the amounts of proteins on the surface layers are shown in Appendix C.2. The relationships between treatment pressure and the amounts of caseins and whey proteins adsorbed on the surface layers of heat/HP-treated recombined milk fat globules are shown in Figures 8.5 and 8.6 respectively.

When heat (80°C for 30 min)/HP treatments were applied to recombined milks after homogenization, the amounts of α_s- and β-caseins adsorbed onto the surface layers of fat globules decreased, whereas the amounts of β-Lg slightly increased as treatment pressure increased. The amounts of κ-casein and α-La did not significantly change with treatment pressure. When these treatments were applied to milks before homogenization, the amounts of caseins and α-La adsorbed onto the surface layers did

not change significantly, whereas the amount of β-Lg slightly increased as treatment pressures increased. The amounts of β-Lg adsorbed onto the surface layers were considerably lower when those treatments were applied before homogenization.

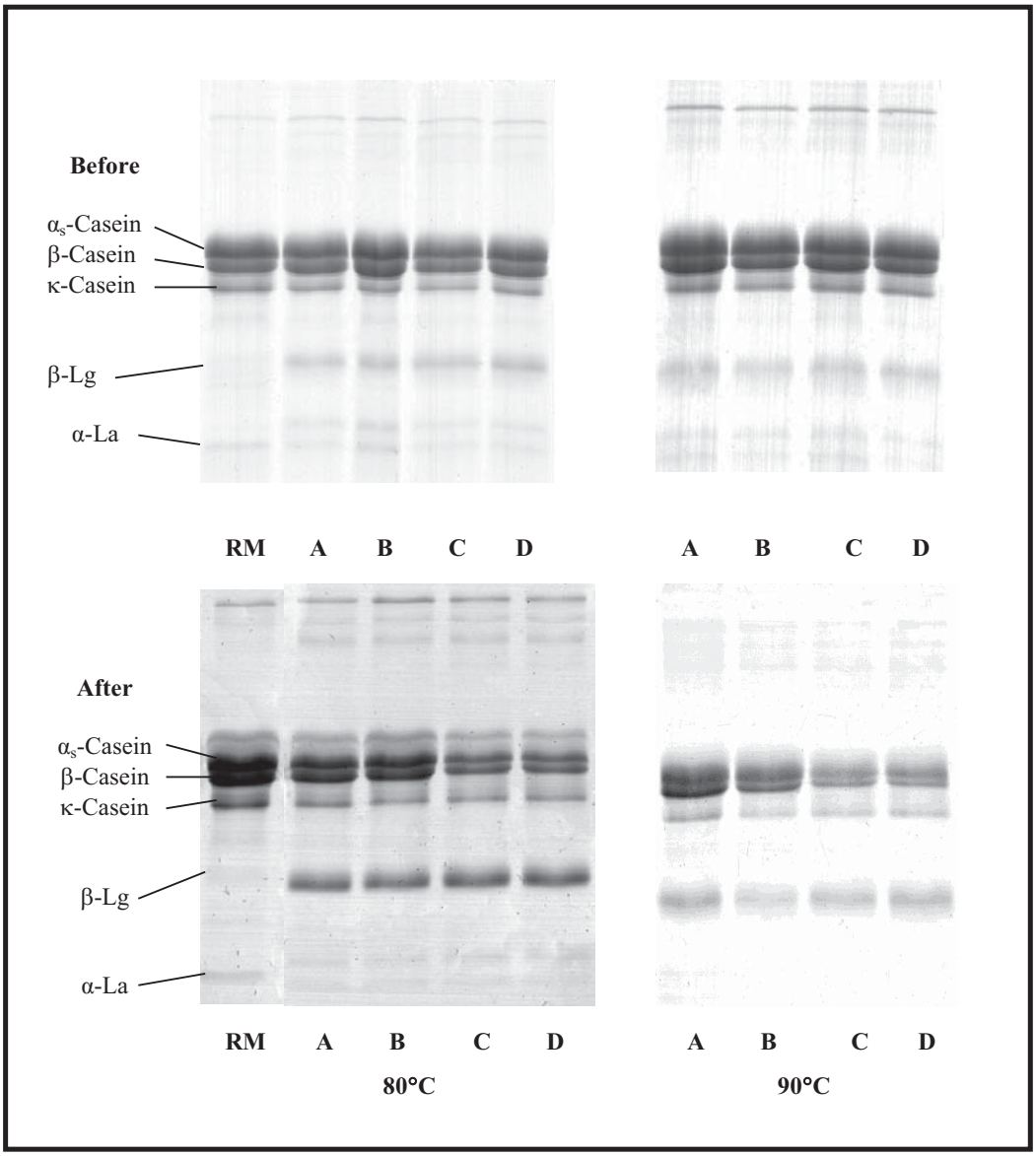


Figure 8.4. SDS-PAGE patterns under reducing conditions of membrane materials isolated from heat/HP-treated recombined milks. HP-treatments were carried out at 20°C for 30 min at (A) 0, (B) 200, (C) 400, and (D) 600 MPa on milk samples that has been heated at 80 or 90°C (for 30 min) either before or after homogenization. RM represents the fat globule surface material of untreated recombined milk. The creams were washed with SMUF.

When the heat treatment applied to milks after homogenization increased from 80°C to 90°C, in general the amount of proteins adsorbed onto the surface layers of fat globules seemed to decrease When heat (90°C for 30 min)/HP treatments were applied to milks before homogenization, the amounts of proteins adsorbed onto the surface layers of fat globules did not change significantly with treatment pressure.

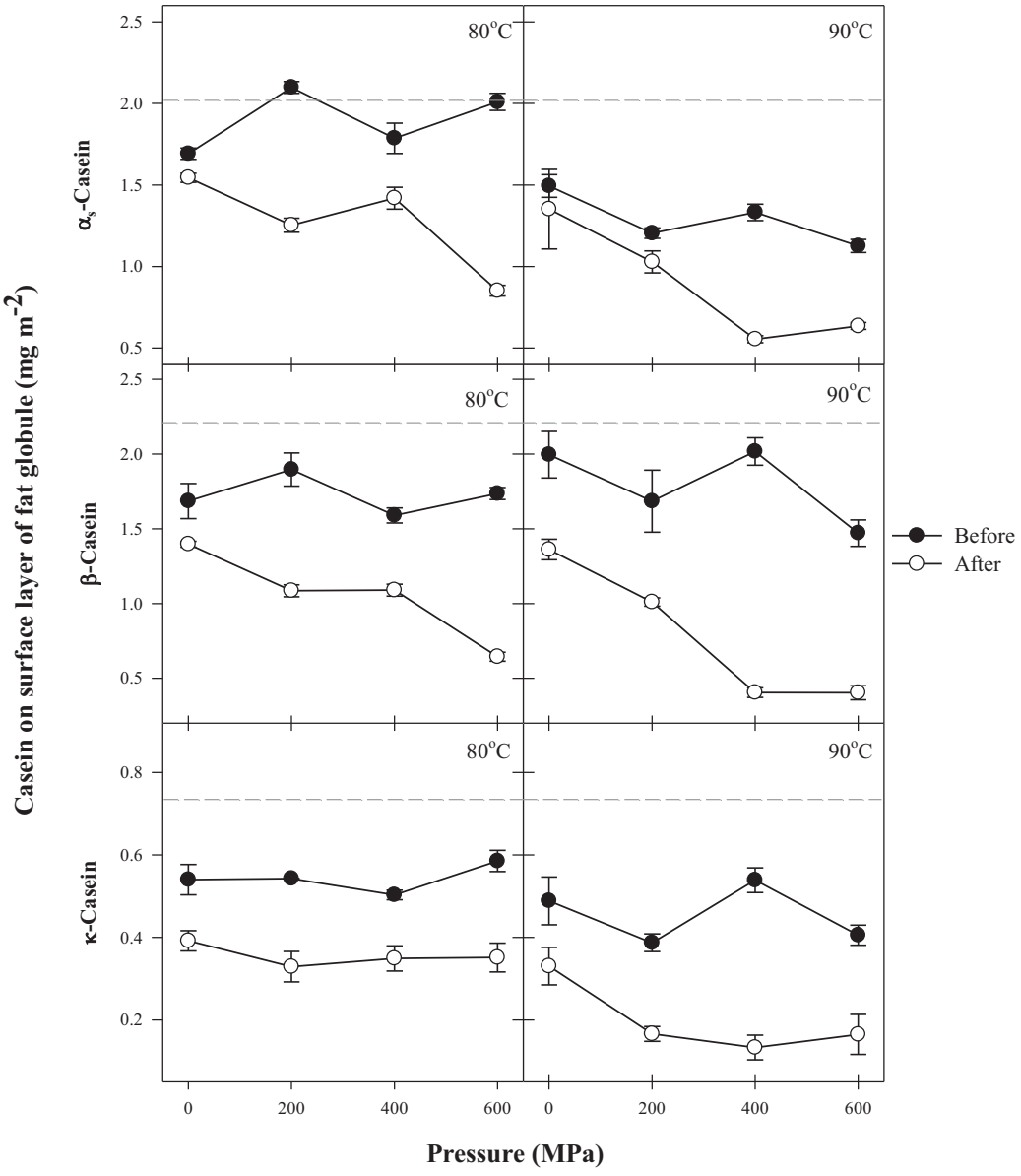


Figure 8.5. The amounts of caseins on the surface layers of heat/HP-treated recombined milk fat globules. HP treatments were applied to recombined milks that had been heated for 30 min either before or after homogenization. Dash lines represent casein on surface layers of untreated recombined milk.

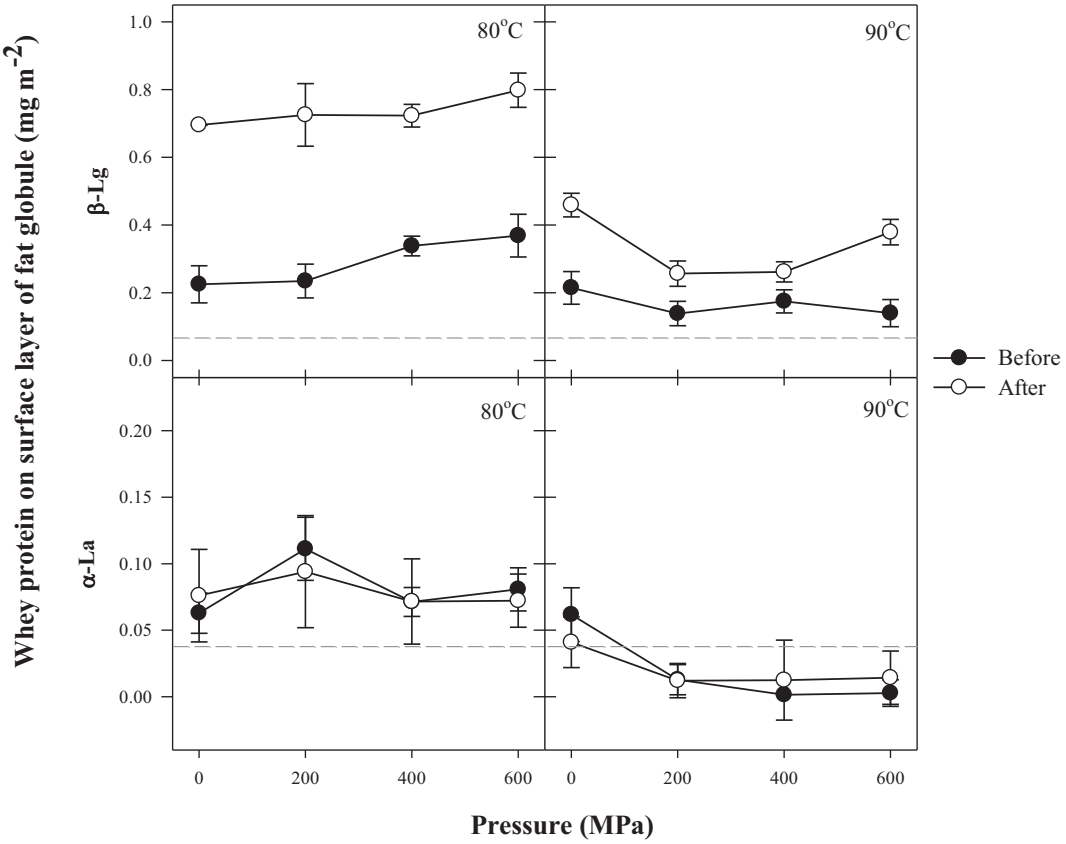


Figure 8.6. The percentages of whey proteins on the surface layers of heat/HP-treated recombined milk fat globules. HP treatments for 30 min were applied to recombined milks that were heated at 80°C or 90°C for 30 min. Dash lines represent whey proteins on surface layer of untreated recombined milk.

However, in milks heated after homogenization (at 90°C), the amount of caseins adsorbed onto the surface layers of fat globules decreased considerably with treatment pressure, whereas whey proteins were largely unaffected. These findings confirmed the previous findings in Chapter 6 that HP treatments applied to recombined milks either before or after homogenization resulted in the decreased in the amounts of caseins on the surface layer, but increased the amounts of whey proteins on the surface layers of fat globules as treatment pressure increased. The increases in whey proteins on the surface layers were consistent with previous studies on heat/HP-treated whole milk. Ye et al. (2004) reported that the amounts of β-Lg associated with MFGM in heated (90°C/15 min) milk or HP-(400 and 800 MPa)/heat (90°C/15 min)-treated milks were

approximately 1.5 $mg \cdot g^{-1}$ fat, which were twice that of HP-treated milk alone. These results suggested that the heat treatments had more pronounced effects on the association of whey proteins on the surface layers of fat globules than the HP treatments.

Figures 8.5 and 8.6 also confirmed that the placement of heat/HP treatment had major effects on the amounts of caseins and whey proteins on the surface layers of fat globules. At 80 and 90°C, the amount of casein (including κ-casein) adsorbed onto the surface layers of fat globules isolated from recombined milk heat/HP-treated before homogenization was higher, whereas the amount of β-Lg was lower than those of recombined milk heat/HP-treated after homogenization. The amount of α-La was similar in milks heated before or after homogenization.

SDS-PAGE patterns under reducing conditions of creams washed with SMUF containing dissociating agents are shown in Figure 8.7. The intensities of protein bands remaining at the fat globule surface after washing the cream in dissociating buffers were markedly higher in recombined milks heat/HP-treated before homogenization compared with recombined milk heat/HP-treated after homogenization. These band intensities were lower in recombined milks heated at 90°C as compared to those heated at 80°C. Very faint bands were observed in samples heated at 80°C and 90°C after homogenisation. Increase in treatment pressure applied to milks that had been heated at 80°C before or after homogenization appeared to increase the amount of β-Lg remaining at the globule surface. The modifications of casein and whey protein induced by heat and HP treatment probably made these proteins on fat globule surface difficult to be dissociated by EDTA...

