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**A comprehensive study on the relative
importance of disulphide and non-covalent
interactions between proteins on the heat-
induced aggregation and functional
property of acid milk gels**

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Abstract

Understanding the interactions between the milk proteins during heat treatment of milk can be employed to manipulate the functional properties of dairy products. The ability to control the functional properties can be beneficial to the dairy industry. When being heated, milk proteins interact via two main types of bonding: disulphide bonds and non-covalent interactions. They are both considered to be important in the properties of heated milks and the resulting milk products. This research aimed to investigate the relative importance of each interaction type on the heat-induced aggregation between the proteins in milk and the functional properties of a milk product in a model food system.

Experiments involved adding low concentrations of a disulphide-bond reducing agent or a thiol blocking reagent to milk systems to either enhance or inhibit the thiol-disulphide exchange reactions between the proteins. The reagent was added to unheated milks, heated milks and unheated milks followed by heating. The effect of modifying the extent of thiol-disulphide exchange reactions between the proteins on the level of proteins participating in intermolecular disulphide bonds, on the degree of interactions between the casein micelles/casein proteins and the whey proteins were investigated. The treated milks were acidified to form acid milk gels of which the rheological properties and the microstructure were examined.

Results demonstrated that the proportion of proteins participating in intermolecular disulphide bonds can be controlled by systematically modifying the thiol-disulphide exchange reactions between the milk proteins. It was shown that the initial interactions between the proteins in milk upon heating were non-covalent and disulphide bonds were subsequently formed to strengthen the bonding between the proteins in the heat-induced aggregates. When the milks were made to acid gels, both types of protein interactions in the milk were equally important in influencing the storage modulus (G') values of the resulting gels with the higher the degree of connections, the higher the G' values. On the other hand, disulphide bonds played a more important role than non-covalent interactions in determining the yield properties of the acid gels. The yield stress values can be increased by increasing the proportion of disulphide bonds in the milk system before acidification or by enhancing the formation of disulphide bonds between the particles during the formation of acid gels.

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List of Symbols

G'	Storage modulus
G''	Loss modulus
pI	Isoelectric point
pKa	The negative base-10 logarithm of the acid dissociation constant (K_a) of a solution. The lower the pKa values, the stronger the acid.
tan δ	Loss tangent, ratio of G''/G'

List of Abbreviations

APS	Ammonium persulphate
CCP	Colloidal calcium phosphate
Cys	Cysteine residue
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
GDL	Glucono- δ -lactone
MF	Micro-fluidic
NEM	N-ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulphate
SH	Thiol group
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
Tris-base	Tris (hydroxymethyl) methylamine
WPE	Whey protein enriched
WPF	Whey protein free
WPI	Whey protein isolate
UV	Ultraviolet

Chapter 1 - Introduction

Milk is a fluid produced by the mammary glands of female mammals and is intended to supply all the nutritional requirements for neonates until weaned. Milk is a readily digested food that is rich in protein, minerals, vitamins and water. The majority of the world's population uses some form of dairy product as a supplement to their daily diet. The conversion from raw milk to commercial milk and milk products can involve many different processing steps such as heating, cooling, evaporation and drying. During these different processes, there are changes in the balance and extent of interactions between the milk proteins and considerable inter-molecular aggregation can occur.

Some form of heat treatment is used in the manufacture of almost all milk products. Traditionally, heating milk was carried out to reduce pathogenic bacteria and inactivate some undesirable enzymes in order to prolong shelf-life and ensure safety. Nowadays, heat treatment is also exploited to achieve other functional benefits. This is because heating milk at sufficiently high temperatures causes the whey proteins to denature (Dannenberg & Kessler, 1988a) and interact with each other and with casein proteins (Oldfield, Singh & Taylor, 1998a). The interactions can be divided into two types: disulphide interactions (Sawyer, Coulter & Jenness, 1963; Oldfield et al., 1998a) and non-covalent interactions (Doi, Ideno, Ibuki & Kanamori, 1983; Haque & Kinsella, 1988).

The extent of these denaturation and interaction reactions during processing is used in commercial dairy processing to control and manipulate the functional properties of dairy products. For example, the heat treatment of milk is exploited in yoghurt manufacture to produce yoghurt with increased firmness and viscosity, and reduced syneresis (Davies, Shankar, Brooker & Hobbs, 1978; Harwalkar & Kalab, 1981; Dannenberg & Kessler, 1988b; Dannenberg & Kessler, 1988c; Mottar, Bassier, Joniau & Baert, 1989). Similarly, the level of heat treatment of milk is used to alter the functional properties of milk, which provides milk ingredients with different end uses depending on the extent of whey protein denaturation and interactions (e.g. high heat milk powder is specifically required for the manufacture of recombined evaporated milk, Morr & Ha, 1993).

The disulphide bonds and non-covalent interactions between the proteins in heat-induced aggregation are well-known. However the relative importance of each interaction type in the protein aggregation reactions in milk and the effect of different types of interactions on the functional properties of the milk and milk products have been not well understood. An in-depth understanding gained from a study of the relative importance of the different protein interactions could lead to the development of innovative milk protein products with targeted, enhanced or unique functional properties.

The aim of this study was to investigate the importance of each interaction type on the heat-induced aggregation between the proteins in milk and the relative influence of these interactions on the functional properties of the milk products in model food systems. Experiments were designed to either enhance or inhibit the thiol-disulphide exchange reactions between the proteins. Consequently, the balance of disulphide bonding and non-covalent interactions between the milk proteins was modified. The effect of these modified interactions on the size of the casein micelles, the degree of interactions between the casein micelles/casein proteins and the whey proteins, and the rheological properties of acid milk gels were examined.

The objectives of the study were:

1. To determine the relative importance of non-covalent interactions and disulphide bonds between the proteins in the formation of heat-induced aggregates in milk
2. To determine the effect of non-covalent and disulphide interactions between the proteins in heated milk on the functional properties of the acid gels prepared from the modified milks
3. To investigate the effect of systematically inhibiting the thiol-disulphide exchange reactions on the degree of non-covalent and disulphide interactions between milk proteins
4. To investigate the effect of systematically enhancing the thiol-disulphide exchange reactions on the degree of non-covalent and disulphide interactions between milk proteins
5. To investigate the functional properties of milk products with modified disulphide bonds and non-covalent interactions between proteins when used in a model dairy food application (e.g. acid gel).

Chapter 2 - Literature review

This literature review covers a basic knowledge of bovine milk and the proteins in milk. Whey protein denaturation and their interactions induced by heating milk are also discussed. The review then focuses on thiol-disulphide exchange reactions in more detail and the effects of modifying these reactions on protein behaviour. Finally a brief review of the literature on acid milk gels and the factors affecting their properties is provided.

2.1 Bovine milk

Milk is produced to feed the neonates from birth to weaning as it is a rich source of proteins, minerals, vitamins, energy and water (Fox, 2009). Milk is designed by nature to be consumed soon after production as it is perishable (Fox, 2003, 2009). As milk is a valuable and versatile food source, humans have developed methods to convert this perishable raw material into a range of more stable products. Traditional products were predominantly fermented including cheeses and yoghurts; whereas more modern science, technology and processing techniques have led to the development of products such as milk powders and milk protein concentrates. Conversion of milk to these types of products allows the value of the milk to be preserved long after its initial production. Bovine milk products are the most common in the commercial dairy industry, hence only the bovine milk will be considered in this review.

2.2 Composition of bovine milk

Bovine milk has about 86% water and 14% dry matter that is either dissolved or suspended in the water phase. Figure 2.1 lists the major components of bovine milk. The majority of the solid content is made up of fat, lactose and proteins while the minority contains trace amounts of vitamins, hormones, enzymes etc. The composition of milk is variable and can be affected by many factors including variation between individual animals, between breeds, seasons, feed, stage of lactation and health of the animal (Fox, 2009).

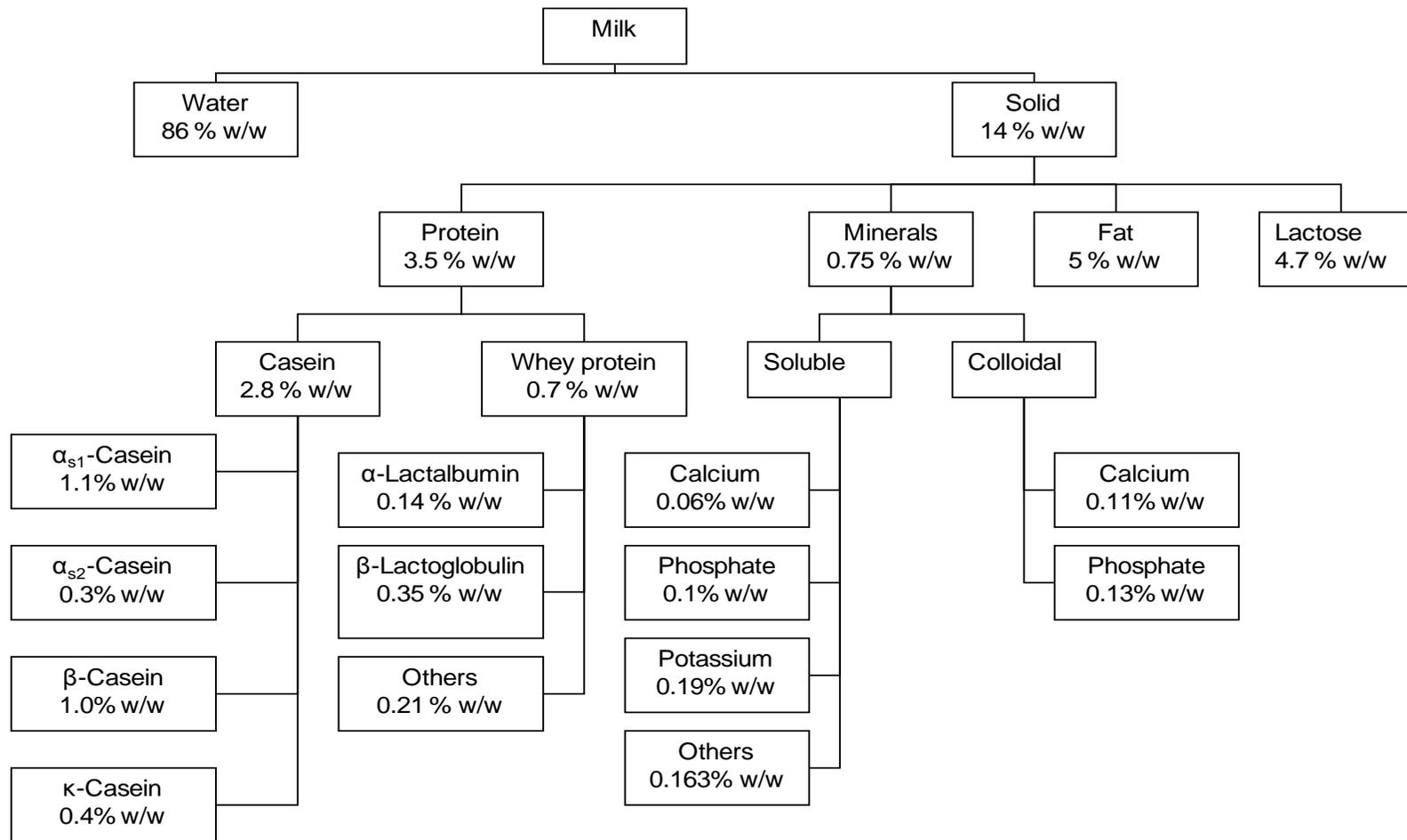


Figure 2.1: The major components of milk and their approximate concentrations. Source Swaisgood (1982, 2003), Walstra (1984), and Fox (2009).

2.3 Proteins

The protein in milk makes up approximately 3 - 3.5 % of the total weight of the milk. There are two main classes of proteins: caseins and whey proteins that respectively contribute for 80 and 20% of the total milk protein (Figure 2.1). In raw milk, caseins are generally defined as the protein that precipitate at pH 4.6 and ~ 20 °C while the native whey proteins remain soluble under these conditions (Walstra & Jenness, 1984; Fox, 2009).

2.3.1 Casein proteins

Casein is made up of four distinct proteins: α_{s1} -, α_{s2} -, β - and κ -casein, of which the molar ratio is about 11:3:10:4, respectively (Walstra, Wouters & Geurts, 2006). Table 2.1 presents some key properties of the caseins.

Table 2.1: Properties of casein proteins. Source: Walstra & Jenness (1984) and Fox (2009).

	α_{s1} -casein	α_{s2} -casein	β -casein	κ -casein
Molecular weight (Da)	~23600	~25200	23983	~19550
Amino acid residues (per molecule)	~199	~207	~209	~169
Phosphoserine residues (per molecule)	8-9	10-14	4-5	1-2
Cysteine residues (per molecule)	0	2	0	2
Proline residues (per molecule)	17	10	35	20
Net charge/residue	-0.10	-0.07	-0.06	-0.02

Caseins have low levels of secondary and tertiary structure. This is partially due to the high level of prolines that are uniformly distributed along the peptide chains. A proline has a ring structure at the N terminus (Figure 2.2) so it restricts the formation of the secondary and tertiary structure of casein molecules as it puts a kink in the protein chain. Due to the lack of high-ordered structure, casein proteins are relatively heat stable.

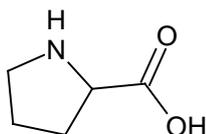


Figure 2.2: Two-dimensional structure of proline.

Caseins are phosphoproteins as they have phosphate residues attached to serine amino acids (phosphoserine, Table 2.1). In α_{s1} -, α_{s2} - and β -caseins, the phosphoserine residues are not distributed uniformly but are found in groups. As they possess negative charge, their grouping together into clusters gives rise to a high charge density (Walstra et al., 2006; Fox, 2009; Horne, 2009). These clusters are able to bind metal ions, particularly Ca^{2+} in the case of milk. The interaction between phosphoserine clusters and Ca^{2+} is important in the association behaviour of caseins in milk and the formation of casein micelles. κ -Casein, on the other hand, contains only one phosphoserine residue, thus κ -caseins cannot bind the high levels of Ca^{2+} that the other caseins do. This feature will be discussed in more detail in Section 2.3.3.

The phosphoserine residues are the main contributors to the hydrophilic residues on the casein molecule. There are also clusters of the hydrophobic residues. The existence of hydrophobic and hydrophilic regions gives rise to the amphiphilic nature of caseins. The hydrophobic regions of different casein molecules can interact with each other, and this hydrophobic interaction also plays an important role in the associating behaviour of caseins, especially the self-association and the structure of the casein micelle.

Only κ -casein and α_{s2} -caseins possess cysteine residues that can form either intra- or inter-molecular disulphide bonds. α_{s2} -Caseins exist as either monomers with intra-molecular disulphide bonds or as disulphide-linked dimers (Rasmussen, Hojrup & Petersen, 1994). κ -Caseins are able to form intermolecular disulphide-bonded oligomers which are reported to have from 1 to 11 monomers (Rasmussen, Hojrup & Petersen, 1992; Rasmussen et al., 1994; Holland, Deeth & Alewood, 2008). Another feature of κ -casein is that it is a glycoprotein with sugar moieties attached to threonine residues, which are predominantly located at the C-terminal polar domain.

2.3.2 Whey proteins

The whey proteins are a collection of proteins of which β -lactoglobulin (~ 50%) and α -lactalbumin (~20%) are the major components (Figure 2.1). Other whey proteins that are also present at significant levels include immunoglobulin (0.8 g L^{-1}), bovine serum albumin (0.4 g L^{-1}) and lactoferrin (0.1 g L^{-1}) (Tremblay, Laporte, Léonil, Dupont & Paquin, 2003). β -Lactoglobulin and α -lactalbumin are typical globular proteins and have well-defined secondary and tertiary structures (Robbins & Holmes, 1970; Sawyer & Holt, 1993; Brownlow et al., 1997). Hence they are prone to heat denaturation (Larson & Jenness, 1952; Roefs & De Kruif, 1994).

2.3.2.1 β -Lactoglobulin

A β -lactoglobulin molecule is made up of 162 amino acids and has a molecular weight of ~ 18.3 kDa. The protein has two common genetic variants, A and B and a number of less common variants (Edwards, Creamer & Jameson, 2009). β -Lactoglobulin can self-associate, and the degree of self-

association is dependent on temperature, pH, protein concentration and other factors like ionic strength (Figure 2.3). The three-dimensional structure of β -lactoglobulin consists of nine antiparallel β -strands and an α -helix (Swaisgood, 1982; Edwards et al., 2009). Eight β -strands make up two β -sheets that form a β -barrel (calyx). The α -helix flanks the calyx on its surface and is followed by the ninth strand, which is an integral part of the dimer interface (Brownlow et al., 1997). At the natural pH of milk and ambient temperatures β -lactoglobulin exists primarily as a dimer; however at higher pH and higher temperatures dimers can dissociate to monomeric species (Figure 2.3). Between pH 3.5 and 5.5, dimers associate with each other to form octamers whereas below pH 3.5, dimers dissociate to monomers due to strong electrostatic repulsive forces (Swaisgood, 1982; Walstra et al., 2006).

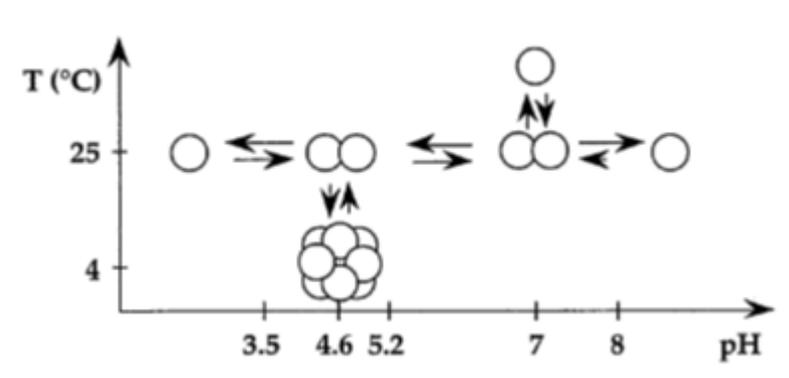


Figure 2.3: Effect of temperature and pH on the self-association of β -lactoglobulin. Source: Elofsson (1996).

A β -lactoglobulin monomer contains two intra-molecular disulphide bonds (Cys⁶⁶-Cys¹⁶⁰ and Cys¹⁰⁶-Cys¹¹⁹) and a free thiol group (-SH, Cys¹²¹) that is buried under the α -helix in the native conformation (Figure 2.4). This thiol group is important because when β -lactoglobulin is denatured, the free thiol group is exposed and can undergo thiol-disulphide exchange reaction with disulphide bonds of other denatured β -lactoglobulin and/or other milk proteins (such as κ -casein and α -lactalbumin) (Purkayastha, Tessier & Rose, 1967; Sawyer, 1967; Morr & Josephson, 1968). As thiol-disulphide exchange reactions between the proteins are an important aspect of this work, this will be discussed in more detail in Section 2.11.

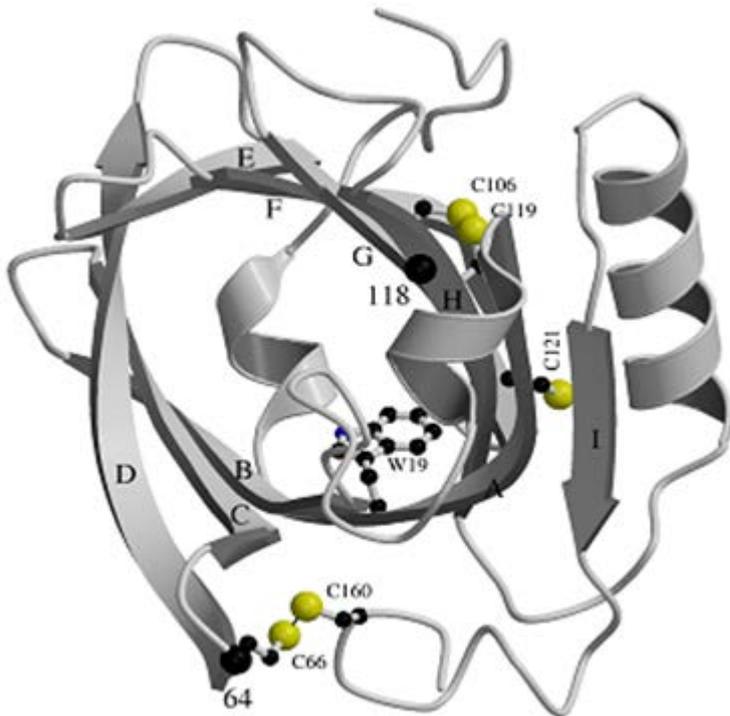


Figure 2.4: The structure of β -lactoglobulin. The α -helix is clearly visible. A-I are the β -strands. The disulphide bonds, C⁶⁶-C¹⁶⁰ and C¹⁰⁶-C¹¹⁹, are highlighted, and so is the free thiol at C¹²¹. Source: Edwards, Creamer and Jameson (2009).

The role of β -lactoglobulin is not known. However, as β -lactoglobulin possesses a hydrophobic cavity (the calyx) that is able to bind small hydrophobic molecules, the function of β -lactoglobulin could be in transporting retinoid species such as vitamin A (Edwards et al., 2009).

2.3.2.2 α -Lactalbumin

An α -lactalbumin molecule has 123 amino acid residues and a molecular weight of ~ 14 kDa. There are two known genetic variants of α -lactalbumin, A and B; however, the A variant is the most commonly found in bovine milk (Swaisgood, 1982). α -Lactalbumin is a metalloprotein as a calcium ion (Ca^{2+}) is an integral part of its molecular structure. Binding Ca^{2+} is important in stabilising the protein conformation as the removal of the ion causes the α -lactalbumin molecule to be more susceptible to denaturation (Swaisgood, 1982; Walstra & Jenness, 1984). The tertiary structure of α -Lactalbumin has a shape of an oblate ellipsoid and consists of two lobes (α and β) divided by a deep cleft, which is the binding site for Ca^{2+} (Figure 2.5, Brew, 2003).

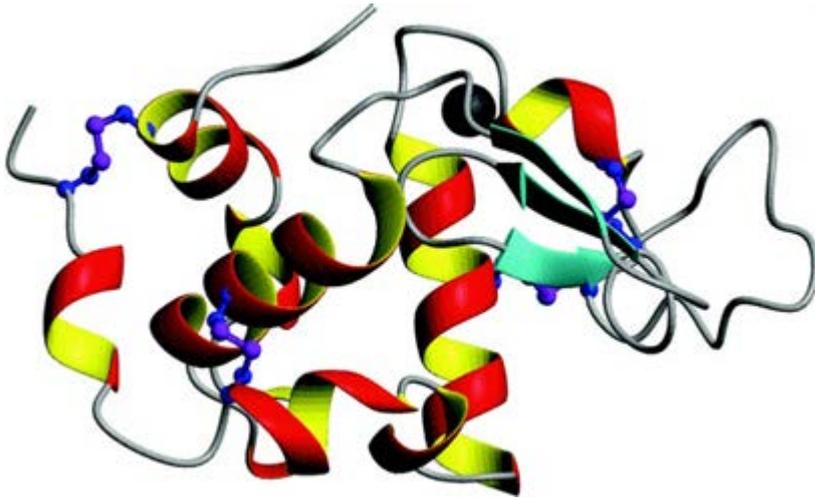


Figure 2.5: The three-dimensional structure of an α -lactalbumin molecule. Cys⁶-Cys¹²⁰, Cys²⁸-Cys¹¹¹, Cys⁶⁰-Cys⁷⁷ and Cys⁷³-Cys⁹⁰, are in blue and the bound calcium ion is represent by the black ball. Source: Edwards (2009).

The α -lobe contains three α -helices and three short 3_{10} -helices, and the β -lobe contains three-stranded β -sheets and a short 3_{10} -helix (The 3_{10} -helix is characterized by having at least two consecutive hydrogen bonds between the main-chain carbonyl oxygen of residue i and the main-chain amide hydrogen of residue $i+3$). α -Lactalbumin contains four intra-molecular disulphide bonds (Cys⁶-Cys¹²⁰, Cys²⁸-Cys¹¹¹, Cys⁶⁰-Cys⁷⁷ and Cys⁷³-Cys⁹⁰) but no free thiol group (Brew, 2003).

α -Lactalbumin is a component of the enzyme lactose synthetase, which catalyses the final step in the biosynthesis of lactose (Fox, 2009). The protein assists in the transfer of galactose to glucose so lactose can be synthesised (Morrissey, 1985).

2.3.2.3 Other whey proteins:

There are several other whey proteins that exist at low levels. Bovine serum albumin, immunoglobulin and iron-binding proteins are the few to be named and will be described briefly below. Nevertheless, these proteins will not be discussed as this work focuses only on the two major whey proteins.

Bovine serum albumin accounts for ~ 6 % of whey proteins. Bovine serum albumin has a molecular weight of ~ 66 kDa, contains seventeen disulphide bonds and one sulphhydryl group (Walstra & Jenness, 1984; Fox, 2003).

Immunoglobulins are antibodies synthesised due to the presence of macromolecular antigens foreign to the animal. The major species of immunoglobulin protein in bovine milk are members of IgG subfamily which give immunity to the young while they are developing their own immune system (Fox, 2009).

There are two iron-binding proteins: lactoferrin and transferrin. Due to these proteins ability to bind iron (e.g. Fe^{3+}) their role is to remove the iron from the serum, making it unavailable to bacteria. Also it has antioxidant, antiviral, anti-inflammatory and anti-carcinogenic activity (Walstra et al., 2006; Fox, 2009).

2.3.3 Casein micelles

In milk, most of the casein proteins are found as macromolecular assemblies that are known as casein micelles. Casein micelles are large highly hydrated (~ 4 g water/g casein) colloidal particles with sizes ranging from about 50 to 500 nm in diameter (average about 200 nm), and consisting of ~ 5000 individual casein proteins (Fox & Brodtkorb, 2008). Other characteristics of the casein micelles are summarized in Appendix 1, Table A.1.

The structure of the casein micelle is a topic of continuing research as the exact structure has not been established. Over the years, models of casein micelles are refined as more information becomes available. Initially, the casein micelles were proposed to be made up of many sub-units called sub-micelles (Slattery & Evard, 1973). Many sub-micelle models have been proposed, but the model that gained most acceptance was the one proposed by Schmidt (1982), and subsequently refined by Walstra (1990, 1999). In this model, each sub-micelle consists of all four casein proteins, interacting with each other via hydrophobic bonds and electrostatic attractions. However, some sub-micelles are rich in κ -casein and some have little κ -casein. The serine phosphate residues of α_{s1} -, α_{s2} - and β -casein are located at the surface of the sub-micelles, and the sub-micelles are bound together by colloidal calcium phosphate (CCP) interacting with these phosphoserine residues, thus forming an integral part of the CCP. As phosphoserine-poor- κ -casein cannot bind to the CCP, sub-micelles with low κ -casein content are found in the centre of the micelle and the ones with a high κ -casein content are located on the micelle surface (Schmidt, 1982). The negatively charged hydrophilic parts of κ -caseins extend from the surface of the casein micelles as flexible "hairs". This hairy layer stabilises the casein micelles from coagulation by means of steric and electrostatic repulsion (Figure 2.6A) (Walstra, 1990). Later, CCP was reported to be present throughout the micelle and included most of the phosphoserine residues of casein proteins (Knoop, Knoop & Wiechen, 1973; Holt, Davies & Law, 1986). Hence, Walstra (1999) proposed a modified casein micelle model in which the CCP were found within the sub-micelles and the hydrophobic interaction was responsible for linking the sub-micelles together (Figure 2.6B).

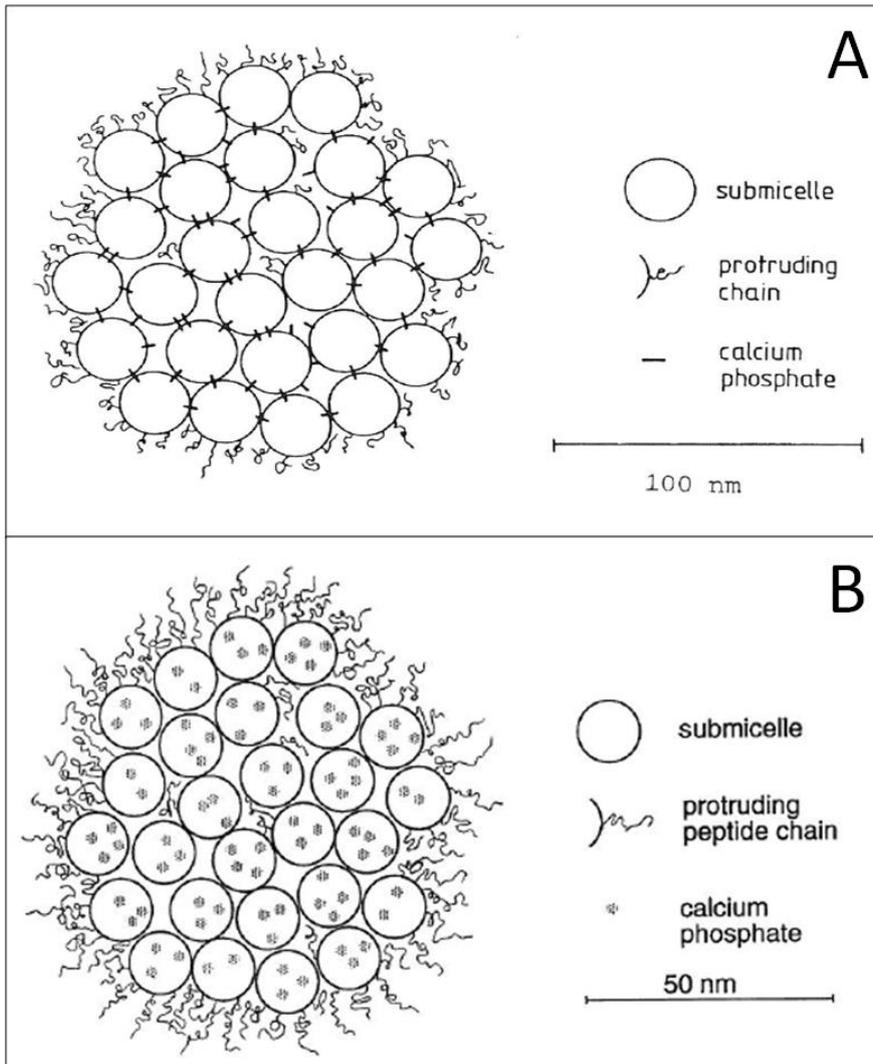


Figure 2.6: Schematic diagram of the sub-micelle model of Walstra in 1990 (A) and in 1999 (B).

In recent years, the existence of sub-micelles in the casein micelle structure has been questioned (Holt & Horne, 1996; Holt, 1998; Horne, 1998; McMahon & McManus, 1998; De Kruif & Holt, 2003). McMahon and McManus (1998) reported a casein micelle structure without sub-units using a novel method, called cryopreparation, to prepare sample for transmission electron microscopy (TEM). Hence the sub-micelles were claimed to be artefacts formed during sample preparation for freeze-fracture TEM. In addition, sub-micelles were not observed by removal of CCP or by addition of high concentration of κ -casein (Holt, 1998). The uneven distribution of κ -casein between two types of sub-micelles was also questioned. It is not fully understood why there should not be only one type of sub-micelle being made up from a mixture of all the casein proteins (Horne, 2006).

The first non-sub-micelle model was the nanocluster model, in which the α_{s1} -, α_{s2} - and β -caseins with phosphoserine clusters interacted with the calcium phosphate nanoclusters, forming a three-dimensional network. The κ -caseins did not interact with the nanoclusters and were located on the

micelle surface (Holt, 1992). This model, however, did not provide a specific role to κ -caseins or how κ -caseins control the micelle size and relied only on CCP to determine the structure of casein micelles.

In 1998, Horne proposed another structure for casein micelles based on the amphiphilic structures of the casein proteins and the two types of interactions between the proteins: hydrophobic interactions and interactions between phosphoserine clusters (in the charged regions) and CCP (Figure 2.7).

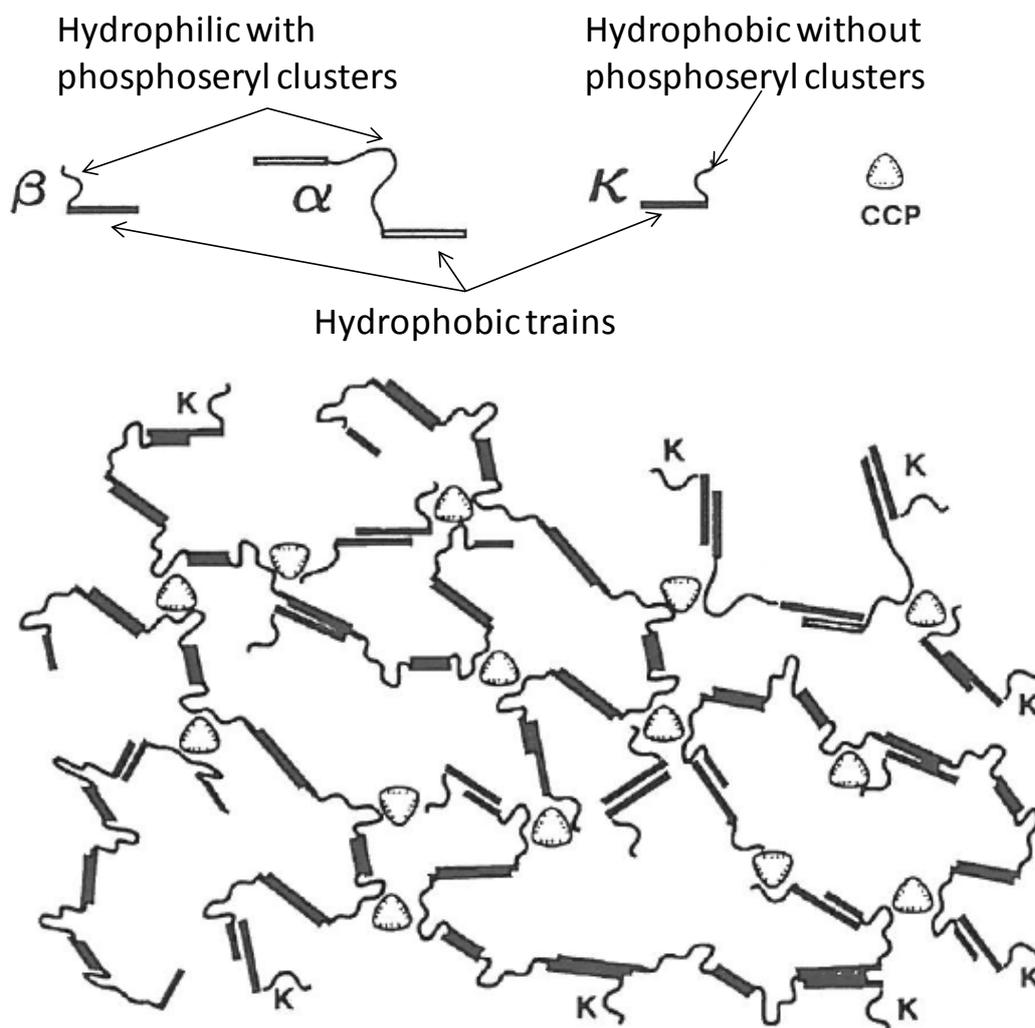


Figure 2.7: Schematic diagram represents the interactions between the caseins in the dual-binding model. The caseins are simplified to the loop/train model. The hydrophobic interactions depicted by the interactions between the trains and the loop containing phosphoserine clusters interact with CCP (Horne, 1998).

Hydrophobic interactions occurred between the hydrophobic regions of α_{s1} , α_{s2} , β - and κ -caseins and resulted in formation of polymers. However, the growth of these polymers was restricted by the strong electrostatic repulsion of the hydrophilic regions, particularly phosphoserine clusters that were highly negatively charged (Swaisgood, 2003). CCP neutralises the negatively charged phosphoserine

residues. Thus it diminishes the electrostatic repulsion between alike-charged regions and the hydrophobic bonds are strengthened. In addition, up to four or more phosphoserine clusters from different casein molecules can interact with one CCP nanocluster, thus linking the caseins together. Hence, CCP acts as the second type of cross-link between the casein proteins (Horne, 1998, 2009). This model is known as dual-binding model. However this model is not strictly a model of the casein micelle structure because it only describes how the caseins interact to assemble into non-submicelle casein micelles.

As with other models of casein micelles, κ -casein is very important in the micelle structure described by the dual-binding model. κ -Casein with one hydrophobic region can associate with the hydrophobic regions of the other casein proteins. However, κ -caseins terminate the growth of any chain or network because the protein does not have a phosphoserine cluster to bind to the CCP, nor another hydrophobic region to extend the chain. As a result, κ -casein is found on the surface of the casein micelles (Figure 2.7, Horne, 1998).

In 2011, Dagleish proposed a refined structure of the casein micelles (Figure 2.8). In this new model, κ -caseins were distributed unevenly on the surface of the casein micelles, leaving clefts where other caseins can be exposed to the serum. This is based on the findings of recent electron microscopic studies showing that the micellar surface contains clefts penetrating the micelles, instead of having a smooth surface (Dagleish, Spagnuolo & Douglas Goff, 2004; Marchin, Puteaux, Pignon & Leonile, 2007; McMahon & Oommen, 2008). In the interior of the casein micelles, α_{s1} -, α_{s2} - and some β -casein aggregate with CCP into sub-assemblies that in turn interact hydrophobically with the rest of the β -casein and κ -casein. This model takes into account the highly hydrated property of the micelles and the ability of β -caseins to exit and re-enter the micelle interior upon cooling and warming milk. Hence the majority of β -caseins are postulated to act as surfactants to stabilise the water in the casein micelles (Dagleish, 2011). The micelle structure in this new model is open and therefore, can explain certain observations that cannot be explained by previous models. These include the dissociation and re-association of β -casein upon temperature changing, the attack on β -casein by trypsin and the attack on caseins by papain (Ashoor, Sair, Olson & Richardson, 1971; Creamer, Berry & Mills, 1977; Le Feunteun & Francois, 2007).

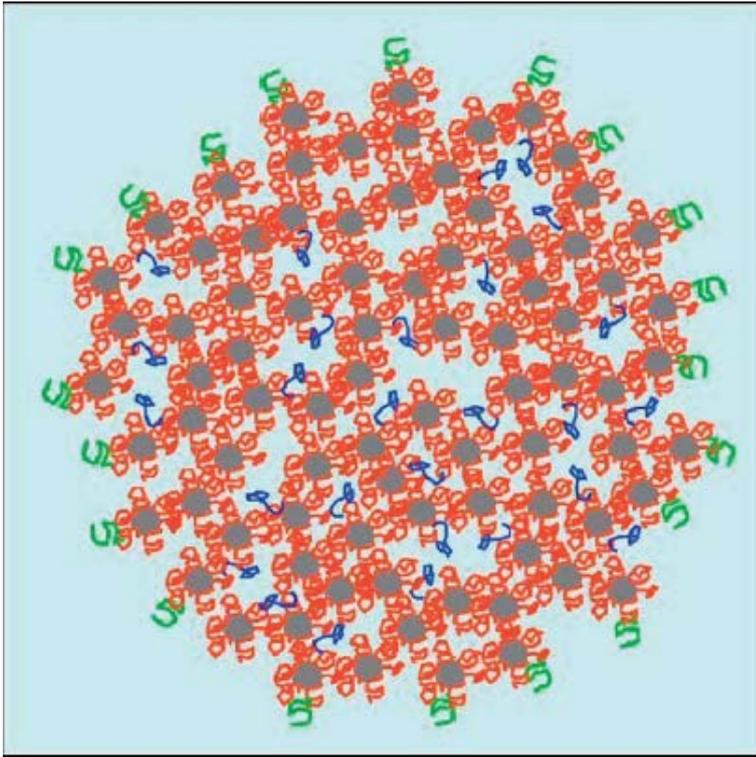


Figure 2.8: Schematic structure of the casein micelle according to Dalglish's model (2011). Grey, calcium phosphate nanoclusters; red, α_{s1} and α_{s2} - casein; blue, β -caseins and green, κ -caseins. The relative sizes of the individual components are not to scale.

2.4 Heat treatment

The manufacture of almost all milk products involves some form of heat treatment. Traditionally heating milk was for food safety and extending shelf-life. In modern days, heat treatment may also serve the purpose of producing specific functional properties in dairy products.

When milk is heated at sufficiently high temperatures, whey proteins are irreversibly denatured, casein proteins can dissociate from the casein micelles depending on environmental conditions and denatured whey proteins can interact with each other and with casein proteins. Many other changes can also occur upon heating milk such as changes in mineral equilibria and the Maillard reactions, etc. However, the protein-related reactions are the focus of this study as they play important roles in determining the functionality of dairy products.

2.4.1 Whey protein denaturation

Definition of 'protein denaturation': A major change from the original native structure, without alteration of the amino acid sequence, i.e., without severance of any of the peptide bonds that join one amino acid to another (Tanford, 1968).

Globular whey proteins can be denatured upon exposure to high temperatures since they possess highly ordered secondary and tertiary structures (Robbins & Holmes, 1970; Brownlow et al., 1997). The schematic of the reaction pathway for the denaturation of whey protein is summarised in Figure 2.9. If the proteins are present in native polymeric form (e.g. dimeric β -lactoglobulin), the polymer will first dissociate into monomers on heating (Figure 2.9a); subsequently, the protein structure unfolds (Figure 2.9b). The unfolded conformation can refold into the native conformation when the denaturing influence is removed. This process is known as reversible denaturation. Otherwise, the unfolded protein can interact with other (denatured) proteins to form aggregates (Figure 2.9c). This is known as irreversible denaturation (De Wit, 1990; Roefs & De Kruif, 1994; Anema, 2009b).

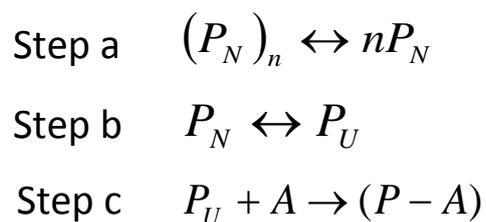


Figure 2.9: Summary of denaturation of globular whey proteins. $(P_N)_n$, polymeric native protein; P_N , native protein; P_U , unfolded protein; A, other protein containing disulphide bonds.

The reversible denaturation of whey proteins has been studied extensively, commonly using differential scanning calorimetry (DSC) (Ruegg, Moor & Blanc, 1977; De Wit & Swinkels, 1980; De Wit & Klarenbeek, 1981; Park & Lund, 1984; De Wit, 1990; Paulsson & Dejmek, 1990). Even though the thermodynamic data obtained from DSC varies amongst studies due to differences in experimental conditions, the denaturation temperatures of major whey proteins obtained from different studies were comparable to each other as long as the conditions were similar (Ruegg et al., 1977; De Wit & Swinkels, 1980; De Wit & Klarenbeek, 1981; Paulsson & Dejmek, 1990). Table 2.2 lists the average values of temperatures at which the proteins denatured and the change in enthalpy (i.e. the energy supplied as heat at a constant pressure) of denaturation of some major whey proteins. Using this method, α -lactalbumin is the most heat labile of the whey proteins whereas β -lactoglobulin is the most heat stable.

Table 2.2: Thermal denaturation temperatures of major whey proteins. Main sources: Kinsella and Whitehead (1989) and Jelen and Rattray (1995).

	T _d (°C)	T _{tr} (°C)	ΔH (kJ/mol)
β-Lactoglobulin	78	83	311
α-Lactalbumin	62	68	253
Bovine serum albumin	70	70	803

T_d = the initial denaturation temperature; T_{tr} = the temperature of the differential scanning calorimetric peak maximum; ΔH = the enthalpy of denaturation.

Even though the reversible denaturation of whey protein is of interest, it is the irreversible denaturation leading to formation of the aggregates (Figure 2.9c) that is the subject of the present work. Therefore, denaturation used in this thesis refers only to the “irreversible denaturation” unless stated otherwise.

Irreversible denaturation involves the unfolding process of protein from native structure to unfolded state, resulting in the exposure of active sites on the proteins. Thus the proteins interact with each other and aggregates are formed (De Wit, 1990; Roefs & De Kruif, 1994). The interactions between the proteins will be discussed further in Section 2.5.

2.4.2 Kinetics of whey protein denaturation

Early studies showed that the heat induced denaturation of whey proteins was dependent on both the temperature and the time of heating (Rowland, 1933; Harland & Ashworth, 1945; Gough & Jenness, 1962; Agrawala & Reuter, 1979). However, subsequent studies indicated that the denaturation reactions of individual whey proteins were, in fact, more complex. The denaturation of α-lactalbumin followed pseudo-first order reaction kinetics (Lyster, 1970; Hillier & Lyster, 1979; Law & Leaver, 1997; Anema, Lee & Klostermeyer, 2006) and denaturation of β-lactoglobulin following ~ 1.5 order reaction kinetics (Dannenberg & Kessler, 1988a). In addition, the rate constants of the denaturation were found to be different above and below critical temperatures (80 °C for α-lactalbumin and 90 °C for β-lactoglobulin).

When the temperatures are below the critical point, the activation enthalpies and activation energies have very high values that can be associated with the breaking of large numbers of bonds when the proteins are unfolding (Table 2.3). Also the positive value of change in the activation entropies confirms that the structure of resultant species at these temperatures is less ordered compared with the structure of the native species. So at this low temperature range, the change in the protein conformation is the rate determining step (Figure 2.9b).

Table 2.3: Change in enthalpy (ΔH), free energy ($\Delta G^\#$), entropy of activation ($\Delta S^\#$) and activation energy (E_A). Source: Dannenberg and Kessler (1988a).

Proteins	Temp (°C)	$\Delta H^\#$ (kJ/mol)	$\Delta G^\#$ (kJ/mol)	$\Delta S^\#$ (kJ/mol)	E_A (kJ/mol)
β -lactoglobulin A	70 – 90	262.28	105.08	0.4452	265.21
	95 – 150	50.8	104.23	-0.1357	54.07
β -lactoglobulin B	70 – 90	277.02	104.34	0.488	279.96
	95 – 150	44.48	103.62	-0.1502	47.75
α -lactalbumin	70 – 80	265.66	108.39	0.4517	268.56
	85 – 150	65.80	110.02	-0.1144	69.01

At temperatures higher than the critical point, the changes of activation enthalpies and activation energies are similar to those of standard chemical reactions (Table 2.3). This means that aggregation reactions between the proteins are the rate determining step at this high temperature range (Figure 2.9c). The negative value of entropy is also an indication of aggregation as the resultant species have a more ordered structure compared with the starting species (Dannenberg & Kessler, 1988a; Anema & McKenna, 1996; Oldfield, Singh, Taylor & Pearce, 1998b).

2.4.3 Factors affecting heat denaturation of whey proteins

The heat denaturation of whey protein depends considerably on the pH at heating. As the heating pH increases to > 6.7, β -lactoglobulin and α -lactalbumin unfold at temperatures lower than the denaturation temperatures at neutral pH, hence aggregation occurs more rapidly (De Wit & Klarenbeek, 1981; Qi, Brownlow, Holt & Sellers, 1995; Law & Leaver, 2000; Tolkach & Kulozik, 2005).

The composition and concentration of milk also affect the denaturation of whey proteins. The rate of denaturation of β -lactoglobulin decreases with the increase of the total solids concentration in milk (Law & Leaver, 1997; Anema, 2000) while the rate of denaturation of α -lactalbumin is not affected by the milk concentration (Anema, 2001). These effects of milk concentration have been related to various components in milk (Anema, 2000; Anema et al., 2006). Increasing the concentrations of milk proteins while keeping other components constant increased the levels of denaturation of both β -lactoglobulin and α -lactalbumin (Law & Leaver, 1997; Anema et al., 2006). On the other hand, increasing the concentrations of non-protein-soluble components, especially lactose, lowered the degree of denaturation of the whey proteins (Anema et al., 2006), but had a greater effect on β -lactoglobulin than α -lactalbumin. When taken together, for α -lactalbumin, the reduced denaturation on increasing non-protein soluble components was almost exactly offset by the increased denaturation induced by higher protein concentrations, hence when milk was concentrated denaturation of α -lactalbumin appeared to be unaffected. In contrast, for β -lactoglobulin, the reduced denaturation on increasing non-protein soluble components was not offset by the increased denaturation induced by

higher protein concentrations, hence β -lactoglobulin denaturation appeared to be retarded when milk concentration was increased (Anema et al., 2006).

2.5 The interactions between milk proteins upon heating

The denaturation of the whey proteins, particularly β -lactoglobulin, is the initial step of a series of aggregation reactions occurring when milk is heated above 70 °C. Unfolded whey proteins expose hydrophobic amino acid residues as well as the disulphide bonds and free thiol groups. The hydrophobic residues can interact with each other via non-covalent interactions whereas the thiol groups and disulphide bonds can undergo thiol-disulphide exchange reactions to form new intra- and inter-molecular disulphide bonds. The denatured whey proteins can interact either with each other or with the caseins. There are other reactions happening due to heating milk at high temperatures such as interactions between proteins and lactose or proteins with fat globule membrane proteins. Amongst all these interactions, the interaction between denatured β -lactoglobulin and κ -caseins is the most studied in milk as it is the interaction that is mainly responsible for the functional properties of the dairy products (Dalgleish, 1990; Jang & Swaisgood, 1990; Corredig & Dalgleish, 1999).

2.6 Interactions between denatured β -lactoglobulin and κ -casein in model systems

Early studies demonstrated that interaction between β -lactoglobulin and κ -casein involved the free thiol group of β -lactoglobulin and that the heat-induced aggregates contained intermolecular disulphide bonds (Zittle, Thompson, Custer & Cerbulis, 1962; Sawyer et al., 1963; Sawyer, 1967, 1969; Smits & van Brouwershaven, 1980). Initially, it was debated whether κ -casein interacted with monomeric-denatured β -lactoglobulin (Euber & Brunner, 1982) or aggregated-denatured β -lactoglobulin (Sawyer, 1969; McKenzie, Norton & Sawyer, 1971). It is probable that both interactions can occur simultaneously, as Cho, Singh and Creamer (2003) proposed that many possible pathways were involved in the formation of aggregates between β -lactoglobulin and κ -casein. When β -lactoglobulin was heated with κ -casein, the free thiol group on denatured β -lactoglobulin became exposed and can initiate thiol-disulphide exchange reactions with other denatured β -lactoglobulin and with κ -casein depending on their concentrations. The products of these reactions ranged from 1:1 β -lactoglobulin: κ -casein complexes to large heterogeneous aggregates (Cho et al., 2003).

2.7 Interactions between whey proteins and caseins in a milk system

2.7.1 Disulphide interactions

In milk, as there are many proteins that could potentially react with one another, therefore the potential interactions between proteins in heated milk is more complicated than those found in model systems. Along with β -lactoglobulin, which is the most abundant whey protein, bovine serum albumin

also has one free thiol group and 17 disulphide bonds. Meanwhile, there are several proteins containing disulphide bonds without free thiol groups: α -lactalbumin, κ -casein and α_{s2} -casein as well as some of the minor whey proteins. These proteins can undergo thiol-disulphide exchange reactions with any protein that has a thiol group exposed. Hence the heat-induced aggregates can contain many different proteins and at different ratios.

In heated milk, denatured β -lactoglobulin forms complexes with κ -casein in a similar manner as their interactions in model systems. The resultant aggregates may also contain other denatured whey proteins and other casein proteins (Slatter & van Winkle, 1952; Smits & van Brouwershaven, 1980; Dalgleish, 1990; Oldfield et al., 1998a; Corredig & Dalgleish, 1999).

When milk is heated at temperatures below 120 °C, interactions between α -lactalbumin and κ -casein can occur only in the presence of β -lactoglobulin (Dalgleish, Mourik & Corredig, 1997a). It is generally accepted that denatured β -lactoglobulin or bovine serum albumin interacts with denatured α -lactalbumin before the combined aggregate interacts with κ -casein (Elfagm & Wheelock, 1978; Dalgleish, 1990; Calvo, Leaver & Banks, 1993; Dalgleish et al., 1997a; Oldfield et al., 1998a; Corredig & Dalgleish, 1999). Meanwhile, interactions between denatured whey protein and α_{s2} -casein, which also contains disulphide bonds, can occur when milk is subjected to ultra-high temperature treatments (Snoeren & van der Spek, 1977; Patel, Singh, Anema & Creamer, 2006) or high hydrostatic pressure treatments (Patel et al., 2006). This indicates that α_{s2} -casein/whey protein interactions probably only occur when the casein micelles were disrupted. As α_{s2} -casein is located in the interior of the casein micelles (Horne, 2006; Dalgleish, 2011), it may not be available for thiol-disulphide interchange reactions unless the casein micelle structure is disrupted.

2.7.2 Non-covalent interactions

Non-covalent interactions refer to non-chemical bonds that include hydrophobic, hydrogen, ionic and van der Waals bonds. Together with the disulphide bonds, non-covalent interactions between the milk proteins were also found to be important in the heat-induced protein aggregates (Mckenzie et al., 1971; Doi et al., 1983; Haque & Kinsella, 1988; Iametti, De Gregori, Vecchio & Bonomi, 1996; Hoffmann & van Mil, 1997; Galani & Apenten, 1999). Some studies reported that the non-covalent interactions between the proteins occurred when formation of disulphide bonds were inhibited (Mckenzie et al., 1971; Hoffmann & van Mil, 1997). In other studies, non-covalent interactions were postulated to be formed at an early stage in the formation of aggregates, followed by formation of disulphide bonds (Haque & Kinsella, 1988; Galani & Apenten, 1999).

2.7.3 Factors affecting the interactions between denatured whey proteins and κ -casein of casein micelles

The degree of interactions between the denatured whey proteins and κ -casein on casein micelles depends on various factors such as heating temperatures (Smits & van Brouwershaven, 1980; Jang & Swaisgood, 1990), time (Dalglish, 1990), heating rate (Oldfield et al., 1998a), concentration of milk components such as salts and whey proteins (Smits & van Brouwershaven, 1980; Dalglish et al., 1997a; Oldfield, Singh & Taylor, 2005) and pH (Creamer, Berry & Matheson, 1978; Smits & van Brouwershaven, 1980).

The pH at heating plays an important role in the properties of heated milk. The profile of heat coagulation time (i.e. the time taken for the proteins in milk to visibly aggregate) as a function of pH when milk is heated at $\sim 140^\circ\text{C}$ shows an increase in stability up to pH ~ 6.6 , followed by decreasing stability to a minimum at about pH 6.9, then an increase in stability again with further increases in pH (Figure 2.10, Rose, 1961).

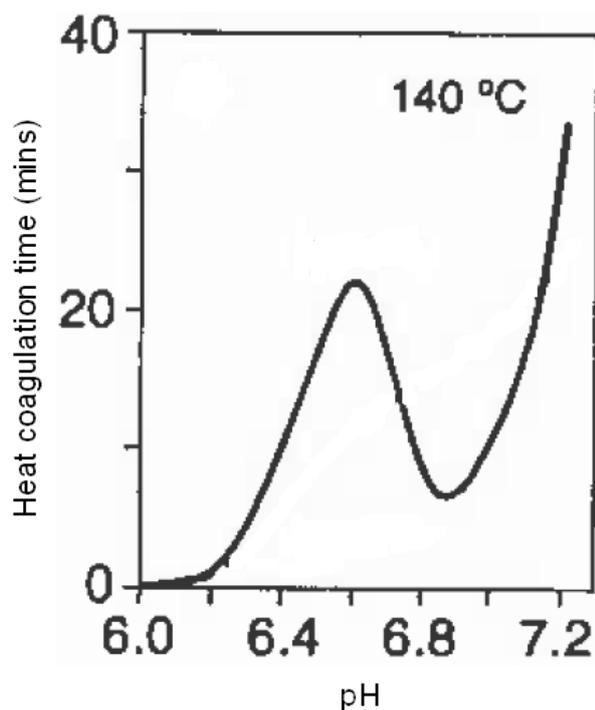


Figure 2.10: An example of a heat coagulation time-pH profile of a typical milk sample. Source: Walstra (1984).

The pH at heating was also found to influence the degree of association of denatured whey proteins on the casein micelles (Creamer et al., 1978; Kudo, 1980). When milk was heated at pH < 6.7 , a high proportion of the denatured whey proteins associated with the casein micelles whereas heating milk at pH > 6.7 resulted in a high level of soluble complexes between the denatured whey protein and κ -casein (Creamer et al., 1978; Kudo, 1980). Subsequent studies reported that pH at heating also

influenced the dissociation of κ -casein from the casein micelles with a corresponding increase in the amount of dissociated κ -casein with increasing pH (Singh & Fox, 1985, 1987).

2.8 The heat-induced dissociation of casein proteins and its relation to the association of denatured whey proteins on casein micelles

The dissociation of casein proteins from casein micelles was thought initially to occur only at temperatures above 90 °C (Morr, 1969; Aoki, Suzuki & Imamura, 1974, 1975; Kudo, 1980; Singh & Fox, 1985). However Anema and Klostermeyer (1997) later found at pH \geq 6.7, κ -casein dissociated from the micelles as soon as the temperature rose above 20 °C (Figure 2.11A). The level of κ -casein dissociation increased with increasing pH \geq 6.7. In addition, at each increase in pH, the amount of dissociated κ -caseins increased with increasing temperatures (Anema & Klostermeyer, 1997).

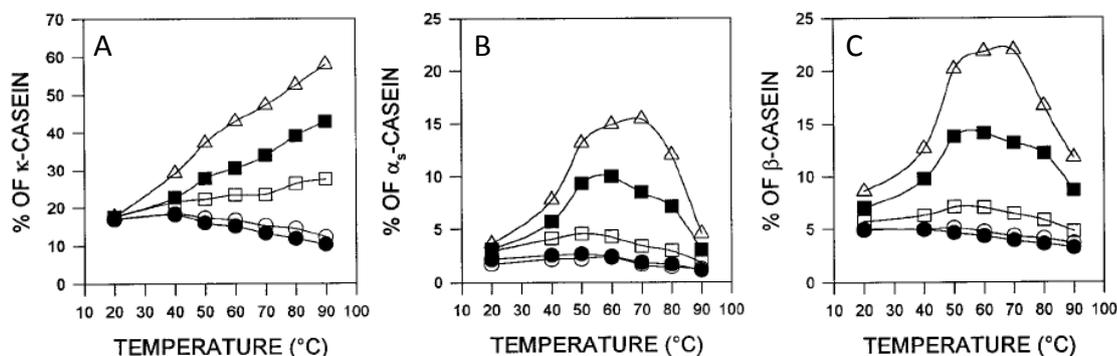


Figure 2.11: Effect of temperature and pH on the percentage of serum proteins. κ -casein (A), α_s -casein (B) and β -casein (C) in the serum phase. The proteins were found in the supernatants obtained from 10% total solid reconstituted skim milk samples. ○, pH 6.3, ●, pH 6.5; □, pH 6.7; ■, pH 6.9; △, pH 7.1 (Anema & Klostermeyer, 1997).

α_s -Caseins (including both α_{s1} - and α_{s2} -casein) and β -caseins were also found to dissociate on heating milk at pH values \geq 6.7, although in a different manner compared to the dissociation of κ -caseins (Anema & Klostermeyer, 1997; Anema, 1998). As the temperature increased, the level of dissociated α_s - and β -caseins increased progressively to a maximum at 70 °C and then decreased at higher temperatures (Figure 2.11B and C). Higher levels of κ -casein were dissociated at each temperature when the pH was increased from 6.7 to 7.1 (Anema & Klostermeyer, 1997; Anema & Li, 2000).

This observation can be related to the interactions between denatured β -lactoglobulin and κ -casein. When whey-protein-free milk was heated, the amount of α_s - and β -caseins that were dissociated from the casein micelles increased with increasing temperature at pH \geq 6.7 and this amount was higher than that of heating standard milk at temperatures above 70 °C (Anema & Li, 2000). It was proposed that on heating casein solutions, all the casein proteins dissociated from the casein micelles and on subsequent cooling, κ -caseins were able to stabilise the soluble α_s - and β -caseins into small soluble

aggregates. When standard milk was heated at temperatures below 70 °C, all the casein proteins dissociated from the casein micelles. However, at temperatures above 70 °C, the whey proteins became denatured and formed aggregates with κ -casein (Zittle et al., 1962; Jang & Swaisgood, 1990; Corredig & Dalgleish, 1999). The complexes between denatured whey proteins and κ -casein were less efficient in stabilising the soluble α_s - and β -caseins, especially in the presence of calcium ions because α_s - and β -caseins can associate strongly with each other via calcium (phosphate) bridging (Section 2.3.3). Therefore, on subsequent cooling, the dissociated α_s - and β -caseins could re-associate with the casein micelles or form larger aggregates that sediment on centrifugation (Anema & Klostermeyer, 1997; Anema & Li, 2000).

The effect of pH at heating on the interactions between denatured whey proteins and casein micelles has been extensively studied to establish the relation between the dissociation of κ -casein and the association of denatured whey proteins on the casein micelles (Smits & van Brouwershaven, 1980; Singh & Fox, 1991; Anema & Klostermeyer, 1997; Oldfield, Singh, Taylor & Pearce, 2000; Anema & Li, 2003a; Vasbinder & De Kruif, 2003). Most recent studies showed that dissociation of κ -casein from the casein micelles increased linearly with increasing pH on heating milk at pH from 6.5 to 7.1 (Rodriguez Del Angel & Dalgleish, 2006; Anema, 2007). Consequently the dissociation of κ -casein significantly determined the distribution of denatured whey protein between the colloidal and serum phases and the level of soluble proteins increased with the increase of pH at heating (Figure 2.12).

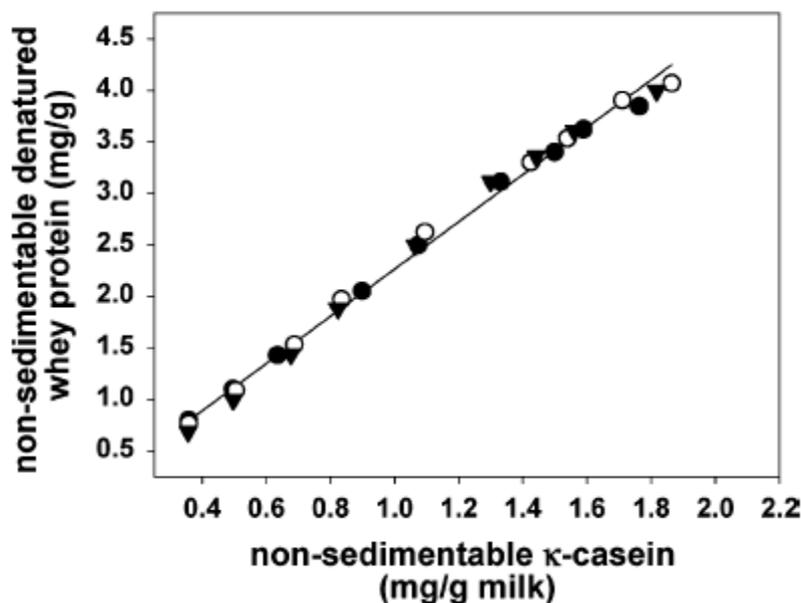


Figure 2.12: Relationship between the denatured whey protein and κ -casein in the serum phase of heated milk. (●), Milk heated at 90 °C for 20 min; (○), 25 min; (▼) and 30 min (Anema, 2007).

2.9 Distribution of denatured whey protein/ κ -casein complexes between colloidal and serum phases

When milk is heated at its natural pH (pH \sim 6.7), the aggregates formed between denatured whey proteins and κ -casein can be found in both serum and colloidal phases (Creamer et al., 1978; Smits & van Brouwershaven, 1980; Oldfield et al., 1998a; Guyomarc'h, Law & Dalgleish, 2003a). It is now generally accepted that the formation of serum aggregates is related to the dissociation of κ -caseins from the casein micelles. However there is still debate on which pathway the formation of aggregates follows.

One research group postulates that on heating milk, the denatured whey proteins first interact with κ -casein on the surface of the casein micelles forming complexes and these complexes are subsequently released from the micelles on continued heating (Figure 2.13 – Pathway A) (Parker, Donato & Dalgleish, 2005; Donato & Dalgleish, 2006). Their studies demonstrated that the addition of sodium caseinate (Parker et al., 2005) or κ -casein (Donato, Guyomarc'h, Amiot & Dalgleish, 2007) into milk prior to heat treatment did not increase the formation of soluble aggregates between denatured whey proteins and κ -casein. These observations were considered evidence that the formation of κ -casein/whey protein aggregates on the surface of the casein micelles occurred first, and then the complexes dissociated from the micelles.

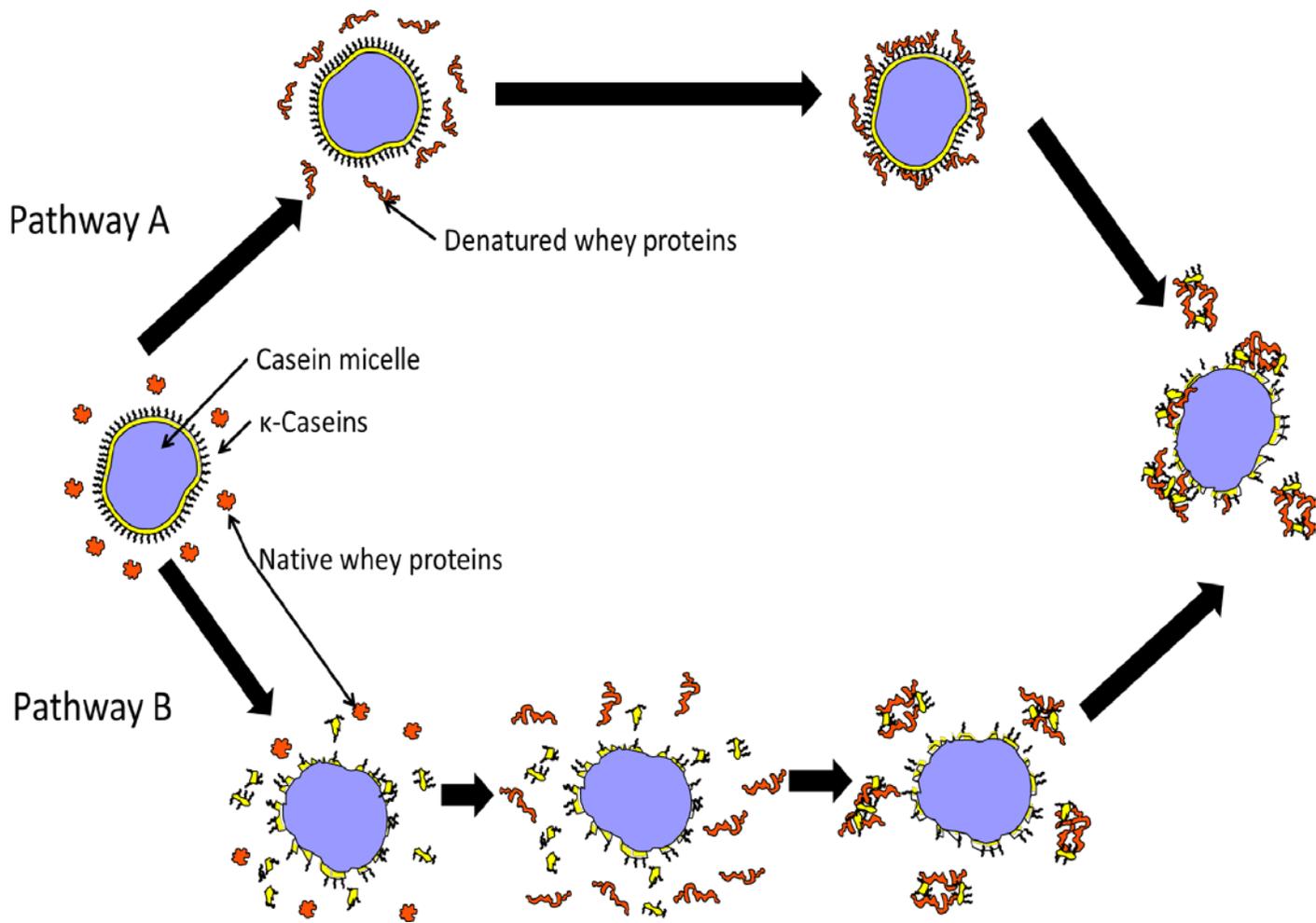


Figure 2.13: The possible pathways of the formation of serum aggregates between denatured whey proteins and κ -casein. Pathway A: whey proteins were denatured and interacted with the κ -casein on the surface of the casein micelles to form aggregates that were later dissociated from the casein micelles. Pathway B: κ -casein dissociated from the casein micelles upon heating milk, probably before whey proteins became denatured. Once whey proteins were denatured, interactions between the whey proteins and κ -casein can occur to form aggregates. Sources: Anema (2008), Donato, Guyomarc'h, Amiot & Dalgleish (2007) and Parker, Donato & Dalgleish (2005).

Other research groups found that κ -casein (and other casein proteins) dissociated from the casein micelles when the temperature was elevated, regardless of the presence of denatured whey protein (Aoki et al., 1974, 1975; Kudo, 1980; Anema & Klostermeyer, 1997). In addition, when κ -casein was added into skim milk before heat treatment, the denatured whey protein interacted preferentially with the added κ -casein that remained in the serum than with the native κ -casein that is located on the casein micelles. This indicates that the reaction between denatured whey protein and κ -casein preferentially occurs in the serum phase (Anema, 2007). Hence it was proposed that κ -casein dissociated from the casein micelles upon heating milk first, then interaction with the denatured whey proteins occurred (Figure 2.13 – Pathway B) (Anema, 2008).

2.10 Description of disulphide bonds and thiol groups in proteins

As disulphide interactions between the milk proteins are the major focus of this thesis, the following section describes the disulphide bonds and thiol groups of proteins. They are also the two main components for the thiol-disulphide exchange reactions that occur between the proteins during heating milk.

2.10.1 Role of disulphide bonds in native proteins

Disulphide bonds are often found in extracellular proteins (Wilkinson & Gilbert, 2004). They play a role in maintaining the highly ordered structure (tertiary and quaternary structures) of the proteins since they covalently link parts of a single polypeptide chains or separate chains within a protein (Torchinsky, 1981). Disulphide bonds also maintain the integrity of the proteins by preventing proteins from interacting with oxidants or proteolytic enzymes (Arolas, Aviles, Chang & Ventura, 2006). As the position of disulphide bonds on the polypeptide chain is determined by the primary structure (Torchinsky, 1981), disulphide bonds stabilise the folded form of proteins by lowering the entropy of the unfolded state instead of determining the structure of folded proteins (Creighton, Zapun & Darby, 1995; Swaisgood, 2005; Arolas et al., 2006). When all the disulphide bonds within a protein are completely broken, the structure will be disrupted and the bioactivity of the protein will be lost (Torchinsky, 1981; Arolas et al., 2006).

2.10.2 Biological formation of disulphide bonds

The formation of disulphide bonds was studied after it was observed that reduced unfolded proteins were able to refold and reform the disulphide bonds (Anfinsen & Haber, 1961; Torchinsky, 1981). The following description of the formation of disulphide bonds therefore is based on the observations of renaturation of reduced unfolded proteins:

The proteins are first synthesised to contain thiol groups (SH) which will then be oxidised into disulphide bonds (Figure 2.14A, Anfinsen & Haber, 1961; Ziegler & Poulsen, 1977; Torchinsky, 1981).

In the endoplasmic reticulum, disulphide bonds are formed arbitrarily between the thiol groups. Then thiol-disulphide exchange reactions occur intra-molecularly in order to rearrange the disulphide bonds until the protein achieves the non-random stable conformation/structure (Figure 2.14B, Creighton et al., 1995; Darby & Creighton, 1995; Wilkinson & Gilbert, 2004). Protein disulphide isomerase catalyses the oxidation of thiol groups to form disulphide bonds and is especially important in the intra-molecular rearrangement of the disulphide bonds.

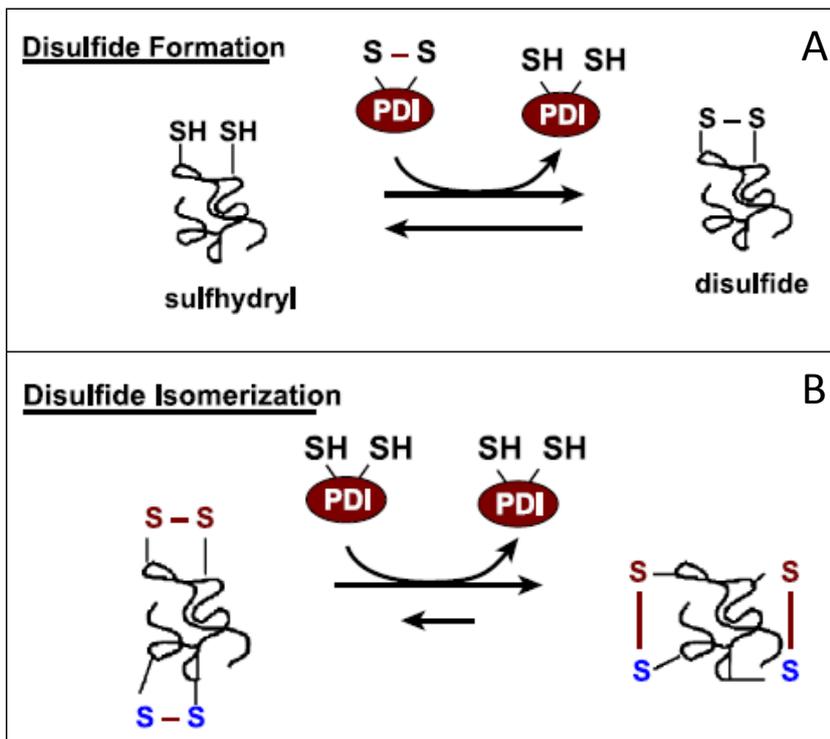


Figure 2.14: Scheme of the formation of disulphide bond (A) and the disulphide isomerisation (B). PDI, protein disulphide isomerase. Source: Wilkinson and Gilbert (2004).

2.10.3 Disulphide bonds in milk proteins

In milk, disulphide bonds are found in the whey proteins and some of the caseins. The whey proteins are typical globular proteins with disulphide bonds located intra-molecularly (Brownlow et al., 1997; Brew, 2003; Fox, 2009). Although not specifically studied (except for α -lactalbumin) it is postulated that disulphide bond formation in the milk whey proteins is by the route as was described in Section 2.10.2 (Creighton et al., 1995).

Unlike the whey proteins, the disulphide bonds in κ -casein and α_{s2} -casein are usually intermolecular to form oligomers and dimers, respectively; albeit some of the κ -casein and α_{s2} -casein are also found as intra-molecular disulphide-bonded monomers (Rasmussen et al., 1994; Farrell, Malin, Brown & Qi, 2006; Holland et al., 2008). Disulphide bonds in monomeric κ -casein and α_{s2} -casein could be formed by the same route as for globular proteins. However the mechanism of intermolecular disulphide bond

formation of those casein proteins has not been fully established. A study into the structure and surface activity of κ -casein in monomeric and polymeric forms indicated that monomeric reduced κ -casein stabilised the aggregates of α_s - and β -casein in order to form casein micelles. Then the reduced κ -caseins underwent oxidation to form intermolecular disulphide bonds (Farrell et al., 2006). The formation of polymeric κ -casein appeared to be terminated by formation of disulphide bonds with small molecules such as glutathione (Talbot & Waugh, 1970).

2.10.4 Thiol groups (-SH) of proteins

The free thiol groups of proteins are important in the formation of intermolecular disulphide bonds between proteins during heating. β -Lactoglobulin and bovine serum albumin are the only two of the major milk proteins containing free thiol groups.

The reactivity of the thiol groups in proteins can be categorised into three types based on how readily they can react with nitroprusside ($\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}] \cdot 2\text{H}_2\text{O}$) and oxidising reagents: 1) readily reacting, 2) sluggishly reacting or 3) “masked” or buried thiol groups. Type 1 thiol groups react easily with nitroprusside and mild oxidants while type 2 reacts only with strong oxidants. The “masked” or buried thiol groups refer to thiol groups that are spatially screened by neighbouring amino acid residues and hence, inaccessible to the reagent. These thiol groups can only be detected when the proteins are completely denatured. Nevertheless these categories do not clearly distinguish thiol types because a thiol could be readily reacting with an oxidising agent after being “unmasked” (Torchinsky, 1981; Jocelyn, 1987).

In the native form, the free thiol group of β -lactoglobulin is situated in the β -structure region of the protein (Figure 2.4). Thus it is away from the disulphide bond Cys⁶⁶-Cys¹⁶⁰ and is separated from Cys¹⁰⁶-Cys¹¹⁹ by a phenyl ring (Brownlow et al., 1997). Hence the cysteine residue at Cys¹²¹ is considered to be a buried thiol and has a low reactivity in the natural state of the protein. The thiol group of bovine serum albumin is also likely to be buried in its native form as well. However, when the protein native structure is disturbed (e.g. by heat or high pressure treatments) the thiol group may be exposed and become reactive towards oxidants (Franklin & Leslie, 1968; Apenten, 1998).

The reactivity of thiol groups can be influenced by the neighbouring amino acid groups. This could either enhance or suppress the reactivity of the thiol group depending on the type of adjacent amino acids or on the interaction between thiol group and amino acid groups. For example, an experiment on the thiol group of alcohol dehydrogenase demonstrated that the thiol group can form hydrogen bonds with neighbouring imidazole groups and thus lead to an increase in electron density on the sulphur atom. As a result, the reactivity of the thiol group increases (Whitehead & Rabin, 1964). The reactivity of the thiol can also be decreased, for example a decrease in reactivity is observed when the thiol group reacts with a negatively charged ion (e.g. iodoacetate) in the presence of negatively charged

neighbouring amino acid groups. The neighbouring negatively charged amino acids inhibit the approach of the anion towards the thiol group. Hence the thiol group reactivity decreases substantially (Heitmann, 1968).

2.11 Thiol-disulphide exchange reactions

2.11.1 Thiol-disulphide exchange reactions between simple molecules

Early studies on the thiol-disulphide exchange reactions between small molecules (six carbon atoms or less including ring structures) showed that the reactions were first order with respect to the disulphide bond ($R''S-SR$) and the thiolate ion ($R'S^-$). The reaction was proposed to start with the ionization of the thiol group ($R'SH$) by a base (OH^-) to form thiolate ions that subsequently break the disulphide bonds (Fava, 1957). Subsequent studies have adopted the generic mechanism in which thiolate ion reacts with disulphide bonds (Figure 2.15) (Whitesides, Lilburn & Szajewski, 1977; Szajewski & Whitesides, 1980; Torchinsky, 1981; Whitesides, Houk & Patterson, 1983; Fernandes & Ramos, 2004; Bach, Dmitrenko & Thorpe, 2008).

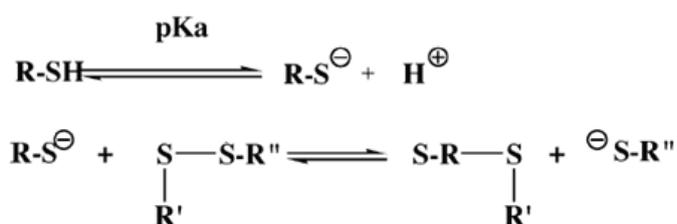


Figure 2.15: Generic reaction mechanism for the thiol-disulphide exchange reaction. Source: Bach, Dmitrenko and Thorpe (2008).

The reaction of thiolate ions with the disulphide bonds has been classified as a substitution nucleophile bimolecular mechanism (S_N2 reaction) as the thiolate ion replaces one of the two sulphurs of the disulphide bonds (Pappas, 1977; Whitesides et al., 1983; Bachrach & Mulhearn, 1996; Fernandes & Ramos, 2004; Bach et al., 2008). The S_N2 reaction was considered to be the most likely mechanism because nucleophilic substitution was found to proceed without any energy barrier and was largely exothermic (Pappas, 1977). The existence of the transition state was confirmed by Whitesides et al. (1983). The activation entropies (ΔS^\ddagger) values were found to be negative, but less negative compared to those of typical S_N2 reactions. This suggested that the reactants were stabilised by the solvent molecules, hence the reactants were more ordered than the transition state. Based on these observations, it was proposed that the charge of the transition state was delocalised and the thiol-disulphide exchange reaction followed the S_N2 reaction of thiolate anion and the disulfide bond (Whitesides et al., 1983). More recently, Fernandes and Ramos (2004) tested different attacking directions for the nucleophile using high-level theoretical calculations and determined that the

nucleophile came into the same plane as the disulphide bond. The transition state, as depicted in Figure 2.16, is a trisulphide anion in which three sulphurs are on an almost 180° plane (Fernandes & Ramos, 2004).

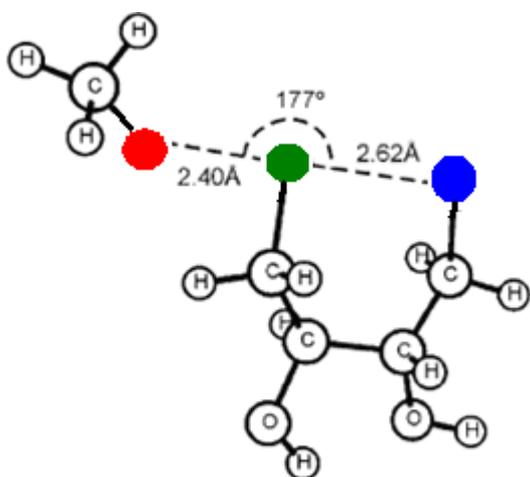


Figure 2.16: The attacking of a thiol along the plane of disulphide bond, resulting in a “straight line” trisulphide anion. Red ball, nucleophilic sulphur; Green ball, central sulphur; Blue ball, leaving sulphur. Source: Fernandes and Ramos (2004).

Studies of the atomic charge on the three sulphurs (Fernandes & Ramos, 2004) showed that the electrons were transferred from the nucleophilic sulphur (red) to the leaving sulphur (blue) without accumulating on the central sulphur (green). Knowing the distribution of charge on the transition state allows one to consider the effect of solvent on the rate of reaction. In a polar solvent, the reactants and products would be stabilised to a greater extent compared to the transition state since the interaction between polar solvent molecules and the charged thiolate ion can decrease the free energy of the thiolate ion. Thus the activation energy becomes large and the rate will be slow. On the other hand, a hydrophobic environment is ideal to accelerate the thiol-disulfide exchange reaction rate because hydrophobic solvent molecules do not affect the free energy of reactants and products like polar solvent molecules do. This could be used to explain the relation between hydrophobic interactions and disulphide bond formation on heating milk proteins. When whey proteins denature, hydrophobic sites become exposed and can come into contact on aggregation of the denatured whey proteins. This may create a hydrophobic environment, favouring the thiol-disulphide exchange reactions, leading to formation of disulphide bonds.