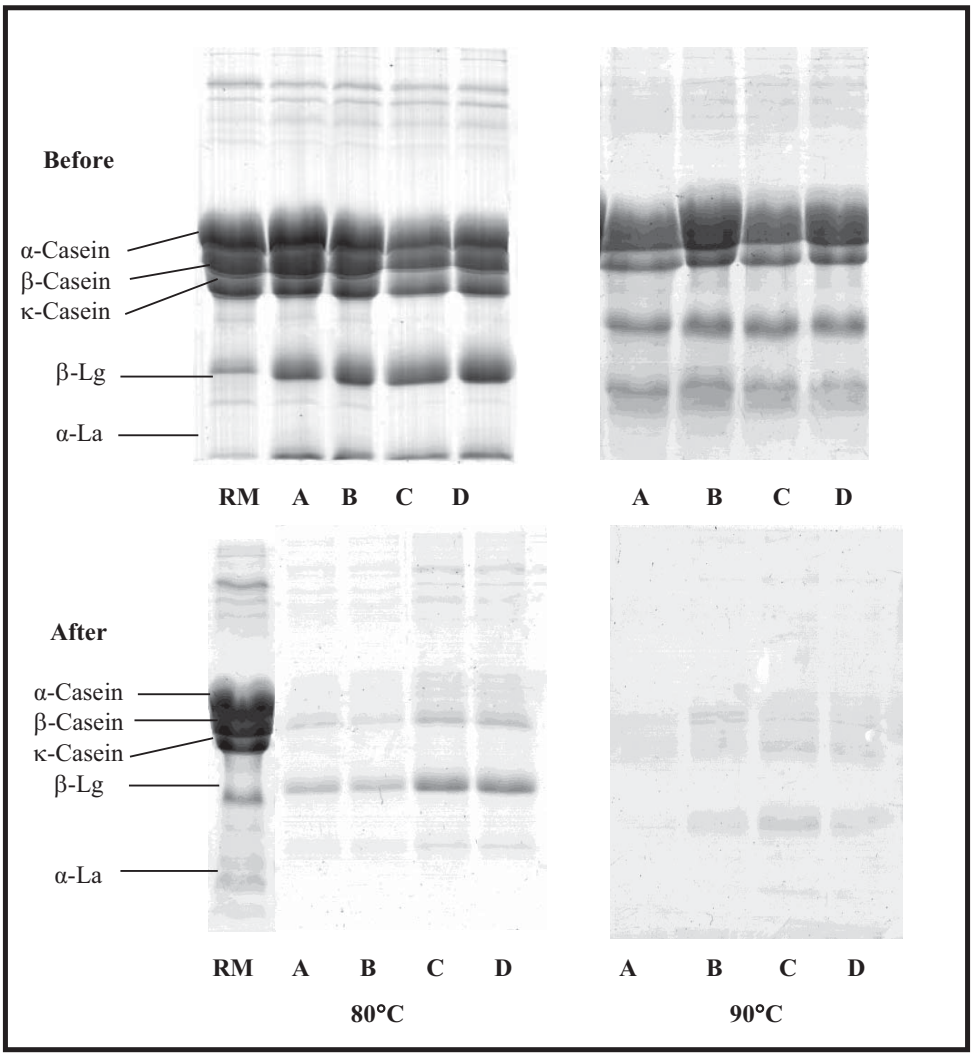


Figure 8.7. SDS-PAGE patterns under reducing conditions of membrane materials isolated from heat/HP-treated recombined milks. The creams were washed with SMUF containing dissociating agents. HP treatments were carried out at 20°C for 30 min at (A) 0, (B) 200, (C) 400, and (D) 600 MPa on milk samples that have been heated at 80 or 90°C either before or after homogenization. RM represents the surface materials of untreated recombined milk.

8.2.3. Acid gelation properties

Both heat and HP treatments decreased the gelation time, increased gelation pH, final *G*' and yield stress values of the acid gels made from recombined milks as discussed in Chapters 5 and 7 for heat and HP treatments respectively. The acid gelation properties of heat/HP-treated recombined milks are shown in Table 8.1.

Table 8.1. *Acid gelation properties of heat/HP-treated recombined milk.*

Milk	Gelation		*G'* (Pa)		Yield	
treatment	pH	Time (h)	30°C	5°C	Strain (%)	Stress (Pa)
Untreated	4.73^{f} (0.00)	1.45^{e} (0.00)	14.20^{f} (.20)	56.07^{g} (2.181)	81.80^{f} (0.00)	107.67^{b,c,d,e} (4.73)
Heat/HP treatments before homogenization						
Heat at 80°C for 30 min and HP-treated at different pressures for 30 min at 20°C						
0 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	234.67^{a} (4.93)	721.00^{a,b} (12.12)	21.17^{c,d} (3.21)	125.00^{b,c,d,e} (19.70)
200 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	256.00^{a,b,c} (3.00)	794.00^{b,c} (3.00)	20.95^{c,d} (0.05)	133.50^{b,c,d,e} (1.50)
400 MPa	5.09^{c} (0.00)	0.68^{b} (0.02)	329.00^{d,e} (19.00)	1075.00^{d,e,f} (55.00)	16.20^{a,b,c,d} (4.70)	147.50^{c,d,e} (46.50)
600 MPa	5.06^{b} (0.01)	0.73^{c} (0.02)	356.00^{e} (50.86)	1131.33^{f} (139.52)	15.40^{a,b,c,d} (2.43)	147.33^{c,d,e} (40.07)
Heat at 90°C for 30 min and HP- treated at different pressures for 30 min at 20°C						
0 MPa	5.14^{e} (0.00)	0.62^{a} (0.00)	238.67^{a} (4.62)	686.33^{a,b} (22.59)	15.67^{a,b,c,d} (2.35)	85.50^{a,b,c} (15.28)
200 MPa	5.12^{d} (0.00)	0.65^{a,b} (0.00)	248.67 (1.15)	725.00^{a,b} (6.56)	21.27^{d}(1.58)	126.00^{b,c,d,e} (9.17)
400 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	316.50^{c,d,e} (23.50)	922.50^{c,d} (65.50)	13.30^{a,b,c,d} (0.70)	103.50^{b,c,d,e} (17.50)
600 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	274.33^{a,b,c,d} (45.06)	835.33^{b,c} (84.32)	11.61^{a,b,c} (1.74)	76.70^{a,b} (22.16)
Heat/HP treatments after homogenization						
Heat at 80°C for 30 min and HP-treated at different pressures for 30 min at 20°C						
0 MPa	5.12^{d} (0.00)	0.65^{a,b} (0.00)	299.00^{a,b,c,d,e} (9.64)	960.33^{c,d,e,f} (25.01)	35.23^{e} (9.33)	249.33^{f} (17.24)
200 MPa	5.05^{a,b} (0.00)	0.75^{c,d} (0.00)	255.50^{a,b,c} (2.50)	933.00^{c,d,e} (2.00)	19.95^{b,c,d} (0.95)	151.50^{d,e} (3.50)
400 MPa	5.04^{a} (0.01)	0.77^{d} (0.02)	313.50^{b,c,d,e} (30.50)	1125.00^{f} (95.00)	10.67^{a,b} (0.74)	105.70^{b,c,d,e} (23.30)
600 MPa	5.04^{a} (0.01)	0.77^{d} (0.02)	289.00^{a,b,c,d} (23.00)	1033.00^{d,e,f} (67.00)	9.44^{a} (0.96)	89.20^{a,b,c,d} (13.80)
Heat at 90°C for 30 min and HP-treated at different pressures for 30 min at 20°C						
0 MPa	5.15^{e} (0.03)	0.62^{a} (0.03)	298.33^{a,b,c,d,e} (4.62)	950.67^{c,d,e} (18.50)	17.03^{a,b,c,d} (3.37)	132.00^{b,c,d,e} (21.93)
200 MPa	5.12^{d} (0.00)	0.65^{a,b} (0.00)	279.50^{a,b,c,d} (6.50)	924.50^{c,d} (21.50)	21.90^{d} (3.80)	163.50^{d} (22.50)
400 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	337.50^{d,e} (23.50)	1100.00^{e,f} (40.00)	11.67^{a,b,c} (1.74)	105.85^{b,c,d,e} (16.15)
600 MPa	5.09^{c} (0.00)	0.68^{b} (0.00)	146.50^{g} (9.50)	554.50^{a} (54.50)	9.00^{a} (.94)	38.40^{a} (0.20)

Note. Standard deviations are in parentheses. [a] Samples with the same superscript letters within the same column are not significantly ($P \leq 0.05$) different from each other.

8.2.3.1. Gelation pH and gelation time

The changes in gelation pH with treatment pressures are shown in Figure 8.8. The gelation pH of heat/HP treated milks was higher and the gelation time was shorter than those of untreated recombined milk. The gelation pH and time of heat/HP-treated milks were in the range of 5.04 - 5.15 and 0.62 - 0.68 h respectively.

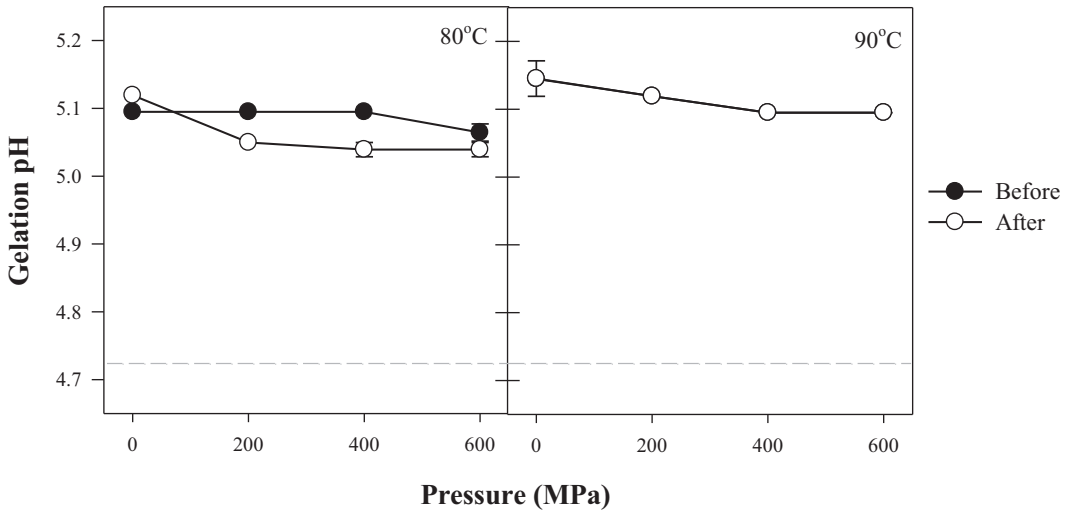


Figure 8.8. Changes in gelation pH as a function of treatment pressures for 30 min at 20°C. HP treatments were applied to milks heated at 80°C or 90°C for 30 min either before or after homogenization. At 90°C, the change in gelation pH is similar for milk sample HP-treated before or after homogenization, i.e., the same line in the Figure. Dash lines represent gelation pH of untreated recombined milk.

At all treatment pressures, recombined milks heated at 80°C for 30 min had lower gelation pH and longer gelation times compared to those of milks heated at 90°C for 30 min. These results confirm that heating milk at temperatures ≥ 70°C resulted in increased gelation pH and decreased gelation time and the effects depend on heating temperature, supporting the findings from Chapter 5 and previous reports (Anema, Lauber, et al., 2005; Lucey, Munro, et al., 1998). The changes in gelation pH and gelation time of heat/HP-treated recombined milks are attributed to the whey protein denaturation and also to the association of denatured whey proteins with casein micelles through the formation of intermolecular disulphide bonds (Singh, 1995). As the pH of acidified heat/HP-treated recombined milk approached the isoelectric point of whey proteins (pH 5.2), denatured whey proteins aggregated and the gelation occurred.

At a heat treatment of 80°C for 30 min, recombined milks that underwent HP treatment before homogenization had a slightly higher gelation pH than those of recombined milks HP treated after homogenization. This was probably due to slightly higher levels

of β-Lg denaturation in recombined milk HP-treated before homogenization (Figure 8.3). At heat treatment of 90°C for 30 min, the gelation pH of recombined milks HP-treated before or after homogenization was not significantly different (Figure 8.8) as both milks had similar levels of whey protein denaturation. However, when HP treatments were applied to heated recombined milk, the gelation pH tended to decrease slightly as treatment pressures increased. Anema (2010b) reported that the gelation pH of skim milks heated (90°C/30 min) either before or after HP (400 MPa/30 min) treatment were not significantly different from the heated milks, but were significantly different from the milk that was only HP-treated.

8.2.3.2. Viscoelastic properties during acidification

The changes in storage modulus (G') with time after GDL addition to heat/HP-treated recombined milks are shown in Figure 8.9. The gelation curves of acid gels made from heat/HP-treated recombined milks were different from those of untreated recombined milks, i.e., the gelation times were shorter and the final G' values were higher.

The rate of increase in G' and the final G' values were significantly different among acid gels made from heat/HP-treated milks. The gelation curves of acid gels made from milks heated at 90°C for 30 min and subsequently HP-treated at 200 MPa for 30 min at 20°C before homogenization were similar to those of heated milks without HP treatment, suggesting that HP treatments before homogenization at this pressure did not have a significant effect on acid gelation properties of heated milks.

The effects of heat/HP treatments on final G' values of acid gels made from heat/HP-treated recombined milks are shown in Figure 8.10. At heat treatment of 80°C for 30 min, increasing treatment pressures before homogenization increased the final G' values, whereas increasing treatment pressures applied after homogenization did not have a significant effect on the final G' values (Figure 8.10, Table 8.1).

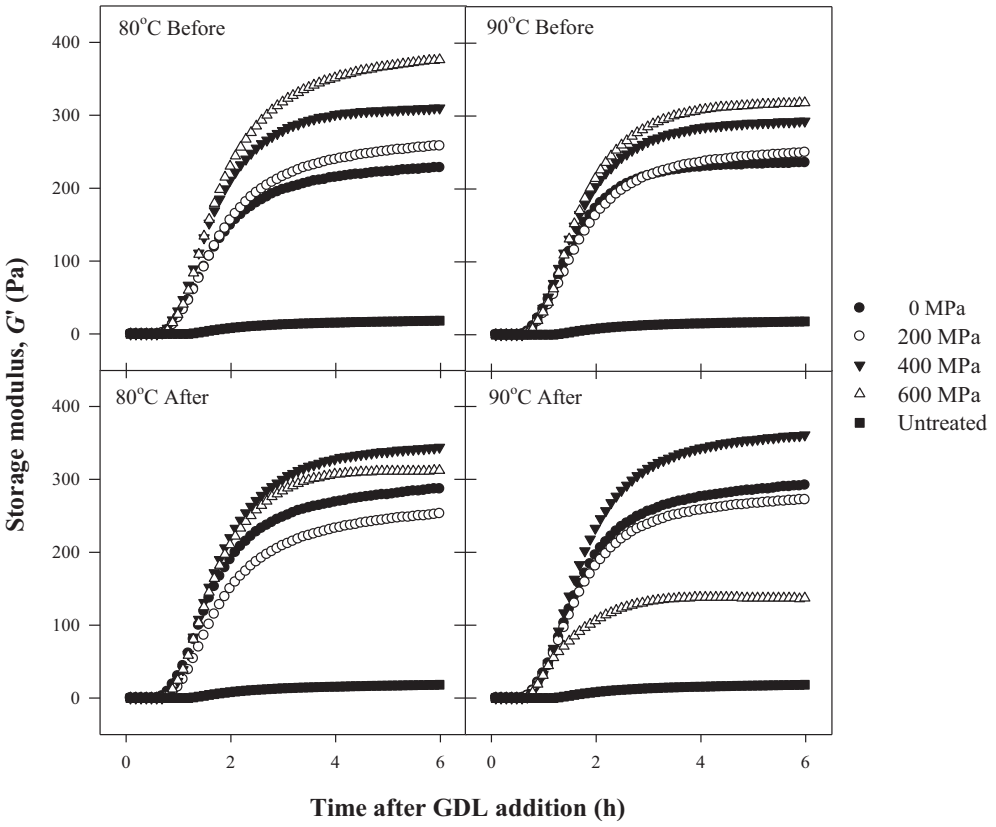


Figure 8.9. Changes in storage modulus, G', with time after GDL addition of heat/HP-treated recombined milks. The milk samples were heat/HP treated either before or after homogenization.

At heat treatment of 90°C for 30 min either before or after homogenization, the final G' values slightly increased as treatment pressures increased from 0 to 400 MPa. However, treatment pressure at 600 MPa resulted in a decrease in the final G' value. Acid gel made from recombined milk heat (90°C/30 min)/HP (600 MPa/30 min/20°C) treated after homogenization had a very low final G' value, i.e., approximately 50% of those made from other heat/HP-treated recombined milks. The lower final G' values of recombined milks that underwent severe heat/HP treatments were in agreement with the findings on heated recombined milks (Chapter 5) and those reported previously on heated skim milks (Anema, Lauber, et al., 2005; Anema & McKenna, 1996; Dannenberg & Kessler, 1988c; Lucey, Teo, et al., 1997).

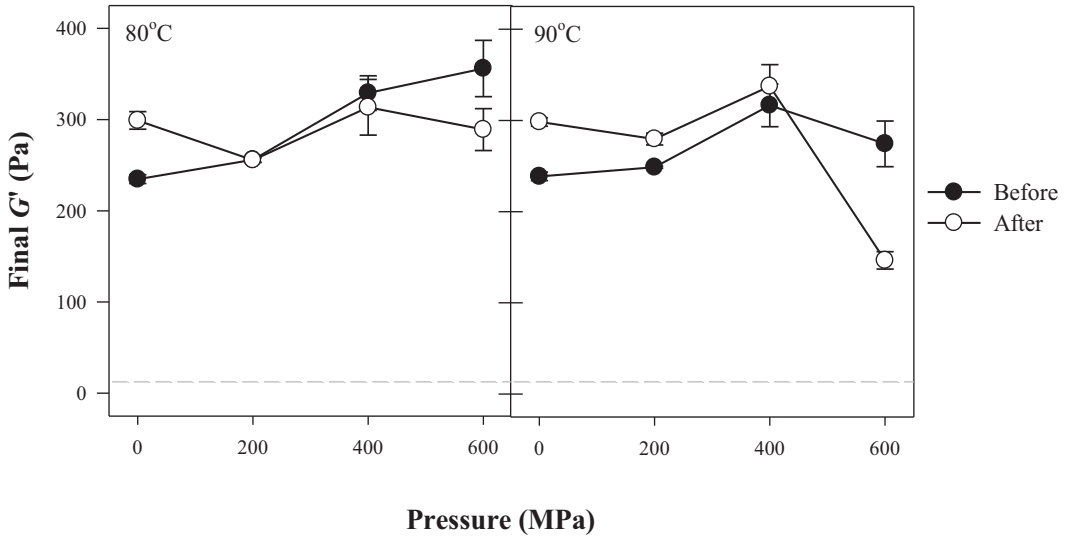


Figure 8.10. Final *G*′ values at 30°C as a function of treatment pressures for 30 min at 20°C. HP treatments were applied to the milks heated at 80°C or 90°C for 30 min either before or after homogenization. Dash line represent final *G*′ values of untreated recombined milk.

When heat treatment beyond that required for denaturing all the whey proteins is applied to skim milks, the final *G*′ value of acid gels decreases (Anema & McKenna, 1996; Dannenberg & Kessler, 1988c; Lucey, Teo, et al., 1997). As both heat and HP treatments denature the whey proteins, the factors responsible for the decrease in final *G*′ values on severe heat/HP treatments are likely to be similar.

Anema Lauber, et al. (2005) reported that for heat (90°C/30 min)/HP (400 MPa/20°C/0-120 min)-treated skim milks, the final *G*′ values increased with pressurizing time. However, prolonged pressurizing time for 120 min resulted in a slight decrease in final *G*′ value. Needs, Stening, et al. (2000) found that the final *G*′ values of acid gels made from HP-treated (600 MPa/15 min) skim milks fortified with whey proteins are markedly higher than those made from heated (85°C/20 min) skim milks. Harte, Luedecke, Swanson, and Barbosa-Canovas (2003a) found that a short HP treatment (5 min) either before or after heat treatment resulted in acid gels with increased firmness compared with those made from heated milks. In contrast, Walsh-O′Grady et al. (2001) found that the firmness of acid gels made from heated milk was similar to those made from HP-treated milk. This study found that the effects of heat/HP treatments on final

G' values of acid gels made from recombined milk depend on treatment temperature and pressure. Both treatments denatured whey proteins and induced the association of denatured whey proteins with casein micelles (Chapters 4 and 6), which in turn affect the acid gelation properties. However, Figures 8.3 and 8.10 implied that the level of whey protein denaturation was not the only contributing factor to the change in final G' value of acid gels made from heat/HP-treated recombined milks. Those factors will be discussed in Section 8.2.3.5.

8.2.3.3. Viscoelastic properties of acid milk gels

The following viscoelastic behaviour for all the acid gels, regardless of heat/HP treatments applied were observed: the plots of G' and G'' against frequency of the applied deformation were straight lines with a slope of approximately 0.14 ± 0.01 on logarithmic scales; G' was greater than G''; the final G' value at 5°C was approximately 3.25 ± 0.28 times of that at 30°C and tan δ were constant with the values of 0.23 ± 0.01. These findings are consistent with Chapters 5 and 7, indicating that the acid gels made from heat/HP-treated recombined milks were elastic, and the proportion of the deformation energy stored and the proportion of the deformation energy lost remained constant.

8.2.3.4. Large deformation rheology of acid gels

After the gel has been formed, the set gels were subjected to strain sweep. Typical strain sweep curves are shown in Figure 8.11. Heat/HP treatments applied to recombined milks have significant effects on yield strain and stress values of acid gels and the effects depend on treatment pressure and temperature. The yield strain and stress values of acid gels made from heat/HP-treated recombined milks as a function of treatment pressures are shown in Figure 8.12. At heat treatment of 80°C for 30 min, increased treatment pressures from 0 to 600 MPa after homogenization resulted in decreased yield strain values. However, increased treatment pressures before homogenization at this heating profile did not significantly affect the yield strain values. At this heating profile, the yield strain values of recombined milks HP treated at 200 – 600 MPa for 30 min before homogenization were slightly higher than those of milks HP-treated after homogenization.

At heat treatment of 90°C for 30 min, there were no significant differences in yield strain values of acid gels made from recombined milks heat/HP-treated before or after homogenization. Increased treatment pressures from 200 to 600 MPa at this heating profile slightly lowered the yield strain values of acid gels. At heat treatment of 80°C for 30 min, increased treatment pressures before homogenization resulted in a slight increase in yield stress values.

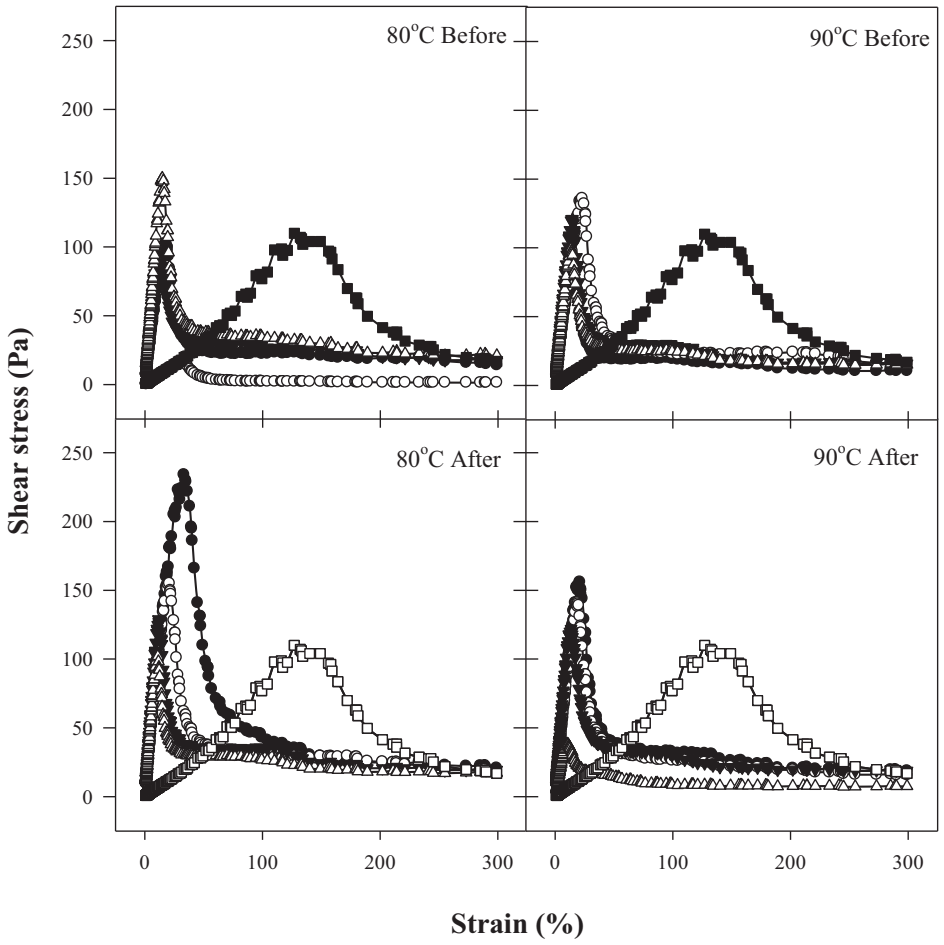


Figure 8.11. Shear stress as a function of strain of acid gels made from untreated (■) and heat/HP-treated recombined milks. Milk samples were heated at 80°C or 90°C for 30 min either before or after homogenization and HP-treated at 20°C for 30 min at 0 (●), 200 (○), 400 (▼), 600 (△) MPa.

However, when heat (80°C/30 min)/HP treatments were applied to the milk samples after homogenization, the yield stress dramatically decreased as the treatment pressure increased. At 80°C, treatment pressure applied after homogenization from 0 to 600 MPa resulted in decreased in yield stress values. However, increased treatment pressure at this temperature before homogenization from 0 to 600 MPa slightly increased the yield stress value. At heat treatment of 90°C for 30 min, increased treatment pressures either before or after homogenization from 0 to 200 MPa resulted in increased yield stress values. Further increase in treatment pressures up to 600 MPa resulted in a dramatic decrease in yield stress values.

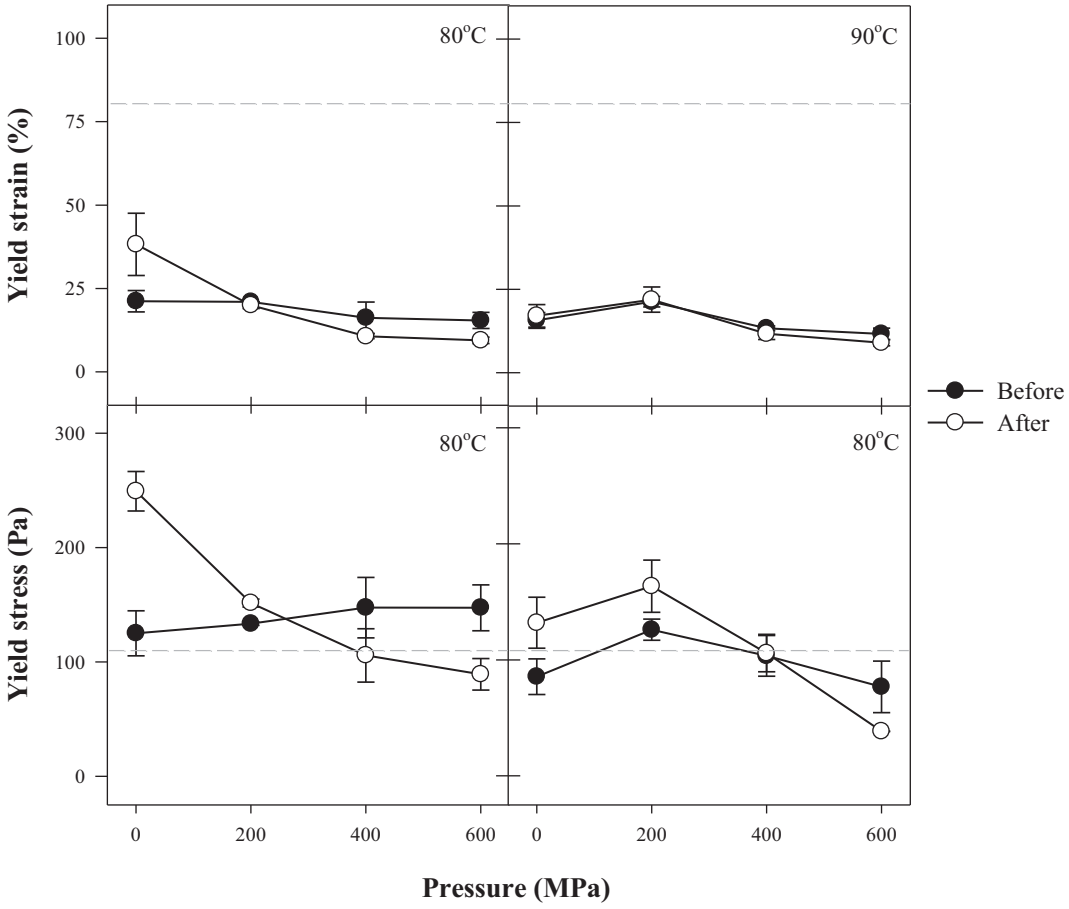


Figure 8.12. Yield strain and stress as a function of treatment pressures for 30 min at 20°C. HP treatments for 30 min were applied to the milks heated at 80°C or 90°C for 30 min either before or after homogenization. Dash lines represent yield strain and stress of untreated recombined milk.

Previous studies on skim milk systems have found that both heat and HP treatments cause the increase in yield stress of acid gels compared to those make from skim milks without any treatment (Anema, 2008c; Considine, et al., 2007; Harte, et al., 2003; Johnston, et al., 1993). A short HP treatment (5 min) either before or after heat treatment produced acid gels with increased yield stress compared with those make from heated milk (Harte, et al., 2003). However, this study found that the combined effects of heat/HP treatments in recombined milks were complicated. These effects were not only dependent on the severity of heat/HP treatments (temperature and pressure), but also on the order of the treatments, which was expected to affect the interactions among milk proteins. In addition, the results implied that the denaturation of whey proteins induced by heat/HP treatments was not the only contributing factor to the difference in yield stress values between acid gels made from recombined milks heat/HP treated before or after homogenization. This finding supports the results from Chapter 7 and previous studies on skim milk systems. Anema et al. (2004) and del Angel and Dalgleish (2006) also reported that the denaturation of the whey proteins in heated skim milk is insufficient to predict the final properties of acid gels. In recombined milk systems, the fat globules surface layers play an important role, which will be discussed in the following sections.

8.2.3.5. Possible mechanisms involved in acid gelation properties of heat/HP-treated recombined milks

(1) Acid gels made from recombined milks heat/HP-treated before homogenization

Heating milks at high temperatures (80°C or 90°C for 30 min) resulted in > 90% of β-Lg denaturation and > 40% of α-La denaturation (with a combined denaturation of > 70% total whey protein denaturation) as shown in Figure 8.3. In heated skim milk only small changes occurred to the structure and size of casein micelles and some caseins, especially a proportion of κ-casein dissociated from the micelles (Anema & Li, 2003a; Considine, et al., 2007). The denatured whey proteins interacted with κ-casein on the surface of casein micelles or with serum-phase κ-casein that had dissociated from the casein micelles during heat treatment (Anema, 2008b; Donato, Alexander, & Dalgleish, 2007). The illustration of heated reconstituted milk is presented in Figure 4.13 (Chapter 4).

Under subsequent HP treatment, the dissociation and rearrangement of casein micelles and further denaturation of whey protein occurred, resulting in smaller non-native casein particles (Anema, 2010b) and higher levels of whey protein denaturation (Considine, et al., 2007; Patel, et al., 2006). The extent of casein micelle dissociation and further whey protein denaturation depend on treatment pressure. Some caseins, i.e., κ- and α_{s2}-caseins were dissociated into the serum. The denatured whey proteins that have been interacted with κ-casein on the surface of casein micelles during heat treatment were also dissociated along with κ-casein into the serum. The denatured whey proteins induced by subsequent HP treatment interacted with κ-caseins on the surface of non-native casein particles. The illustration of heat/HP-treated reconstituted milk is shown in Figure 8.13.