The thiol-disulphide exchange reaction, specifically the attacking of thiolate to the disulphide bond, was reported to follow the Brønsted relation (Whitesides et al., 1977; Szajewski & Whitesides, 1980). The rate of reaction depends on the pKa of the thiol. Since the reaction involves the attacking of the thiolate ion to the disulphide bond (Fava, 1957), the reaction rate depends on the nucleophilic

reactivity of the thiolate which in turns depends on how readily a proton is dissociated from a thiol (Whitesides et al., 1977; Szajewski & Whitesides, 1980). Studies on the relation between the pK_a and the logarithm of reaction rate (k) and the Brønsted coefficient (β) found that the thiol-disulphide exchange reaction rate was fastest when the thiol pK_a was similar in value with the pH of the medium in which the reaction occurred (Whitesides et al., 1977; Shaked, Szajewski & Whitesides, 1980; Szajewski & Whitesides, 1980).

2.11.2 Thiol-disulphide exchange reactions in protein systems

Thiol-disulphide exchange reactions in protein systems may be similar to that described for the reaction between small molecules, as depicted in Figure 2.15. However, the mechanism for this reaction in proteins has not been fully established. Based on a theoretical study, Bachrach and Mulhearn (1996) postulated that thiol-disulphide reactions for large molecules with steric constraints like proteins would most probably undergo S_N2 mechanism.

Recently, Bach et al. (2008) carried out a theoretical study on the transition state for the thiol-disulphide exchange reaction and found that the transition state structure had energy lower than the isolated reactants. In addition the transition state did not represent the coordination of disulphide bonds that were broken and formed simultaneously. Although they confirmed there was a trisulphide anion in which the charge was distributed evenly across three sulphur atoms (Figure 2.16), the observation that the energy was lower than the reactants suggested that S_N2 may not be the mechanism. Instead it was suggested that the mechanism was an addition-elimination process with a stable intermediate (Bach et al., 2008).

The rate of reactions involving thiol reagents and disulphide bonds of macro-molecules (i.e. proteins and enzymes) was first order for both the thiol reagent and the disulphide bonds (Shaked et al., 1980), as was also observed in reactions between small molecules (Fava, 1957). The thiol-disulphide exchange reactions between macro-molecules were also found to follow the Brønsted relation (Shaked et al., 1980; Bulaj, Kortemme & Goldenberg, 1998). In protein systems, while the pK_a still plays an important role in determining the rate of the thiol-disulfide exchange reaction (Shaked et al., 1980; Singh & Whitesides, 1991; Bulaj et al., 1998; DeCollo & Lees, 2001), factors such as steric and electrostatic effects also influence the reaction rate (Whitesides et al., 1977). An example of a steric effect is the reactions between proteins and glutathione. These reactions were found to be slower than that predicted by the Brønsted relation because glutathione is a bulky molecule, making it more difficult to access the disulphide bonds on the proteins (Shaked et al., 1980). The importance of electrostatic effects was shown in the two following examples. First, the amino acid groups adjacent to the cysteine residues could change the pK_a of the nucleophilic thiol group. For example, if the neighbouring amino acids are positively charged, they will help in stabilizing the thiolate ion, hence

deprotonation is favoured (i.e. pK_a will decrease). Secondly, the neighbouring amino acid groups of disulphide bonds can affect the rate of the reaction. For example, positively charged neighbouring amino acid groups promote the approach of the negatively charged thiolate ion whereas negatively charged neighbouring amino acid groups inhibit it. In addition, negatively charged neighbouring groups may not result in the release of the central sulphur (Shaked et al., 1980; Bulaj et al., 1998; Jensen, Hansen & Winther, 2009).

In conclusion the thiol-disulphide exchange reaction in protein systems is more complex than for reactions between small molecules, even though the kinetics follows the Brønsted relation in a similar manner as for small molecules. As proteins are large molecules containing many amino acid groups with highly ordered structures, the rate of reaction will be affected by the pK_a of sulphur atoms involved as well as electrostatic effects, steric effects and the properties of newly formed disulphide bonds.

2.12 Controlling the thiol-disulphide exchange reactions

The kinetics of the thiol-disulphide exchange reaction between proteins is understood; hence this reaction can possibly be controlled, either by inhibiting or enhancing the reaction. As the thiol-disulphide exchange reactions are first order for both disulphide bonds and thiol groups, the research presented in this thesis attempted to control the ratio of thiol groups to disulphide bonds in the milk systems, thus altering the ratio of reagents available to participate in the reaction. Two possible means of controlling include: 1) reducing the disulphide bonds to increase the number of thiol groups in the system and concomitantly decrease the number of disulphide bonds; 2) blocking the thiol groups in order to decrease the available thiol groups in the system.

2.12.1 Thiol blocking reagents

Thiol blocking reagents have often been used to determine the importance of the thiol groups in the formation of aggregates via thiol-disulphide exchange reactions during heating whey-protein-containing solutions. By reacting with the thiol blocking reagents, the protein thiol groups became unavailable for the thiol-disulphide exchange reactions. The most commonly used reagent is N-ethylmaleimide (NEM) (Zittle et al., 1962; Sawyer et al., 1963; Purkayastha et al., 1967; Sawyer, 1967; Morr & Josephson, 1968; Harwalkar, 1986; Hashizume & Sato, 1988; Shimada & Cheftel, 1988; Xiong, Dawson & Wan, 1993; Goddard, 1996; Hoffmann & van Mil, 1997). Alternatively, reagents such as hydrogen peroxides, iodoacetamide and 5-5'-dithiobis-(2-nitrobenzoic acid) have also been used (Hashizume & Sato, 1988; Iametti et al., 1996; Manderson, Hardman & Creamer, 1999; Özer, Grandison, Robinson & Atamer, 2003; Creamer et al., 2004).

2.12.2 The effects of thiol blocking reagents on the heat denaturation of thiol-containing whey proteins

When β -lactoglobulin in buffer solution was treated with thiol blocking reagents, the protein became more susceptible to reversible and irreversible heat denaturation (Xiong et al., 1993; Hoffmann & van Mil, 1997; Burova, Choiset, Tran & Haertle, 1998; Jayat et al., 2004). Differential scanning calorimetric studies demonstrated that β -lactoglobulin with blocked thiols unfolded at lower temperatures compared to untreated β -lactoglobulin (Xiong et al., 1993; Hoffmann & van Mil, 1997; Burova et al., 1998). Figure 2.17 showed that the transition temperature decreased with increasing levels of thiol-blocking reagent.

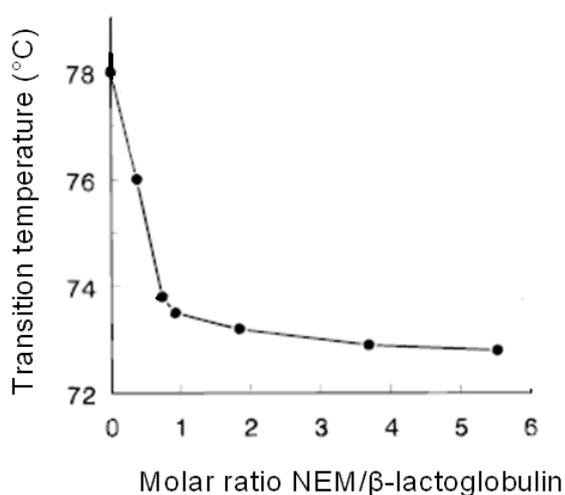


Figure 2.17: Effects of NEM/ β -lactoglobulin molar ratio on the temperatures at which β -lactoglobulin unfolded. Source: Hoffmann and van Mil (1997).

2.12.3 The effects of thiol blocking reagents on the interactions between proteins upon heat treatment

In model protein systems, when the thiol groups of β -lactoglobulin were blocked by NEM, heat-induced aggregates were still formed but did not contain any intermolecular disulphide bonds. Instead the aggregates were formed by non-covalent interactions between the proteins (Sawyer, 1967; Xiong et al., 1993; Hoffmann & van Mil, 1997; Havea, Watkinson & Kuhn-Sherlock, 2009). The formation of non-covalent aggregates was especially enhanced when thiol-blocked β -lactoglobulin was heated at low pH (Hoffmann & van Mil, 1997; Mounsey & O'Kennedy, 2007).

When concentrated whey protein solutions containing thiol-blocking reagents were heated at sufficiently high temperatures and time, heat-induced gels were formed and these contained non-covalent interactions between the proteins. It was shown that the hardness (examined by compression

tests) of the gels formed from treated solutions were lower than that of control protein gels whereas, the storage modulus (G') values of gels made from treated solutions were higher than that of control gels (Matsudomi, Rector & Kinsella, 1991; Havea et al., 2009). It was concluded that intermolecular disulphide bonds were not formed in the heat-induced gels made from solutions containing thiol blocking reagents. Instead the degree of intermolecular non-covalent interactions increased markedly compared to gels made from protein solutions without thiol blocking reagent (Matsudomi et al., 1991; Havea et al., 2009).

When milk was treated with thiol blocking reagents then subsequently heated at $\text{pH} \leq 5.5$, the gel hardness (measured by penetration tests) of the resulting acid-heat-induced gels decreased with increasing concentrations of the thiol blocking reagents (Figure 2.18) (Hashizume & Sato, 1988). Interestingly, Goddard (1996) found that the gel hardness decreased progressively with the increase of NEM concentrations up to a certain level, after which the gel hardness was very low and independent of NEM concentrations (Figure 2.18B). Like in model systems, the decrease in hardness of acid-heat induced milk gels was the result of thiol blocking reagents inhibiting the formation of intermolecular disulphide bonds.

The storage modulus values (G') of acid-heat-induced gels prepared from NEM-treated milk were reported to be lower than those of control gels (Goddard, 1996). Since disulphide-bonds contributed to the elasticity of gels (represented by the storage modulus, Shimada & Cheftel, 1989), the low storage modulus values indicated that disulphide bonds were not the main factor in the formation of the acid-heat-induced NEM-treated gels.

Furthermore, decreasing the amount of thiol groups in milk also affected the rheological properties of the resulting yoghurts. The complex modulus (G^*) of yoghurts prepared from milks treated with hydrogen peroxide were lower whereas the $\tan \delta$ values (G''/G') were higher than those of yoghurts made from control milk (Özer et al., 2003). This indicated that the storage modulus values of yoghurt made from hydrogen peroxide-treated milk were lower than that made from control milk. Thus the result was consistent with previous studies (Hashizume & Sato, 1988; Goddard, 1996) and suggested that disulphide bonds may not be required for the formation of protein gels but were required to strengthen the gel network structure.

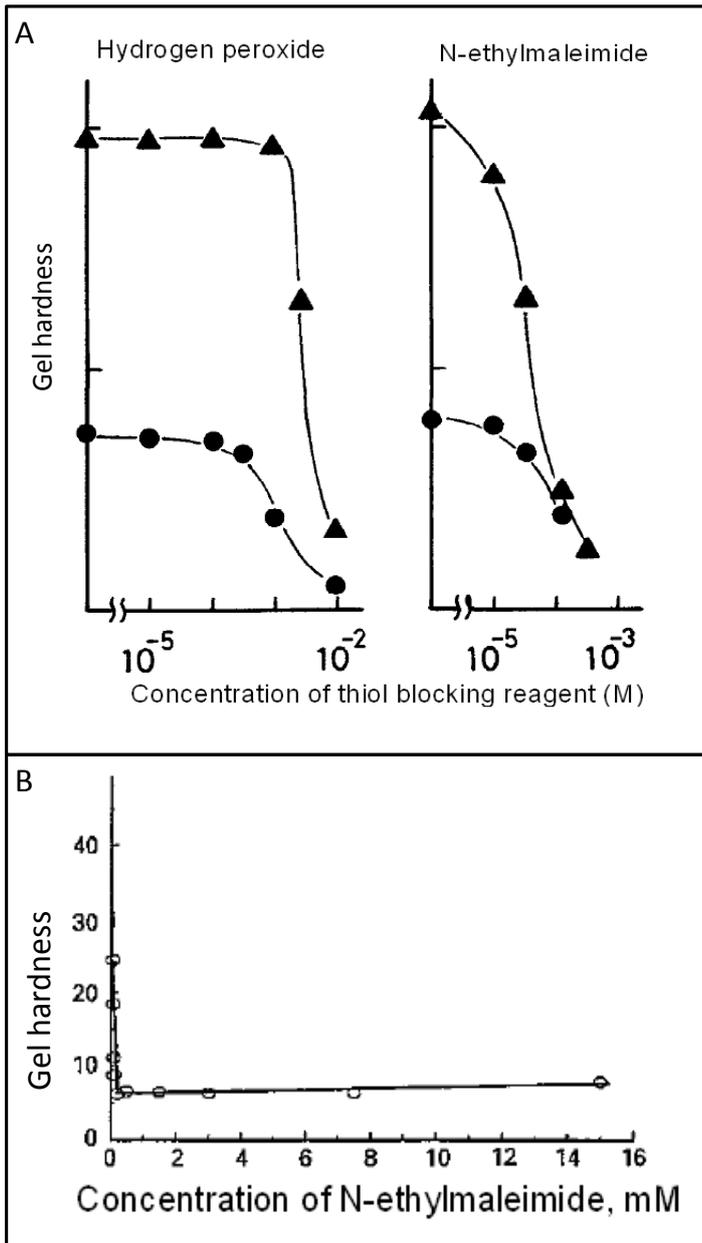


Figure 2.18: The effects of thiol blocking reagents on the gel hardness of acid-heat-induced milk gels. A, results of Hashizume and Sato (1988), skim milk can be unheated (\blacktriangle) or heated at 80 °C for 30 min (\bullet) prior to adding thiol blocking reagents, gels were formed by heating glucono- δ -lactone (GDL)-treated milk at 80 °C for 60 min. B, results of Goddard (1996), gels were formed by incubating skim milk (250 g solids/L) with GDL overnight (25 °C) then heating at 60 °C for 1 h followed by cooling milk to 25 °C for 1 h.

2.12.4 Disulphide-bond reducing agents

Disulphide-bond reducing agents have been used extensively to examine whether or not heat-induced aggregates contained intermolecular disulphide bonds. The most commonly used disulphide reducing agents are β -mercaptoethanol and dithiothreitol (DTT). DTT is considered to be a stronger reducing

agent with a significantly higher equilibrium constant compared to β -mercaptoethanol (1.3×10^4 M versus 1 M) (Hansen & Winther, 2009), probably because the formation of oxidised DTT is driven by favourable steric and entropic effects (Cleland, 1964; Jensen et al., 2009). However thiols of β -mercaptoethanol and DTT have similar pK_a values, 9.5 and 9.2/10.1 (DTT has two thiol groups), respectively (Jocelyn, 1987), thus the thiol-disulphide exchange reaction between protein disulphide bonds and either β -mercaptoethanol or DTT should be chemically similar.

The reduction of disulphide bonds in milk is complex because there are several proteins that contain disulphide bonds. β -Lactoglobulin, α -lactalbumin and bovine serum albumin have two, four and 17 intra-molecular disulphide bonds, respectively. Amongst the minor whey proteins, lactoferrin and the immunoglobulins also contain intra-molecular disulphide bonds. κ -Caseins are present as intermolecular disulphide-linked oligomers and α_{s2} -casein can be found in disulphide-linked dimeric forms as well as a monomer with an intra-molecular disulphide bond. Researchers often used an amount of disulphide bond reducing agent that far exceeded the level required to completely reduce the disulphide bonds existing in protein solutions. However there are a few studies that used lower concentrations of reducing agents. The following sections summarise the results of research with both excess and lower levels of disulphide-bond reducing agents.

2.12.5 The effects of disulphide-bond reducing agents on properties of proteins and the heat stability of protein systems

The reduction of disulphide bonds has been studied in a whey protein-free milk (WPF milk) system in order to examine how the reduction of κ -casein disulphide bonds affected the heat stability of the WPF milk (Aoki & Kako, 1984; Singh & Fox, 1987). The heat coagulation time of the WPF milk decreased substantially with the increase of β -mercaptoethanol concentrations (Aoki & Kako, 1984). In addition, the levels of soluble caseins (caseins that were not sedimented out during ultracentrifugation of the heated WPF milk) increased with the increase of β -mercaptoethanol concentrations (Aoki & Kako, 1984; Singh & Fox, 1987). Even though the two studies used different techniques (e.g. centrifuging condition) on WPF milks of different concentrations, the results are consistent between the studies. Both studies found that as κ -casein was reduced by β -mercaptoethanol, this weakened the intermolecular interactions between κ -caseins and other casein proteins. This decreased the stability of casein micelles and promoted the formation of soluble casein when the WPF milks were heated at $> 100^\circ\text{C}$ (Aoki & Kako, 1984; Singh & Fox, 1987).

The reduction of the disulphide bonds in κ -casein also affected the heat stability of milk systems (Singh & Fox, 1987). It was shown that heating milk with added β -mercaptoethanol resulted in the dissociation of κ -casein in a similar manner as heating milk at high pH and temperatures. Hence it was suggested that the reduction of disulphide bonds of κ -casein caused the κ -casein to bind less strongly

to other caseins in the micelles (Singh & Fox, 1987), which agreed with the studies on model systems (Aoki & Kako, 1984; Singh & Fox, 1987).

2.12.6 The effects of disulphide-bond reducing agents on the protein interactions

In β -lactoglobulin systems, the addition of low concentrations of disulphide reducing reagents increased the hardness (measured by penetration tests) of heat-induced gels but higher levels of disulphide reducing agents resulted in a pronounced decrease in gel hardness (Matsudomi et al., 1991). In another study, Wada and Kitabatake (2000, 2001) showed that addition of low levels of disulphide reducing agent into a thiol-blocked β -lactoglobulin solution generated thiol groups. The newly formed thiol groups initiated thiol-disulphide exchange reactions, forming disulphide-linked polymers. However adding excess levels of disulphide reducing agents reduced all the disulphide bonds, hence disulphide-linked polymers could not be formed (Wada & Kitabatake, 2001). These studies demonstrated that while thiol-disulphide exchange reactions can be inhibited by complete reduction of all the existing disulphide bonds, the reactions can be enhanced by controlling the ratio of thiol groups to disulphide bonds. In contrast, Havea et al. (2004; 2009) found that both the hardness and the storage modulus of heat-induced gels made from whey protein solutions with extensive reduction were higher than those of heat-induced control gels. The authors proposed that while disulphide formation was inhibited, non-covalent interactions between the proteins with exposing hydrophobic regions can be greatly enhanced.

In milk systems, there are some reports showing that milk treated with reducing agent formed a weaker (low hardness) acid-heat-induced gel than milk without reducing agent (Figure 2.19A) (Hashizume & Sato, 1988) whereas, in other reports, addition of disulphide-bond reducing agent resulted in harder acid-heat-induced milk gels compared to control gels (Figure 2.19B) (Goddard, 1996; Surel & Famelart, 2003). In early studies, it was proposed that thiol-disulphide exchange reactions were inhibited due to the reduction of disulphide bonds, regardless of the concentrations of disulphide reducing agent (Hashizume & Sato, 1988). In contrast, in the later studies, it was proposed that low concentrations of disulphide-bond reducing agents may have resulted in an increased number of available thiol groups. This in turn promoted formation of intermolecular disulphide bonds (Goddard, 1996; Surel & Famelart, 2003). Hence, an increase in gel hardness and storage modulus was observed (Goddard, 1996). However, further addition of reducing agents caused complete reduction of all disulphide bonds, thus preventing thiol-disulphide exchange reactions and causing the gel hardness to decrease slightly (Figure 2.19B). Nevertheless, Surel and Famelart (2003) indicated that despite the lack of disulphide bonds, non-covalent interactions are still important in determining the hardness of the acid-heat-induced gels. Hence the gels made from treated milks were still harder than those made from control milks (Goddard, 1996; Surel & Famelart, 2003).

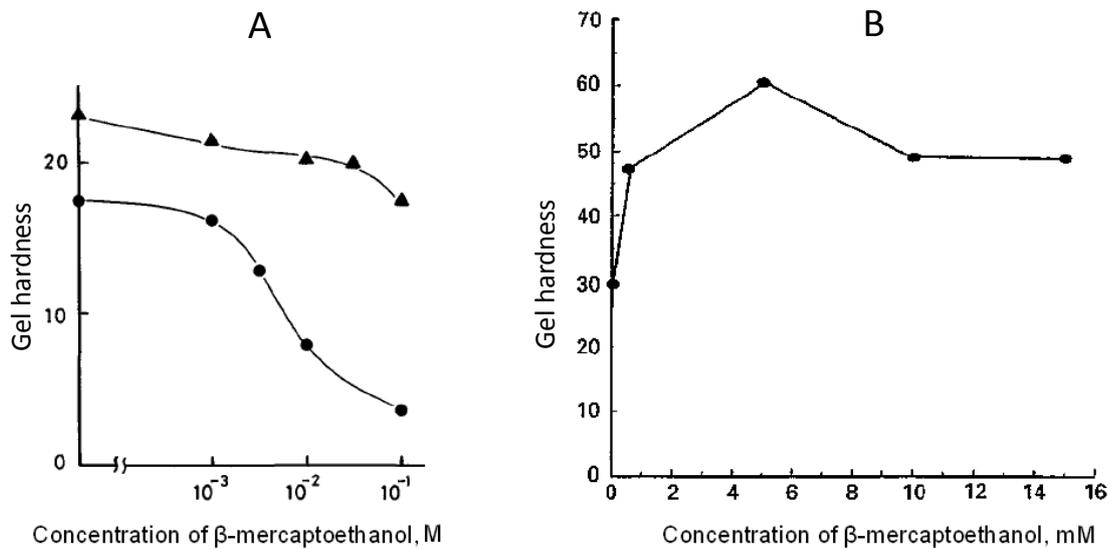


Figure 2.19: The effect of β -ME concentrations on the gel hardness. **A**, results of Hashizume and Sato (1988), milk can be unheated (\blacktriangle) or heated (\bullet) prior to adding disulphide reducing agents. **B**, results of Goddard (1996).

2.13 Acid gelation of milk

Acid milk gels are a model for set yoghurts, which are produced by acidifying the milk. During acidification, the pH of the system is decreased until the isoelectric point of the casein is approached. This leads to neutralising of the charge and the collapse of the κ -casein hairy layer on the casein micelles. Consequently, the casein micelles lose their stability and aggregate, developing chains and clusters that link together to form a network structure (De Kruif, 1997; Lucey & Singh, 1998). The work reported in this study intends to use the acid milk gel as a model dairy application to examine the effect and importance of protein interactions on the rheological and structural properties of dairy products.

2.13.1 The effects of heat-induced protein interactions on the properties of acid milk gels

The acid gels made from pre-heated milk have a higher storage modulus than those made from unheated milk (Figure 2.20, Davies et al., 1978; Mottar et al., 1989; Lucey, Teo, Munro & Singh, 1997; Lucey & Singh, 1998; Lucey, Tamehana, Singh & Munro, 1998). Heating milk results in the denaturation of whey protein (see Section 2.4) and formation of whey protein- κ -casein aggregates that can be located in both the colloidal and serum phases of the milk (see Sections 2.7 – 2.9). It has been shown that the firmness of the acid gels increased when the level of denatured whey protein in heated milk increased (Dannenberg & Kessler, 1988c; Anema, Lee, Lowe & Klostermeyer, 2004b).

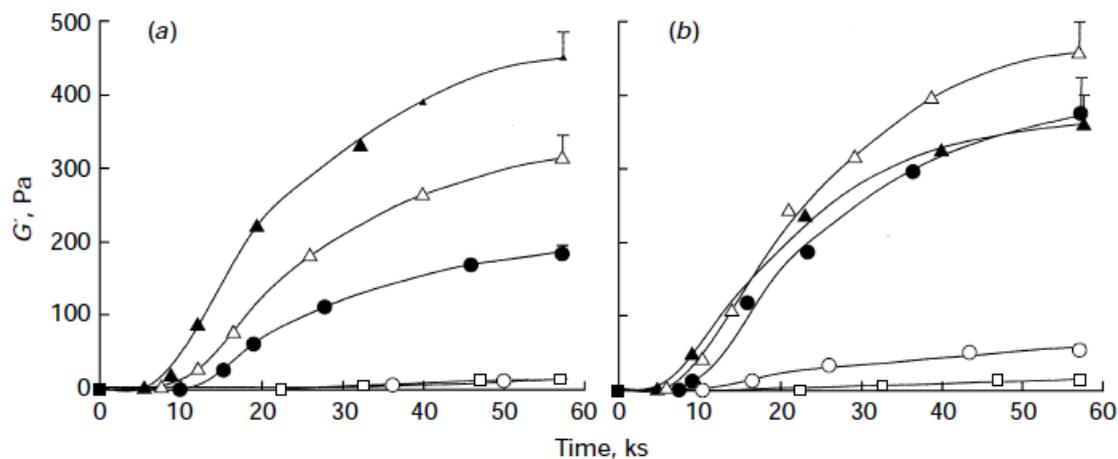


Figure 2.20: The change of G' over time during acidification of skim milk with GDL at 30 °C. Milks were heated for (a) 15 or (b) 30 min at, ○, 75; ●, 80; △, 85; ▲, 90 °C. □, Unheated skim milk. Source: Lucey (1997).

Moreover, disulphide interactions between denatured whey protein and κ -casein were proposed to be an important factor in producing a firm gel. Lucey (1998) found that the storage modulus values of acid gels made from heated milk decreased with added thiol blocking reagents (Figure 2.21).

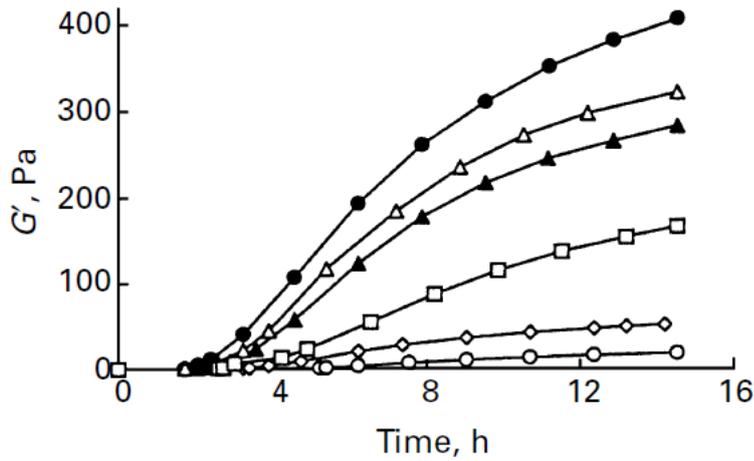


Figure 2.21: The change of G' over time during acidification of NEM-treated skim milk. Acid milk gels made at 30 °C with GDL. Gels were made from ○, unheated reconstituted skim milk and reconstituted milks with NEM at ●, 0; △ 5; ▲, 10; and □, 20 mM added prior to heating milk at 80 °C for 30 min. ◇, Fresh milk with 20 mM NEM added prior to heating milk. Source: Lucey (1998).

As acid gelation of heated milk also involved denatured whey proteins, the resulting gel network was assumed to have a high level of inter-connections with a greater number of participating proteins (Lucey et al., 1998; Schorsch, Wilkins, Jones & Norton, 2001; Anema et al., 2004b; Famelart, Tomazewski, Piot & Pezennec, 2004). The confocal microscopic images of the acid gels made from unheated and heated milks illustrated that the gel network of heated milk was more cross-linked and denser than that of unheated milk (Figure 2.22).

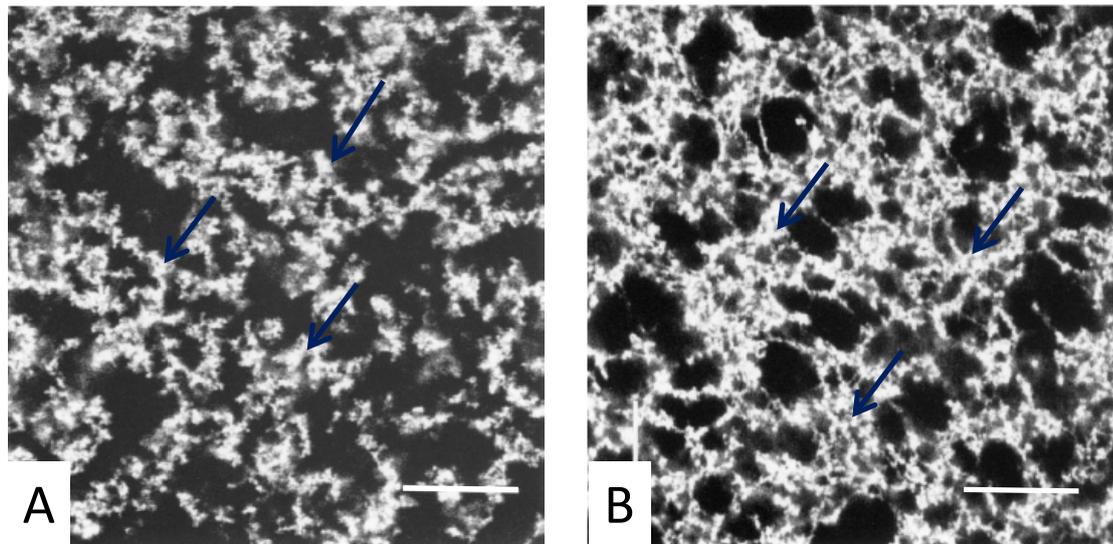


Figure 2.22: Confocal scanning laser micrographs of acid milk gels made at 30 °C by acidifying milks with 1.3% GDL. A, unheated milk and B, heated milk (80 °C for 30 min). Blue arrows point to milk proteins, scale bar: 20 μ m. Source: Lucey (1999b).

In addition, denatured whey proteins that associated with casein micelles contribute to the early gelation (i.e. the gel is formed at higher pH values) of heated milk compared with gelation of unheated milk (Figure 2.20, Lucey et al., 1998; Vasbinder, van Milk, Bot & de Kruif, 2001; Anema, Lowe & Lee, 2004a; Famelart et al., 2004; Vasbinder, Van de Velde & De Kruif, 2004). It has been proposed that the heat-induced complexes of caseins with denatured whey proteins aggregate at a higher pH than the casein micelles since whey proteins had higher isoelectric points than casein micelles (pI of β -lactoglobulin was ~ 5.3 and pI of casein micelles was ~ 4.6). This accounted for the increase in the gelation pH when heated skim milk was acidified (Lucey et al., 1998; Alting, De Jongh, Visschers & Simons, 2002; Anema et al., 2004a; Vasbinder et al., 2004). Others have proposed that the association of the denatured whey proteins on the surface of the casein micelles increased the hydrophobicity of the micelles. Consequently, the particles may begin to associate at pH values higher than their isoelectric points (Famelart et al., 2004; Jean, Renan, Famelart & Guyomarc'h, 2006; Guyomarc'h, Renan, Chatriot, Gamarre & Famelart, 2007).

While the interactions between denatured whey proteins and casein micelles are well-known to influence the rheological properties of the acid milk gels, it should be noted that factors such as pH at heating, the location of the heat-induced aggregates (either in the serum or in the colloidal phase), size of the aggregates and the concentration of the protein can also determine the acid gel properties as well because they are related to the protein interactions (see Section 2.7.3) (Mottar et al., 1989; Lucey, Singh & Munro, 1999a; Puvanenthiran, Williams & Augustin, 2002; Anema et al., 2004b; Anema et al., 2004a; Famelart et al., 2004; Vasbinder et al., 2004; Lakemond & van Vliet, 2008a; Donato & Guyomarc'h, 2009; Morand, Guyomarc'h & Famelart, 2011).

2.13.2 The effects of protein interactions during gelation on the properties of acid milk gels

The interactions between proteins after heating and subsequent cooling, and especially during gelation have been seldom reported. A few reports showed that when thiol blocking reagents were added to heated milks before acidification, the resulting acid milk gels had lower storage modulus values as well as lower hardness than those made from heated control milk (Figure 2.23, Lucey et al., 1998; Vasbinder, Alting, Visschers & de Kruif, 2003; Lakemond & van Vliet, 2008b). Hence it was proposed that the formation of disulphide bonds during acid gelation of milk were as important as those formed during the heating of milk in determining the rheological properties of acid milk gels (Lucey et al., 1998; Vasbinder et al., 2003).

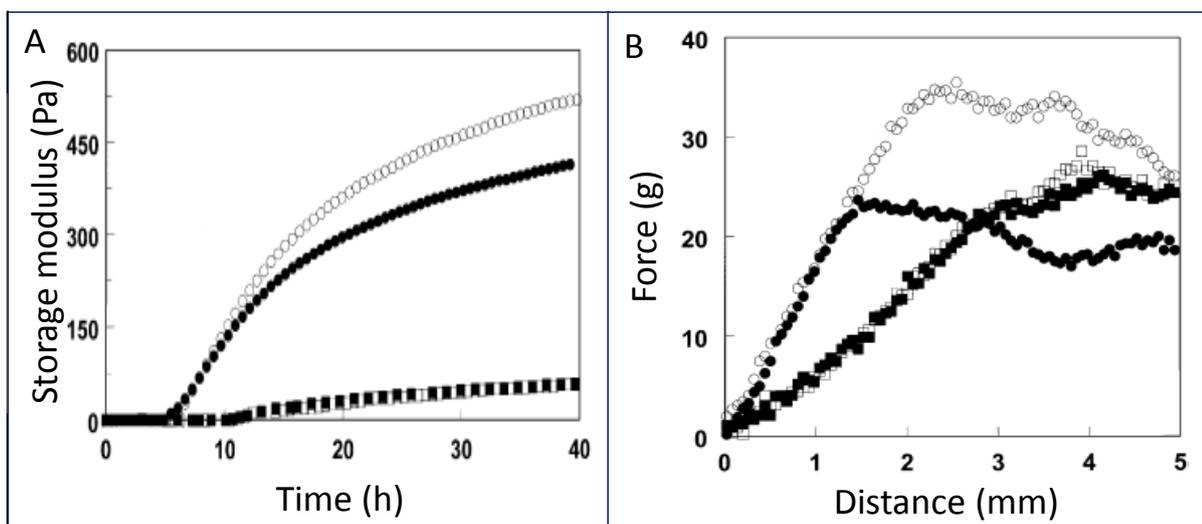


Figure 2.23: Effect of thiol blocking reagents on A, the storage modulus and B, the large deformation properties (i.e. gel hardness) of acid gels. Acid gels were prepared from unheated (■□) and heated (●○) milks in the absence (open symbols) and presence of NEM (closed symbols). Source: Vasbinder (2003).

2.14 Conclusions

On heating milk, intermolecular disulphide and non-covalent interactions are formed between denatured whey proteins and κ -casein. The formation of intermolecular disulphide bonds via the thiol-disulphide exchange reactions involves a free thiol group from one protein, which is initially mainly sourced from β -lactoglobulin, and a disulphide bond from another protein. The non-covalent interactions can be hydrophobic, hydrogen, ionic and van der Waals. When the formation of disulphide bonds between the proteins is completely inhibited during heating by adding excess quantity of thiol reagents (disulphide reducing and thiol blocking reagents), non-covalent interactions are reported to become predominant in the system.

The importance of the heat-induced interactions between the proteins on the functional properties of milk based products has been widely reported in the literature. It was often concluded that intermolecular disulphide bonds greatly influenced the functional properties of acid milk gels. When intermolecular disulphide bond formation was prevented on heating milk, the resulting acid gels were markedly weaker than gels prepared from heated milk. Nevertheless, these gels were still stronger than those prepared from unheated milks due to the presence of non-covalent interactions between the proteins.

Previous studies tended to use a large excess of thiol reagents to investigate the effects of inhibiting or enhancing disulphide bond formation on the protein interactions. In addition, most research was carried out on model systems or whey protein systems. Reports on the effects of adding thiol reagents systematically to skim milk on the functional properties of milk products are scarce. Hence this research was carried out to investigate the effects of changing the balance between disulphide and non-covalent interactions on the protein aggregation and the rheological properties of milk products. Ultimately, the aim was to understand the importance of each interaction type (disulphide and non-covalent) on the protein aggregation and the functional properties of milk products.

Chapter 3 - Materials and methods

The work in this thesis has been divided into two main streams: (1) addition of a disulphide-bond reducing agent (β -mercaptoethanol) to promote thiol-disulphide exchange reactions and (2) addition of a thiol blocking reagent (N-ethylmaleimide, NEM) to inhibit the exchange reactions. For each reagent, the experiments were carried out as described below:

- The reagents were added to (a) unheated milks, (b) heated milks and (c) unheated milks that were subsequently heated.
- For treated samples, the percentage of proteins in the serum or colloidal phase and the percentage proteins participating in disulphide bonds of the total protein present in milk were determined using gel electrophoretic techniques.
- The size of the casein micelles in the treated samples was examined.
- The treated samples were acidified to form acid gels of which the rheological and microstructural properties were investigated.

β -Mercaptoethanol and NEM were the chosen reagents because they are commonly used in other studies. The materials and techniques used for analysis in this research are described below.

3.1 Materials

3.1.1 Reconstituted skim milk solutions

Reconstituted skim milk samples of 10% total solids (w/w) were prepared by adding the appropriate quantity of low-heat skim milk powder (Fonterra Co-operative Group, New Zealand) to Milli-Q water (UltraPur water system, Australia; resistivity $\geq 18 \text{ M}\Omega \text{ cm}^{-1}$). The milk samples were stirred for at least 6 h at room temperature and were then kept in the cold room at $4 \pm 0.5 \text{ }^\circ\text{C}$ before further use. Reconstituted skim milk was used within four days after preparation. Skim milk at $20 \pm 1 \text{ }^\circ\text{C}$ had pH of ~ 6.7 . This was considered to have similar composition and concentration to that of fresh skim milk.

Whey protein enriched skim milk was prepared by adding whey protein isolate (WPI 895, Fonterra Co-operative Group, New Zealand) to reconstituted skim milk. Addition of 1.15 g of whey protein isolate to 100 g of skim milk raised the protein concentration in skim milk from ~ 3.5 to $\sim 4.5\%$.

A small quantity of sodium azide ($\sim 0.01\%$ w/v) was added to the milk samples as a preservative.

3.1.2 Fresh skim milk

Fresh skim milk was obtained from the commercial Anchor Trim milk (Fonterra Co-operative Group, New Zealand). The freshly collected milk was skimmed, pasteurized at ~ 72 °C for 16 s then chilled at 4.0 ± 0.5 °C. The fat content in Anchor Trim milk is less than 0.1% and its pH was ~ 6.65 at 20°C. Sodium azide ($\sim 0.01\%$ w/v) was also added to the fresh skim milk as a preservative. The milk was used within four days from the day the milk was taken from the commercial bottle.

3.1.3 Protein solutions

κ -Casein was purchased from Sigma (St Louis, MO, USA) and β -lactoglobulin was isolated from fresh bovine whole milk using the method described by Manderson, Hardman & Creamer (1998). The β -lactoglobulin preparation was essentially 100% pure on a protein basis; however, the κ -casein was estimated to be about 85% pure with contaminants of α_{s1} -casein ($\sim 6\%$ w/w) and β -casein ($\sim 5\%$) as well as small amounts of peptide material. As the major impurities do not contain disulphide bonds, they were not expected to influence the results of this study. In solution, κ -casein exists as polymers.

κ -Casein or β -lactoglobulin solutions were prepared by dissolving 25 mg of each protein with 5 mL of Milli-Q water. To prepare a mixed solution of β -lactoglobulin and κ -casein, individual β -lactoglobulin and κ -casein solutions were made up first by dissolving 50 mg of the protein with 5 mL of Milli-Q water. Then the two individual solutions were mixed together at a ratio of 1:1.

The protein solutions were stirred for ~ 2 h at ambient temperature and were adjusted to pH 7.0 ± 0.2 with 0.1 M NaOH. They were used within 8 h of preparation. The concentration of the protein solutions was estimated using ultraviolet-spectrophotometry and known extinction coefficients; the technique will be described in detail in Section 3.2. Table 3.1 summarises the concentrations of the protein solutions that were used in the experiments that are described in Chapter 8.

Table 3.1: The estimated concentrations of the protein solutions that were used in the experiments described in Chapter 8.

	Individual solution		Mixed solution	
	mg mL ⁻¹	mol mL ⁻¹	mg mL ⁻¹	mol mL ⁻¹
κ-Casein	4.5 ± 0.2	~2.4×10 ⁻⁷	4.4 ± 0.1	~2.3×10 ⁻⁷
β-Lactoglobulin	4.2 ± 0.3	~2.3×10 ⁻⁷	4.1 ± 0.2	~2.2×10 ⁻⁷

3.1.4 Chemicals

Below is the list of key chemicals that were used for this research and their suppliers.

Table 3.2: The list of chemicals and their supplier.

Supplier	Chemicals
Bio-Rd Laboratories, Hercules, CA, U.S.A.	Acrylamide Ammonium persulphate (APS) Tetramethylethylenediamine (TEMED)
Fisher scientific UK, Leics, U.K.	Propan-2-ol Sodium dodecyl sulphate (SDS)
Merck KGaA, Damstadt, Germany	Amido black Glacial acetic acid Glycine Tris (hydroxymethyl) methylamine (Tris-base)
Sigma – Aldrich Co., St Louis, MO, U.S.A.	β-Mercaptoethanol Glucono-δ-lactone (GDL) N-ethylmaleimide (NEM)

3.2 Ultraviolet (UV)-spectrophotometry

The concentration of the pure protein solutions was measured using a Jasco V-560 UV/Vis Spectrometer (Jasco Corporation, Tokyo, Japan). The protein solutions were diluted 10 fold (10 μL of the protein solution with 90 μL of Milli-Q water). The diluted sample was inserted in a quartz cell that was held in the cell-holder of the UV/Vis spectrometer. Then the absorbance of the proteins at wavelength of 280 nm was measured as the proteins in solution absorb ultraviolet light with absorbance maxima at 280 nm. The absorbance of protein at 280 nm is

mainly due to the amino acids tryptophan, tyrosine and cysteine. The absorption coefficient is specific for each protein depending on the relative concentrations of these three amino acids. The absorbance at 320 nm was also measured as the background scattering. The concentration of the protein can be calculated using Equation 3.1.

$\text{Concentration} = \frac{A_{280} - (A_{320} \times 1.7)}{\text{Absorptivity}} \times \text{Dilution factor} \times 10$	Equation 3.1
<p>$A_{280/320}$ = absorbance of working solutions read against Milli-Q blank at 280 or 320 nm</p> <p>The 1.7 factor corrects for the wavelength dependence of the scattering of particles at 280 and 320 nm.</p> <p>Absorptivity of β-lactoglobulin and κ-casein was 9.4 and 9.5 mm² mg⁻¹, respectively (Fox, 1982)</p> <p>10 = for 10% solution</p>	

3.3 Heat treatment of milk samples

Six millilitre aliquots of milk were transferred to 8 mL clear glass vials with rubber lined screw caps (Wheaton, Biolab, U.S.A.) and then heated at 80 ± 0.1 °C for 30 min in thermostatically controlled silicone-oil bath. It took ~ 2 min for the sample to reach 80 °C from 5 °C. During heating, the samples were held horizontally in a rack that was rocked also horizontally at 20 cycles per minute. After heating, the sample vials were rapidly cooled (~ 2 min) to below 20 °C using cold tap water.

3.4 Centrifugation of milk samples

Milk samples (1 mL) were pipetted into 1.5 mL Eppendorf tubes and were centrifuged at 21,000 *g*, 25 °C for 60 min using a bench centrifuge (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany) to separate the colloidal (pellet) and the serum (supernatant) proteins. The supernatant was separated from the pellets by carefully pouring the liquid out of the Eppendorf tubes. The protein composition of the original milks and their supernatants was then determined by electrophoresis as described in Section 3.6.

3.5 Preparing samples to measure the protein denaturation

Sodium acetate buffer (0.2 M, pH 3.95) was prepared by dissolving 16.4 g of sodium acetate powder in 600 mL Milli-Q water using a magnetic stirrer. Once the powder was completely dissolved, the pH of the solution was adjusted to pH 3.95 using 6 M HCl. The volume was then made up to 1 L in a volumetric flask and the buffer was stored at 20 °C.

Milk samples were mixed with sodium acetate buffer at ratio of 1:1 to bring the pH of milk to pH 4.6. This caused the acid precipitation of casein proteins and denatured whey proteins.

The mixture was then centrifuged as described in Section 3.4. The supernatant was carefully separated from the pellets and the level of proteins remaining in the supernatants (which were considered to be native whey proteins) was determined by electrophoresis as described in Section 3.6.

3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis is a widely used technique in various fields involving proteins (e.g. biochemistry, genetics etc.). This technique is used to separate proteins according to their electrophoretic mobility, which is a function of the length of a polypeptide chain and its charge. SDS-PAGE, in particular, employed SDS (an anionic detergent) to disrupt the hydrophobic and hydrogen bonds in the proteins while inserting negative charge to the protein (Andrews, 1990). This means that all the proteins in the SDS-environment retain only their primary structure and have a large negative charge with a constant charge-to-mass ratio. Hence, in SDS-PAGE, the proteins are separated based on their molecular mass.

SDS-PAGE was performed using a Bio-Rad mini-gel slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, U.S.A) following the method of (Laemmli, 1970) as described by Manderson et al. (1998) and Havea, Singh, Creamer & Campanella (1998).

3.6.1 Preparation of stock solutions for SDS-PAGE

- **30% Acrylamide solution:** Thirty grams of a mixture of 37.5:1 of acrylamide and N,N'-methylene-bis-acrylamide were added to approximately 60 mL of Milli-Q water. The sample was stirred until completely dissolved. The sample was transferred to a volumetric flask and the volume was brought up to 100 mL with Milli-Q water. The solution was stored in an amber bottle at 4 °C for up to one month.
- **10% (w/v) APS:** APS (100 mg) was dissolved in 1.0 mL of Milli-Q water. The solution was prepared fresh each day.
- **10% (w/v) SDS:** SDS (10 g) was dissolved in Milli-Q water with gentle stirring and the volume was made up to 100 mL in a volumetric flask. The solution was kept at room temperature.

- **Bromophenol Blue 0.4% (w/v):** Bromophenol blue (1.6 g) was dissolved in approximately 7 mL of 0.1 M NaOH. The volume was made up to 400 mL with Milli-Q water in a measuring cylinder. The solution was kept at room temperature.
- **Resolving buffer (1.5 M Tris-HCl buffer):** Tris-base (18.15 g) was dissolved in approximately 60 mL of Milli-Q water. The pH was adjusted to 8.8 using 6 M HCl. The volume was made up to 100 mL in a volumetric flask. The buffer was stored at 4 °C for up to one month.
- **Stacking buffer (0.5 M Tris-HCl buffer):** Tris-base (6.0 g) was dissolved in approximately 60 mL of Milli-Q water in a beaker. The pH was adjusted to 6.8 using 6 M HCl. The volume was made up to 100 ml in a volumetric flask and the buffer was stored at 4 °C for up to one month.
- **SDS Sample buffer:** The buffer was prepared by combining 500 mL of Milli-Q water, 125 mL of 0.5 M Tris-HCl buffer (pH 6.8), 100 mL glycerol, 200 mL of 10% SDS and 25 mL of 0.4% bromophenol blue solution. This was stored at 4 °C for up to one month.
- **SDS Electrode stock buffer (5× concentration):** Tris-base (15 g), glycine (72 g) and SDS (5 g) were combined and dissolved in Milli-Q water to make up a volume of 1 L in a measuring cylinder. The pH was 8.6 (± 0.2) as was not adjusted. The buffer was stored at 4°C. For each electrophoresis run, 80 mL of the stock buffer was diluted to 400 mL with Milli-Q water.

3.6.2 Preparation of SDS resolving gel

To prepare four resolving gels, Milli-Q water (3.00 mL), 1.5 M Tris-HCl buffer (3.75 mL) and 30% acrylamide solution (7.95 mL) were mixed and degassed for 15 min in a Buchner flask with moderate stirring under vacuum. After degassing, 150 μ L of 10% (w/v) SDS solution, 7.5 μ L of TEMED and 75 μ L of 10% (w/v) APS were added to the solution and mixed by gentle swirling. The contents (3 mL per gel) were poured between the electrophoresis casting plates (Bio-Rad Laboratories, Richmond, CA, U.S.A). A small quantity of water-saturated butan-2-one was added on top of the resolving gel solution to form an upper layer, which ensured a straight gel surface. The acrylamide solution was allowed to polymerise at room temperature for about 1 h. The butan-2-one solution was carefully poured off and residual butan-2-one was removed from the gel surface with pieces of filter paper. The gel was now ready for the addition of the stacking gel.

3.6.3 Preparation of SDS stacking gel

To prepare four stacking gels, a mixture of Milli-Q water (6.10 mL), 0.5 M Tris-HCl buffer (2.5 mL) and 30% acrylamide solution (1.3 mL) was prepared and degassed for 15 min in a Buchner flask with moderate stirring. After degassing, 100 μ L of 10% (w/v) SDS solution, 10 μ L of TEMED and 50 μ L of 10% (w/v) APS were added and the solution stirred by gentle swirling. The stacking gel solution was poured on top of the set resolving gel between the glass plates until it reached the top and slightly overflowed. A well former (10- or 15-well plastic comb) was inserted between the plates to form appropriate wells for the samples. The gel was allowed to polymerise at room temperature for at least 12 h and then the gels were stored at 4 °C for up to 5 days.

3.6.4 Sample treatment

The skim milk samples were diluted 1:40 and the supernatants were diluted 1:20, by volume, with the SDS sample buffer. The milk and supernatant samples were analysed under non-reduced and reduced conditions. Non-reduced conditions indicated no additional β -ME was added to samples whereas reduced conditions involved addition of an excess of β -ME (~ 20 μ L) to 1 mL of diluted sample (concentration of β -ME in 1 mL of diluted sample would be 2.85 M), and then heating the sample at ~ 100 °C for 10 min.

The samples containing the native whey proteins (obtained after acid precipitation of the casein and denatured whey proteins) were diluted 1:10, by volume with the SDS sample buffer. The samples were analysed under reduced conditions.

3.6.5 SDS gel electrophoresis running conditions

Two gels were placed in a gel holder and then placed in an electrode buffer chamber and SDS electrode buffer (diluted) was used to fill the inner buffer chamber and excess overflowed into the outer chamber. The prepared samples (10 μ L) were injected into the wells of the gel using a Hamilton syringe. In each gel, there were at least two control samples. Once the gels were loaded with samples, the electrophoresis was started using a Bio-Rad power supply unit (Bio-Rad model 1000/500). The voltage, current, and power were set at 210 V, 70 mA and 6.5 W, respectively, per two gels. The gels were run for approximately 1.1 h or until the bromophenol blue dye band reached the bottom of the gels. The power was then turned off.

When there were both fully-reduced samples and non-reduced samples on the same gel, a transition lane was required to separate the former from the latter samples (Red rectangle in Figure 3.1). The reason for this was because any non-reduced sample loaded next to the fully-reduced sample would become partially reduced also. Therefore, a non-reduced sample was

loaded in two consecutive lanes after the fully-reduced samples and one lane is the transition lane. A blank lane was not used because the reducing agent could travel through the blank lane toward the first non-reduced sample.

3.6.6 Staining and destaining

- **Staining solution:** Amido Black 10 B Dye (Merck KGaA, Darmstadt, Germany) (1 g) was dissolved in 250 mL isopropanol, 100 mL glacial acetic acid and 650 mL of Milli-Q water. The stain was stirred for at least 4 h before use.
- **Destaining solution:** Glacial acetic acid was mixed with Milli Q water to make up a 10 % v/v solution.

The gels were removed from the casting assembly and were placed in plastic containers containing about 50 mL of staining solution. The gels in the containers were shaken on a rocking table for at least 12 h to ensure uniform staining. To destain, the staining solution was discarded and replaced with 100 mL of destaining solution. After an hour, the destaining solution was replaced by fresh solution (100 mL) then the solution was refreshed twice with a 2 h interval between each change or until a clear background was achieved.

Once destained, the gels were scanned using an Image Scanner III (GE Healthcare, Bio-Sciences AB, Sweden). A typical protein pattern on a SDS-PAGE gel is shown in Figure 3.1.

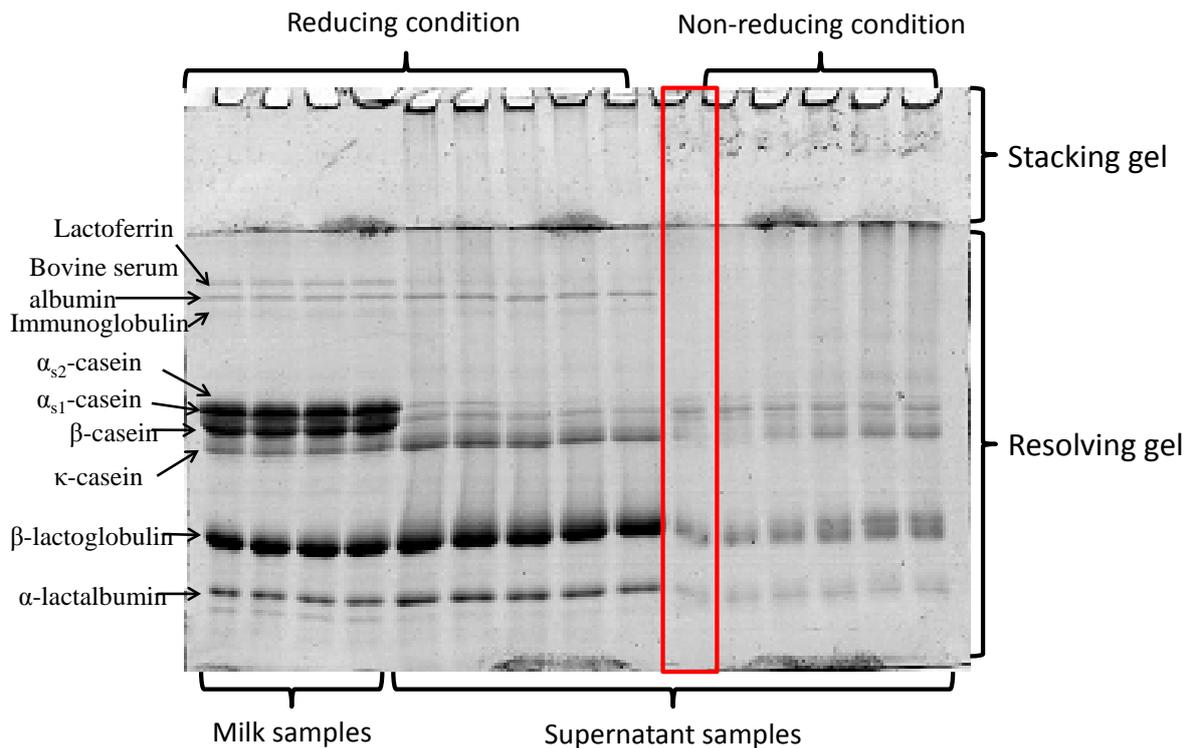


Figure 3.1: An example of a SDS-PAGE gel with the patterns of proteins in milk samples in reducing condition and supernatant samples in both non-reducing and reducing conditions. Red rectangle indicates the transition lane between the fully-reduced and non-reduced samples.

3.6.7 Determination of the protein interactions and distributions

The intensity of the protein band on a SDS-PAGE gel can be integrated into volume unit using Molecular Dynamics ImageQuant integration software (GE Healthcare, Bio-Sciences AB, Sweden) and is related to the quantity of that protein in the sample. The gels of reduced supernatant samples gave information on the proteins present in the supernatant, i.e. the serum proteins (Sup Reduced). The gels of non-reducing milk samples gave information on the proteins that were not participating in intermolecular disulphide bonds (Milk Non-reduced). The gels of non-reducing supernatant samples gave information on the proteins that were not involved in disulphide bonds (Sup Non-reduced).

The quantity of each protein in the experimental milk and supernatant samples are determined as a percentage of that protein in the original milk samples (fully reduced milk = 100%). Table 3.3 describes how to obtain the percentage of proteins in colloidal or serum phases and the percentage of proteins participating in disulphide or non-covalent bonds.

Table 3.3: Methods to calculate the percentage of proteins in colloidal or serum phases and disulphide-linked proteins.

Serum proteins without disulphide bonds	Serum proteins with disulphide bonds	Colloidal proteins with disulphide bonds	Colloidal proteins without disulphide bonds
Sup Non-reduced	[Sup Reduced] - [Sup Non-reduced]	100 - [Sup Reduced] - ([Milk Non-reduced] - [Sup Non-reduced])	[Milk Non-reduced] - [Sup Non-reduced]

3.6.8 Determination of the protein denaturation

The quantity of β -lactoglobulin and α -lactalbumin in the acid precipitate samples was determined as a percentage of the same protein in the supernatant (obtained after acid precipitation of the unheated skim milk). This represents the percentage of native whey protein in the samples.

3.7 Micro-fluidic chip SDS polyacrylamide gel electrophoresis (MF-electrophoresis)

MF-electrophoresis is a miniaturised electrophoresis technique that works on the same principle as traditional SDS-PAGE. The proteins are denatured and imparted with a negative charge through interacting with SDS molecules. The proteins are moved across the chip in microfluidic channels that are filled with a sieving polymer in order to separate the proteins according to their size. All steps that are involved in SDS-PAGE, such as sample loading, electrophoretic separation, staining, destaining, and detection/integration are also performed in MF-electrophoresis. However as MF-electrophoresis was designed to miniaturise the processes, in this case chemical and analytical, to very small dimensions, this technology can shorten analysis time significantly and automate the process of analysis. In addition, it requires very small sample volumes, and is therefore ideal for expensive or rare samples (Goetz et al., 2004; Jeong et al., 2005; Anema, 2009a).

MF-electrophoresis was performed using an Agilent 2100 Bioanalyzer system and the associated Protein 80 chip kits (Agilent Technologies, Waldbronn, Germany). The kits contain the chips on which electrophoresis was performed, the gel matrix solution, a protein dye concentrate solution, a marker protein/sample buffer solution and a protein molecular mass ladder solution. The gel matrix and destain solutions were prepared according to the protocols supplied with the chips. However the marker protein/sample buffer was modified from the protocol because the sample preparation required very small volumes of the buffer (2 μ L) and the sample (4 μ L) and this introduced large errors in the analysis. Hence a modified buffer was

developed in the Food Science Lab of Fonterra (Palmerston North, New Zealand) to eliminate the requirement of using very small volumes. The detailed preparation of the reagents is described in Section 3.7.1.

3.7.1 Solution preparations

- *MF-electrophoresis sample buffer*: A mixture of Milli Q water (250 mL), 0.5 M Tris-HCl buffer (62.5 mL) and 10% (w/v) SDS (50 mL) was prepared. Lactoferrin (1.0 mL of 0.3 mg mL⁻¹ solution) was added to 150 mL of the sample buffer before further use. Lactoferrin is used as the upper marker while SDS is the lower marker. Excess SDS appears as a peak at low molecular weights in the electropherograms and this SDS peak was used as the lower marker. These markers correspond to the highest and lowest molecular mass proteins in the protein ladder.
- *Gel-dye*: The gel-dye solution was prepared by centrifuging the gel matrix solution (670 µL) provided in the kit at 2500 *g* for 20 min. The dye (25 µL) was added to the centrifuged gel matrix solution and mixed well by vortexing for 20 s. The gel-dye solution was light sensitive and kept in a darkened container at 5 °C when not in use.
- *Gel destain*: The gel destain solution was prepared by centrifuging the provided gel matrix solution (670 µL) at 2500 *g* for 20 min. The gel destain solution was also kept in a darkened container at 5 °C when not in use.
- *Ladder solution*: An aliquot of the ladder solution (6 µL, provided in the kit) was mixed thoroughly with Milli-Q water (84 µL). The diluted ladder solution was prepared fresh on the day of performing MF-electrophoresis.

The layout of a chip is shown in Figure 3.2. There are a total of 16 wells on the chip, 10 for samples, 1 for the protein ladder, 4 for gel-dye solution and 1 for destain solution. In a typical run, a new chip is primed with gel-dye matrix by injecting gel dye into well G1 followed by applying air pressure (1 mL) for 1 min using a gas syringe and the supplied priming station. Once primed, the remaining wells are filled with the appropriate solutions: gel dye (wells G2 – G4, 12 µL each), gel destain (well DS, 12 µL), ladder (well L, 6 µL) and samples (wells S1-10, 6 µL each), as shown in Figure 3.2. For each chip, two control samples and eight experimental samples were run on the ten available wells. Each chip was repeated at least twice and each sample was prepared at least twice. Once loaded, the chip was inserted into the machine. Closing the lid allowed the insertion of electrodes into each of the wells within the chip. The electrophoresis was started and the analysis took approximately 30 min for all ten samples. This procedure and the preliminary peak identification and integration were performed automatically by the Agilent 2100 expert software (Agilent Technologies, Waldbronn,

Germany). The automatic peak identification and integration was not usually accurate enough so was manually refined within the software.

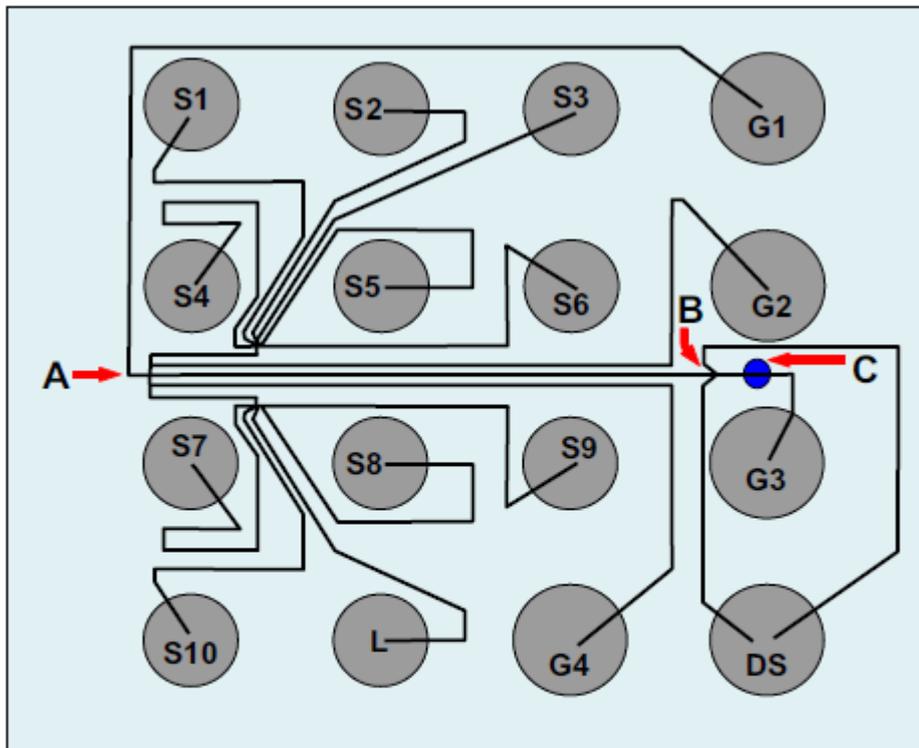


Figure 3.2: Layout of the wells and channels in a typical microfluidic electrophoresis chip. The wells designated G1 – G4 are loaded with gel/dye matrix, DS is loaded with destain solution. The wells designated S1 – S10 are filled with the samples under study, and the well designated L is loaded with the protein molecular weight standards. The separations channel (A), position of destaining (B) and position of the detection window (C) are highlighted with arrows. Source: Anema (2009a).

3.7.2 Interpreting the electropherograms obtained from MF-electrophoresis

The MF-electropherograms of the control (non-reduced) and fully reduced skim milk are shown in panels A and B of Figure 3.3, respectively. The quantity of the protein in the sample is related to the peak area of that protein on the electropherograms.

In the control (non-reduced) milk, the majority of κ -casein is disulphide-linked to form oligomers while small quantity of κ -casein appeared as monomers with an intra-molecular disulphide bond (Talbot & Waugh, 1970; Rasmussen et al., 1992; Holland et al., 2008). Hence the level of monomeric κ -casein is low (peak VI in Figure 3.3A).

In fully reduced milk, the disulphide bonds were broken, thus the level of monomeric κ -casein was at its maximum (peak VI in Figure 3.3B). Similarly, α_{s2} -casein naturally occurs as a mixture

of monomers (with one intra-molecular disulphide bond) or as disulphide-linked dimers (Rasmussen et al., 1994). Hence, in non-reduced milk electropherograms, both monomeric and dimeric α_{s2} -casein were observed (peaks V and VII in Figure 3.3A, respectively); whereas in fully reduced milk, only monomeric α_{s2} -casein was detected (peak V in Figure 3.3B). A small peak for VII was still detected in fully reduced milk. This peak is probably bovine serum albumin because this runs at a similar position to dimeric α_{s2} -casein.

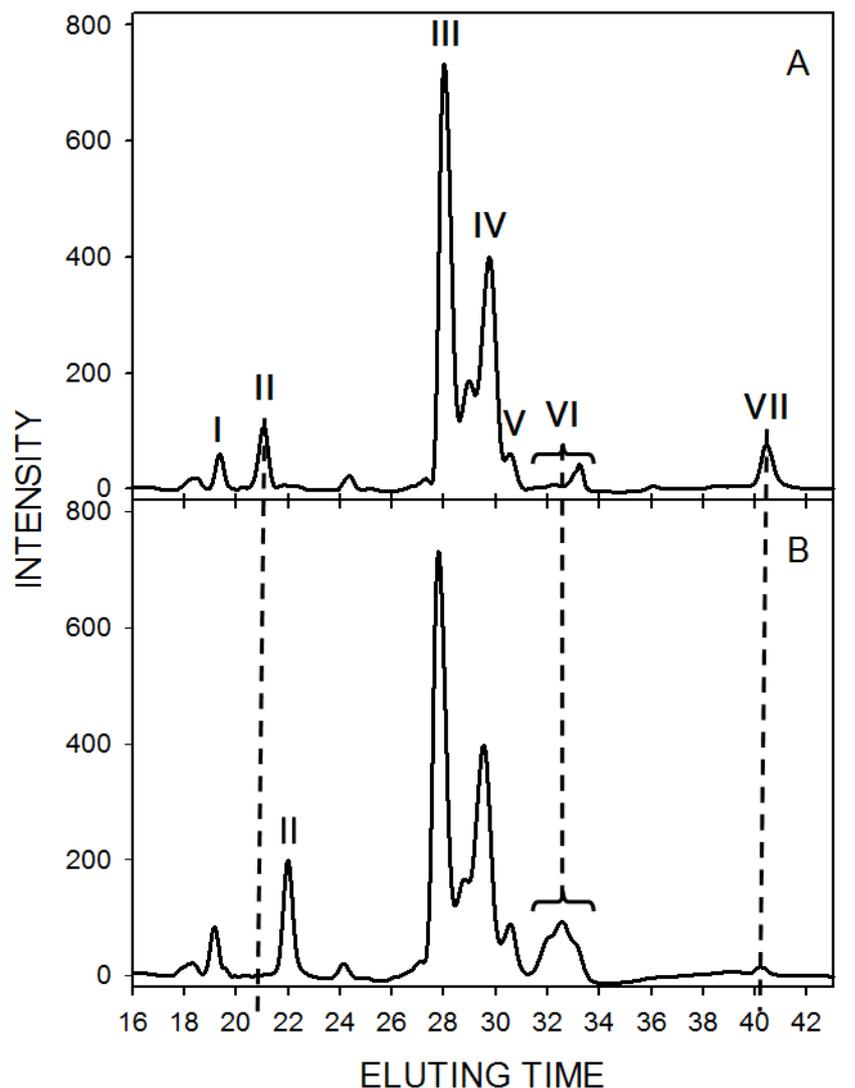


Figure 3.3: Typical electropherograms obtained from MF-electrophoresis, of non-reduced (A) and fully reduced (B) skim milk. I, α -lactalbumin; II, β -lactoglobulin; III, β -casein; IV, α_{s1} -casein; V, monomeric α_{s2} -casein; VI, κ -casein and VII, dimeric α_{s2} -casein.

In the non-reduced milk, β -lactoglobulin and α -lactalbumin contain intra-molecular disulphide bonds (Brownlow et al., 1997; Brew, 2003), whereas these bonds are disrupted upon fully reducing the milk. While the reduction of disulphide bonds did not affect the mobility of α -

lactalbumin (Peak I in Figure 3.3), the non-reduced and reduced states of β -lactoglobulin had different electrophoretic mobilities (peak II in Figure 3.3), as also observed in a previous study using traditional SDS-PAGE (Griffith, 1972). One of the two disulphide bonds (Cys⁶⁰-Cys¹⁶⁰) in β -lactoglobulin was reported to be more reactive to thiol groups than the other (Davidson & Hird, 1967). When the concentration of disulphide-bond reducing agent was not sufficient to reduce all of the disulphide bonds in the system, it was possible that β -lactoglobulin can be partially reduced (i.e. only one disulphide bond was broken). It was not possible to distinguish between partially reduced β -lactoglobulin and fully reduced β -lactoglobulin (i.e. both disulphide bonds were cleaved) using the current method.

On the basis of this analysis, the reduction of disulphide bonds of β -lactoglobulin and κ -casein can be examined by monitoring the peak area of the reduced β -lactoglobulin and the monomeric κ -casein. The increase in the peak area corresponds to the increase in amount of reduced β -lactoglobulin/monomeric κ -casein. The intensity of the reduced protein in the treated sample was divided by the intensity of that protein in the control sample, multiplying by 100 to give the percentage of the reduced protein.

3.8 Measurement of the size of the casein micelles

Size and polydispersity index of particles in skim milk were measured using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK). Milk samples were diluted 100 fold with Ca-imidazole buffer (20 mM imidazole, 5 mM CaCl₂.H₂O, 30 mM NaCl, pH 7.0) and measurements were performed at 20 °C, as was described previously (Anema & Klostermeyer, 1997). The dispersant (Ca-imidazole buffer) was considered to have the properties of water with a refractive index of 1.330 and viscosity of 1.0031 cP (Anema & Klostermeyer, 1997). Measurements of the dynamics of the scattered light were collected at a scattering angle of 173°. As the particles in the dispersion scatter light, the Brownian motion of the scattering particles produces intensity fluctuations in the signal. The average diffusion coefficients were calculated from these changes of intensity and were then translated to average particle diameters using the Stokes-Einstein relationship for spheres (Anema & Li, 2003b).

The measured particles in this study were casein micelles. Polydispersity index is defined as the weight-averaged molecular weight divided by the number-averaged molecular weight. This gives information of the width of the molecular weight distribution (IUPAC), hence the dispersion of sizes.

3.9 Measurement of the zeta potential of the casein micelles

The Malvern Zetasizer Nano-ZS was also used to measure the zeta potential of the casein micelles in skim milk samples prepared as in Section 3.8. The zeta potential is defined as follows.

When a particle with a charged surface is suspended in solution, the counter ions, ions of opposite charge to that of the particle, in the solution will re-distribute and concentrate at the surface of the particle. The liquid layer surrounding the particle exists as two parts: an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a slipping plane inside which the ions and particles form a stable entity. When a particle moves, ions within the boundary move with it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary is the zeta potential (Figure 3.4).

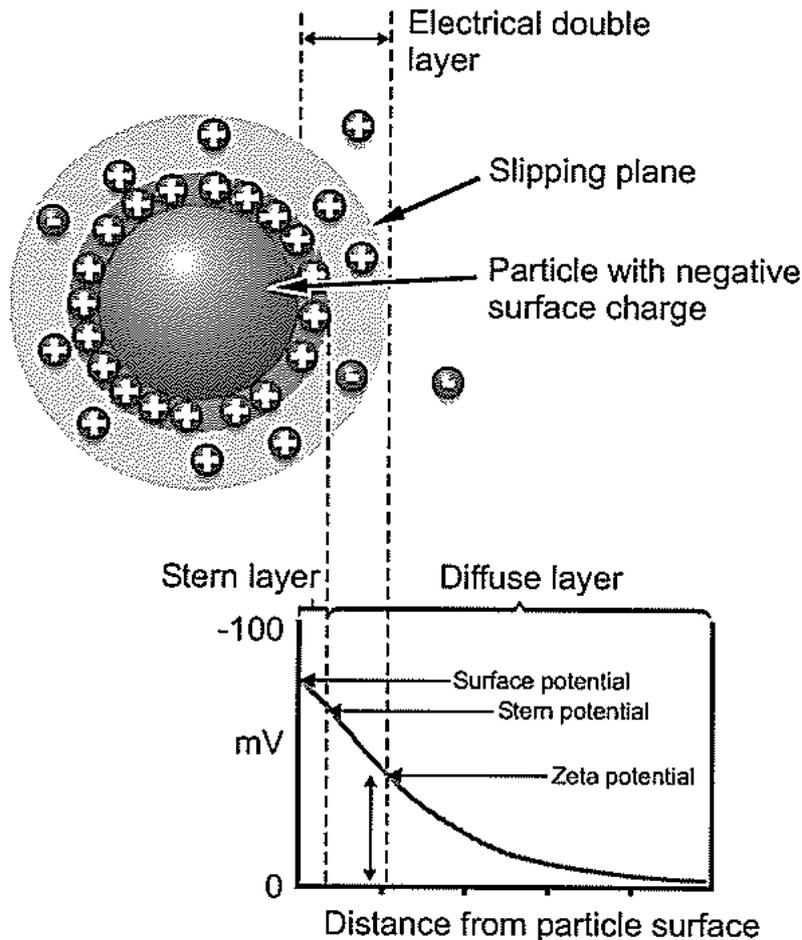


Figure 3.4: Schematic representation of zeta potential of a particle. Source: Instruction Manual of Malvern-Instrument (Malvern-Instruments, 2007).

In the Malvern Zetasizer Nano-ZS, the electrophoretic mobility of the particle in solution is measured under an applied electric field of 40 V. The software is programmed to use the Henry equation, which can be found in Instruction manual of Malvern-Instrument (Malvern-Instruments, 2007), to calculate the zeta potential. The Ca-imidazole buffer had a dispersant dielectric constant of 80.4.

3.10 Preparation and properties of acid gels

3.10.1 Preparation of acid gels

Skim milk samples were acidified using glucono- δ -lactone (GDL) at a level of 2% (w/w) and at 30 °C for 3 h. During acidification, GDL is hydrolysed to form gluconic acid that is a weak acid and dissociates further, resulting in a gradual reduction in the pH of the system (De Kruif, 1997). Preliminary experiments were carried out to monitor the change in pH as a function of time in systems with different concentrations of GDL being added to skim milk. It was found

that with 2% GDL and at 30 °C, after 3 h, the pH of skim milk decreased from pH ~6.7 to pH ~4.4, which was typically the pH used as an endpoint for the fermentation in the manufacturing of yoghurt. The change of pH on acidification was similar in all treated milks (Figure 3.5A).

Figure 3.5A showed the gradual change of pH of milk system from pH ~ 6.7 to pH ~ 4.4 during 3 h gelation with GDL. As the pH decreases exponentially as a function of gelation time, the change of pH was plotted as a function of the log of gelation time (Figure 3.5B). An equation was derived from the relation of pH and log time (Equation 3.2) and was used to calculate the pH at time of interest (e.g. gelation pH).

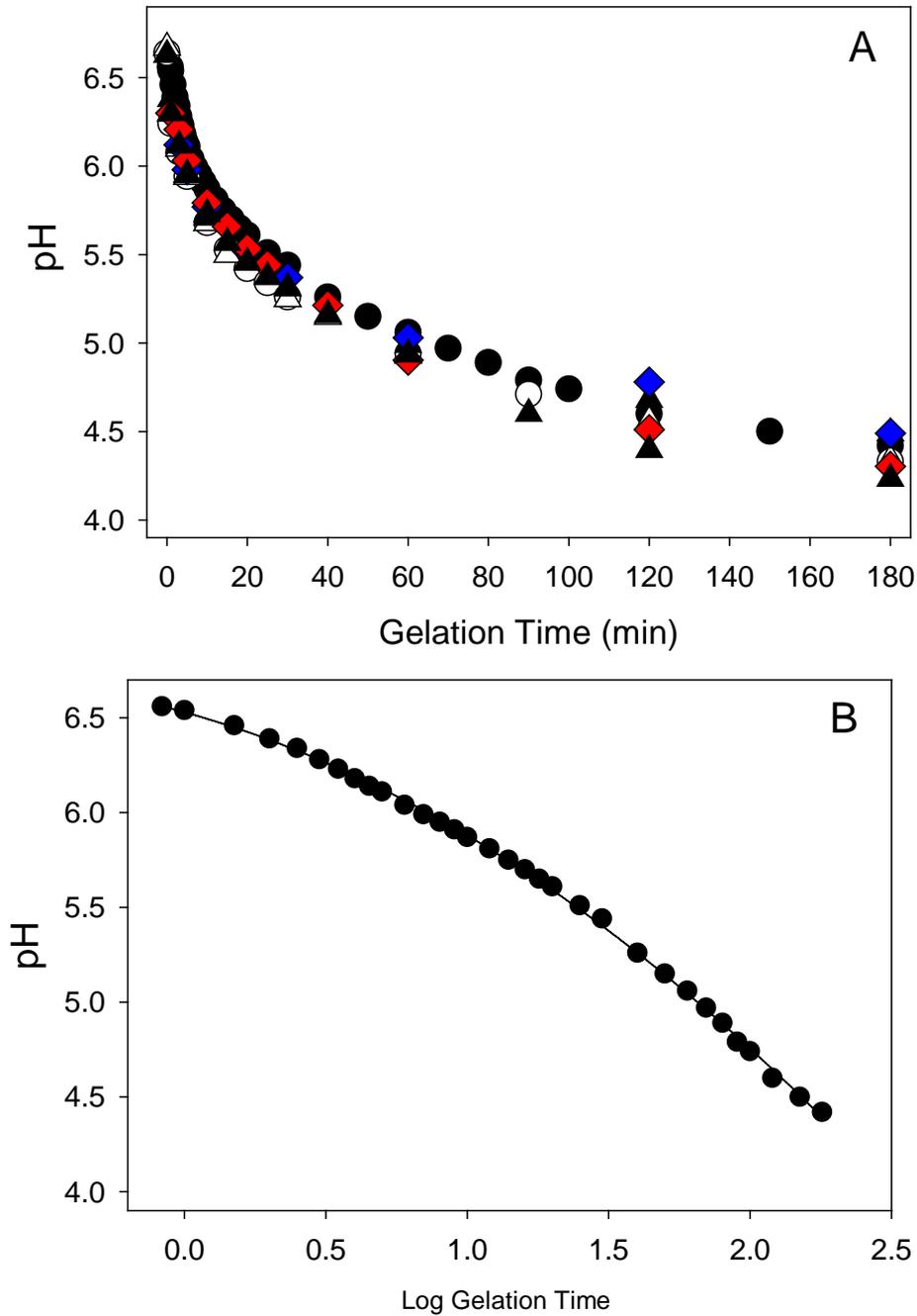


Figure 3.5: The change of pH as a function of gelation time (A) and of log of gelation time (B). The data is the average of two replicates. ▲, unheated skim milk; ●, heated skim milk; △, unheated WPE skim milk; ○, heated WPE skim milk; ◆, skim milk heated in the presence of β-mercaptoethanol; ◇, skim milk heated in the presence of NEM.

$$pH = -0.2373(\log, t)^2 + -0.4123(\log, t) + 6.5287$$

Equation 3.2

3.10.2 Rheological measurements

Acid gels, as models for yoghurt, are viscoelastic as they exhibit both solid and liquid-like properties (Ross-Murphy, 1984). The solid and liquid-like components can be separated by performing a small amplitude oscillatory test using a rheometer with a set geometry arrangement such as a cone and plate, parallel plates or a cup and bob arrangement (Goodwin & Hughes, 2008). The geometry is made to oscillate about a central point with a sinusoidal angular velocity at low amplitude (strain) while the shear stress is measured (Bourne, 2002). The stress develops in direct response to the applied strain. If the strain has an oscillating value with time, the stress must also be oscillating with time. Thus there are two sigmoidal waveforms. Typical stress responses of various material types to an applied (driving) strain are shown in Figure 3.6. This test is non-destructive when the amplitude of the strain is small (i.e. within the linear viscoelastic region).

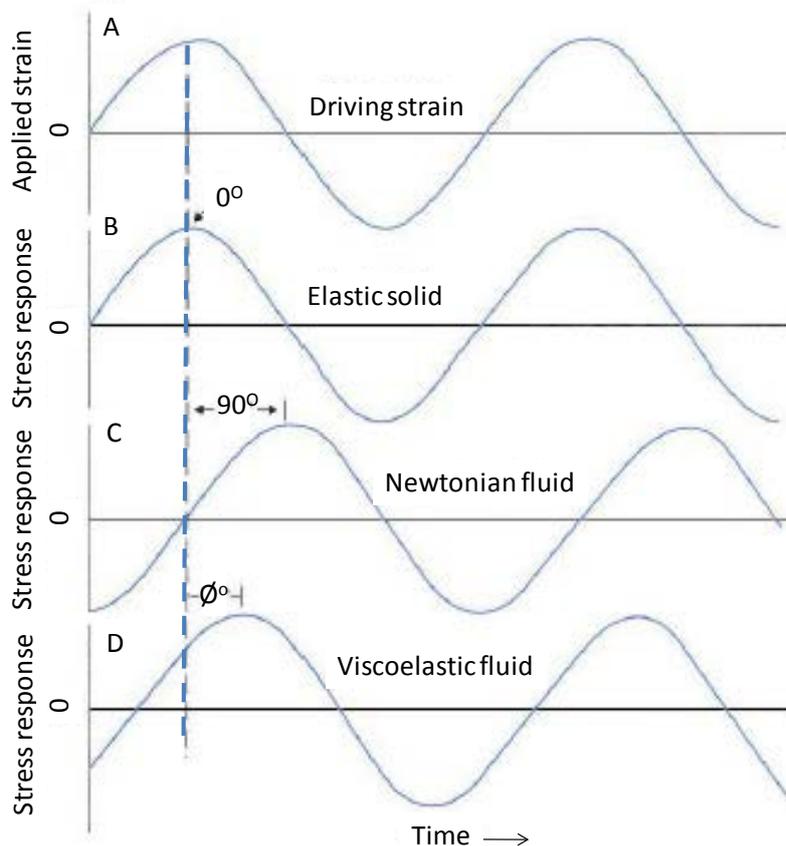


Figure 3.6: The principle of oscillation rheology. Applied strain versus time on various types of materials. Elastic solid has a stress response being in phase with the driving strain (i.e. 0 degree) while the Newtonian fluid has a stress response exactly out of phase with the driving strain (i.e. 90 degree). ϕ° indicates the degree out of phase (between 0 and 90 degree) of viscoelastic solid. Source: Bourne (2002).