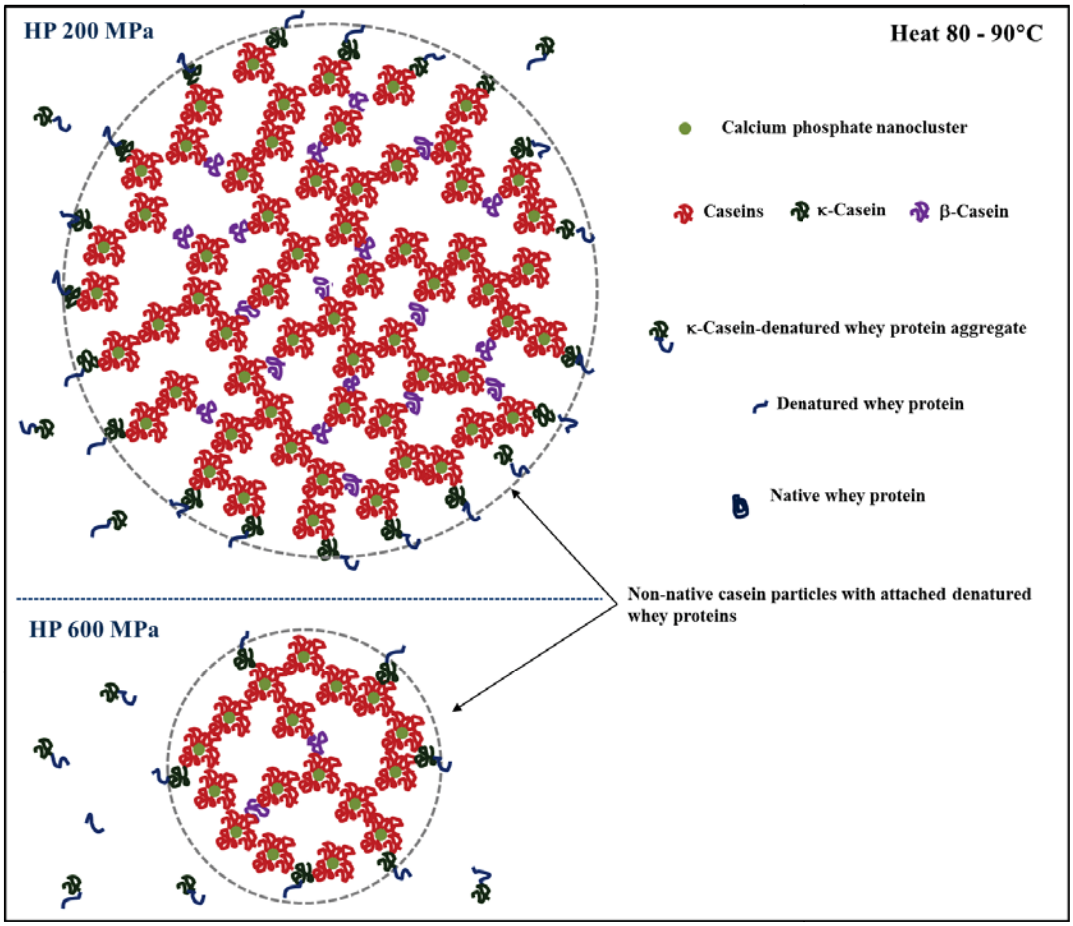


Figure 8.13. Schematic illustration of heat/HP-treated reconstituted milk. The relative sizes of individual components are not to scale.

When heat/HP-treated reconstituted milks were homogenized with milk fat, the non-native casein particles dissociated caseins (with attached denatured whey proteins) and denatured whey proteins were adsorbed onto the surface layers of fat globules. It appears that many of these protein species were directly adsorbed at the globule surface as shown in Figure 8.7. As a result, the proportions of different proteins adsorbed onto the fat globule surface layers of recombined milk heat/HP-treated before homogenization are different from those of untreated, heated, and HP-treated recombined milks. The illustration of heat/HP-treated recombined milk is shown in Figure 8.14. When heat/HP-treated (before homogenization) recombined milks were acidified with GDL, the non-native casein micelles including those on the surface layers of fat globules are involved in the formation of a protein gel network. The general gel formation process is expected to be similar to that of HP-treated skim milk systems reported by Anema (2010b), except that fat globules will also take part in the gel formation process.

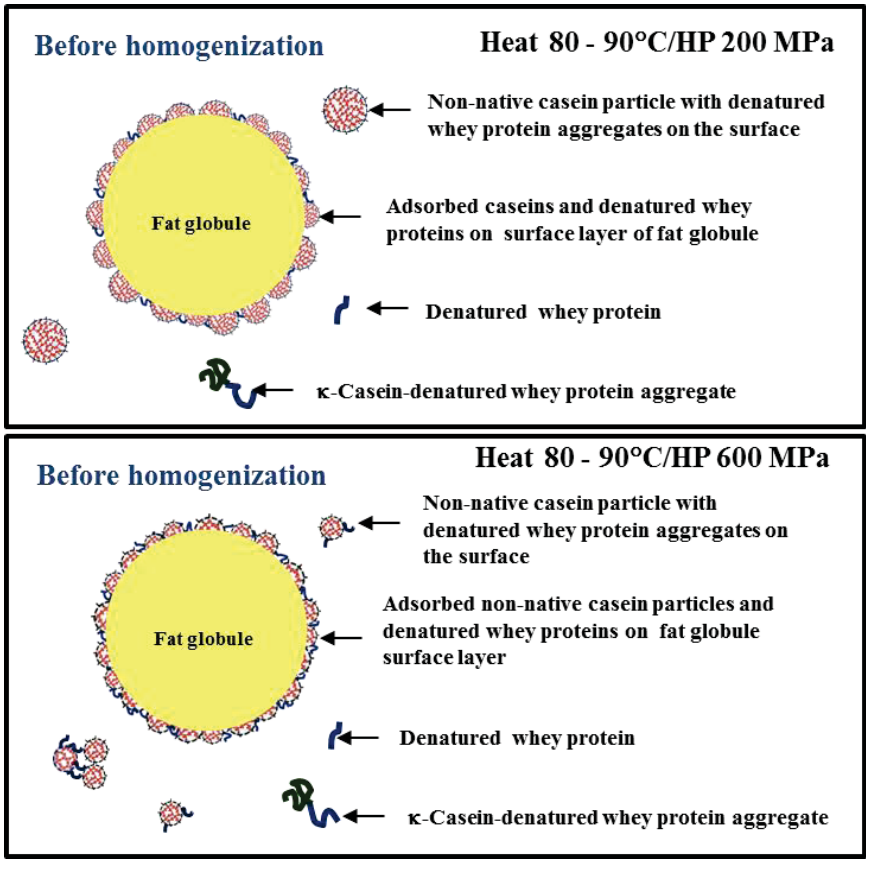


Figure 8.14. Schematic illustration of recombined milk heat/HP-treated before homogenization. The relative sizes of individual components are not to scale.

The non-native casein particles in heat/HP-treated recombined milk started to fuse as the pH of acidified milk was lowered, resulting in an increased in size of aggregated particles. At pH lower than the gelation point, the colloidal particles would have the size increased almost to the size of original casein micelles in untreated or heated recombined milks and the particle fuses into chains and clusters. The denatured whey proteins complexes are interacted with the protein strands. The heat/HP-treated recombined milk fat globules which contained interactive materials, i.e., casein micelles and denatured whey proteins, are also interacted within the protein network. The possible structure of acid gels made from recombined milk heat/HP-treated before homogenization is illustrated in Figure 8.15.

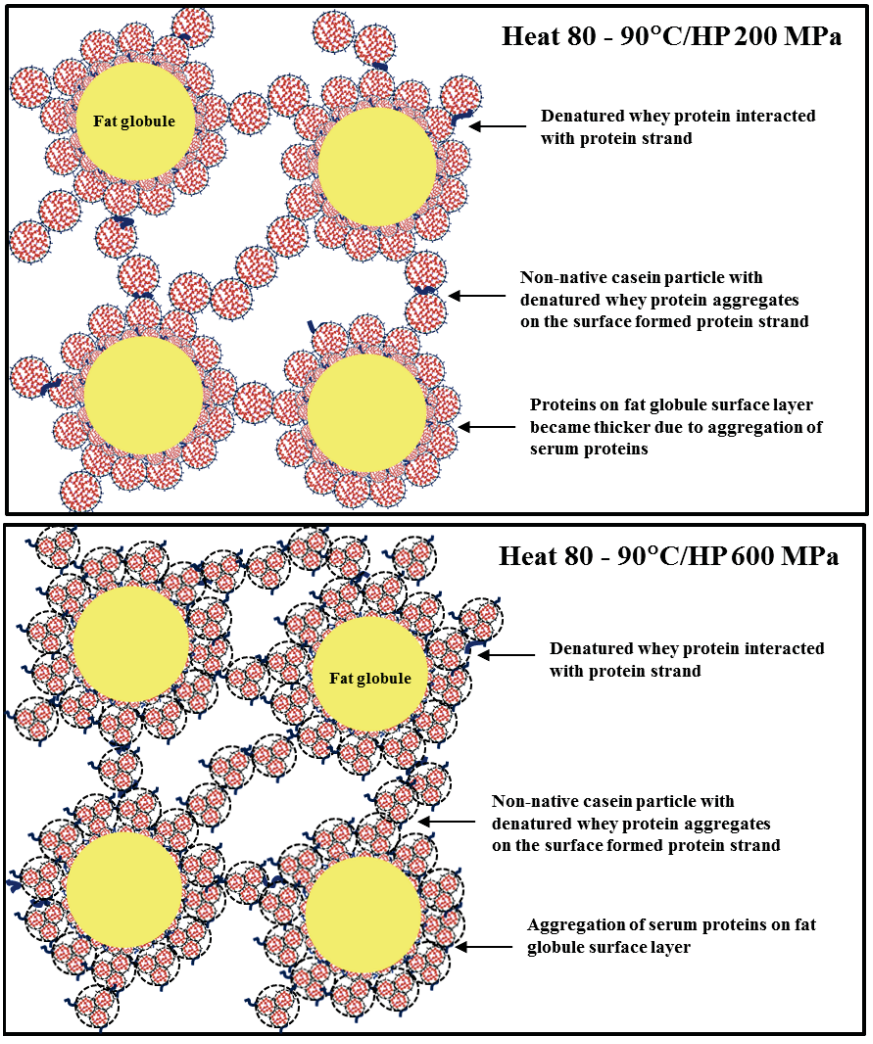


Figure 8.15. Schematic illustration of acid milk gel made from recombined milk heat/HP-treated before homogenization. The relative sizes of individual components are not to scale.

(2) Acid gels made from recombined milks heat/HP-treated after homogenization

As discussed in Chapter 4, the surface layers of recombined milk fat globules are composed mainly of caseins (Figure 4.2). When heat treatments at 80°C or 90°C were applied to recombined milks after homogenization, the whey proteins in the serum were denatured (Figure 8.3), whereas, the casein micelles were not significantly changed in their size and internal structure, even though some caseins (especially κ-casein) were dissociated from the micelles (Considine, et al., 2007). The denatured whey proteins interacted with κ-casein on the surfaces of casein micelles (Patel & Creamer, 2009) or remained in the serum or interacted with the surface layers of fat globules (Chapter 4). The illustration of recombined milk heated after homogenization is shown in Figure 4.15 (Chapter 4).

The subsequent HP treatment applied to heated recombined milks dissociated the casein micelles, both in the serum and on the surface layers of fat globules and further denatured whey proteins in the serum. As a consequence, the non-native casein particles with smaller size were formed and some caseins were dissociated from the surface layers of fat globules into the serum. The decrease in casein load at the globule surface indicated dissociation of caseins, with increase in pressure. The denatured whey proteins that had been associated with casein micelles during heat treatment were also dissociated along with caseins under HP treatment, but at the same time more denatured whey proteins from the serum were deposited on the globule surface. Compared to recombined milk heat/HP-treated before homogenization, the amount of caseins was lower, but the amount of whey proteins was higher on fat globule surface layer of recombined milk heat/HP-treated after homogenization. The illustration of heat/HP-treated recombined milk is shown in Figure 8.16.

When heat/HP-treated recombined milks were acidified with GDL, the non-native casein micelles formed the protein gel network. The dissociated κ-caseins with attached denatured whey proteins and the denatured whey protein were integrated into the protein gel matrix. The fat globules of heat/HP-treated recombined milk were also integrated into the proteins gel matrix as they contained interactive materials, i.e., non-native casein particles and denatured whey proteins. The fat globules in this system contained relatively thin surface layers and low concentration of caseins. The possible

illustration of acid gel structure of recombined milk heat/HP-treated after homogenization is shown in Figure 8.17.

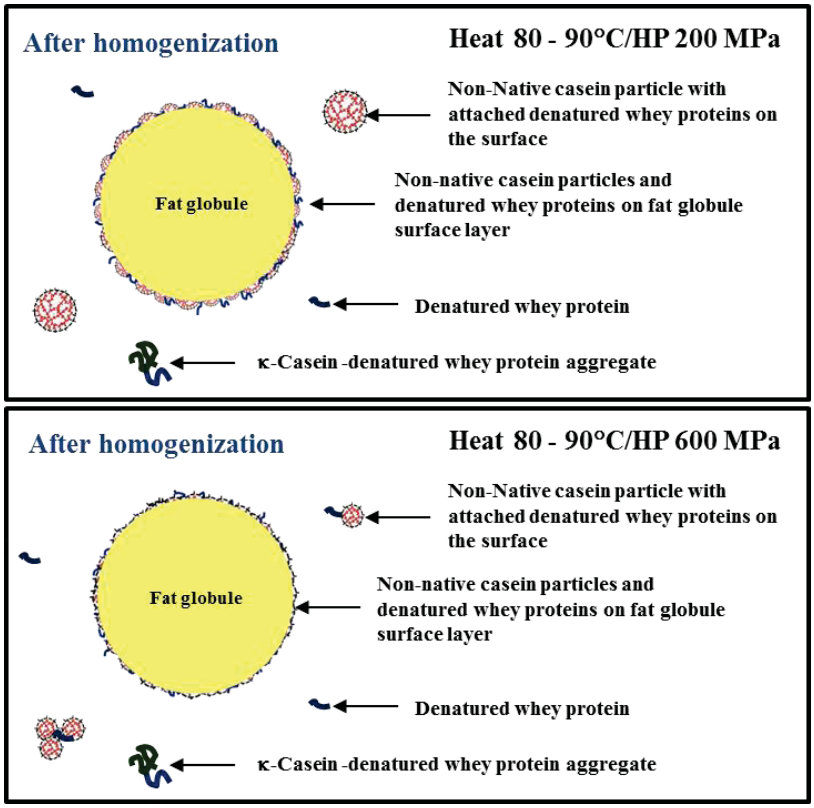


Figure 8.16. Schematic illustration of recombined milk heat/HP-treated at 200 and 600 MPa after homogenization. The relative sizes of individual are not to scale.

It is interesting to note that the differences in the state and proportions of caseins at the fat globule surfaces (shown in Figures 8.14 and 8.16) resulted in the differences in final G' (Figure 8.10) and yield stress (Figure 8.12) values of recombined milks heat/HP-treated before or after homogenization. In other words, the differences in behaviour of final G' and yield stress values were dependent on the order of heat/HP treatments, especially at heat treatment of 80°C. When HP treatments were applied to reconstituted milks heated (80°C/30 min) before homogenization, the final G' values increased, whereas the yield stress values did not significantly change with increased treatment pressure. However, different effects were observed when HP treatments were applied to recombined milks heated (80°C/30 min) after homogenization. At the latter conditions, the final G' values were approximately constant (Figure 8.10), whereas the yield stress values decreased (Figure 8.12) with increased treatment pressure.

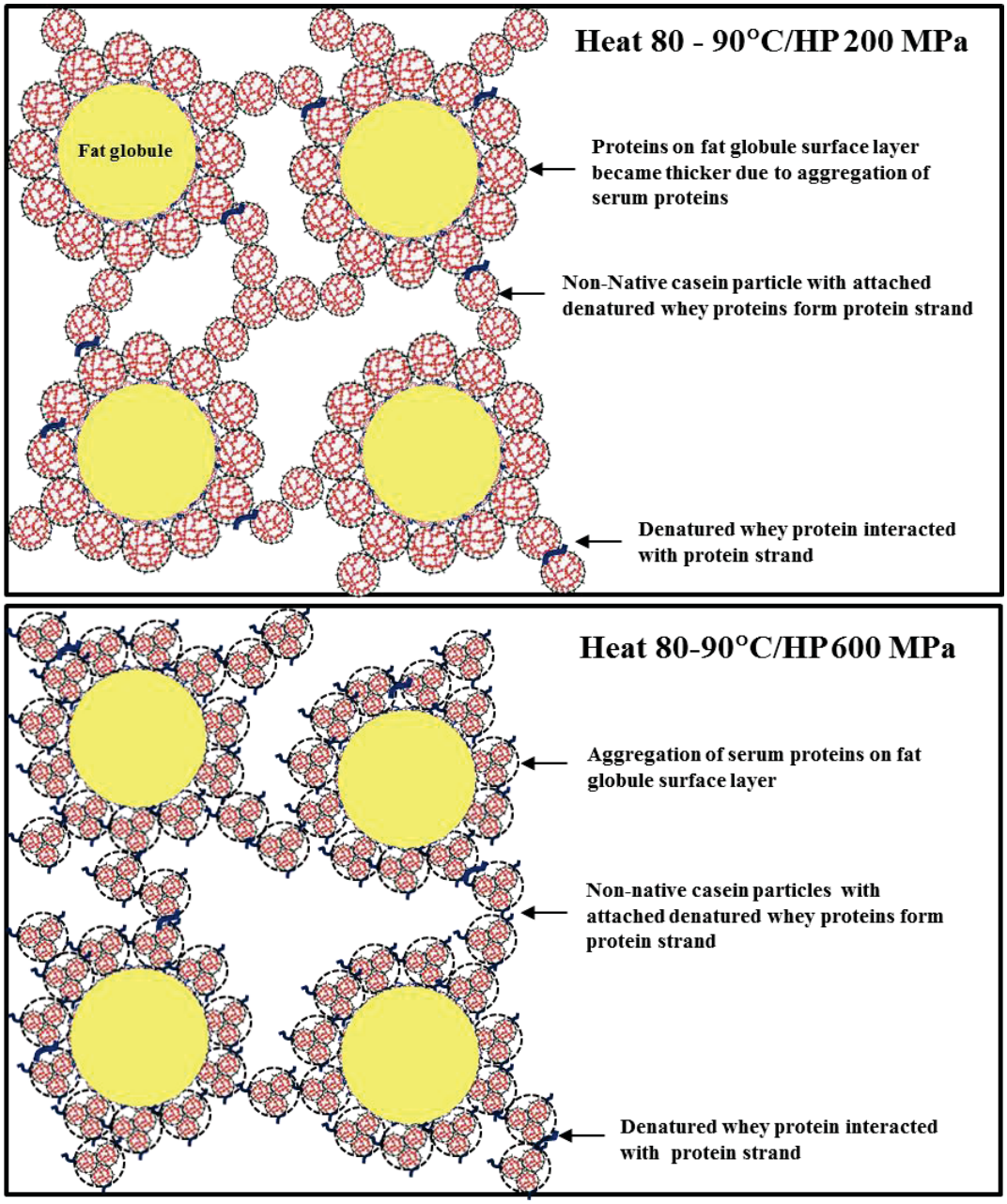


Figure 8.17. Schematic illustration of acid milk gel made from recombined milk heat/HP-treated at 200 and 600 MPa after homogenization. The relative sizes of individual components are not to scale.

At heat treatment of 90°C, the effects of heat/HP treatments applied to milk samples before or after homogenization seem to be similar. Under these conditions, increasing treatment pressures from 200 MPa to 400 MPa resulted in increasing final G' values, however when the treatment pressure was increased to 600 MPa, the final G' values decreased. The yield stress values decreased with increasing treatment pressure (200

MPa to 600 MPa) applied to milk samples that had been heated at 90°C for 30 min (Figure 8.12). The effects of treatment pressure were more pronounced when heat/HP treatments were applied after homogenization. Under extreme conditions (90°C/30 min) and HP (600 MPa), the lower values of final G' and yield stress found in acid gels made from recombined milk heated after homogenization compared with those of milks underwent heat/HP treatments before homogenization might reflect differences in the reactivity of fat globule surfaces.

Figures 8.10 and 8.12 show the differences in behaviour of final G' and yield stress depending on the order of treatment, especially at 80°C. Milk heated at 80°C and HP-treated at 200 – 600 MPa before homogenization resulted in little change in yield stress value as pressure increased, but resulted in increasing final G' values. Recombined milk heated at 80°C and HP-treated at 200 – 600 MPa after homogenization resulted in decreasing yield stress values as pressure increased, the final G' values remained relatively constant.

8.3. Conclusions

Heat/HP treatments applied to recombined milks either before or after homogenization denatured the whey proteins, altered the surface layers of recombined milk fat globules, and changed the acid gelation properties. These effects were dependent on heating temperature, treatment pressure, and placement of heat/HP treatments. The interactions between modified milk proteins present in the serum and those adsorbed at the fat globule surfaces play an important role in the formation and structure of acid gels.

CHAPTER 9

OVERALL CONCLUSIONS AND RECOMMENDATIONS

9.1 Introduction

Recombined milks form the basis for the production of several dairy products. This is particularly important in countries where the milk production is insufficient to meet the demand for dairy products. Recombined milks are essentially oil-in-water emulsions, made by dispersing milk fat into reconstituted skim milk using homogenization. The composition and properties of the fat globule surface layers, which are markedly affected by processing treatments, play an important role in determining the properties of the recombined dairy products. This is largely due to the fact that fat globules in recombined milk are covered with adsorbed layers of milk proteins and they behave as large protein particles. Consequently, these globules take part in all protein reactions, such as coagulation during heating, acidification and renneting.

The effects of various processing conditions, such as the intensity of homogenization, heat and high pressure treatments on the fat globule surface layers have been studied previously, as described in the literature review. Most studies have focused on pressure-induced changes in skim milk and whole milk systems, including denaturation of whey proteins, changes in casein micelles, and natural fat globule membranes. However, the effects of high pressure treatments on recombined milk systems, particularly on the state of fat globules, have not been studied before. No information is available on HP-induced changes in the surface layers of fat globules and their interactions with milk proteins. Limited information on gelation properties of acid gel prepared from HP-treated recombined milk is available. Further understanding of these aspects would allow better control of the texture, microstructure and quality of dairy products.

In this study, the effects of heat treatment, HP treatment and combined heat and HP treatments on whey protein denaturation, surface layers of fat globules and acid gelation properties of milks are explored. The surface layers of recombined milk fat globules are composed of caseins and whey proteins, which are different from those of whole milk, thus the changes and interactions with milk proteins as well as the microstructure and

rheological properties of acid gel of HP-treated recombined milks are expected to differ from those of HP-treated whole milks. The characteristics of recombined milk and dairy products, in particularly texture and nutrition value, also differ depending on whether heat treatment, HP treatment and combined heat and HP treatments are applied before or after homogenization.

9.2 Comments and conclusions

Heat treatment at temperatures higher than 70°C applied to milk either before or after homogenization resulted in: (1) denaturation of whey proteins; (2) significant changes in the amounts proteins adsorbed onto the surface layers of fat globules; and (3) major changes in rheological properties of acid gels. These effects were dependent mainly on heating temperature and holding time; and to a lesser extent on whether heat treatments were applied before or after homogenization. The amount of denatured whey proteins adsorbed onto the surface layers became higher with increase in heating temperature and holding time; this was most likely due to the interactions of denatured whey proteins with caseins adsorbed on the surface layers of fat globules. The amounts of caseins and denatured whey proteins adsorbed directly at the interface of fat globules of milk heated before homogenization were significantly higher than those adsorbed on fat globules of recombined milk heated after homogenization. Under both heating conditions, the fat globule size did not significantly change with heating temperature and holding time.

The acid gels prepared from recombined milks heated at higher temperature (72 - 80°C) and longer holding time (0 – 30 min) had shorter gelation time, higher gelation pH, final G' and yield stress values compared with those heated at lower temperature and shorter holding time. These changes were attributed to the denaturation of whey proteins and their interactions with protein strands formed by the association of casein micelles with attached denatured whey proteins (i.e., bound casein micelles). The denatured whey proteins interacted with κ-casein on the surface of casein micelles resulting in an increase in the isoelectric point of these casein particles. However, applied severe heat treatments beyond the level required to denature all the whey proteins (i.e., temperature higher than 80°C) resulted in lower final G' and yield stress values of acid gel.

Compared with the effects of conventional heat treatment, HP treatment also induced significant changes in milk proteins. Casein micelles have been found to dissociate and reassociate into non-native casein particles; the micelle size became smaller and the internal structure differed from those of native casein micelles (Anema, 2008b). These changes are dependent on treatment pressure, temperature and holding time. Anema found that at 200 MPa, the size and internal structure of non-native casein particles was only slightly different from those of native casein micelles, whereas at higher pressure (400 and 600 MPa) the size reduced by ~50% and some caseins, especially κ- and α_{S2}-caseins dissociated from the micelles into the serum. Significant changes have also been shown to occur to whey proteins as they are denatured (Considine, et al., 2007; Patel & Creamer, 2009). In recombined milk systems, HP induced denaturation of α-La and β-Lg started at 600 and 200 MPa respectively and the denaturation levels increased as treatment pressure, temperature and time increased. The application of HP treatment either before or after homogenization did not have a significant effect the on denaturation level of whey proteins. The denatured whey proteins associated with κ-casein on the surface of casein micelles and in the serum or interacted with other whey proteins, all of which resulted in denatured whey protein aggregates (Patel & Creamer, 2009). At low treatment pressure (200 MPa), the denatured whey protein did not form large protein aggregates, whereas at high treatment pressure (600 MPa), it polymerized into larger aggregates (Patel, 2007).

The size of fat globules of HP-treated recombined milk did not significantly change with HP treatment. When HP treatment was applied to milk before homogenization, the non-native casein particles and denatured whey proteins induced by HP treatment were adsorbed onto the surface layers of fat globules during homogenization. In this system, the caseins on the surface layers of fat globules were present as discrete non-native casein particles. However, when HP treatment was applied to recombined milk after homogenization, the surface layers of fat globules had already formed and were composed primarily of casein micelles. As a result, the fat globules behaved like large casein particles. During HP treatment, the casein micelles on the surface layer dissociated into non-native casein particles. At very high treatment pressure (600 MPa) most of the caseins on the surface layers dissociated, leaving only small amounts of casein present as a layer on the surface of fat globules. Thus, the different nature of surface layers of recombined milks that underwent HP treatment before or after

homogenization resulted in different interactions between the proteins on the surface layers with milk proteins present in the serum. In addition, the amounts and composition of milk proteins on the surface layers of fat globules differed between recombined milks HP-treated before and after homogenization, with the proteins directly adsorbed at the interface of fat globules being significantly lower in recombined milk HP treated after homogenization. Increasing treatment pressure, temperature and time resulted in a decrease in the amount of caseins, but the amount of denatured whey proteins adsorbed onto the surface layers of fat globules appeared to increase. A possible explanation for is that the HP treatment caused dissociation of casein from the fat globule resulting in lower amount of casein detected. The interaction of denatured whey proteins, resulting from HP treatment, with caseins on the surface layer of the fat globules could have resulted in higher amounts of whey protein being detected on the surface of the fat globules..

Acid gels prepared from HP-treated recombined milk, applied both before and after homogenization, had lower gelation time and higher gelation pH, final G' and yield stress values compared with those prepared from untreated recombined milk and the effects were more pronounced with increased treatment pressure, temperature and holding time. These effects were attributed to the structure of non-native casein particles, the HP-induced denaturation of whey proteins as well as the interactions between denatured whey proteins and protein strands. In acid gel prepared from HP-treated recombined milk, the gel matrix was formed by the association of non-native casein particles with attached denatured whey proteins. In this system, the denatured whey proteins appeared to strengthen the gel matrix by the interactions with protein strands via disulphide bonds. The fat globules were incorporated into the gel matrix as they contained the interactive materials, i.e., non-native casein particles and denatured whey proteins on the surface layers.

The placement of HP treatment applied to milk, either before or after homogenization, resulted in different final G' and yield stress values, even though these milks contained similar levels of whey protein denaturation, indicating different interactions between milk proteins. The acid gel structure appears to be dependent upon factors in addition to whey protein denaturation. Recombined milk HP-treated after homogenization had higher non-native casein particles present in the serum which provided more protein

species to take part in forming cross-links and the protein strands. The differences in the amount and proportion of caseins and denatured whey proteins adsorbed onto the surface layers of fat globules had significant effects on the structure of acid gels. In recombined milk HP-treated after homogenization, especially at higher pressure (600 MPa), the proteins were present as a layer on the surface layers of fat globules, which might provide more sites for the interactions with protein strands and denatured whey proteins, resulting in higher final G' and yield stress values compared to those of recombined milk HP-treated before homogenization.

The combined heat and HP treatments applied to recombined milk resulted in more pronounced effects on denaturation of whey proteins, amounts of caseins and denatured whey proteins adsorbed onto the surface layers of fat globules and also structure of acid gels. These effects were mainly dependent on the severity of the heat treatment applied.

An interesting potential benefit of HP on protein ingredients could be that at certain processing conditions (pressure, time, temperature) there may be inactivation of pathogens but little or no change in protein structures and functionality or potentially enhanced functionality. This would allow creation of new food ingredients and beverages with enhanced food safety and functionality.

9.3 Concluding remarks

HP treatment has significant effects on recombined milk, especially on the fat globule surface layer. These changes differ from those induced by heat treatment and have significant effects on acid gel properties. The denaturation of whey proteins, the structure of non-native casein particles and the interactions between denatured whey proteins and proteins strands are involved in determining the structure of acid gels.

The application of HP treatment applied to milk either before or after homogenization did not have a significant effect on the denaturation levels of whey proteins, but the proportions of caseins and denatured whey proteins adsorbed onto the surface layers of fat globules between those milks were different, which had a significant impact on the interactions with milk proteins and the structure of acid gels. The combined heat and HP treatments applied to recombined milk system induced changes in both casein micelles and whey proteins but the effects depends mainly on heat treatment.

9.4 Recommendations for further study

It was found in this study that there were considerable differences in the compositions of surface layers of fat globules of recombined milks after heat, HP or heat/HP-treatments. PAGE techniques used in this study revealed the proportions of adsorbed individual caseins and whey proteins on the globule surface, and also identified the proteins directly adsorbed at the surface (after EDTA/urea treatments). The denaturation profiles of whey proteins were also determined, but the state of unadsorbed casein micelles and whey proteins was not determined. It would be useful to know the size distributions, structures and compositions of unadsorbed casein micelles and whey proteins after heat or HP treatments. This could be determined using light scattering, transmission electron microscopy and PAGE techniques after separation of fat globules (with adsorbed proteins) from the unadsorbed protein material. This unadsorbed protein material has an important role in forming the gel matrix in acid gels. Confocal scanning microscopy, measurement of acid gels permeability, and the thickness of the adsorbed casein micelles layer by dynamic light scattering should be employed in further studies. This could provide greater understanding of the role of the surface layer of fat globule and the microstructure of acid gel in the physical properties of gels prepared from heat or HP-treated recombined milk.

The effects of placement of heat treatment before or after homogenisation on the rheological properties of acid gels revealed some interesting findings. This is probably only partly related to modification of fat globule surfaces, as pointed out in the present study. Further fundamental work is required to unravel the complex mechanisms involved; this may involve studying the structure of the fat globule surface in more depth, using confocal microscopy with different probes, atomic force microscopy and other surface probing techniques.

The preliminary work on the effects of combined HP and heat treatments on fat globules and acid gelation properties needs to be expanded further. Initially this could involve assessing the effects of a wider range of processing conditions (e.g. combined low or medium heat treatment with HP treatments). This approach might be useful for the production of new dairy products with better nutritional value, and fresh-like attributes with novel textures.

The present study focussed only on rheological properties of acid recombined milk gels, which are relevant to the manufacture of recombined yoghurt and cottage-cheese like products. The concepts and methods developed in this study could be applied to study other commercially-important properties of milk products, such as rennet coagulation and heat stability.