The storage modulus (G'), loss modulus (G'') and $\tan \delta$ (G''/G') were derived from the shear stress and shear strain. The stress component that is in phase with the shear strain is defined as the storage modulus (G') that is the ratio of the shear stress (which is in phase with the shear strain) to the strain (Equation 3.3a) (Bourne, 2002). The stress component that is 90° out of phase with the shear strain is defined as the loss modulus (G'') which is the ratio of the shear stress out of phase with the strain, to the strain (Equation 3.3b) (Bourne, 2002). A perfect elastic material has the responding stress being exactly in phase with the applied strain (Figure 3.6B) (Goodwin & Hughes, 2008). A purely viscous liquid (Newtonian liquid) has the responding stress being exactly 90° out of phase with the applied strain (Figure 3.6C) (Goodwin & Hughes, 2008). The $\tan \delta$ is the ratio of the loss modulus to the storage modulus (Equation 3.3c) (Bourne, 2002).

$G' = \frac{\sigma'}{\gamma}$	Equation 3.3a	
$G'' = \frac{\sigma''}{\gamma}$	Equation 3.3b	σ' = shear stress in phase σ'' = shear stress 90° out of phase
$\tan \delta = \frac{G''}{G'}$	Equation 3.3c	γ = strain

In this study, the rheological measurements were carried out on a stress-controlled rheometer, the Physica UDS200 (Anton Paar GmbH, Graz, Austria). In most of the measurements, a cone (50 mm, 2°) and plate geometry were used; however in a particular study (Chapter 5), a cup and bob geometry (25 mm) was used. The reason for switching the geometry will be explained in detail in Chapter 5 and Appendix 4. Unless stated, a cone and plate arrangement was employed in the rheological measurements.

Prior to the rheological measurements, GDL was added to a milk sample and the sample was stirred for 30 s. An aliquot of the sample (1.1 mL) was immediately transferred to the rheometer plate. The rheometer cone was lowered to the required gap between cone and plate (the same procedure was used for the cup and bob geometry except the sample volume was 19 mL). A thin layer of sunflower oil was added to the edge of the cone and plate gap to prevent water evaporation.

The development of the acid gel structure was monitored using the oscillation mode at a frequency of 0.1 Hz and a strain of 0.5%. The temperature was maintained at 30 ± 1 °C during acidification. Measurements were taken every 18 s for 3 h. A typical gelation curve showing the changes in G' values over a period of 3 h is presented in Figure 3.7. The gelation point is where

the G' value started to show an increasing trend and is often the first G' value that is ≥ 1 Pa. The final G' value is the last point at the 180th min.

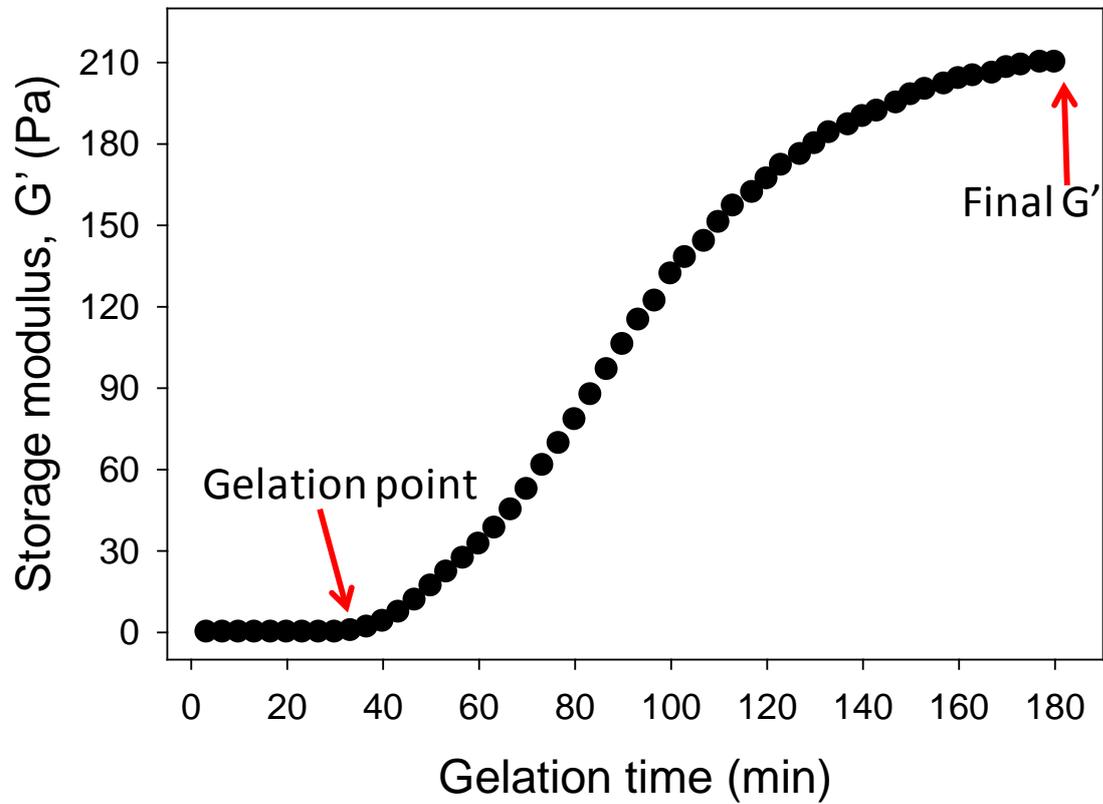


Figure 3.7: The change of the storage modulus over time after 2% GDL was added to heated skim milk.

Once the acid gels were formed, a temperature sweep was performed. The temperature of the sample was decreased from 30 to 5 °C at a rate of 1 °C min⁻¹. The G' values of the acid gels were monitored, also using 0.1 Hz frequency and 0.5% strain. Once at 5 °C, the gels were subjected to a low (0.005 s⁻¹) and constant shear rate up to the yield point of the gel. This is when the gel was broken, observed by the decrease in the shear stress values (Figure 3.8).

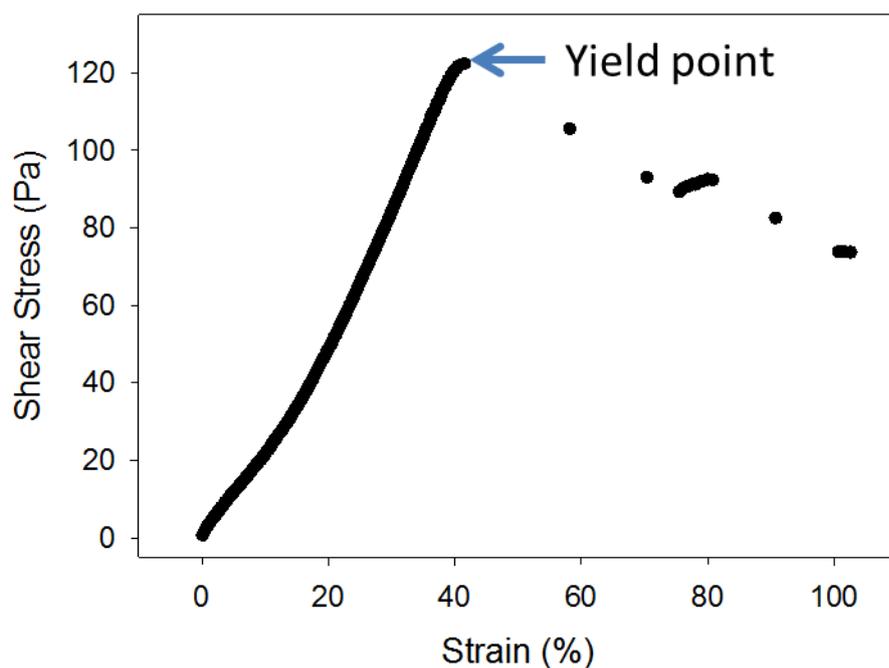


Figure 3.8: Typical changes of shear stress and strain values of the acid gel samples that were subjected to a constant shear rate.

3.10.3 Confocal scanning laser microscopy

The two-dimensional microstructure of the acid gels was examined using confocal scanning laser microscopy. A scanning laser confocal microscope works based on two principal ideas: point-by-point illumination of the sample and the rejection of out-of-focus light. The internal workings of a confocal microscope are depicted in Figure 3.9. Laser light (blue line) is projected from the laser source, directed by a dichroic mirror (this mirror passes light longer than a certain wavelength while reflects light shorter than that wavelength) towards a pair of mirrors that can rotate, allowing the light to scan in the x and y directions (Semwogerere & Weeks, 2005). The light then passes through the microscope objective and excites the sample that has a fluorescent probe bound to the component of interest. The fluoresced light from the sample (green line) travels back the direction from which the laser light has passed. When the fluoresced light passes through the dichroic mirror, it continues passing through a pinhole placed in the conjugate focal (hence the term confocal) plane of the sample. The pinhole rejects any out-of-focus light arriving from the sample. The light that emerges from the pinhole is finally measured by a detector such as a photomultiplier tube (PMT).

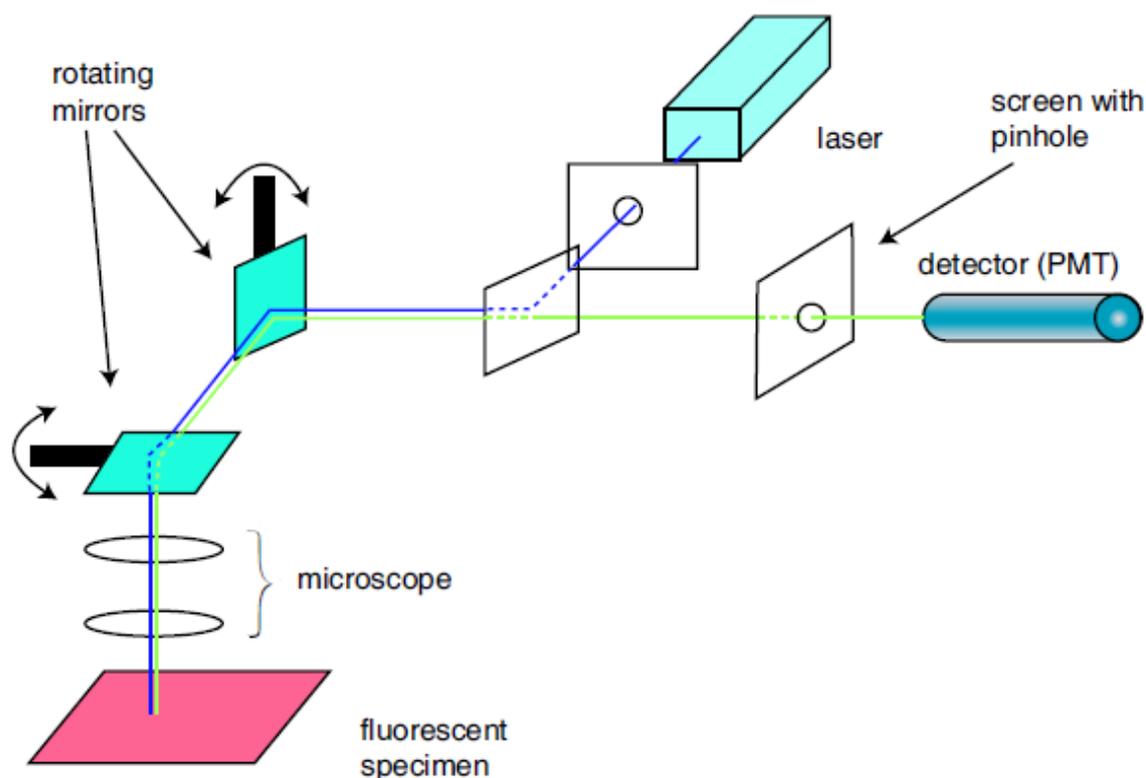


Figure 3.9: Basic setup of a confocal microscope. Light from the laser is scanned across the specimen by the scanning mirrors. Optical sectioning occurs as the light passes through a pinhole on its way to the detector. Source: Semwogerere and Weeks (2005).

Nile blue dye (1% w/v in water) was added to selected milk samples (20 μ L dye per 5 g of milk) before addition of GDL. The milk sample with added dye and GDL was deposited into the concave region of a special glass slide, and a cover slip was immediately placed on top, ensuring no air was trapped. The slides with sample were transferred to an incubator set at 30 ± 0.5 °C. After 3 h, the gelled samples were examined using a Zeiss LSM510 Meta confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany) using the 63 \times objective. For the protein imaging, the excitation wavelength was 633 nm and the emission band was collected at 650-735 nm. Samples were imaged 5 μ m below the surface. This methodology is adapted from Ji et al. (2011).

3.11 Statistical analysis

All experiments were carried out in duplicate (at least) for both sample treatments and sample analyses. Standard deviations and pooled standard deviations were used where appropriate to indicate the variability between repeated experiments or measurements. Any significant difference between the means was determined using one way ANOVA and correlations were determined by regression in Minitab 15 Statistical software (Minitab Inc, USA).

Chapter 4 - Effects of N-ethylmaleimide concentrations on the properties of unheated skim milk

4.1 Introduction

In bovine milk, β -lactoglobulin is the main source of free thiol groups that can initiate thiol-disulphide exchange reactions upon heating (Zittle et al., 1962; Sawyer et al., 1963; Lowe et al., 2004). In many published research findings, an excess level of thiol blocking reagent (e.g. NEM, iodoacetamide and hydrogen peroxide) was used to block all the free thiol groups of β -lactoglobulin, hence upon heating, formation of new intermolecular disulphide bonds is eliminated (Zittle et al., 1962; Sawyer et al., 1963; Purkayastha et al., 1967; Sawyer, 1967; Özer et al., 2003). NEM has been used extensively in many studies involving β -lactoglobulin because it reacts readily with the free thiol groups of proteins at pH 6.5 – 7.0 (Gregory, 1955). The interaction between thiol groups and NEM results in a thioether containing a strong C-S bond and is therefore irreversible (Figure 4.1). For these reasons, in the studies reported in this thesis, NEM will be used as the thiol-blocking reagent.



Figure 4.1: The interaction between the thiol group on a protein with the thiol blocking reagent N-ethylmaleimide (NEM). The newly formed bond in a thioether is a strong C-S bond (red bond).

There are numerous studies investigating the effect of NEM on the interactions between whey protein or β -lactoglobulin in solution (Shimada & Cheftel, 1988; Matsudomi et al., 1991; Xiong et al., 1993; Hoffmann & van Mil, 1997; Havea et al., 2009) and a few studies on the effect of NEM on the interactions between proteins in milk and milk products (Mckenzie et al., 1971; Hashizume & Sato, 1988; Goddard, 1996; Vasbinder et al., 2003; Lakemond & van Vliet, 2008a). While most of the previous studies used NEM concentrations higher than the concentration of free thiol groups in milk, Hashizume and Sato (1988) and Goddard (1996) worked with NEM concentrations ≤ 1 mM and found that the hardness (measured by a penetration test) of the acid-heat-induced gels decreased as the concentrations of NEM increased up to 0.2 mM

(Goddard, 1996) or 1 mM (Hashizume & Sato, 1988). The work reported in this thesis will work with a range of NEM concentrations ≤ 0.8 mM, aiming to cover a ratio of NEM to free thiol groups from below to above 1.

Previous studies investigated the effects of blocking the thiol groups of β -lactoglobulin on the properties of proteins or the properties of acid-heat-induced gels of systems that had been heated in the presence of NEM (Hashizume & Sato, 1988; Shimada & Cheftel, 1988; Matsudomi et al., 1991; Xiong et al., 1993; Goddard, 1996; Hoffmann & van Mil, 1997; Havea et al., 2009). However, there have been no reports on the effects of blocking the thiol groups of β -lactoglobulin in unheated milks on the properties of the milks and the resulting milk products. One can indeed suppose that adding NEM to unheated milks does not affect their acid gelation properties since thiol groups of β -lactoglobulin are buried in the native structure (Brownlow et al., 1997), hence disulphide interactions between β -lactoglobulin and other proteins cannot occur. However non-covalent interactions may be possible since NEM has some effects on the secondary structure of pure β -lactoglobulin (Wada, Fujita & Kitabatake, 2006).

Despite the anticipated results, the experiments in this chapter involved investigating the effect of NEM on the protein interactions in unheated skim milk and unheated WPE skim milk. The acid gels were prepared from the treated milks and their properties were examined using rheological measurements and confocal microscopy. The aim of these experiments was to confirm that blocking the thiol groups of native β -lactoglobulin did not have any effects on the protein interactions and the properties of the resulting acid gels. In addition, the results of this study will provide detailed information on the protein status in milks containing NEM before heat treatment, as control samples for later comparison with milk heated in the presence of NEM (Chapter 5).

4.2 Materials and methods

Skim milk and whey-protein-enriched (WPE) skim milk were prepared as described in Section 3.1.1.

Diluted NEM (1% w/v) was added to give NEM concentrations of 0.08, 0.24, 0.4 and 0.6 mM in skim milk and 0.24, 0.4, 0.6 and 0.8 mM in WPE skim milk. The ratio of NEM to thiol groups of β -lactoglobulin in skim milk and WPE skim milk was calculated based on the average composition of skim milk (of which $\sim 0.35\%$ was β -lactoglobulin) and whey protein isolate (of which 69% was β -lactoglobulin). Hence skim milk had 0.35% and WPE skim milk had 1.14% β -lactoglobulin. Table 4.1 summarised the concentrations of NEM and their corresponding ratio of NEM to free thiol groups.

Table 4.1: The ratio of NEM to free thiol groups (-SH) of β -lactoglobulin in skim milk and WPE skim milk corresponding to the concentration of NEM used.

Concentration of NEM (mM)	Ratio of NEM to -SH in skim milk	Ratio of NEM to -SH in WPE skim milk
0.08	0.4	0.1
0.24	1.3	0.4
0.4	2.1	0.6
0.6	3.1	1.0
0.8	4.2	1.3

The treated milk was shaken on a vortex mixer for 10 s and then left to react at 20 ± 0.5 °C in a thermostatically controlled water bath for one hour. The lids of the containers containing the milks were closed tightly to minimise any contact with oxygen.

In order to determine the appropriate holding time of reaction between NEM and the milk proteins, the extent of inhibition of the irreversible heat-denaturation of α -lactalbumin and β -lactoglobulin was assayed at different incubation times, until a plateau was found. NEM (0.6 mM) was added to skim milk that was then held for different times up to 6 h at 20 ± 1 °C before heating. After heating, the percentage of α -lactalbumin and β -lactoglobulin remaining as native protein (i.e. soluble at pH 4.6) was determined. Figure 4.2 showed that the percentage of native α -lactalbumin and β -lactoglobulin in skim milk heated in the presence of NEM was significantly higher than that in control heated skim milk ($p < 0.05$). However the holding time did not have a significant effect on the percentage of native whey proteins ($p > 0.05$). This indicated that the reaction between free thiol groups and β -lactoglobulin occurred rapidly.

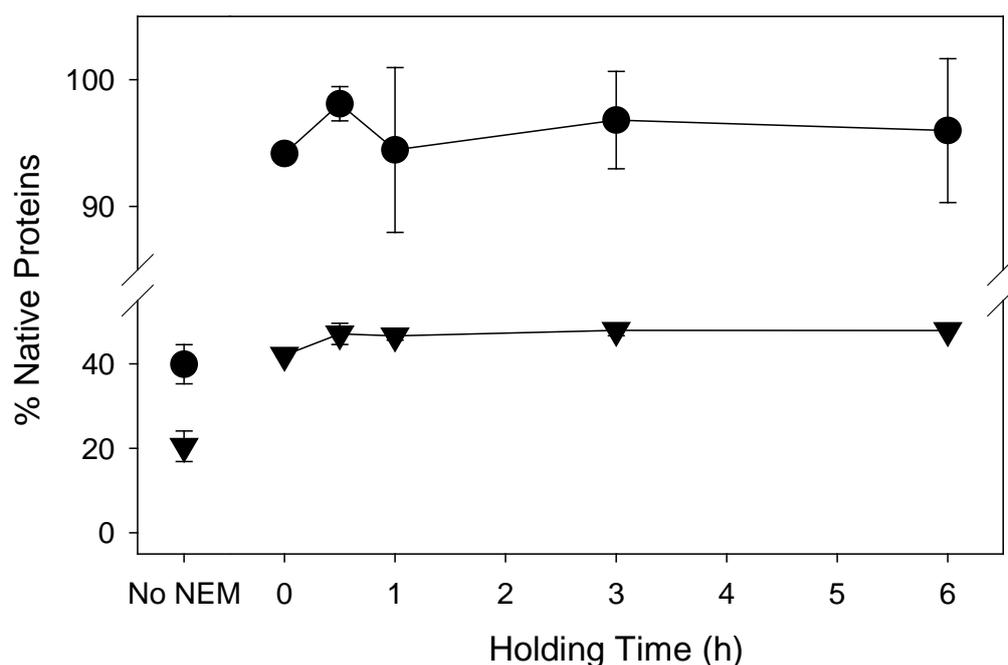


Figure 4.2: Effects of reaction time between 0.6 mM NEM and skim milk on the proportion of native proteins remaining after heat treatment (80 °C for 30 min). ●, α-lactalbumin; ▼, β-lactoglobulin. Each data point is the average of two replicates. Error bars represent the standard deviation.

Many previous studies used one hour as a reaction time between β-lactoglobulin and NEM (Morr & Josephson, 1968; Xiong et al., 1993; Goddard, 1996; Hoffmann & van Mil, 1997; Anema & Li, 2000; Havea et al., 2009). It was also reported that NEM is able to interact with other amino acids (e.g. amine groups) when the holding time exceeds 2 h (Smyth, Blumenfeld & Konigsberg, 1964; Hansen & Winther, 2009). Throughout this thesis, when NEM was added to milk, the milk was held for 1 h at 20 ± 1 °C before further analysis or treatments.

After addition of NEM to the milks, the level of proteins participating in disulphide bonds and the proportion of proteins in the serum were investigated using SDS-PAGE (as described in Section 3.6). The size of the casein micelles in the milks were also examined using light scattering (as described in Section 3.8).

The NEM-treated milks were acidified to form acid gels of which the rheological properties and microstructure were investigated using rheometry and confocal microscopy (as described in Sections 3.10.2 and 3.10.3).

4.3 Results

4.3.1 Effects of NEM concentrations in unheated milks on the protein interactions

In unheated milks, interactions between whey proteins and κ -casein were not expected to occur and the addition of NEM was not expected to change this situation. Figure 4.3 shows the SDS-PAGE patterns for the monomeric α -lactalbumin, β -lactoglobulin, κ -casein, β -casein, α_{s1} - and α_{s2} -casein in the skim milk and WPE skim milk samples under non-reducing conditions. The proteins bands were identified by comparison with the SDS-PAGE patterns previously reported by Anema and Klostermeyer (1997) and were marked on the left-hand side of the control sample (Lane a). While the band intensities in the control samples (Lane a) represent the total amount of proteins in the studied milk, the band intensities in the other samples (Lanes b to g) represent the amount of proteins that did not participate in disulphide interactions.

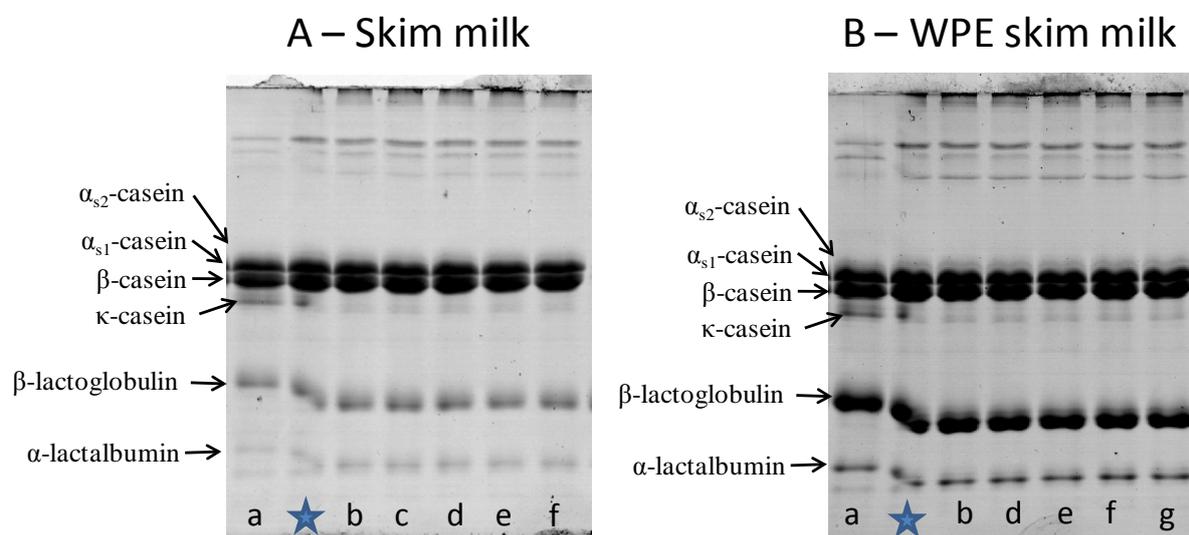


Figure 4.3: SDS-PAGE patterns of unheated skim milk (A) and WPE skim milk (B) with added NEM. The PAGE pattern shows: control samples (i.e. control untreated milks, Lane a), milks with: 0 mM (Lane b), 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g) added NEM. The milk samples were diluted with SDS sample buffer at ratio of 1 to 40 and were not reduced except for the control samples. Blue star represents the transition lane between reduced and non-reduced samples.

The bands representing the α -lactalbumin and β -lactoglobulin under non-reducing conditions (Lanes b to g) are shifted to a lower position than the proteins under reducing conditions (Lane a), indicating that the non-reduced whey proteins have a faster electrophoretic mobility than the reduced proteins. Under non-reducing conditions, the intra-molecular disulphide bonds of the whey proteins are still intact, thus their structure are more compact than when the disulphide bonds are broken (Griffith, 1972). As a result, non-reduced proteins with disulphide

bonds have a faster mobility than the reduced proteins without disulphide bonds (Figure 4.3). Despite the different mobilities, the bands representing the whey proteins have the same intensities in both non-reduced and reduced conditions, indicating that the total level of monomeric whey proteins was not affected by the addition of NEM (Figure 4.3).

As for κ -casein, Figure 4.3 shows that the level of monomeric κ -casein in milk under non-reducing condition was very low (Lanes b to g) compared to the total level of monomeric κ -casein in the reduced milk (Lane a). This is in agreement with previous reports showing that the majority of κ -casein is present in milk as oligomers linked by intermolecular disulphide bonds (Rollema, Brinkhuis & Vreeman, 1988; Holland et al., 2008). In both skim milk and WPE skim milk under non-reducing condition, the bands of monomeric κ -casein have the same low intensity, regardless of NEM concentrations (Lanes b to g, Figure 4.3). This demonstrates that adding NEM did not affect the interactions of κ -casein.

In milk, the other caseins, α_{s1} -, α_{s2} - and β -casein, are assembled together by non-covalent bonds and calcium phosphate clusters within the casein micelles (Horne, 1998). Except for α_{s2} -casein which can have intermolecular disulphide bonds (Rasmussen et al., 1994), the other caseins are not involved in any disulphide interactions. The same band intensity across the gel pattern was observed for α_{s1} - and β -casein; hence all of α_{s1} - and β -casein were present as monomers under both non-reduced and reduced condition and regardless of the NEM addition. Due to the low concentration of α_{s2} -casein and the sensitivity of SDS-PAGE, the α_{s2} -casein band cannot be integrated properly, and was often not adequately separated from the α_{s1} -casein band.

Overall, the SDS-PAGE patterns demonstrated that the addition of NEM to unheated milks did not affect the amount of proteins participating in disulphide bonds. In both skim milk and WPE skim milk, the percentage of disulphide-linked α -lactalbumin or β -lactoglobulin was integrated to be below 10%, but not at absolute 0% at any NEM concentrations. Whereas, the percentages of disulphide-linked κ -casein were $76 \pm 2\%$ in skim milk and $80 \pm 4\%$ in WPE skim milk, regardless of the NEM concentrations, and were not significantly different from each other ($p > 0.05$, Figure 4.4).

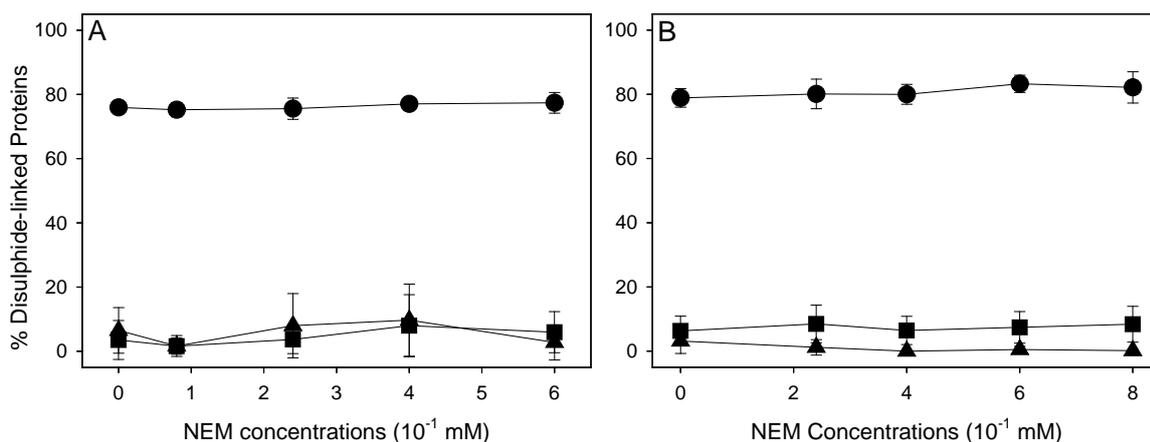


Figure 4.4: Effects of NEM concentrations on the percentage of individual proteins (individual protein from non-reduced SDS-PAGE divided by total for that protein present from reduced SDS-PAGE) participating in intermolecular disulphide bonds. α -lactalbumin (▲), β -lactoglobulin (■) and κ -casein (●). Unheated skim milk (A) and WPE skim milk (B). Each data point is the average of two to four replicates. Error bars represent the standard deviation.

4.3.2 Effects of NEM concentrations in unheated milks on protein distributions

The distribution of the proteins between the serum and colloidal phases was determined by examining the level of proteins remaining in the supernatant of milk, obtained by centrifuging milk for 1 h at 21 000 *g* (as described in Section 3.4), using SDS-PAGE. The protein bands in the SDS-PAGE patterns (Figure 4.5) are also identified by comparison with previous reports (Anema & Klostermeyer, 1997) and marked on the left-hand side of the control sample (Lane a, Figure 4.5). As the control milk sample was more diluted than the serum samples (1:20 and 1:40 dilutions with SDS-sample buffer, respectively), the protein bands of the serum samples (Lanes b to g) may appear darker than those of the control milk sample (Lane a). However this does not mean that there were higher levels of protein in the serum than in milk. When integrating, the band intensities were corrected according to the dilution factors.

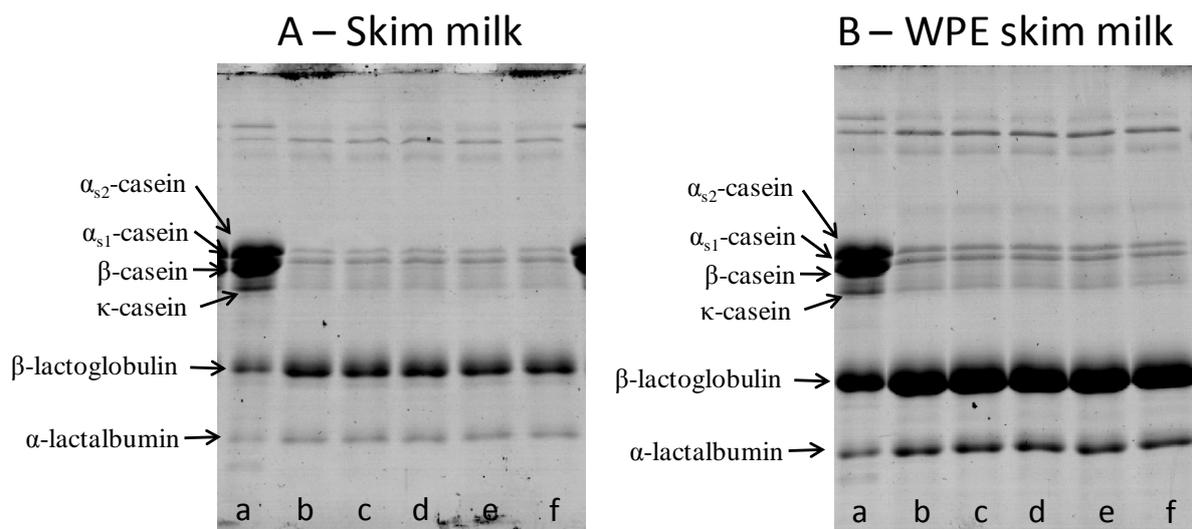


Figure 4.5: SDS-PAGE patterns of supernatant samples obtained from centrifugation of unheated skim milk (A) and WPE skim milk (B). The PAGE pattern shows: control samples (i.e. control untreated milks, Lane a), supernatant of milks with different NEM concentrations: 0 mM (Lane b), 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The milk sample (Lane a) and supernatant samples (Lane b – g) were diluted with SDS sample buffer at ratio of 1:40 and 1:20, respectively. All the samples were fully reduced (i.e. all of the existing disulphide bonds in the samples were broken).

In both skim milk and WPE skim milk, the bands for α -lactalbumin, β -lactoglobulin and the caseins appear the same in all supernatant samples regardless of the NEM concentrations (Lanes b to g, Figure 4.5). This demonstrates that the distribution of the proteins between colloidal and serum phases was not affected by the presence of NEM in the unheated milks.

The quantitation of the band intensities provides the percentage of the each protein in the serum relative to the total amount of that protein in the milk, as is shown in Figure 4.6. In skim milk, the serum κ -casein, α -lactalbumin, β -lactoglobulin and other caseins combined were $14 \pm 3\%$, $86 \pm 7\%$, $94 \pm 4\%$ and $3.4 \pm 0.2\%$, respectively (Figure 4.6A). In WPE skim milk, the percentages were $17 \pm 2\%$ for κ -casein, $99 \pm 1\%$ for α -lactalbumin, $98 \pm 2\%$ for β -lactoglobulin and $6.5 \pm 0.6\%$ for other caseins combined (Figure 4.6B).

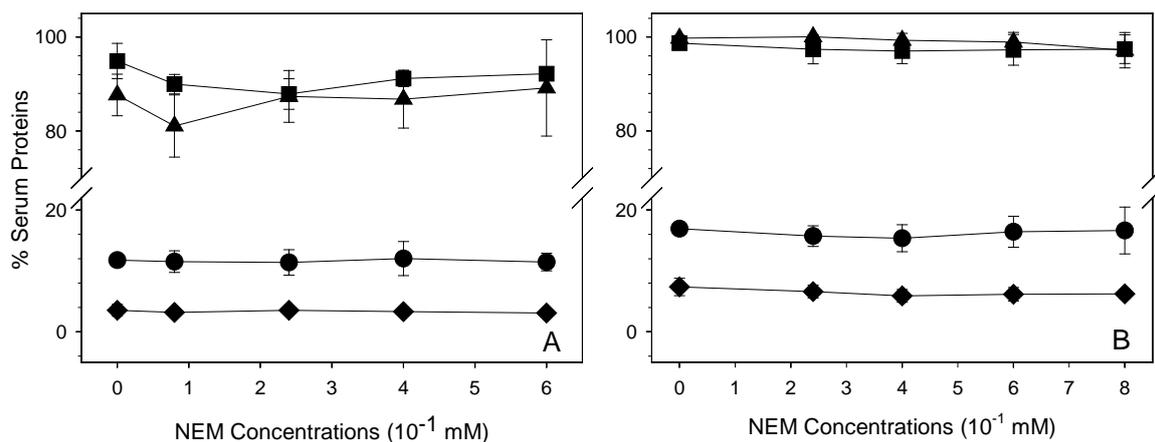


Figure 4.6: Effects of NEM concentrations on the percentage of individual proteins in the serum phase. α -lactalbumin (▲), β -lactoglobulin (■), κ -casein (●) and other caseins (◆). Unheated skim milk (A) and unheated WPE skim milk (B). Each data point is the average of two to four replicates. Error bars represent the standard deviation.

As adding NEM did not affect the distribution of the proteins, it did not significantly affect the size of the casein micelles in skim milks in both the skim milk and the WPE skim milk, except at the highest addition levels of in the WPE skim milk ($p > 0.05$, Figure 4.7). In both skim milk and WPE skim milk, the average size of the casein micelles was 207 ± 2 nm whereas, the size of the casein micelle in WPE skim milk with 0.8 mM added NEM was 210 ± 2 nm. The reason of this slight increase was unclear.

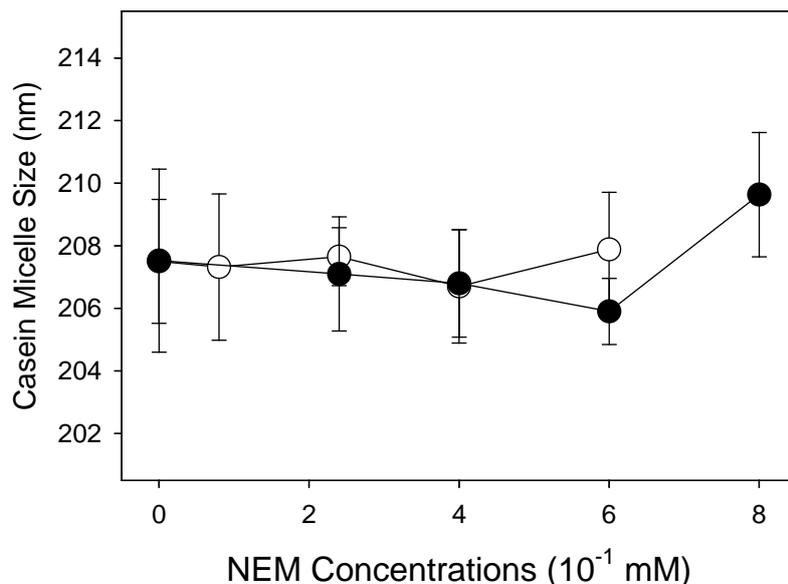


Figure 4.7: Effects of NEM concentrations on the diameter of the casein micelles in unheated skim milk (○) and WPE skim milk (●). Each data point is an average of two to four replicates. Error bars represent the standard deviation.

4.3.3 Effects of NEM concentrations in unheated milk on the rheological properties of the acid gels

Acid gels were prepared at 30 °C by acidifying (using 2% GDL) the unheated milks that had been treated with different concentrations of NEM for one hour (as described in Section 3.10.1). Figure 4.8 showed the increase of G' values over time on acidification of unheated skim milk and WPE skim milk with 0 and 0.6 mM added NEM. These gelation curves for the skim milk and WPE skim milk with 0.6 mM added NEM represented the typical change of G' values during the acid gelation of all milks treated with NEM. They showed that the gelation of unheated skim milk and WPE skim milk with added NEM had a similar profile to that of control milks (Figure 4.8).

Figure 4.8 also showed that the curves of acid gelation of skim milk and WPE skim milk had different shapes, gelation points and final G' values. The gelation point of skim milk acid gels occurred after ~ 60 min and of WPE skim milk gels occurred after ~ 71 min, corresponding to gelation pH values of ~ 5.0 and 4.9, respectively ($p > 0.05$). The final G' values of skim milk gels were 14 ± 1 Pa and of WPE skim milk gels were 10 ± 1 Pa ($p < 0.05$).

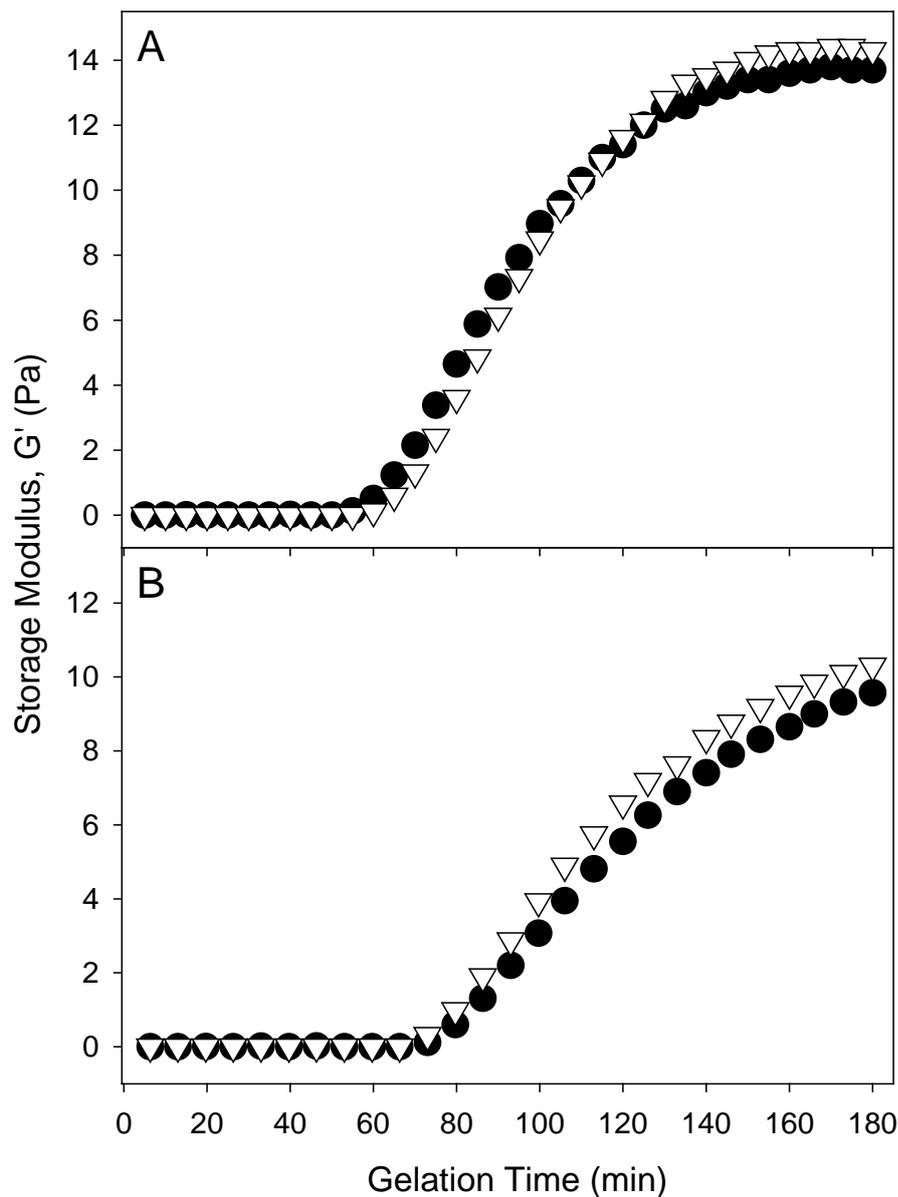


Figure 4.8: The change of the storage modulus on the formation of the acid gels over time. Unheated skim milk (A) and WPE skim milk (B) that had been treated with 0 mM (●) and 0.6 mM NEM (▽) were acidified by 2% of GDL at 30 °C.

The effect of different NEM concentrations on the final G' values at 30 °C was summarised in Figure 4.9. Overall, addition of NEM did not markedly change the final G' values of acid gels for both skim milk and WPE skim milk. The final G' values of skim milk only acid gels ranged from 13 ± 1 Pa to 18 ± 2 Pa ($p > 0.05$) whereas; those of WPE skim milk acid gels were 10 ± 2 Pa. The G'' values were also not affected by the addition of NEM as $\tan \delta$ values (G'' to G' ratio) were the same in all milks (Appendix 2, Figure A.1).

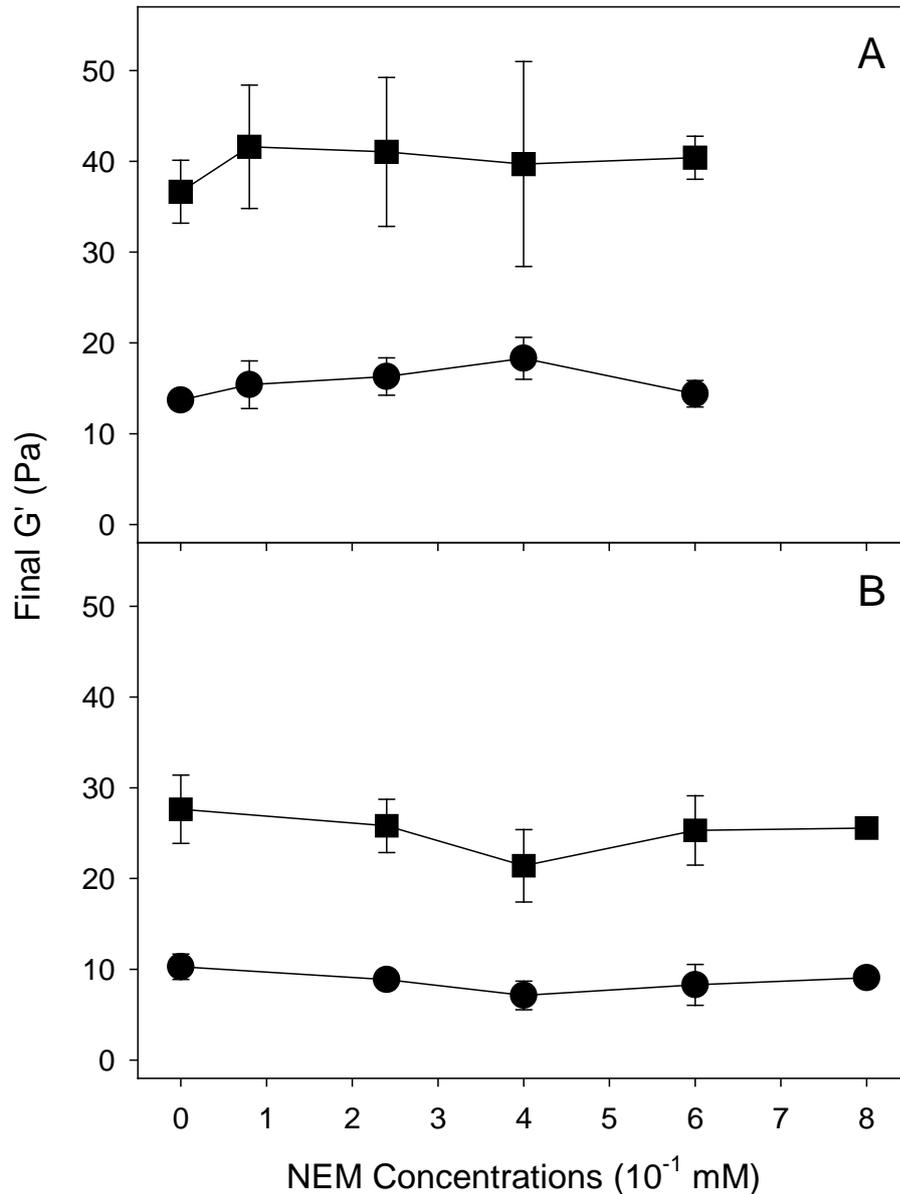


Figure 4.9: The effects of NEM concentrations on the final G' of acid gels made from skim milk (A) and WPE skim milk (B). ●, G' at 30 °C and ■, G' of gels after being cooled to 5 °C; Each data point is the average of two to four replicates. Error bars represent the standard deviation.

Once the acid gels were formed, the temperature of the gels was dropped gradually from 30 to 5 °C (1 °C min⁻¹). There was no significant difference between the G' values of control acid gels and those prepared from milks with added NEM, regardless of the NEM concentrations and whey protein concentrations ($p > 0.05$). Nevertheless, on average, the G' values of acid gels prepared from unheated skim milks with added NEM were about 5 Pa (i.e. 13%) higher than those of control acid gels (Figure 4.9). The G' values of acid gels made from unheated WPE skim milk with added NEM were mostly 2 Pa lower than those of control acid gels (7% decrease); except at 0.4 mM NEM, the G' values were 5 Pa lower than the control (18% decrease, Figure

4.9). This is consistent with the G' values of acid gels at 30 °C as the G' values of acid gels with 0.4 mM NEM were lower than the rest (Figure 4.9). The G' values at 5 °C were higher than those at 30 °C by ~ 20 Pa for both skim milk and WPE skim milk gels ($p < 0.05$).

At 5 °C, the acid gels were subjected to a constant rotation with a shear rate of 0.005 s⁻¹ and the shear stress and strain were monitored. Under constant shear rate, the shear stress values increased with strain to a maximum and then decreased. This maximum in shear stress was the yield point at which the gels broke. The strain and shear stress values at this yield point were called the yield strain and yield stress. Figure 4.10 showed the change of shear stress as a function of strain and Table 4.2 summarised the yield strain and yield stress values.

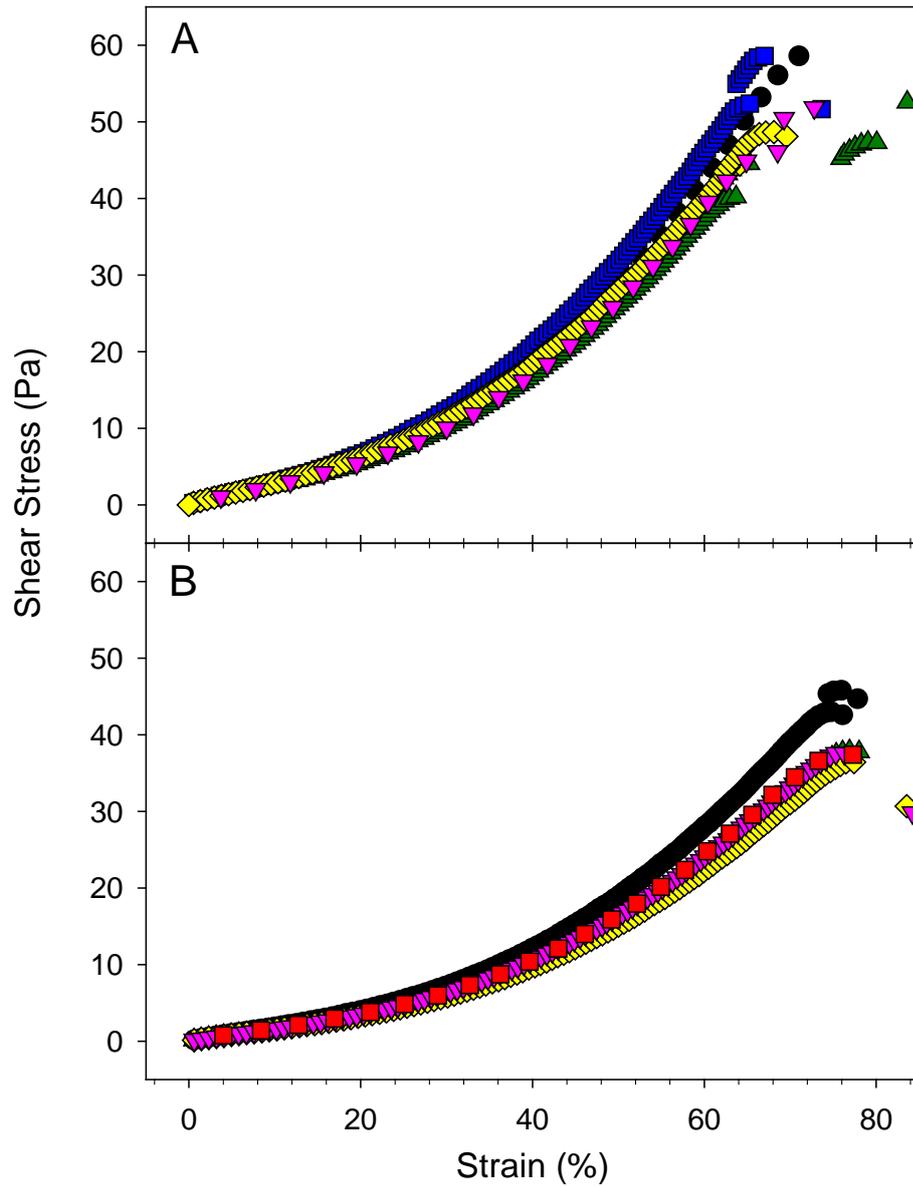


Figure 4.10: The change of shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^\circ\text{C}$ and analysed at $5 \text{ }^\circ\text{C}$. The acid gels were prepared from (A) unheated skim milk and (B) unheated WPE skim milk with different concentrations of added NEM. ●, 0 mM; ■, 0.08 mM; ▲, 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM; ■, 0.8 mM NEM. Each data point is the average of two to five replicates. Error bars are not shown for clarity.

Table 4.2: The values of the yield stress (Pa) and strain (%) of acid gels when subjected to constant shear rate of 0.005 s⁻¹ at 5 °C. Acid gels were prepared from unheated skim milk and WPE skim milk with added NEM of different concentrations. The values are the mean value ± standard deviation.

NEM Concentrations (mM)	Unheated skim milk		Unheated WPE skim milk	
	Yield Strain (%)	Yield Stress (Pa)	Yield Strain (%)	Yield Stress (Pa)
0	72.8 ± 1.6	58.6 ± 1.7	80.0 ± 1.9	45.8 ± 2.0
0.08	67.0 ± 0.0	58.6 ± 0.0	-	-
0.24	68.8 ± 3.7	52.9 ± 3.9	78.0 ± 4.0	37.8 ± 3.9
0.4	68.1 ± 11.2	48.7 ± 11.2	77.4 ± 3.3	36.4 ± 3.3
0.6	72.8 ± 3.1	51.9 ± 1.9	76.3 ± 5.1	37.6 ± 5.1
0.8	-	-	78.9 ± 1.2	37.4 ± 1.3

In unheated skim milk, adding NEM of 0.08 mM did not affect the yield stress value. However, the yield stress values of acid gels with NEM concentrations ≥ 0.24 mM were lower than those with lower NEM concentrations (Figure 4.10A and Table 4.2). The yield stress values of acid gels containing 0.24 and 0.6 mM NEM were 10% lower than those of the control gels. In addition, the average yield stress values of acid gels made from milks with 0.4 mM NEM was 16% lower than the average values of the control. The standard deviation of these yield stress values was high (± 11.2 Pa, Table 4.2); therefore the shear stress values of gels with 0.4 mM NEM cannot be considered as significantly different from those of gels with other NEM concentrations. The yield strain values were not significantly affected by the addition of NEM to unheated skim milk.

In unheated WPE skim milk, the yield stress values of acid gels containing NEM were significantly lower (by $\sim 17\%$) than those of control gels (Table 4.2 and Figure 4.10B). However there was no significant difference between the yield stress values of acid gels containing different concentrations of NEM. This is very interesting because even though the G' values of acid gels were not affected by the presence of NEM, the acid gels containing NEM were more prone to breakage at large deformation than those without NEM (Figure 4.10B). Similarly to acid skim-milk gel, adding NEM to unheated WPE skim milk did not affect the yield strain of the resulting acid gels.

4.3.4 Effects of NEM concentrations in unheated milks on the microstructure of acid gels

Confocal microscopic images of the set acid gels prepared from milk samples that were treated with 0 and 0.6 mM NEM are shown in Figure 4.11 and 4.12. As the addition of NEM did not affect the final G' values of acid gels, it also did not affect the appearance of the microstructure of the acid gels. The acid gel network of all the milk samples consisted of connected clusters (shown as green on the confocal images), regardless of the whey protein concentration and the NEM concentration. There were no apparent differences between the acid gels to distinguish control acid gels from acid gels prepared from NEM-treated milks.

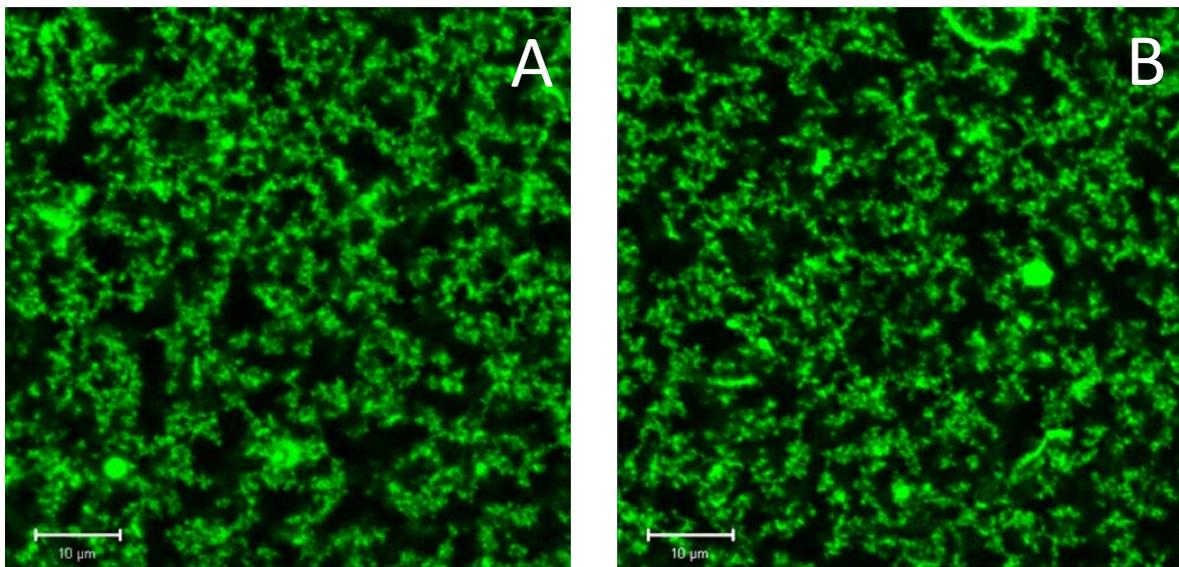


Figure 4.11: Confocal microstructural images of acid gels made from skim milk treated with 0 mM (A) and 0.6 mM (B) NEM. Scale bar is 10 µm.

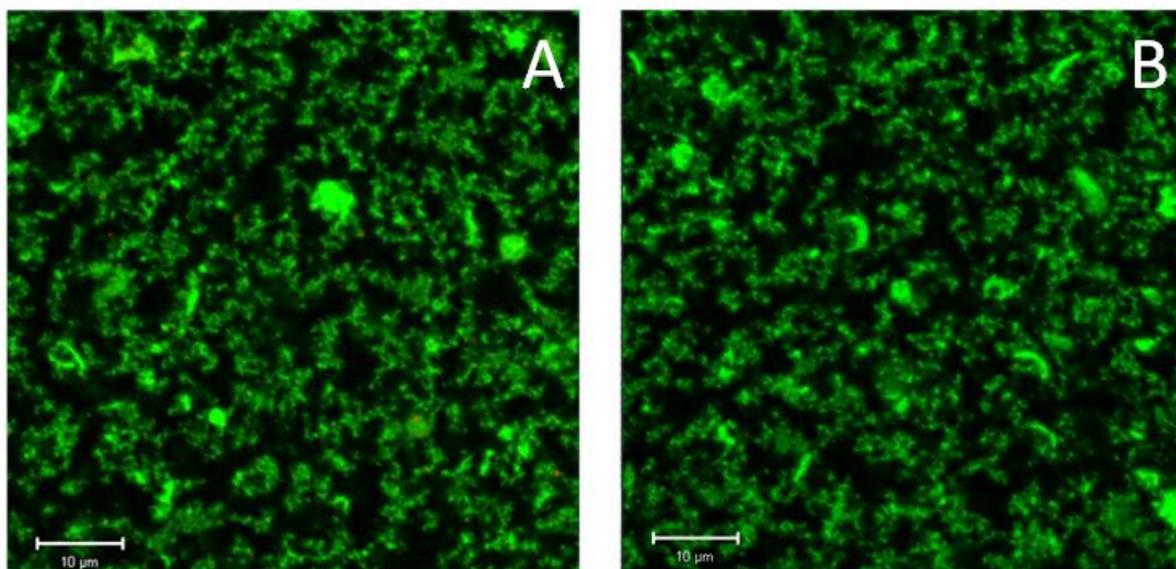


Figure 4.12: Confocal microstructural images of acid gels made from WPE skim milk treated with 0 mM (A) and 0.6 mM (B) NEM. Scale bar is 10 μm .

4.4 Discussion

Rasmussen (1992) reported that 10 - 15 % of the total κ -casein was present as monomers, with the rest as disulphide bonded oligomers in skim milk. In the current study, $\sim 20\%$ of κ -casein was found to be monomeric. This slightly higher level may be due to the different sources of milk and different techniques of detecting the monomeric κ -casein.

Theoretically, 100% of β -lactoglobulin and α -lactalbumin are native in unheated skim milks (Brownlow et al., 1997; Brew, 2003) and under SDS-PAGE conditions, all these whey proteins should appear as monomers. However the skim milk powder used in this study had been exposed to heat ($\sim 70 - 72$ °C) during the processes of pasteurisation and drying. Thus it is possible that thiol-disulphide exchange reactions occurred during these processes, leading to the interactions between α -lactalbumin, β -lactoglobulin and κ -casein. Consequently, there may already be a small amount of disulphide bonded α -lactalbumin and/or β -lactoglobulin present.

Similarly, due to the process of making skim milk powder, the interactions between whey proteins and κ -casein may lead to the association of the whey proteins with the casein micelles (Smits & van Brouwershaven, 1980; Dalglish, 1990). Hence the proportion of whey protein in the serum was < 100 %, as observed in unheated milks in this study (Figure 4.6). Whey proteins that are associated with the casein micelle can be found in skim milk powder but not in whey protein isolate. Therefore, even though the absolute amount of colloidal whey protein in both skim milk and WPE skim milk is the same, the proportion of colloidal whey protein in skim milk would be higher than in the WPE skim milk (Figure 4.6).

4.4.1 Effects of NEM concentrations on protein interactions and distributions between serum and colloidal phases in unheated milks

In bovine milk, NEM can interact with the free thiol groups of β -lactoglobulin and bovine serum albumin. As β -lactoglobulin is the main source of free thiol groups, only the effect of blocking the thiol of β -lactoglobulin will be discussed.

At 20 °C, the β -lactoglobulin structure is native, hence whether or not the thiol groups were blocked by addition of NEM; the thiol groups were expected to be buried in the folded structure. As a result, thiol-disulphide exchange reactions between β -lactoglobulin, κ -casein and α -lactalbumin should not occur, as was confirmed in Figure 4.4, which showed a low percentage of intermolecular disulphide-linked β -lactoglobulin and α -lactalbumin. In addition, the levels of proteins in the serum phase were not affected by addition of NEM to unheated milks (Figure 4.6 and 4.7). This also confirmed a lack of interactions between the whey proteins and κ -casein because if there were interactions, the level of serum whey protein would be decreased (Oldfield et al., 1998a; Anema, 2007). Hence under non-heating conditions, the NEM-treated skim milk and WPE skim milk were expected to have the same properties of milks without NEM (control samples).

4.4.2 Effects of NEM concentrations on rheological and microstructural properties of acid gels made from unheated milks

During acidification of unheated skim milk, the charges that maintain the κ -casein brushes (on the casein micelles) are proposed to progressively neutralised leading to their shrinkage and then collapsing (Tuinier & De Kruif, 2002). This leads to the destabilisation of the casein micelles. The casein micelles then come together to aggregate, forming chains and clusters. The clusters and chains subsequently link together to form a network (De Kruif, 1997; Lucey & Singh, 1998), as observed in Figure 4.11 and Figure 4.12. This leads to the increase in G' values over time during gelation, as shown in Figure 4.8. The network structure of acid gels made from unheated skim milk consisted of connected-chains and clusters of aggregating casein micelles and the native whey protein acted as inactive fillers (Lucey & Singh, 1998; Schorsch et al., 2001). Therefore, the dynamic moduli (storage and loss modulus) of the acid gels (during and after formation at 30 °C without deformation) were postulated to be determined by the number and strength of the bonds between the casein particles, the structure of the particles and the spatial distribution of the strands made up by these casein particles (Roefs, De Groot-Mostert & van Vliet, 1990; Roefs & van Vliet, 1990).

In the present study, acid gels prepared from unheated skim milk had low final G' values (typically < 20 Pa), as also observed in previous reports (Lucey et al., 1998; Vasbinder et al.,

2003). The acid gels prepared from WPE skim milk had lower gelation pH and final G' values than those prepared from skim milk, regardless of NEM concentrations (Figure 4.8 and 4.9). In WPE skim milk, the high concentration of whey proteins may sterically hinder the approach of, and the connections between, the casein micelles. Nevertheless, it should be noted that the difference in G' values between unheated skim milk only gels and WPE skim milk gels was small, especially compared to when the milks were heated.

The unheated NEM-treated milks were expected to produce acid gels that had similar moduli as gels prepared from control milks due to the lack of interactions between whey proteins and κ -casein (Figure 4.4). The rheological results presented in Figure 4.8 and Figure 4.9 showed that this hypothesis was correct. The similar microstructure of the acid gels made from skim milks without and with added NEM (Figure 4.11 and Figure 4.12) further supported the hypothesis and clearly showed the gel network consisted of chains and clusters of aggregating casein micelles.

As mentioned earlier, the milks used in this study may contain low levels of denatured β -lactoglobulin and α -lactalbumin that were already involved in inter-molecular disulphide bonds and may contain exposed thiol groups. Attachment of NEM onto those thiol groups may insert a bulk hindrance on such a protein. This can sterically hinder the interactions between the proteins during acidification. As a result, NEM-containing gels may consist of fewer connections and may be broken more readily than control gels, as observed when comparing the yield properties (Figure 4.10).

4.5 Conclusions

Under non-heating conditions, the addition of NEM to skim milk and WPE skim milk resulted in the blocking of the thiol groups of β -lactoglobulin. This blocking had no effect on the distribution of caseins and whey proteins between serum and colloidal phases, as was expected. As a result, when the milks with added NEM were acidified to form acid gels, the firmness and the microstructure of those acid gels were similar to those of gels made from milks without NEM. However, since low levels of denatured whey proteins with exposed thiol groups may exist, blocking those thiol groups with NEM may decrease the degree of connections between the proteins in acid gels containing NEM. Hence these gels had slightly lower yield stresses than control gels.

The findings in this chapter provided the control data to compare with experiments for NEM-treated skim milks that were subsequently heated at 80 °C for 30 min. This chapter confirmed that addition of NEM without heat treatment has no significant effect on the protein interactions

that can occur in milk, and limited effects on the properties of acid gels prepared from the treated milks.

Chapter 5 - Effects of heating milks in the presence of NEM on the milk properties

5.1 Introduction

Adding NEM to unheated milks blocked the free thiol groups of native β -lactoglobulin and did not influence the intermolecular disulphide bonding between the native whey proteins and caseins proteins (Chapter 4). Since blocking the thiol groups of β -lactoglobulin was often used to prevent the thiol-disulphide exchange reactions between the milk proteins that can occur during heating milk, the following study investigated the effects of heating milks that had been treated with low concentrations of NEM on the protein interactions and the properties of the resulting acid gels.

It has been demonstrated that intermolecular disulphide bonds formed between the proteins are important in determining the firmness of acid gels prepared from the heated milks (Davies et al., 1978; Lucey et al., 1998; Guyomarc'h, Queguiner, Law, Horne & Dalgleish, 2003b; Famelart et al., 2004). There have been many studies investigating the effect of completely inhibiting the formation of intermolecular disulphide bonds between the proteins in milk or model systems during heating on the rheological properties of gels formed from the systems studied (Sawyer, 1967; Hashizume & Sato, 1988; Matsudomi et al., 1991; Xiong et al., 1993; Goddard, 1996; Hoffmann & van Mil, 1997; Havea et al., 2009). The details of the previous findings were summarised in Section 2.12.3.

In general, the previous studies used excess levels of thiol blocking reagent such as NEM to completely block all of the available free thiol groups in the protein/milk systems. It was proposed that upon the elimination of intermolecular disulphide bonds, non-covalent interactions between the proteins became dominant and the total degree of connections may be higher in systems without disulphide bonds than in systems with disulphide bonds (Matsudomi et al., 1991; Hoffmann & van Mil, 1997; Havea et al., 2009). Consequently, heat-induced whey protein gels prepared from NEM-containing systems exhibited higher G' values compared to the control heat-induced gels (Matsudomi et al., 1991; Havea et al., 2009). In contrast, other researchers have found that the acid-heat-induced gels made from skim milk with added NEM had lower G' than the gels made from control milk (Hashizume & Sato, 1988; Goddard, 1996). Non-covalent interactions were stated as being responsible for the formation of the gel network (Goddard, 1996).

There are no detailed reports on the effects of very low concentrations of NEM (i.e. below the level required to block all the available thiol groups in milk) on the interactions between the proteins and on the properties of acid gels that were prepared by acidifying the treated and subsequently heated milk. In the experiments reported in this chapter, the effects of heating milks with added NEM on the interactions and distributions of the milk proteins between the colloidal and serum phases were investigated using gel electrophoresis. NEM concentrations ranging from 0.08 to 0.8 mM was added to skim milk and WPE skim milk. This range covered a ratio of NEM to free thiol groups from below to above 1. Subsequently, acid gels were prepared from the treated milks and the firmness and the microstructure of the acid gels were examined using rheological measurements and confocal microscopy, respectively.

5.2 Materials and methods

Skim milk and WPE skim milk were prepared as described in Section 3.1.1.

Diluted NEM (1% w/v) was added to give NEM concentrations of 0.08, 0.24, 0.4 and 0.6 mM in skim milk and 0.24, 0.4, 0.6 and 0.8 mM in WPE skim milk. The ratio of NEM to thiol groups of β -lactoglobulin in skim milk and WPE skim milk was calculated based on the average composition of skim milk and whey protein isolate. The ratio ranged from 0.4 to 3.1 mol NEM per mol β -lactoglobulin in skim milk and from 0.4 to 1.3 mol NEM per mole β -lactoglobulin in WPE skim milk (Table 4.1).

After adding the NEM to the milk, the lid of the containers was tightly closed to minimise oxidation. The treated milk was shaken on a vortex mixer for 10 s and then left to react at 20 ± 1 °C in a thermostatically controlled water bath for one hour. After 1 h, subsamples of the treated milks were heated at 80 ± 0.1 °C for 30 min as described in Section 3.3. After heating, the samples in the vials were rapidly cooled in cold tap water for ~ 2 min until they were at ambient temperature and were then analysed immediately.

Analyses involved examining the level of α -lactalbumin and β -lactoglobulin remaining native after the treatment, the level of proteins participating in disulphide bonds and the distribution of the proteins between the colloidal and serum phases. This was done using SDS-PAGE (as described in Section 3.6). The size of the casein micelles was measured using dynamic light scattering (as described in Section 3.8).

After heat treatment, subsamples of the milks were acidified using 2% GDL to form acid gels. The rheological properties of the milks during acidification were monitored. Especially in this study, the rheological properties of acidified **WPE skim milk** were examined using a **cup and bob geometry**. The reason was that when using a cone and plate geometry, the G' values of the

acidified WPE skim milk treated with 0.8 mM NEM, after a period of increasing, decreased suddenly. This indicated that the formation of the acid gels was being disrupted (Appendix 3, Figure A.5). This sudden decrease in G' did not occur when using a cup and bob geometry and it was unclear why the breakage of gels was only observed in the cone and plate geometry and not the cup and bob geometry.

In addition, the microstructure of the set gels was also investigated. The details of the rheological and microstructural methods can be found in Sections 3.10.2 and 3.10.3.

5.3 Results

5.3.1 Effects of heating NEM-treated milks on protein denaturation and interactions

Since the reaction between NEM and β -lactoglobulin leads to a decrease in the number of thiol groups available for thiol-disulphide exchange reactions, less α -lactalbumin and β -lactoglobulin were expected to be irreversibly denatured, thus a higher level of whey protein was expected to be native. Figure 5.1 showed the SDS-PAGE patterns of β -lactoglobulin and α -lactalbumin remaining in their native states (i.e. proteins remaining soluble at pH 4.6, determined as described in Section 3.5) in the heated milks. The bands corresponding to β -lactoglobulin and α -lactalbumin were identified by comparison with the SDS-PAGE patterns reported previously (Havea et al., 1998; Patel, 2007) for whey protein solutions analysed using similar methods. The bands were marked appropriately on the left-hand side of the control samples (Lane a) on the SDS-PAGE patterns (Figure 5.1).

The intensities of β -lactoglobulin and α -lactalbumin bands in control heated milks (Lane b) were markedly lower than those of the whey proteins in unheated milks (Lane a). The intensities of these protein bands progressively increased with the increase of the NEM concentration (Lanes c to g). The proportion of native α -lactalbumin and β -lactoglobulin as a function of NEM concentration is summarised in Figure 5.2.

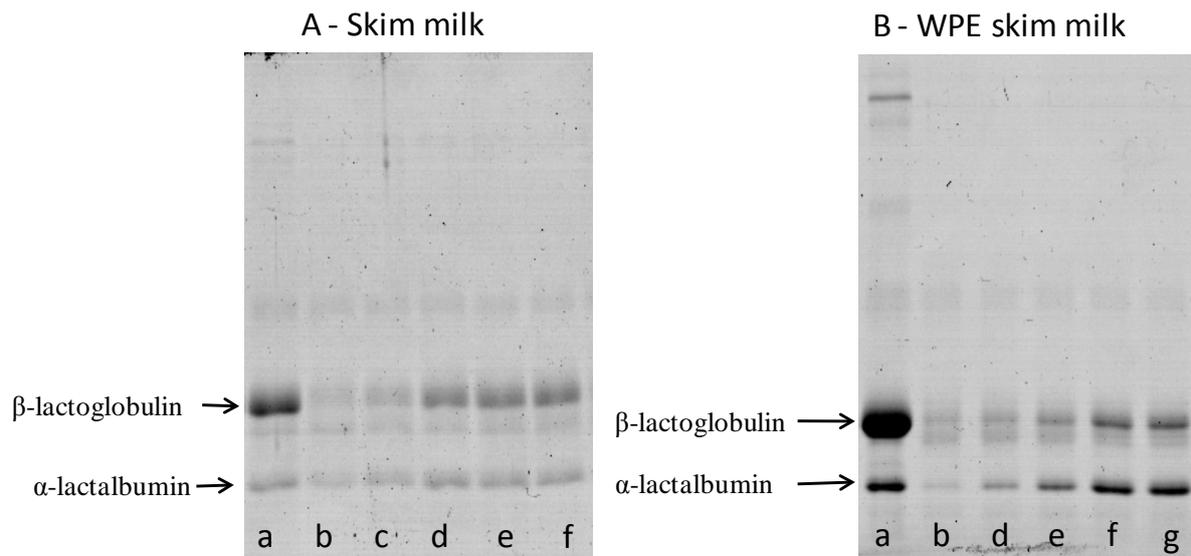


Figure 5.1: SDS-PAGE patterns of proteins remaining soluble after acid precipitation (i.e. native proteins) of skim milk and WPE skim milk that had been heated in the presence of NEM. Control sample (i.e. native protein in unheated milks, Lane a), control heated milks (Lane b) and milk heated in the presence of NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The samples were diluted with SDS sample buffer at a ratio of 1 to 10 and were fully reduced.

In heated skim milk without NEM, there were $37 \pm 10\%$ α -lactalbumin and $16 \pm 7\%$ β -lactoglobulin that remained in its native state (Figure 5.2A). These values agree with previous reports in which $\sim 30\%$ of α -lactalbumin and $\sim 20\%$ of β -lactoglobulin remained as native protein after skim milk was heated at $80\text{ }^{\circ}\text{C}$ for 30 min (Anema & Li, 2003b). As the concentration of added NEM was raised up to 0.24 mM, the proportion of native α -lactalbumin and β -lactoglobulin increased significantly up to $85 \pm 9\%$ and $41 \pm 7\%$, respectively ($p < 0.05$). Higher concentrations of NEM did not show any significant increase in the native forms of the proteins ($p > 0.05$, Figure 5.2A).

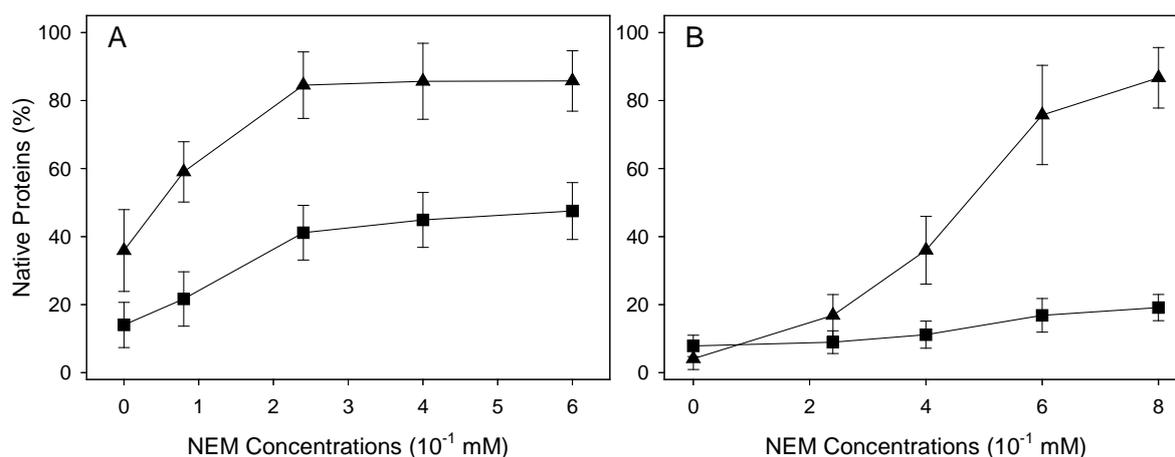


Figure 5.2: Effects of NEM concentrations on the percentage of individual whey protein that remained native after heating skim milk (A) and WPE skim milk (B) in the presence of NEM. ▲, α-lactalbumin; ■, β-lactoglobulin. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

The percentages of native α-lactalbumin and β-lactoglobulin in heated WPE skim milk increased significantly ($p < 0.05$, Figure 5.2B) with increasing NEM concentrations. The percentage of native α-lactalbumin increased from $4.7 \pm 3.4\%$ to $87 \pm 9\%$ while the percentage of native β-lactoglobulin increased from $7.9 \pm 3.1\%$ to $19 \pm 4\%$. In both skim milks (control and WPE) with added NEM, the percentage of native α-lactalbumin was always higher than the percentage of β-lactoglobulin (Figure 5.2). The results also showed that the percentage of native α-lactalbumin was at the highest as soon as the NEM to thiol ratio was above 1 (Table 4.1).

As mentioned above, blocking thiol groups of β-lactoglobulin may prevent the whey proteins (and ultimately κ-casein) from participating in thiol-disulphide exchange reactions, thus the proportion of proteins involved in intermolecular disulphide bonds was expected to decrease. Figure 5.3 showed the SDS-PAGE patterns of the major proteins in milks under non-reducing condition. The proteins bands were identified by comparison with the SDS-PAGE patterns previously reported by Anema and Klostermeyer (1997) and were marked on the left-hand side of the control sample (Lane a). While the band intensities in the control samples (Lane a) represented the total amount of proteins in the milk, the band intensities in the other samples (Lanes b to g) represent the amount of proteins that did not participate in disulphide interactions. These proteins can either be monomeric or participate in non-covalent interactions.

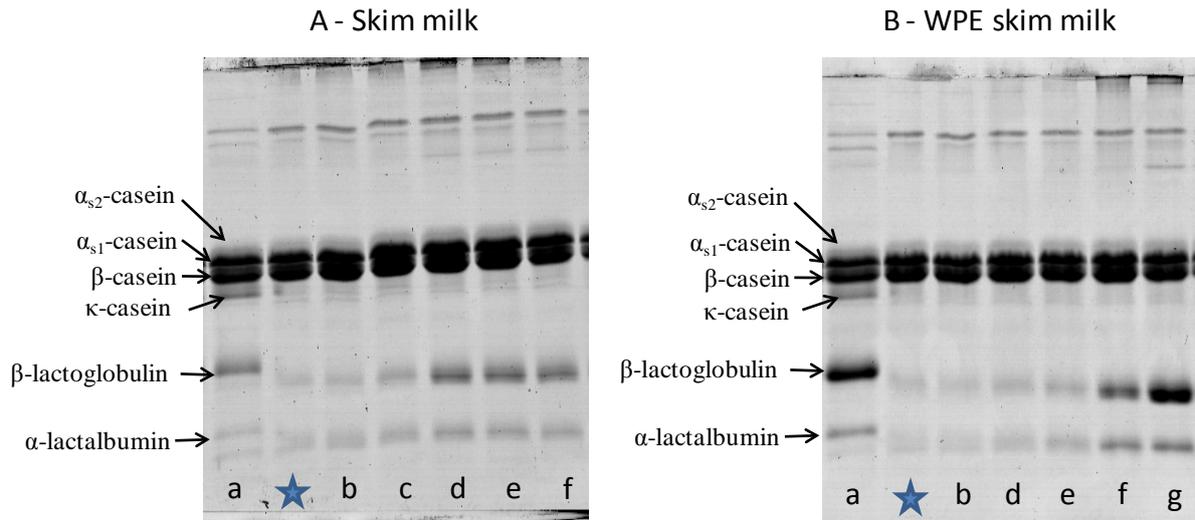


Figure 5.3: SDS-PAGE patterns of skim milk (A) and WPE skim milk (B) heated in the presence of NEM. Control samples (i.e. control untreated milks, Lane a), milks heated without added NEM (Lane b) and milk heated with added NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The milk samples were diluted with SDS sample buffer at ratio of 1 to 40 and were not reduced except for the control samples being fully reduced. Blue star was the transition lane between reducing and non-reducing conditions.

The intensities of whey protein and κ -casein bands were markedly low in control heated milks (Lane b, Figure 5.3). Heating milks in the presence of NEM did not cause any visual change in the κ -casein band intensity. In contrast, in both skim milk and WPE skim milk, the intensities of whey protein bands increased with the increase of NEM concentrations (Lanes c to g, Figure 5.3). This indicated that adding NEM affected the proportion of α -lactalbumin and β -lactoglobulin involved in disulphide bonds but did not affect κ -casein. The band intensities were integrated and the percentage of disulphide-linked protein over the total of each protein in milk is presented in Figure 5.4.

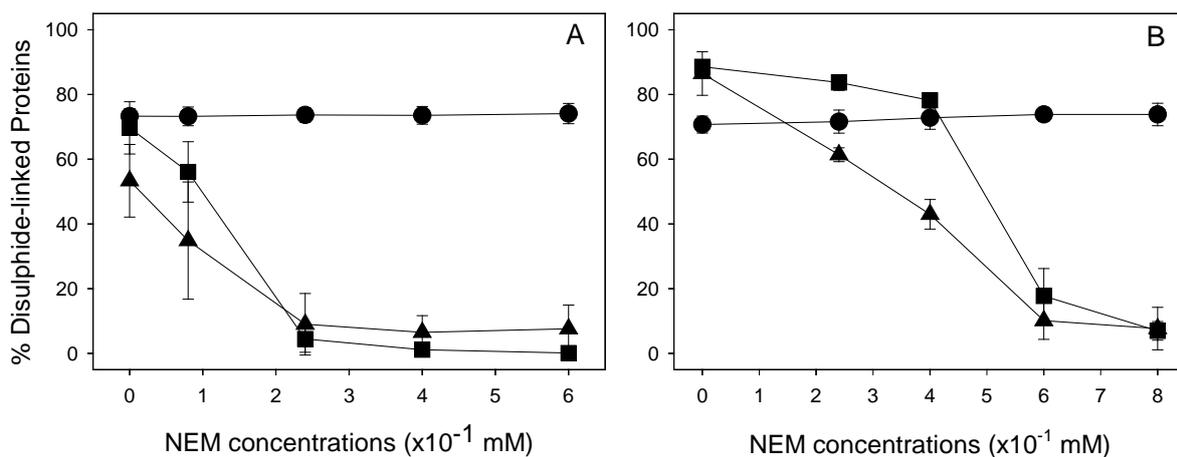


Figure 5.4: Effects of NEM concentrations on the percentage of individual protein participating in intermolecular disulphide bonds in heated skim milk (A) and WPE skim milk (B). ▲, α-lactalbumin; ■, β-lactoglobulin; ●, κ-casein; each data point is the average of two to four replicates. The error bars represent the standard deviation.

In skim milk, the percentage of disulphide-linked whey protein (combination of disulphide-linked α-lactalbumin and β-lactoglobulin) decreased significantly from $53 \pm 11\%$ in untreated heated milk to $7.6 \pm 7.0\%$ at 0.24 mM NEM ($p < 0.05$). At higher NEM concentrations, very low levels of α-lactalbumin and β-lactoglobulin were involved in intermolecular disulphide bonds. The percentage of κ-casein involved in disulphide bonds was not affected by the presence of NEM and remained at $74 \pm 3\%$ (Figure 5.4A).

In WPE skim milk, the percentage of disulphide-linked α-lactalbumin decreased significantly down to $6.5 \pm 5.1\%$ when NEM concentrations increased from 0 to 0.6 mM ($p < 0.05$, Figure 5.4B). The percentage of disulphide-linked β-lactoglobulin decreased slightly from $88.6 \pm 1.7\%$ to $78.2\% \pm 2.4\%$ as NEM concentrations increased from 0 to 0.4 mM and then decreased significantly to $17.7 \pm 8.5\%$ when the NEM concentration increased to 0.6 mM. At 0.8 mM NEM, very low levels of α-lactalbumin and β-lactoglobulin participated in intermolecular disulphide bonds (Figure 5.4B). As observed in skim milk, the percentage of disulphide-linked κ-casein was not affected by the addition of NEM.

5.3.2 Effects of heating NEM-treated milks on the protein distribution between colloidal and serum phases and on the casein micelle size

After centrifugation of treated milks, the obtained supernatants were analysed using reduced SDS-PAGE to determine the level of proteins in the serum phase. SDS-PAGE patterns of skim milk supernatants showed that the intensity of monomeric κ-casein band decreased as the NEM concentrations increased to levels ≥ 0.24 mM whereas the bands of other caseins did not change

in intensity upon addition of NEM. The intensities of whey protein bands also increased at NEM concentrations ≥ 0.24 mM (Figure 5.5A).

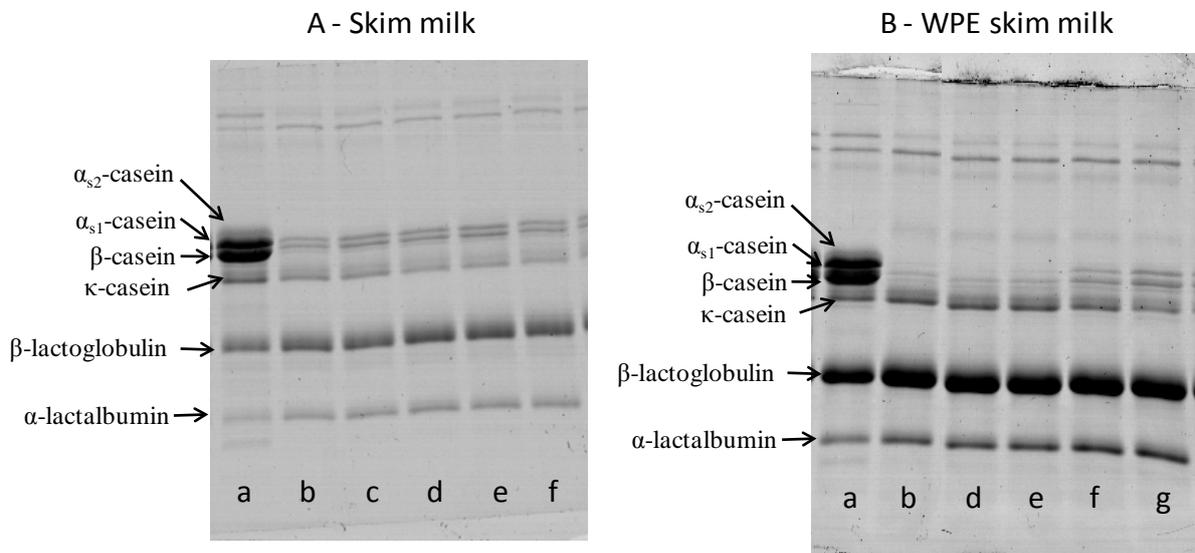


Figure 5.5: SDS-PAGE patterns of supernatant samples obtained from centrifugation of skim milk (A) and WPE skim milk (B) that were heated (80 °C, 30 min) in the presence of NEM. Control samples (i.e. control untreated milks, Lane a), supernatant of milks heated without added NEM (Lane b) and with added NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The milk sample (Lane a) and supernatant samples (Lanes b to g) were diluted with SDS sample buffer at ratio of 1:40 and 1:20, respectively. All the samples were fully reduced.

In the SDS-PAGE pattern of WPE skim milk supernatants, the monomeric κ -casein band decreased in intensity whereas the α_s - and β -casein bands increased in intensity at NEM concentrations ≥ 0.6 mM. There was no pronounced effect of heating NEM-treated WPE milk on the level of β -lactoglobulin in the serum phase, whereas the level of serum α -lactalbumin increased as the NEM concentration increased. The effects of NEM concentrations on the percentage of proteins in the serum phase were determined based on the integration of the protein band intensities and the results are summarised in Figure 5.6.

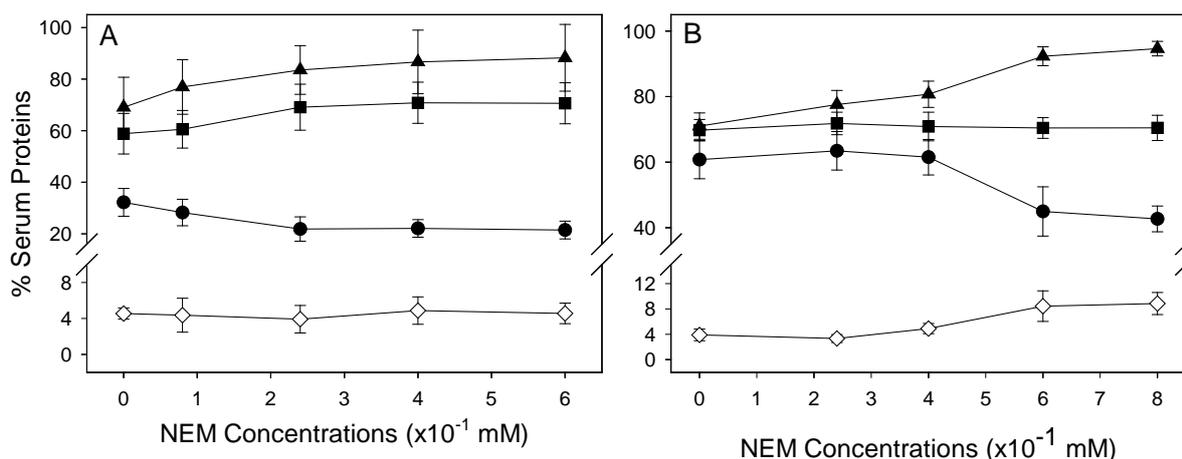


Figure 5.6: Effects of NEM concentrations on the percentage of serum protein in skim milk (A) and WPE skim milk (B). Milks were heated at 80 °C for 30 min in the presence of NEM. ▲, α -lactalbumin; ■, β -lactoglobulin; ●, κ -casein; ◇, other casein proteins. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

In skim milk, as the NEM concentration increased up to 0.24 mM, the percentage of serum α -lactalbumin increased from $69 \pm 12\%$ to $84 \pm 9\%$ ($p < 0.05$) while the percentage of serum β -lactoglobulin increased from $59 \pm 8\%$ to $69 \pm 9\%$ ($p < 0.05$, Figure 5.6A). In contrast, the percentage of serum κ -casein decreased from $32 \pm 5\%$ to $22 \pm 5\%$ ($p < 0.05$). As the NEM concentration was increased further, the percentage of serum α -lactalbumin, β -lactoglobulin and κ -casein did not change anymore. The percentage of serum α_s - and β -casein was not affected by the addition of NEM (Figure 5.6A).

In WPE skim milk, as NEM concentrations increased from 0 to 0.8 mM, the percentage of serum α -lactalbumin increased significantly from $71 \pm 4\%$ to $95 \pm 2\%$ ($p < 0.05$) while the percentage of serum β -lactoglobulin was not affected ($p > 0.05$, Figure 5.6B). On average, the percentage of serum β -lactoglobulin in different milks was $71 \pm 4\%$. The percentage of serum κ -casein did not change when the added NEM concentration was ≤ 0.4 mM and then decreased significantly from $62 \pm 5\%$ to $43 \pm 4\%$ after addition of 0.6 mM NEM ($p < 0.05$). This percentage showed very little change when higher concentrations of NEM was added (Figure 5.6B). In contrast to κ -casein, the percentage of serum α_s - and β -casein increased slightly but significantly from $3 \pm 1\%$ to $8 \pm 2\%$ when the NEM concentrations increased between 0.24 and 0.6 mM ($p < 0.05$, Figure 5.6B).

5.3.3 Effects of heating NEM-treated milks on the casein micelle size

The size of the casein micelles in heated skim milks increased from 190 ± 1 nm to 198 ± 2 nm as the NEM concentration increased up to 0.4 mM ($p < 0.05$, Figure 5.7A). At higher concentrations

of NEM, the size did not change further. The polydispersity index was found to increase with increasing concentrations of NEM as well ($p < 0.05$, Figure 5.7B).

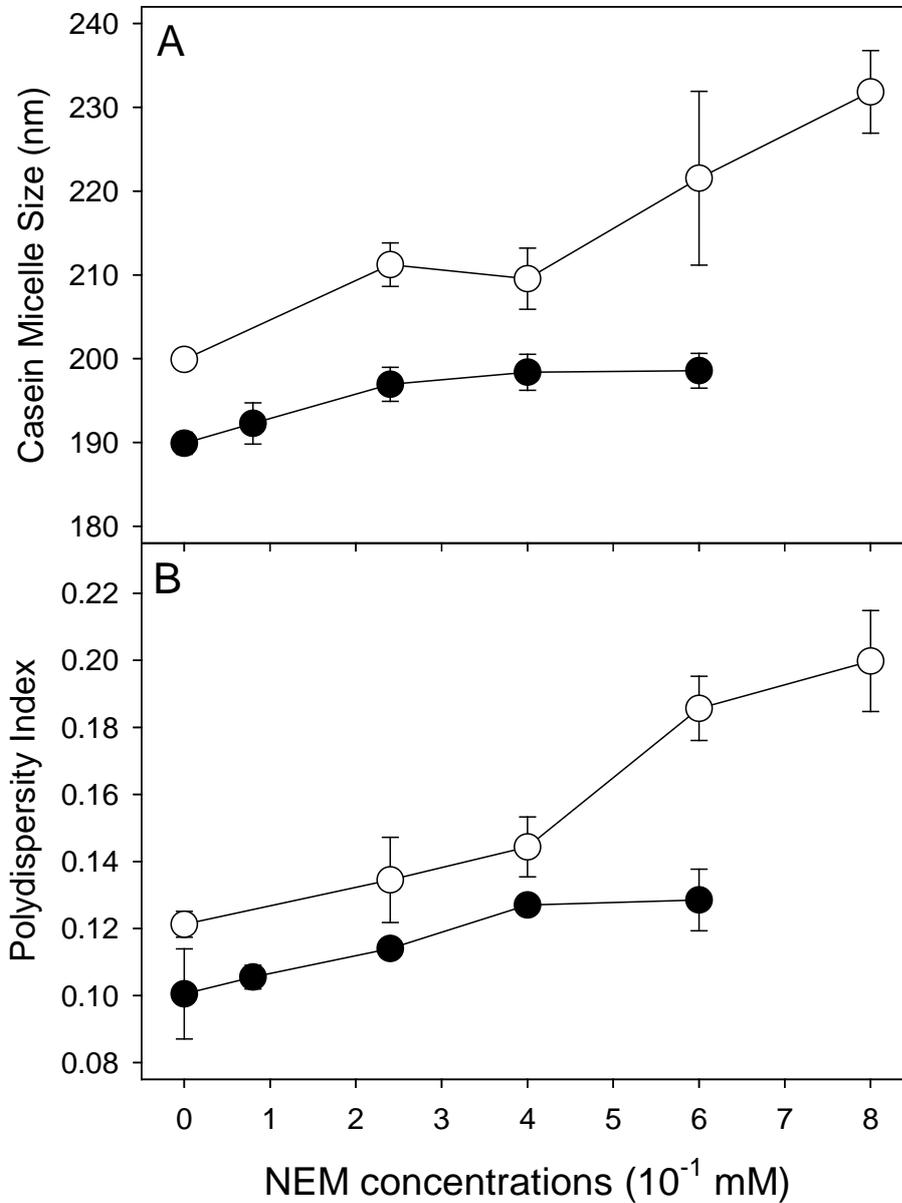


Figure 5.7: Effects of NEM concentrations on the size (A) and the polydispersity index (B) of casein micelles in skim milk (●) and WPE skim milk (○). Milks were treated with NEM prior to heat treatment. Each data point is an average of two to four replicates. The error bars represent the standard deviation.

For the WPE skim milks, the size of the casein micelles increased significantly from 200 ± 1 to 232 ± 5 nm with the increase in NEM concentrations ($p < 0.05$, Figure 5.7A). When NEM was added prior to heating, the increase in the casein micelle size was more pronounced in WPE

skim milk than in only skim milk. The polydispersity index increased progressively with NEM concentrations but the increase was only significant when the NEM concentration increased from 0.4 to 0.6 mM (Figure 5.7B).

5.3.4 Effects of heating NEM-treated milks on the rheological properties of acid gels

Acid gels were made by acidifying the milk samples with 2% GDL over a 3 h period at 30 °C. The effect of 0.6 mM NEM on the change of G' values during the formation of acid gels is shown in Figure 5.8.

When skim milk heated in the presence of 0.6 mM NEM was acidified, the increasing values of G' during gelation followed the same trends as that of the control heated skim milk, but at slightly lower G' values. Nevertheless, the heated skim milks (without and with added NEM) gelled at similar pH (i.e. gelation point) (Figure 5.8A). By contrast, when the WPE skim milk heated in the presence of 0.6 mM NEM was acidified, the changing values of G' followed the same trend as that of the control heated WPE skim milk, but the gelation point occurred earlier and the G' increased to higher values than that of control sample (Figure 5.8B). A summary of the effects of NEM on the gelation points and the final G' values of the acid gels is shown in Figure 5.9.

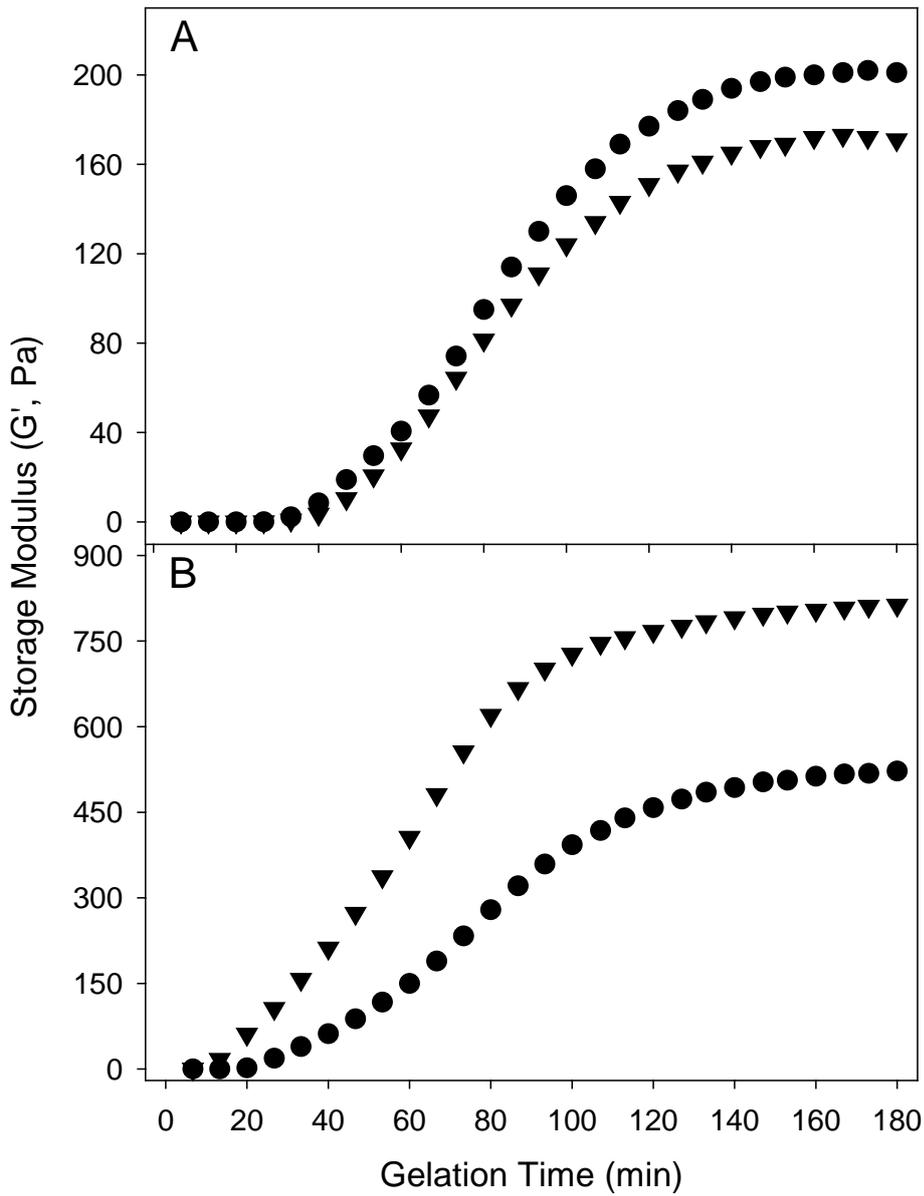


Figure 5.8: Typical changes of the storage modulus during the formation of acid gels after 2% GDL was added to skim milk (A) and WPE skim milk (B). ●, 0 mM NEM and ▼, 0.6 mM NEM.

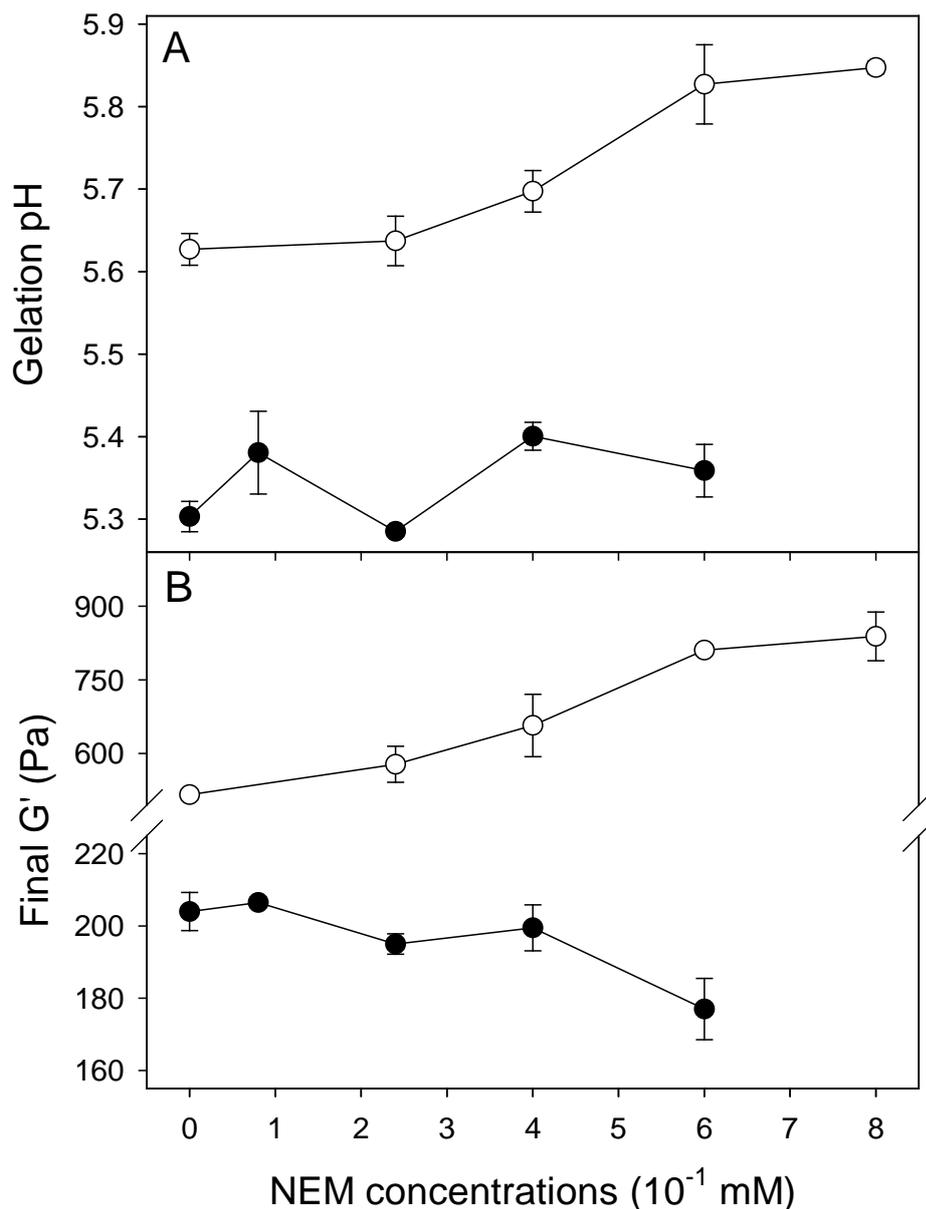


Figure 5.9: Effects of NEM concentrations on the gelation pH (A) and the final G' values (B) of acid gels made from skim milk (●) and WPE skim milk (○) that were heated in the presence of NEM. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

The gelation pH of skim milk that had been heated in the presence of NEM did not change with the NEM concentrations. The gelation pH values fluctuated between pH 5.3 and 5.4 (Figure 5.9A). The gelation pH of WPE skim milk heated in the presence of NEM increased significantly from pH 5.63 ± 0.02 to pH 5.85 ± 0.01 with an increase in NEM concentrations (Figure 5.9A).

The final G' values (ranging from 195 to 201 Pa), in skim milk heated with NEM, were not affected when NEM concentrations were ≤ 0.4 mM and decreased significantly to 177 ± 9 Pa at

0.6 mM NEM ($p < 0.05$, Figure 5.9B). The final G' values of acid gels made from WPE skim milk heated with NEM increased significantly from 516 ± 9 Pa to 838 ± 50 Pa when NEM concentrations increased from 0 to 0.8 mM ($p < 0.05$, Figure 5.9B).

The changes of $\tan \delta$ values (G''/G') during formation of the acid gels are presented in Figure 5.10. In skim milk, the $\tan \delta$ values of acid gels made from skim milk heated with NEM were slightly lower than those of gels made from milk heated without NEM during the early stages of gelation (< 80 min, Figure 5.10A). After which, the $\tan \delta$ values of both gels were similar (within standard deviations, Figure 5.10A).

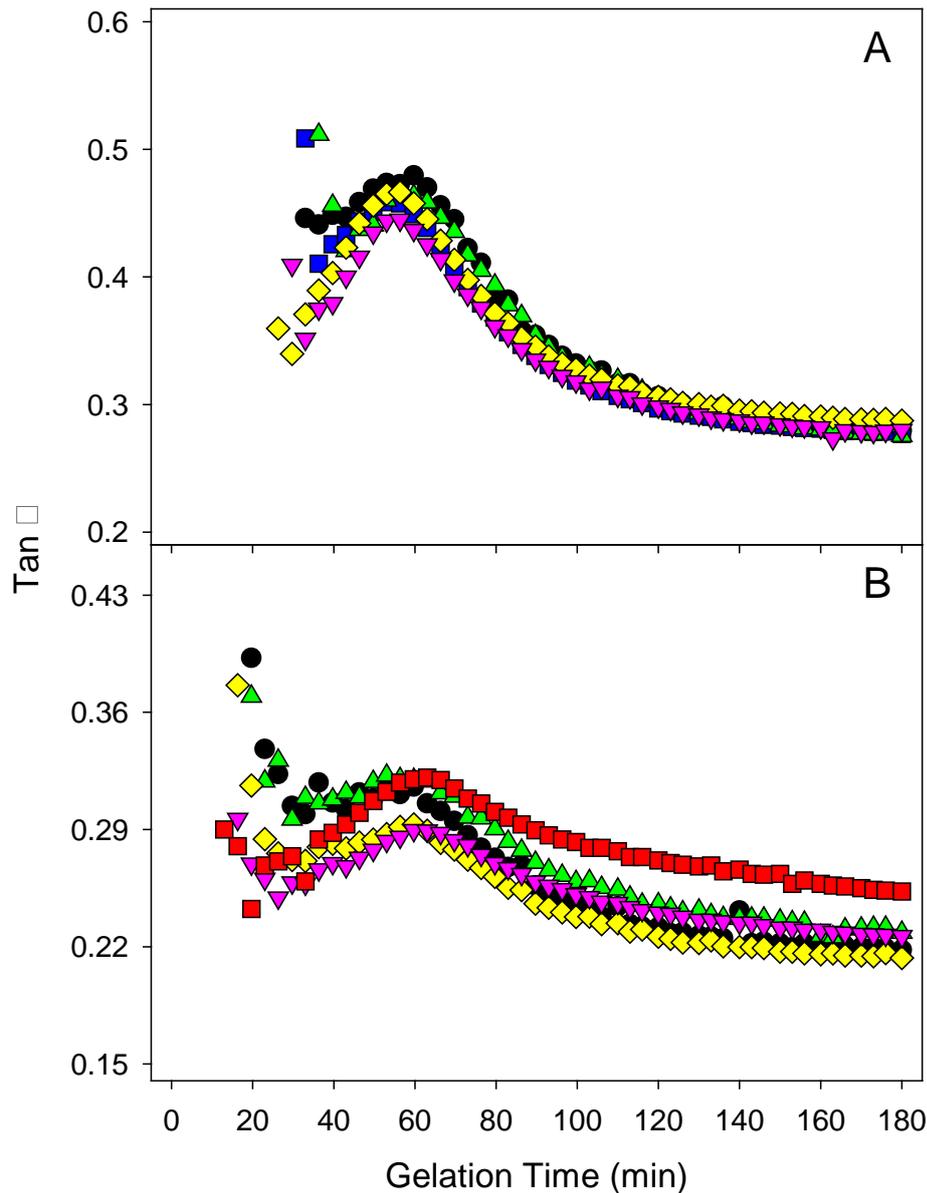


Figure 5.10: The changes in $\tan \delta$ values during gelation of skim milk (A) and WPE skim milk (B). Milks were heated in the presence of various NEM concentrations: ●, 0 mM; ■, 0.08 mM; ▲, 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM and ■, 0.8 mM. Each point is the average of two to four replicates. Error bars were not presented to simplify the graph.

In WPE skim milk, there was no general trend to describe the effects of NEM on the changing $\tan \delta$ values during gelation (Figure 5.10B). The profile of acid gels with 0.24 mM NEM was similar to that of control gel in the early stages (< 80 min) then the $\tan \delta$ values became higher than those of control gel. At 0.4 and 0.6 mM NEM, the $\tan \delta$ values were lower than those of control gels in the early stage (< 80 min). Subsequently, the $\tan \delta$ values of 0.4 mM NEM-containing-gels became comparable with those of the control while those of 0.6 mM NEM-containing-gels were higher than those of the control (Figure 5.10B). At 0.8 mM, the $\tan \delta$ values were lower than

those of the control during the first 60 min of gelation; they then reached comparable maximum values as the control and finally remained higher than those of the control as the gelation continued. This indicated that viscous properties relative to elastic ones were higher in the WPE-milk gels at 0.8 mM than in any other samples.

Once the acid gels had been formed (3 h at 30 °C), the temperature of the acid gels was dropped to 5 °C at a rate of 1 °C min⁻¹. The G' values at 5 °C of the acid gels as a function of NEM concentrations are presented in Figure 5.11. In skim milk, the G' values at 5 °C of acid gels containing NEM were on average slightly lower than those of control acid gels, which were 408 ± 42 Pa ($p > 0.05$, Figure 5.11A). This meant that the G' values of skim-milk gels were about 2 times higher at 5 °C than at 30 °C.

In WPE skim milk, the G' values increased progressively from 890 ± 148 Pa to 1198 ± 181 Pa as the NEM concentrations increased from 0 to 0.8 mM (increase by 35%, $p > 0.05$, Figure 5.11A). The final G' values at 5 °C were plotted as a function of the final G' at 30 °C (Figure 5.11B). The relationship between the G' at 30 °C and those at 5 °C was linear with a gradient of 1.0. The results indicate that regardless of the NEM concentration added to WPE skim milk, the G' values of the acid milk gels at 5 °C were ~ 386 Pa higher than those at 30 °C (Figure 5.11B).

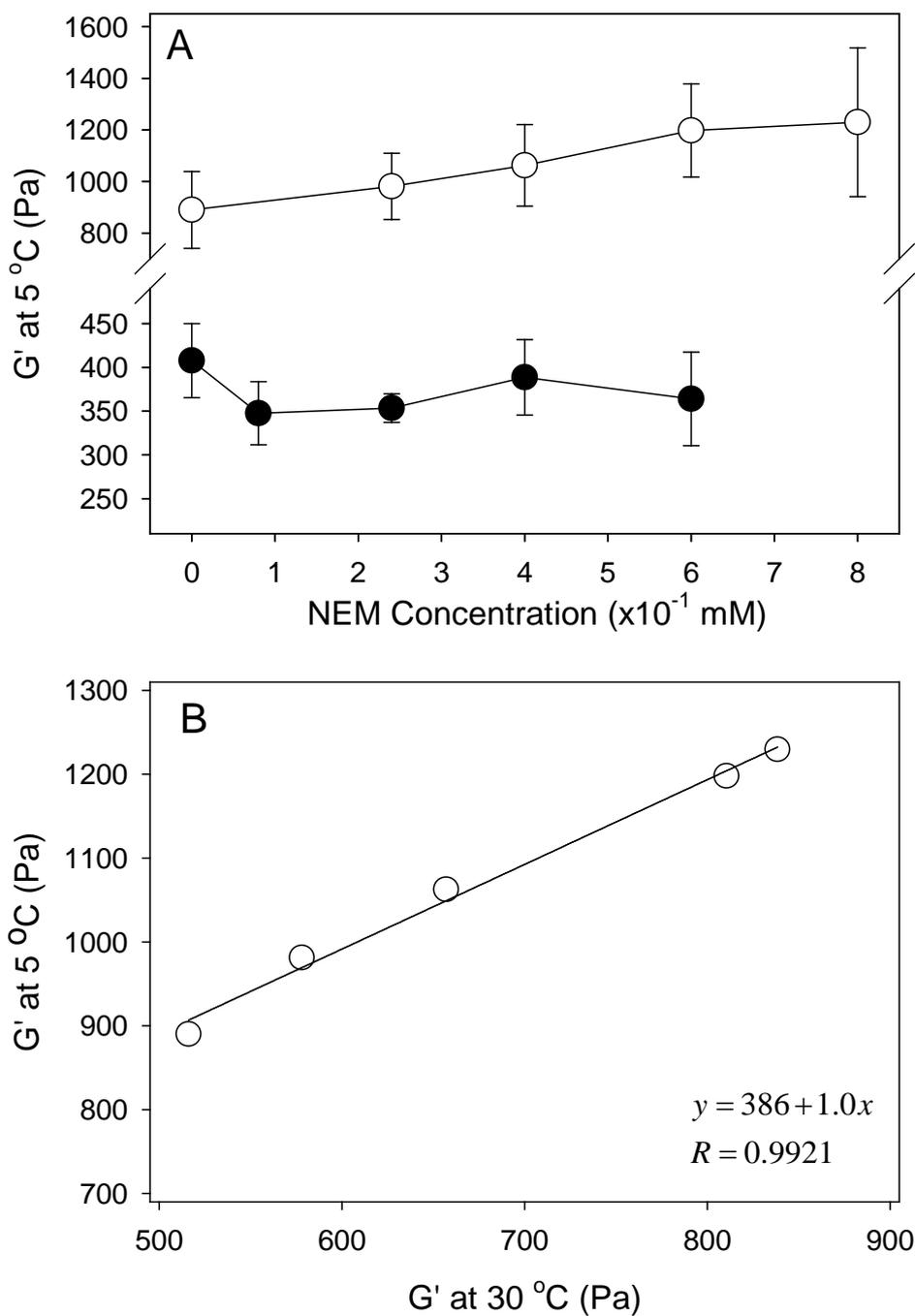


Figure 5.11: A: Effects of NEM concentrations on the G' of acid gels at 5 °C. B: the relationship between the G' values at 30 °C and at 5 °C of WPE-milk gels. Acid gels were prepared from skim milk (●) and WPE skim milk (○) that had been heated in the presence of NEM and formed at 30 °C. Subsequently, the temperature of the acid gels was decreased to 5 °C (1 °C/min). Each data point is the average of two to four replicates. Error bars represent the standard deviation.

The yield properties of the acid gels were examined by monitoring the change of shear stress as a function of strain when the gels were subjected to a constant rotational shear at a rate of

0.005 s⁻¹. Due to the constant shear, the strain and shear stress of the acid gels increased progressively until the acid gels could not withstand the shear any longer and broke. The maximum shear stress and the strain at this maximum were taken as the yield point. The change of shear stress as a function of strain is shown in Figure 5.12 and the values of yield strain and stress are summarised in Table 5.1.

In skim milk, while the yield strain was not affected on the addition of NEM, the yield stress values were lower in acid gels containing NEM compared to those without NEM ($p < 0.05$, Table 5.1). The yield stress decreased gradually as the NEM concentration increased from 0 to 0.24 mM, then increased slightly as the NEM concentration increased further (Figure 5.12A).

In WPE skim milk, both the yield strain and yield stress decreased with the increase of NEM concentration. At 0.8 mM NEM, the yield stress was 34% while the yield strain was 23% of those from control WPE skim milk acid gels (Table 5.1 and Figure 5.12B). This decrease was more pronounced than the effect observed in acid gels prepared from skim milk. For the skim milk, the lowest yield stress was observed in acid gels containing 0.24 mM and this stress was 64% of the yield stress of control skim milk acid gel (Table 5.1).

Table 5.1: Yield stress (Pa) and strain (%) of acid gels when subjected to constant shear rate of 0.005 s⁻¹ at 5 °C. Acid gels were prepared from skim milk and WPE skim milk that had been heated in the presence of NEM. The values are the mean value of two to four replicates ± standard deviation.

NEM concentrations (mM)	Skim milk		WPE skim milk	
	Yield strain (%)	Yield Stress (Pa)	Yield strain (%)	Yield Stress (Pa)
0	42 ± 2	122 ± 3	61 ± 5	314 ± 8
0.08	43 ± 2	111 ± 2	-	-
0.24	39 ± 3	78 ± 3	66 ± 17	299 ± 30
0.4	41 ± 1	91 ± 1	33 ± 8	203 ± 3
0.6	45 ± 2	95 ± 2	17 ± 3	116 ± 5
0.8	-	-	14 ± 3	107 ± 5

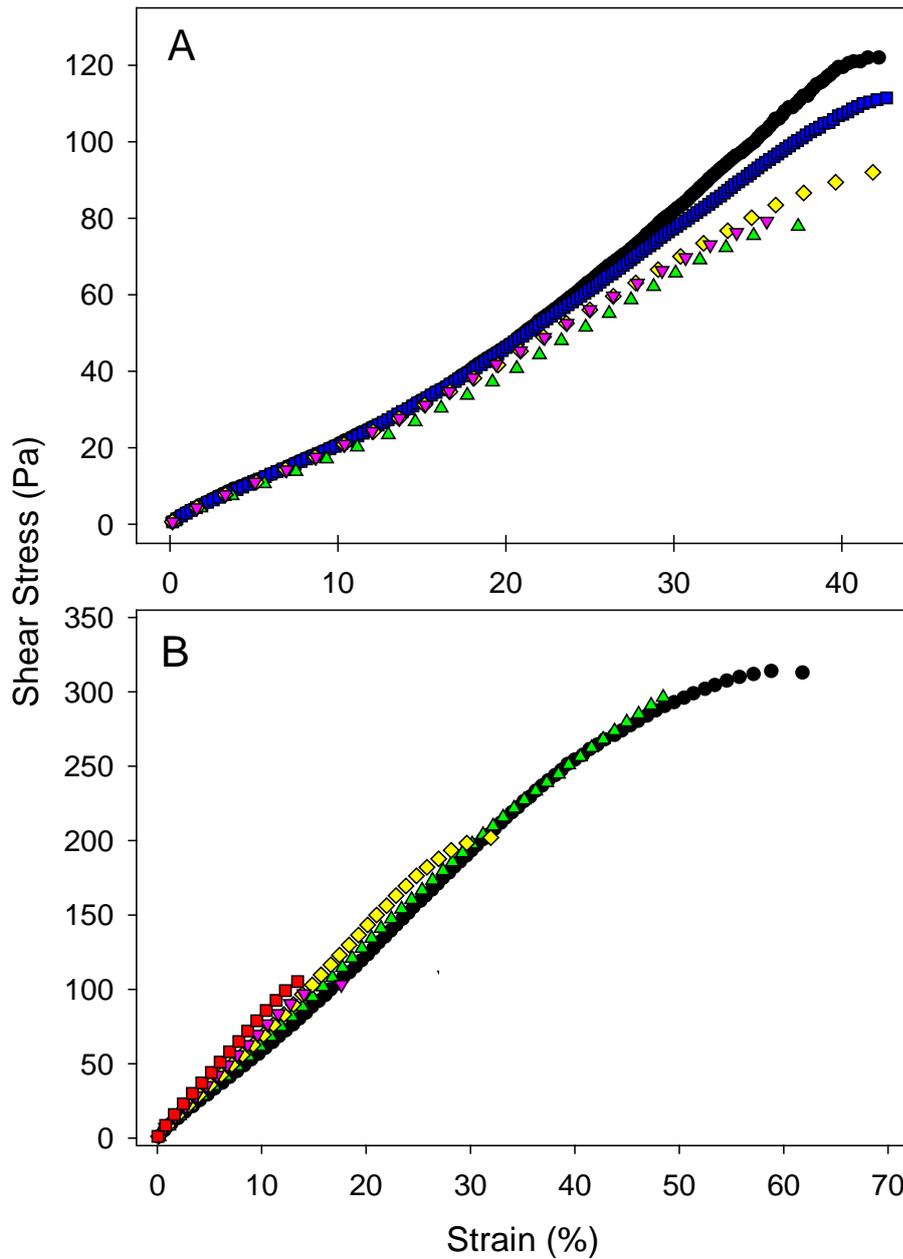


Figure 5.12: Shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^\circ\text{C}$ and analysed at $5 \text{ }^\circ\text{C}$. The acid gels were prepared from skim milk (A) and WPE skim milk (B) that was heated with different concentrations of NEM. ●, 0 mM; ■, 0.08 mM; ▲, 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM; ■, 0.8 mM. The last point was the stress and strain before the gel was broken. Each point is the average of two to four replicates. Error bars represent the standard deviation.

5.3.5 Effects of heating NEM-treated milks on the microstructure of the acid gels

Confocal microscopic images of the set acid gels of milk samples that were treated with 0.24 and 0.6 mM NEM were obtained to compare with those of the acid gels of control heated milk (Figure 5.13). Even though the final G' values of acid gels made from skim milk heated with 0 and 0.24 mM NEM were not significantly different (201 ± 1 and 195 ± 3 Pa, respectively), the microstructure of acid gels made from skim milk heated with 0.24 mM NEM (Figure 5.13B) had larger open spaces (black areas) than that of acid gels made from heated control skim milk (Figure 5.13A). The strands in Figure 5.13B were thinner compared with those in Figure 5.13A, despite the levels of connectivity and the length of the strands between the two gels being similar. The microstructure of acid gels made from skim milk heated with 0.6 mM NEM (Figure 5.13C) consisted of a higher number of large open spaces compared to the microstructure of acid gels made from skim milk heated with lower NEM concentrations (Figure 5.13A and B).

The microstructure of the acid gels showed that the networks of gels made from NEM-treated-heated WPE skim milk were denser and had fewer open spaces than those made from control heated skim milk (Figure 5.14), corresponding with the increase in the G' values (Figure 5.9B). There were no obvious differences between the microstructures of acid gels that were prepared from WPE skim milk heated in the presence of various NEM concentrations.

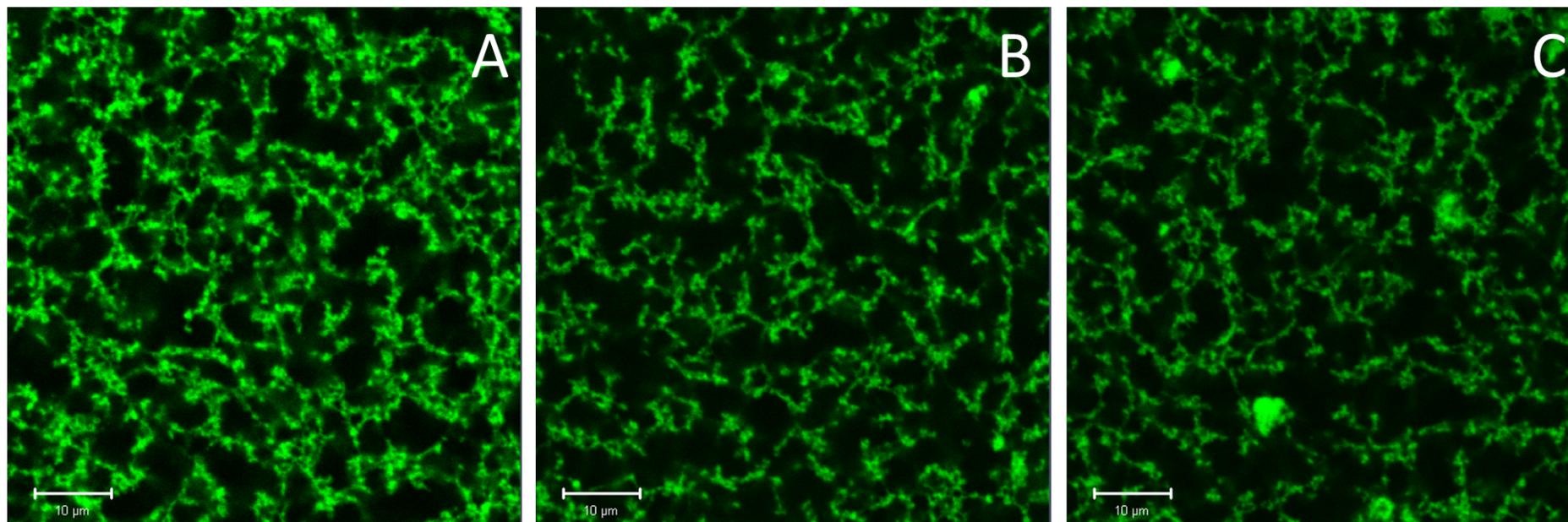


Figure 5.13: Confocal microstructural images of acid gels made from skim milk heated with 0 (A), 0.24 (B) and 0.6 (C) mM NEM. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μm .

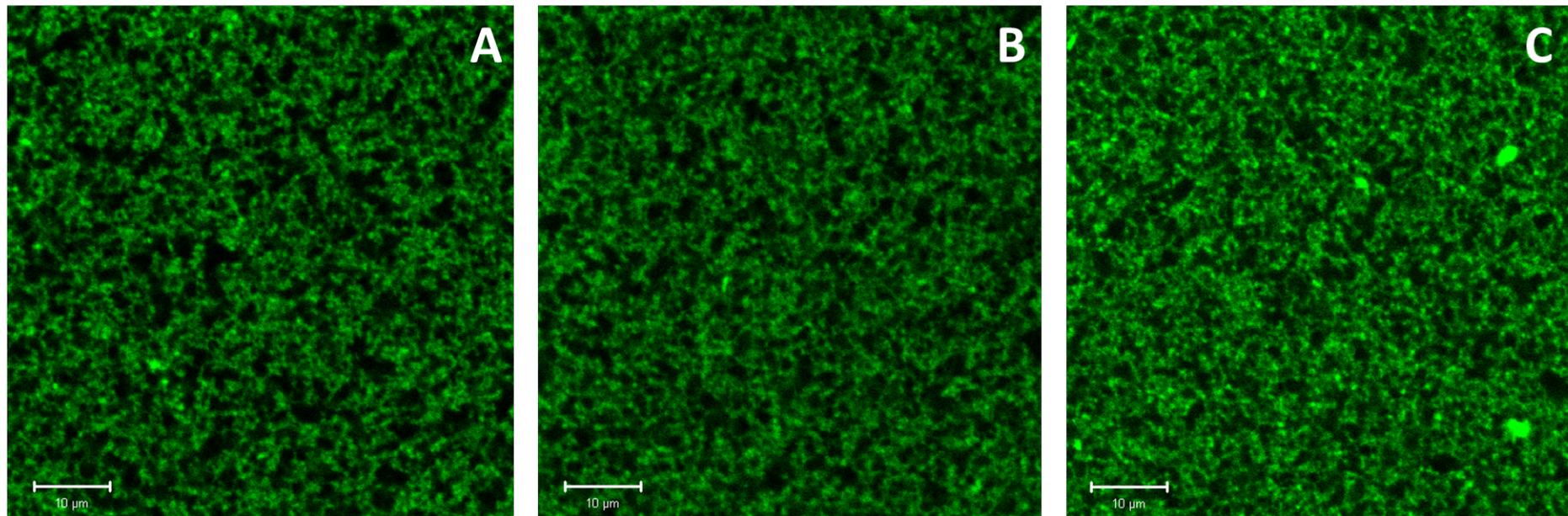


Figure 5.14: Confocal microstructural images of acid gels made from WPE skim milk heated with 0 (A), 0.4 (B) and 0.8 (C) mM NEM. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar was 10 μm .

5.4 Discussion

5.4.1 Effects of heating NEM-treated milks on the protein denaturation, interactions and distributions

In this chapter, the results of skim milk and WPE skim milk are discussed separately because unlike previous chapter, the effects of NEM on the rheological properties of the acid gels was significantly different between skim milk and WPE skim milk.

5.4.1.1 Skim milk

When the free thiol groups of β -lactoglobulin reacted with NEM, this blocked the thiol and made it unavailable for thiol-disulphide exchange reactions. Hence the proteins cannot aggregate via intermolecular disulphide interactions (Sawyer et al., 1963; Purkayastha et al., 1967) and mostly underwent reversible denaturation (Tanford, 1968; Ruegg et al., 1977; De Wit & Klarenbeek, 1984). This meant that even when the whey protein structure unfolded during heat treatment, the protein structure could refold to its native form upon cooling (Chaplin & Lyster, 1986). Hence, the increased proportion of native α -lactalbumin in skim milk (Figure 5.2A) suggested that as the NEM concentration increased, α -lactalbumin molecules did not interact via covalent bonding with other proteins in milk. Furthermore, the fact that α -lactalbumin remained native after heating milk also suggested that α -lactalbumin did not interact non-covalently with other proteins either.

The proportion of β -lactoglobulin that remained as native protein (i.e. underwent reversible denaturation) was only 40 % (Figure 5.2A). This has been similarly observed in previous studies on β -lactoglobulin in protein solutions (Iametti et al., 1996; Hoffmann & van Mil, 1997). Iametti et al. (1996) found that up to 50% of β -lactoglobulin in the protein solution was denatured even when the thiol groups were completely blocked by iodoacetamide (compared to 96% β -lactoglobulin being denatured in the untreated solution). Hoffmann and van Mil (1997) observed large aggregates on the native gels when β -lactoglobulin solution was heated with NEM, implying that β -lactoglobulin was irreversibly denatured and aggregated even though the thiol groups were blocked.

In milks heated in the presence of NEM, it was expected that the level of whey protein participating in intermolecular disulphide bonds would decrease with the increase of NEM concentrations because the level of native whey protein was observed to increase with the increase of NEM concentration (Figure 5.2). The percentage of disulphide-linked β -lactoglobulin in the heated milks decreased from 71% to 6.5% as the NEM concentration increased up to 0.24 mM (which corresponded to the ratio of NEM to thiol group of 1.2). The level of disulphide aggregated β -lactoglobulin remained low at higher NEM concentrations (Figure 5.4A), as

expected. As for α -lactalbumin, which does not contain free thiol groups, this protein can interact via thiol-disulphide exchange reactions with other proteins only in the presence of thiol groups, which are mainly sourced from β -lactoglobulin in milk (Calvo et al., 1993; Dalgleish, Senaratne & Francois, 1997b; Angel de la Fuente, Singh & Hemar, 2002; Oldfield et al., 2005). Thus, with the decrease in the percentage of disulphide-linked β -lactoglobulin, the percentage of disulphide-linked α -lactalbumin was also greatly reduced: there was $7 \pm 7\%$ disulphide-linked α -lactalbumin in 0.6 mM-NEM-heated skim milk compared to $57 \pm 9\%$ disulphide-linked α -lactalbumin in control heated skim milk (Figure 5.4A), as was also expected.

β -Lactoglobulin was found to be denatured but did not participate in thiol-disulphide interactions (Figure 5.2 and Figure 5.4). This suggested that β -lactoglobulin was involved in aggregates via non-covalent interactions, even when the ratio of NEM to free thiol groups was above 1 (0.24mM) in skim milk (Table 4.1). Many studies have reported that aggregates can still be formed between denatured β -lactoglobulin and casein micelles when the thiol groups of β -lactoglobulin were completely blocked (Mckenzie et al., 1971; Xiong et al., 1993; Hoffmann & van Mil, 1997; Boye, Ma & Ismail, 2004).

In addition, a portion of the denatured β -lactoglobulin (50% of denatured β -lactoglobulin or 30% of total β -lactoglobulin in skim milk) was not found in the serum. Hence this portion of β -lactoglobulin was assumed to associate with the casein micelles (i.e. being located in the colloidal phase. Figure 5.6A). As for κ -casein, even though the percentage of serum κ -casein in NEM-treated heated milk decreased to 22% compared to 32% in heated skim milk without added NEM (Figure 5.6A), this percentage was still higher than that in unheated skim milk ($14 \pm 3\%$, Figure 4.6, Chapter 4). Furthermore, the percentage of disulphide-linked κ -casein polymers remained the same (on average $74 \pm 3\%$ of κ -casein was present as disulphide-linked polymers, Figure 5.4A) regardless of the decrease in level of disulphide-linked whey protein. These electrophoretic results demonstrated that in heated NEM-treated skim milk, a portion of κ -casein dissociated from the casein micelles. In addition a portion of β -lactoglobulin interacted and associated with the casein micelles, without forming intermolecular disulphide bonds with the κ -casein.

Even though the proportion of colloidal whey protein decreased with the increase of the NEM concentrations, the size of the casein micelles increased by up to 4% (Figure 5.7A). In previous studies, an increase in the size of the casein micelles (by $\sim 12\%$) was proposed to be related to an increase in the level of colloidal whey protein (by $\sim 70\%$, Anema & Li, 2003b, a). A similar increase in casein micelle size was also argued to be possibly due to the partial aggregation of the casein micelles. However the latter reason was not considered likely because the

polydispersity index did not increase as the size increased (Journink, 1992; Anema & Li, 2003b, a). In this study, the increase in the casein micelle size was accompanied by an increase in the polydispersity index (Figure 5.7B). This implied that when skim milk was heated in the presence of NEM, some casein micelles aggregated with each other, leading to an increase in the apparent average size. As a result, the size distribution of the casein micelle increased, leading to an increase in the polydispersity index (Figure 5.7B).

5.4.1.2 Whey-protein-enriched skim milk

In the heated WPE skim milk, the percentage of disulphide-linked α -lactalbumin and β -lactoglobulin decreased significantly when the NEM concentrations increased to 0.8 mM (to a ratio of NEM to thiol groups above 1, Figure 5.4B). This demonstrated that heating WPE skim milk in the presence of NEM markedly reduced the thiol-disulphide exchange reactions during heating, as was also observed in skim milk heated with NEM (Figure 5.4A).

The increase in the percentage of native α -lactalbumin in heated NEM-treated milk indicated that the majority of α -lactalbumin underwent reversible denaturation (Figure 5.2B). This meant that α -lactalbumin did not interact via disulphide bonds and may not have interacted non-covalently with other proteins either. On the other hand, while very low levels of β -lactoglobulin were disulphide-linked (Figure 5.4B), the percentage of native β -lactoglobulin in WPE skim milk heated with 0.8 mM NEM was only 19% (Figure 5.2B). In addition, the percentage of serum β -lactoglobulin was not affected when WPE skim milk was heated in the presence of NEM whereas the percentage of serum α -lactalbumin increased to 95% of the total α -lactalbumin present in WPE skim milk with the increase of NEM concentrations (Figure 5.6B). The percentage of serum κ -casein decreased slightly with an increase of NEM concentration (Figure 5.6B), but this percentage was still higher than that found in unheated WPE skim milk ($17 \pm 2\%$, Figure 4.6, Chapter 4). This implied that when almost all of the thiol groups of β -lactoglobulin in WPE skim milk were blocked, β -lactoglobulin still denatured and interacted with itself and probably with κ -casein, but not with α -lactalbumin.

In the heated WPE milk, the addition of NEM caused less κ -casein but more α_s - and β -casein to dissociate from the casein micelle (Figure 5.6B). An early study reported that α_s - and β -casein dissociated from the casein micelles and can be stabilised as small aggregates by κ -casein in the serum when being exposed to high temperatures up to 70 °C (Anema & Klostermeyer, 1997). Above 70 °C, the aggregates formed from denatured β -lactoglobulin and κ -casein are less capable to stabilise the α_s - and β -casein than native κ -casein, causing these casein proteins to re-associate with the colloidal phase (Anema & Klostermeyer, 1997). In this study, when disulphide bonds between β -lactoglobulin and κ -casein cannot be formed due to the blocking of

thiol groups, the serum κ -casein may be available to stabilise the dissociated α_s - and β -casein. As a result, higher level of α_s - and β -casein were found in the serum compared with the heated WPE skim milk (Figure 5.6B), in agreement with a previous study (Anema & Li, 2000).

The size of the casein micelles in the heated WPE skim milks markedly increased with the increase of NEM concentrations and this was accompanied by an increase in the polydispersity index (Figure 5.7). The increase in size of casein micelles in WPE skim milk was more pronounced than that observed in skim milk. As the general percentage of colloidal protein decreased as the NEM concentration increased (Figure 5.6B), the increase in casein micelle size in this study cannot be related to an association of protein on the casein micelle. This increase in size could be related to the partial aggregation of the casein micelles in milk, as postulated in 5.4.1.1.

5.4.1.3 The proposed mechanism of interactions between the proteins in milks heated in the presence of NEM

When skim milk and WPE skim milk were heated at 80 °C in the presence of low concentrations of NEM, α -lactalbumin and β -lactoglobulin unfolded and some κ -, α_s - and β -casein dissociated from the casein micelles. While β -lactoglobulin with blocked thiol groups may not be able to initiate thiol-disulphide exchange reactions with disulphide bond-containing proteins, the unfolded β -lactoglobulin may expose other active (e.g. hydrophobic, hydrogen, etc.) sites; hence they may interact with other milk proteins via non-covalent interactions. Dissociated casein proteins, especially κ -casein, also containing hydrophobic residues, can readily associate with unfolded β -lactoglobulin. This could lead to the formation of heat-induced aggregates. However, these aggregates would have different compositions than those found in control heated milks.

5.4.2 Effects of heating NEM-treated milks on the rheological and microstructural properties of acid gels

5.4.2.1 Skim milk

The gelation pH of acid gels made from skim milk heated in the presence of NEM was similar to those of acid gels made from control heated skim milk (~ pH 5.3, Figure 5.9A). It is well-known that the gelation pH of acid gels prepared from heated milk was higher than that of gels made from unheated skim milk (Lucey et al., 1998; Vasbinder et al., 2001; Anema et al., 2004b). This increase of gelation pH has been correlated to the denaturation of the whey proteins and the formation of the whey protein/ κ -casein complexes (Section 2.13.1). In this study, the high gelation pH of skim milk heated in the presence of NEM implied that the system contained denatured whey proteins, which agreed with the electrophoretic results (Figure 5.2A), and these denatured proteins did participate in the gel network.

The final G' values of the acid gels made from NEM-treated-heated skim milk were lower by 12% compared with those of heated control milks but higher than those of unheated control milks (which were 10 ± 1 Pa, Figure 4.9 in Chapter 4). This was in agreement with previous studies (Lucey et al., 1998; Vasbinder et al., 2003).

The yield stress of the acid gels decreased by 22% with the increase of NEM concentration up to 0.6 mM (Figure 5.12A). These values were still higher than those of acid gels prepared from unheated skim milks (59 ± 2 Pa, Chapter 4). When the gels were subjected to a constant shear, the factors determining the breaking point of the gels include the number of bonds between the proteins per cross-sectional area in the gel network, the strength of the bonds and the curvature of the strands in the network (van Vliet, van Dijk, Zoon & Walstra, 1991; van Vliet & Walstra, 1995; Lakemond & van Vliet, 2008b). In this study, the decrease in yield stress was likely to be caused by the lack of intermolecular disulphide bonds since stronger forces were required to break disulphide bonds, of which energy was markedly higher than non-covalent bonds (51 kcal mol^{-1} versus $<10 \text{ kcal mol}^{-1}$) (Chou & Buehler, 2009). As the yield strain of the skim milk acid gels did not change markedly on the addition of NEM (Figure 5.12A and Table 5.1), this suggested that the relative curvature of the individual strands within the gel network was similar for all acid gel samples.

The rheological results also suggested that despite the lack of intermolecular disulphide bonds, non-covalent interactions between the denatured β -lactoglobulin and κ -casein in skim milk heated in the presence of NEM influenced the G' values and the yield stresses of the acid gels prepared from NEM-treated heated skim milk. That was why these acid gels had higher G' values and yield stress when compared to acid gels prepared from unheated skim milks in which intermolecular interactions between the whey protein and κ -casein were not present.

Confocal images were also consistent with the observed G' : the gels of lower G' (Figure 5.13C) consisted of thinner strands and larger pores compared to the gels of higher G' (Figure 5.13B). However, due to the denaturation of whey proteins and the probable aggregations of the proteins via non-covalent bonding, the degree of connections between the proteins in the gels made from NEM-treated-heated skim milk (Figure 5.13B) was still higher than that in unheated skim milk (Figure 4.11 in Chapter 4). The microstructure of NEM-treated-heated skim milk gels consisted of connected linear strands of proteins (Figure 5.13); whereas the microstructures of unheated skim milk gels consisted of connected clusters (Figure 4.11 in Chapter 4).

5.4.2.2 Whey-protein-enriched skim milk

In WPE skim milk, the gelation pH and final G' values increased with increasing concentrations of added NEM. This was opposite to the results observed when skim milk was treated with NEM (Figure 5.9).

The gelation pH of acid gels made from heated WPE skim milk was higher than that of gels made from unheated WPE skim milk because of the presence of denatured whey proteins (in particular β -lactoglobulin, Figure 5.2B). As the percentage of colloidal β -lactoglobulin was the same in both WPE skim milk heated without and with added NEM, the increase in gelation pH with the increase of NEM could be due to the properties of β -lactoglobulin. Without intermolecular disulphide bonding, denatured β -lactoglobulin may be flexible and expose many hydrophobic sites (Havea et al., 2009). It was found that increasing the hydrophobicity of the colloidal heat-induced aggregates increased the gelation pH (Famelart et al., 2004; Morand, Dekkari, Guyomarc'h & Famelart, 2012). Therefore, as the pH was lowered during gelation, milks containing fewer disulphide-linked β -lactoglobulin and/or higher concentrations of denatured β -lactoglobulin with exposed hydrophobic sites may start to gel earlier than milks with higher level of disulphide-linked β -lactoglobulin (Figure 5.9A). Furthermore, the concentration of denatured β -lactoglobulin was an important factor as the increase in the gelation pH was only observed in WPE skim milk but not in skim milk.

The rheological properties of acid gels obtained in this study suggested that intermolecular disulphide interactions between the proteins in milk may not be the main factor influencing the G' of the acid gel. Instead, non-covalent interactions between denatured β -lactoglobulin and κ -casein may influence the magnitude of the G' values of acid gels prepared from WPE skim milk heated in the presence of NEM, as was also proposed for acid gels prepared from NEM-treated skim milk (Section 5.4.2.1). The increase in G' values with the increase of NEM concentrations also implied that the total degree of interactions between the proteins may be substantially higher in the WPE-treated-heated milk system than in heated WPE milk without NEM even though in the former system there was a lack of intermolecular disulphide bonds between κ -casein and denatured whey proteins. This argument contrasts with previous studies claiming that disulphide interactions between the proteins in heated milk greatly contribute to the magnitude of the G' values of the acid gels (Mottar et al., 1989; Lucey et al., 1997; Van Vliet, Lakemond & Visschers, 2004).

Unlike acid gels made from skim milk heated in the presence of NEM, the $\tan \delta$ profiles during the acidification process of acid gels made from WPE skim milk changed with the increase of NEM concentrations, though the change did not follow any patterns (Figure 5.10). There were

two noticeable differences between the control acid gels of WPE skim milk and the acid gels prepared from the WPE skim milk heated in the presence of 0.8 mM NEM. Firstly, the difference in the maximum $\tan \delta$ values and the final $\tan \delta$ values were much smaller in NEM-containing gels. Secondly, the final $\tan \delta$ values of acid gels made from 0.8 mM NEM-containing WPE skim milk were higher than those of acid gels with NEM concentrations < 0.8 mM. The higher $\tan \delta$ value suggested that the G'' to G' ratio was greater. As acid gels prepared from 0.8 mM NEM-containing WPE skim milk had G' values markedly higher than acid gels with NEM concentrations < 0.8 mM, the higher $\tan \delta$ value also suggested that the former gels have a substantially higher G'' values than the latter gels.

The addition of whey protein isolate into skim milk before heating was reported to lead to the formation of firmer acid gels (Lucey et al., 1999a), which agreed with the current study. The results of this study also agreed with those of Graveland-Bikker and Anema (2003) that α -lactalbumin concentration did not affect the G' of acid gels but the concentration of β -lactoglobulin was the important factor influencing the G' values. This study demonstrated that the firmness of acid gels can be increased (Figure 5.9B) despite the majority of α -lactalbumin remaining native (Figure 5.2B). In WPE skim milk, the increase in concentration of β -lactoglobulin may have provided more sites for bonding between the proteins and also a greater number of particles that can participate in the gel network. As a result, the gel matrix contained high quantities of participating proteins and a high degree of interactions between the proteins, hence high G' values. This is supported by the microstructure images of the acid gels as shown in Figure 5.14.

Despite the increase in G' values, the gels made from WPE skim milk heated with NEM became more prone to breakage at large deformation, evidenced by the decrease in both yield stress and strain values when the concentration of NEM was increased (Figure 5.12B and Table 5.1). The yield strain was dependent on factors such as the degree of curvature of the strands as the strands within the gel network first need to be straightened and then stretched until the strands or the bonds within the strands are ruptured. Hence the yield strain increases with the increase in strand curvature (van Vliet & Keetels, 1995; Lakemond & van Vliet, 2008b). In this study, the decrease in yield strain of acid gels made from NEM-treated-heated WPE skim milk indicated a decrease in the strand curvature.

The decrease in yield stresses values with the increase of NEM concentrations demonstrated the decrease in the proportion of intermolecular disulphide bonds in the WPE skim milk, which agreed with the electrophoretic results (Figure 5.4). This also supported the claim that non-covalent interactions may be the predominant interactions between the proteins in the milk

systems containing NEM since non-covalent bonds had markedly lower bond energy than disulphide bonds.

5.5 Conclusions

When skim milk was heated in the presence of NEM, the majority of the β -lactoglobulin denatured and participated in non-covalent interactions. Without thiol-disulphide exchange reactions, κ -casein interacted with β -lactoglobulin to a lesser extent than when thiol-disulphide reactions could occur. The decrease in the amount of denatured β -lactoglobulin and in the level of interactions, especially disulphide-interactions, between the proteins was responsible for the decrease in the final G' values of the resulting acid gels.

When skim milk was enriched with whey proteins, despite the increase in NEM concentrations, there was higher percentage of denatured β -lactoglobulin in WPE skim milk than in skim milk. The high quantity of denatured β -lactoglobulin, with numerous exposed hydrophobic, hydrogen and ionic sites, interacted non-covalently with each other and κ -casein. This may have led to the dominance of the non-covalent interactions in the milk system while the proportion of intermolecular disulphide interactions was reduced. Subsequently, the acid gels made from NEM-treated-heated WPE skim milk had higher G' values than those made from control WPE milk. This gave strong evidence that the gel firmness was not solely dependent on the disulphide interactions between κ -casein and β -lactoglobulin.

Nevertheless, the lack of intermolecular disulphide bonds between the proteins in both skim milk and WPE skim milk was responsible for the easy breakage of the resulting acid gels when subjected to large deformation. The yield stress values of the acid gels decreased with the increase in the addition level of NEM to skim milk and WPE skim milk before heating.

Chapter 6 - Effects of adding NEM to heated milks on the protein interactions and acid gels properties

6.1 Introduction

The results presented in Chapter 5 demonstrated that blocking the thiol groups of β -lactoglobulin inhibited the thiol-disulphide exchange reactions between the proteins on heating skim milk and WPE skim milk. However, despite the inhibition of the formation of new intermolecular disulphide bonds between whey proteins and κ -casein in heated milks, the G' values of acid gels prepared from WPE skim milk heated in the presence of NEM increased markedly with the increase of NEM concentration. This suggested that non-covalent interactions between the proteins can contribute greatly to the G' values of the acid gels. The next question was how the inhibition of thiol-disulphide exchange reactions during acidification affected the rheological properties of acid gels made from heated milks, knowing that intermolecular disulphide bonds already existed in the system.

There have been a number of studies reporting that disulphide bonds can be formed during acid gelation and that the formation of these bonds during acidification contributed to the high G' values of the resulting acid milk gels (Hashizume & Sato, 1988; Lucey et al., 1998; Vasbinder et al., 2003) and acid whey protein gels (Alting, Hamer, De Kruif & Visschers, 2000; Alting, Hamer, De Kruif, Paques & Visschers, 2003). It was found that when thiol blocking reagents (e.g. NEM) were added to heated milks or whey protein solutions prior to acidification, the produced acid gels had lower G' values than those made from control samples. These findings were observed in very different systems, Alting et al. (2000; 2003) studied whey protein solutions of $> 4.5\%$ protein concentrations, Hashizume (1988) examined gels prepared by heating milk at acidic pH and Vasbinder (2003) and Lucey (1998) prepared acid gels over a period of 20 and 15 h, respectively.

Few studies used low NEM concentrations (i.e. below 1:1 ratio with thiol groups of β -lactoglobulin). Therefore the aim of the experiments reported in this chapter was to investigate whether or not thiol-disulphide exchange reactions occurred during the formation of acid gels and also to clarify the importance of disulphide bonds in determining the firmness of the gels during acidification. Firstly, the protein interactions and distributions were examined after 1 h of adding NEM to heated milks. Subsequently, acid gels were prepared from treated milks and rheological measurements and confocal microscopy were used to examine the firmness and the microstructure of the resulting acid gels.

6.2 Materials and methods

Skim milk and WPE skim milk were prepared as described in Section 3.1.1.

Skim milk and WPE skim milk were heated at 80 ± 0.1 °C for 30 min (Section 3.3). Diluted NEM (1% v/v) was added to heated skim milk to give NEM concentrations of 0.08, 0.24, 0.4 and 0.6 mM in skim milk (i.e. 0.4 to 3.1 mol NEM per mol β -lactoglobulin, Table 4.1) and 0.24, 0.4, 0.6 and 0.8 mM in WPE skim milk (i.e. 0.4 to 1.3 mol NEM per mol β -lactoglobulin, Table 4.1). The treated milk was shaken on a vortex mixer for 10 s and then left to react at 20 ± 0.1 °C in a thermostatically controlled water bath for 1 h. The milk container was closed tightly to reduce any oxidation.

The levels of α -lactalbumin, β -lactoglobulin and κ -casein involving in intermolecular disulphide bonds and the levels of proteins in the serum phase were examined using SDS-PAGE (as described in Section 3.6). The size of the casein micelles were monitored using dynamic light scattering (as described in Section 3.8).

The milks were then acidified to form acid gels of which the rheological properties and microstructure were investigated using low amplitude rheology (as described in Section 3.10.2) and confocal microscopy (as described in Section 3.10.3).

6.3 Results

6.3.1 Effects of NEM concentrations on the protein interactions and distribution of proteins between the colloidal and serum phases

The proportion of native β -lactoglobulin and α -lactalbumin (i.e. proteins remaining soluble at pH 4.6, as described in Section 3.5) in heated skims were determined using SDS-PAGE. It is clear that the amount of native whey proteins remaining in heated milk (Figure 6.1a, lanes b to g) and WPE milk (Figure 6.1b, Lanes b to g) were markedly less than those in the unheated milk and WPE milk (Lane a in Figure 6.1a and b respectively), as expected. This demonstrated that α -lactalbumin and β -lactoglobulin were denatured upon heating milks, in agreement with previous reports (Dannenberg & Kessler, 1988a; Oldfield et al., 1998b). When NEM was subsequently added to the heated skim milk and WPE milk (Lanes c to g in Figure 6.1A and B, respectively), the band intensities of native whey proteins were visually similar.

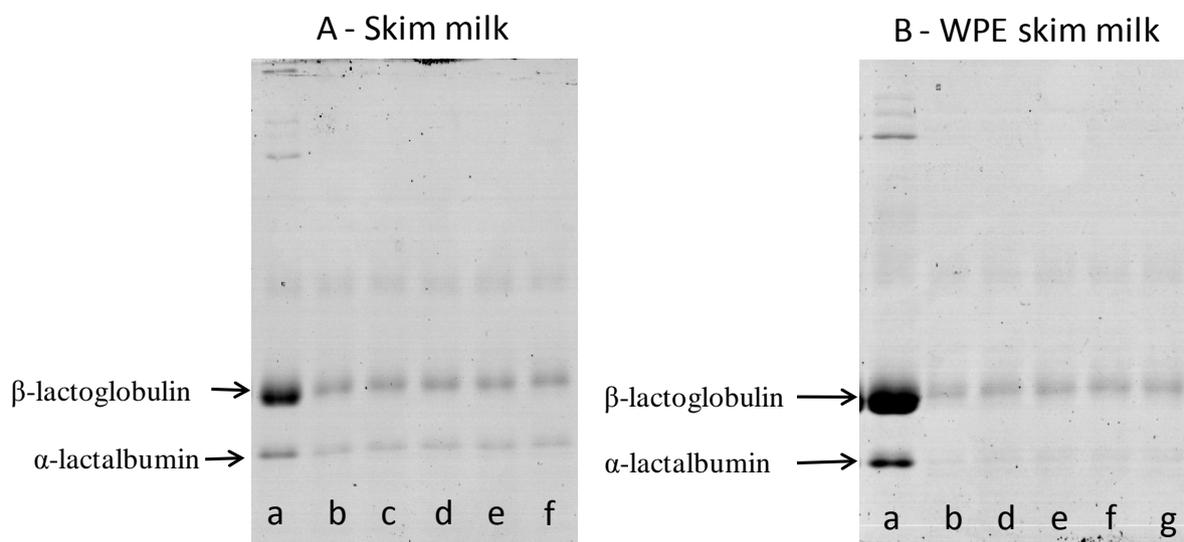


Figure 6.1: SDS-PAGE patterns of proteins remaining soluble after acid precipitation (i.e. native proteins) from heated skim milk and WPE skim milk with added NEM. Control sample (i.e. native protein in unheated skim milk or WPE skim milk, Lane a); control heated milks (Lane b); milk heated with added NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The samples were fully reduced.

The integration of the band intensities and comparison with the total native whey proteins in control unheated milks showed that the addition of NEM at any concentration did not significantly affect the percentage of native α -lactalbumin or β -lactoglobulin remaining in the heated milk and WPE milk ($p > 0.05$, Figure 6.2). In heated skim milk regardless of the NEM concentrations, the average percentage of native α -lactalbumin and β -lactoglobulin over the total of that protein present was $41 \pm 6\%$ and $23 \pm 4\%$, respectively (Figure 6.2A). In heated WPE skim milk, there was on average, $6 \pm 2\%$ native α -lactalbumin and $6 \pm 1\%$ native β -lactoglobulin (Figure 6.2B).

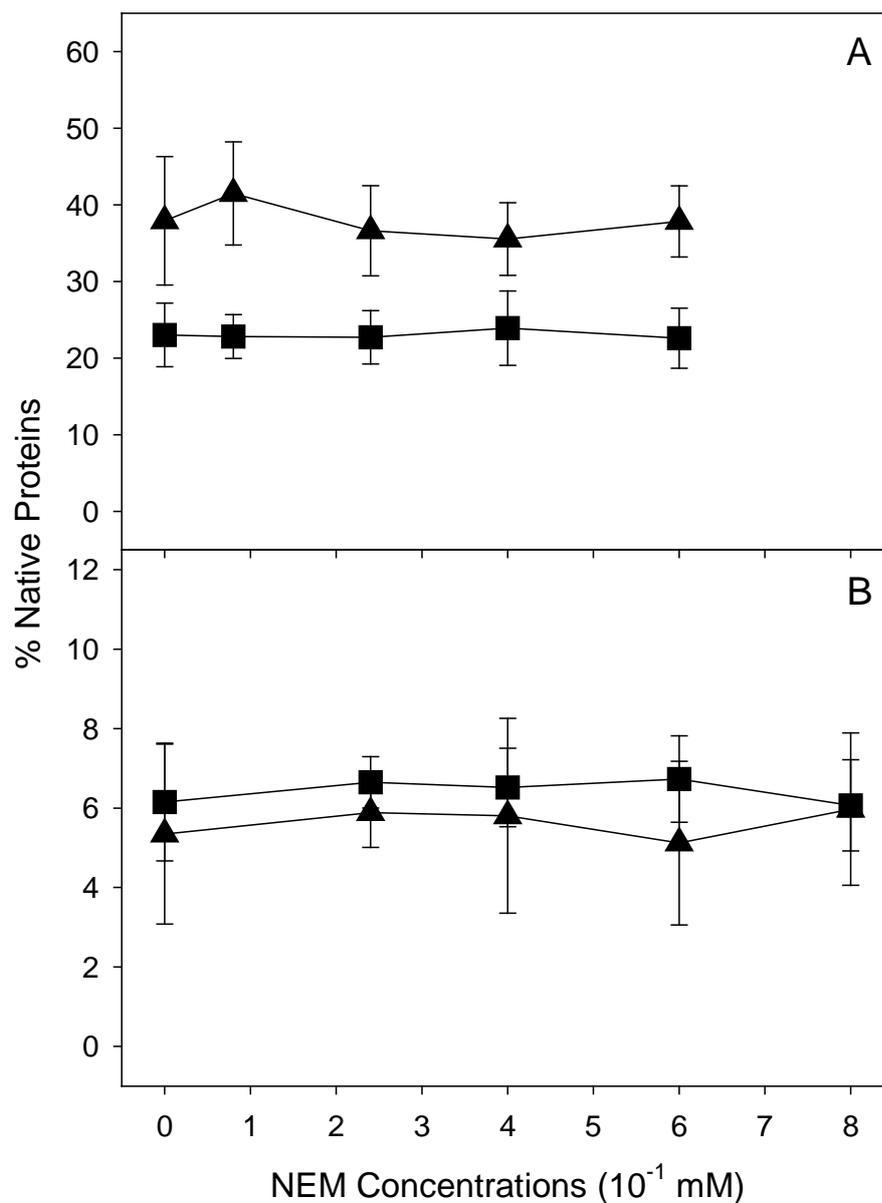


Figure 6.2: Effects of NEM concentrations on the percentage of native whey protein in heated skim milk (A) or WPE skim milk (B). ▲, α -lactalbumin; ■, β -lactoglobulin; each data point is an average of two to four replicates. The error bars represent the standard deviation.

Figure 6.3 shows the SDS-PAGE patterns of samples of heated skim milk (Figure 6.3A) and WPE skim milk (Figure 6.3B) with added NEM. The control heated and untreated sample (Lane a) was fully reduced, thus the band intensities represented the total amount of proteins in the milk. All other samples (Lanes b to g) were not reduced, and therefore the band intensities represent the amount of proteins that did not participate in disulphide interactions. These proteins can either be monomeric or participate in aggregates via non-covalent interactions.

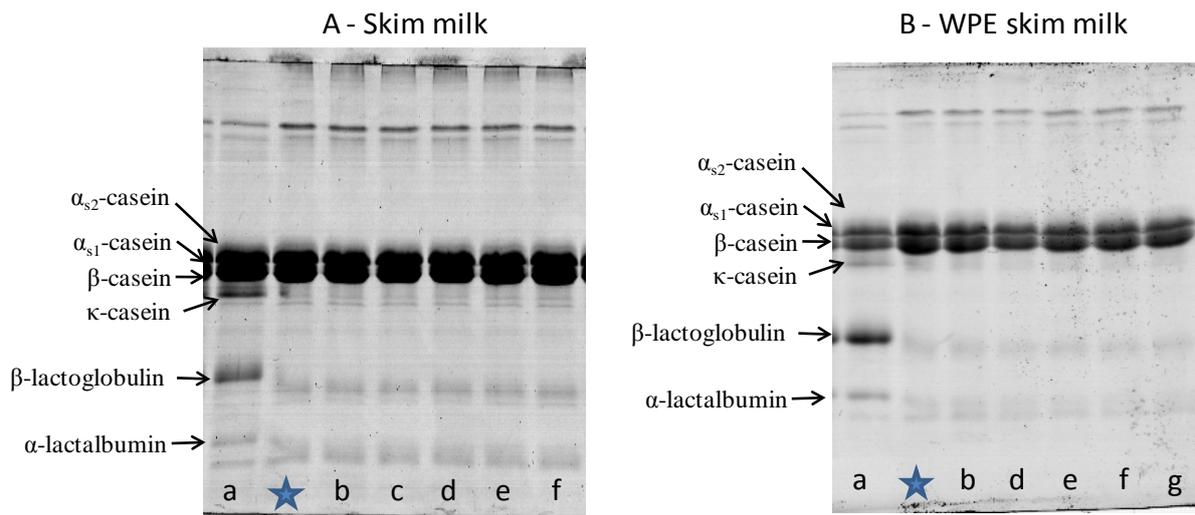


Figure 6.3: SDS-PAGE patterns of samples of heated skim milk (A) or heated WPE skim milk (B) followed by addition of NEM. Control samples (i.e. control untreated milks, Lane a), control heated milks heated (Lane b) and milk heated with added NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The milk samples were diluted with SDS sample buffer at ratio of 1 to 40 and were not reduced except for the control samples being fully reduced. Blue star was the transition lane between reducing and non-reducing conditions.

The SDS-PAGE patterns of milk samples showed that very low levels of monomeric α -lactalbumin, β -lactoglobulin and κ -casein were observed in heated milks (Figure 6.3). This showed that these proteins were largely involved in intermolecular disulphide interactions. The band intensities were integrated and the percentage of disulphide-linked protein over the total of each protein in milk is presented in Figure 6.4.