APPENDIX A DATA FOR CHAPTER 4

Appendix A.1 *Whey protein denaturation of heat-treated recombined milk*

Milk treatment	Whey proteins denaturation (%)		
	α-La	β-Lg	Total[a]
Untreated	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)
Heated after homogenization			
72°C/15 s	3.10 (0.59)	3.90 (1.58)	3.71 (1.34)
140°C/5 s	28.17 (2.70)	100.00 (0.00)	82.03 (0.93)
72°C			
0 min	4.27 (1.35)	5.28 (1.07)	5.04 (0.78)
10 min	8.15 (1.99)	14.88 (1.92)	13.30 (1.64)
20 min	13.85 (3.32)	24.49 (4.04)	21.99 (3.73)
30 min	19.89 (2.61)	27.93 (3.05)	26.04 (2.97)
76°C			
0 min	4.66 (1.08)	10.39 (1.98)	9.18 (1.57)
10 min	9.31 (1.47)	27.46 (3.96)	23.61 (3.51)
20 min	19.86 (3.01)	40.84 (2.67)	36.32 (1.18)
30 min	35.40 (1.39)	58.95 (4.86)	53.99 (4.52)
80°C			
0 min	7.08 (0.40)	17.97 (1.04)	15.23 (0.49)
10 min	13.03 (2.07)	50.52 (1.86)	41.16 (2.58)
20 min	32.52 (3.33)	89.41 (4.33)	74.52 (2.86)
30 min	39.25 (3.08)	92.77 (2.74)	77.14 (2.24)
90°C			
0 min	12.08 (0.92)	42.42 (3.31)	35.96 (2.32)
10 min	49.80 (5.27)	94.39 (2.05)	84.90 (0.68)
20 min	81.96 (4.73)	95.50 (0.71)	92.63 (0.91)
30 min	84.85 (0.49)	97.55 (2.13)	93.10 (0.78)
100°C			
0 min	15.15 (1.82)	48.21 (0.22)	39.94 (0.28)
10 min	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)
20 min	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)
30 min	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)
Heated before homogenization			
72°C/15 s	3.16 (0.52)	3.96 (0.44)	3.72 (0.28)
140°C/5 s	32.92 (1.57)	92.04 (2.63)	74.55 (1.87)
72°C/30 m	13.83 (1.28)	17.34 (3.21)	16.28 (2.22)
76°C/30 m	47.75 (3.12)	62.57 (6.89)	58.07 (5.67)
80°C/30 min	55.37 (3.70)	100.00 (0.00)	86.78 (1.22)
90°C/30 min	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)
100°C/30 min	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)

Note. Standard deviations are in parentheses. [a] Total whey proteins = β-Lg + α-La. *The holding time presented here are exclude cooling time for approximately 2 min to cool milk samples from heating temperature to 20°C using ice water bath. Therefore, the whey protein denaturation at 0 min at any given temperature was occurred during cooling period.

Appendix A.2 *Protein and fat contents of cream washed with SMUF and some characteristics of fat globules of recombined milks heated after homogenization.*

Heat Treatment	Washed Cream		Fat globule				
	Protein (%)	Fat (%)	$d_{3,2}$ (μm)		Surface area ($m^2 \cdot g^{-1}$)		Protein load ($mg \cdot m^{-2}$)
			Water	SDS/EDTA	Water	SDS/EDTA	
72°C/15 s	2.88 (0.05)	50.52 (1.37)	0.53 (0.01)	0.54 (0.00)	11.29 (0.10)	11.05 (0.01)	5.16
140°C/5 s	3.06 (0.01)	59.23 (0.53)	0.55 (0.01)	0.55 (0.00)	11.34 (0.52)	10.97 (0.02)	4.70
72°C							
0 min	5.07 (0.10)	55.35 (5.37)	0.56 (0.00)	0.55 (0.01)	10.73 (0.02)	11.00 (0.04)	8.33
10 min	5.28 (0.29)	54.11 (1.68)	0.56 (0.01)	0.55 (0.00)	10.68 (0.05)	10.93 (0.03)	8.93
20 min	4.96 (0.26)	56.46 (2.13)	0.56 (0.00)	0.55 (0.00)	10.71 (0.04)	10.98 (0.02)	8.01
30 min	4.68 (0.42)	40.63 (6.26)	0.56 (0.01)	0.55 (0.00)	10.77 (0.06)	10.94 (0.03)	10.52
76°C							
0 min	4.25 (0.73)	61.08 (2.37)	0.56 (0.00)	0.55 (0.00)	10.71 (0.05)	10.91 (0.02)	6.39
10 min	4.86 (0.31)	60.23 (1.59)	0.56 (0.00)	0.55 (0.00)	10.72 (0.07)	10.97 (0.04)	7.36
20 min	5.18 (0.25)	59.45 (2.68)	0.56 (0.01)	0.55 (0.00)	10.80 (0.08)	10.93 (0.05)	7.98
30 min	5.18 (0.15)	60.15 (3.41)	0.56 (0.01)	0.55 (0.01)	10.83 (0.05)	10.61 (0.52)	8.11
80°C							
0 min	4.17 (0.43)	50.92 (2.90)	0.55 (0.00)	0.54 (0.01)	10.89 (0.03)	11.21 (0.02)	7.30
10 min	4.16 (0.42)	50.80 (3.79)	0.56 (0.00)	0.54 (0.01)	10.68 (0.05)	11.20 (0.03)	7.31
20 min	4.58 (0.14)	47.76 (3.13)	0.55 (0.00)	0.54 (0.00)	10.90 (0.02)	11.12 (0.01)	8.63
30 min	4.27 (0.11)	48.39 (4.44)	0.55 (0.00)	0.54 (0.00)	10.82 (0.01)	11.20 (0.01)	7.88
90°C							
0 min	4.54 (0.14)	50.82 (3.54)	0.56 (0.00)	0.54 (0.00)	10.72 (0.02)	11.14 (0.01)	8.02
10 min	4.15 (0.06)	42.21 (1.38)	0.55 (0.00)	0.54 (0.00)	10.86 (0.05)	11.05 (0.03)	8.90
20 min	4.51 (0.12)	47.25 (1.39)	0.55 (0.00)	0.54 (0.00)	10.88 (0.04)	11.04 (0.00)	8.65
30 min	4.20 (0.03)	52.47 (1.75)	0.55 (0.00)	0.54 (0.00)	10.89 (0.04)	11.17 (0.01)	7.16
100°C							
0 min	4.21 (0.04)	53.95 (1.65)	0.56 (0.00)	0.55 (0.02)	10.75 (0.04)	10.95 (0.33)	7.13
10 min	4.76 (0.18)	52.20 (3.38)	0.55 (0.00)	0.54 (0.00)	10.89 (0.04)	11.13 (0.00)	8.20
20 min	4.24 (0.02)	48.95 (4.55)	0.55 (0.00)	0.54 (0.00)	10.91 (0.01)	11.06 (0.01)	7.83
30 min	4.57 (0.16)	46.71 (2.51)	0.55 (0.00)	0.54 (0.00)	10.91 (0.08)	11.08 (0.01)	8.82

Note. Standard deviations are in parentheses.

Appendix A.3 *Percentage of individual proteins on surface layers of fat globules isolated from recombined milks heated before or after homogenization. The cream was washed with SMUF*

Heat treatment	Individual protein on surface layer of recombined milk fat globule (%)				
	α_S-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	40.52 (2.90)	40.93 (4.65)	16.56 (3.77)	0.41 (0.71)	1.59 (2.28)
Heated after homogenization					
72°C/15 s	43.06 (1.42)	36.99 (1.32)	17.03 (0.81)	0.00 (0.00)	2.92 (0.30)
140°C/5 s	39.25 (2.00)	40.44 (1.98)	10.98 (0.60)	0.62 (0.93)	8.71 (0.33)
72°C					
0 min	45.57 (2.38)	38.23 (1.70)	14.86 (1.25)	0.26 (0.51)	1.09 (0.17)
10 min	43.42 (1.10)	35.68 (0.36)	14.28 (0.79)	0.47 (0.14)	6.16 (0.51)
20 min	39.62 (1.07)	36.57 (0.76)	13.93 (0.22)	1.22 (0.31)	8.67 (0.30)
30 min	39.65 (1.15)	34.66 (1.38)	13.80 (0.55)	1.54 (0.31)	10.34 (0.78)
76°C					
0 min	38.83 (1.72)	43.54 (1.25)	14.31 (0.41)	0.14 (0.35)	3.17 (0.21)
10 min	36.79 (1.25)	42.27 (1.15)	12.98 (0.59)	0.22 (0.55)	7.73 (0.52)
20 min	35.40 (1.57)	41.17 (1.29)	11.95 (0.72)	0.35 (0.86)	11.12 (0.40)
30 min	34.75 (2.18)	41.23 (1.65)	11.26 (0.35)	0.45 (1.05)	12.30 (0.57)
80°C					
0 min	41.97 (4.44)	34.31 (1.94)	13.23 (1.17)	0.00 (0.00)	10.49 (3.80)
10 min	40.65 (1.57)	32.96 (2.84)	10.76 (0.37)	0.00 (0.00)	15.64 (1.75)
20 min	38.71 (0.97)	35.59 (3.47)	10.07 (1.16)	0.00 (0.00)	15.63 (3.82)
30 min	39.62 (0.97)	36.40 (1.88)	9.46 (0.82)	0.00 (0.00)	14.53 (2.71)
90°C					
0 min	43.63 (1.62)	33.95 (1.06)	10.46 (1.35)	0.74 (1.29)	11.20 (2.05)
10 min	42.36 (1.20)	35.14 (1.34)	9.04 (0.17)	0.00 (0.00)	13.46 (0.31)
20 min	44.03 (0.02)	34.79 (0.52)	8.95 (0.75)	0.00 (0.00)	12.22 (0.20)
30 min	34.22 (3.73)	27.38 (0.57)	27.38 (0.57)	0.00 (0.00)	11.03 (2.59)
100°C					
0 min	42.15 (2.42)	35.37 (2.65)	9.62 (0.81)	0.00 (0.00)	12.86 (0.57)
10 min	41.79 (1.58)	35.26 (1.67)	8.93 (0.60)	0.00 (0.00)	14.02 (0.52)
20 min	40.83 (5.54)	32.13 (4.63)	14.90 (10.32)	0.00 (0.00)	12.14 (0.21)
30 min	37.06 (5.58)	30.18 (4.88)	21.04 (10.98)	0.00 (0.00)	11.72 (2.19)
Heated before homogenization					
72°C/15 s	45.21 (1.47)	39.21 (1.21)	14.91 (0.64)	0.14 (0.25)	0.53 (0.91)
140°C/5 s	37.92 (0.69)	35.82 (1.69)	12.14 (0.67)	1.46 (0.31)	12.65 (1.31
72°C/30 min	42.22 (2.71)	33.15 (4.39)	15.18 (2.14)	1.25 (1.11)	8.19 (3.46)
76°C/30 min	42.00 (0.54)	35.24 (3.06)	13.45 (1.02)	1.39 (1.96)	7.93 (0.61)
80°C					
0 min	42.18 (0.49)	43.68 (2.27)	10.25 (1.37)	0.38 (0.34)	3.51 (0.99)
10 min	43.92 (2.54)	44.29 (1.85)	8.23 (1.29)	0.62 (0.46)	2.94 (0.69)
20 min	44.99 (1.44)	41.57 (0.98)	8.35 (1.65)	0.13 (0.27)	4.96 (1.04)
30 min	39.91 (3.04)	40.89 (2.53)	11.60 (1.92)	1.45 (1.51)	6.15 (0.80)
90°C					
0 min	41.56 (2.57)	41.73 (0.28)	9.88 (1.44)	1.16 (1.65)	5.68 (0.80)
10 min	43.94 (0.25)	42.58 (0.71)	8.74 (1.06)	0.61 (0.86)	4.13 (0.96)
20 min	44.03 (0.24)	40.31 (1.32)	10.15 (0.94)	0.74 (1.04)	4.78 (0.89)
30 min	42.58 (3.13)	40.10 (1.41)	11.20 (1.57)	1.81 (1.69)	4.32 (1.55)
100°C/30 min	42.27 (3.61)	34.54 (4.29)	14.02 (3.86)	2.79 (2.50)	6.37 (1.65)

Note. Standard deviations are in parentheses.

Appendix A.4 *Percentage of individual protein at the interface of fat globule isolated from recombined milks heated either before or after homogenization. The cream was washed with SMUF containing dissociation agents.*

Milk treatment	Individual protein at the interface of recombined milk fat globule (%)*				
	α-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	35.00 (2.95)	33.76 (2.33)	22.31 (2.33)	2.17 (1.11)	6. 16 (976)
Heat after homogenization					
72°C/15 s	26.00 (1.17)	29.07 (1.86)	23.74 (1.05)	4.47 (0.91)	16.72 (0.37)
140°C/5 s	8.40 (1.01)	8.25 (2.46)	28.52 (1.05)	6.13 (0.97)	48.70 (2.38)
72°C					
0 min	24.78 (0.61)	29.29 (1.56)	23.18 (1.03)	3.90 (0.60)	18.85 (0.84)
10 min	5.07 (0.58)	6.96 (1.63)	33.53 (3.14)	9.34 (0.89)	45.10 (2.40)
20 min	3.28 (1.34)	6.12 (1.70)	33.19 (2.70)	10.78 (1.24)	46.62 (3.04)
30 min	3.22 (2.27)	7.42 (1.39)	32.18 (3.02)	9.53 (0.95)	47.65 (3.11)
76°C					
0 min	7.83 (1.24)	5.70 (0.66)	40.62 (1.21)	8.44 (1.11)	37.40 (1.29)
10 min	6.12 (1.03)	4.69 (0.41)	33.74 (0.88)	7.92 (0.96)	47.53 (0.78)
20 min	1.62 (2.23)	0.53 (1.19)	33.25 (1.01)	9.84 (1.20)	54.75 (1.84)
30 min	1.52 (2.31)	1.06 (2.10)	31.43 (1.44)	11.34 (1.09)	54.65 (2.58)
80°C					
0 min	4.90 (0.99)	7.52 (1.52)	31.14 (2.07)	5.49 (0.41)	50.96 (4.17)
10 min	0.00 (0.00)	0.00 (0.00)	48.36 (6.29)	0.00 (0.00)	51.64 (6.29)
20 min	0.00 (0.00)	0.00 (0.00)	51.10 (0.03)	0.00 (0.00)	48.90 (0.03)
30 min	0.00 (0.00)	0.00 (0.00)	55.65 (1.43)	0.00 (0.00)	44.35 (1.43)
90°C					
0 min	0.00 (0.00)	0.00 (0.00)	38.90 (2.84)	0.00 (0.00)	61.10 (2.84)
10 min	0.00 (0.00)	0.00 (0.00)	51.73 (2.72)	0.00 (0.00)	48.27 (2.72)
20 min	0.00 (0.00)	0.00 (0.00)	42.73 (3.47)	0.00 (0.00)	57.27 (3.47)
30 min	0.00 (0.00)	0.00 (0.00)	43.49 (4.60)	0.00 (0.00)	56.51 (4.60)
100°C					
0 min	0.00 (0.00)	0.00 (0.00)	34.89 (1.43)	0.00 (0.00)	65.11 (1.43)
10 min	0.00 (0.00)	0.00 (0.00)	47.22 (2.40)	0.00 (0.00)	52.78 (2.40)
20 min	0.00 (0.00)	0.00 (0.00)	44.29 (2.62)	0.00 (0.00)	55.71 (2.62)
30 min	0.00 (0.00)	0.00 (0.00)	41.95 (1.59)	0.00 (0.00)	58.05 (1.59)
Heat before homogenization					
72°C/15 s	37.74 (2.23)	31.43 (3.34)	20.96 (2.15)	2.05 (0.25)	7.82 (0.96)
140°C/5 s	20.69 (1.16)	21.01 (2.14)	21.77 (1.76)	2.65 (0.42)	33.88 (1.58)
72°C/30 m	39.49 (0.51)	27.02 (1.67)	18.67 (1.44)	2.30 (0.26)	12.52 (0.48)
76°C/30 m	33.62 (1.23)	23.07 (0.84)	21.34 (1.20)	3.77 (0.19)	18.20 (0.67)
80°C					
0 min	32.88 (3.29)	31.62 (2.28)	16.81 (0.26)	6.21 (0.52)	12.48 (0.51)
10 min	31.31 (1.29)	27.20 (0.84)	17.86 (0.19)	8.53 (0.49)	15.11 (0.36)
20 min	27.15 (2.89)	23.33 (1.83)	19.94 (0.69)	6.84 (0.90)	22.75 (2.44)
30 min	30.32 (2.94)	29.75 (2.72)	19.06 (1.14)	5.56 (1.19)	15.30 (3.58)
90°C					
0 min	37.15 (2.88)	31.48 (0.85)	15.91 (0.55)	4.56 (1.08)	10.90 (2.12)
10 min	31.00 (0.70)	27.61 (2.87)	19.98 (0.69)	6.86 (1.39)	14.55 (1.49)
20 min	41.69 (0.59)	33.08 (0.98)	11.48 (1.09)	3.87 (0.95)	9.88 (1.65)
30 min	33.82 (3.50)	29.79 (2.17)	18.50 (2.19)	6.09 (1.48)	11.80 (1.27)
100°C/30 min	40.34 (3.06)	29.04 (1.95)	18.54 (0.23)	4.66 (0.25)	7.41 (1.10)