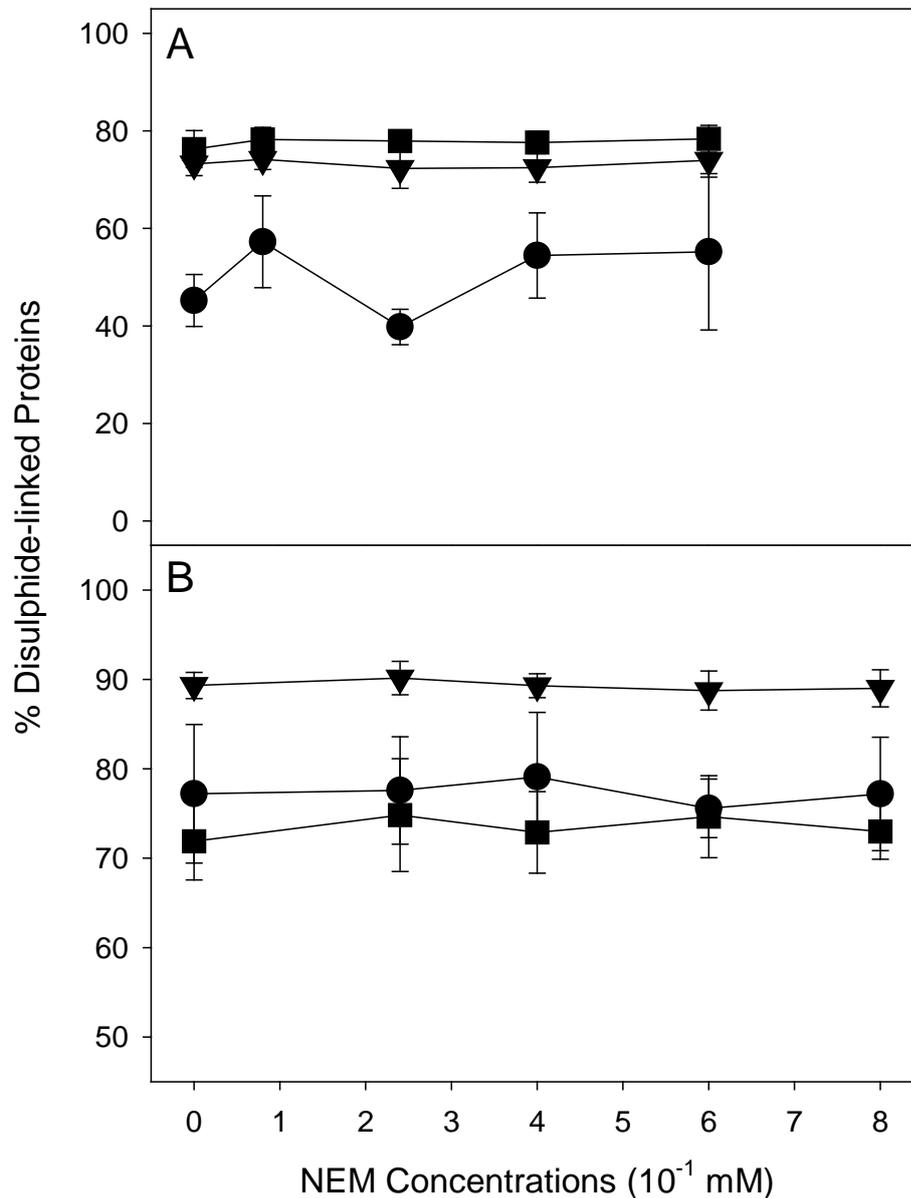


Figure 6.4: Effects of NEM concentrations on the percentage of disulphide-linked individual protein in heated skim milk (A) or WPE skim milk (B). ●, α -lactalbumin; ▼, β -lactoglobulin; ■, κ -casein. Each data point is the average of two to four replicates. The error bars represent the standard deviation.

Adding NEM to heated milks did not significantly affect the proportions of α -lactalbumin, β -lactoglobulin and κ -casein involved in intermolecular disulphide bonds ($p > 0.05$, Figure 6.4). In heated skim milk, the average percentage of disulphide-linked α -lactalbumin, β -lactoglobulin and κ -casein was $52 \pm 12 \%$, $72 \pm 3 \%$ and $78 \pm 3 \%$, respectively (Figure 6.4A). In heated WPE skim milk, the average total percentage of disulphide-linked α -lactalbumin was $77 \pm 6\%$, β -lactoglobulin was $89 \pm 2 \%$ and κ -casein was $74 \pm 5 \%$ (Figure 6.4B).

After centrifugation of the treated milks, the obtained supernatants were analysed using SDS-PAGE to determine the proportion of proteins in the serum phase. The SDS-PAGE visually showed that addition of NEM did not change the proportions of whey proteins and casein proteins located in the serum phases (Figure 6.5).

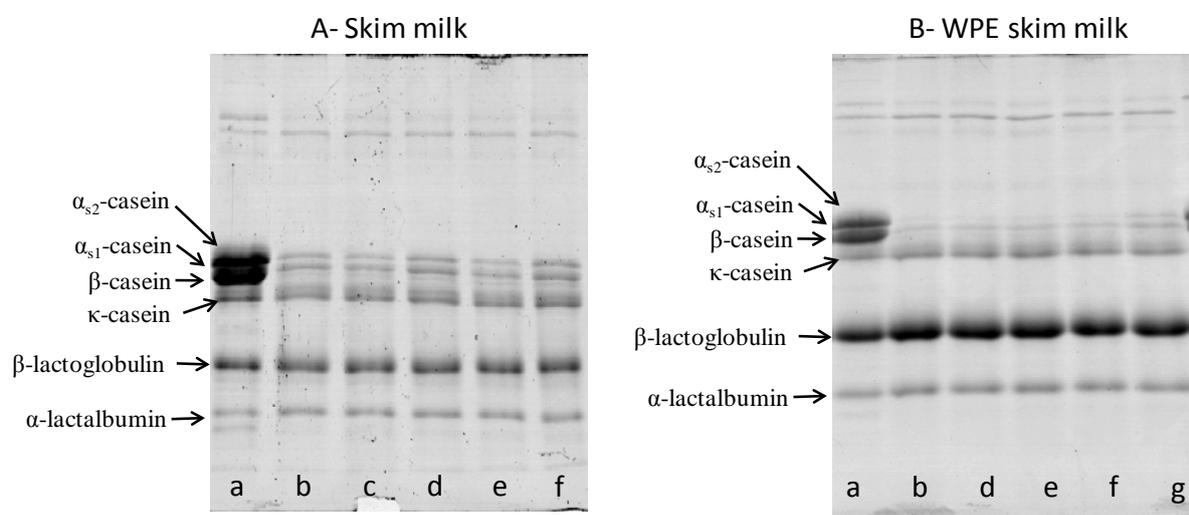


Figure 6.5: SDS-PAGE patterns of supernatant samples obtained from centrifugation of heated skim milk (A) and heated WPE skim milk (B) followed by addition of NEM. Control samples (i.e. control untreated milks, Lane a), supernatant of control heated milks (Lane b) and milk heated with added NEM: 0.08 mM (Lane c), 0.24 mM (Lane d), 0.4 mM (Lane e), 0.6 mM (Lane f) and 0.8 mM (Lane g). The milk sample (Lane a) and supernatant samples (Lanes b to g) were diluted with SDS sample buffer at ratio of 1:40 and 1:20, respectively. All the samples were fully reduced.

Based on the integration of the protein band intensities, the percentage of proteins in the serum and colloidal phases and the percentage of disulphide-linked proteins in each phase were calculated (as described in Section 3.6.7) and the results are presented in Figure 6.6, Figure 6.7 and Figure 6.8. The distribution of α -lactalbumin, β -lactoglobulin and κ -casein between the serum and colloidal phases did not change on addition of NEM in both heated skim milk ($p > 0.05$, Figure 6.6) and heated WPE skim milk ($p > 0.05$, Figure 6.8). Although some variation in serum α -lactalbumin was observed; the standard deviation values are large and therefore the changes were not significant ($p > 0.05$, Figure 6.6A).

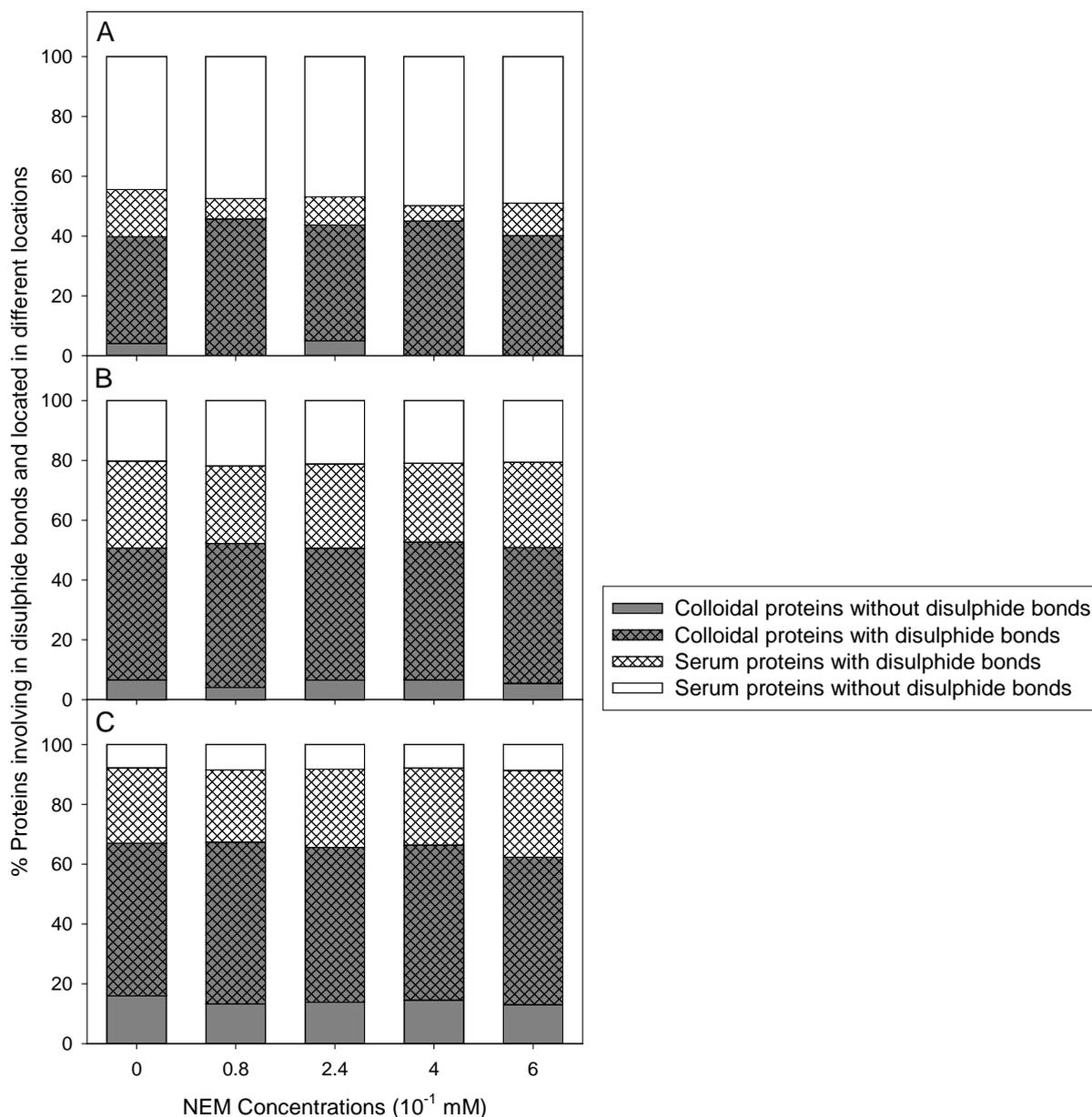


Figure 6.6: Effects of NEM concentrations in heated skim milk on the distribution of the proteins between the serum and colloidal phases and on the participation of the proteins in intermolecular disulphide bonds. A: α -lactalbumin; B: β -lactoglobulin and C: κ -casein. Y-axis is the percentage of individual protein. The levels of protein located in the colloidal phase are represented by grey columns and the levels of protein located in serum phase are represented by white columns. Each set is the average of two to four replicates.

Overall, regardless of NEM concentrations, the average percentage of serum α -lactalbumin was $57 \pm 3\%$ in heated skim milk and $76 \pm 2\%$ in heated WPE (Figure 6.6A and Figure 6.8A). The average percentage of serum β -lactoglobulin was $49 \pm 1\%$ in heated skim milk and $67 \pm 1\%$ in heated WPE skim milk (Figure 6.6B and Figure 6.8B). The average percentage of serum κ -casein was $34 \pm 2\%$ and $67 \pm 2\%$ in heated skim milk and heated WPE skim milk (Figure 6.6C and

Figure 6.8C), respectively. The average percentage of other caseins was $5.4 \pm 0.2\%$ in heated skim milk (Figure 6.7A) and $5.4 \pm 0.6\%$ in WPE skim milk (Figure 6.7B).

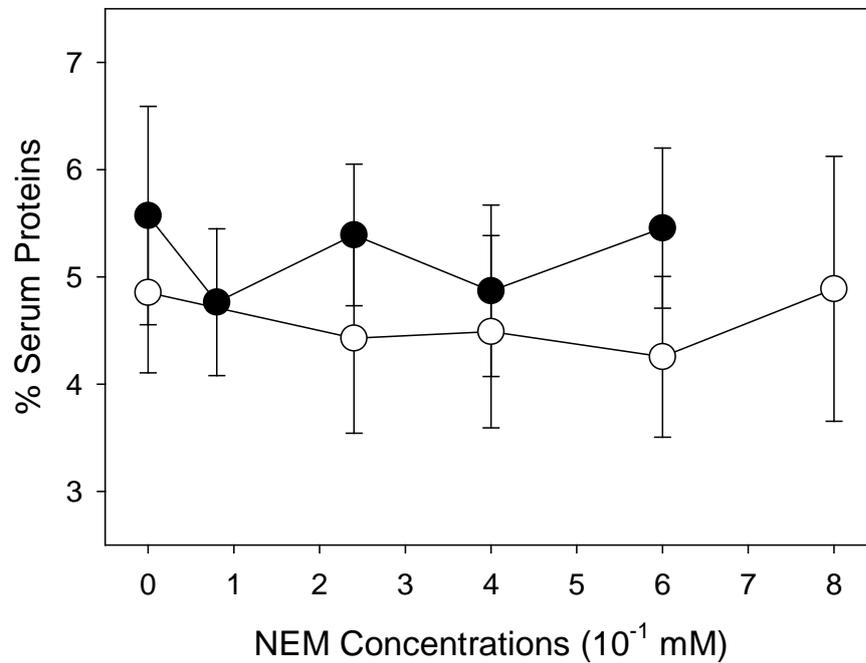


Figure 6.7: Effects of NEM concentrations on the percentage of other caseins (i.e. a combination of α_{s1} -, α_{s2} - and β -casein) in the serum over the total of those proteins present in heated skim milk (●) and heated WPE skim milk (○). Each data point is the average of two to four replicates. Error bars represent standard deviation.

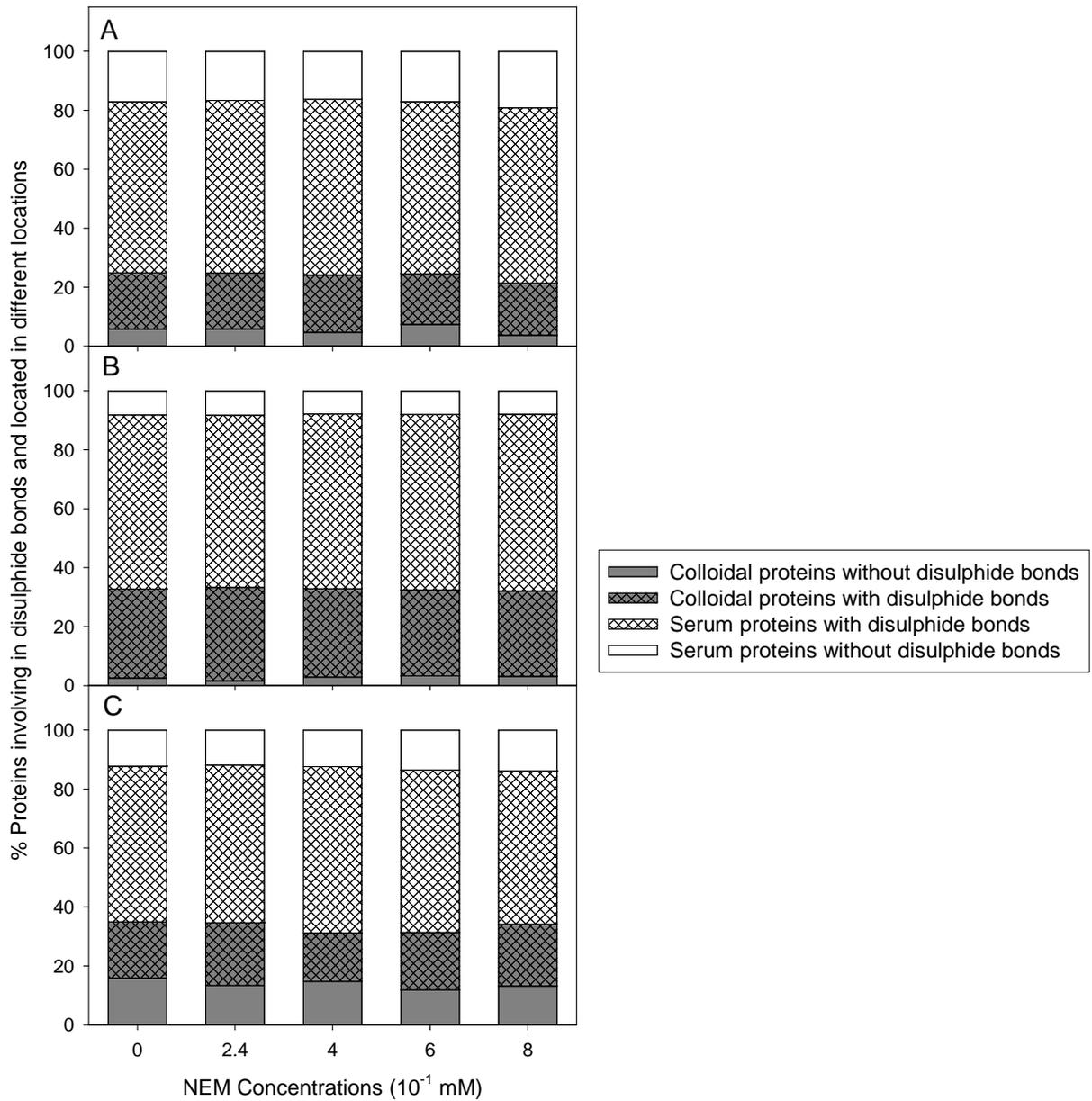


Figure 6.8: Effects of different NEM concentrations in heated WPE skim milk on the distribution of the proteins between the serum and colloidal phases and on the participation of the proteins in intermolecular disulphide bonds. A: α -lactalbumin; B: β -lactoglobulin and C: κ -casein. Y-axis is the percentage of individual protein over the total for that protein present in sample. The levels of protein located in the colloidal phase are represented by grey columns and the levels of protein located in serum phase are represented by white columns. Each set is the average of two to four replicates.

The size of the casein micelles in heated skim milk without and with added NEM remained at approximately 190 ± 2 nm. The size of the casein micelles in heated WPE skim milk decreased from 214 ± 3 nm in the sample without added NEM to 211 ± 1 nm at 0.24 mM NEM ($p < 0.05$, Figure 6.9). The casein micelle size did not change further at higher NEM concentrations ($p < 0.05$).

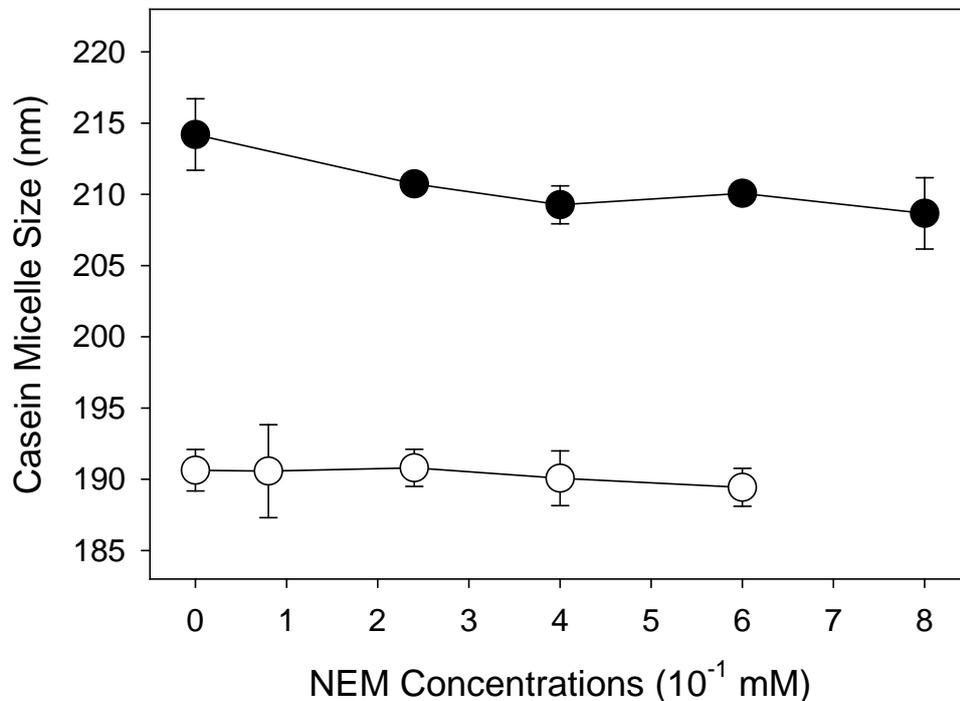


Figure 6.9: Effects of NEM concentrations on the size of the casein micelle in heated skim milk (○) and heated WPE skim milk (●). Each data point is an average of two to five replicates. The error bars represent the standard deviation.

6.3.2 Effects of adding NEM to heated milks on the rheological properties of the resulting acid gels

The treated milks were made into acid gels using 2% w/w GDL. The gels were formed at 30 °C over a period of 3 h. The typical changes of G' during acidification of milks are shown in Figure 6.10. The changes of G' values during gelation of heated skim milk and heated skim milk with added NEM were similar (Figure 6.10A). In contrast, the changes of G' values during gelation of heated WPE skim milk without and with added NEM were different to each other and to the gelation curve for heated skim milk (Figure 6.10B). Firstly, with no added NEM, the G' of WPE skim milk during gelation increased to markedly higher values than those of skim milk only. Secondly, when 0.6 mM NEM was added to WPE skim milk, the G' values during gelation were lower than those of the control WPE skim milk but still higher than those of skim milk (Figure 6.10).

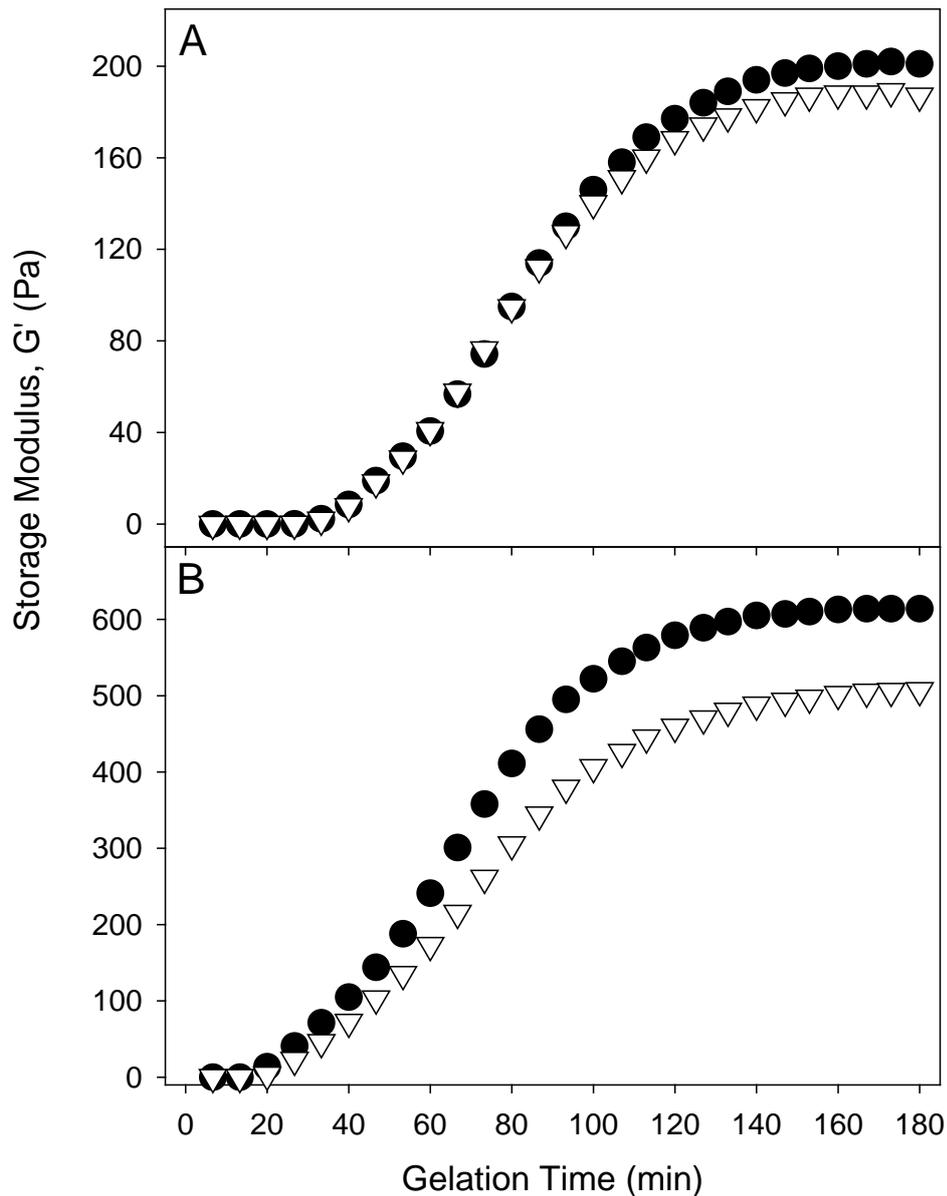


Figure 6.10: The typical increase of the G' values during formation of acid gels. A, heated skim milk; B, heated WPE skim milk; ●, 0 mM; ▽, 0.6 mM NEM.

The effects of different concentrations of NEM on the final G' values of acid gels made from heated skim milks is summarised in Figure 6.11. In heated skim milk, adding NEM did not significantly affect the final G' of the resulting acid gels ($p > 0.05$, Figure 6.11). In heated WPE skim milk, the final G' values of acid gels made from NEM-containing heated milks were significantly lower than those of control acid gels ($p < 0.05$, Figure 6.11). However, the increase in the concentration of NEM did not significantly affect the final G' values.

The gelation pH was also monitored and was found to be similar for each milk type, regardless of the NEM concentrations (even at 0 mM). The gelation pH of heated skim milk was $\text{pH } 5.40 \pm$

0.04 and of heated WPE skim milk it was $\text{pH } 5.66 \pm 0.04$. The changes of $\tan \delta$ values (G''/G') during gelation process of heated milks were not affected by the addition of NEM (Figure A.2, Appendix 2).

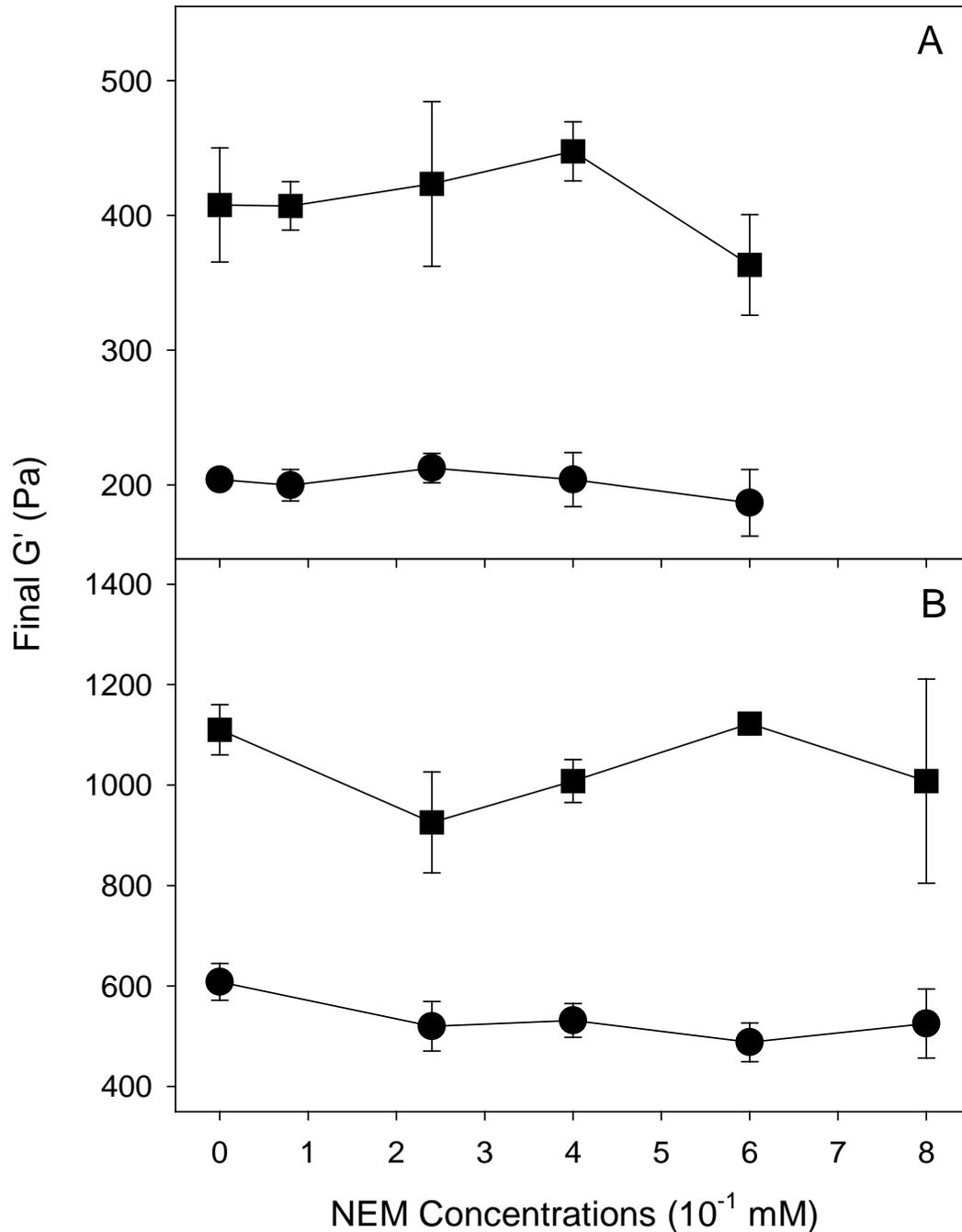


Figure 6.11: Effects of NEM concentrations on the final G' of acid gels made from heated skim milk (A) and heated WPE skim milk (B). ●, G' at 30 °C and ■, G' of gels after being cooled to 5 °C; Each data point is the average of two to four replicates. Error bars represent the standard deviation.

Once the gels had formed, the temperature was lowered to 5 °C (1 °C min⁻¹). The G' values at 5 °C of acid gels made from heated skim milks (without and with added NEM) as a function of NEM concentrations were summarised in Figure 6.11. At 5 °C, the presence of NEM in heated skim milk did not affect the G' values of acid gels ($p > 0.05$, Figure 6.11A), as also observed for gels at 30 °C.). In heated WPE skim milk, the G' values at 5 °C of acid gels containing NEM were significantly lower than those of acid gels made from heated WPE skim milk without added NEM. However, there was no significant difference between the G' values at 5 °C of acid gels containing NEM ≥ 0.24 mM ($p > 0.05$, Figure 6.11B). The relationship between G' at 30 °C and those at 5 °C of both skim milk and WPE skim milk gels was almost linear with the G' at 5 °C being ~ 1.9 times higher than those at 30 °C, regardless of the NEM addition concentration (Figure 6.11).

At 5 °C, the yield properties of acid gels were examined by monitoring the shear stress and strain of the gels that were subjected to a constant rotational shear at a rate of 0.005 s⁻¹. Due to the constant shear, the strain and the shear stress of the acid gels increased until the acid gels broke, resulting in sudden decrease of shear stress. The change of shear stress as a function of strain is shown in Figure 6.12 and the values of maximum shear stress and the strain at this point are summarised in Table 6.1.

In heated skim milk, acid gels made from milks with added NEM had a maximum shear stress $\sim 13\%$ lower than those made from milks without NEM (Figure 6.12A, Table 6.1). However the gels were broken at a similar strain, irrespective of their NEM content ($40 \pm 1\%$, Table 6.1). In heated WPE skim milk, the shear stress values of acid gels made from heated milks with added NEM were markedly lower (by 69%) than those of acid gels made from heated milks without NEM (Figure 6.12B and Table 6.1). In addition, the acid gels made from heated milks with added NEM were broken at lower strain ($\leq 36\%$) than those made from heated milk without NEM ($64 \pm 3\%$, Table 6.1).

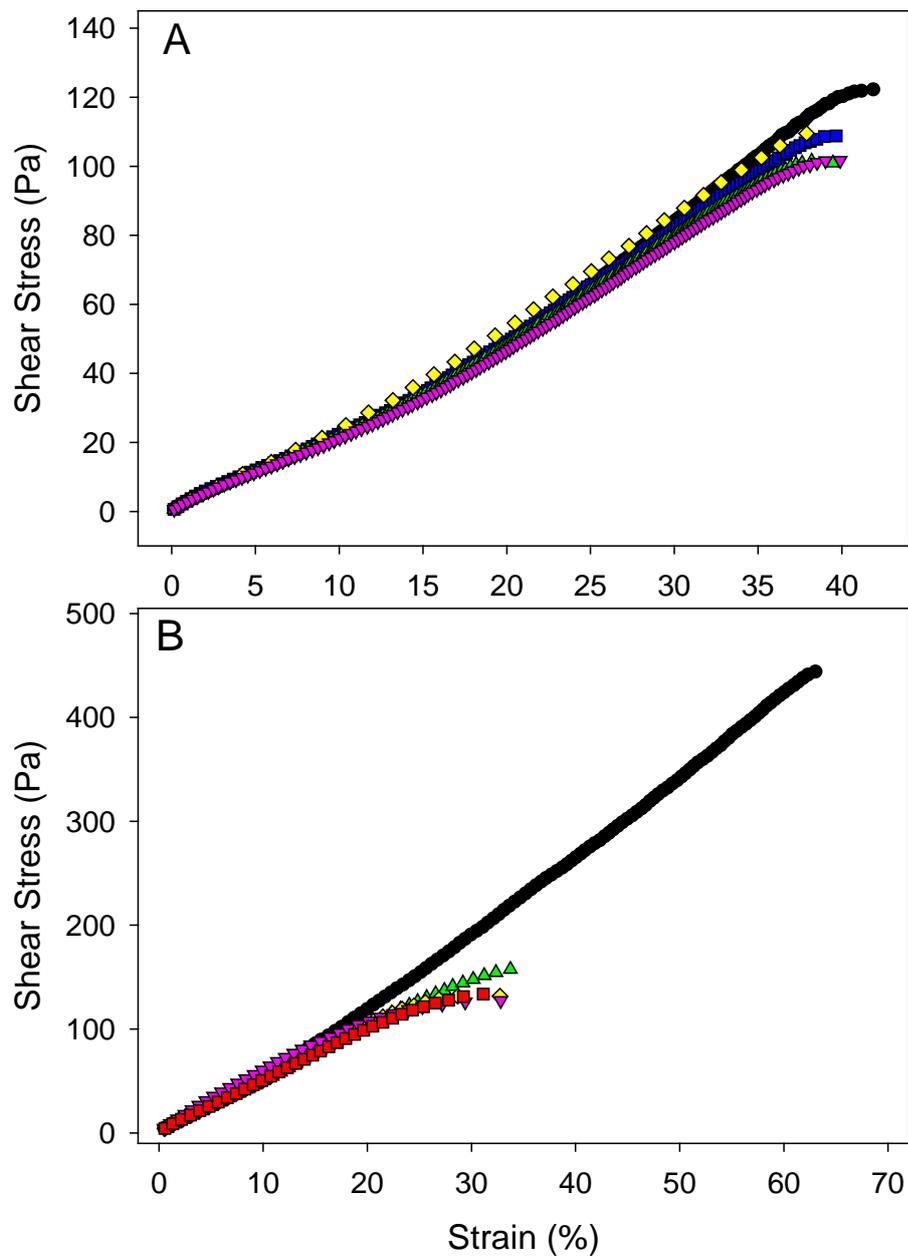


Figure 6.12: Shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^\circ\text{C}$ and analysed at $5 \text{ }^\circ\text{C}$. The acid gels were prepared from heated skim milk (A) and heated WPE skim milk (B) with different concentrations of NEM. ●, 0 mM ; ■, 0.08 mM; ▲ 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM and ■, 0.8 mM. Each point is the average of four to six replicates. Error bars were not shown to simplify the graph.

Table 6.1: Effects of NEM concentrations on the values of the yield strain (%) and yield stress (Pa) of acid gels when subjected to constant shear rate. Acid gels were prepared from heated skim milk and heated WPE skim milk followed by addition of different concentrations of NEM. The values are the mean value of two to four replicates \pm standard deviation.

Concentrations of NEM (mM)	Skim milk		WPE skim milk	
	Yield strain (%)	Yield stress (Pa)	Yield strain (%)	Yield stress (Pa)
0	42 \pm 3	122 \pm 1	64 \pm 2	445 \pm 10
0.008	40 \pm 3	109 \pm 3	—	—
0.024	40 \pm 4	101 \pm 5	36 \pm 5	159 \pm 5
0.4	40 \pm 2	112 \pm 2	31 \pm 8	133 \pm 8
0.6	40 \pm 3	102 \pm 3	36 \pm 4	126 \pm 5
0.8	—	—	33 \pm 4	135 \pm 4

6.3.3 Effects of adding NEM to heated milks on the microstructure of the acid gels

The structure of acid gels was examined using confocal microscopy. The confocal images of acid gels made from heated skim milks (without and with added whey proteins) followed by addition of 0, 0.24 and 0.6 mM NEM are shown in Figure 6.13 and Figure 6.14 for the skim milks and WPE milks respectively. The network for the acid gels prepared from heated skim milk with added NEM (Figure 6.13B-C) consisted of large open pores (black areas) that were larger in size than those in acid gels prepared from control heated skim milk indicating a less compact structure (Figure 6.13A). In heated WPE skim milk, the network of acid gels prepared from milks without and with added NEM appeared to be similar, being the same density in terms of protein aggregates and having pores of similar size (Figure 6.14).

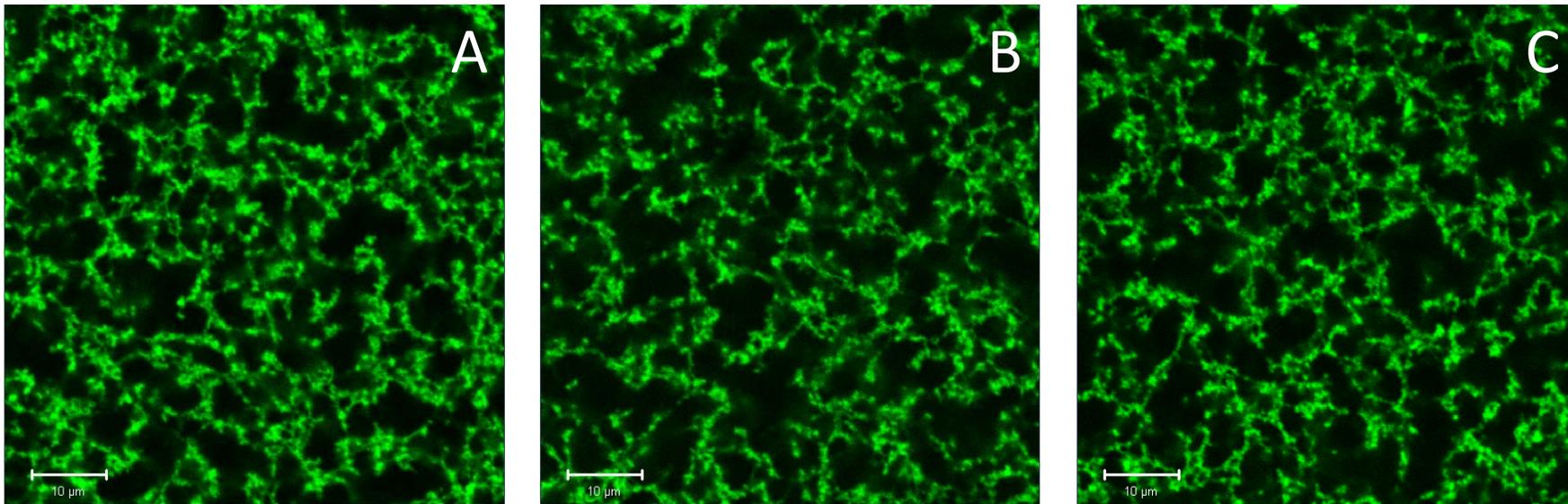


Figure 6.13: Effects of NEM concentrations on the microstructure of the acid gels prepared from heated skim milk with 0 mM (A), 0.24 mM (B) and 0.6 mM (C) added NEM. Scale bar is 10 μm.

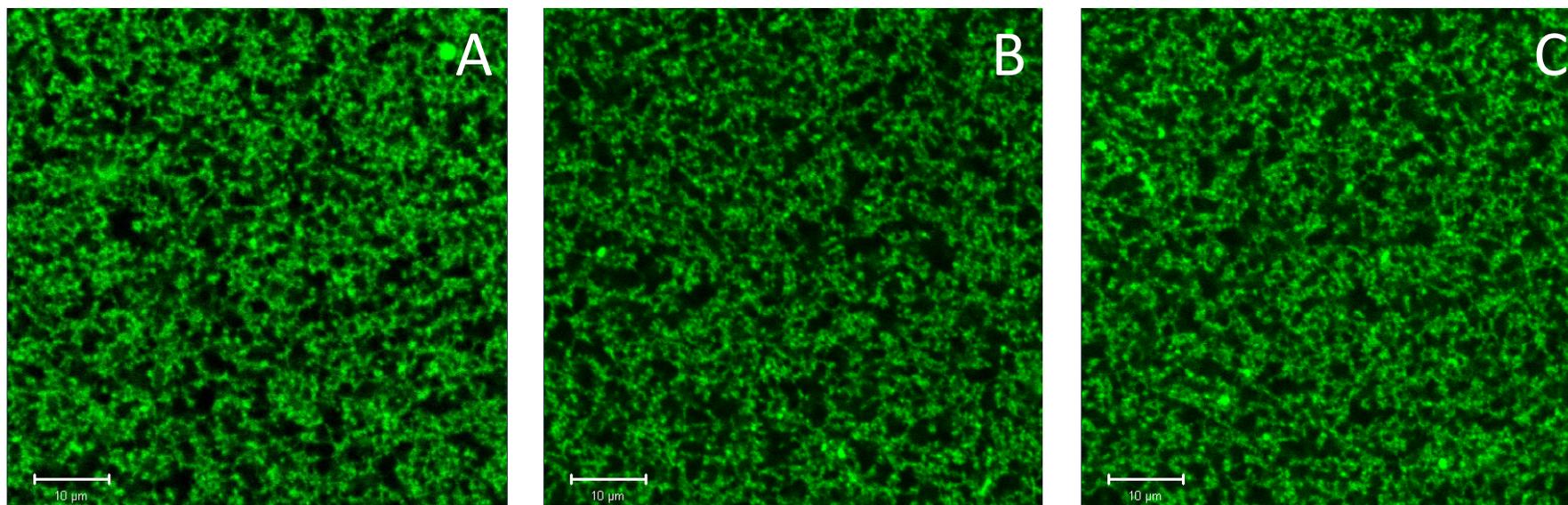


Figure 6.14: Effects of NEM concentrations on the microstructure of the acid gels prepared from heated WPE skim milk with 0 mM (A), 0.24 mM (B) and 0.6 mM (C) added NEM. Scale bar is 10 μm .

6.4 Discussion

6.4.1 Effects of adding NEM to heated milks on the protein interactions and distribution between colloidal and serum phases

The denaturation of whey proteins can occur when milks are exposed to temperatures ≥ 70 °C (Anema & Li, 2003b). As adding NEM to heated milks did not change the percentage of native whey proteins (Figure 6.2), this implied that addition of NEM at 20 °C neither inhibited nor promoted further denaturation of the whey proteins in heated milks. Based on the electrophoretic results (Figure 6.4, Figure 6.6, Figure 6.7 and Figure 6.8), it was shown that adding NEM to heated milks did not affect the interactions between the proteins, nor the distribution of the proteins between the serum and colloidal phases.

6.4.2 Effects of adding NEM to heated milks on the rheological properties and microstructure of acid gels

In previous studies, NEM was added to whey protein solutions and milks after heat treatment and before acid gelation. The G' values and the hardness of the gels decreased with the increase in NEM concentrations (Lucey et al., 1998; Alting et al., 2000; Alting et al., 2003; Vasbinder et al., 2003). It was also found that once the acid gels were solubilised in SDS solution without disulphide reducing agent, the aggregates in acid gels without NEM were larger than those in acid gels with NEM. Therefore, thiol-disulphide exchange reactions were proposed to occur during acid gelation, leading to formation of intermolecular disulphide bonds that increased the firmness (G') and hardness (measured by penetration tests) of the acid gels (Alting et al., 2000; Alting et al., 2003; Vasbinder et al., 2003).

In this study, the addition of NEM to heated skim milk did not significantly affect the final G' values of the resulting acid gels prepared from the heated skim milk (Figure 6.11A), which is contradictory to the previous findings of Vasbinder et al. (2003) and Lucey et al. (1998). Even though the acid gels prepared from heated skim milk with added NEM had a yield stress $\sim 13\%$ lower than those made from the control heated milk, they still had a markedly higher yield stress value than those made from unheated skim milk (which was 59 ± 2 Pa as shown in Chapter 4). This result was also in contrast with those from the study of Vasbinder et al. (2003) who reported that acid gels made from heated milk containing NEM had yield properties comparable to gels prepared from unheated milks.

In heated WPE skim milk, the final G' values of acid gels decreased (by $\sim 20\%$) when acid gels were prepared in the presence of NEM, regardless of the concentration of NEM added (Figure 6.11B). The NEM-containing acid gels broke at lower stress and strain values than gels without NEM (Figure 6.12B). The yield stress values of acid gels with NEM were not as low as those from

acid gels prepared from unheated WPE skim milk (46 ± 2 Pa, as shown in Table 4.2). Although this effect on the yield properties was similar to what was observed in gels made from heated skim milk, the decrease in gel yield properties was much greater in the heated WPE skim milk than in heated skim milk (Table 6.1).

The contrasting findings between previous studies and this study may be due to the conditions of gelation. In previous studies, the gelation process occurred over a period of 15 to 24 h at 20 °C (Lucey et al., 1998; Alting et al., 2000; Alting et al., 2003; Vasbinder et al., 2003) whereas in this study, the acid gelation took place at 30 °C, over 3 h. In addition, lower GDL concentrations were used in previous studies than in this current study. This affected the rate of pH decreasing during gelation.

A test was carried out to monitor the change of the pH under the gelation conditions of previous studies. The pH was found to decrease at a much slower rate compared with this study and reached \sim pH 5.9 after 3 h. It was found that thiol-disulphide exchange reactions between whey proteins were slower at acidic pH (Shimada & Cheftel, 1989; Hoffmann & van Mil, 1999). The reason was that the thiol group was protonated and this reduced its reactivity compared with the un-protonated form found under neutral or alkaline conditions. Therefore, thiol-disulphide exchange reactions during acidification were likely to happen to a greater extent in previous studies than in this study because the acidification process occurred over a markedly longer period of time and the pH of the system remained relatively high for a longer time in previous studies than in this study.

Despite the different magnitude of effects in this study compared with the previous ones, the rheological analyses in this study did show that blocking the thiol groups in heated skim milk and WPE skim milk caused the yield stress (and yield strain) values of acid gels to decrease. This suggested that blocking thiol groups prevented thiol-disulphide exchange reactions between the proteins during acid gelation. Consequently, new disulphide bonds may not be formed between the particles that participated in the gelation. Since disulphide bonds are stronger than non-covalent bonds, the prevention of the formation of new disulphide bonds between particles may cause the resulting acid gels to be easily broken, as summarised in Table 6.1.

Overall, in this study, it can be understood that low levels of thiol-disulphide exchange reactions between proteins can occur during acidification and using NEM to prevent these exchange reactions had a bigger effect on yield properties (where the bonds need to be broken) than on the storage modulus (i.e. the low amplitude properties where bonds are not broken) of the resulting acid gels. Furthermore, adding NEM to skim milk and WPE skim milk after heat

treatment (this Chapter) had little effect on the rheological properties of acid gels when compared to adding NEM to milk before heating (Chapter 5). This indicated that the thiol-disulphide exchange reactions during acidification may play only a minor role in the acid gel firmness and may have a bigger influence on the yield properties of the gels.

The effects of preventing the formation of new disulphide bonds during gelation on the final G' values and the yield stress values were more pronounced in gels formed from heated WPE skim milk than those from heated skim milk. In WPE skim milk, due to the high protein concentration, the proteins can be “physically” closer together than in skim milk where the protein can be more scattered. Hence, thiol-disulphide exchange reactions can occur more readily during gelation of WPE skim milk compared to skim milk. Indeed, Alting et al. (2000; 2003) found that disulphide bonds were formed only during acid gelation of whey protein solutions consisting > 4.5% protein concentrations. When the thiol-disulphide exchange reactions were prevented during gelation, the number of newly formed disulphide bonds may decrease more drastically in WPE skim milk gels than in skim milk only gels. As a result, the effect of NEM on the acid gel properties was more pronounced in WPE skim milk gels than in skim milk only gels.

6.5 Conclusions

In heated skim milk and WPE skim milk, interaction of NEM with the thiol groups may prevent the thiol-disulphide exchange reactions between the proteins during acid gelation. Consequently, disulphide bonds between neighbouring particles may not be formed. The formation of new disulphide bonds may not greatly influence the G' values of the resulting acid gels but definitely contributed to the yield properties of the acid gels. Therefore, the acid gels made from heated skim milk and WPE skim milk with added NEM had lower yield properties than those made from control heated milks.

Chapter 7 - Effects of adding low β -mercaptoethanol concentrations to unheated milks on the protein interactions and the acid gel properties

7.1 Introduction

From the results presented in the previous Chapters, it was demonstrated that blocking the thiol groups in milk system prevented thiol-disulphide exchange reactions on heating milk and protein interactions (either non-covalently or covalently) during the acid gelation of milks. The prevention of the formation of new intermolecular disulphide bonds may result in gels that are broken more easily under large deformation, but without a dramatic decrease in the gel storage modulus. This led to the work described in the next part of this thesis in which the effect of enhancing the thiol-disulphide exchange reactions on protein interactions in milks and properties of the resulting acid gels was investigated.

Thiol-disulphide exchange reactions can be enhanced by introducing free thiol groups into protein systems, which can be achieved by breaking **a few** of the existing disulphide bonds of the proteins (Hashizume & Sato, 1988; Kella, Yang & Kinsella, 1989; Goddard, 1996; Bazinet, Lamarche, Boulet & Amiot, 1997; Sural & Famelart, 2003) or by chemically or genetically adding thiol groups to the proteins (Kim, Olson & Richardson, 1990; Lee, Cho & Batt, 1993). In this thesis, a disulphide reducing agent, β -mercaptoethanol, was used to increase the ratio of thiol groups to disulphide bonds in the milk systems.

Unlike the thiol blocking reagent, which interacted mostly with β -lactoglobulin (Zittle et al., 1962; Hoffmann & van Mil, 1997), a disulphide reducing agent can interact with any milk protein that contains disulphide bonds including κ -casein, α_{s2} -casein, β -lactoglobulin and α -lactalbumin. The protein disulphide bonds can be either intra-molecular (e.g. those in α -lactalbumin and β -lactoglobulin) or intermolecular (e.g. those in κ -casein oligomers). In α_{s2} -casein, disulphide bonds can exist in both intra- and inter-molecular forms depending on whether α_{s2} -casein is in a monomeric state or a dimeric state.

Even though all of the disulphide bonds in milk can be reduced by an excess of disulphide reducing agent (Purkayastha et al., 1967; Sawyer, 1967), it is not widely reported whether or not all of the disulphide bonds of different milk proteins are equally reactive towards the reducing agent. There are studies comparing the reactivity of the disulphide bonds within α -lactalbumin and β -lactoglobulin toward disulphide reducing agents. It was found that the disulphide bond Cys⁶-Cys¹²⁰ of α -lactalbumin is reduced more readily by dithiothreitol than the

other three disulphide bonds of the protein (Ku wajima, Ikeguchi, Sugawara, Hiraoka & Sugai, 1990), whereas the disulphide bond Cys⁶⁶ – Cys¹⁶⁰ of β -lactoglobulin is more reactive toward the reducing agents than the other bond (Davidson & Hird, 1967). However the relative reactivity of the disulphide bonds on different milk proteins has not been established.

In addition, the reduction of disulphide bonds of the milk proteins was mostly carried out at temperatures > 70 °C (Goddard, 1996; Havea et al., 1998 as an example; Wada & Kitabatake, 2001). It was unclear if heat is required to assist with the reduction of the disulphide bonds of the milk proteins. Therefore, the aim of the first part of this study was to investigate whether or not the disulphide reducing agents interacted equally with the disulphide bonds on different milk proteins under non-heating conditions. Low levels of the disulphide reducing agent (< 43 mM of β -mercaptoethanol) were added to unheated skim milk. Since gel electrophoresis (SDS-PAGE and MF-electrophoresis) can only detect the reduction of β -lactoglobulin and κ -casein in milk (see Sections 3.6 and 3.7), the levels of reduced β -lactoglobulin and monomeric κ -casein were monitored as a function of time after adding the reducing agent. Monomeric κ -casein consists of both the native κ -casein with an intra-molecular disulphide bond (Rasmussen et al., 1994) and the reduced κ -casein with two free thiol groups. The changes in the distribution of the proteins between the colloidal and serum phases were also determined with time after adding the reducing agent.

In addition, acid gels were made from the unheated skim milk treated with added disulphide reducing agent. The firmness and the microstructure of the gels were investigated by rheological measurement and confocal microscopy, respectively.

7.2 Materials and methods

Skim milk and WPE skim milk were prepared as described in Section 3.1.1.

In this study, fresh skim milk (Section 3.1.2) was used when the reduction of the disulphide bonds of β -lactoglobulin and κ -casein was examined using MF-electrophoresis. The whey protein in fresh skim milk did not have any lactosylation, thus the electropherograms have a better resolution with α -lactalbumin and β -lactoglobulin shown as one single peak each. Nevertheless, unless specifically stated, reconstituted skim milks were used in other analyses in this study.

Diluted β -mercaptoethanol (10% v/v) was added to give β -mercaptoethanol concentrations of 1.4, 4.3, 7.1, 17 and 43 mM in unheated skim milk and 1.4, 4.3 and 7.1 mM in unheated WPE skim milk. The ratio of β -mercaptoethanol to the disulphide bonds in the milks is listed in Table 7.1. The calculation was based on the composition of the skim milk and whey protein isolate

that were estimated to have 1.1 mM and 2.6 mM disulphide bonds, respectively. The treated milk was shaken on a vortex mixer for 10 s and then left to react at 20 °C in a thermostatically controlled water bath for up to 12 h. The milk container was sealed to minimise any oxidation.

Table 7.1: The corresponding ratio of β -mercaptoethanol to disulphide bonds existing in skim milk and WPE skim milk corresponding to the β -mercaptoethanol concentrations used in this study.

Concentrations of β-mercaptoethanol (mM)	Ratio of β-mercaptoethanol to disulphide bonds in skim milk	Ratio of β-mercaptoethanol to disulphide bonds in WPE skim milk
1.4	1.3	0.6
4.3	3.7	1.6
7.1	6.2	2.7
17	15	-
43	38	-

The reduction of disulphide bonds of β -lactoglobulin and κ -casein as a function of time and a function of β -mercaptoethanol concentrations were investigated using MF-electrophoresis (as described in Section 3.7). The distribution of the proteins between the colloidal and serum phases was examined using SDS-PAGE (as described in Section 3.6). The sizes of the casein micelles were monitored using size measurement method, which is described in Section 3.8.

After defined times, subsamples of the milks were slowly acidified to form acid gels. The rheological properties of the milks during acidification were monitored. In addition, the rheological properties and the microstructure of the set gels were also investigated. The details of the rheological and microstructural methods can be found in Section 3.10.2 and 3.10.3.

7.3 Results

7.3.1 Effects of reaction time and β -mercaptoethanol concentrations – by MF-electrophoresis method

7.3.1.1 Skim milk

Figure 7.1 and Figure 7.2 are the examples of the MF-electropherograms showing the relative changes in the amount of reduced β -lactoglobulin and monomeric κ -casein on addition of β -mercaptoethanol to unheated skim milk.

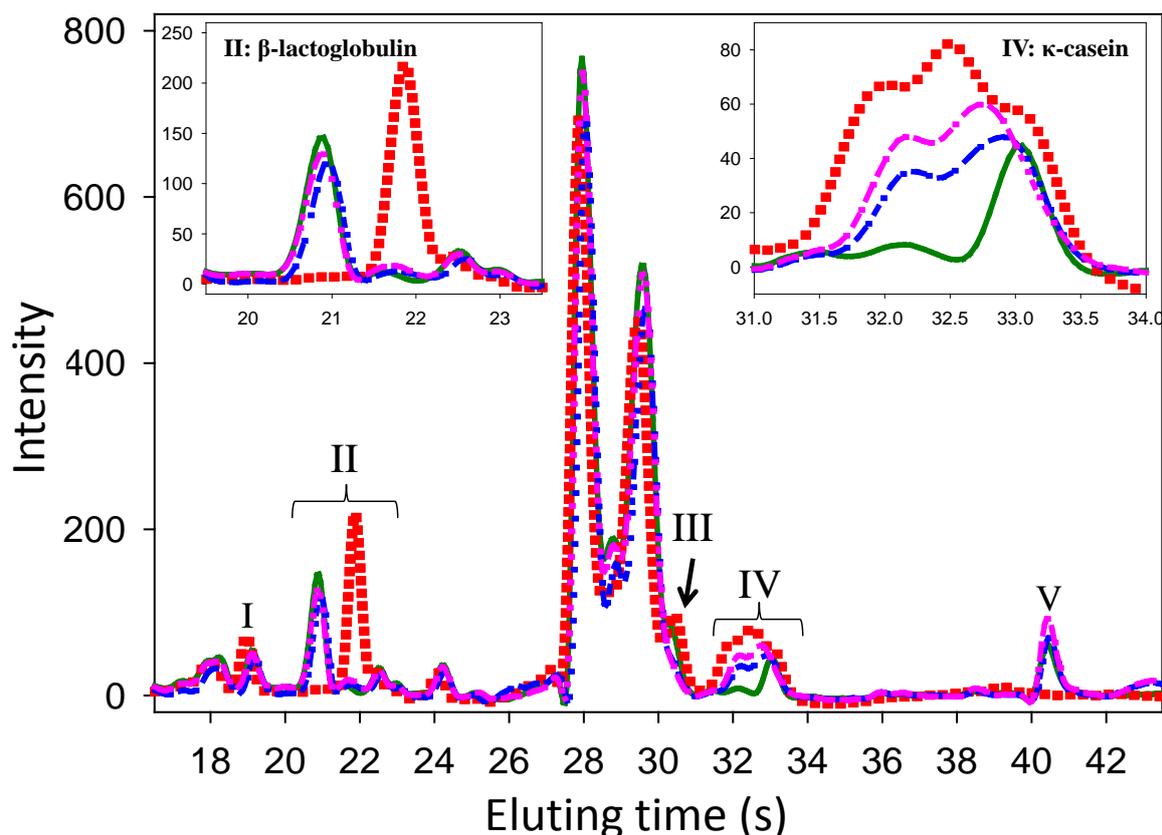


Figure 7.1: MF-electropherograms of fully reduced control milk (red dotted line), non-reduced control milk (green line) and milk reacted with 7.1 mM β -mercaptoethanol for 1 h (blue dashed line) and 6 h (pink dashed line). I, α -lactalbumin; II, β -lactoglobulin; III, monomeric α_{s2} -casein; IV, κ -casein and V, dimeric α_{s2} -casein.

On addition of 7.1 mM β -mercaptoethanol to skim milk, the peak area of monomeric κ -casein increased progressively and markedly as the reaction time increased from 1 to 6 h (Insert IV of Figure 7.1) whereas the peak area of reduced β -lactoglobulin was hardly changed (Insert II of Figure 7.1). This indicated the reduction of only κ -casein disulphide bonds and that more reduction occurred at longer reaction times.

Figure 7.2 showed that the peak areas of reduced β -lactoglobulin (Peak II) and monomeric κ -casein (Peak IV) increased as the β -mercaptoethanol concentrations in skim milk increased. The increase in the proportion of monomeric κ -casein was more pronounced than the increase in the proportion of reduced β -lactoglobulin. This indicated that the disulphide bonds of κ -casein were reduced more readily than those of β -lactoglobulin.

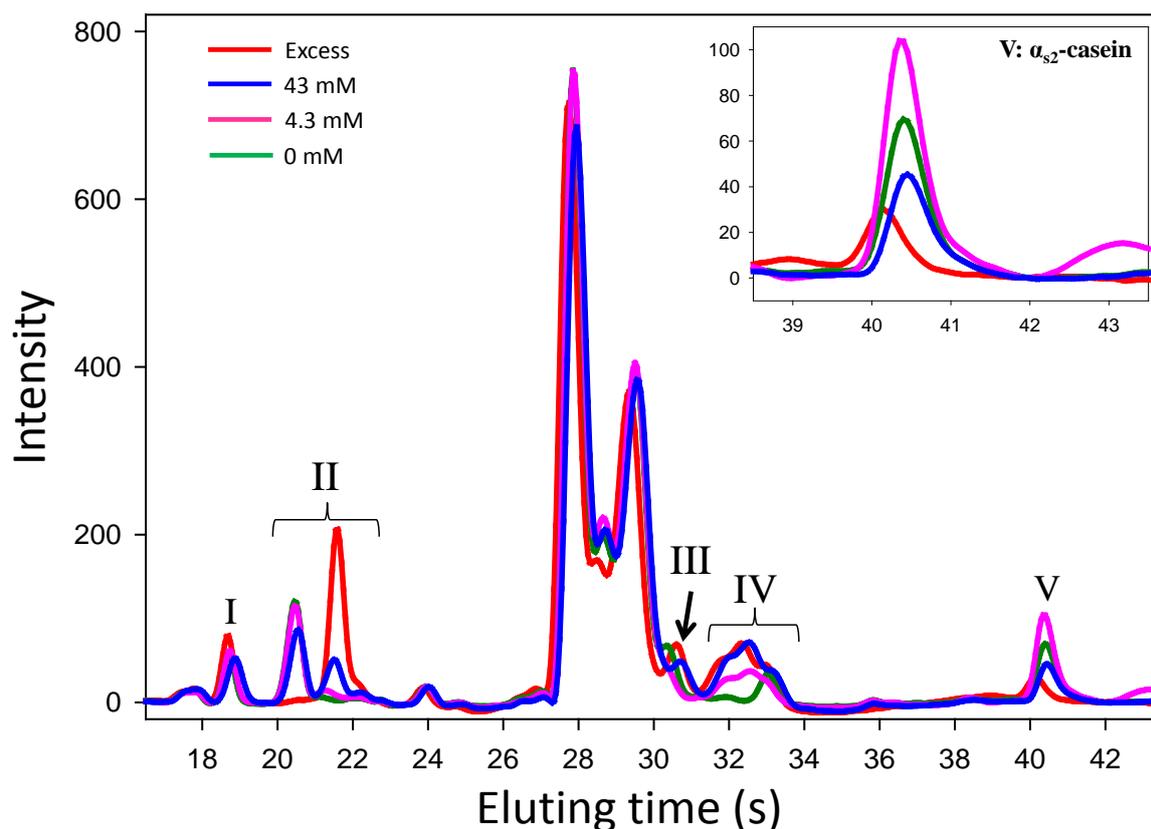


Figure 7.2: MF-electropherograms of skim milk that had been reacted with different levels of β -mercaptoethanol (indicated by the coloured lines) for 3 h. I, α -lactalbumin; II, β -lactoglobulin; III, monomeric α_{s2} -casein; IV, κ -casein and V, dimeric α_{s2} -casein.

In bovine milk, α -lactalbumin and α_{s2} -casein, two of the major milk proteins, also contain disulphide bonds (Rasmussen et al., 1994; Brew, 2003). As already mentioned in Section 3.7.2, the reduced and non-reduced α -lactalbumin cannot be differentiated (Peak I in Figure 7.2) using MF-electrophoresis whereas the monomeric and dimeric α_{s2} -casein can be distinguished (peaks III and V in Figure 7.2, respectively). Due to the location of α_{s2} -casein at the interior of the casein micelles, disulphide bonded interactions between α_{s2} -casein and whey proteins were reported to only occur upon disruption of the casein micelles (Patel et al., 2006; Considine, Patel, Anema, Singh & Creamer, 2007). In this study, it was expected that β -mercaptoethanol, a small molecule (MW: 78.13 g mol⁻¹), could penetrate into the casein micelles and interact with the disulphide bonds of α_{s2} -casein. Hence the reduction of disulphide bonds of α_{s2} -casein can be examined by monitoring the change in intensity of the monomeric α_{s2} -casein (peak III) or of the dimeric α_{s2} -casein peak (peak V) as a function of β -mercaptoethanol concentrations. As the monomeric α_{s2} -casein peak overlaps with other casein peaks, monitoring the change of the α_{s2} -casein peak

intensity was not accurate. Hence an attempt was made to monitor the change of the dimeric α_{s2} -casein peak.

The area of α_{s2} -casein peak, however, increased when the β -mercaptoethanol concentration increased from 0 to 4.3 mM then decreased with further increases of the β -mercaptoethanol concentration (Inset V of Figure 7.2). A possible reason why the level of dimeric α_{s2} -casein increased when the intermolecular disulphide bonds of α_{s2} -casein were supposed to be broken could be that interactions between β -mercaptoethanol and dimeric α_{s2} -casein can cleave only one disulphide bond at a time. Hence the breakage of one out of two intermolecular disulphide bonds may loosen the structure of the dimer. This may allow higher portion of SDS molecules to attach, hence higher proportion of dye molecules to bind to the partially broken dimer than to an intact dimer. As a result, a partially broken α_{s2} -casein dimer may give a more intense signal than an intact one. Due to this complexity, the reduction of α_{s2} -casein cannot be accurately monitored. Hence only the reduction of β -lactoglobulin and κ -casein was examined.

The effects of reaction time and β -mercaptoethanol concentrations on the reduction of disulphide bonds of β -lactoglobulin and κ -casein are summarised in Figure 7.3. The percentage of reduced β -lactoglobulin (relative to the total amount of β -lactoglobulin present in the system) remained very low ($< 7\%$) at β -mercaptoethanol concentrations ≤ 7.1 mM, regardless of the reaction time (Figure 7.3A). At β -mercaptoethanol concentrations of 17 and 43 mM, the percentage of reduced β -lactoglobulin increased significantly from $1.7 \pm 0.2 \%$ at 0 mM β -mercaptoethanol up to $8.9 \pm 0.6\%$ and $21 \pm 3\%$, respectively after 3 h ($p < 0.05$, Figure 7.3A). As the reaction time increased beyond 3 h, no further β -lactoglobulin was observed.

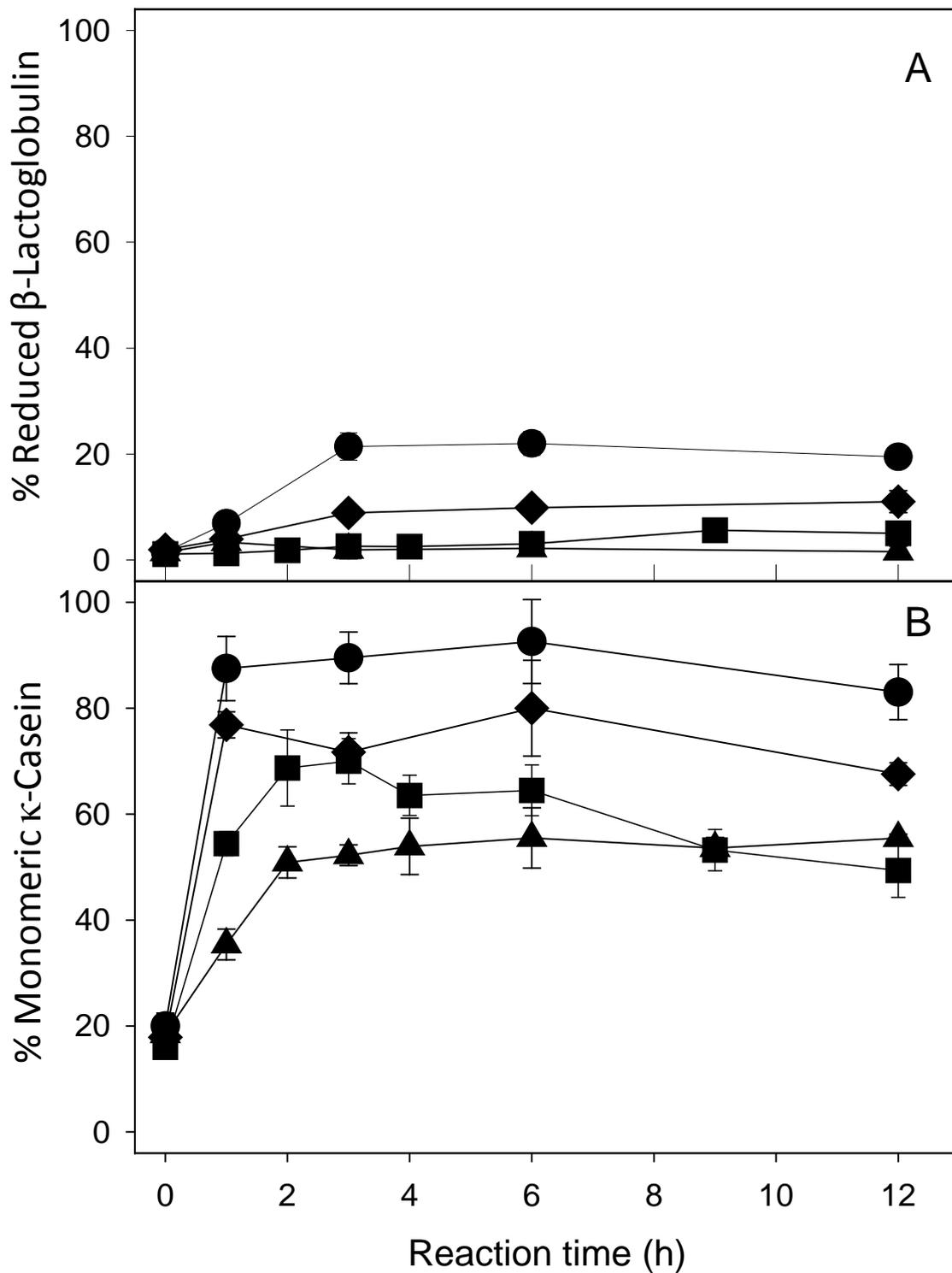


Figure 7.3: The effects of reaction time on the percentage of reduced individual proteins over the total of that protein in unheated control skim milk at varying concentrations of β -mercaptoethanol. β -lactoglobulin (A) and monomeric κ -casein (B): ▲, 4.3 mM; ■, 7.1 mM; ◆, 17 mM and ●, 43 mM. Analysis used MF-electrophoresis.

At a β -mercaptoethanol concentration of 4.3 mM, the percentage of monomeric κ -casein (relative to the total amount of κ -casein present) increased from $18 \pm 1\%$ to $51 \pm 2\%$ after 2 h ($p < 0.05$), then no further reduction occurred. At a β -mercaptoethanol concentration of 7.1 mM, the κ -casein reduction also occurred after 2 h and increased from $16 \pm 1\%$ to $70 \pm 4\%$ during this time; after which the percentage of monomeric κ -casein decreased gradually as the reaction time was increased to 12 h (Figure 7.3B). This decrease could be due to a number of factors including the re-oxidation of free thiol groups to reform disulphide bonds, or the interaction between a newly formed thiol group on a monomeric κ -casein and an existing disulphide bond on another protein (via thiol-disulphide exchange reactions) to aggregate the κ -casein. Interestingly only this concentration of β -mercaptoethanol (7.1mM) showed the increase then decrease in the monomeric κ -casein as the reaction progressed (Figure 7.3B). At β -mercaptoethanol concentrations ≥ 17 mM, the reduction of κ -casein disulphide bonds occurred only over 1 h; faster than at β -mercaptoethanol concentration of ≤ 7.1 mM, and remained unchanged as reaction time increased further.

Figure 7.3 showed that at time ≥ 1 h, the percentage of monomeric κ -casein and reduced β -lactoglobulin increased as the concentration of β -mercaptoethanol increased from 4.3 to 43 mM. In addition, the percentage of κ -casein reduced was always higher than the percentage of β -lactoglobulin reduced, regardless of the β -mercaptoethanol concentration and reaction time. This indicates that the disulphide bonds of κ -casein were reduced in preference to those of β -lactoglobulin.

The effect of reaction time on the reduction of κ -casein and β -lactoglobulin showed that up to 3 h was required for the reaction to be complete at any concentration of β -mercaptoethanol. The effects of reaction time on the size of the casein micelles in skim milk and the final G' values of the acid gels prepared from the treated milks were also examined. The casein micelle size and the final G' values decreased after 1 to 3 h of reaction time, depending on the concentrations of β -mercaptoethanol (Figure A.6 and Figure A.7, Appendix 4). Hence further investigations were carried out by adding β -mercaptoethanol to milk, holding for 3 h at 20.0 ± 0.5 °C before analyses or further treatments.

7.3.1.2 WPE skim milk

In skim milk, κ -casein and β -lactoglobulin are present at similar concentrations (0.4 and 0.35%, respectively). In WPE skim milk, the concentration of β -lactoglobulin was ~ 3 times higher than that of κ -casein. The interaction between β -mercaptoethanol and the milk proteins was therefore investigated in WPE skim milk to verify whether β -lactoglobulin can be reduced preferentially to κ -casein by β -mercaptoethanol when present in higher levels.

The effect of reaction time on the reduction of κ -casein and β -lactoglobulin was investigated. Figure 7.4 showed the relative change in the peak area of reduced β -lactoglobulin and monomeric κ -casein when 0, 7.1 mM and an excess amount of β -mercaptoethanol was added to the unheated WPE skim milk. The peak area of reduced β -lactoglobulin in WPE skim milk with 7.1 mM added β -mercaptoethanol was low (Insert II of Figure 7.4), even after 6 h. In contrast, the peak area of monomeric κ -casein in the same sample markedly increased after 1 h and decreased slightly as the reaction time increased to 6 h (Insert IV of Figure 7.4).

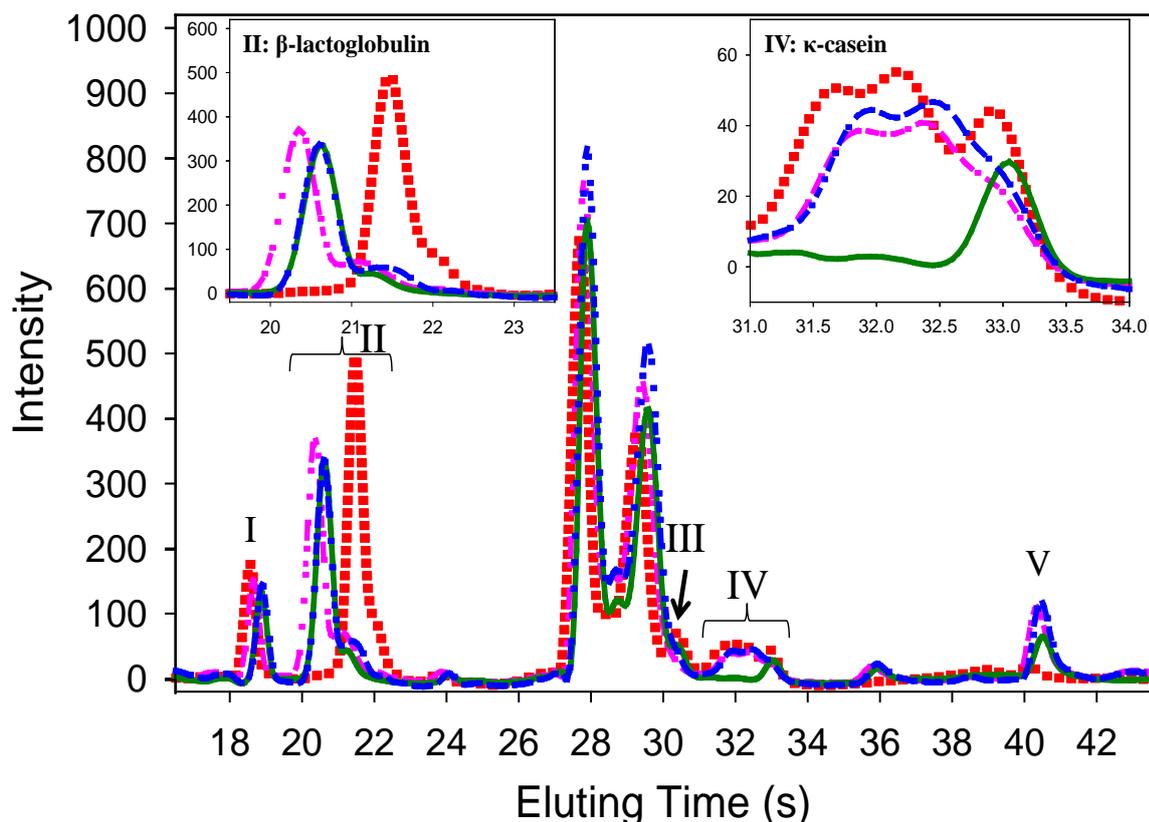


Figure 7.4: MF-electropherograms of control WPE skim milk (green line), fully reduced control WPE skim milk (red dotted line) and WPE skim milk that had been treated with 7.1 mM β -mercaptoethanol for 1 h (blue dashed line) and 6 h (pink dashed line). I, α -lactalbumin; II, β -lactoglobulin; III, monomeric α_{s2} -casein; IV, κ -casein and V, dimeric α_{s2} -casein.

The percentage of reduced individual proteins in unheated WPE skim milk as a function of reaction time is shown in Figure 7.5. The percentage of monomeric κ -casein increased significantly after 1 h of β -mercaptoethanol addition. As the reaction time was prolonged beyond 1 h, this percentage then decreased gradually ($p < 0.05$). However, the decrease in percentage of monomeric κ -casein beyond 1 h was small; it only decreased from $51 \pm 3\%$ to $42 \pm 5\%$ between 1 and 5 h. The percentage of reduced β -lactoglobulin remained low ($< 5\%$) over

a reaction period of 6 h. Even though the percentage of reduced β -lactoglobulin appeared to decrease after 1 h of reaction, the change was not significant ($p > 0.05$, Figure 7.5).

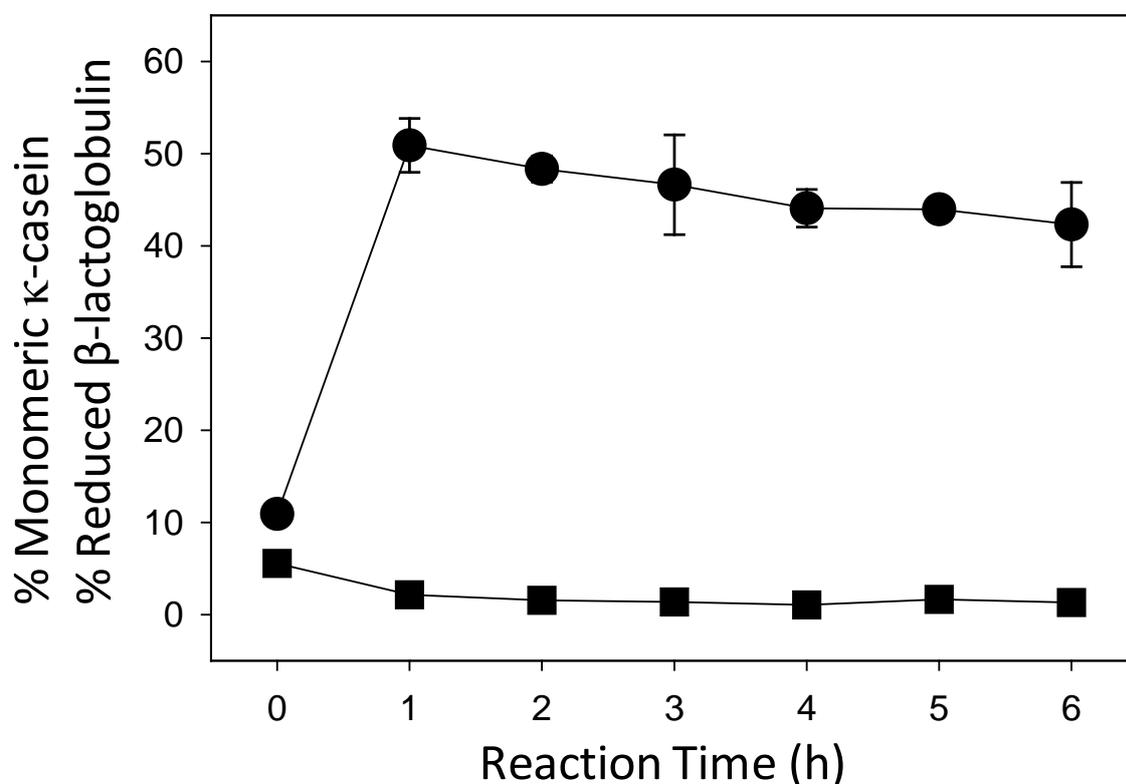


Figure 7.5: The effect of reaction time on the percentage of reduced β -lactoglobulin (■) and monomeric κ -casein (●) over the total of that protein in unheated WPE skim milk treated with 7.1 mM β -mercaptoethanol. Analysis used MF-electrophoresis and each data point is the mean value of two to four replicates, the error bar is the standard deviation.

In order to keep the study consistent with the experiments carried out with skim milk, further investigations on WPE skim milk were carried out as follows: after addition of β -mercaptoethanol, the milk was held for 3 h at 20.0 ± 0.5 °C before analyses or further treatments. Figure 7.6 showed the electropherograms of selected milk samples that had been treated with different concentrations of β -mercaptoethanol for 3 h. While the change of the peak area of reduced β -lactoglobulin was not obvious (Peak II), the peak area of monomeric κ -casein increased progressively with the increase of β -mercaptoethanol concentration (Peak IV in Figure 7.6). As mentioned in Section 7.3.1.1, the reduction of α -lactalbumin and α_{s2} -casein cannot be detected accurately, hence was not monitored.

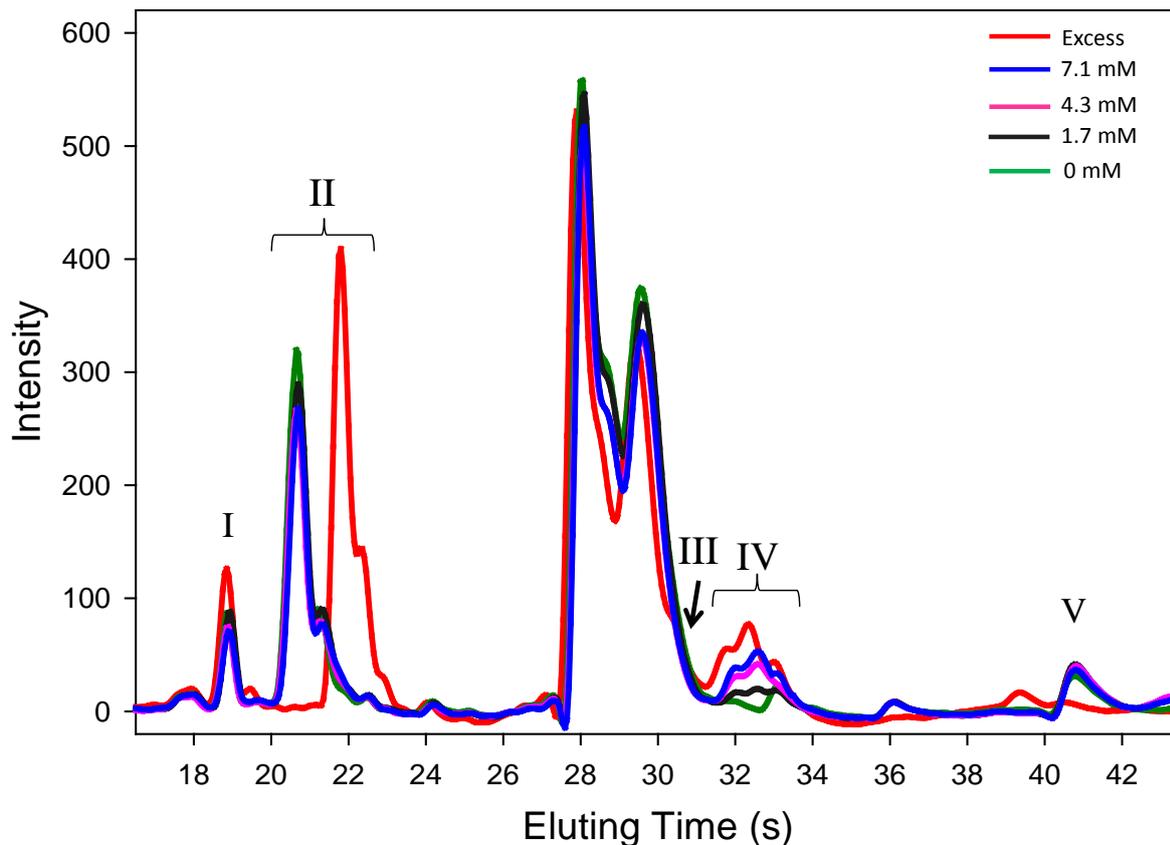


Figure 7.6: MF-electropherograms of WPE skim milk that had been treated with different levels of β -mercaptoethanol (indicated by the coloured lines) for 3 h. I, α -lactalbumin; II, β -lactoglobulin; III, monomeric α_{s2} -casein; IV, κ -casein and V, dimeric α_{s2} -casein.

The effects of β -mercaptoethanol concentrations on the reduction of κ -casein and β -lactoglobulin in the WPE milk is summarised in Figure 7.7. As the β -mercaptoethanol concentration increased from 0 to 7.1 mM, the percentage of reduced κ -casein increased from $11 \pm 4\%$ to $43 \pm 2\%$. The percentage of reduced β -lactoglobulin was much lower compared with the percentage of reduced κ -casein at any β -mercaptoethanol concentration (Figure 7.7). At 7.1 mM β -mercaptoethanol, the percentage of reduced β -lactoglobulin was only $5.3 \pm 3.9\%$. In general, the results show that the percentage of monomeric κ -casein and reduced β -lactoglobulin increased with the increase of β -mercaptoethanol concentrations and that disulphide bonds of κ -casein were reduced in preference than those of β -lactoglobulin. This is consistent with what observed in unheated skim milk (Figure 7.3).

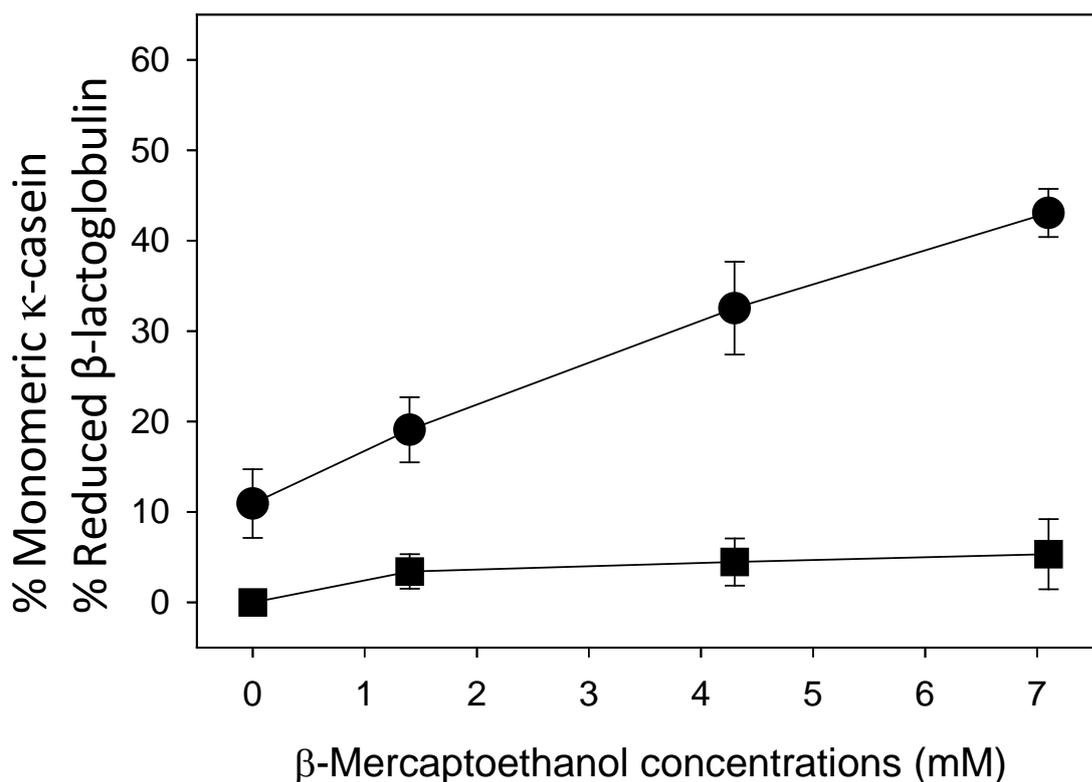


Figure 7.7: The effect of β -mercaptoethanol concentrations on the percentage of reduced β -lactoglobulin (■) and κ -casein (●) over the total of that protein in WPE skim milk after 3 h of addition of β -mercaptoethanol. Each data point is the average of two to four replicates. The error bars are the standard deviation of the replicates.

7.3.2 Effects of reaction time and β -mercaptoethanol concentrations - evaluation by SDS-PAGE method

MF-electrophoresis can monitor the reduction of the disulphide bonds of κ -casein, β -lactoglobulin and α_{s2} -casein; the traditional SDS-PAGE method was also used to examine this reduction reaction. Figure 7.8 showed the SDS-PAGE patterns of proteins being present as monomers in both unheated milks that were treated with 7.1 mM β -mercaptoethanol for various times. The proteins bands were identified by comparison with the SDS-PAGE patterns previously reported by Anema and Klostermeyer (1997) and were marked on the left-hand side of the gel. Unfortunately, only the reduction of disulphide bonds of κ -casein can be clearly detected by the SDS-PAGE method. The bands representing non-reduced and reduced β -lactoglobulin were not clearly separated; hence it was difficult to quantitatively integrate.

Figure 7.8 clearly showed that the intensity of the κ -casein band in both skim milk and WPE skim milk increased as the reaction time increased from zero to 3 h, then did not change further

as the reaction was prolonged. This agrees with the observation made by MF-electrophoresis (Figure 7.3B).

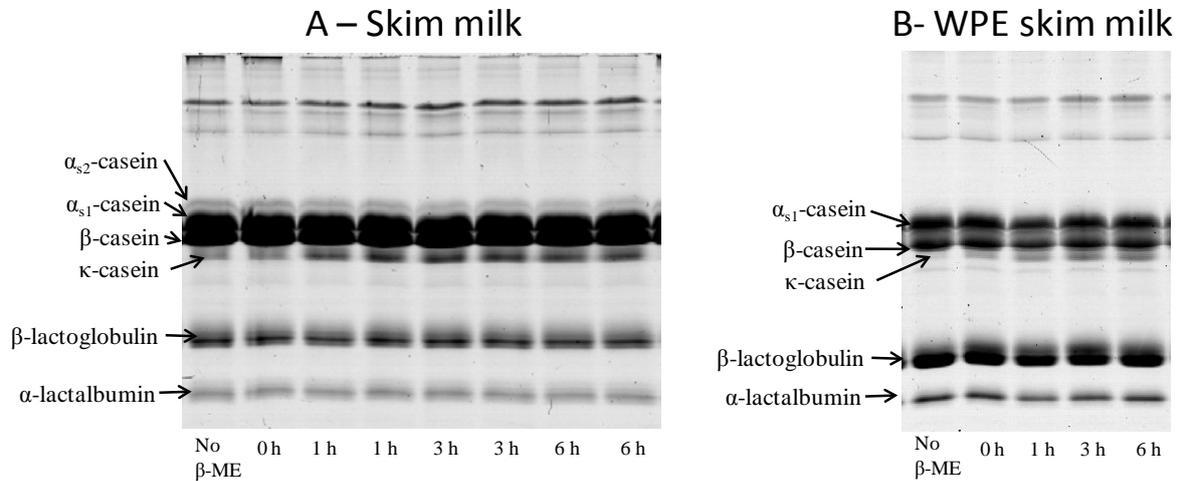


Figure 7.8: The SDS-PAGE patterns showing the effect of reaction time on the reduction of proteins in unheated skim milk (A) and WPE skim milk (B) that were treated with 7.1 mM β -mercaptoethanol. The milk samples were diluted with SDS sample buffer at ratio of 1 to 40 and were not further reduced. The duplicate times in (A) were replicate wells.

The effect of β -mercaptoethanol concentrations on the reduction of milk proteins was also examined using SDS-PAGE and the gels are shown in Figure 7.9. When different concentrations of β -mercaptoethanol were added to unheated milks and held for 3 h, the SDS-PAGE patterns showed that the band intensity of monomeric κ -casein increased with the increase of β -mercaptoethanol concentrations. This is also consistent with the observation made by MF-electrophoresis (Figure 7.3B).

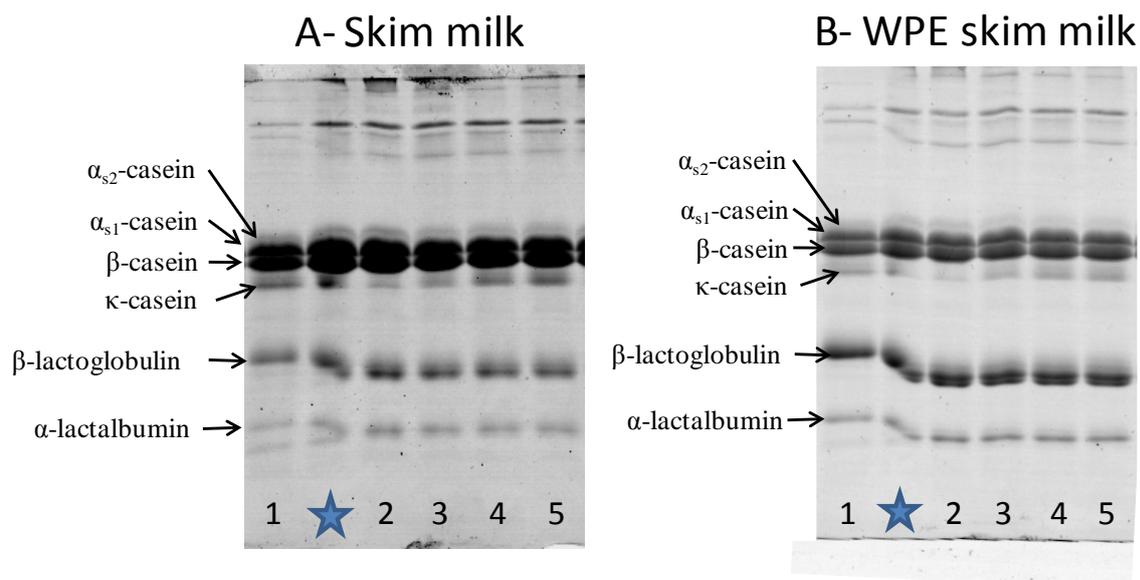


Figure 7.9: The SDS-PAGE patterns showing the effect of β -mercaptoethanol concentrations on the reduction of disulphide bonds of proteins in unheated skim milk (A) and WPE skim milk (B). Milks were treated with β -mercaptoethanol for 3 h at 20 °C. Control samples (i.e. control unheated milks, Lane 1), milks treated with zero mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk samples were diluted with SDS sample buffer at ratio of 1 to 40 and were not reduced except for the control samples being fully reduced.

7.3.3 Effects of β -mercaptoethanol concentrations on the amount of protein in the serum phase of skim milk and WPE skim milk

The effect of adding β -mercaptoethanol to unheated milks on the amount of protein found in the serum phase was investigated using SDS-PAGE (Figure 7.10). The SDS-PAGE patterns showed that addition of β -mercaptoethanol to both skim milk and WPE skim milk did not change the intensities of the α -lactalbumin and β -lactoglobulin bands. The intensities of κ -, β - and α_s -casein bands in both milks were higher in β -mercaptoethanol-treated milks than in untreated milks (Figure 7.10). However the band intensities of casein proteins in β -mercaptoethanol-treated milks were the same amongst the samples, regardless of β -mercaptoethanol concentrations.

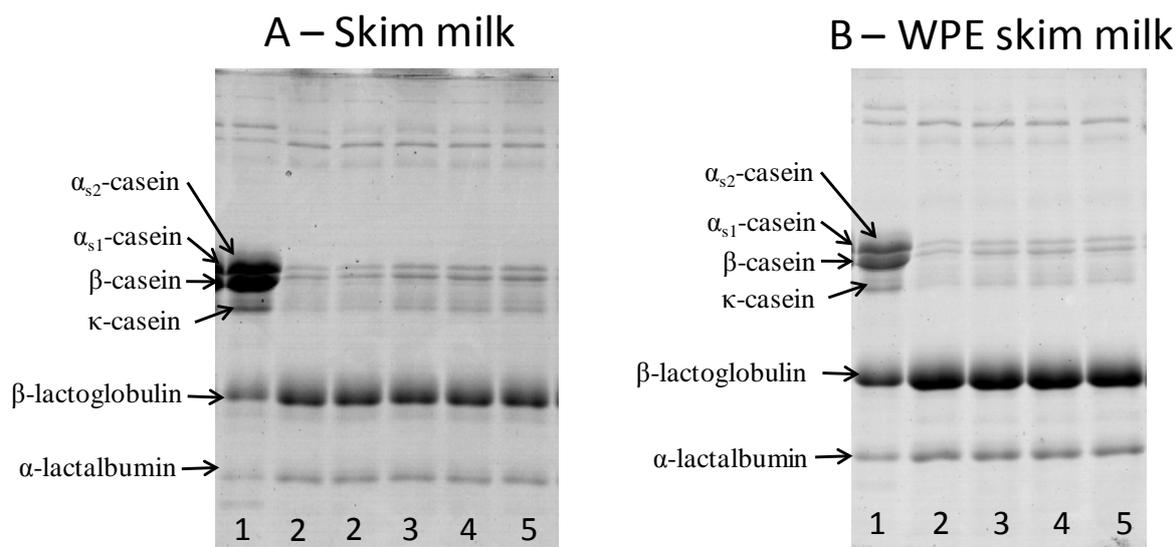


Figure 7.10: The SDS-PAGE patterns showing the effects of β -mercaptoethanol concentrations on the level of proteins in the serum phase of unheated skim milk (A) and WPE skim milk (B). Milks were treated with β -mercaptoethanol for 3 h at 20 °C. Control samples (i.e. control unheated milks, Lane 1), supernatant of milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk sample (Lane 1) and supernatant samples (Lane 2-5) were diluted with SDS sample buffer at ratio of 1:40 and 1:20, respectively. All the samples were fully reduced.

Quantification of the protein bands from the gels shown in Figure 7.10 are presented in Figure 7.11. The amounts of proteins in the serum phase are presented as percentage of the total amounts of each protein in the skim milk. There was $96 \pm 2\%$ of α -lactalbumin and $98 \pm 1\%$ of β -lactoglobulin remaining in the serum at any β -mercaptoethanol concentrations (Figure 7.11A). The percentage of serum κ -casein increased significantly from $14 \pm 3\%$ at zero mM β -mercaptoethanol to $22 \pm 2\%$ at 1.4 mM β -mercaptoethanol ($p < 0.05$, Figure 7.11A). As β -mercaptoethanol concentrations increased further, the percentage of serum κ -casein increased only slightly ($p > 0.05$). As for the other caseins (α_{s1} -, α_{s2} - and β -casein), the presence of β -mercaptoethanol did not change their percentage in serum phases as their average serum proportion was $5 \pm 1\%$ (Figure 7.11A).

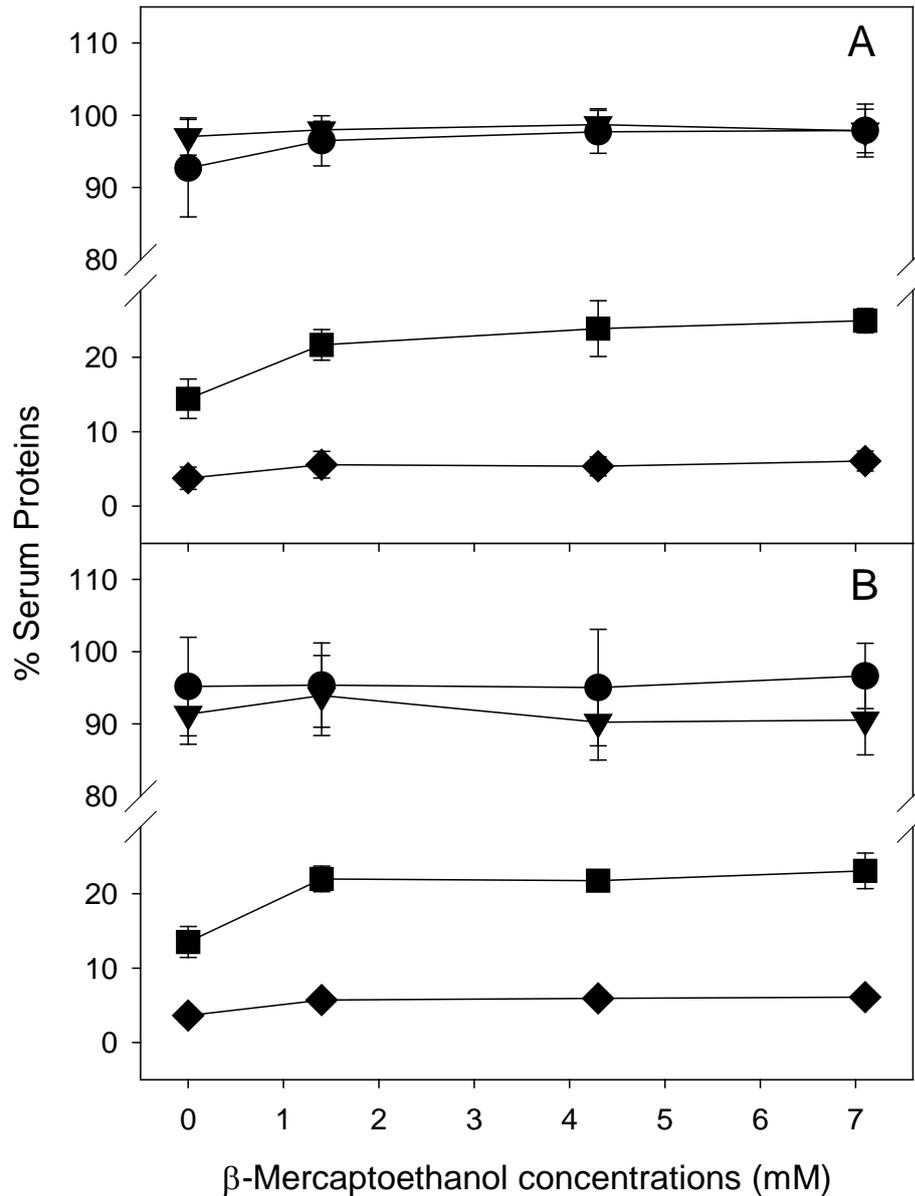


Figure 7.11: The effect of β -mercaptoethanol concentrations on the percentage of individual proteins in the serum phase over the total of that protein present in unheated milk. Skim milk (A) and unheated WPE skim milk (B). Milks were treated with β -mercaptoethanol for 3 h at 20 °C. ▼, α -lactalbumin; ●, β -lactoglobulin; ■, κ -casein; ◆, other caseins. Each data point is the average of two to four replicates. The error bars are the standard deviation of the replicates.

In WPE skim milk, the average percentage of serum α -lactalbumin was $96 \pm 6\%$ and of β -lactoglobulin was $92 \pm 5\%$ (Figure 7.11B). The percentage of serum κ -casein increased significantly from $14 \pm 2\%$ to $22 \pm 2\%$ as β -mercaptoethanol concentration increased from 0 to 1.4 mM ($p < 0.05$), as also observed in skim milk. Interestingly, in WPE skim milk, the level of serum α_{s1-} , α_{s2-} and β -caseins also increased slightly from $4 \pm 1\%$ to $6 \pm 1\%$ as the concentration of β -mercaptoethanol increased from 0 to 1.4 mM (Figure 7.11B). As the β -mercaptoethanol

concentration increased, there was no further increase in serum κ -casein and other caseins (Figure 7.11B). The proportions of serum proteins of both unheated skim milk and WPE skim milk in this study are comparable with those presented in Chapter 4 (Figure 4.6).

The increase in the percentage of serum κ -casein in unheated skim milk and all casein proteins in WPE skim milk indicated that some dissociation of the casein proteins from the casein micelles was occurring (Figure 7.11). The size of the casein micelles were examined (Figure 7.12). In control unheated skim milk, the average diameter of the casein micelles was 205.3 ± 0.2 nm. The size of the casein micelle decreased slightly but significantly to 201.6 ± 0.9 nm as the β -mercaptoethanol concentrations increased to 4.3 mM, but did not change further at 7.1 mM β -mercaptoethanol (Figure 7.12).

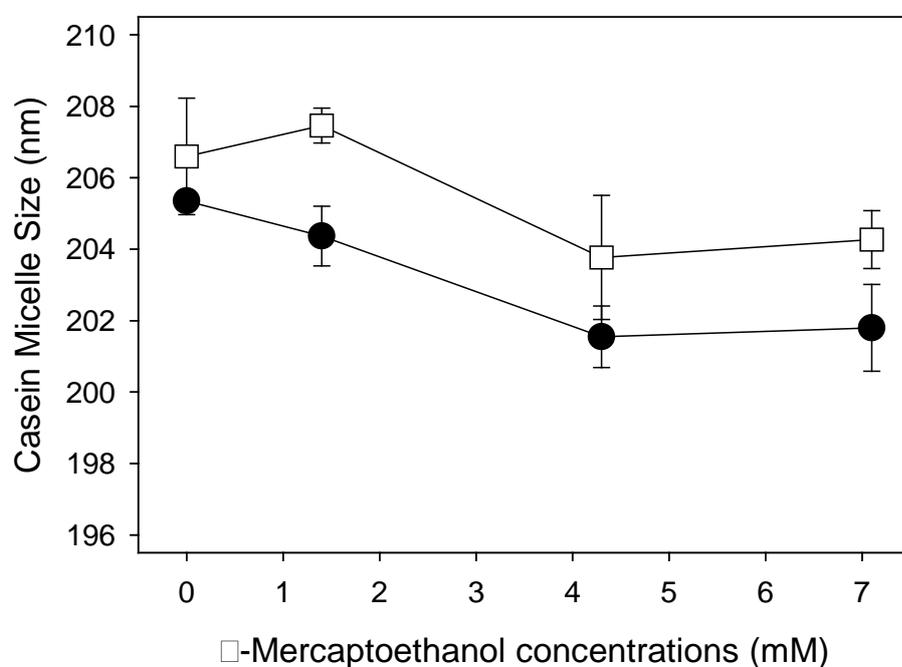


Figure 7.12: The effect of β -mercaptoethanol concentrations on the diameter of casein micelles in unheated skim milk (●) and WPE skim milk (□). Each data point is the average of two to four replicates. The error bars are the standard deviation.

In WPE skim milk, the size of the casein micelles was not affected by the addition of 1.4 mM β -mercaptoethanol but decreased significantly by 3 nm when the level of added β -mercaptoethanol was increased to 4.3 mM. The micelle size did not change further when the concentration of β -mercaptoethanol was increased above 4.3 mM (Figure 7.12).

Preliminary experiments were carried out to examine the zeta potential of the casein micelles of selected samples. The results show that despite the reduction of κ -casein on the surface of the

casein micelles, the zeta potential was not affected (Figure A.8 in Appendix 5). Hence further tests on zeta potential were not pursued.

7.3.4 Effects of β -mercaptoethanol concentrations on the rheological properties of the acid milk gels

Acid gels were prepared by adding 2% GDL to unheated milks that had been treated with β -mercaptoethanol for 3 h. The gels were formed at 30 °C over a period of 3 h. The curves showing the typical change of G' as a function of time during acidification for milks without and with 7.1 mM added β -mercaptoethanol are shown in Figure 7.13. The curve for acid gels made from milks with 7.1 mM added β -mercaptoethanol represents the typical acid gelation behaviour of unheated milks treated with β -mercaptoethanol (Figure 7.13B). The gelation of milks with added β -mercaptoethanol had a similar profile to that of control milks although the final G' reached was markedly lower than that observed in the controls.

The curves for the acid gelation of control milks showed a continuous increase in G' values after the gelation point, which occurred after ~ 60 min (skim milk) and ~ 71 min (WPE skim milk) and, corresponded to a gelation pH of ~ 5.0 and 4.9 , respectively (Figure 7.13A). However it was difficult to determine the exact gelation point of acid gels made from milks treated with β -mercaptoethanol due to the fluctuation of the G' values (Figure 7.13B). Thus the gelation point was assumed to be the point after which the G' values start to continuously increased and did not return to the minimum value. Based on this assumption, the gelation points of β -mercaptoethanol-treated skim milks occurred between the 67th and 72th minute (Figure 7.13B), corresponding to pH 4.9 ± 0.1 . Hence the addition of β -mercaptoethanol to skim milk or WPE skim milk did not affect the gelation point.

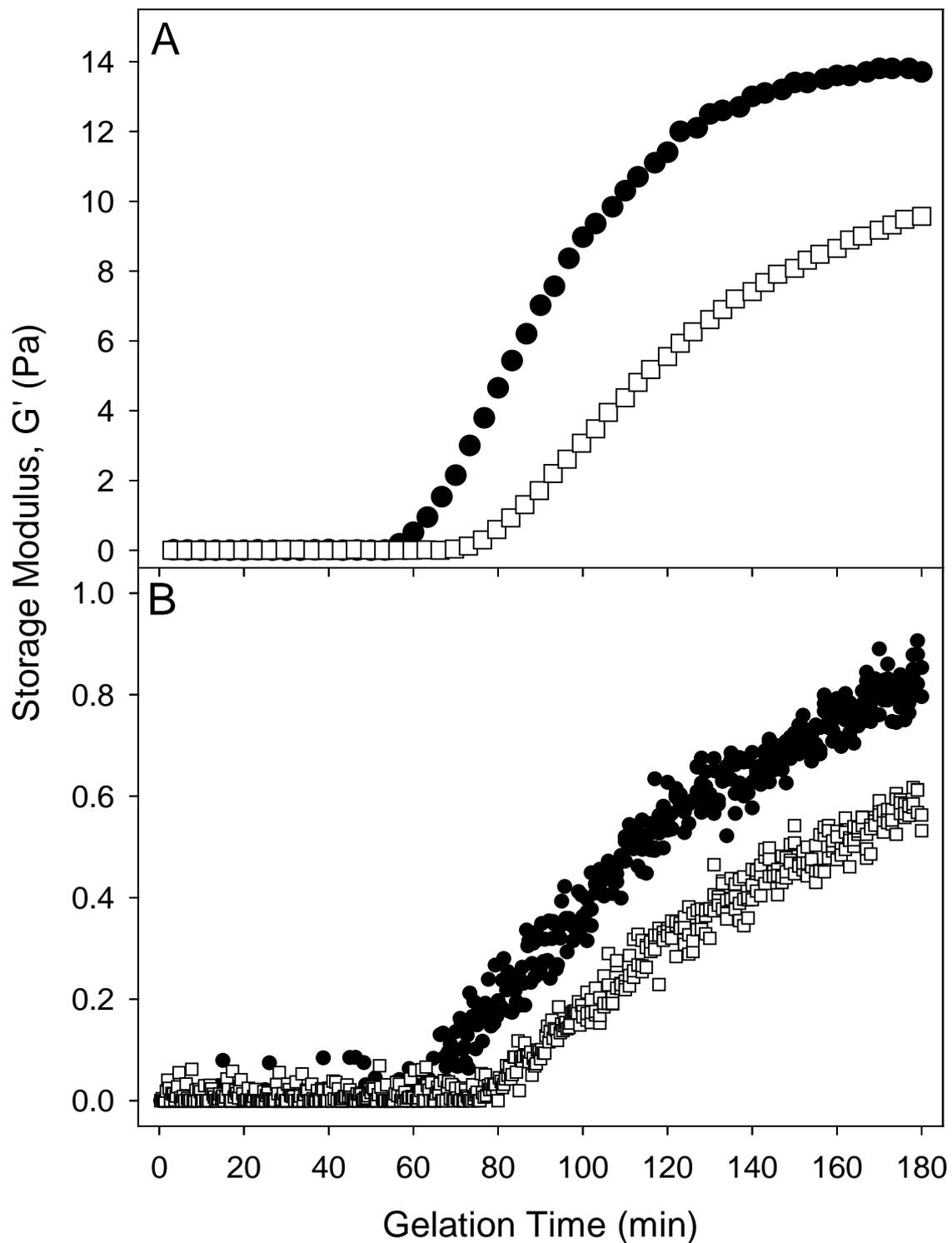


Figure 7.13: Typical changes of the storage modulus as a function of gelation time for unheated skim milk (●) and unheated WPE skim milk (□). A, control milks and B, milks treated with 7.1 mM β -mercaptoethanol for 3 h.

The final G' values were plotted against β -mercaptoethanol levels (Figure 7.14). In unheated skim milk, the final G' values decreased significantly from 13.7 ± 1.0 Pa to 1.3 ± 0.2 Pa as the β -

mercaptoethanol concentrations increased from 0 to 1.4 mM, and then remained very low at higher β -mercaptoethanol concentrations (Figure 7.14). In unheated WPE skim milk, the final G' values decreased significantly from 10.3 ± 1.4 Pa to 1.3 ± 0.08 Pa then to 0.50 ± 0.3 Pa as β -mercaptoethanol concentrations increased from 0 to 1.4 then to 4.3 mM ($p < 0.05$). At 7.1 mM, the final G' values were 0.51 ± 0.07 Pa (Figure 7.14). In general, adding β -mercaptoethanol to unheated milks had the same effect in both skim milk and WPE skim milk: resulting in the formation of very weak acid gels (Figure 7.14).

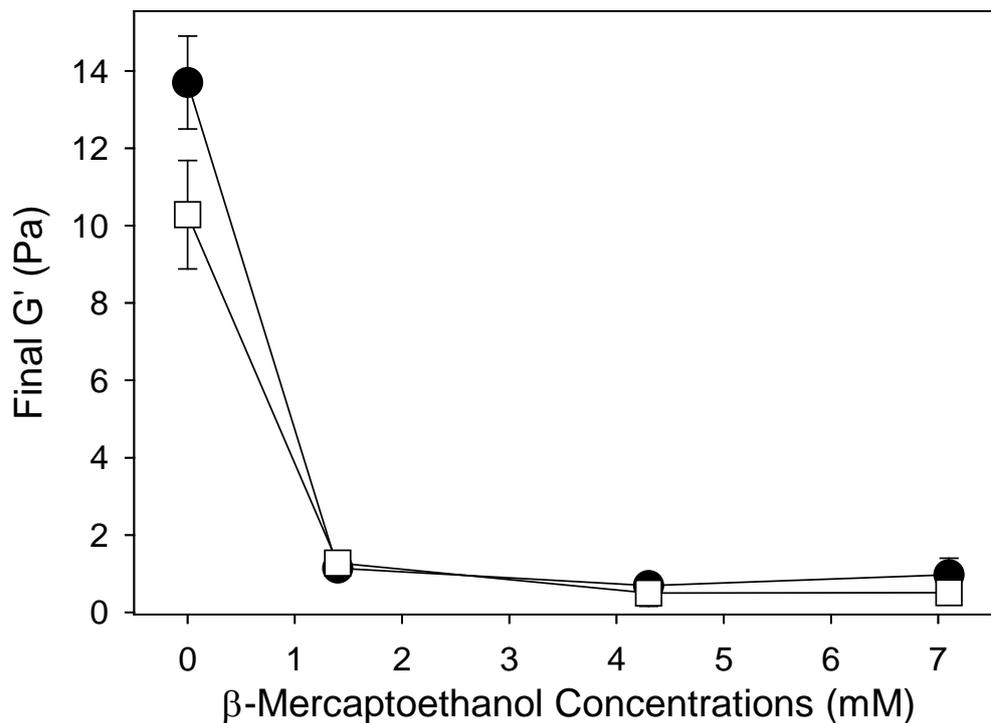


Figure 7.14: The effect of β -mercaptoethanol concentrations on the final G' values of acid gels. ●, skim milk; □, WPE skim milk. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

The change of the $\tan \delta$ values during the acid gelation process showed that the acid gels prepared from β -mercaptoethanol-treated milks had very low G'' values as well as very low G' values. These gels were neither more elastic nor more viscous compared to those prepared from control milks as the $\tan \delta$ values were similar at all pH (Appendix 2, Figure A.3).

The yield properties at 5 °C of selected acid gels were examined by monitoring the shear stress and strain when the acid gels were subjected to a constant rotational shear at a rate of 0.005 s^{-1} . As the shear was constantly applied, the strain and shear stress of the acid gels increased until the acid gels could not withstand the shear any longer and broke. This was demonstrated by the

maximum shear stress point (Figure 7.15). The acid gels prepared from β -mercaptoethanol-treated milks were broken at lower shear stress and strain than those prepared from control milks (Table 7.2). This indicated that the β -mercaptoethanol-containing gels were very weak and broke much more easily than the control gels. The yield stress values of acid gels prepared from β -mercaptoethanol-treated milks decreased significantly to < 1.5 Pa while for control milk gels their yield stress values were > 50 Pa ($p < 0.05$, Table 7.2).

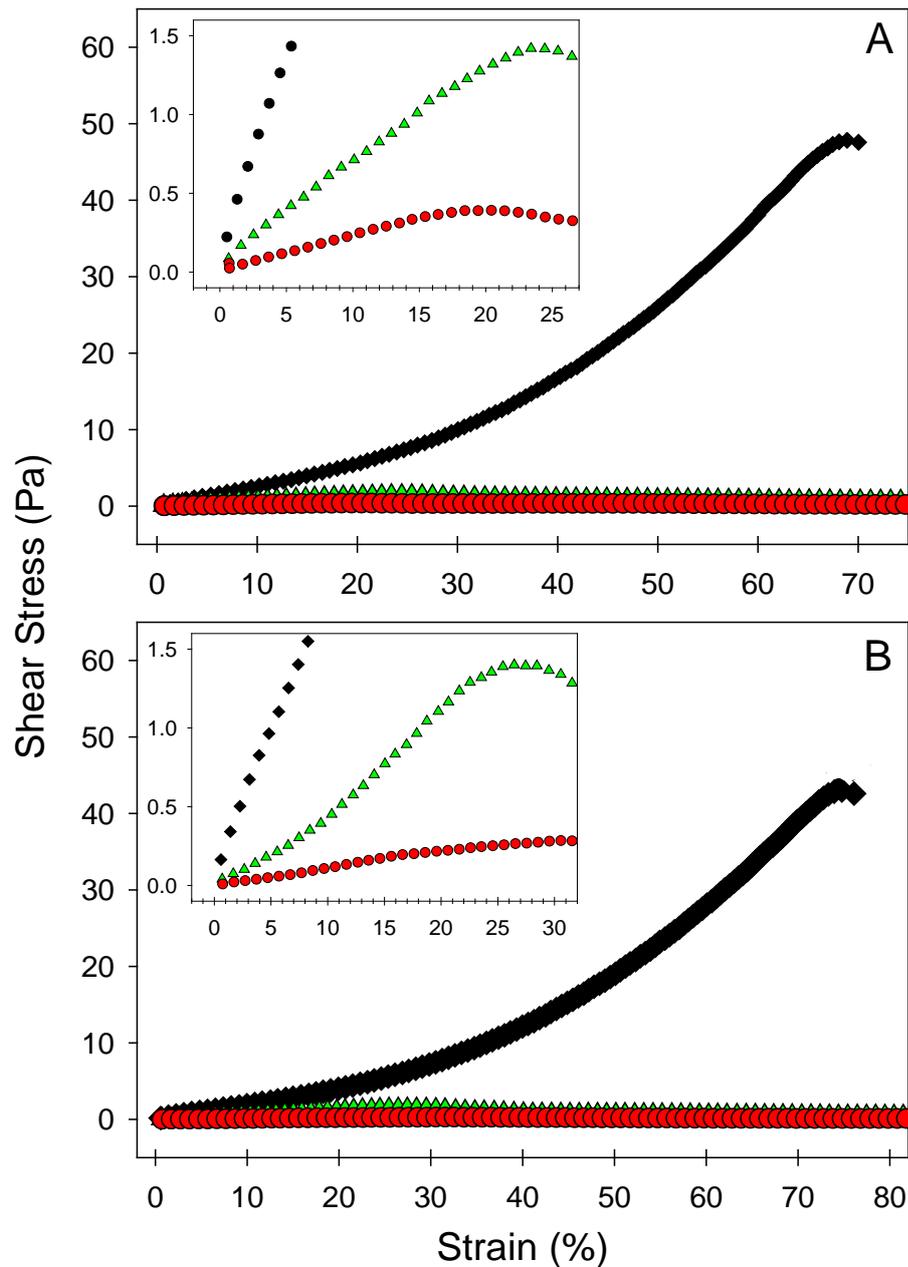


Figure 7.15: Shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^\circ\text{C}$ and analysed at $5 \text{ }^\circ\text{C}$. The acid gels were prepared from unheated skim milk (A) and unheated WPE skim milk (B) that had been treated with zero mM (\blacklozenge), 1.4 mM (\blacktriangle) and 7.1 mM (\bullet) β -mercaptoethanol for 3 h. Each data point is the average of two to four replicates. The inserts showed the zoom-in of the change of shear stress of β -mercaptoethanol-containing milk gels.

Table 7.2: The effect of β -mercaptoethanol concentrations on the yield shear stress and yield strain values of acid gels. Acid gels were prepared from unheated skim milk and WPE skim milk that were treated with β -mercaptoethanol for 3 h. The values are the mean value of two to four replicates \pm standard deviation.

β-mercaptoethanol Concentrations (mM)	Unheated skim milk		Unheated WPE skim milk	
	Yield Strain (%)	Yield Shear Stress (Pa)	Yield Strain (%)	Yield Shear Stress (Pa)
0	72.8 \pm 1.6	58.6 \pm 1.7	80.0 \pm 1.9	45.8 \pm 2.0
1.4	23.4 \pm 1.6	1.4 \pm 1.6	25.5 \pm 0.1	1.39 \pm 0.08
7.1	20.4 \pm 0.2	0.39 \pm 0.26	30.6 \pm 0.1	0.28 \pm 0.02

7.3.5 Effect of β -mercaptoethanol concentrations on the microstructure of the acid milk gels

In general, all the examined acid gels consisted of a network of connected clusters, consistent with observations made by Lucey (1999a) on acid gels prepared from unheated skim milk. The microstructure of acid gels made from β -mercaptoethanol-treated skim milk (Figure 7.16B and C) was a little different from those of the control acid gels (Figure 7.16A). The network of the control was denser, more interconnected and had pores of a smaller size than that of β -mercaptoethanol-containing gels. The pores became larger and the gel network became sparser and contained fewer clusters with the increase of β -mercaptoethanol concentration. This change was progressive as Figure 7.16 showed that the gel with 1.4 mM β -mercaptoethanol (Figure 7.16B) was intermediate between the control gel (zero mM β -mercaptoethanol, Figure 7.16A) and the gel with 7.1 mM β -mercaptoethanol (Figure 7.16C).

The acid gels prepared from β -mercaptoethanol-treated WPE skim milk were also slightly different from the control WPE skim milk gels (Figure 7.17). The pores appeared larger and there appeared to be somewhat fewer interconnections in the acid gels made from WPE skim milk treated with 7.1 mM β -mercaptoethanol (Figure 7.17C) than in acid gels of WPE skim milk with lower β -mercaptoethanol concentrations (Figure 7.17A and B). Similarly to skim milk gels, the gel with 1.4 mM β -mercaptoethanol (Figure 7.17B) was the intermediate between the control gel (zero mM β -mercaptoethanol, Figure 7.17A) and the gel with 7.1 mM β -mercaptoethanol (Figure 7.17C).

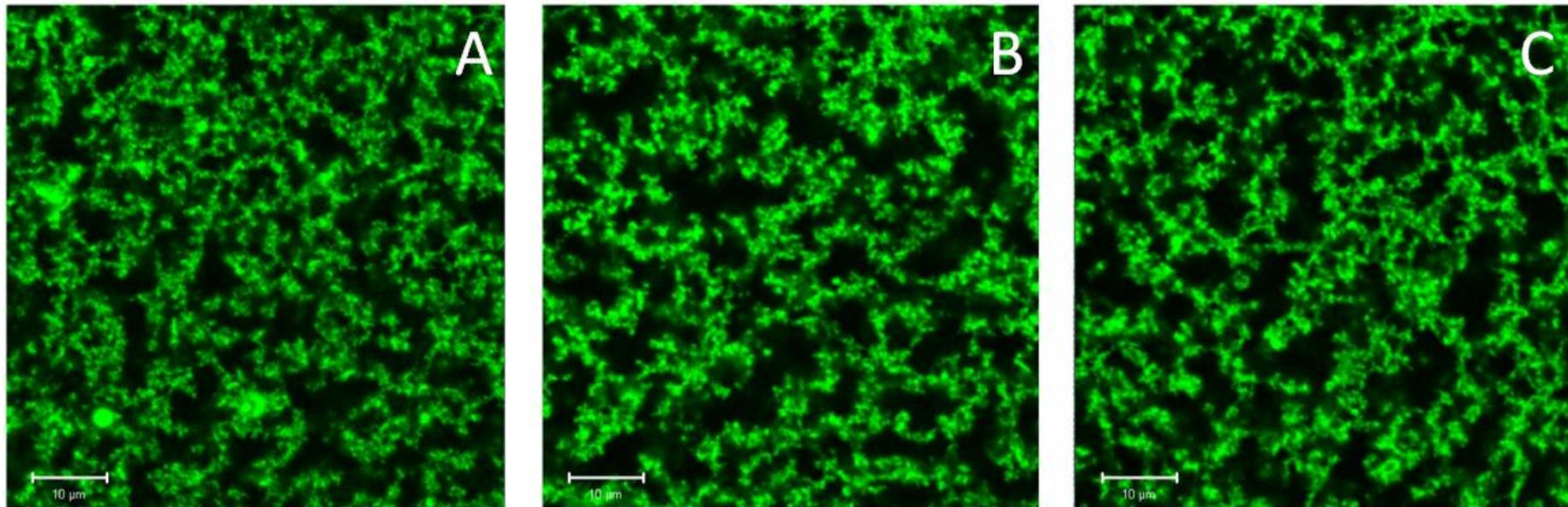


Figure 7.16: The confocal micrographs of acid gels made from unheated skim milk that was treated with 0 (A), 1.4 (B) and 7.1 (C) mM β -mercaptoethanol. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μ m.

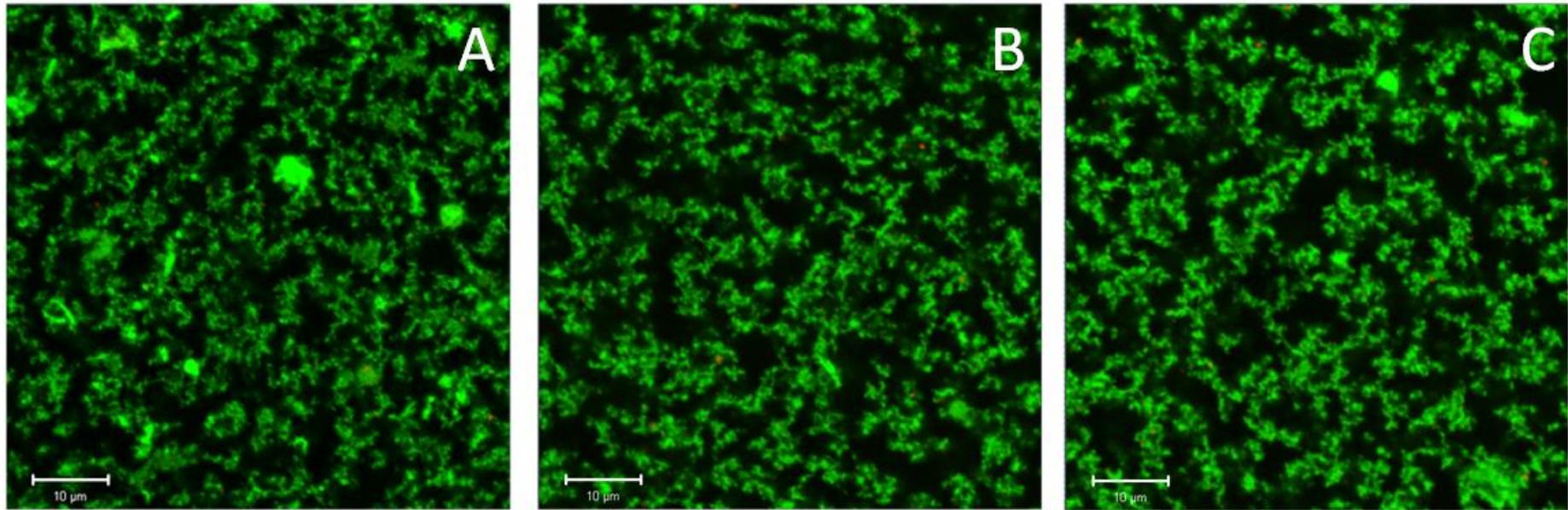


Figure 7.17: The confocal micrographs of acid gels made from unheated WPE skim milk that was treated with zero (A), 1.4 (B) and 7.1 (C) mM β -mercaptoethanol. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μ m.

7.4 Discussion

7.4.1 Effects of reducing the protein disulphide bonds on the protein properties in unheated milks

In unheated control and WPE skim milk, the disulphide bonds of κ -casein were reduced in preference to those of β -lactoglobulin, regardless of the concentrations of the whey proteins in the milk and of the concentrations of β -mercaptoethanol (Figure 7.3 and Figure 7.7, respectively). Due to the complexity of interpreting the peak representing the dimeric α_{s2} -casein, the reduction of disulphide bonds of α_{s2} -casein could not be compared with that of κ -casein to determine which protein was reduced preferentially by β -mercaptoethanol. Even though the reduction of the β -lactoglobulin disulphide bonds was not as fast as those of κ -casein, this reduction can be accelerated by increasing the β -mercaptoethanol concentrations (Figure 7.3A).

κ -Casein exists as disulphide-linked oligomers of various sizes because the two cysteine residues (Cys¹¹ and Cys⁸⁸) can form intermolecular disulphide bonds in a random manner between κ -casein monomers (Rasmussen et al., 1992). κ -Casein has an amphiphilic nature with hydrophobic and hydrophilic regions (Horne, 1998). The cysteine residues are located in the hydrophobic region, and this region is associated with the interior of the casein micelle. The hydrophilic regions project into the serum and stabilise the casein micelles (Holt & Dalgleish, 1986; Holt & Horne, 1996). However, this layer is porous; therefore denatured β -lactoglobulin and small molecules (such as β -mercaptoethanol) can diffuse through to interact with the disulphide bonds of κ -casein (Guyomarc'h et al., 2003a). As for α_{s2} -casein, in view of the model of the casein micelle structure proposed by Dalgleish (2011), the micelle structure is porous and there are spaces on the micelle surface where κ -casein are not located and other caseins can be exposed to the materials in the serum (Figure 2.8). Therefore, despite the interior location of α_{s2} -casein in the casein micelles, β -mercaptoethanol can approach and reduce their disulphide bonds (Figure 7.7).

Native β -lactoglobulin has two intra-molecular disulphide bonds, Cys¹⁰⁶-Cys¹¹⁹ and Cys⁶⁶-Cys¹⁶⁰ (Brownlow et al., 1997). The Cys⁶⁶-Cys¹⁶⁰ disulphide bond is located near the C-terminal and near the surface of the protein structure. Hence this disulphide bond is more accessible than the Cys¹⁰⁶-Cys¹¹⁹ (Davidson & Hird, 1967). However both disulphide bonds of β -lactoglobulin were considered to be less accessible to β -mercaptoethanol than the intermolecular disulphide bonds of κ -casein, hence the lower proportion of β -lactoglobulin was reduced than κ -casein at any β -mercaptoethanol concentration and reaction time (Figure 7.3).

Once reduced, the majority of the κ -casein (~78% at 7.1 mM β -mercaptoethanol) remained associated with the other casein proteins on the casein micelles (in the colloidal phase Figure 7.11). Considering the biosynthesis of casein micelles, it has been proposed that initially monomeric κ -casein (with free thiol groups) stabilised the aggregates of α - and β -caseins during the formation of the casein micelles. The oxidation of thiol groups of κ -casein to form disulphide bonds occurred upon exposure of the casein micelles to an oxidising environment, such as air during milking (Talbot & Waugh, 1970; Farrell et al., 2006). In addition, the size of the casein micelles decreased by only 3 to 4 nm in both skim milk and WPE skim milk with the addition of β -mercaptoethanol (Figure 7.12). This suggested that the casein micelle “integrity” was not markedly affected by the reduction of κ -casein disulphide bonds. Hence, the results from this study demonstrated that κ -casein was associated with other caseins in the polymeric form and remained in the casein micelles after being reduced, which was consistent with previous studies (Nakai, Wilson & Herreid, 1965; Talbot & Waugh, 1970; Farrell et al., 2006).

However, the question is why a small amount of monomeric κ -casein still dissociated from the casein micelles, as there was a higher percentage of serum κ -casein in β -mercaptoethanol - treated milks than in control milks (Figure 7.11). This could be explained by the location of the κ -casein on the surface of the casein micelle. The micelle surface appears to consist of cylindrical or tubular structures, as shown in Figure 2.8 and Figure 7.18 (Dalglish et al., 2004; Dalglish, 2011). The authors hypothesised that κ -casein was located at the end of those cylinders/tubules, which agreed with previous reports from the same group that κ -casein was not distributed evenly across the surface of the casein micelle (Dalglish, 1998). κ -Casein polymers can be depicted as tufts (like grass), distributed randomly on the surface of the casein micelles. It is possible that most of the κ -casein in these “tufts” interacted with the other casein proteins via non-covalent bonds. A small portion of κ -casein may be sterically constrained, hence cannot interact with other caseins, but can only bind to other κ -casein by intermolecular disulphide bonds. Consequently, once the disulphide bonds were cleaved, this portion of κ -casein may be released from the tuft of κ -casein into the serum.

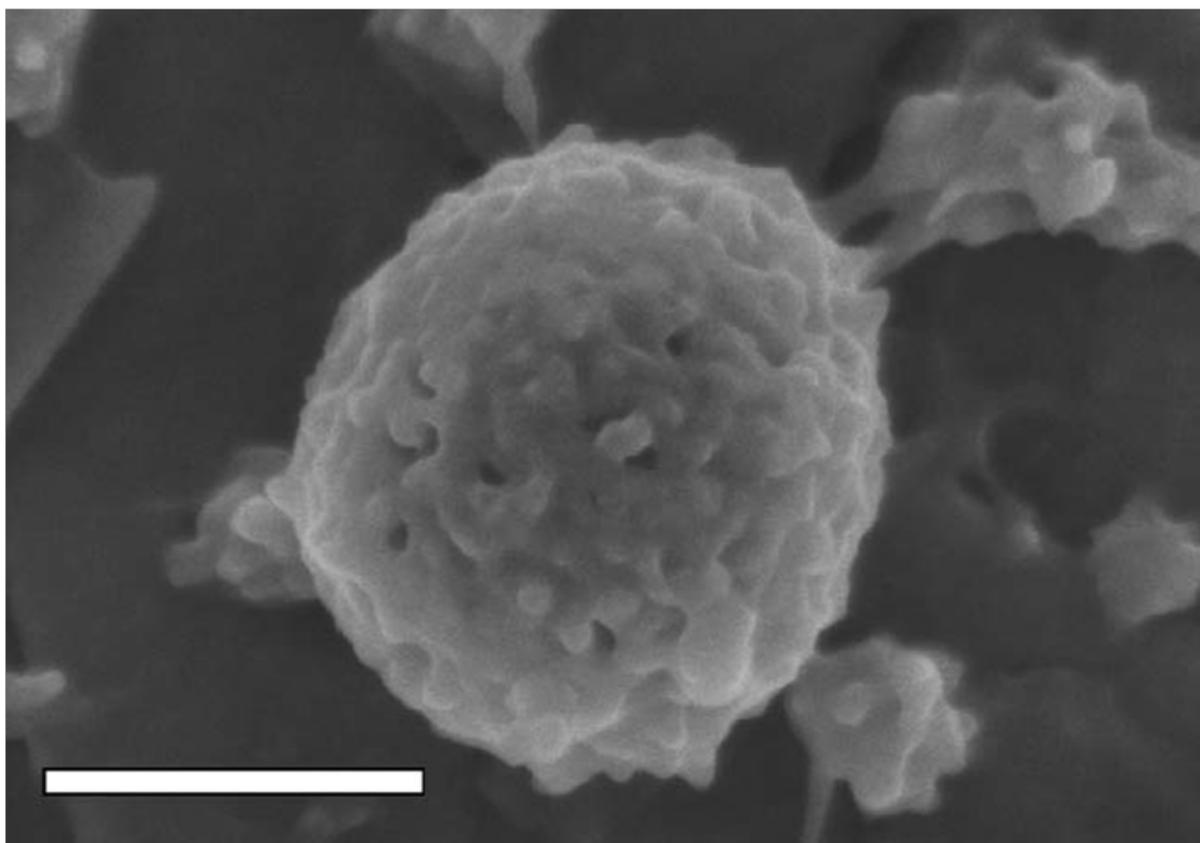


Figure 7.18: Electron micrograph of an individual casein micelle, obtained from the technique of field-emission scanning electron microscopy. Samples were prepared on a carbon substrate (flat or foliated background) and fixed as described in the article. No coating techniques were employed. The scale bar in the figure represents 200 nm (Dagleish et al., 2004).

The decrease in size of the casein micelles in unheated milks can be caused by the dissociation of κ -casein from the casein micelles (Figure 7.12). However the proportion of dissociated κ -casein was relatively low to be responsible for ~ 4 nm change in diameter of the casein micelle. Thus other factors such as a relaxation of the monomeric κ -casein after the reduction of disulphide bonds may also partly account for the size reduction. In its native form, κ -casein was described as a polyelectrolyte brush on the surface of the casein micelles (De Kruif & Zhulina, 1996). Native polymeric κ -casein may appear as a “brush” because they were assembled into dense tufts, causing the charge to repel neighbouring molecules (Figure 7.19A, Milner, 1991). When the polymeric κ -casein was broken into monomers in β -mercaptoethanol-treated milks, individual κ -casein molecules can diffuse apart and the monomeric κ -casein can change to a “mushroom” state (Figure 7.19B, Walstra, 2002). Being in the “mushroom” state, the macropeptide (hair) of κ -casein does not stretch out or extend out as much as compared to the “brush” state. Consequently, the κ -casein in β -mercaptoethanol-treated milks can appear to be shorter than those in the control milks, causing the casein micelle in the β -mercaptoethanol-treated milks to appear smaller than those in control milks (Figure 7.12).

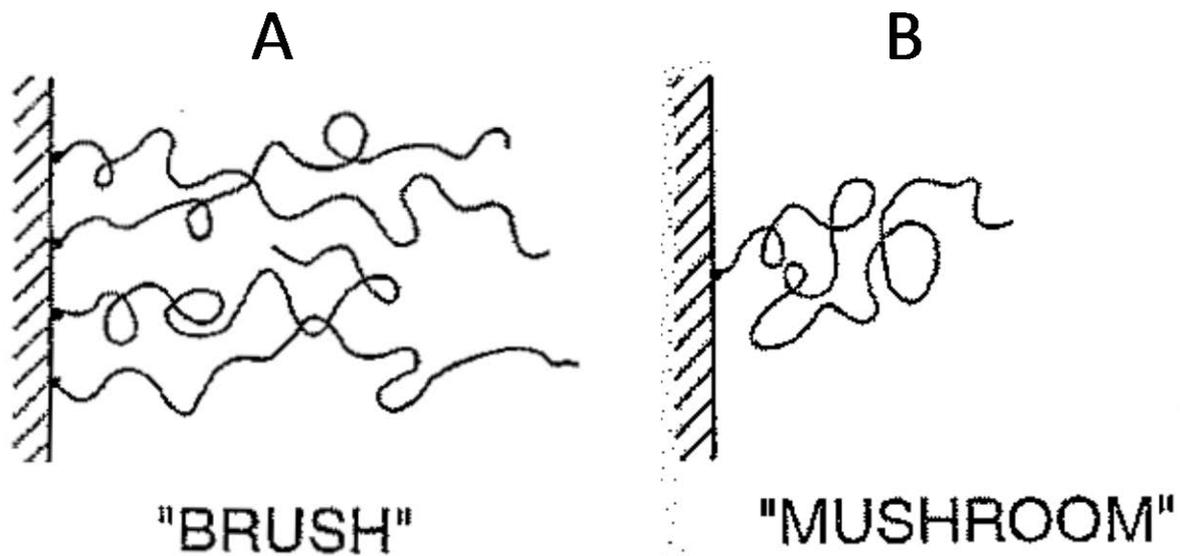


Figure 7.19: The schematic of molecule in the brush (A) and mushroom (B) state. Source: Walstra (2002).

In most cases, the percentage of monomeric κ -casein remained unchanged after reaching a maximum level (Figure 7.3). However, in solutions with 7.1 mM β -mercaptoethanol, this percentage decreased after the maximum reduction of disulphide bonds had occurred (Figure 7.3 and Figure 7.5). This suggested that new protein disulphide bonds were reformed via either re-oxidation or through thiol-disulphide exchange reactions. As mentioned in Chapter 4 (Section 4.4), low levels of denatured disulphide-linked whey proteins may be in the skim milk powder and whey protein isolate powder used to prepare skim milk and WPE skim milk in this study. After being reduced, monomeric κ -casein with free thiol groups can initiate thiol-disulphide exchange reactions with the disulphide bonds of the denatured whey proteins, forming new intermolecular disulphide bonds between κ -casein and these whey proteins. As a result, the proportion of monomeric κ -casein could decrease as the reaction time was prolonged.

Nevertheless, the decrease in level of monomeric κ -casein with reaction time was observed only at certain β -mercaptoethanol concentrations. The reason could be that at higher β -mercaptoethanol concentrations, there was sufficient disulphide reducing agent to continue to break the newly formed disulphide bonds, hence keeping the κ -casein reduced. On the other hand, when the concentration of β -mercaptoethanol was at lower concentrations, the level of monomeric κ -casein with free thiol groups was not high. Hence the chance for interactions between monomeric κ -casein and disulphide bonds of the denatured whey proteins was lower.

As a result, thiol-disulphide exchange reactions were not likely to occur and the level of monomeric κ -casein was maintained.

7.4.2 Effects of reducing disulphide bonds on the rheological properties acid gels made from unheated milks

Addition of β -mercaptoethanol did not markedly change the microstructure of the acid gels (Figure 7.16 and Figure 7.17) but significantly decreased the acid gel firmness (Figure 7.14). Although the casein micelles are aggregated largely through non-covalent interactions to form acid gels (De Kruif, 1997), the inter-molecular disulphide bonds of κ -casein must contribute to the structure of the gel as their reduction weakened the gels. As disulphide bonds have a higher bond energy compared to non-covalent bonds (Chou & Buehler, 2009) and can help to maintain the integrity of the casein micelle, the control milk gels with intact κ -casein disulphide bonds had higher gel firmness (Figure 7.14) and higher yield stress (Figure 7.15). When the intermolecular disulphide bonds of κ -casein were reduced, not only the number of disulphide bonds was diminished, the proteins in the system can also be easily re-arranged, even at lowest strain. Hence the acid gels made from β -mercaptoethanol-treated milks became weaker (Figure 7.14) and were readily broken in large deformation experiments (Figure 7.15).

The decreasing firmness of acid gels due to addition of β -mercaptoethanol may be caused by the degree of interactions between the casein proteins in the gel network. In some reports, the casein micelles were proposed to act as hard spheres and retain their integrity during gelation (Zoon, van Vliet & Walstra, 1988; De Kruif, 1997; Van Vliet et al., 2004). The decrease in size of the casein micelles led to smaller contact junctions between the particles in the casein gels (Zoon et al., 1988; Roefs & van Vliet, 1990). This corresponded to the decrease in the degree of interactions between the proteins in the neighbouring casein micelles. In this study, the casein micelle size decreased due to the reduction of κ -casein disulphide bonds. This may cause the decrease in firmness of acid gels containing β -mercaptoethanol.

In other reports, the casein micelles were proposed to change their internal structures and the casein proteins dissociated from the casein micelles during gelation (Dalglish & Law, 1988; Horne, 1999, 2003). This led to interactions between the casein proteins from different casein micelles (Figure 7.20A and B). In this study, when polymeric κ -casein was reduced to monomers, the hydrophobic and other non-covalent interactions between κ -casein and other caseins can be increased (Figure 7.20C) as these interactions may be restricted by the disulphide bonds in the κ -casein polymers (Figure 7.20A). The increase in interactions between casein proteins in the same micelle may limit the interactions between the casein proteins from

different casein micelles on gelation (Figure 7.20D). Hence, overall, the degree of interactions between particles in the gel network was decreased, leading to weaker acid gels.

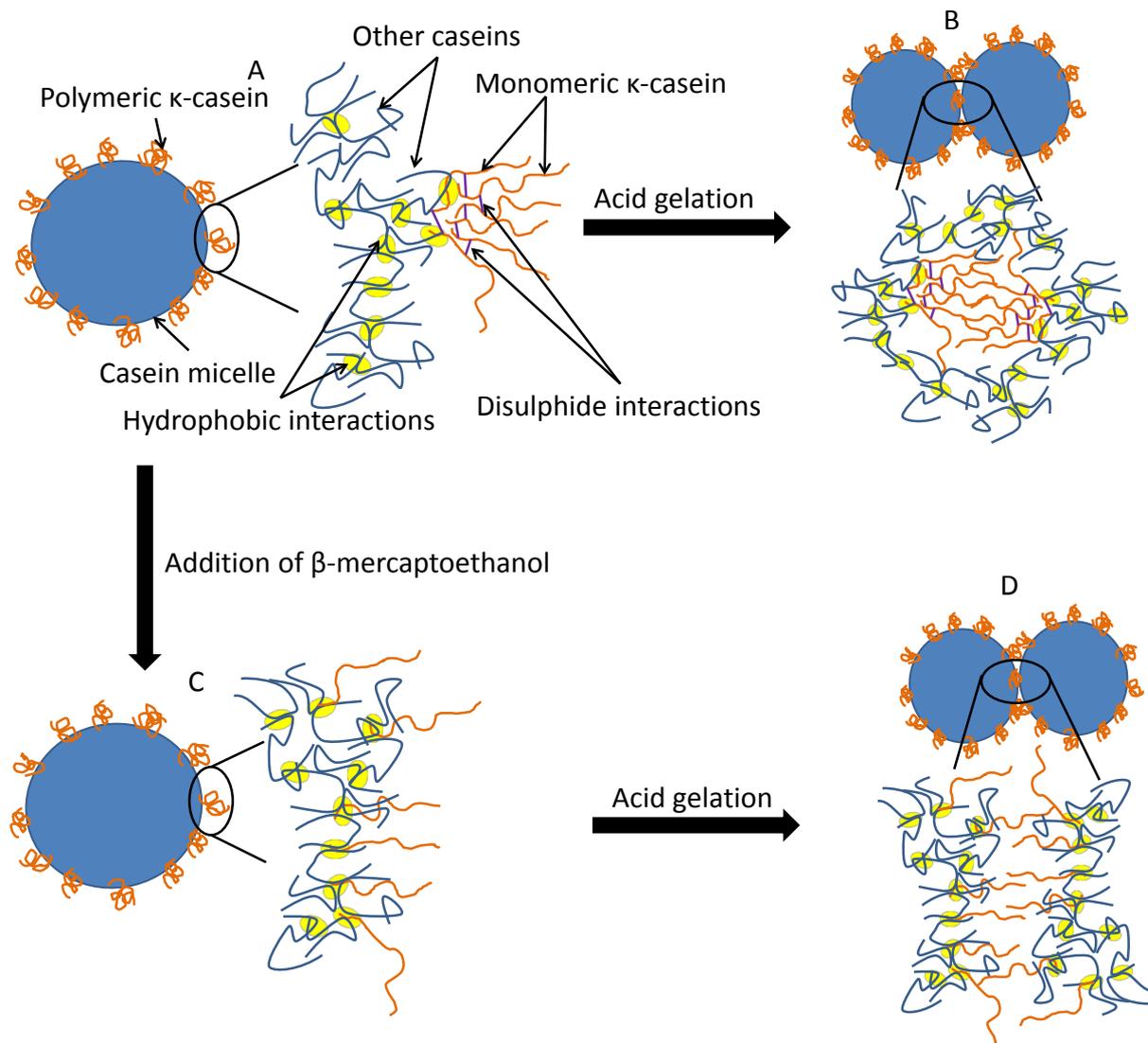


Figure 7.20: Possible interactions between the proteins in unheated milks and in the resulting acid gels. A, control milks; B, control acid gels; C, milks with added β -mercaptoethanol; D, acid gels containing β -mercaptoethanol.

7.5 Conclusions

The disulphide bond reducing agent β -mercaptoethanol reacted with κ -casein and β -lactoglobulin in unheated skim milk. However it required up to 3 h at 20 °C for the reaction to complete at all concentrations of reducing agent tested. In unheated skim milk, the disulphide bonds of κ -casein were reduced in preference to those of β -lactoglobulin, regardless of the concentrations of whey protein in the milk. The intermolecular disulphide bonds of κ -casein on the surface of the casein micelles were considered to be more accessible than those of native

β -lactoglobulin, which are intra-molecular. Reducing κ -casein disulphide bonds to monomers with free thiol groups did not initiate thiol disulphide exchange reactions with native whey protein, at neutral or acidic pH. The monomeric κ -casein can be considered stable and inert to native folded whey proteins.

The findings of this chapter provided a starting point for the further work in which β -mercaptoethanol was added to unheated skim milk, holding for 3 h before the reduced milk undergoing heat treatments.

Chapter 8 - Effects of β -mercaptoethanol on κ -casein and β -lactoglobulin in pure protein systems

8.1 Introduction

The addition of low concentrations of β -mercaptoethanol to unheated skim milk cleaved the disulphide bonds of κ -casein in preference to those of β -lactoglobulin. This affected the functional properties of milks, as shown in Chapter 7. It was concluded that the intermolecular disulphide bonds of κ -casein were more accessible to β -mercaptoethanol than the intramolecular disulphide bonds of β -lactoglobulin. However it was uncertain whether the preferential reduction of κ -casein over β -lactoglobulin was also due to differences in the rate of reduction.

Therefore, the aim of this study was to determine the propensity and rates of reduction of κ -casein and β -lactoglobulin when the proteins were isolated from each other. The reduction of disulphide bonds in individual protein solutions (as well as in binary system) was examined when low concentrations of β -mercaptoethanol were added to the solutions and incubated for various holding times at various temperatures.

8.2 Materials and methods

κ -Casein ($\sim 2.4 \times 10^{-4}$ mol L⁻¹), β -lactoglobulin ($\sim 2.3 \times 10^{-4}$ mol L⁻¹), and the mixed β -lactoglobulin/ κ -casein ($\sim 2.3 \times 10^{-4}$ mol L⁻¹ κ -casein and $\sim 2.2 \times 10^{-4}$ mol L⁻¹ β -lactoglobulin) solutions were prepared as described in Section 3.1.3.

Diluted β -mercaptoethanol (10%, v/v) was added to the solutions to give concentrations of 1.4 to 7.1 mM in κ -casein solutions or 17 to 71 mM in β -lactoglobulin solutions. Higher β -mercaptoethanol concentrations were added to β -lactoglobulin solutions because it was shown in Chapter 7 that the reduction of β -lactoglobulin was observed at β -mercaptoethanol ≥ 17 mM.

In the mixed solution, concentrations of 7.1 mM or 17 mM of β -mercaptoethanol was added. The protein solutions with added β -mercaptoethanol were shaken on a vortex mixer for 10 s and then left to react at 20 ± 0.5 °C in a thermostatically controlled water bath for up to 24 h.

The effect of temperature on the reduction of κ -casein and β -lactoglobulin was examined by adding 1.7 mM β -mercaptoethanol to the κ -casein solution, 17 mM to the β -lactoglobulin solution, and 7.1 and 17 mM to the mixed κ -casein/ β -lactoglobulin solution. The treated protein solutions were held in a thermostatically controlled water bath at 20, 30, 40 and 50 ± 0.5 °C for up to 6 h.

The reduction of disulphide bonds of β -lactoglobulin or κ -casein as a function of time, β -mercaptoethanol concentrations, and temperature was monitored using MF-electrophoresis. The details of the methods and techniques can be found in Chapter 3.

8.3 Results

The MF-electropherograms of κ -casein and β -lactoglobulin under non-reducing and reducing conditions are shown in Figure 8.1. The peaks of β -lactoglobulin and κ -casein were observed at a similar position in control and fully reduced milk systems (Figure 7.1). When κ -casein was fully reduced, the area of κ -casein peak was high, indicating the proportion of monomeric κ -casein was high as the intermolecular disulphide bonds were cleaved (Figure 8.1A). This proportion was taken as 100% reduction of the κ -casein. The percentage of monomeric κ -casein in non-reduced solutions was, therefore, calculated to be under 5% of the total κ -casein. This was in agreement with the percentages found in unheated skim milk because most of κ -casein is disulphide-bonded to form oligomers (Rollema et al., 1988; Holland et al., 2008).

The non-reduced and reduced states of β -lactoglobulin are presented by two different peaks on the MF-electropherograms (Figure 8.1). The difference in electrophoretic mobility of non-reduced and reduced β -lactoglobulin has also been observed in skim milk (Chapter 7) and in samples from previous studies that used traditional SDS-PAGE (Pitt-Rivers & Impiombato, 1968; Griffith, 1972; Wada & Kitabatake, 2001). As mentioned previously (Section 3.7.2), it is possible that only one of the two disulphide bonds of β -lactoglobulin was reduced. However, it is not possible to distinguish between partially reduced, non-reduced or fully reduced β -lactoglobulin using the current method. Thus the β -lactoglobulin peak as observed in this study in the presence of excess β -mercaptoethanol was assumed to represent the fully reduced β -lactoglobulin with both disulphide bonds being cleaved. Based on this analysis, the progressive reduction of disulphide bonds of β -lactoglobulin and κ -casein can be examined by monitoring the increases in intensity of the monomeric κ -casein peak and of the reduced β -lactoglobulin peak (Figure 8.1).

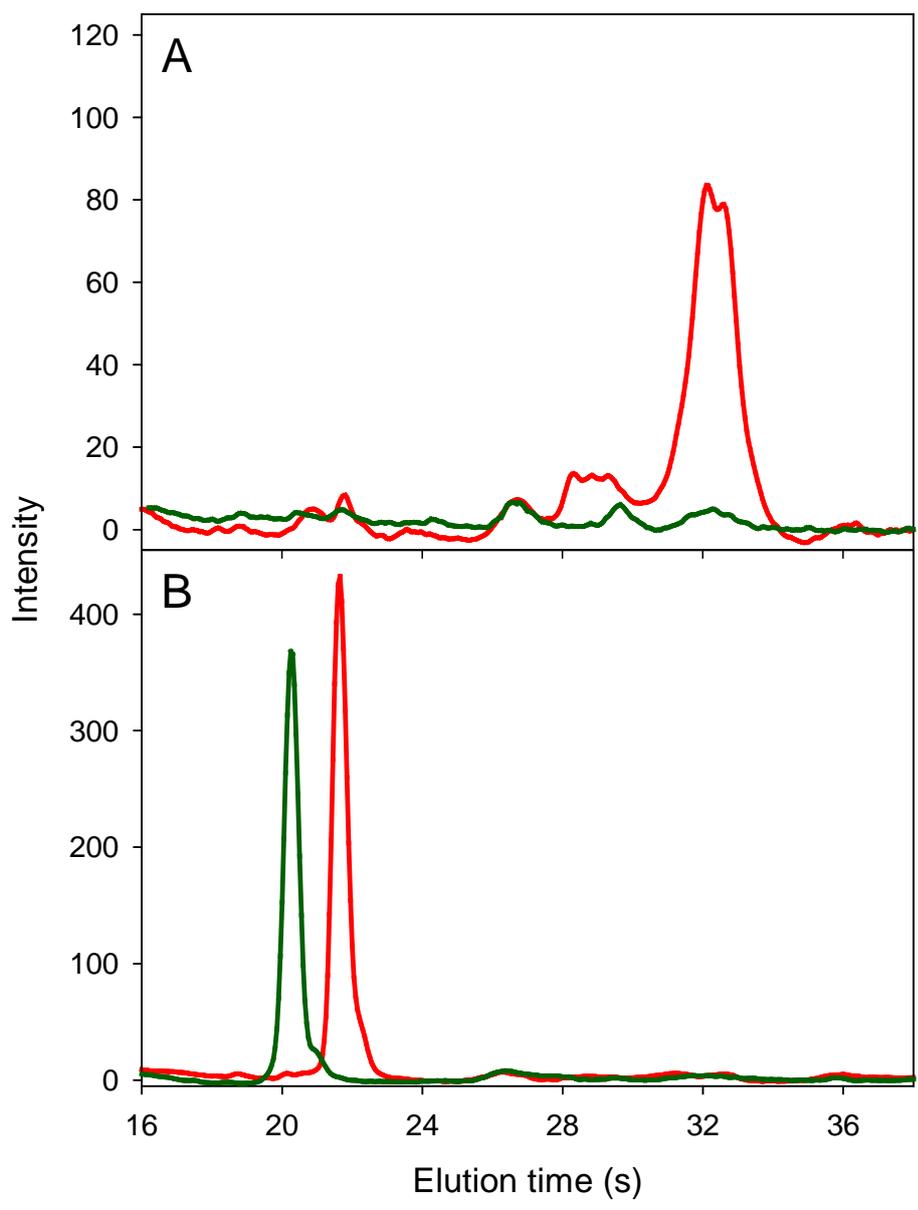


Figure 8.1: MF-electropherograms of κ -casein (A) and β -lactoglobulin (B) solutions. Red, reducing condition; green, non-reducing condition.

8.3.2 The reduction of disulphide bonds in κ -casein and β -lactoglobulin solutions

Selected electropherograms are presented to show the effect of β -mercaptoethanol concentrations and reaction time on the reduction of κ -casein and β -lactoglobulin (Figure 8.2A and B). At any reaction time, the area of the monomeric κ -casein peak increased with the increase in β -mercaptoethanol concentrations. In addition, at any β -mercaptoethanol concentration, the peak area of monomeric κ -casein was larger after 6 h of reaction time than after 1 h.

The percentage of the κ -casein that was monomeric (i.e. was not disulphide-bonded) was quantified and the results are summarised in Figure 8.2C. When low concentrations of β -mercaptoethanol (≤ 7.1 mM) were added to the κ -casein solution at 20 °C, the reduction of the protein disulphide bonds occurred over 4 to 5 h, after which no further reduction occurred (Figure 8.2C). The percentage of monomeric κ -casein increased with increasing concentrations of β -mercaptoethanol so that after 6 h, this percentage was $26 \pm 3\%$, $44 \pm 3\%$ and $46 \pm 4\%$ for 1.4, 4.3, and 7.1 mM β -mercaptoethanol, respectively.

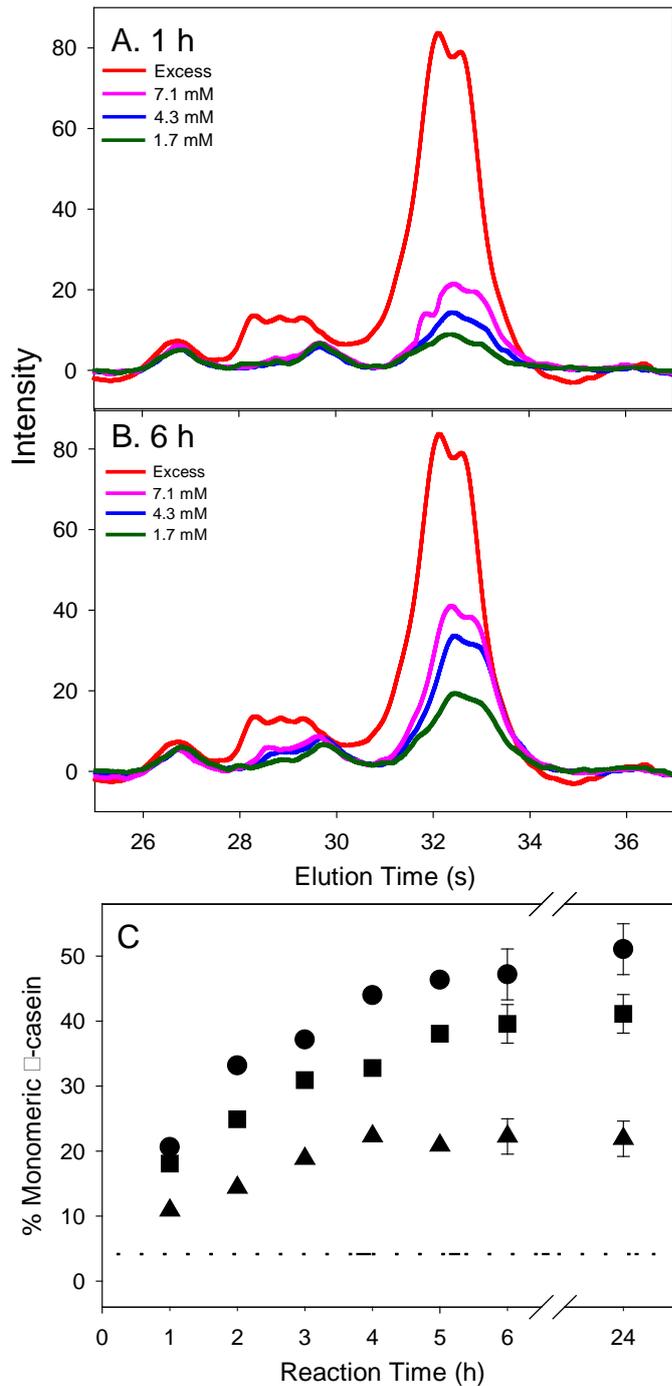


Figure 8.2: Effects of β -mercaptoethanol concentrations and reaction times on the reduction of κ -casein. A & B, MF-electropherograms of κ -casein after reacting with various β -mercaptoethanol concentrations for 1 h and 6 h; C: Percentage of monomeric κ -casein over the total of that protein present in the solution; ▲, 1.4 mM; ■, 4.3 mM; ●, 7.1 mM β -mercaptoethanol; dash line, the percentage of monomeric κ -casein in control solution. Each data point is the average of two to three replicates. Error bars represent the pooled standard deviation.

The change in the peak intensity of reduced β -lactoglobulin followed a similar pattern to the changes seen with the monomeric κ -casein peak at different concentrations of β -

mercaptoethanol. At any reaction time, the peak area of reduced β -lactoglobulin increased with the increase of β -mercaptoethanol concentrations (Figure 8.3A and B). The increase in peak area of reduced β -lactoglobulin was accompanied by a decrease in the peak area of non-reduced β -lactoglobulin. At any β -mercaptoethanol concentration, the peak area of reduced β -lactoglobulin was higher after 6 h (Figure 8.3B) than after 1 h (Figure 8.3A) of reaction time.

The effects of reaction time and β -mercaptoethanol concentrations on the percentage of reduced β -lactoglobulin over the total of that protein present in the solution is summarised in Figure 8.3C. The percentage of reduced β -lactoglobulin increased progressively with the β -mercaptoethanol concentrations so that after a reaction time of 6 h, this percentage increased from $11 \pm 1\%$ to $22 \pm 4\%$ as the level of β -mercaptoethanol increased from 14 to 71 mM. In addition, Figure 8.3C showed that the reduction of β -lactoglobulin continued for up to 24 h.