Note. Standard deviations are in parentheses. *Calculated from the percentage of each proteins from the total bound proteins on at the interface of fat globules

APPENDIX B DATA FOR CHAPTER 6

Appendix B.1 *Whey protein denaturation of HP-treated recombined milks*

Milk treatment	Whey proteins denaturation (%)		
	α-La	β-Lg	Total[1]
Untreated	0.00[a] (0.00)	0.00[a] (0.00)	0.00[a] (0.00)
HP treatment at 10°C after homogenization at different pressurizing times and pressures			
200 MPa			
5 min	0.00[a] (0.00)	3.26[a] (0.63)	2.43[a] (0.44)
15 min	0.00[a] (0.00)	5.17[a] (0.60)	3.88[a] (0.40)
30 min	0.00[a] (0.00)	14.81[b] (0.64)	11.18[c] (0.53)
60 min	0.00[a] (0.00)	24.09[d] (0.38)	18.22[d,e] (0.36)
400 MPa			
5 min	0.00[a] (0.00)	23.21[c,d] (2.13)	18.43[d,e] (1.83)
15 min	0.00[a] (0.00)	38.82[e] (2.28)	29.38[f] (1.85)
30 min	0.00[a] (0.00)	51.95[f] (0.85)	39.30[g] (1.30)
60 min	0.00[a] (0.00)	69.34[h] (2.11)	52.48[h,i] (1.72)
600 MPa			
5 min	5.23[b] (2.42)	39.77[e] (3.65)	30.46[f] (2.27)
15 min	9.35[b,c] (5.45)	59.89[g] (3.08)	48.01[h] (3.56)
30 min	14.46[d] (4.32)	72.57[h,i] (2.47)	58.89[j,k,l] (3.25)
60 min	16.93[d] (1.86)	81.62[j,k] (0.58)	66.34[m,n,o] (0.36)
HP treatment at 20°C after homogenization at different pressurizing times and pressures			
200 MPa			
5 min	0.00[a] (0.00)	3.83[a] (0.51)	2.97[a] (0.42)
15 min	0.00[a] (0.00)	5.49[a] (0.43)	4.24[a,b] (0.30)
30 min	0.00[a] (0.00)	14.79[b] (1.24)	11.48[c] (0.85)
60 min	0.00[a] (0.00)	29.53[d] (2.49)	22.88[e] (2.13)
400 MPa			
5 min	0.00[a] (0.00)	53.62[f,g] (1.83)	40.90[g] (1.70)
15 min	0.00[a] (0.00)	73.96[h,i] (0.40)	56.41[i,j,k] (1.21)
30 min	0.00[a] (0.00)	80.95[j,k] (1.28)	61.74[k,l,m] (0.81)
60 min	0.00[a] (0.00)	88.56[l] (1.63)	67.53[n,o] (0.09)
600 MPa			
5 min	7.34[b] (0.28)	75.69[h,I,j] (1.43)	57.19[i,j,k] (1.25)
15 min	12.72[c,d] (2.44)	86.99[k,l] (1.42)	66.88[m,n,o] (1.94)
30 min	14.59[d] (1.31)	100.00[n] (0.00)	76.87[p,q,r] (0.68)
60 min	26.38 (4.20)	100.00[n] (0.00)	80.08[r,s] (0.88)
HP treatment at 40°C after homogenization at different pressurizing times and pressures			
200 MPa			
5 min	na[2]	na	na
15 min	0.00[a] (0.00)	5.90[a] (0.20)	4.41[a,b] (0.17)
30 min	0.00[a] (0.00)	17.21[b,c] (0.57)	12.69[c,d] (0.36)
60 min	0.00[a] (0.00)	29.48[d] (0.66)	22.01[e] (0.33)
400 MPa			
5 min	0.00[a] (0.00)	85.94[k,l] (1.56)	63.74[l,m,n] (1.32)
15 min	0.00[a] (0.00)	96.41[m,n] (6.22)	71.51[o,p] (4.84)
30 min	0.00[a] (0.00)	96.70[m,n] (5.72)	71.72[o,p] (4.48)
60 min	0.00[a] (0.00)	97.15[m,n] (4.93)	72.06[o,p] (3.89)
600 MPa			
5 min	42.07[e] (1.66)	91.53[l,m] (0.41)	78.53[q,r] (0.16)
15 min	44.49[e] (0.96)	99.83[n] (0.17)	85.28[s,t] (0.16)
30 min	46.64[e,f] (0.58)	100.00[n] (0.00)	85.97[t] (0.19)
60 min	50.18[f] (2.29)	100.00[n] (0.00)	86.90[t] (0.63)
HP treatment at 20°C before homogenization at different pressures for 30 min			
200 MPa	0.00[a] (0.00)	14.31[b] (0.73)	9.97[b,c] (0.42)
400 MPa	0.00[a] (0.00)	77.11[i,j] (1.27)	53.52[h,i,j] (0.42)
600 MPa	13.61[c,d] (0.38)	100.00[n] (0.00)	73.31[p,q] (0.76)

Note. [1] Total = α-La + β-Lg. [2] na = not analysis. [a] Samples with the same superscript letters within the same column are not significantly ($P \leq 0.05$) different from each other

Appendix B.2 *Protein and fat content of cream washed with SMUF and d_{32}, surface area and protein load of HP-treated recombined milk fat globules*

Milk treatment	Washed Cream		Fat globule				
	Protein (%)	Fat (%)	d_{32} (µm)		Surface area ($m^2 \cdot g^{-1}$)		Protein load ($mg \cdot m^{-2}$)
			Water	SDS/EDTA	Water	SDS/EDTA	
Untreated	2.58 (0.05)	59.32 (0.93)	0.58 (0.01)	0.58 (0.00)	10.37 (0.07)	10.41 (0.01)	4.18
HP treatment at 10°C after homogenization at different pressurizing times and pressures							
200 MPa							
5 min	na	na	0.60 (0.01)	0.59 (0.00)	9.95 (0.06)	10.13 (0.02)	na
15 min	na	na	0.60 (0.01)	0.59 (0.00)	9.92 (0.04)	10.20 (0.02)	na
30 min	na	na	0.60 (0.00)	0.58 (0.00)	9.97 (0.01)	10.34 (0.01)	na
60 min	na	na	0.60 (0.00)	0.58 (0.00)	10.03 (0.03)	10.36 (0.02)	na
400 MPa							
5 min	na	na	0.60 (0.00)	0.58 (0.00)	9.98 (0.05)	10.42 (0.03)	na
15 min	na	na	0.61 (0.01)	0.58 (0.00)	9.92 (0.02)	10.38 (0.04)	na
30 min	na	na	0.60 (0.00)	0.58 (0.00)	10.00 (0.04)	10.34 (0.03)	na
60 min	na	na	0.60 (0.00)	0.58 (0.00)	9.85 (0.17)	10.28 (0.01)	na
600 MPa							
5 min	na	na	0.61 (0.00)	0.58 (0.01)	9.77 (0.01)	10.27 (0.02)	na
15 min	na	na	0.61 (0.00)	0.60 (0.01)	9.78 (0.02)	10.08 (0.02)	na
30 min	na	na	0.61 (0.00)	0.59 (0.01)	9.84 (0.01)	10.15 (0.13)	na
60 min	na	na	0.61 (0.00)	0.59 (0.01)	9.86 (0.02)	10.19 (0.13)	na
HP treatment at 20°C after homogenization at different pressurizing times and pressures							
200 MPa							
5 min	na	na	0.56 (0.00)	0.56 (0.00)	10.69 (0.02)	10.74 (0.04)	na
15 min	2.82 (0.04)	58.45 (1.90)	0.57 (0.00)	0.56 (0.00)	10.56 (0.01)	10.77 (0.02)	4.48
30 min	2.70 (0.05)	59.09 (1.47)	0.56 (0.00)	0.55 (0.00)	10.69 (0.02)	10.97 (0.01)	4.16
60 min	2.92 (0.01)	58.50 (0.20)	0.59 (0.00)	0.58 (0.00)	10.18 (0.01)	10.37 (0.02)	4.82
400 MPa							
5 min	na	na	0.57 (0.01)	0.56 (0.01)	10.62 (0.02)	10.81 (0.01)	na
15 min	2.39 (0.03)	66.30 (0.34)	0.58 (0.00)	0.57 (0.00)	10.36 (0.00)	10.52 (0.01)	3.42
30 min	2.33 (0.03)	65.46 (0.98)	0.59 (0.00)	0.57 (0.00)	10.15 (0.06)	10.45 (0.01)	3.40
60 min	2.24 (0.03)	65.46 (1.54)	0.62 (0.01)	0.58 (0.00)	9.60 (0.03)	10.37 (0.03)	3.30
600 MPa							
5 min	na	na	0.56 (0.00)	0.54 (0.01)	10.69 (0.05)	11.03 (0.02)	na
15 min	2.47 (0.04)	64.79 (0.31)	0.62 (0.01)	0.58 (0.00)	9.58 (0.10)	10.37 (0.01)	3.68
30 min	2.63 (0.00)	62.78 (0.69)	0.59 (0.01)	0.58 (0.00)	10.15 (0.07)	10.43 (0.01)	4.01
60 min	2.88 (0.06)	62.02 (1.14)	0.61 (0.01)	0.57 (0.00)	9.78 (0.12)	10.51 (0.02)	4.41
HP treatment at 40°C after homogenization at different pressurizing times and pressures							
200 MPa							
5 min	na	na	na	na	na	na	na
15 min	4.34 (0.10)	62.23 (2.66)	0.58 (0.01)	0.59 (0.01)	10.33 (0.15)	10.25 (0.06)	6.81
30 min	4.69 (0.06)	61.75 (0.35)	0.58 (0.01)	0.58 (0.02)	10.41 (0.07)	10.39 (0.39)	7.31
60 min	4.56 (0.02)	57.72 (3.46)	0.56 (0.00)	0.59 (0.00)	10.70 (0.03)	10.23 (0.01)	7.72
400 MPa							
5 min	4.56 (0.64)	49.20 (5.57)	0.64 (0.01)	0.57 (0.00)	9.45 (0.04)	10.49 (0.04)	8.83
15 min	4.35 (0.15)	53.32 (2.60)	0.66 (0.00)	0.58 (0.00)	9.05 (0.02)	10.38 (0.04)	7.86
30 min	4.41 (0.06)	53.01 (2.76)	0.67 (0.00)	0.57 (0.00)	8.96 (0.04)	10.47 (0.01)	7.95
60 min	4.85 (0.43)	55.54 (4.34)	0.68 (0.00)	0.58 (0.00)	8.84 (0.01)	10.30 (0.02)	8.48
600 MPa							
5 min	3.82 (0.10)	62.26 (2.38)	0.74 (0.01)	0.59 (0.02)	7.95 (0.10)	10.10 (0.19)	6.08
15 min	4.17 (0.04)	66.15 (3.22)	0.86 (0.06)	0.54 (0.02)	7.02 (0.48)	11.05 (0.29)	5.71
30 min	4.33 (0.06)	70.79 (2.03)	0.90 (0.01)	0.55 (0.01)	6.71 (0.03)	10.84 (0.25)	5.64
60 min	4.20 (0.05)	62.61 (2.51)	1.03 (0.09)	0.55 (0.01)	5.87 (0.51)	10.87 (0.16)	6.17

Note. Standard deviations are in parenthesis. na = Not analysis.

Appendix B.3 *Percentage of protein on the surface layers of fat globules isolated from HP-treated recombined milks.*

Milk treatment	Proteins on surface layer of recombined milk fat globule (%)*				
	α_s-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	39.76 (1.62)	43.57 (2.19)	14.40 (1.09)	0.77 (0.72)	1.50 (0.99)
HP at 10°C					
200 MPa					
5 min	45.51 (1.25)	36.37 (1.02)	14.46 (0.23)	1.09 (0.33)	2.57 (0.21)
15 min	46.54 (0.84)	35.15 (0.64)	13.35 (0.42)	1.09 (0.31)	3.86 (0.23)
30 min	48.54 (0.71)	33.11 (0.94)	11.98 (0.71)	1.16 (0.36)	5.22 (0.33)
60 min	48.69 (0.93)	31.67 (0.80)	11.38 (0.81)	1.04 (0.24)	7.22 (0.65)
400 MPa					
5 min	48.13 (0.52)	29.99 (0.71)	14.35 (0.73)	1.65 (0.16)	5.89 (0.48)
15 min	46.88 (1.64)	29.77 (1.27)	13.14 (1.19)	1.34 (0.21)	8.86 (0.68)
30 min	46.43 (0.57)	29.26 (0.54)	11.69 (0.73)	1.31 (0.08)	11.31 (0.36)
60 min	47.27 (1.31)	28.01 (0.48)	10.72 (0.77)	1.17 (0.14)	12.84 (0.50)
600 MPa					
5 min	45.81 (0.63)	30.59 (1.47)	12.02 (0.80)	1.33 (0.08)	10.26 (0.74)
15 min	44.78 (0.53)	32.63 (0.38)	13.09 (0.48)	1.78 (0.30)	7.72 (0.31)
30 min	45.88 (1.36)	30.65 (1.27)	11.53 (0.52)	1.42 (0.05)	10.51 (0.66)
60 min	45.50 (1.61)	29.64 (1.57)	11.72 (0.45)	1.41 (0.30)	11.75 (0.30)
HP at 20°C					
200 MPa					
5 min	44.48 (0.99)	39.83 (0.77)	12.54 (0.94)	0.72 (0.35)	2.44 (0.35)
15 min	45.10 (1.07)	38.48 (0.80)	12.09 (0.86)	0.63 (0.38)	3.70 (0.33)
30 min	46.20 (1.01)	36.60 (1.34)	11.16 (0.72)	0.48 (0.33)	5.56 (0.32)
60 min	44.91 (2.02)	36.96 (2.74)	10.71 (1.74)	0.44 (0.25)	6.98 (2.38)
400 MPa					
5 min	42.01 (1.87)	34.03 (2.40)	11.94 (1.09)	0.56 (0.12)	11.47 (0.16)
15 min	42.90 (1.21)	34.26 (1.43)	10.67 (0.75)	0.14 (0.22)	12.02 (0.74)
30 min	43.07 (1.38)	32.15 (0.79)	10.31 (0.70)	0.41 (0.33)	14.07 (1.14)
60 min	43.80 (0.78)	33.94 (2.06)	10.34 (0.70)	0.17 (0.22)	11.75 (1.94)
600 MPa					
5 min	43.09 (1.14)	32.99 (1.95)	11.19 (0.58)	0.83 (1.12)	11.90 (0.63)
15 min	42.98 (1.05)	34.58 (1.26)	10.89 (0.48)	0.40 (0.62)	11.15 (0.58)
30 min	42.60 (1.02)	34.22 (1.01)	10.87 (0.77)	0.42 (0.63)	11.90 (0.34)
60 min	44.19 (0.80)	33.72 (1.04)	10.62 (0.72)	0.60 (0.83)	10.87 (0.27)
HP at 40°					
200 MPa					
15 min	46.58 (0.69)	39.45 (0.80)	10.11 (0.84)	0.50 (0.35)	3.36 (0.34)
30 min	46.09 (1.04)	40.33 (0.91)	9.43 (0.64)	0.41 (0.29)	3.73 (0.29)
60 min	46.48 (1.32)	39.80 (1.00)	8.92 (0.51)	0.46 (0.39)	4.34 (0.40)
400 MPa					
5 min	45.83 (3.64)	36.52 (1.82)	7.03 (1.16)	0.72 (1.05)	9.90 (1.86)
15 min	45.40 (1.78)	34.98 (2.58)	7.86 (1.13)	1.36 (1.26)	10.40 (1.56)
30 min	47.50 (3.10)	36.22 (1.38)	7.19 (1.30)	0.72 (1.45)	8.37 (1.77)
60 min	46.39 (2.41)	35.82 (1.24)	7.66 (0.78)	1.05 (1.34)	9.07 (1.44)
600 MPa					
5 min	45.12 (0.68)	37.52 (1.43)	7.24 (0.87)	0.00 (0.00)	10.12 (0.92)
15 min	45.47 (1.64)	36.43 (1.83)	7.46 (0.94)	0.66 (1.23)	9.98 (1.02)
30 min	46.02 (1.47)	36.39 (1.47)	7.40 (1.13)	0.39 (0.96)	9.80 (0.96)
60 min	46.94 (1.86)	36.08 (1.84)	7.31 (0.72)	0.00 (0.00)	9.67 (0.95)
HP at 20°					
200 MPa	40.77 (2.49)	36.89 (1.01)	14.25 (0.50)	1.15 (1.06)	6.93 (1.07)
400 MPa	35.64 (1.96)	33.04 (1.43)	12.86 (0.78)	0.27 (0.47)	18.19 (1.60)
600 MPa	37.46 (3.54)	33.72 (0.78)	13.55 (1.01)	0.88 (1.61)	14.39 (2.11)