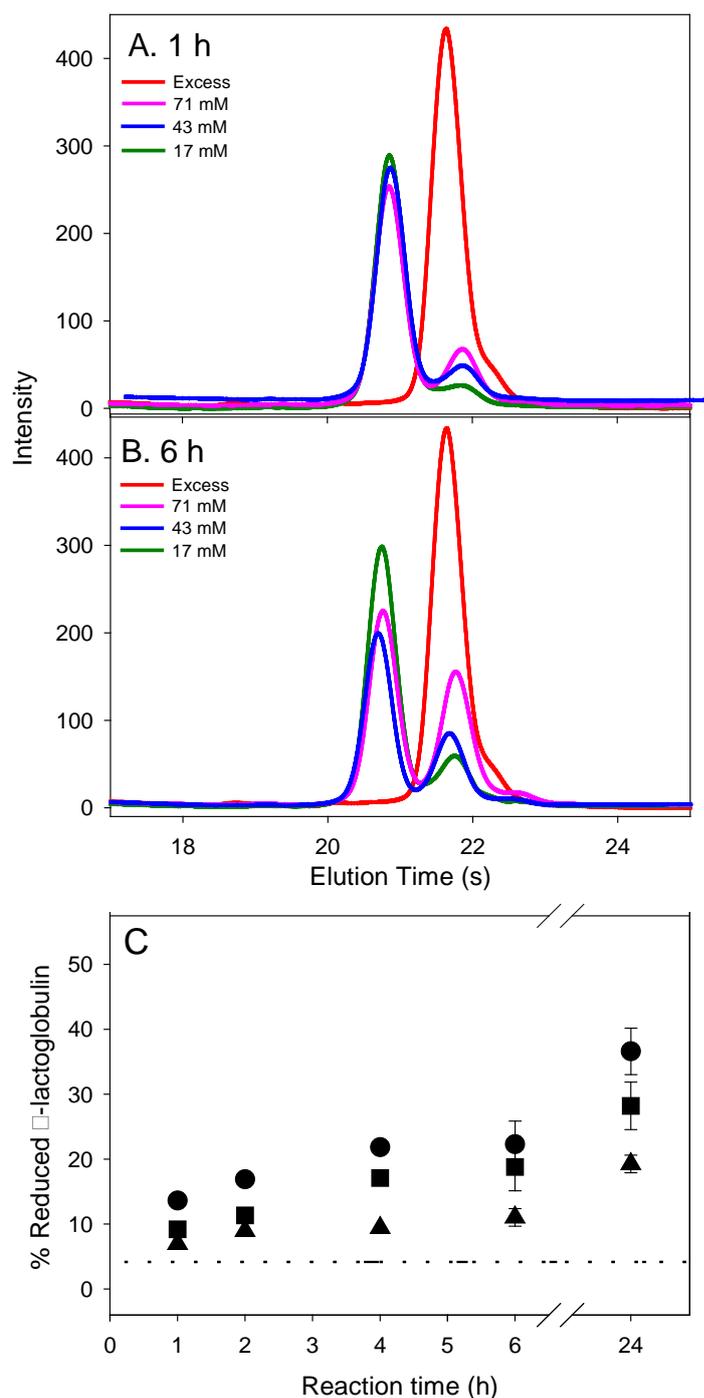


Figure 8.3: Effects of β -mercaptoethanol concentrations and reaction times on the reduction of β -lactoglobulin. A & B, typical MF-electropherograms of β -lactoglobulin after reacting with various β -mercaptoethanol concentrations for 1 h and 6 h, 1st peak, non-reduced β -lactoglobulin; 2nd peak, reduced β -lactoglobulin; C: Percentage of reduced β -lactoglobulin over the total level of that protein present in the solution; \blacktriangle , 14 mM; \blacksquare , 43 mM; \bullet , 71 mM β -mercaptoethanol; dashed line, the percentage of reduced β -lactoglobulin in control solution. Each data point is the average of two to three replicates. Error bars represent the pooled standard deviation.

8.3.3 Effects of temperature on the reduction of disulphide bonds in κ -casein and β -lactoglobulin solutions

The temperature of the solution was increased up to 50 °C to investigate the effect of temperature on the reduction of the disulphide bonds of κ -casein and β -lactoglobulin. In a preliminary experiment, various β -mercaptoethanol concentrations (1.4 to 71 mM) were added to κ -casein or β -lactoglobulin solution that were then held at 30 or 50 °C for 1 and 6 h. Figures 8.4 and 8.5 show the changes in the peak area of monomeric κ -casein and reduced β -lactoglobulin, respectively.

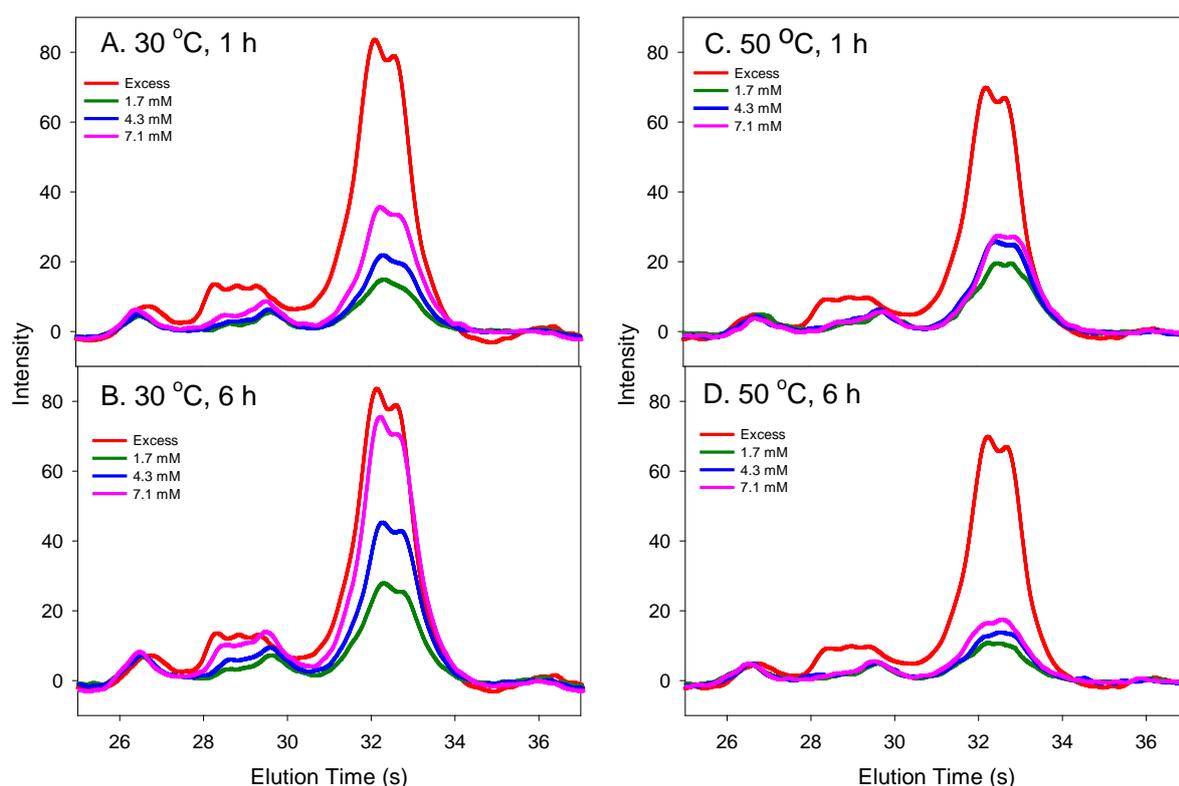


Figure 8.4: MF-electropherograms showing the effects of β -mercaptoethanol concentrations, reaction times and temperature on the reduction of κ -casein. κ -casein was incubated with various β -mercaptoethanol concentrations for 1 or 6 h at 30 °C (A and B) or 50 °C (C and D).

At 30 and 50 °C, the peak area of monomeric κ -casein increased with the increase of β -mercaptoethanol concentrations at either reaction time (Figure 8.4). This indicates that the proportion of monomeric κ -casein increased with β -mercaptoethanol concentration, as already observed in solutions at 20 °C (Figure 8.2). At 50 °C, the peak area of monomeric κ -casein decreased for all concentrations of β -mercaptoethanol as the reaction time increased from 1 to 6 h (Figure 8.4C and D). This is contrary to what was observed at 20 and 30 °C as at these lower temperatures, the peak area of monomeric κ -casein at 6 h was higher than that at 1 h (Figure 8.2 and Figure 8.4A and B).

The peak of reduced β -lactoglobulin increased with the increasing concentrations of β -mercaptoethanol, regardless of the reaction time and temperature (Figure 8.5). Based on this preliminary experiment, 7.1 mM and 17 mM β -mercaptoethanol were the chosen concentrations to be studied in κ -casein and β -lactoglobulin solutions, respectively. The solutions were maintained at 30, 40 and 50 °C for different periods of time. Figure 8.6 summarises the results of the impact of reaction time and temperature on the reduction of pure β -lactoglobulin or κ -casein in solution.

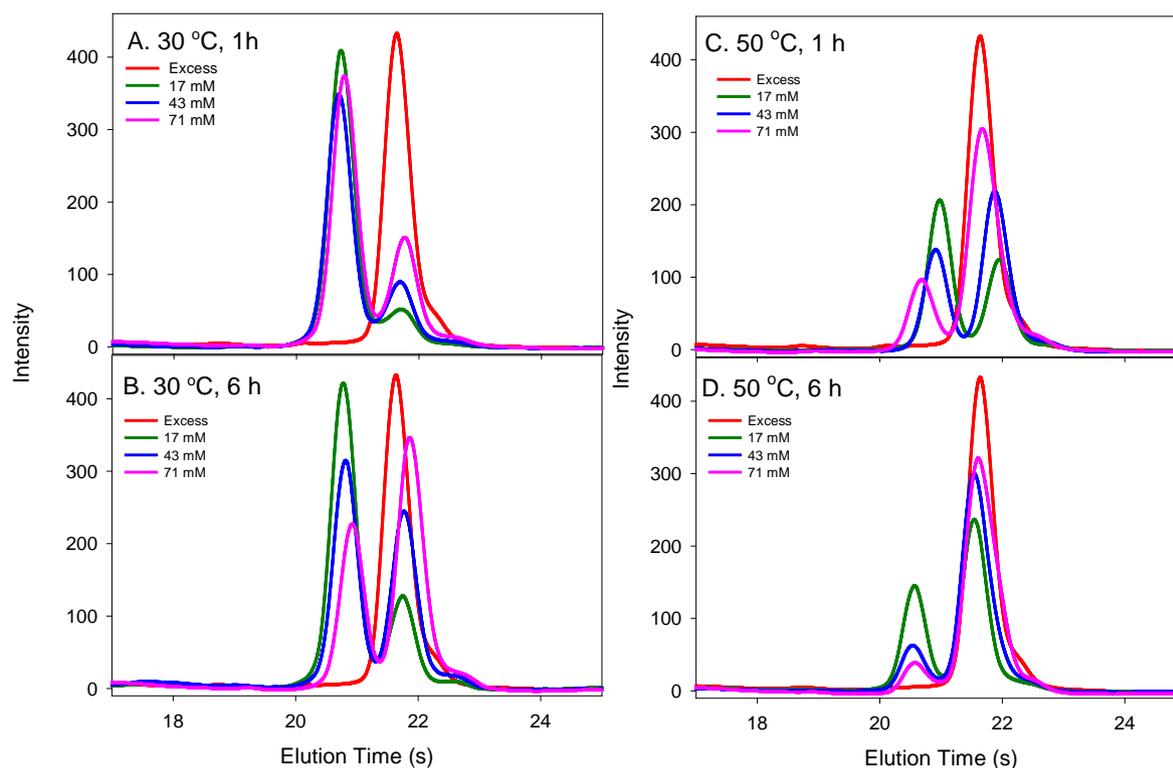


Figure 8.5: MF-electropherograms showing the effects of β -mercaptoethanol concentrations, reaction times and temperature on the reduction of β -lactoglobulin. β -lactoglobulin was incubated with various β -mercaptoethanol concentrations for 1 and 6 h at 30 °C (A and B) and for 1 and 6 h at 50 °C (C and D).

The reduction of the disulphide bonds in κ -casein by 7.1 mM β -mercaptoethanol was greatly affected by temperature (Figure 8.6A). At 20 and 30 °C, the percentage of monomeric κ -casein increased with reaction time and was higher at 30 °C than at 20 °C at any reaction time. At 40 and 50 °C, the percentage of κ -casein increased during the first 2 h and then decreased as the reaction time was prolonged beyond 2 h. The decrease was more pronounced at 50 °C than at 40 °C.

As for β -lactoglobulin, the percentage of reduced β -lactoglobulin increased with the increase of temperature from 20 to 50 °C at all reaction times. After 6 h, the percentage of reduced β -lactoglobulin increased from $11 \pm 1\%$ at 20 °C to $38 \pm 2\%$ at 50 °C (Figure 8.6B).

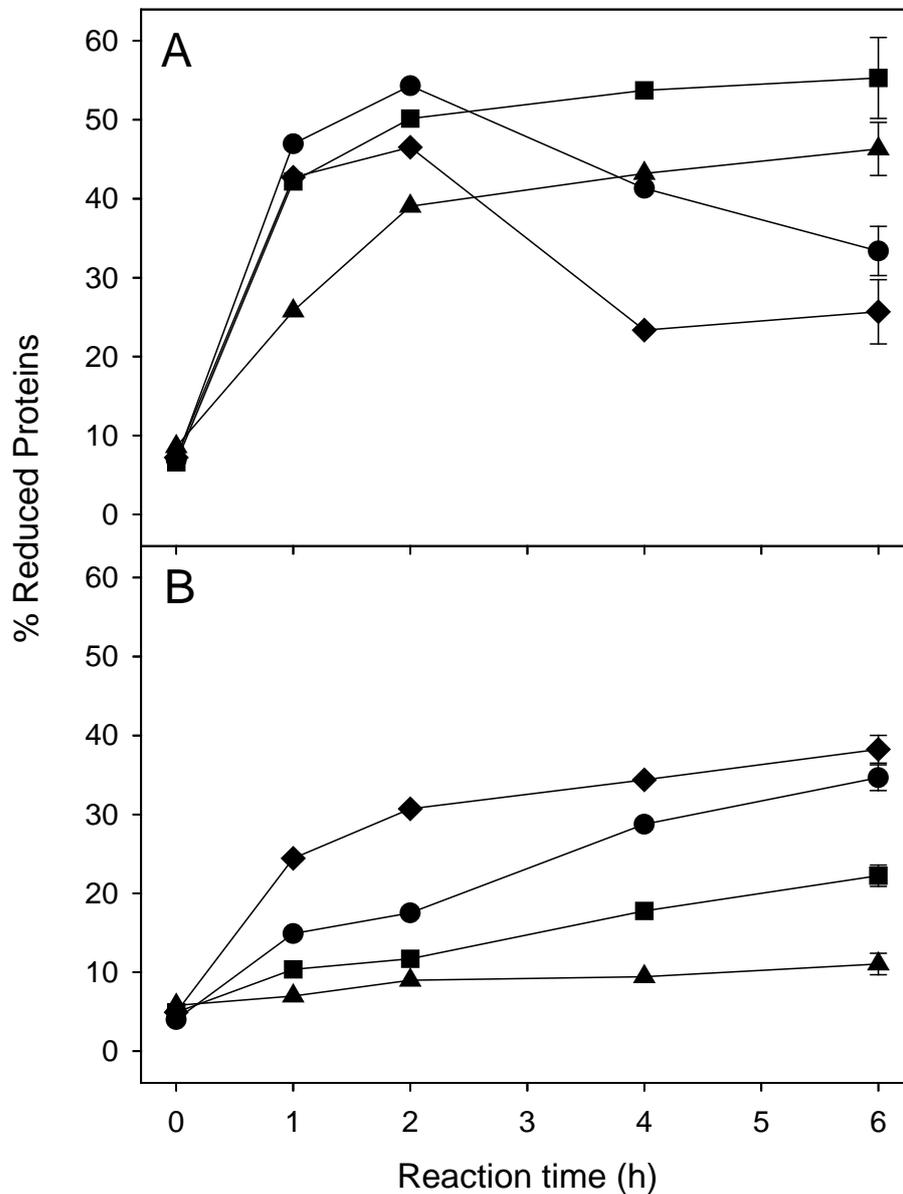


Figure 8.6: Effects of reaction time and temperature on the reduction of the proteins. A, κ -casein treated with 7.1 mM β -mercaptoethanol; B, β -lactoglobulin treated with 17 mM β -mercaptoethanol. ▲, 20 °C; ■, 30 °C; ●, 40 °C; ◆, 50 °C. Each data point is the average of two to three repetitions. Error bars represent the pooled standard deviation of the data set of each temperature.

8.3.4 The reduction of protein disulphide bonds in β -lactoglobulin/ κ -casein binary mixtures

The mixed solution of β -lactoglobulin and κ -casein was treated with 7.1 mM and 17 mM β -mercaptoethanol for different reaction times at temperatures ranging from 20 to 40 °C. The typical electropherograms of the change of reduced β -lactoglobulin and monomeric κ -casein peaks are shown in Figure A.9 in Appendix 6. Figure 8.7 summarises the effects of reaction time and temperature on the percentage of reduced β -lactoglobulin and monomeric κ -casein over the total protein present.

When 7.1 mM β -mercaptoethanol was added to the mixture of β -lactoglobulin and κ -casein, the percentage of monomeric κ -casein increased with reaction time and peaked after 2 h (Figure 8.7A). This percentage was higher at 30 °C (46%) than at 20 or 40 °C (41%). After 2 h, the percentage of monomeric κ -casein remained almost constant at 20 and 30 °C but decreased to 27% at 40 °C. In contrast to κ -casein, the percentage of reduced β -lactoglobulin was lower than 10% regardless of the reaction temperature and the reaction time (Figure 8.7A).

When 17 mM of β -mercaptoethanol was added to the β -lactoglobulin/ κ -casein mixture, the percentage of monomeric κ -casein increased significantly over the first 2 h ($p < 0.05$) and the increase was most pronounced at 30 °C. The proportion of monomeric κ -casein decreased as the reaction was prolonged beyond 2 h at all temperatures. The decrease was more pronounced at 40 °C than at the lower temperatures (Figure 8.7B). A higher proportion of β -lactoglobulin was reduced with 17 mM β -mercaptoethanol (Figure 8.7B) when compared with 7.1 mM β -mercaptoethanol (Figure 8.7A). Temperature had only a small effect on the reduction of β -lactoglobulin with a slightly higher percentage of reduced β -lactoglobulin as the temperature was increased from 20 °C to 40 °C (Figure 8.7B).

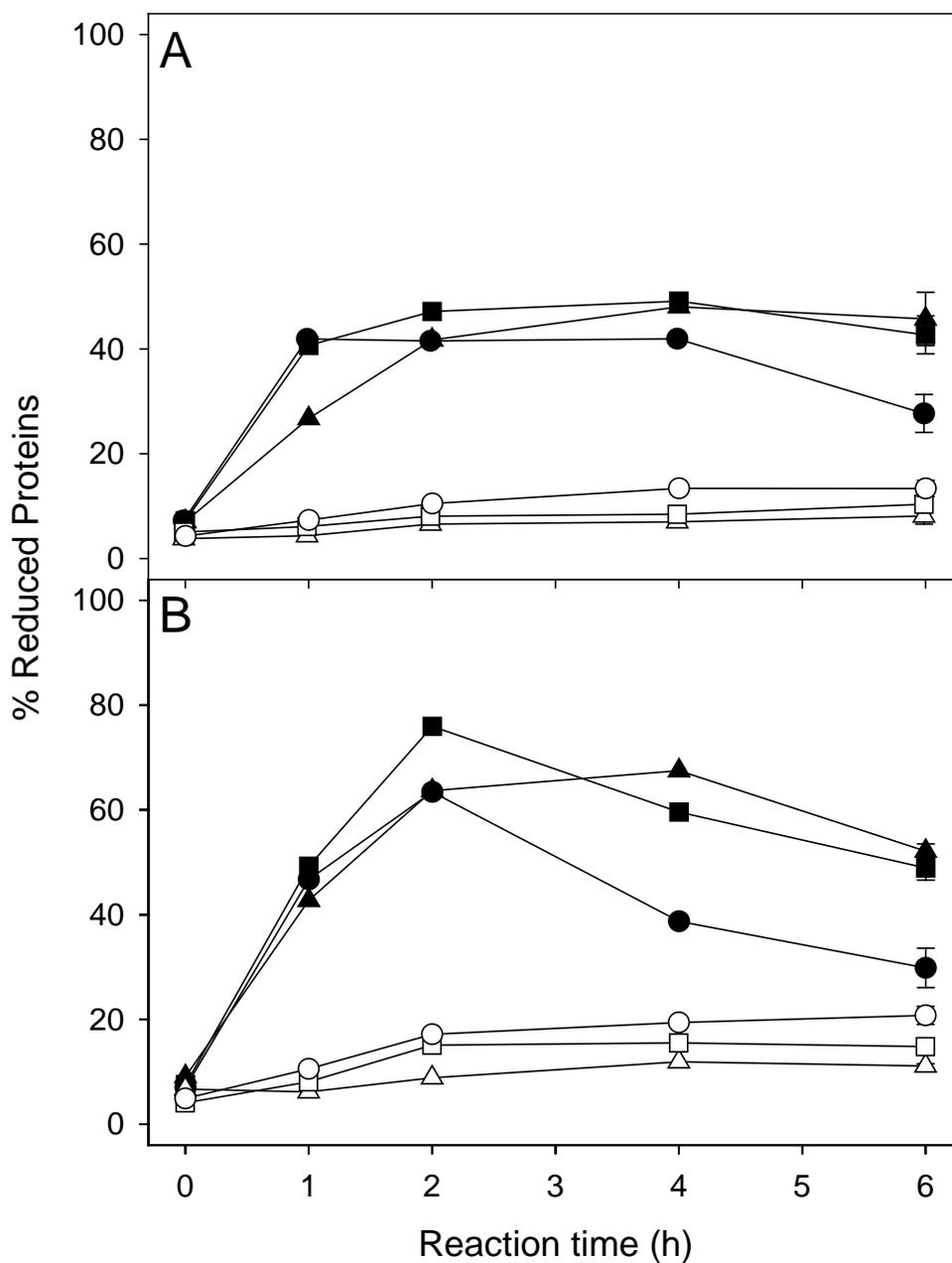


Figure 8.7: Effects of reaction time and temperature on the percentage of monomeric κ -casein (closed symbols) and reduced β -lactoglobulin (open symbols) over the total of that protein in mixed solutions. A: 7.1 mM β -mercaptoethanol; B: 17 mM β -mercaptoethanol. ▲, 20 °C; ■, 30 °C; ●, 40 °C. Experimental points were the average of two to three replicates. Error bars represent pooled standard deviation of the data set of each temperature.

8.4 Discussion

The results presented in this chapter demonstrated that κ -casein was reduced in preference to β -lactoglobulin, regardless of whether the proteins were in separate solutions (Figure 8.2 and Figure 8.3) or in mixed protein solutions (Figure 8.7). As mentioned in Chapter 7, κ -casein contains two cysteine residues (Cys¹¹ and Cys⁸⁸) that formed intermolecular disulphide bonds in a random manner (Rasmussen et al., 1992). As a consequence, κ -casein is found in the form of disulphide-linked oligomers of various sizes (Holland et al., 2008). Isolated κ -casein polymers are proposed to form soap-like micelles (Swaisgood, 2003). κ -Casein is amphiphilic and has a hydrophobic region and a hydrophilic charged region (Horne, 1998). In isolated κ -casein micelles the hydrophobic regions are associated with the core of the micelles, whereas the hydrophilic regions project into the serum and stabilise the micelles (Farrell et al., 2004).

As the cysteine residues are in the hydrophobic region, they are associated with the core of the soap-like micelles. Previous studies on model systems demonstrated that unfolded β -lactoglobulin, considered to be a large molecule, was able to initiate thiol-disulphide exchange reactions with κ -casein (Zittle et al., 1962; Cho et al., 2003). This indicated that the surface of the κ -casein micelles was porous. Therefore a small molecule such as β -mercaptoethanol (molecular weight of 78.13 g mol⁻¹) should be able to easily approach and reduce the disulphide bonds, as was observed in this study (Figure 8.2).

Unlike κ -casein, the disulphide bonds of native β -lactoglobulin are intra-molecular (Brownlow et al., 1997). As discussed in 7.4.1, one of the two disulphide bonds (Cys⁶⁶-Cys¹⁶⁰) is more accessible to disulphide reducing agent than the other (Cys¹⁰⁶-Cys¹¹⁹) (Davidson & Hird, 1967). However, higher concentrations of β -mercaptoethanol and long periods of time were required to completely reduce β -lactoglobulin to the same extent as required to reduce κ -casein (Figure 8.3). Hence, both the intra-molecular disulphide bonds of native β -lactoglobulin were considered less accessible to β -mercaptoethanol compared with the intermolecular disulphide bonds of κ -casein.

At 20 °C, the percentage of reduced κ -casein increased after 6 h of reaction, with this percentage remaining constant after 6 or 24 h (Figure 8.2C). This suggested that the newly formed thiol groups on κ -casein were stable and did not undergo subsequent thiol-disulphide exchange reactions or oxidation. As the temperature of reaction was increased ≥ 40 °C, a decrease of the percentage of monomeric κ -casein occurred after the longer reaction times (> 3 h, Figure 8.6A and Figure 8.7). This indicated that an oxidation reaction may occur between κ -casein containing free thiol groups. Alternatively, monomeric κ -casein with free thiol groups can

initiate thiol-disulphide exchange reactions with disulphide-linked κ -casein (i.e. the κ -casein that had not been reduced by β -mercaptoethanol) to re-form polymeric κ -casein.

The question is why oxidation or thiol-disulphide exchange reactions between κ -casein occurred more readily at temperatures ≥ 40 °C than at 20 °C. This could be because the rate of collisions between the molecules increased with the increase in temperature. When the temperature was raised, the Brownian motion of κ -casein increased. Hence the rate of collisions between the proteins was higher, resulting in higher chance of interacting between the proteins.

Alternatively, the free energy of the polymeric κ -casein and monomeric κ -casein can be altered on increasing the temperature of the reaction and the reaction equilibrium was shifted. As shown in Figure 8.8, at low temperatures, it required lower energy for molecule A to react and become molecule B, hence the reaction went forward. However at higher temperature, it was the opposite. It took less energy for molecule B to react and become molecule A, thus the reaction went backward. In this study, polymeric κ -casein can be molecule A and monomeric κ -casein can be molecule B. The reduction of polymeric κ -casein to form monomers may be favoured at temperatures below 40 °C and the oxidation of the monomeric κ -casein to become polymers may be favoured at higher temperatures.

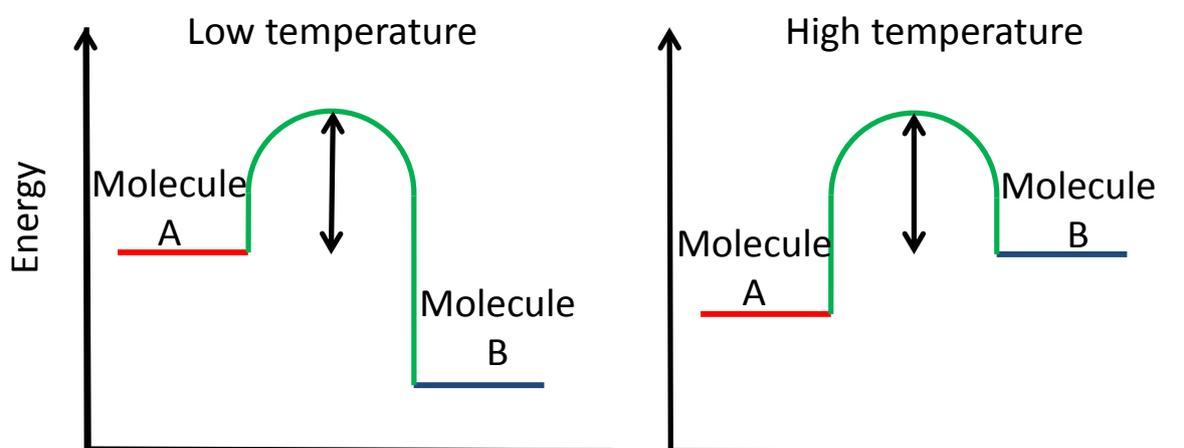


Figure 8.8: Schematic plot of the energy of a reacting system at different temperatures.

The reduction of β -lactoglobulin increased with increasing temperatures (Figure 8.6B and Figure 8.7). As mentioned above, the Brownian motion of the proteins and β -mercaptoethanol would increase when the temperature was raised. This may lead to an increase in rate of collisions between the protein and the reducing agent. The free energy of the β -lactoglobulin may also be increased due to the increase of temperatures. Hence the reduction rate increased since it may require less energy to transform native β -lactoglobulin to reduced β -lactoglobulin at high temperatures.

In addition, while the irreversible denaturation of β -lactoglobulin occurred at $> 70\text{ }^{\circ}\text{C}$ (Ruegg et al., 1977; De Wit & Swinkels, 1980), β -lactoglobulin has been observed to undergo reversible conformational change at temperatures $< 60\text{ }^{\circ}\text{C}$ by differential scanning calorimetric examination (Owusu-Apenten & Chee, 2004) and by intrinsic fluorescence measurement (Cairolì, Iametti & Bonomi, 1994). It is conceivable that in this study, β -lactoglobulin may partially unfold at temperatures $> 30\text{ }^{\circ}\text{C}$, especially at long holding time (up to 6 h). This may make the disulphide bonds more accessible to the reducing agent. Furthermore, it is known that on heating, the dimer β -lactoglobulin was first dissociated into monomers before the protein structure unfolds (Figure 2.9) (De Wit, 1990; Roefs & De Kruif, 1994; Anema, 2009b). Subsequently, at temperatures $> 30\text{ }^{\circ}\text{C}$, the majority of β -lactoglobulin can exist as monomers. It is possible that the disulphide bonds of β -lactoglobulin were more accessible when the proteins were monomers than when they were dimers. Hence, the partial unfolded structure and the monomeric state of β -lactoglobulin at temperatures $> 30\text{ }^{\circ}\text{C}$ may account for the increased percentage of reduced β -lactoglobulin at these temperatures (Figure 8.6B and Figure 8.7).

8.5 Conclusions

The disulphide reducing agent β -mercaptoethanol reacted with κ -casein more preferentially than β -lactoglobulin. As the micelle structure of the κ -casein polymer in the solution can be porous, the disulphide bonds of κ -casein may be more accessible towards β -mercaptoethanol. The disulphide bonds of β -lactoglobulin were relatively less accessible because of its native folded structure. Consequently, the disulphide bonds of κ -casein can be selectively reduced in the mixture of β -lactoglobulin and κ -casein by using low concentrations of β -mercaptoethanol. In addition, monomeric κ -casein with free thiol groups was found to be stable at $20\text{ }^{\circ}\text{C}$ for up to 24 h. However, if the temperature was raised to above $40\text{ }^{\circ}\text{C}$, oxidation may occur, which resulted in a decrease in the proportion of monomeric κ -casein.

Chapter 9 - Effects of heating milks in the presence of β -mercaptoethanol on the protein interactions and acid gel properties

9.1 Introduction

The results in Chapter 7 showed that adding β -mercaptoethanol to unheated milks reduced the majority of the disulphide bonds in κ -casein and possibly some of the disulphide bonds in the β -lactoglobulin and α -lactalbumin. The newly formed free thiol groups were expected to initiate thiol-disulphide exchange reactions on heating the reduced milks. Consequently, a higher proportion of disulphide bonds were expected to be formed in heated milks.

Previously, Hashizume & Sato (1988) and Goddard (1996) added up to 100 mM of β -mercaptoethanol to skim milk. Gels were formed from the treated milks by firstly lowering the pH followed by heat treatment. It was observed that the final firmness of the acid-heat-induced gels increased at low concentrations of β -mercaptoethanol (< 10 mM), and then decreased markedly at higher β -mercaptoethanol concentrations. At β -mercaptoethanol concentrations of ≥ 10 mM, there was a larger proportion of reducing agent compared to the total number of disulphide bonds in the milk. Hence all of the disulphide bonds were considered to be broken, resulting in the decrease in the firmness of acid-heat-induced gels (Hashizume & Sato, 1988; Goddard, 1996).

Other than the studies of Goddard (1996) and Hashizume (1988) on acid-heat-induced gels, there are no other studies examining the effect of heating milk in the presence of low concentrations of β -mercaptoethanol (< 10 mM) on the interactions between the proteins and on the acid gels made by slowly acidifying the treated milk. The aim of the work presented in this chapter was to investigate the effect of β -mercaptoethanol on protein interactions in heated milks.

From the experiments presented in Chapter 5, it was shown that even though the thiol-disulphide exchange reactions were inhibited by the addition of NEM to the milk, acid gels prepared from WPE skim milk heated in the presence of NEM had G' higher than that of those prepared from the heated control. It would be interesting to investigate the rheological properties of the acid gels prepared from milks that had been heated in the presence of β -mercaptoethanol, as the thiol disulphide exchange reactions were expected to be enhanced. The microstructure of the acid gels was also examined. Finally, an attempt was made to explain how

the reduction of disulphide bonds affected the mechanism of aggregations and the observed change in the functional properties.

9.2 Materials and methods

Skim milk and WPE skim milk were prepared as described in Section 3.1.1.

Diluted β -mercaptoethanol (10% v/v) was added to unheated skim milk and WPE skim milk to give β -mercaptoethanol concentrations of 1.4, 4.3 and 7.1 mM. The treated milk was shaken on a vortex mixer for 10 s and then left to react at 20 ± 0.05 °C in a thermostatically controlled water bath for 3 h. The milk container was tightly closed to minimise oxidation.

After 3 h, subsamples of treated milk were heated at 80 ± 0.1 °C for 30 min as described in Section 3.3. After heating, the samples in the vials were rapidly cooled in cold tap water until the samples were below 30 °C. The samples were analysed immediately after the cooling.

The composition of the milk and the distribution of the protein between the colloidal and serum phases were determined using SDS-PAGE (Section 3.6). SDS-PAGE under non-reducing conditions was used to determine the protein and its level not being involved in intermolecular disulphide bonds. The size of the casein micelles were monitored using light scattering method, which was described in Section 3.8.

At defined times after heating, subsamples of the milks were slowly acidified to form acid gels. The rheological properties of the milks during acidification were monitored. In addition, the rheological properties and the microstructure of the set gels were also investigated.

The details of the rheological and microstructural methods can be found in Section 3.10.2 and 3.10.3 respectively.

9.3 Results

9.3.1 Effects of heating β -mercaptoethanol-treated milks on the denaturation of the whey proteins

Addition of β -mercaptoethanol reduced the disulphide bonds of the proteins in milk to form free thiol groups. Thus, the thiol-disulphide exchange reactions were expected to occur more readily in milk that was heated in the presence of β -mercaptoethanol. Consequently, the proportion of whey proteins participating in the exchange reactions was expected to increase and the percentage of native whey proteins was expected to decrease.

The level of denaturation of whey proteins in skim milk was examined using SDS-PAGE. Figure 9.1 showed the SDS-PAGE patterns of β -lactoglobulin and α -lactalbumin remaining in their

native states. The intensity of β -lactoglobulin and α -lactalbumin bands decreased markedly with the increase of β -mercaptoethanol concentrations, especially the α -lactalbumin bands in β -mercaptoethanol-treated-heated milks almost completely disappeared (Lanes 3 and 4, Figure 9.1).

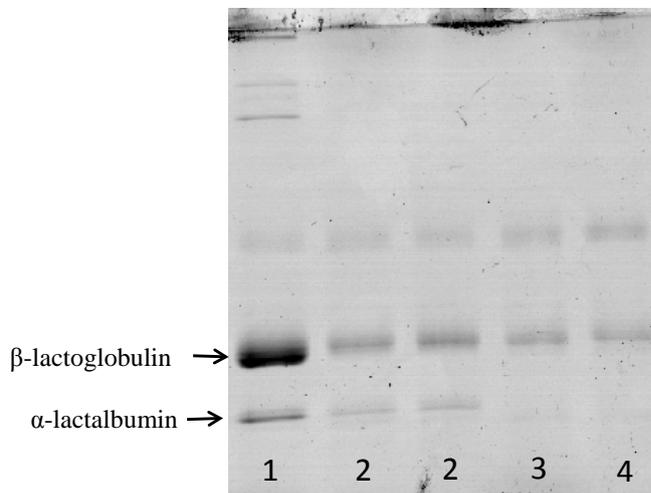


Figure 9.1: SDS-PAGE patterns of native proteins of selected samples of skim milks that had been heated (80 °C, 30 min) in the presence of β -mercaptoethanol. Control sample (i.e. native proteins in unheated skim milk, Lane 1); control heated skim milk (Lanes 2); milks heated in the presence of 1.4 mM (Lane 3) and 7.1 mM (Lane 4) β -mercaptoethanol.

Considering the trace amounts of native whey proteins in the acid precipitate samples, the more sensitive MF-electrophoresis method was used to quantify the decrease of native whey protein levels in the milks after addition of β -mercaptoethanol and subsequent heat treatments. Figure 9.2 showed the relative change in the level of native β -lactoglobulin and α -lactalbumin observed by MF-electrophoresis.

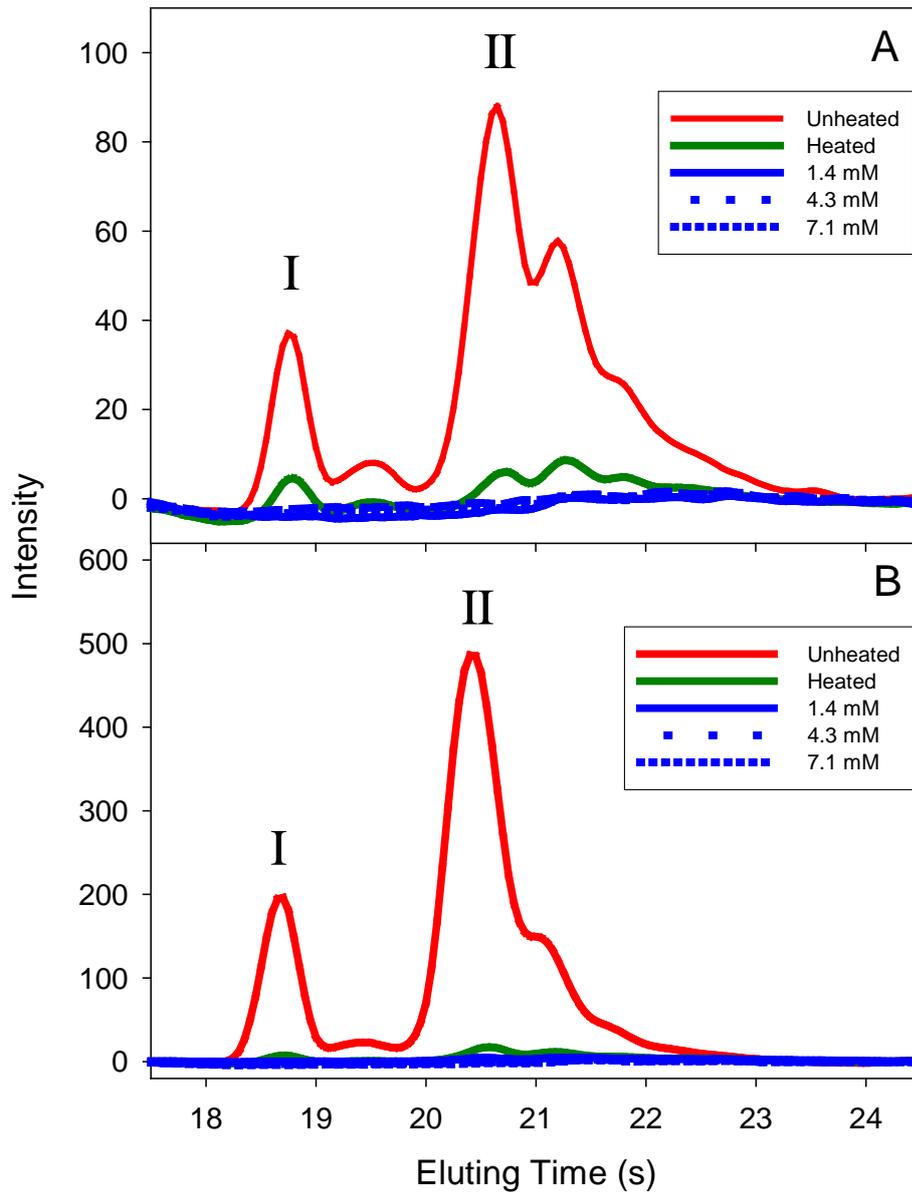


Figure 9.2: MF-electropherograms showing the effects of heating milks in the presence of β -mercaptoethanol on the level of native whey proteins. Skim milk (A) and WPE skim milk (B). Red line, unheated milks; green line, heated milks; blue lines, milks heated in the presence of various β -mercaptoethanol concentrations. I, α -lactalbumin; II, β -lactoglobulin.

In heated β -mercaptoethanol-treated milks, the peaks of α -lactalbumin and β -lactoglobulin were very small and close to the baseline at zero. This indicated that the proportions of native α -lactalbumin and β -lactoglobulin in these milks were very low and markedly lower than those in the control heated milks. The remaining native whey protein levels are summarised in Table 9.1.

Table 9.1: Proportion of α -lactalbumin and β -lactoglobulin remaining native in milks that were heated in the presence of β -mercaptoethanol. n/d: not-detected.

β-mercaptoethanol concentrations	Skim milk		WPE skim milk	
	α -lactalbumin	β -lactoglobulin	α -lactalbumin	β -lactoglobulin
0	39%	25%	5.6%	5.7%
1.4	n/d	8.4%	n/d	2.3%
4.3	n/d	7.6%	n/d	2.0%
7.1	n/d	6.7%	n/d	1.2%

The proportion of native β -lactoglobulin in both skim milk and WPE skim milk decreased from about 25% to less than 10% as the β -mercaptoethanol concentration increased, whereas that of native α -lactalbumin decreased to essentially zero. This indicated that a higher proportion of whey protein was involved in the heat-induced aggregation when milk was reduced prior to heating.

9.3.2 Effects of heating β -mercaptoethanol-treated milks on protein interactions and distributions

Measuring the concentration of the major whey proteins and κ -casein that participated in intermolecular disulphide bonds and the distribution of proteins between the colloidal and serum phases provides evidence of the formation and location of the heat-induced aggregates between κ -casein and the whey proteins. The samples of skim milk and WPE skim milk that had been treated with 0 to 7.1 mM β -mercaptoethanol for 3 h and then heated at 80 °C for 30 min were examined using SDS-PAGE under non-reducing conditions to determine the level of protein that was not participating in inter-molecular disulphide bonds. The samples of the supernatants (obtained from centrifuging the milk) were also investigated using SDS-PAGE under both reducing and non-reducing conditions to determine the level of proteins in the serum phase and whether or not these proteins were involved in disulphide interactions.

Figure 9.3 shows the SDS-PAGE patterns of proteins that did not participate in intermolecular disulphide bonds after milks were heated in the presence of β -mercaptoethanol. In skim milk, the band intensities of α -lactalbumin and β -lactoglobulin decreased markedly when the β -mercaptoethanol concentration increased from zero (Lane 2) to 1.4 mM (Lane 3 of Figure 9.3), and then increased as the β -mercaptoethanol concentrations increased further. The band intensity of α -lactalbumin was still lower at 7.1 mM β -mercaptoethanol than at 0 mM β -mercaptoethanol; whereas the band intensity of β -lactoglobulin at 7.1 mM β -mercaptoethanol

was similar to that at 0 mM β -mercaptoethanol. The intensity of the κ -casein band appeared to be unchanged at β -mercaptoethanol concentration ≤ 4.3 mM (Lane 3 and 4) and increased at 7.1 mM β -mercaptoethanol (Lane 5, Figure 9.3A). However after integration of the band intensity it was found that the percentage of monomeric κ -casein increased progressively with the increase of the β -mercaptoethanol concentration.

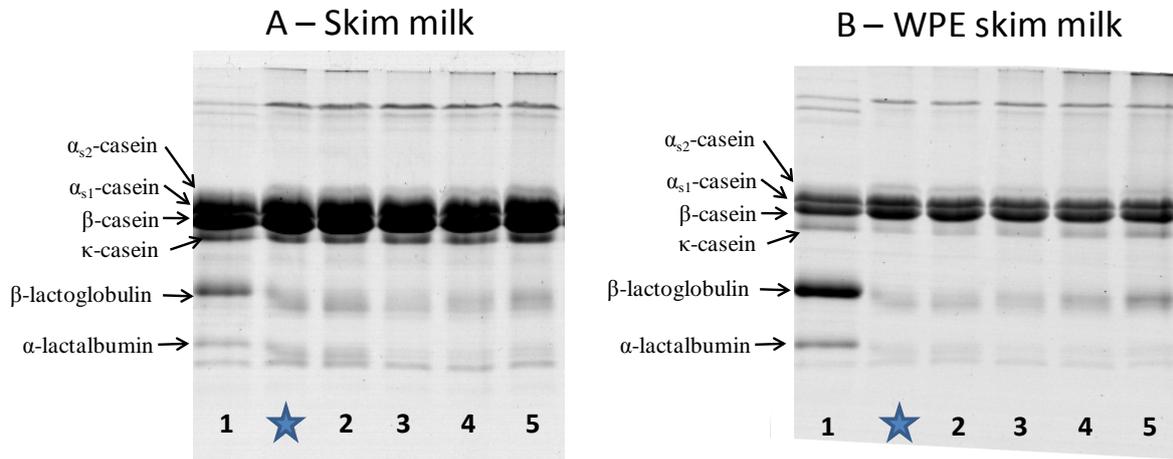


Figure 9.3: SDS-PAGE patterns showing the effects of β -mercaptoethanol concentrations on the level of proteins that were not participating in intermolecular disulphide bonds. Skim milk (A) and WPE skim milk (B). The milks were treated with β -mercaptoethanol for 3 h before being heated at 80 °C for 30 min. Control samples (i.e. control unheated milks, Lane 1); milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk samples were diluted with SDS sample buffer at a ratio of 1 to 40 and were not reduced except for the control samples being fully reduced. Blue star lane was a repeat of the sample in lane 2.

In WPE skim milk, the intensity of the α -lactalbumin band was low, regardless of the concentration of β -mercaptoethanol. The band intensities of β -lactoglobulin and κ -casein appeared to increase with the increase of β -mercaptoethanol concentrations (Figure 9.3B). This indicated that less β -lactoglobulin and κ -casein were involved in disulphide interactions upon adding β -mercaptoethanol.

Figure 9.4 shows the SDS-PAGE patterns of proteins in the milk supernatants, analysed under reducing conditions. The proteins present in the SDS-PAGE gels are those remaining in the serum phase of the milk samples. In skim milk, the intensity of α -lactalbumin and β -lactoglobulin bands decreased as the β -mercaptoethanol concentration increased. It was clear that the band at 7.1 mM β -mercaptoethanol was less intense than that at 0 mM β -mercaptoethanol (Lanes 5 and 2, respectively Figure 9.4A). This indicates that fewer whey proteins remained in the serum phase when skim milk was heated with β -mercaptoethanol. The

intensities of the casein protein bands did not appear to be affected by the addition of β -mercaptoethanol.

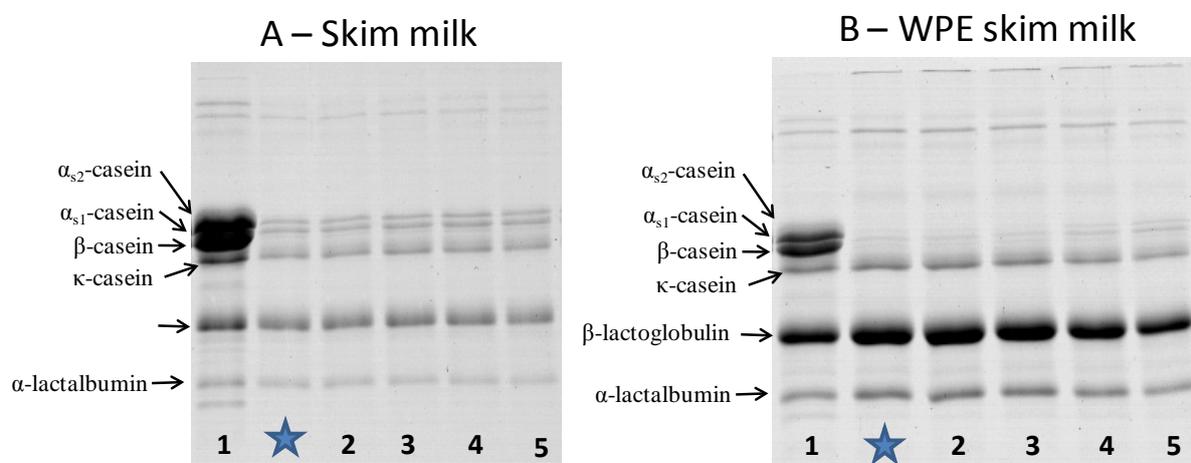


Figure 9.4: SDS-PAGE patterns showing the effect of β -mercaptoethanol concentration on the serum proteins in skim milk (A) and WPE skim milk (B). The milks were treated with β -mercaptoethanol for 3 h before being heated at 80 °C for 30 min. Control sample (i.e. control unheated milks, Lane 1); supernatant of milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk sample (Lane 1) and supernatant samples (Lanes 2 to 5) were diluted with SDS sample buffer at a ratio of 1:40 and 1:20, respectively. All the samples were fully reduced. Blue star lane was a repeat of the sample in lane 2.

In WPE skim milk, the α -lactalbumin and β -lactoglobulin bands became less intense as the concentration of β -mercaptoethanol increased. Similarly to skim milk, this indicated that fewer whey proteins were found in the serum phase when milk was heated with β -mercaptoethanol. The intensity of κ -casein band decreased with the increase in β -mercaptoethanol concentration. So unlike skim milk, when WPE skim milk was heated in the presence of β -mercaptoethanol, fewer κ -casein monomers were dissociated from the casein micelles. Interestingly, the bands of β - and α_{s1} -casein increased in intensity and became more distinguishable at β -mercaptoethanol concentration of 7.1 mM (Lane 5 in Figure 9.4B). This suggested the dissociation of β - and α_{s1} -casein from the casein micelles increased slightly when β -mercaptoethanol-treated WPE skim milk was heated.

Figure 9.5 shows the SDS-PAGE patterns of proteins that were present in the serum phase and were not disulphide-linked. In both skim milk and WPE skim milk, the band of α -lactalbumin almost disappeared in samples containing β -mercaptoethanol (Lanes 3 to 5, Figure 9.5). The band intensity of β -lactoglobulin decreased as β -mercaptoethanol concentration increased from

zero to 1.4 mM (Lanes 2 and 3), then increased progressively as the β -mercaptoethanol concentration increased further (Lanes 4 and 5).

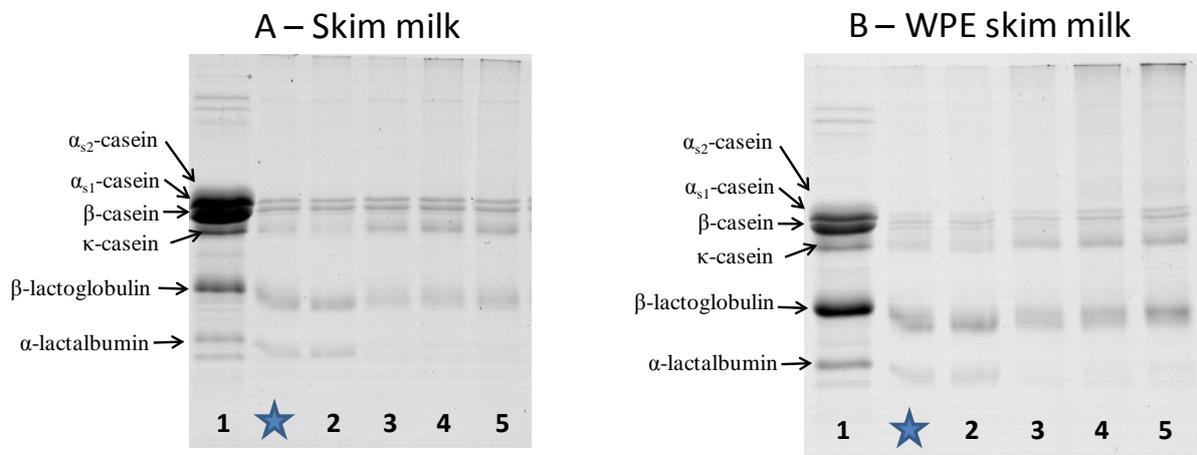


Figure 9.5: SDS-PAGE patterns showing the effect of β -mercaptoethanol concentrations on the proteins that were not disulphide-linked in the serum phase of skim milk (A) and WPE skim milk (B). The milks were treated with β -mercaptoethanol for 3 h before being heated at 80 °C for 30 min. Control samples (i.e. control unheated milks, Lane 1); supernatant of milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk sample (Lane 1) and supernatant samples (Lanes 2 to 5) were diluted with SDS sample buffer at a ratio of 1:40 and 1:20, respectively. The milk sample was fully reduced and the supernatant samples were not. Blue star lane was the transition between reducing and non-reducing conditions.

The band intensity of κ -casein in both skim milk and WPE skim milk was clearly higher in β -mercaptoethanol-containing samples (Lanes 3 to 5) than in samples without β -mercaptoethanol (Lane 2 in Figure 9.5). In particular, the band intensity for κ -casein increased with the increase of β -mercaptoethanol concentration in the WPE skim milk (Figure 9.5B). As for the other caseins, the bands for α_s - and β -casein in skim milk did not change in intensity at different β -mercaptoethanol concentrations (Figure 9.5A) whereas in WPE skim milk these protein bands appeared to be higher in intensity as the β -mercaptoethanol concentration increased (Figure 9.5B). The SDS-PAGE patterns clearly indicated that a high level of serum α -lactalbumin was involved in disulphide bonding and a high proportion of κ -casein was not involved in disulphide bonding.

Integration of the band intensities provided the percentage of each protein participating in disulphide bonds and located in the serum phase. The percentage of colloidal proteins was calculated assuming that if a protein was not detected in the serum phase, then that protein

would be in the colloidal phase (Section 3.6.7). The average data are presented in Figures 9.6 to 9.8.

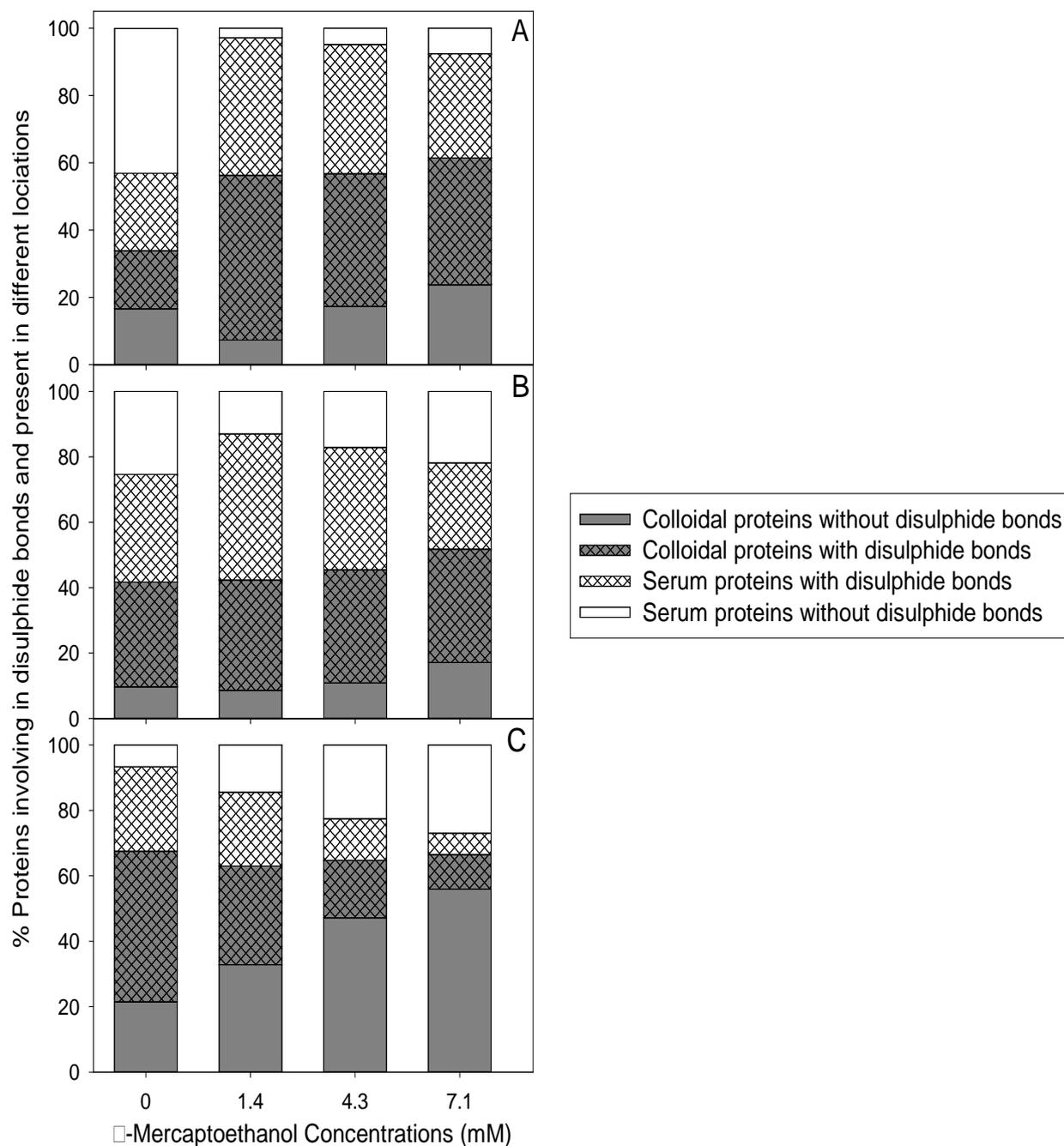


Figure 9.6: Effects of β -mercaptoethanol concentrations on the distribution of the proteins between the colloidal and serum phases and the levels of protein participating in disulphide bonds in skim milk. A: α -lactalbumin; B: β -lactoglobulin and C: κ -casein. The levels of protein located in the colloidal phase are represented by grey columns and the levels of protein located in serum phase are represented by white columns. The levels of protein participating in disulphide bonds are represented by “crossed hatched” columns in both colloidal and serum phases. The data was the average of two to four replicates.

In skim milk heated in the presence of β -mercaptoethanol, the proportion of α -lactalbumin participating in disulphide bonds increased upon addition of 1.4 mM β -mercaptoethanol (Figure 9.6A). The increase in the proportion of α -lactalbumin involved in intermolecular disulphide bonding was more pronounced in the colloidal than in the serum phase (Figure 9.6A). The total proportion of α -lactalbumin involved in disulphide bonds then decreased gradually when the β -mercaptoethanol concentration increased from 1.4 to 4.3 and then to 7.1 mM. The percentage of disulphide-linked α -lactalbumin was always greater in the skim milk with added β -mercaptoethanol ($70 \pm 7\%$ at 7.1 mM β -mercaptoethanol) than that in skim milk without added β -mercaptoethanol ($46 \pm 7\%$, Figure 9.6A).

The proportion of β -lactoglobulin involved in intermolecular disulphide bonding followed the same trend as α -lactalbumin. The level of disulphide-linked β -lactoglobulin increased at low concentrations of added β -mercaptoethanol, then decreased as the β -mercaptoethanol concentration increased (Figure 9.6B). However, the increase in the proportion of disulphide-linked β -lactoglobulin when 1.4 mM β -mercaptoethanol was added was not as pronounced as the increase of disulphide-linked α -lactalbumin. At 7.1 mM β -mercaptoethanol, the percentage of disulphide-linked β -lactoglobulin was $61 \pm 6\%$, which was not significantly different from that percentage in control heated skim milk ($65 \pm 5\%$, $p > 0.05$). In addition, the increase then decrease in the proportion of disulphide-linked β -lactoglobulin was mostly due to the changes in the disulphide-linked β -lactoglobulin in the serum phase as the proportion of disulphide-linked β -lactoglobulin in the colloidal phase did not change significantly for all the β -mercaptoethanol concentrations (Figure 9.6B).

Unlike the major whey proteins, the percentage of disulphide-linked κ -casein decreased gradually and significantly from $72 \pm 4\%$ to $17 \pm 1\%$ ($p < 0.05$) as the β -mercaptoethanol concentration increased from 0 to 7.1 mM (Figure 9.6C). The decrease in the percentage of disulphide-linked κ -casein was observed in both the colloidal and serum phases.

Regarding the distribution of the proteins, upon addition of 1.4 mM β -mercaptoethanol, the percentage of α -lactalbumin that was in the serum decreased significantly from $58 \pm 5\%$ in control heated skim milk to $49 \pm 3\%$ ($p < 0.05$, Figure 9.6A). This percentage did not change markedly when the β -mercaptoethanol concentration increased to 4.3 mM but decreased further to $43 \pm 3\%$ when β -mercaptoethanol was increased to 7.1 mM. The percentage of serum β -lactoglobulin decreased gradually from $58 \pm 3\%$ to $48 \pm 5\%$ as the β -mercaptoethanol concentration increased from 0 to 7.1 mM (Figure 9.6B). In contrast, the addition of β -mercaptoethanol did not significantly change the distribution of κ -casein and other caseins

between the colloidal and serum phases ($p > 0.05$; Figure 9.6C and Figure 9.7). The average percentage of serum κ -casein was $37 \pm 3\%$ and the other caseins were $4 \pm 1\%$.

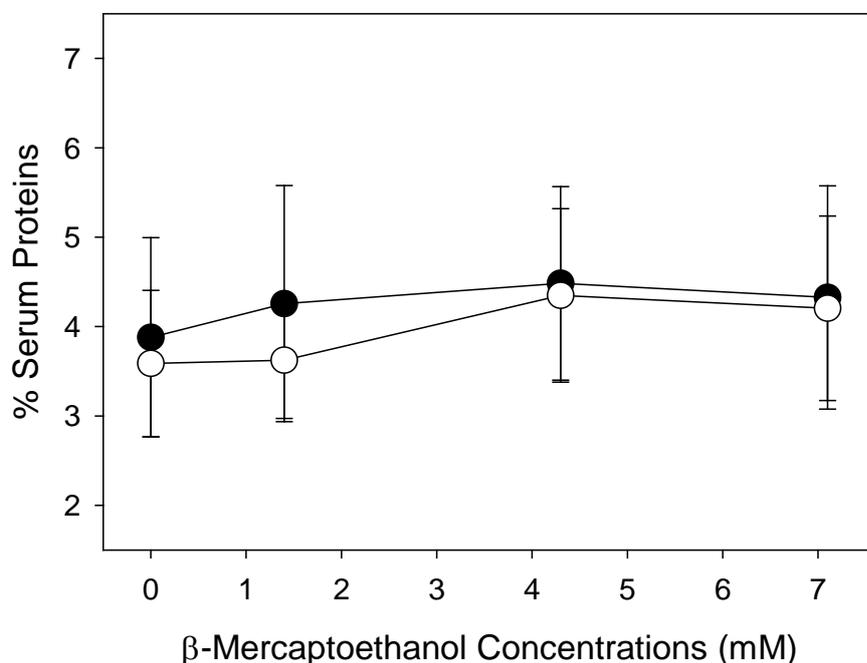


Figure 9.7: The effect of heating milks in the presence of β -mercaptoethanol on the percentage of other casein proteins in the serum. Skim milk (A) and WPE skim milk (B). Other caseins include α_{s1} , α_{s2} - and β -casein. Each data point is the average of two to four replicates. Error bars represent standard deviation.

In heated WPE skim milk, the percentage of α -lactalbumin participating in disulphide bonding increased significantly from 87 ± 2 to $96 \pm 3\%$ upon addition of 1.4 mM β -mercaptoethanol ($p < 0.05$, Figure 9.8A). This percentage then decreased gradually with further additions of β -mercaptoethanol and reached $91 \pm 3\%$ at 7.1 mM β -mercaptoethanol, which was not significantly different from $87 \pm 2\%$ in control heated WPE skim milk ($p > 0.05$). The percentage of disulphide-linked β -lactoglobulin was $88 \pm 5\%$ in the control WPE skim milk and this level did not change significantly at 1.4 mM β -mercaptoethanol. It then decreased to $75 \pm 4\%$ at 7.1 mM, which was significantly lower than the $88 \pm 5\%$ observed in the WPE skim milk with 0 mM β -mercaptoethanol ($p < 0.05$, Figure 9.8B). The percentage of disulphide-linked κ -casein decreased significantly from $77 \pm 5\%$ to $27 \pm 4\%$ when β -mercaptoethanol concentration was raised from 0 to 7.1 mM ($p < 0.05$, Figure 9.8C). Even though the decrease was significant ($p < 0.05$), it was much less than that in heated skim milk where the level decreased from 71% to 17% (Figure 9.6C).

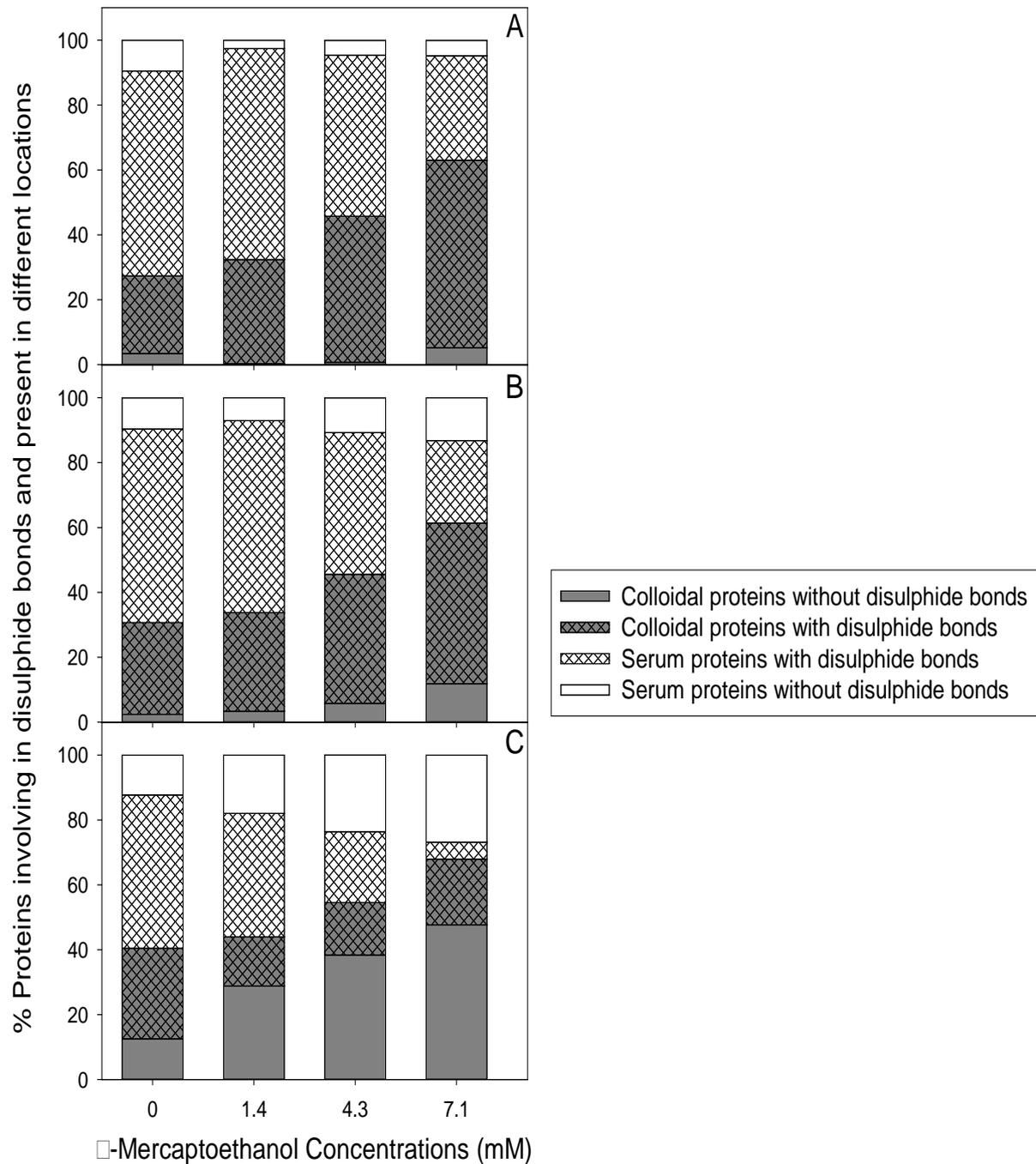


Figure 9.8: Effects of β -mercaptoethanol concentrations on the distribution of the proteins between the colloidal and serum phases and the levels of protein participating in disulphide bonds in WPE skim milk. A: α -lactalbumin; B: β -lactoglobulin and C: κ -casein. The levels of protein located in the colloidal phase are represented by grey columns and the levels of protein located in serum phase are represented by white columns. The levels of protein participating in disulphide bonds are represented by “crossed hatched” columns in both colloidal and serum phases. The data was the average of two to four replicates.

In heated WPE skim milk, the proportion of α -lactalbumin and β -lactoglobulin that remained in the serum phase decreased with the increase of β -mercaptoethanol concentrations (Figure 9.8A and B), as was also observed in skim milk (Figure 9.6A and B). The percentage of serum α -lactalbumin decreased from $71 \pm 3\%$ to $36 \pm 6\%$ ($p < 0.05$, Figure 9.8A) while the percentage of serum β -lactoglobulin decreased from $69 \pm 4\%$ to $39 \pm 4\%$ ($p < 0.05$, Figure 9.8B). The decrease in the percentage of serum β -lactoglobulin in heated WPE skim milk was more pronounced than that in heated skim milk (Figure 9.6B). The percentage of κ -casein found in the serum decreased with the increase of β -mercaptoethanol (Figure 9.8C). At 0 mM β -mercaptoethanol in WPE skim milk, the percentage of serum κ -casein was $40 \pm 6\%$. At 7.1 mM β -mercaptoethanol in WPE skim milk, the percentage of serum κ -casein decreased to $31 \pm 2\%$ (Figure 9.8C), not significantly different from $35 \pm 3\%$ in heated skim milk (Figure 9.6C). In contrast, the distribution of other caseins between the colloidal and serum phases was not affected by the presence of β -mercaptoethanol and remained at $4 \pm 1\%$ (Figure 9.7).

9.3.3 Effects of heating β -mercaptoethanol-treated milks on the size of the casein micelles

In both milks, the size of the casein micelles increased progressively as the concentration of β -mercaptoethanol increased from 1.4 to 7.1 mM. However, the increase in the casein micelle size in heated WPE skim milk was about five times greater compared to the increase in heated skim milk (Figure 9.9A).

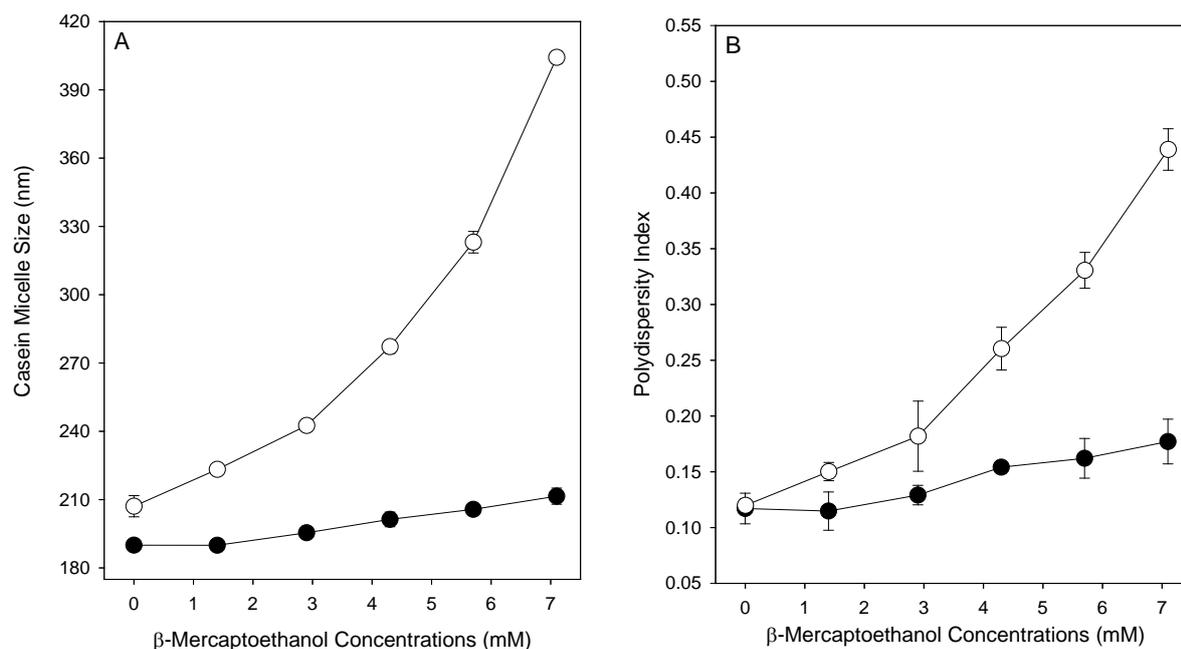


Figure 9.9: Effects of β -mercaptoethanol concentrations on the size (A) and the polydispersity index (B) of the casein micelles in heated skim milk (●) and heated WPE skim milk (○). Each data point is an average of two to four replicates. Error bars represent the standard deviation.

Without β -mercaptoethanol, the casein micelle diameter in heated skim milk was 190 ± 0.5 nm while in heated WPE skim milk it was found to be 207 ± 5 nm. As the β -mercaptoethanol concentration increased up to 7.1 mM, the size of the casein micelles increased up to 212 ± 4 nm and 406 ± 1 nm in skim milk and WPE skim milk, respectively. While the casein micelle size increased by 22 nm in heated skim milk, the micelle size increased by 198 nm in heated WPE skim milk, which was almost a two fold increase compared to its original size.

The polydispersity index in both milks also increased with the increase in size of the casein micelles. The polydispersity index in heated skim milk changed from 0.12 ± 0.01 to 0.18 ± 0.03 ($p < 0.05$) whereas in heated WPE skim milk it changed from 0.12 ± 0.01 to 0.44 ± 0.02 ($p < 0.05$; Figure 9.9).

9.3.4 Effects of heating β -mercaptoethanol-treated milks on the rheological properties of acid gels

The development of G' during the acidification of skim milk with 4.3 and 7.1 mM added β -mercaptoethanol followed the same trend as that of skim milk heated without β -mercaptoethanol (Figure 9.10A). However, the gelation point (gelation pH, which was considered as the first G' value ≥ 1 Pa) occurred earlier and the G' increased to higher values in heated skim milks with added β -mercaptoethanol than in the control heated skim milk (Figure 9.10A).

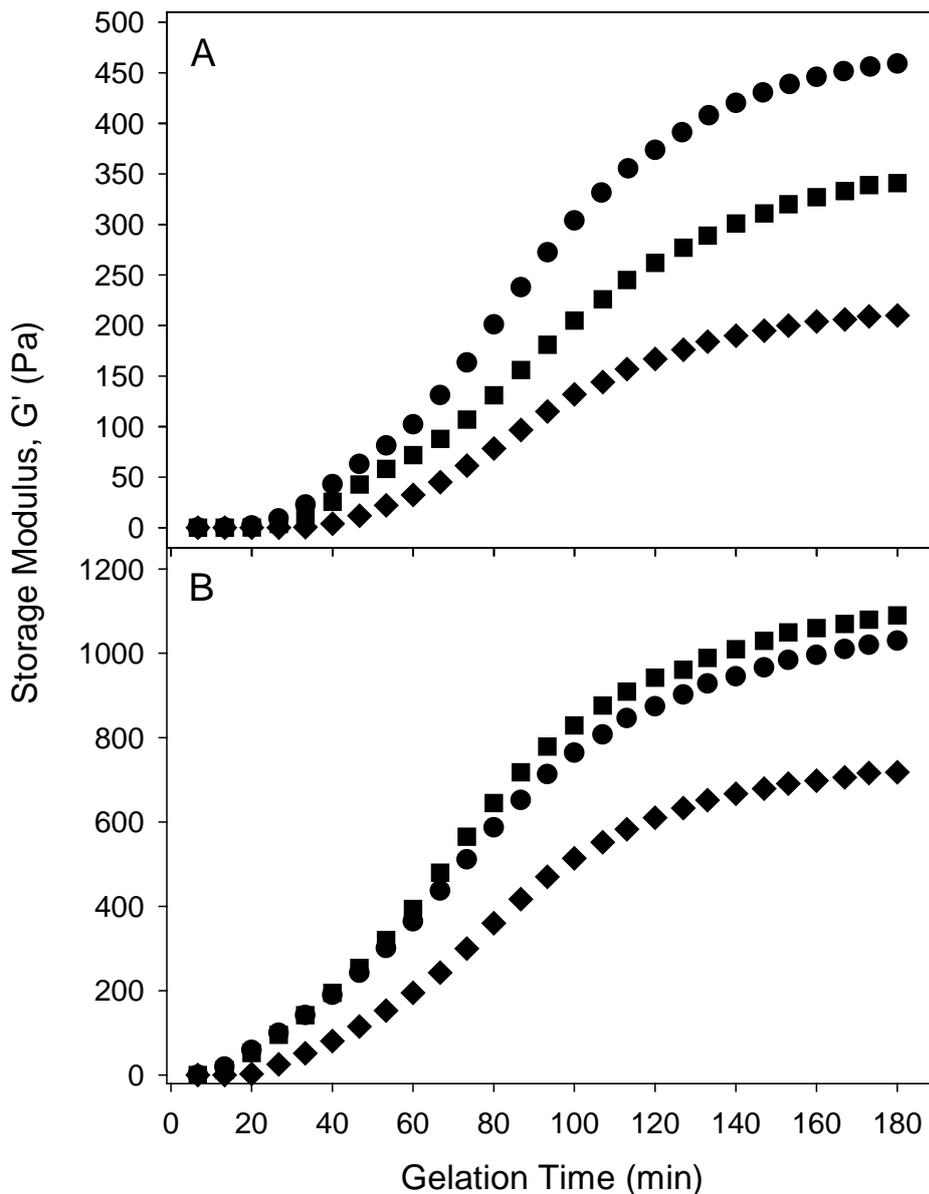


Figure 9.10: Effects of β -mercaptoethanol concentrations on the typical change of G' during acidification of skim milk (A) and WPE skim milk (B). \blacklozenge , 0 mM; \blacksquare , 4.3 mM; \bullet , 7.1 mM β -mercaptoethanol.

When the heated WPE skim milk (control or with added β -mercaptoethanol) were acidified, the increase of G' followed the same trend as that of heated skim milk. Similarly to heated skim milk, the addition of β -mercaptoethanol resulted in earlier gelation and higher final G' values than that of the control (Figure 9.10).

The effects of heating β -mercaptoethanol-treated milks on the gelation pH and the final G' values of milks are summarised in Figure 9.11. For both skim milk and WPE skim milk, the gelation pH and the final G' values were higher in acid gels prepared from β -mercaptoethanol-treated-heated milks than in acid gels prepared from control heated milks.

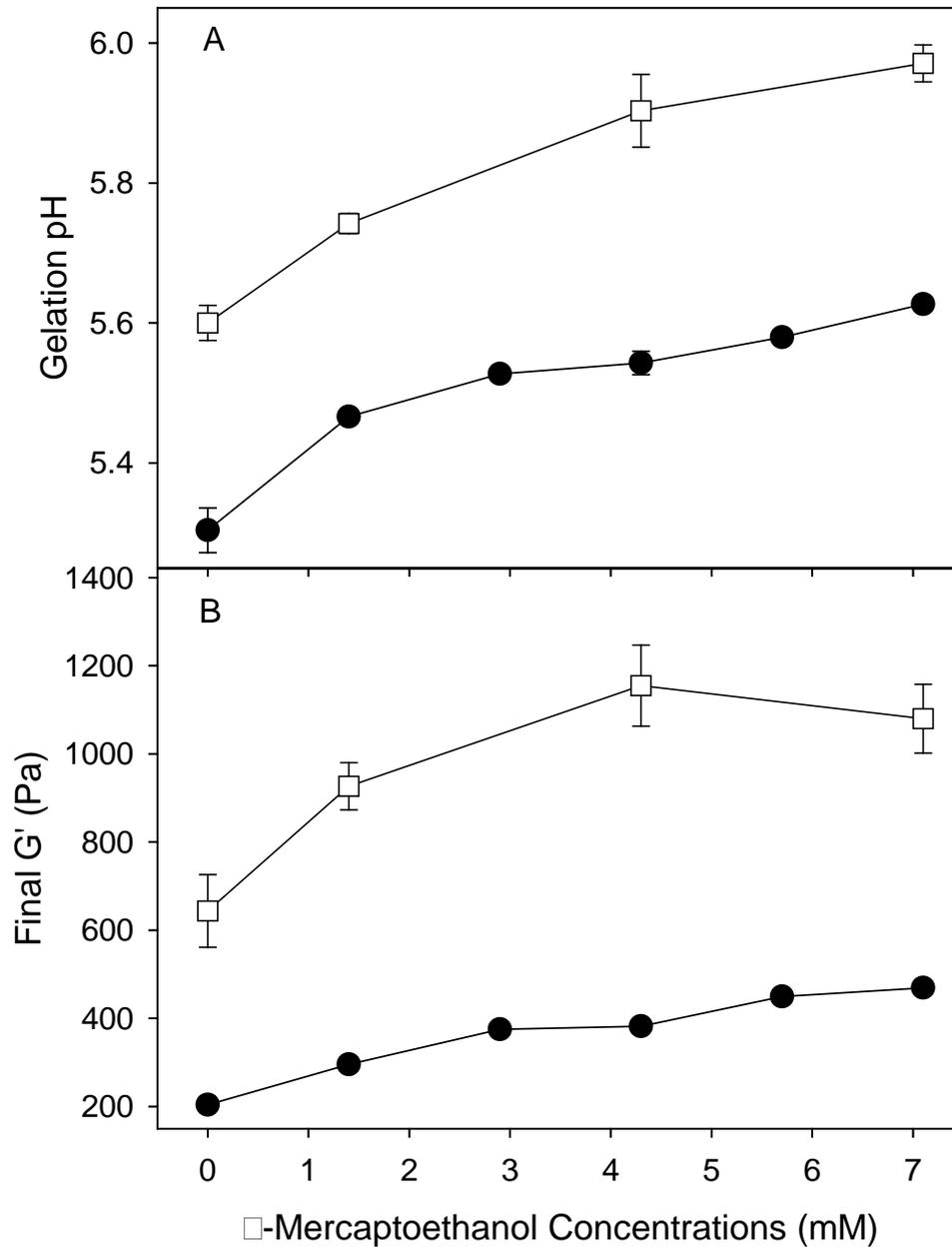


Figure 9.11: Effects of β -mercaptoethanol concentrations on the gelation pH (A) and the final G' values (B) of acid gels made from skim milk (●) and WPE skim milk (□). Milks were treated with β -mercaptoethanol for 3 h before heat treatment. Each data point is the average of two to six replicates. Error bars represent the standard deviation.