Note. Standard deviations are in parenthesis. * Calculated from percentage of individual proteins of total bound proteins on the surface layer of fat globule.

Appendix B.4 *Percentage of protein at the interface of fat globules isolated from HP-treated recombined milks.*

Milk treatment	Protein at the interface of recombined milk fat globule (%)*				
	α_s-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	26.62 (2.63)	33.36 (1.90)	23.38 (1.40)	3.52 (0.27)	13.11 (0.88)
HP at 10°C after homogenization					
200 MPa					
5 min	30.40 (1.33)	33.85 (1.07)	20.68 (0.64)	4.57 (0.25)	10.50 (0.70)
15 min	30.63 (1.33)	28.09 (1.63)	20.32 (0.77)	4.57 (0.27)	16.39 (0.68)
30 min	27.61 (1.36)	24.86 (1.15)	21.95 (1.62)	4.38 (0.41)	21.20 (0.95)
60 min	27.56 (2.22)	22.67 (1.67)	19.53 (1.31)	4.35 (0.59)	25.89 (2.05)
400 MPa					
5 min	26.61 (1.14)	21.43 (1.67)	24.20 (0.53)	3.98 (0.60)	23.78 (1.16)
15 min	22.22 (1.55)	17.72 (2.14)	26.82 (1.33)	2.05 (0.18)	31.19 (2.63)
30 min	25.83 (1.92)	17.81 (1.75)	21.90 (0.68)	3.48 (0.36)	30.98 (3.43)
60 min	25.15 (1.05)	17.76 (1.19)	20.84 (1.11)	2.94 (0.18)	33.31 (1.81)
600 MPa					
5 min	28.37 (0.94)	21.90 (1.51)	23.17 (0.80)	3.57 (0.40)	22.99 (1.24)
15 min	24.26 (1.32)	20.37 (1.24)	24.41 (0.62)	2.51 (0.22)	28.45 (1.20)
30 min	25.21 (1.51)	18.24 (1.70)	22.40 (0.41)	4.14 (0.29)	30.01 (0.55)
60 min	24.15 (1.27)	16.96 (1.38)	22.91 (0.92)	4.49 (0.45)	31.49 (0.88)
HP at 20°C after homogenization					
200 MPa					
5 min	33.67 (2.15)	29.95 (3.30)	21.42 (1.31)	2.98 (0.30)	11.99 (1.00)
15 min	35.03 (2.03)	28.13 (1.74)	19.38 (0.83)	3.37 (0.20)	14.09 (1.38)
30 min	31.67 (2.14)	24.26 (1.41)	17.97 (1.16)	3.76 (0.32)	22.34 (2.03)
60 min	22.56 (1.71)	15.21 (0.66)	19.33 (1.35)	3.54 (0.52)	39.35 (1.72)
400 MPa					
5 min	24.23 (3.21)	16.05 (2.77)	18.46 (0.81)	3.13 (0.54)	38.12 (4.64)
15 min	23.23 (0.84)	17.66 (1.55)	18.02 (0.55)	2.97 (0.20)	38.11 (1.89)
30 min	22.60 (1.74)	17.25 (0.58)	17.37 (0.38)	2.44 (0.09)	40.34 (1.59)
60 min	21.80 (0.60)	15.15 (1.05)	17.50 (0.54)	2.44 (0.16)	43.11 (1.50)
600 MPa					
5 min	25.22 (2.11)	19.77 (1.66)	18.94 (2.08)	3.30 (0.63)	32.77 (1.73)
15 min	24.73 (1.58)	17.88 (1.11)	19.96 (1.46)	4.11 (0.40)	33.32 (0.96)
30 min	20.23 (1.76)	14.47 (0.90)	21.20 (1.77)	4.34 (0.47)	39.77 (1.24)
60 min	23.64 (1.00)	18.80 (0.72)	20.43 (1.37)	4.28 (0.44)	32.85 (1.14)
HP at 40°C after homogenization					
200 MPa					
15 min	16.21 (1.01)	11.25 (0.96)	27.48 (1.89)	7.96 (0.13)	37.10 (0.88)
30 min	20.08 (0.65)	16.03 (0.78)	24.08 (1.16)	5.71 (1.16)	34.09 (1.47)
60 min	17.40 (0.43)	10.32 (0.31)	24.16 (0.10)	6.52 (0.58)	41.61 (0.80)
400 MPa					
5 min	10.55 (0.49)	5.10 (1.15)	15.88 (1.68)	2.64 (0.36)	65.82 (2.17)
15 min	11.67 (0.95)	5.71 (0.56)	17.98 (1.15)	2.66 (1.50)	61.97 (1.10)
30 min	11.51 (0.28)	6.06 (0.36)	18.78 (1.35)	3.75 (0.42)	59.90 (1.71)
60 min	13.96 (0.80)	7.32 (0.80)	19.09 (1.33)	3.96 (0.39)	55.66 (1.84)
600 MPa					
5 min	11.45 (0.86)	6.31 (0.43)	18.41 (1.12)	6.79 (0.85)	57.04 (1.67)
15 min	13.86 (0.63)	7.73 (0.68)	17.87 (1.14)	8.16 (1.23)	52.38 (1.89)
30 min	12.17 (0.65)	6.30 (0.71)	17.89 (0.35)	8.82 (1.80)	54.82 (1.09)
60 min	12.20 (0.60)	6.21 (0.17)	18.88 (1.64)	9.31 (1.58)	53.41 (1.92)
HP 20°C before homogenization					
200 MPa	29.02 (1.12)	27.26 (0.92)	20.99 (0.58)	1.68 (0.26)	21.05 (1.15)
400 MPa	16.38 (0.84)	19.25 (0.62)	20.01 (0.66)	0.45 (0.34)	43.91 (1.95)
600 MPa	26.17 (0.96)	24.95 (0.78)	19.07 (0.22)	1.86 (0.51)	27.95 (0.67)

Note. Standard deviations are in parentheses. * Calculate from percentage of individual protein of total bound proteins at the interface of fat globule.

APPENDIX C DATA FOR CHAPTER 8

Appendix C.1 *Whey protein denaturation of heat/HP-treated recombined milks.*

Milk treatment	Whey protein denaturation (%)		
	α-La	β-Lg	Total[1]
Untreated	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Heat/HP treatments before homogenization			
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C			
0 MPa	35.39[a] (0.24)	96.51[c] (3.49)	70.46[a] (1.33)
200 MPa	39.02[a,b] (2.69)	99.85[c] (0.15)	73.98[a,b] (0.66)
400 MPa	39.55[a,b] (0.62)	99.66[c] (0.26)	74.08[a,b] (0.15)
600 MPa	46.46[a,b] (6.04)	96.35[c] (3.66)	75.13[a,b] (4.20)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C			
0 MPa	87.91[c] (3.36)	96.72[c] (0.38)	94.24[d,e] (1.36)
200 MPa	92.34[c] (7.66)	97.27[c] (2.73)	95.82[d,e] (4.18)
400 MPa	91.61[c] (8.39)	99.75[c] (0.25)	97.35[e] (2.28)
600 MPa	92.00[c] (6.80)	98.72[c] (1.28)	96.72[e] (1.07)
Heat/HP treatments after homogenization			
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C			
0 MPa	41.76[a,b] (6.67)	83.34[a] (1.42)	74.44[a,b] (7.63)
200 MPa	40.75[a,b] (2.46)	85.64[a,b] (2.10)	75.03[a,b] (5.39)
400 MPa	52.04[b] (1.19)	91.61[a,b,c] (3.06)	82.98[b,c] (3.47)
600 MPa	51.24[b] (5.41)	94.75[b,c] (3.56)	85.70[c,d] (3.69)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C			
0 MPa	84.85[c] (0.49)	96.37[c] (3.63)	92.25[c,d,e] (1.52)
200 MPa	79.77[c] (4.46)	93.55[b,c] (6.45)	88.24[c,d,e] (1.92)
400 MPa	80.53[c] (1.91)	95.89[c] (4.11)	90.36[c,d,e] (1.13)
600 MPa	92.91[c] (7.09)	95.27[c] (4.73)	94.63[d,e] (5.38)

Note. Standard deviations are in parentheses. [1] Total = α-La + β-Lg. [a] Samples with the same superscript letters within the same column are not significantly ($P \leq 0.05$) different from each other.

Appendix C.2 *Percentage of protein on the surface layer of fat globule isolated from heat/HP-treated recombined milks.*

Milk treatment	Protein on surface layer of recombined milk fat globule (%)				
	α_s-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	39.76 (1.62)	43.57 (2.19)	14.40 (1.09)	1.50 (0.99)	0.77 (0.72)
Heat/HP treatments before homogenization					
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	39.86 (3.60)	40.51 (4.24)	12.88 (0.34)	1.47 (0.21)	5.27 (0.77)
200 MPa	43.00 (4.68)	38.81 (2.54)	11.12 (0.25)	2.27 (1.03)	4.80 (1.36)
400 MPa	41.62 (1.16)	37.11 (1.88)	11.73 (0.16)	1.66 (0.28)	7.88 (0.60)
600 MPa	42.05 (2.11)	36.32 (0.70)	12.25 (0.60)	1.69 (0.46)	7.70 (1.76)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	33.48 (3.97)	25.46 (2.78)	19.47 (0.20)	9.61 (0.36)	11.98 (0.64)
200 MPa	41.10 (4.79)	25.31 (5.28)	16.10 (0.81)	7.53 (0.15)	9.97 (0.17)
400 MPa	30.46 (3.78)	24.13 (5.48)	20.99 (3.22)	9.03 (1.12)	15.39 (0.41)
600 MPa	36.70 (2.71)	26.13 (3.97)	18.48 (1.87)	6.46 (0.37)	12.24 (0.64)
Heat/HP treatments after homogenization					
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	37.63 (4.86)	34.03 (2.15)	9.55 (2.14)	1.85 (0.60)	16.94 (0.02)
200 MPa	35.63 (2.02)	30.59 (3.52)	9.55 (0.74)	2.63 (0.45)	21.61 (5.24)
400 MPa	34.30 (2.34)	25.15 (1.00)	13.04 (1.69)	2.85 (0.74)	24.65 (2.30)
600 MPa	31.33 (0.43)	23.71 (0.41)	12.94 (1.08)	2.66 (0.13)	29.36 (0.93)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	37.97 (4.72)	38.61 (4.32)	9.31 (0.86)	1.16 (0.54)	12.95 (0.21)
200 MPa	47.06 (2.85)	36.98 (3.22)	6.13 (1.02)	0.45 (0.46)	9.38 (0.91)
400 MPa	40.32 (1.72)	30.25 (2.57)	8.83 (3.65)	1.03 (0.64)	19.57 (1.97)
600 MPa	39.23 (3.45)	25.86 (4.19)	9.81 (2.91)	1.05 (0.88)	24.04 (3.79)

Note. Standard deviations are in parentheses. *Calculate from percentage of individual protein of total bound proteins on the surface layer of fat globule.

Appendix C.3 *Percentage of protein at the interface of fat globule isolated from heat/HP-treated recombined milks.*

Milk treatment	Protein at the interface of fat globule (%)*				
	α_s-Casein	β-Casein	κ-Casein	α-La	β-Lg
Untreated	26.62 (2.63)	33.36 (1.90)	23.38 (1.40)	13.11 (0.88)	3.52 (0.27)
Heat/HP treatments before homogenization					
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	36.48 (1.31)	29.24 (0.34)	16.35 (1.46)	5.37 (0.87)	12.57 (0.51)
200 MPa	32.96 (1.23)	27.16 (0.46)	17.63 (1.21)	6.76 (1.52)	15.49 (1.00)
400 MPa	25.83 (1.42)	23.86 (3.15)	20.43 (1.93)	6.03 (1.62)	23.86 (1.35)
600 MPa	27.75 (2.01)	25.61 (1.53)	18.31 (1.00)	5.33 (1.50)	23.00 (0.82)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	33.48 (3.97)	25.46 (2.78)	19.47 (0.20)	9.61 (0.36)	11.98 (0.64)
200 MPa	41.10 (4.79)	25.31 (5.28)	16.10 (0.81)	7.53 (0.15)	9.97 (0.17)
400 MPa	30.46 (3.78)	24.13 (5.48)	20.99 (3.22)	9.03 (1.12)	15.39 (0.41)
600 MPa	36.70 (2.71)	26.13 (3.97)	18.48 (1.87)	6.46 (0.37)	12.24 (0.64)
Heat/HP treatments after homogenization					
Heated at 80°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	11.60 (0.43)	7.94 (0.92)	25.87 (0.66)	7.86 (1.02)	46.73 (1.71)
200 MPa	14.93 (0.66)	8.50 (0.47)	20.63 (0.59)	8.50 (0.98)	47.44 (0.44)
400 MPa	18.52 (2.94)	8.34 (0.40)	17.63 (0.84)	6.45 (0.18)	49.06 (2.33)
600 MPa	16.48 (3.01)	6.51 (0.02)	19.64 (0.83)	6.92 (0.23)	50.45 (1.97)
Heated at 90°C for 30 min and HP-treated at difference pressures for 30 min at 20°C					
0 MPa	22.58 (1.66)	30.53 (0.50)	34.45 (1.72)	2.69 (0.60)	9.75 (0.44)
200 MPa	25.01 (1.00)	23.04 (1.80)	17.49 (2.17)	1.67 (1.53)	32.79 (1.32)
400 MPa	18.53 (1.29)	15.90 (1.02)	20.21 (1.07)	1.22 (0.69)	44.14 (1.22)
600 MPa	17.76 (0.41)	21.14 (1.86)	26.44 (0.98)	2.08 (1.01)	32.59 (0.52)

Note. Standard deviations are in parentheses. * Calculate from percentage of individual protein of total bound proteins at the interface of fat globule.

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