The gelation point of acidified heated skim milk occurred at $\text{pH } 5.30 \pm 0.03$ at 0 mM β -mercaptoethanol and increased significantly to $\text{pH } 5.63 \pm 0.01$ at 7.1 mM β -mercaptoethanol (Figure 9.11A). The final G' values reached 469 ± 10 Pa in heated skim milk with 7.1 mM added β -mercaptoethanol, compared with 204 ± 5 Pa in heated control skim milk (an increase of > 2 fold, Figure 9.11B).

As for WPE skim milk, the pH values at the gelation point increased from $\text{pH } 5.60 \pm 0.03$ to $\text{pH } 5.97 \pm 0.03$ with the increase of β -mercaptoethanol concentration ($p < 0.05$, Figure 9.11A). The final G' values increased significantly from 644 ± 82 Pa to 1155 ± 92 Pa as the β -mercaptoethanol concentrations was raised from 0 to 4.3 mM (increase of 1.8 fold, $p < 0.05$). However, unlike the heated skim milk, when the added β -mercaptoethanol concentration was increased further up to 7.1 mM, the final G' did not increase any further but appeared to decrease (1080 ± 78 Pa; Figure 9.11B).

The change of $\tan \delta$ during acidification of milks was monitored and the results are shown in Figure 9.12.

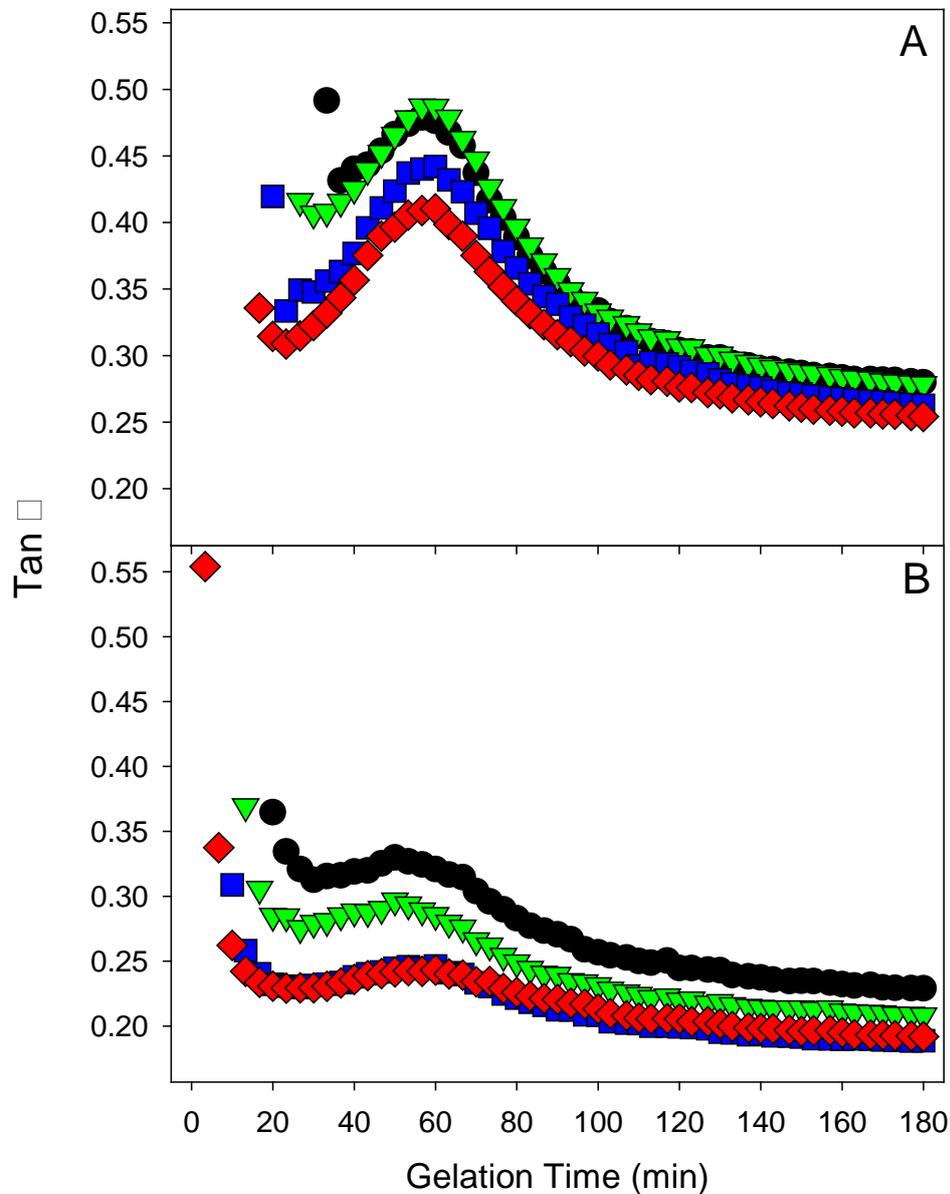


Figure 9.12: Effects of β -mercaptoethanol concentrations on the change of $\tan \delta$ during acidification of skim milk (A) and WPE skim milk (B). Milks were treated with β -mercaptoethanol for 3 h and heated at 80 °C for 30 min. The β -mercaptoethanol concentrations were 0 mM (●), 1.4 mM (▼), 4.3 mM (■) and 7.1 mM (◆). Each data point is the average of two to six replicates. Error bars are not present for simplification.

$\tan \delta$ values, taken as the ratio of G'' over G' , of acid gels prepared from control skim milk and skim milk with 1.4 mM β -mercaptoethanol showed similar curves over the gelation time. However as the β -mercaptoethanol concentration increased to 4.3 and 7.1 mM, the $\tan \delta$ values

became lower than those of the control gel (Figure 9.12A). This indicated that the gels prepared from β -mercaptoethanol-treated milks were more elastic than the control gels.

In WPE skim milk, the $\tan \delta$ values of acid gels prepared from milks containing β -mercaptoethanol were lower than those of control acid gels (Figure 9.12B). The acid gels prepared from milk heated with 4.3 and 7.1 mM β -mercaptoethanol had the same $\tan \delta$ profile during gelation whereas the acid gels prepared from milk heated with 1.4 mM β -mercaptoethanol had a profile that was intermediate between the control gel and gels with higher β -mercaptoethanol concentrations (Figure 9.12B). Similarly to skim milk gels, this result indicated that the acid gels became more elastic with the increase of β -mercaptoethanol concentration added to WPE skim milk before heat treatment.

The temperature of the acid gels was dropped from 30 to 5 °C at a rate of 1 °C min⁻¹. The G' values of the acid gels at 5 °C were higher than those at 30 °C, at any β -mercaptoethanol concentration. In skim milk, the G' values at 5 °C increased progressively and significantly from 400 ± 41 Pa to 718 ± 30 Pa ($p < 0.05$) as β -mercaptoethanol concentration increased from zero to 7.1 mM (Figure 9.13A). In WPE skim milk, the G' values at 5 °C increased significantly from 928 ± 28 Pa to 1824 ± 172 Pa ($p < 0.05$) as β -mercaptoethanol concentration increased from zero to 4.3 mM. The G' values then appeared to decrease to 1713 ± 254 Pa ($p > 0.05$) as β -mercaptoethanol concentration increased to 7.1 mM (Figure 9.13A).

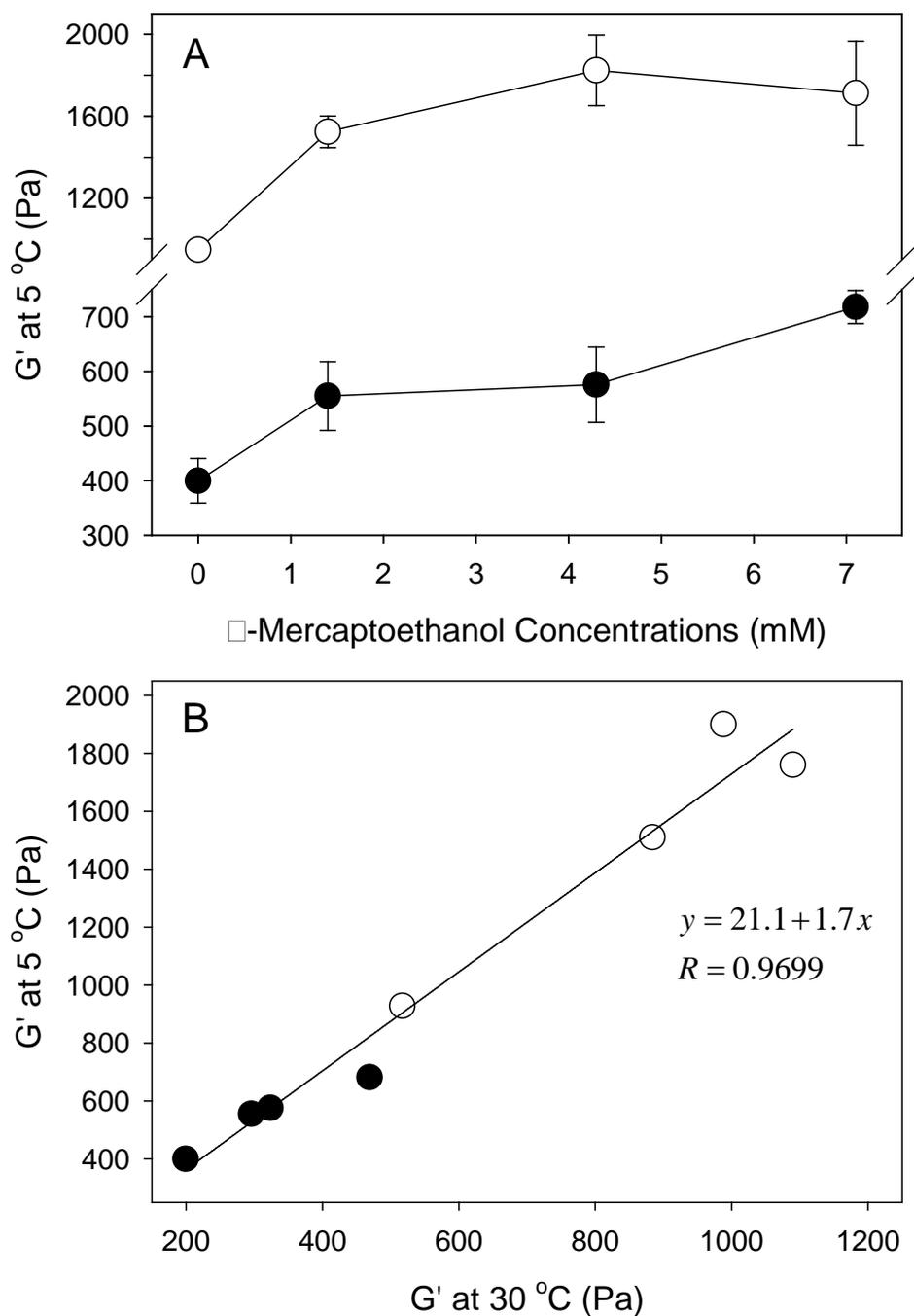


Figure 9.13: A, Effects of β -mercaptoethanol concentrations on the G' values of acid gels at 5 °C. B, The relationship between the final G' values at 5 °C and the final G' values at 30 °C. ●, skim milk; ○, WPE skim milk. Each data point is the average of two to four replicates.

In Figure 9.13B, the G' values at 5 °C were plotted as a function of the G' values at 30 °C. As observed in previous chapters, the relationship between the G' at 30 °C and those at 5 °C was linear. The gradient of the slope was 1.7. This linear relationship indicated that G' of the acid

gels increased by 1.7 times when the temperature was decreased from 30 to 5 °C, regardless of the milk under study or the concentration of added β -mercaptoethanol (Figure 9.13B).

The yield stress properties of the acid gels at 5 °C were examined by monitoring the change of shear stress and strain of the gels when subjected to a constant shear rate (0.005 s^{-1}). In skim milk, the yield strain (strain values at the yield point) of the acid gels prior to being broken decreased from $42 \pm 3 \%$ to $32 \pm 5 \%$ as the β -mercaptoethanol concentration increased from zero to 7.1 mM (Table 9.2 and Figure 9.14A). The yield stress (maximum shear stress values), on the other hand, increased from 122 ± 1 to 144 ± 4 Pa as β -mercaptoethanol concentration increased from 0 to 1.4 mM. These values then decreased to 129 ± 5 Pa at 4.3 mM β -mercaptoethanol and did not change significantly at higher β -mercaptoethanol concentrations ($p > 0.05$, Figure 9.14A). The results indicated that the acid gels prepared from skim milk heated with 1.4 mM β -mercaptoethanol were harder to break than the control skim milk gels, and those prepared from skim milk heated with higher β -mercaptoethanol levels had similar yield properties to the control ones.

In WPE skim milk, the effect of β -mercaptoethanol was more dramatic than what was observed in skim milk. The yield stress decreased significantly from 725 ± 11 Pa to 140 ± 3 Pa as β -mercaptoethanol concentration increased from 0 to 7.1 mM. The yield strain also decreased markedly with the increase of β -mercaptoethanol. This indicated that the acid gels became easier to break with the higher the β -mercaptoethanol concentrations in WPE skim milk before heating,

Table 9.2: Yield stress (Pa) and stain (%) of acid gels when subjected to constant shear rate. Acid gels were prepared from skim milk and WPE skim milk that had been heated in the presence of β -mercaptoethanol. The values are the mean value of two to four replicates \pm standard deviation.

β -mercaptoethanol Concentrations (mM)	Heated skim milk		Heated WPE skim milk	
	Yield Strain (%)	Yield Stress (Pa)	Yield Strain (%)	Yield Stress (Pa)
0	42 ± 3	122 ± 1	99 ± 12	725 ± 11
1.4	37 ± 4	144 ± 4	71 ± 7	643 ± 7
4.3	37 ± 4	129 ± 5	25 ± 3	272 ± 3
7.1	32 ± 5	133 ± 4	14 ± 2	140 ± 3

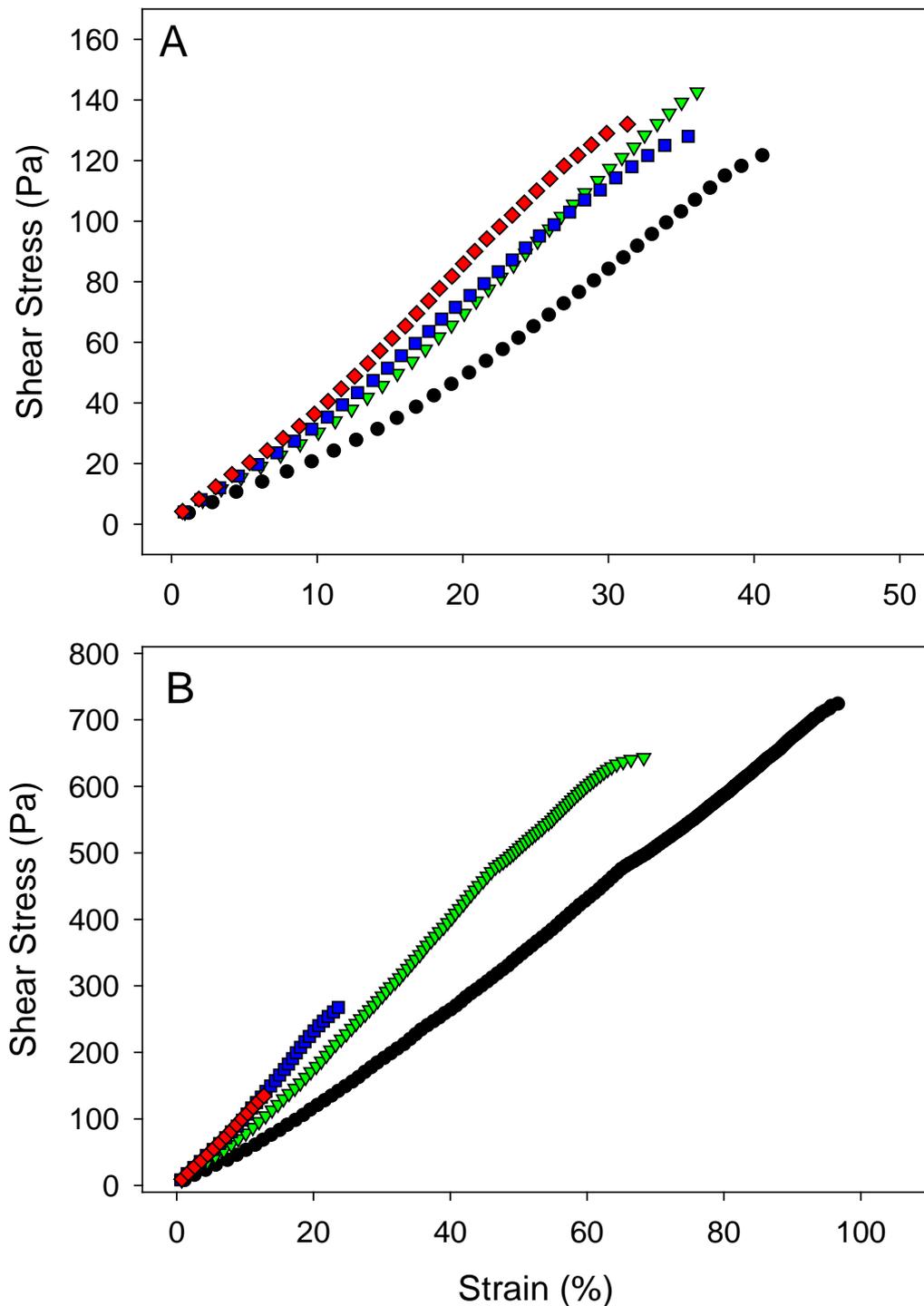


Figure 9.14: Shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^\circ\text{C}$ and analysed at $5 \text{ }^\circ\text{C}$. The acid gels were prepared from skim milk (A) and WPE skim milk (B) that were heated in the presence of different concentrations of β -mercaptoethanol. ●, 0 mM; ▼, 1.4 mM; ■, 4.3 mM; ◆, 7.1 mM β -mercaptoethanol. Each point is the average of four to six replicates. Error bars were not shown to simplify the graph.

9.3.5 Effects of heating β -mercaptoethanol-treated milks on the microstructure of acid gels

For acid gels prepared from skim milk, it appeared that as the concentration of β -mercaptoethanol increased, the acid gel network became slightly denser with less pores (black areas). In addition, the number of strands per unit area in the acid gel networks increased and the network appeared slightly more inter-connected when the β -mercaptoethanol concentrations increased (Figure 9.15).

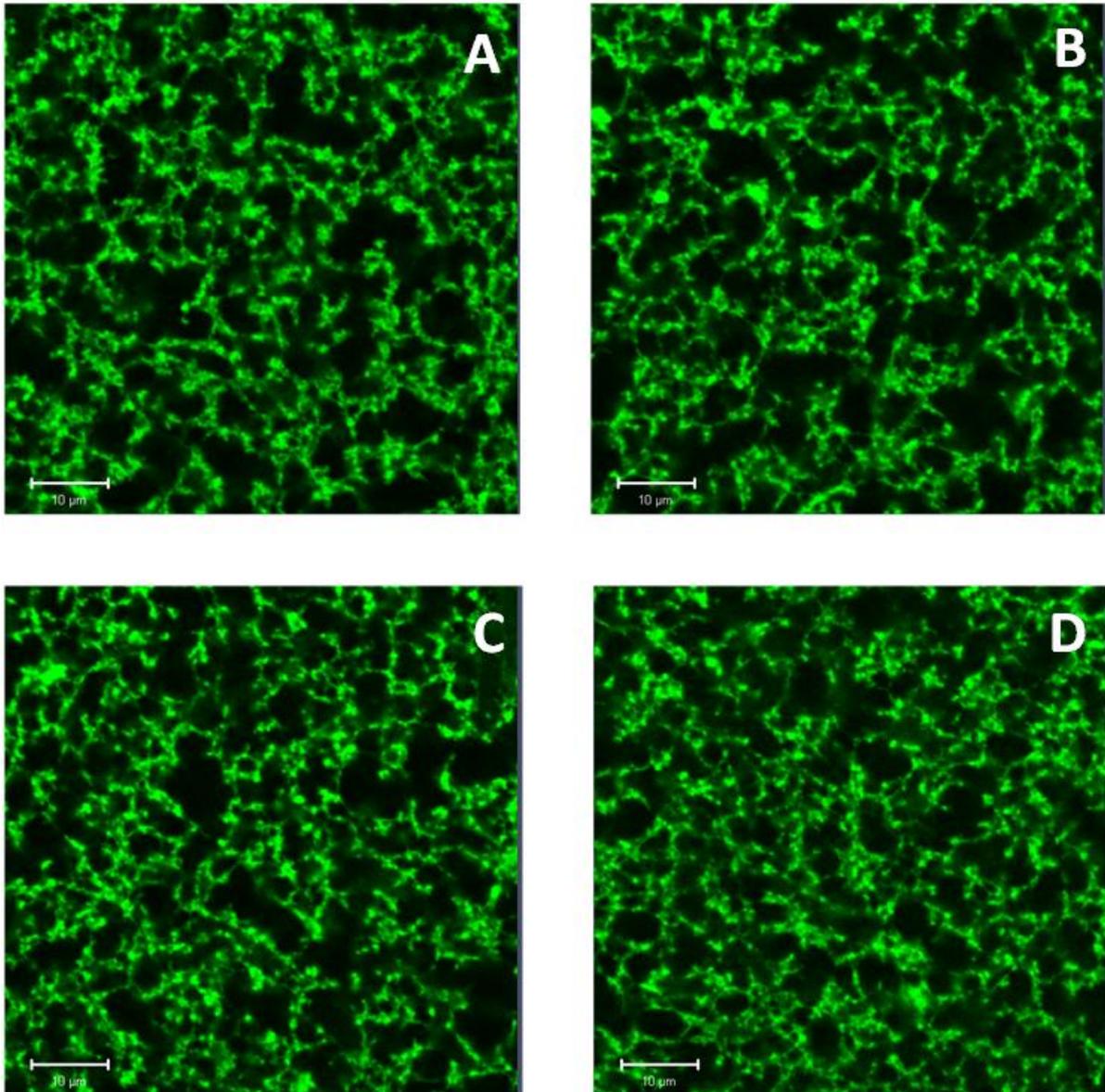


Figure 9.15: Effects of β -mercaptoethanol concentrations on the confocal microstructure of acid gels made from skim milk. Milks were heated in the presence of 0 (A), 1.4 mM (B), 4.3 mM (C) and 7.1 mM (D) β -mercaptoethanol. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μm .

The confocal images of the microstructures of the acid gels made from selected WPE skim milk samples showed that the gel network became very dense as the β -mercaptoethanol concentration increased (Figure 9.16). In the acid gels made from milks treated with 0 and 1.7 mM β -mercaptoethanol, the strands can still be observed (Figure 9.16A and B) whereas in the acid gels made from milks treated with 4.3 and 7.1 mM β -mercaptoethanol, the gel network was so dense that the strands could not be distinguished as individual strands (Figure 9.16C and D).

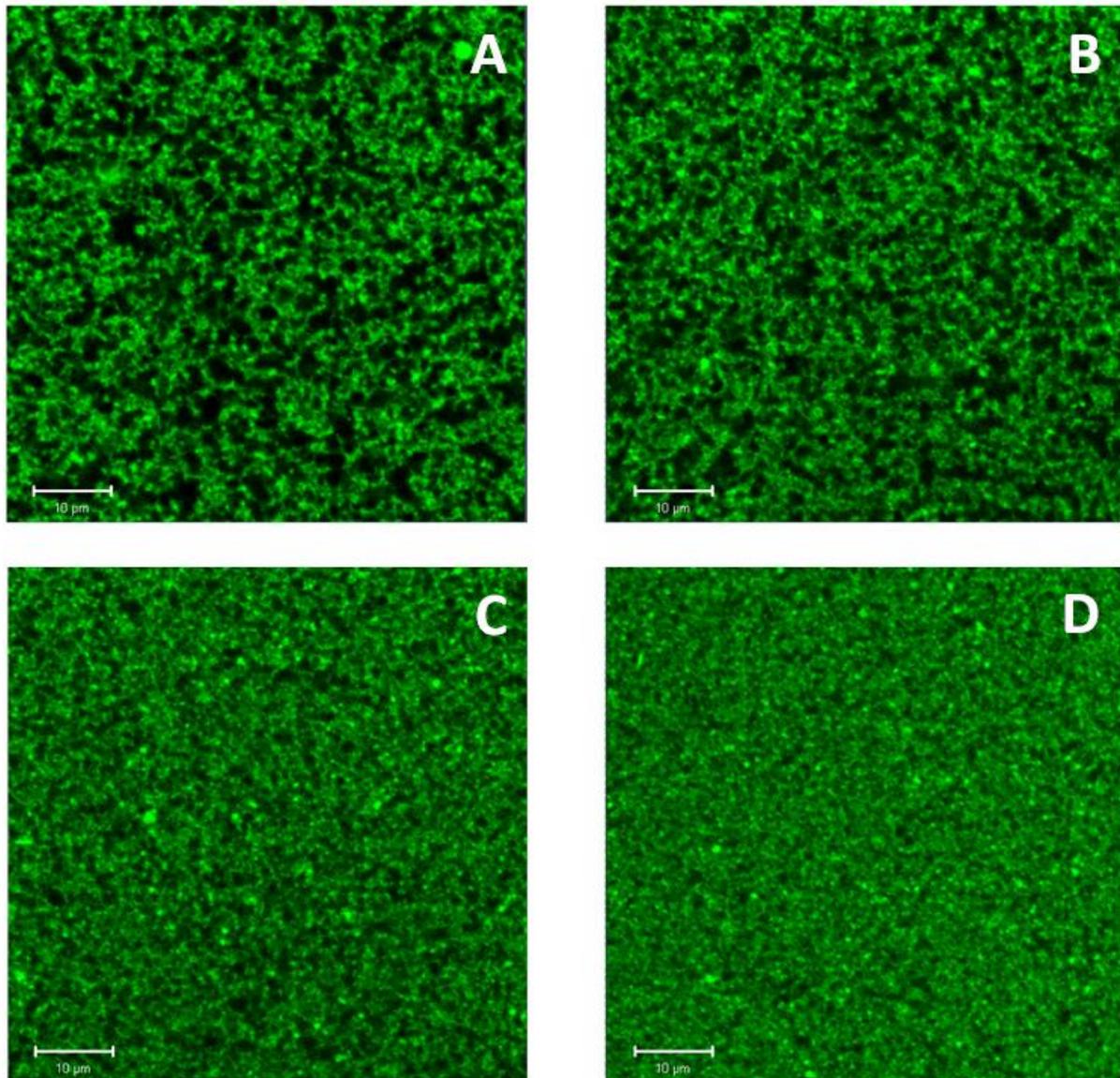


Figure 9.16: Effects of β -mercaptoethanol concentrations on the microstructure of acid gels made from WPE skim milk. Milks were heated in the presence of 0 (A), 1.4 mM (B), 4.3 mM (C) and 7.1 mM (D) β -mercaptoethanol. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μ m.

The connections between the proteins in the gels from the milks with higher concentrations of β -mercaptoethanol appeared to be very high, resulting in much less open spaces/pores (black

areas) when compared with the lower concentrations of β -ME. When comparing the acid gels made from heated skim milk (Figure 9.11B and Figure 9.15) to those made from heated WPE skim milk (Figure 9.11B and Figure 9.16), the milks with added β -mercaptoethanol had markedly higher final G' values and the gel networks were much denser with higher numbers of protein strands and higher number of connections between the strands. Interestingly, it was clear in this study that the acid gels with higher G' values had a much denser network than those with lower G' . This was also observed in the results presented in Chapter 5 in which the acid gels prepared from NEM-treated-heated WPE skim milk had higher G' values and denser network than those made from control heated WPE skim milk (Section 5.3.5).

9.4 Discussion

9.4.1 Effects of heating β -mercaptoethanol-treated milks on the protein interactions

In Chapter 7, it was found that after adding β -mercaptoethanol to unheated skim milk and holding for 3 h, up to $70 \pm 4\%$ of the naturally occurring polymeric κ -casein was converted into monomeric κ -casein. It was also found that α_{s2} -casein dimers were reduced to monomers, potentially containing free thiol groups (Figure 7.2). As for whey proteins, at least one of the two disulphide bonds of β -lactoglobulin was reduced; however, at a very low level (Figure 7.3 in Chapter 7). Even though the reduction of α -lactalbumin cannot be monitored using MF-electrophoresis or traditional SDS-PAGE (Chapter 7), it was considered conceivable that at least one of disulphide bonds of α -lactalbumin (Cys⁶ – Cys¹²⁰) was reduced in milks that had been heated with β -mercaptoethanol. This was because the Cys⁶ – Cys¹²⁰ bond of α -lactalbumin was found to react almost instantly with the disulphide reducing agent, dithiothreitol, while the other three disulphide bonds of α -lactalbumin were about 140 times slower to react (Kuwajima et al., 1990). Hence in milks with added β -mercaptoethanol, the quantity of thiol groups was higher than that in control milks and these thiols groups could potentially be found in κ -casein, α_{s2} -casein, β -lactoglobulin and α -lactalbumin. As a result, the thiol-disulphide exchange reactions could be initiated by any of these proteins and many reactions could occur simultaneously.

The following section discusses the results presented in this chapter and describes two possible scenarios that may be occurring in milks that were heated after being reduced with β -mercaptoethanol. In one scenario, κ -casein may be the initiator of the thiol-disulphide exchange reactions and the second scenario may be that α -lactalbumin and β -lactoglobulin were the initiators.

9.4.1.1 Scenario 1 – κ -casein as the initiator of the thiol-disulphide exchange reactions

In skim milk and WPE skim milk heated in the presence β -mercaptoethanol, monomeric κ -casein may become the initiator of the thiol-disulphide exchange reactions as it was observed that a markedly higher level of α -lactalbumin and β -lactoglobulin was observed to be located in the colloidal phase (along with κ -casein) compared with that found in the heated milks without added β -mercaptoethanol (Figure 9.6 and Figure 9.8). If κ -casein was the initiator, this would lead to reactions between κ -casein and α -lactalbumin directly, which can normally occur only in the presence of β -lactoglobulin (Calvo et al., 1993). This scenario may also explain the increase in the proportion of intermolecular disulphide-linked α -lactalbumin, especially at low concentrations of β -mercaptoethanol (Figure 9.6 and Figure 9.8).

However, κ -casein acting as the exchange reaction initiator did not explain the fact that the proportion of intermolecular disulphide-linked κ -casein was lower after heating than before heating. In skim milk with 7.1 mM added β -mercaptoethanol, the percentage of disulphide-linked κ -casein was 30% before heating (Figure 7.3) and 17% after heating (Figure 9.6). In WPE skim milk with 7.1 mM added β -mercaptoethanol, the percentage of disulphide-linked κ -casein was 57% before heating (Figure 7.7) and 27% after heating (Figure 9.8). If κ -casein was the reaction initiator, the total percentage of disulphide-linked κ -casein would be expected to increase after heating. This was only observed for the colloidal κ -casein in the WPE skim milk, as the β -mercaptoethanol concentration increased the percentage of disulphide-linked κ -casein in colloidal phase increased from $18 \pm 3\%$ to $24 \pm 7\%$ (Figure 9.8C). However, overall, the total percentage of disulphide-linked κ -casein decreased in both skim milk and WPE skim milk on the addition of β -mercaptoethanol. The reason for this could possibly be that only a very low number of κ -casein molecules could initiate the intermolecular disulphide bonding so that an increase in the percentage of disulphide-linked κ -casein was not observed.

The formation of colloidal whey protein/ κ -casein aggregates in milks heated with β -mercaptoethanol can be described as follows. Firstly, the κ -casein polymers were reduced to monomeric κ -casein containing free thiol groups (Process 1 in Figure 9.17).

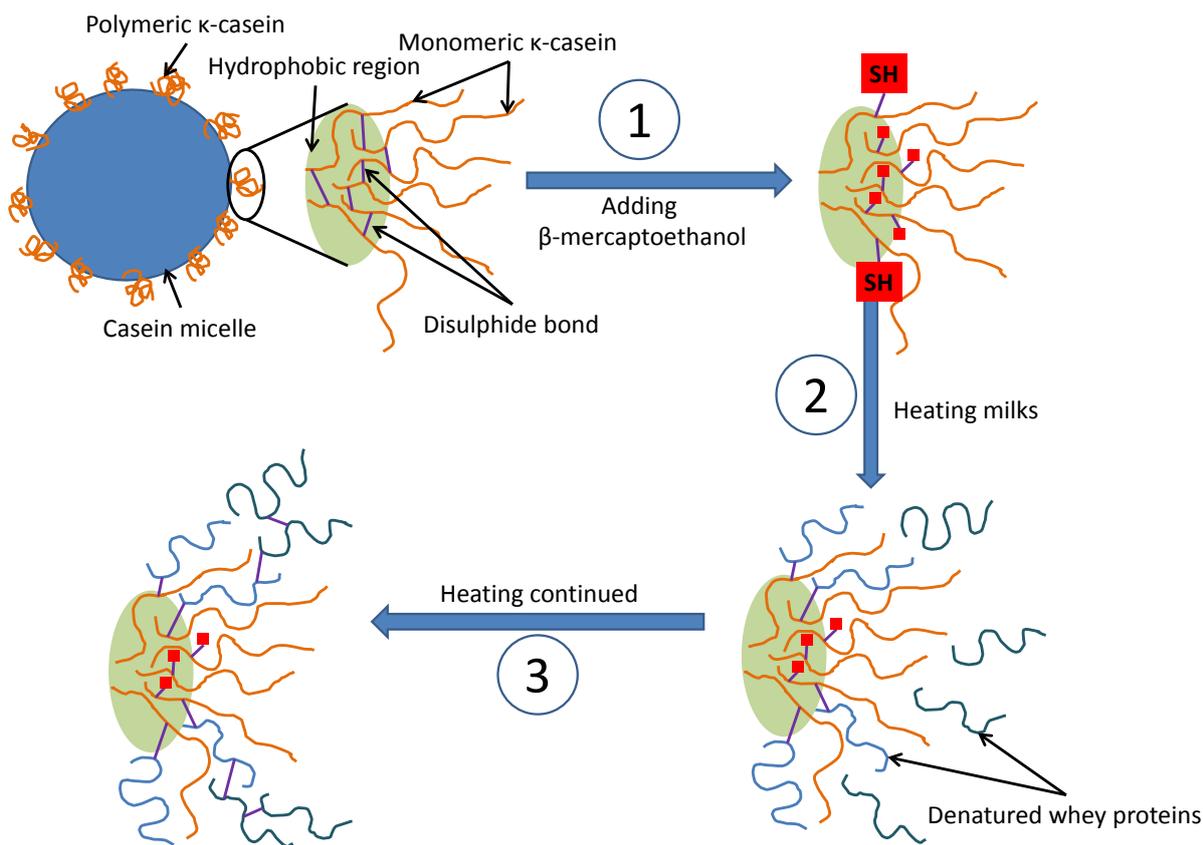


Figure 9.17: A scheme representing the possible mechanism of interactions between the proteins with κ -casein being the initiator of the thiol-disulphide exchange reactions in milk that was heated in the presence of β -mercaptoethanol.

Upon heating, a proportion of the monomeric κ -casein with free thiol groups initiated thiol-disulphide exchange reactions with denatured whey proteins, especially with α -lactalbumin (Process 2 in Figure 9.17). The remainder of the monomeric κ -casein may not be able to approach other proteins and unable to participate in disulphide bonding because they may have been shielded from the approaching whey protein by denatured whey proteins that were already attached to the casein micelles. Nevertheless the initial interactions formed an anchor for whey proteins on the surface of the casein micelles. These proteins with free thiol groups can continue to propagate disulphide interactions with other proteins that contain disulphide bonds in the serum or on other casein micelles (Process 3 in Figure 9.17).

9.4.1.2 Scenario 2 – α -lactalbumin and β -lactoglobulin as initiators of the thiol-disulphide exchange reactions

In the reduced milks, beside monomeric κ -casein, disulphide bonds of α -lactalbumin could have been broken and α -lactalbumin may consist of free thiol groups. These α -lactalbumin proteins may also initiate thiol-disulphide exchange reactions, along with β -lactoglobulin that was the natural initiator. In fact, if α -lactalbumin was the reaction initiator, the direct interactions

between κ -casein and α -lactalbumin would occur as well, resulting in a higher percentage of colloidal α -lactalbumin in milks treated with β -mercaptoethanol than in milks without β -mercaptoethanol, as was observed (Figure 9.6A and Figure 9.8A).

This second scenario explaining the interactions between the proteins could be attributed to (partially) reduced α -lactalbumin and β -lactoglobulin, which may contain at least one or two free thiol groups, respectively. The reason why the number of thiol increased by one was because when a molecule of β -mercaptoethanol interacted with a disulphide bond, β -mercaptoethanol formed a new disulphide bond with one of the sulphurs belonging to the initial disulphide bond while the other sulphur left to form a thiol group (Figure 2.15). The partially reduced α -lactalbumin and β -lactoglobulin can initiate the thiol-disulphide exchange reactions with other proteins that contain disulphide bonds. Since the number of free thiol groups in skim milk increased with the addition of β -mercaptoethanol and the rate of the thiol-disulphide exchange reactions was first order for thiol groups (Shaked et al., 1980), the rate of exchange reactions would be expected to increase. As a result, in β -mercaptoethanol-treated skim milk and WPE skim milk many aggregates could be formed simultaneously and each aggregate could consist of higher numbers of free thiol groups compared with those in control heated milks. However, the increase in the number of thiol groups was accompanied by a decrease in the number of disulphide bonds. Hence, even though a large number of aggregates were formed in β -mercaptoethanol-treated milks, they were expected to be smaller in size than the aggregates in milks without β -mercaptoethanol. This point is schematically depicted in Figure 9.18.

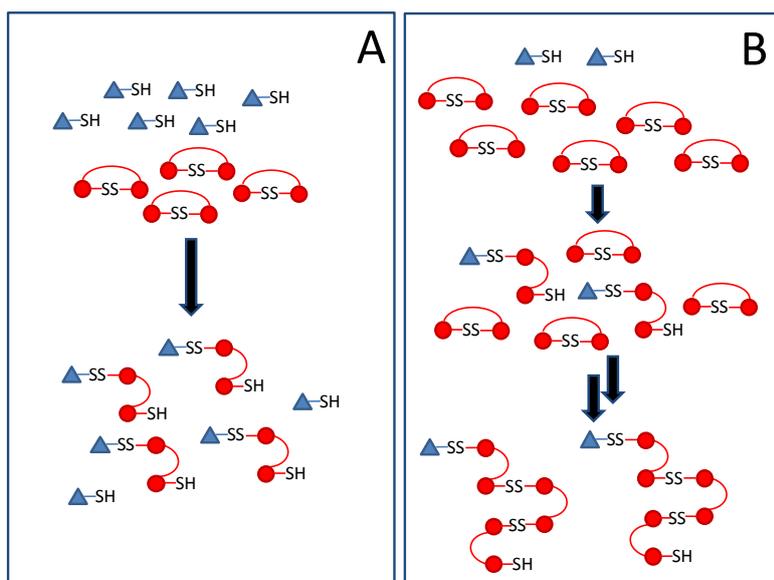


Figure 9.18: Schematic mechanisms of the formation of aggregates in different milks that contained different ratio of thiol groups to disulphide bonds. A, A greater number of thiol groups than disulphide bonds; B, A greater number of disulphide bonds than thiol groups.

9.4.1.3 Proposed mechanism of interactions between the proteins in reduced-heated milks

The two proposed scenarios described could occur simultaneously as κ -casein, α -lactalbumin and β -lactoglobulin all potentially had free thiol groups. The following section will elaborate further on the mechanism of interactions between the proteins in milks that were reduced prior to heating.

Examining the distribution of the proteins between the colloidal and serum phases and the size of the casein micelles may also provide information on the mechanism of interactions between the proteins. An increase in the proportion of whey protein associated with the casein micelles was observed when the pH was lowered towards pH \sim 6 at heating, when the whey protein concentration was increased or when milk was heated at 75 °C (Smits & van Brouwershaven, 1980; Corredig & Dalgleish, 1996; Dalgleish et al., 1997a; Oldfield et al., 1998a; Corredig & Dalgleish, 1999; Oldfield et al., 2000; Anema & Li, 2003b, a). In the studies of Anema and Li (2003b, a), the increase in the size of the casein micelle was found to be coupled with the increase in the level of whey protein being associated with the casein micelles. This relationship was also observed in this study (Figure 9.6, Figure 9.8 and Figure 9.9). At 7.1 mM β -mercaptoethanol, the total percentage of colloidal whey protein was 55 % and 63% in heated skim milk and heated WPE skim milk, respectively (Figure 9.6 and Figure 9.8). Correspondingly, the size of the casein micelles increased by \sim 22 nm (\sim 11% increase in size) and \sim 198 nm (96% increase in size) in heated skim milk and heated WPE skim milk, respectively (Figure 9.9).

Anema and Li (2003b) found an increase of 30 nm (14% increase in size) when 70% of whey protein was associated with the casein micelles upon heating skim milk at pH 6.50 (which was lower than pH 6.7 of the milk systems in this study). The increase in the casein micelle size was thought to be caused by the association of whey protein with the casein micelles or by the partial aggregation between the casein micelles. However, the polydispersity index of the casein micelles was found to remain constant despite the increase in size (Jeurnink, 1992; Anema & Li, 2003b, a). Hence, it was unlikely that the increase in the casein micelle size was due to the partial aggregation of the casein micelles (Anema & Li, 2003b, a). A different study, in which the κ -casein was hydrolysed by chymosin before heat treatment, found that the size of the casein micelles increased by up to ~ 36 nm when up to 80% of whey proteins were associated with the casein micelles (Renan, Guyomarc'h, Chatriot, Gamerre & Famelart, 2007).

In this study, even though the proportion of colloidal protein in heated skim milk and WPE skim milk was lower than previously reported, the increase of the casein micelle sizes in heated skim milk was comparable with the previous work while in WPE skim milk the casein micelles doubled in size compared to untreated milk, thus markedly higher than previously reported. The volumes of the casein micelles were estimated based on the measured diameter of the casein micelles. The results showed that at 7.1 mM, the casein micelle volume increased by 1.4 fold in skim milk and by 7.4 fold in WPE skim milk (Table A.2 in Appendix 7). In addition, the polydispersity index of the casein micelles was found to increase with the increase of the casein micelle size (Figure 9.9B). Hence, it is likely that in this study, the increase in size of the casein micelles in heated skim milk was related to both the association of the whey proteins with the casein micelles and the partial aggregation between the casein micelles, particularly in WPE skim milk.

As proposed in scenario 1 (Section 9.4.1.1), thiol-disulphide exchange reactions may be initiated at the surface of the casein micelles and then continued outwards away from the micelle surface. The whey proteins attached on to the surface of a casein micelle can interact with those in the serum and/or with those on the surface of different casein micelles. This may be the cause of the partial aggregation of the casein micelles. Alternatively, as proposed in scenario 2 (section 9.4.1.2), the aggregates in the serum can consist of free thiol groups that can continue to propagate thiol-disulphide exchange reactions with other proteins in the serum or on the surface of the casein micelles. Hence, these aggregates can act as the connection between casein micelles. As a result, the casein micelles can be connected via a number of serum aggregates, resulting in the partial aggregation of casein micelles.

A further point about the interaction mechanism was about the role of reduced κ -casein. Even though the majority of κ -casein was not participating in intermolecular disulphide bonds, the proportion of serum κ -casein in heated skim milk was not affected by the presence of β -mercaptoethanol (Figure 9.6C). Whereas, the level of serum κ -casein decreased with the increase of β -mercaptoethanol in heated WPE skim milk (Figure 9.8C) but was still higher than that found in unheated milks (Chapters 4 and 7). This indicated that κ -casein dissociated from the casein micelles when heated, regardless of whether or not κ -casein was disulphide-linked. This also agreed with previous studies (Anema & Klostermeyer, 1997; Anema, 2008).

Early studies reported a dependence of the size of whey-protein aggregates on the κ -casein concentration (Dalglish, 1990; Beaulieu, Pouliot & Pouliot, 1999). Recently, Guyomarc'h et al (2009) reported that in whey protein solutions at the same ionic strength and calcium levels as in bovine milks, the denatured whey proteins continue to aggregate to form large particles. However in the presence of κ -casein or caseinate, the aggregate size was limited. In this study, while the κ -caseins that dissociated from the casein micelles were polymeric in control heated milk, the dissociated κ -caseins were monomeric in β -mercaptoethanol-treated-heated milk. Monomeric κ -casein may be a much more efficient stabiliser than polymeric κ -casein because monomeric κ -casein can have a higher degree of freedom to stabilise the surface of the micelle or aggregates. Therefore, polymeric κ -casein can only stabilise a small surface area, thus aggregates were large in size and fewer in number (Figure 9.19A). In contrast, in reduced milk, monomeric κ -casein can stabilise a much larger surface area. As a result, the aggregates can be smaller and there may be a large number of these aggregates in the serum (Figure 9.19B). This ability of monomeric κ -casein to stabilise large surface areas can be related to the formation of numerous small aggregates, as proposed in scenario 2 (9.4.1.2).

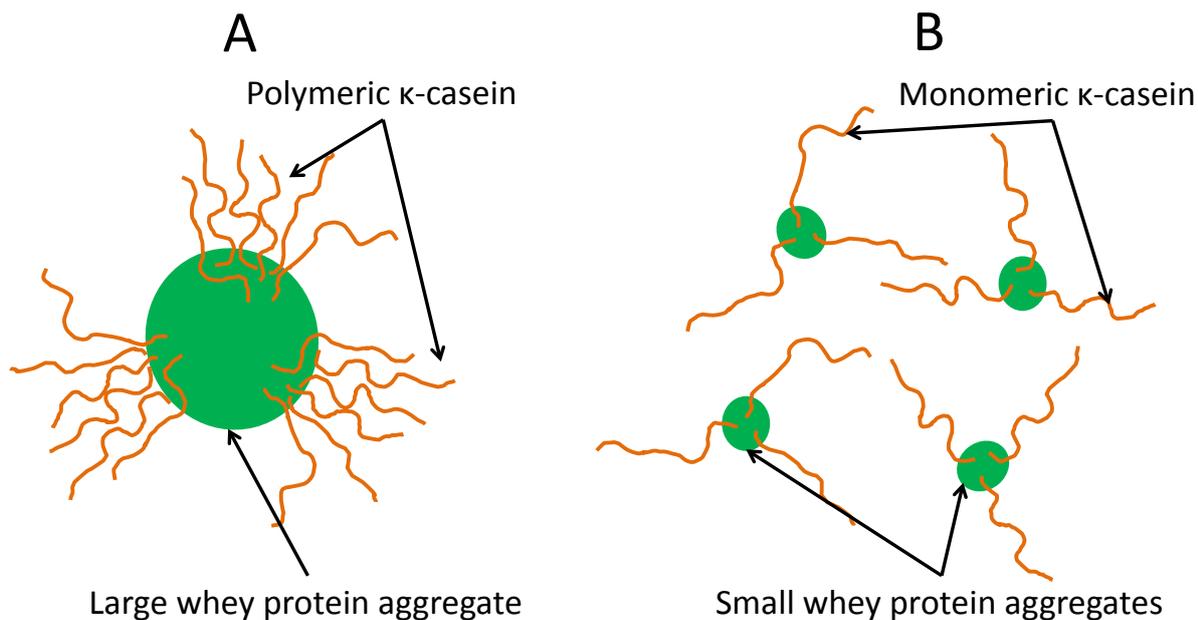


Figure 9.19: Schematic mechanism of stabilisation the whey protein aggregates by the κ -casein. A, polymeric κ -casein stabilised large whey protein aggregates; B, monomeric κ -casein stabilised small whey protein aggregates.

In summary, both scenarios 1 and 2 could occur simultaneously when reduced milks were heated. On the surface of the casein micelles, thiol-disulphide exchange reactions could be initiated by κ -casein. Disulphide bonds could propagate between casein micelles, resulting in the aggregation of the casein micelles. Concurrently in the serum phase, a high level of whey proteins could initiate the exchange reactions, leading to the formation of numerous small-sized aggregates.

9.4.2 Effects of heating β -mercaptoethanol-treated milks on the rheological properties of acid gels

9.4.2.1 Effects on the gelation pH

In this study, the increase in gelation pH may be due to the denaturation of whey proteins, the amount of whey-protein-containing aggregates in the serum phase and the increase in the hydrophobicity on the casein micelles, as was proposed in previous studies (Mottar et al., 1989; Lucey et al., 1997; Lucey et al., 1998; Vasbinder et al., 2001; Puvanenthiran et al., 2002; Anema et al., 2004a; Famelart et al., 2004; Vasbinder et al., 2004; Renan et al., 2006).

In the proposed interaction mechanism (Section 9.4.1.3), the milk that was heated in the presence of β -mercaptoethanol may contain a higher number of aggregates in the serum phase. Hence this milk gelled at higher pH than milk containing fewer aggregates, agreeing with previously reported results (Puvanenthiran et al., 2002; Anema et al., 2004a; Renan et al., 2006).

In addition, the heat-induced aggregates in milks containing β -mercaptoethanol were expected to contain a higher proportion of β -lactoglobulin than α -lactalbumin because of a higher level of α -lactalbumin found in the colloidal phase (Figure 9.6 and Figure 9.8). Since β -lactoglobulin had a higher pI value than α -lactalbumin (pI 5.2 and 4.9, respectively), the serum aggregates, which contained a lower level of α -lactalbumin, may have a higher pI. As a result, milks, which had a higher level of serum aggregates consisting of higher concentrations of β -lactoglobulin than α -lactalbumin due to β -mercaptoethanol, would have a higher gelation pH.

Furthermore, the hydrophobicity on the surface of the casein micelles may increase with the increase of β -mercaptoethanol concentrations because higher proportion of α -lactalbumin and β -lactoglobulin were found to associate with casein micelles (Figure 9.6 and Figure 9.8). This can cause the milks to gel at higher pH, as also observed previously (Famelart et al., 2004; Morand et al., 2012). Another factor that can contribute to the early gelation is the partial aggregation of the casein micelles. As discussed in Section 9.4.1.3, the casein micelles with whey-protein aggregates in milks heated in the presence of β -mercaptoethanol may be partially aggregated. This may also lead to an early gelation when the pI of the whey proteins was approached.

9.4.2.2 Effects on the final G' values

The increase in final G' values of acid gels prepared from β -mercaptoethanol-treated-heated milks can be related to the number of particles that can participate in the gel network. In previous studies, when the pH of milk at heating was raised from 6.5 to 7.1, the levels of protein particles found in the serum were reported to increase (Anema & Klostermeyer, 1997; Anema & Li, 2000, 2003a; Vasbinder & De Kruif, 2003) and the final G' values of the subsequent acid gels also increased (Vasbinder & De Kruif, 2003; Anema et al., 2004b; Lakemond & van Vliet, 2008a). This implied that the increase in the levels of protein particles in the serum may increase the final G' of the acid gels. In this study, even though the proportion of whey protein in the serum of milks decreased with the increase of β -mercaptoethanol, the number of serum aggregates may have increased as depicted in Figure 9.18 (Scenario 2). This may lead to an increase in the number of particles taking part in the structure of the gel during acidification and the degree of connection between the particles can be increased substantially, thus producing higher G' values. This was supported by the microstructure of acid gels made from milks heated with β -mercaptoethanol, which showed a denser network with a high number of connections compared to the acid gels of milk heated without β -mercaptoethanol (Figure 9.15 and Figure 9.16). The microstructure of the acid gels made from β -mercaptoethanol treated milks also related with the high final G' values of the gels, which had also been shown in previous research,

where a high number of connections led to high final G' in acid gels (Lucey et al., 1998; Guyomarc'h, Jemin, Tilly, Madec & Famelart, 2009b).

9.4.2.3 Effects on the $\tan \delta$ values

The $\tan \delta$ values of acid gels made from skim milk and WPE skim milk became lower as the addition level of β -mercaptoethanol increased (Figure 9.12). This was not consistent with the findings in Chapter 5 (Experiments on milks that were heated in the presence of NEM), in which $\tan \delta$ values increased with the increase of final G' values. This meant that in Chapter 5, the viscous property (G'') increased with the increase of elastic property (G') but in this Chapter, the viscous property either was not affected by β -mercaptoethanol or decreased with the increase of β -mercaptoethanol concentrations.

9.4.2.4 Effects on the yield properties

The effect of heating milk in the presence of β -mercaptoethanol on the yield properties of skim milk acid gels differ to those of WPE skim milk acid gels. For acid gels made from skim milk, the yield stress values of acid gels containing β -mercaptoethanol were higher than those of control acid gels but were not significantly different from each other; and the yield strain values were not markedly affected by the presence of β -mercaptoethanol (Table 9.2). For acid gels made from WPE skim milk, the yield stress and strain values decreased progressively with the increase of β -mercaptoethanol (Table 9.2).

The different observations between skim milk only gels and WPE skim milk gels could be related to the proportion of disulphide bonds in the systems. In skim milk, the total proportion of disulphide-linked whey protein in β -mercaptoethanol-treated milk was higher than that of control milk (Figure 9.6), hence contributing to the higher yield stresses and strain of the gels. In WPE skim milk, the total proportion of disulphide-linked whey protein decreased progressively as β -mercaptoethanol increased above 1.4 mM (Figure 9.8). As discussed in Chapter 5 (Milks heated in the presence of NEM), the lack of intermolecular disulphide bonds between denatured whey proteins and κ -casein can be the cause of the easy breakage of the acid gels when subjected to large deformation. Nevertheless, as the level of disulphide bonds in WPE skim milk in this study decreased only slightly, this cannot contribute to the substantial decrease in yield stress of the acid gels.

In addition, in Chapter 5, when acid gels prepared from WPE skim milk with different addition level of NEM were sheared, the yield stress values decreased with the increase of NEM concentrations but before the yield point, the shear stress values were found the same between samples at any given strain (Figure 5.12). In this chapter, at any given strain before the yield point, the shear stress values increased with the increase of β -mercaptoethanol concentrations

(Figure 9.14B). This also suggested that the decrease in total level of intermolecular disulphide bonds in the WPE skim milk was not the main reason causing the resulting acid gels to be more prone to breakage at large deformation. Alternatively, the easy breakage of acid gels made from WPE skim milk with added β -mercaptoethanol can be related to the curvature of the strands. The decrease in yield strain values with the increase of β -mercaptoethanol concentrations suggested a decrease in the curvature of the strands in the network (Figure 9.14B). This means that the strands can be ruptured more easily as the additional level of β -mercaptoethanol increased, hence lower yield stress and strain values.

9.5 Conclusions

When skim milk and WPE skim milk were heated in the presence of low levels of a disulphide reducing agent, besides denatured β -lactoglobulin produced during heating, κ -casein and α -lactalbumin may act as initiators of the thiol-disulphide exchange reactions with other proteins containing disulphide bonds. Subsequently, the rate of thiol-disulphide exchange reactions between the proteins increased. As a result, there were higher concentrations of α -lactalbumin and β -lactoglobulin associating with the casein micelles as well as a substantially larger number of small aggregates being formed in the serum.

When the treated milk was subsequently made into acid gels, there were large quantities of aggregates that participated in the structure of the network during acid gelation. Therefore the degree of connections between small aggregates was markedly increased, reflected by the dense gel microstructural network. As a result, the acid gels prepared from milk heated in the presence of β -mercaptoethanol had substantially higher G' values than those made from control heated milks. This study also demonstrated that the firmness of the acid gels can be increased without increasing the total number of disulphide bonds in the milk system, as was also observed in Chapter 5.

Chapter 10 - Effects of β -mercaptoethanol on protein interactions and rheological properties of acid gels of heated milk

10.1 Introduction

The results in Chapter 6 showed that inhibition of thiol-disulphide exchange reactions during acidification did not affect the G' values but affected the yield properties of the acid gels. From the results in Chapter 9, it was shown that increasing the free thiol groups in milks increased the thiol-disulphide exchange reactions during heat treatment of milks. The next question to answer was whether or not thiol-disulphide exchange reactions can be enhanced during acid gelation by adding free thiol groups to heated milks and how the modified interactions between the proteins affected the rheological properties of the resulting acid gels.

The results in Chapter 7 and 8 showed that the intermolecular disulphide bonds of native polymeric κ -casein were more accessible to β -mercaptoethanol than intra-molecular disulphide bonds of native β -lactoglobulin. Addition of up to 7.1 mM β -mercaptoethanol to unheated skim milk reduced up to 70% of the total κ -casein to monomers but less than 10% of β -lactoglobulin was reduced (Chapter 7). In this chapter, experiments will be described where β -mercaptoethanol was added to skim milk and WPE skim milk that had been heated at 80 °C for 30 min. In heated milks, the majority of β -lactoglobulin and κ -casein have participated in intermolecular disulphide bonds (Jang & Swaisgood, 1990; Lowe et al., 2004). Hence the first aim of the experiments in this Chapter was to determine whether or not disulphide bonds of κ -casein were still reduced in preference to those of β -lactoglobulin by β -mercaptoethanol and secondly, to determine how accessible the disulphide bonds of denatured β -lactoglobulin to β -mercaptoethanol were compared with those of native β -lactoglobulin.

Similarly to the experiments in Chapter 7, after addition of β -mercaptoethanol, the percentages of reduced β -lactoglobulin and monomeric κ -casein were monitored as a function of time. Subsequently, the effect of β -mercaptoethanol concentrations on the distribution of the proteins between the colloidal and serum phases and the levels of proteins participating in intermolecular disulphide bonds was determined.

Finally, the effects of the modified protein interactions on the firmness and microstructure of the acid gels made from the heated skim milks and WPE skim milks were investigated.

10.2 Materials and methods

Skim milk and WPE skim milk were prepared as described in Section 3.1.1. Fresh skim milks (as described in Section 3.1.2) were used when the reduction of the disulphide bonds of β -lactoglobulin and κ -casein was examined using microfluidic electrophoresis (MF-electrophoresis). The whey protein in fresh skim milk did not have any lactosylation, thus the electropherograms have a better resolution with α -lactalbumin and β -lactoglobulin being present as one single peak each. Nevertheless, unless specifically stated, reconstituted skim milks were used throughout this study.

Milks were heated at 80 ± 0.1 °C for 30 min. A detailed description of the methodology can be found in Section 3.3. After heating, the milks were rapidly cooled in cold tap water for ~2 min until the temperature dropped below ~30 °C prior to further treatments.

Diluted β -mercaptoethanol (10% v/v) was added to heated skim milk to give β -mercaptoethanol concentrations of 1.4, 4.3 and 7.1 mM. The treated milk was shaken with a vortex mixer for 10 s and then left to react at 20 ± 0.1 °C in a thermostatically controlled water bath for up to 6 h. The milk container was sealed to minimise oxidation.

Analyses involved monitoring the reduction of disulphide bonds of β -lactoglobulin and κ -casein as a function of time and a function of the β -mercaptoethanol concentrations using both MF-electrophoresis (as was described in Section 3.7) and traditional SDS-PAGE (as was described in Section 3.6). The distribution of the proteins between the colloidal and serum phases was also examined, using SDS-PAGE (as was described in Section 3.6) and the size of the casein micelles was measured as a function of β -mercaptoethanol concentrations, using dynamic light scattering (as was described in Section 3.8).

After defined times, subsamples of the milks were slowly acidified to form acid gels. The rheological properties of the milks during acidification were monitored. In addition the rheological properties and the microstructure of the set gels were also investigated. The details of the rheological and microstructural methods can be found in Section 3.10.2 and 3.10.3.

10.3 Results

10.3.1 Effects of reaction time on the reduction of protein disulphide bonds

In Figure 10.1, the electropherograms of fully reduced heated fresh skim milk showed maximum intensities of the reduced β -lactoglobulin and κ -casein, taken as 100%. In non-reduced heated skim milk, the peaks of the reduced β -lactoglobulin and monomeric κ -casein were at their minimum intensities since the majority of β -lactoglobulin, α -lactalbumin and κ -casein were involved in intermolecular disulphide bonds (Figure 10.1, Corredig & Dalgleish, 1999; Lowe et al., 2004).

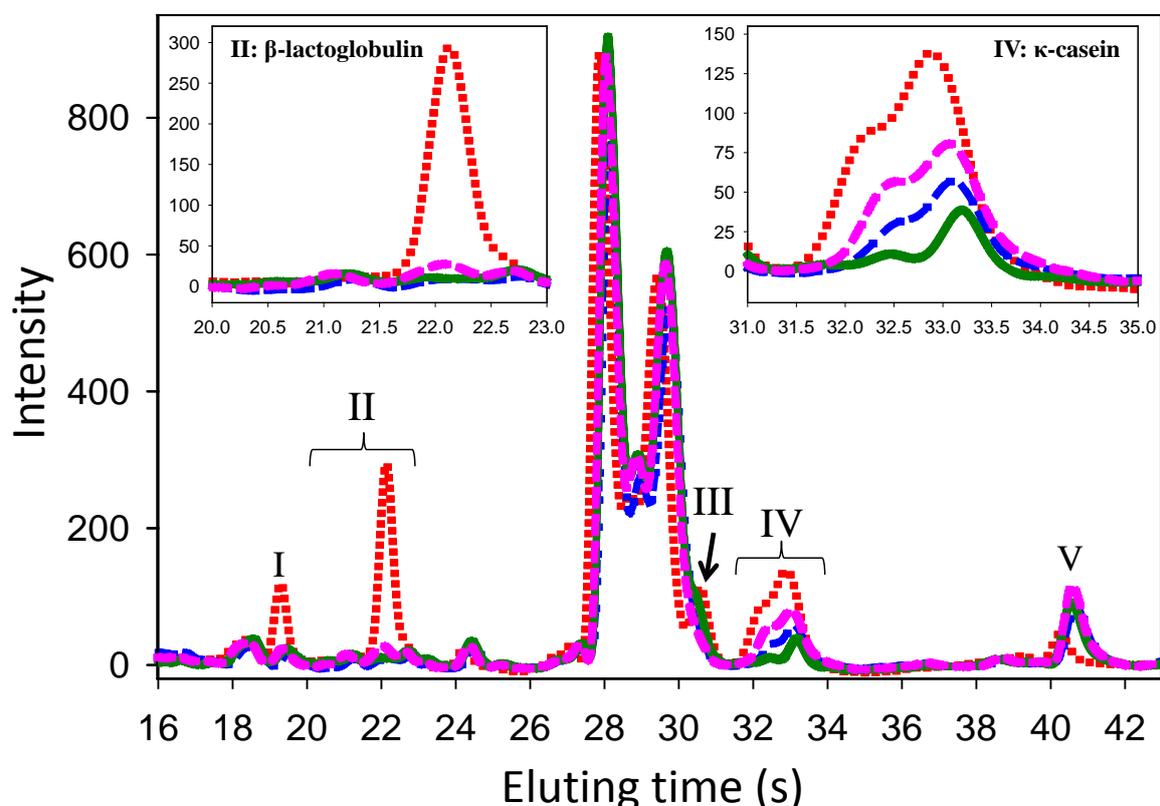


Figure 10.1: Electropherograms of fully reduced skim milk (red dotted line), non-reduced heated skim milk (green line) and heated skim milk that had been reacted with 7.1 mM β -mercaptoethanol for 1 h (blue dashed line) and 6 h (pink dashed line). Peak I, α -lactalbumin; Peak II, β -lactoglobulin; Peak III, monomeric α_{s1} -casein; Peak IV, κ -casein; Peak V, dimeric α_2 -casein.

When 7.1 mM of β -mercaptoethanol was added to heated skim milk, the level of monomeric κ -casein increased progressively as the reaction time increased from 1 to 6 h. After 6 h, $\sim 50\%$ of κ -casein was reduced to monomers (Insert IV, Figure 10.1) while a very low level of β -lactoglobulin was reduced (Insert II, Figure 10.1). This demonstrates that the reaction

between β -mercaptoethanol and disulphide bonds of κ -casein and β -lactoglobulin occurred over time, as also observed in unheated skim milk (Chapter 7). In addition, the electropherograms also indicated that κ -casein was reduced much more readily than β -lactoglobulin or α -lactalbumin, even in heated milk where the whey proteins were denatured.

The effect of reaction time on the reduction of disulphide bonds of β -lactoglobulin and κ -casein was monitored and the results are summarised in Figure 10.2. The percentage of reduced β -lactoglobulin did not change much with time and remained at $< 15\%$ at all β -mercaptoethanol concentrations (Figure 10.2A). Regarding the reduction of κ -casein as a function of time, at 1.4 mM β -mercaptoethanol, the percentage of monomeric κ -casein increased gradually from $19.5 \pm 0.7\%$ to $32.4 \pm 2.5\%$ over 6 h. At 4.3 mM β -mercaptoethanol, the reduction of κ -casein occurred over 2 h and increased from $15.7 \pm 2.0\%$ to $38.1 \pm 1.6\%$; after which no further reduction occurred. At 7.1 mM, the percentage of monomeric κ -casein increased significantly from $18.7 \pm 1.4\%$ to $55.7 \pm 3.5\%$ ($p < 0.05$) over 3 h then increased slightly further to $62.5 \pm 3.5\%$ as the reaction time increased to 6 h (Figure 10.2B). In general, at any reaction time, the proportion of monomeric κ -casein increased with the increase of β -mercaptoethanol concentration. In addition, the percentage of monomeric κ -casein was always higher than the percentage of reduced β -lactoglobulin regardless of the β -mercaptoethanol concentration and reaction time (Figure 10.2). This indicates that the disulphide bonds of κ -casein were reduced in preference to those of β -lactoglobulin.

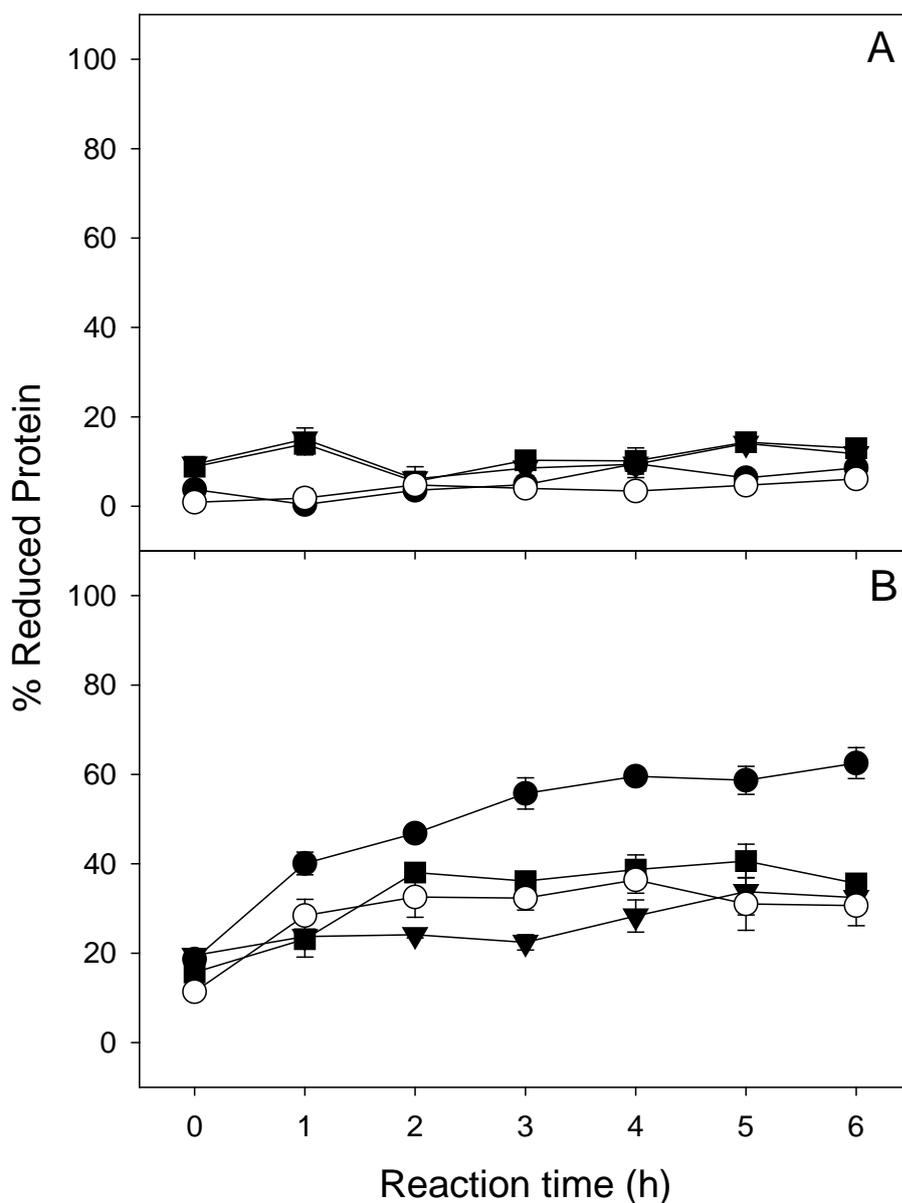


Figure 10.2: Effects of reaction time on the percentages of reduced β -lactoglobulin (A) and monomeric κ -casein (B) over the total of that protein in heated skim milk (closed symbol) and heated WPE skim milk (open symbols). ▼, 1.7 mM; ■, 4.3 mM; ●○, 7.1 mM β -mercaptoethanol. Analysis used MF-electrophoresis and each data point is the mean value of two to four replicates, the error bar is the standard deviation.

The effect of time on the reduction of κ -casein and β -lactoglobulin was also examined in WPE skim milk and the results are also shown in Figure 10.2. Similarly to the heated skim milk, the percentage of reduced β -lactoglobulin in the WPE milk remained low at any β -mercaptoethanol concentration and was not affected by the reaction time (Figure 10.2A). In contrast, the

percentage of monomeric κ -casein increased significantly from $11 \pm 1\%$ to $33 \pm 5\%$ over 2 h of reaction, after which no further reduction occurred (Figure 10.2B).

Overall, the impact of reaction time on the reduction of κ -casein and β -lactoglobulin in heated skim milk and WPE skim milk demonstrated that more than 2 h was required for the reaction to go to completion at any β -mercaptoethanol concentration. Since the reaction time between β -mercaptoethanol and the proteins in unheated milk was set at 3 h (Chapter 7), an appropriate reaction time for the heated skim milks would also be 3 h. Further investigations were carried out by adding β -mercaptoethanol to heated (reconstituted) skim milks (without and with added whey protein) and holding for 3 h at 20.0 ± 0.5 °C before analyses or further treatments.

10.3.2 Effects of β -mercaptoethanol concentrations on protein interactions and distribution between colloidal and serum phases

The percentage of proteins that were not participating in disulphide bonds was examined using SDS-PAGE under non-reducing condition and the SDS-PAGE patterns of the proteins are presented in Figure 10.3.

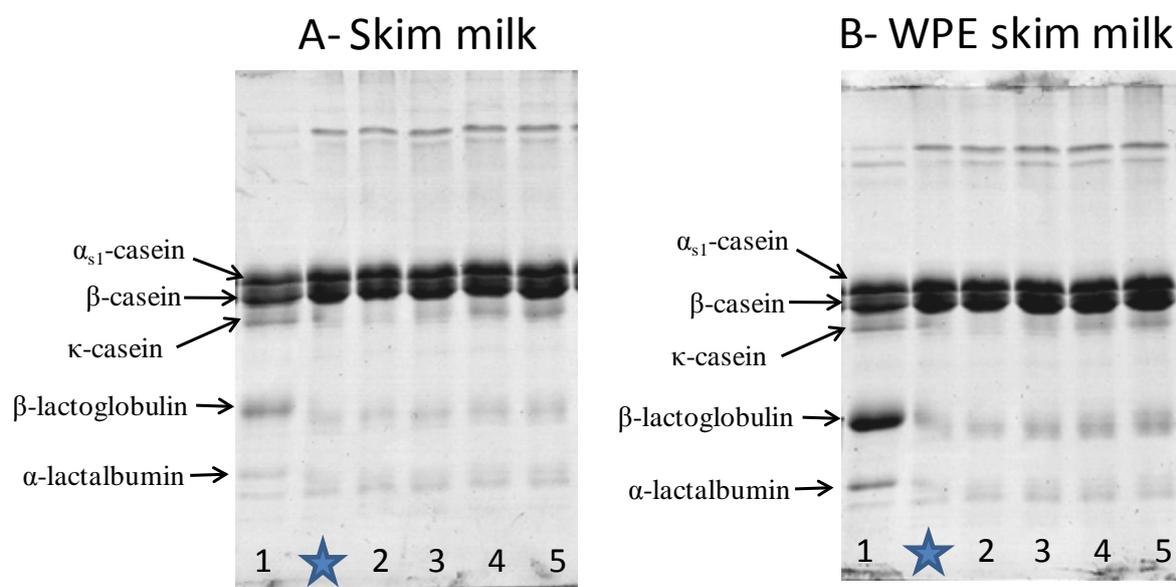


Figure 10.3: SDS-PAGE patterns showing the effects of β -mercaptoethanol concentrations on the level of proteins that were not participating in inter-molecular disulphide bonds in heated skim milk (A) and heated WPE skim milk (B). Control samples (i.e. control unheated milks, Lane 1), heated milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk samples were diluted with SDS sample buffer at a ratio of 1 to 40 and were not reduced except for the control samples being fully reduced. The lane with the blue star is the transition between the reducing and non-reducing conditions.

In both skim milk and WPE skim milk, the bands representing α -lactalbumin, β -lactoglobulin and κ -casein increased in intensity as the concentration of β -mercaptoethanol increased from 0 to 7.1 mM with the increase being more pronounced in κ -casein and β -lactoglobulin than in α -lactalbumin (Figure 10.3). This indicates that the level of reduced proteins increased with the increase of β -mercaptoethanol concentration and the disulphide bonds of β -lactoglobulin and κ -casein were reduced in preference to those of α -lactalbumin.

Figure 10.4 showed the SDS-PAGE patterns of the total proteins present in the serum phases and the proteins in the serum phase that were not involved in disulphide bonds. In both milks, the SDS-PAGE patterns of the supernatant analysed under reducing condition shows that the intensities of α -lactalbumin, β -lactoglobulin and κ -casein bands did not change with the increase of β -mercaptoethanol concentrations. This indicated that the proportion these proteins in the serum phase was not affected by the addition of β -mercaptoethanol. The levels of the other caseins in the serum from skim milk were low. The β -casein band was not affected by β -mercaptoethanol whereas the α_{s1} -casein band appeared to disappear as the β -mercaptoethanol concentration increased ≥ 1.4 mM (Figure 10.4A). In WPE skim milk, the level of α_{s1} - and β -caseins in the serum was low and did not change as a function of β -mercaptoethanol concentrations (Figure 10.4B).

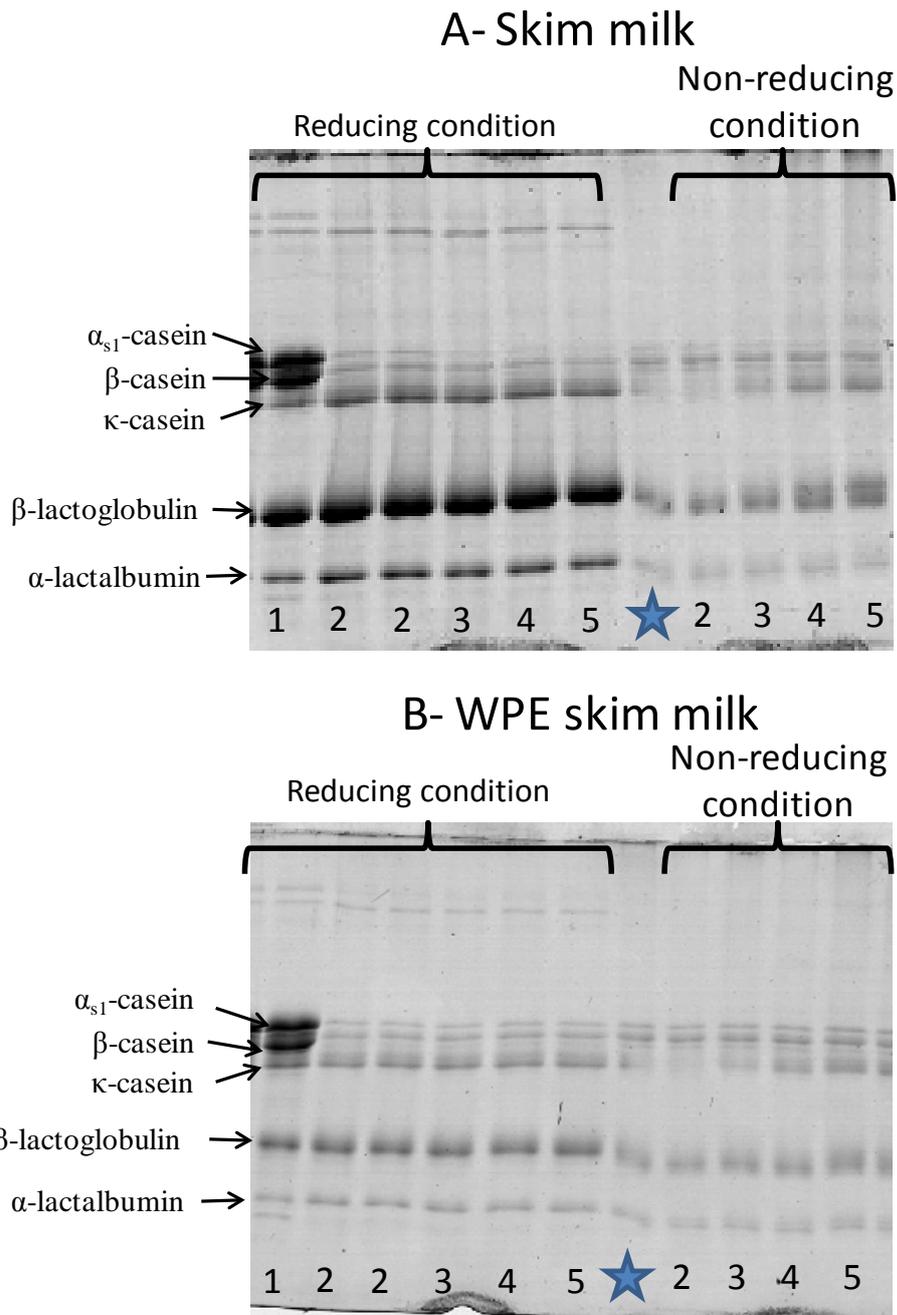


Figure 10.4: SDS-PAGE patterns showing the effects of β -mercaptoethanol concentrations on the levels of proteins in the serum phase and the serum proteins that were not disulphide-linked. Skim milk (A) and WPE skim milk (B). Control samples (i.e. control unheated milks, Lane 1), supernatant of milks treated with 0 mM (Lane 2), 1.4 mM (Lane 3), 4.3 mM (Lane 4) and 7.1 mM (Lane 5) β -mercaptoethanol. The milk sample (Lane 1) and supernatant samples (Lane 2-5) were diluted with SDS sample buffer at ratio of 1:40 and 1:20, respectively. The lane with the blue star was the transition between reducing and non-reducing conditions.

SDS-PAGE patterns of the serum phase analysed under non-reducing condition showed that in both milks, the intensity of β -lactoglobulin and κ -casein bands increased with the increase of

β -mercaptoethanol concentrations. The intensities of the bands of α -lactalbumin, α_{s1} - and β -caseins did not change on the addition of β -mercaptoethanol (Figure 10.4). This indicated that the disulphide bonds of serum β -lactoglobulin and κ -casein were reduced by β -mercaptoethanol while α -lactalbumin and other caseins were hardly affected.

After integrating the gels, the average data showing the interactions of the proteins and the distributions of the proteins between the serum and colloidal phases are summarised in Figure 10.5, Figure 10.6 and Figure 10.7. The percentage of colloidal protein was calculated based on the assumption that if a protein was not found in the serum phase, that protein would be found in the colloidal phase (Section 3.6.7).

In heated skim milk, as the concentrations of β -mercaptoethanol increased from 0 to 7.1 mM, the percentage of reduced α -lactalbumin increased from $39 \pm 6\%$ to $52 \pm 10\%$ ($p > 0.05$, Figure 10.5A); the percentage of reduced β -lactoglobulin increased from $27 \pm 7\%$ to $40 \pm 8\%$ ($p > 0.05$, Figure 10.6B) and the percentage of monomeric κ -casein increased from $23 \pm 5\%$ to $84 \pm 7\%$ ($p < 0.05$, Figure 10.6C).

The percentage of reduced β -lactoglobulin and monomeric κ -casein was significantly higher for the results shown in Figure 10.5 than those shown in Figure 10.2. A summary of the percentage of reduced proteins in heated skim milk with 7.1 mM added β -mercaptoethanol (reaction time: 3 h) is presented in Table A.3 (Appendix 8) to show the different figures obtained from MF-electrophoresis and SDS-PAGE. The difference could be due to the technique used for analysing (MF-electrophoresis versus SDS-PAGE), the way of integrating the results (peak area in MF-electrophoresis versus band intensity in SDS-PAGE) and the type of milk (fresh skim milk versus reconstituted skim milk). Despite the difference in specific numerical results, the trend was still consistent: greater β -mercaptoethanol concentrations resulted in higher levels of protein being reduced. In addition, at any β -mercaptoethanol concentration, the percentage of monomeric κ -casein relative to the total concentration of κ -casein in skim milk was higher than the percentage of reduced α -lactalbumin or β -lactoglobulin relative to the total concentration of that protein in skim milk (Figure 10.2 and Figure 10.5).

Regarding the effects of adding β -mercaptoethanol to heated skim milk on the distribution of the proteins, the average percentage of serum α -lactalbumin was $58 \pm 6\%$, of β -lactoglobulin was $54 \pm 3\%$, of κ -casein was $40 \pm 6\%$ and of other caseins was $5 \pm 1\%$ (Figure 10.5 and Figure 10.6).

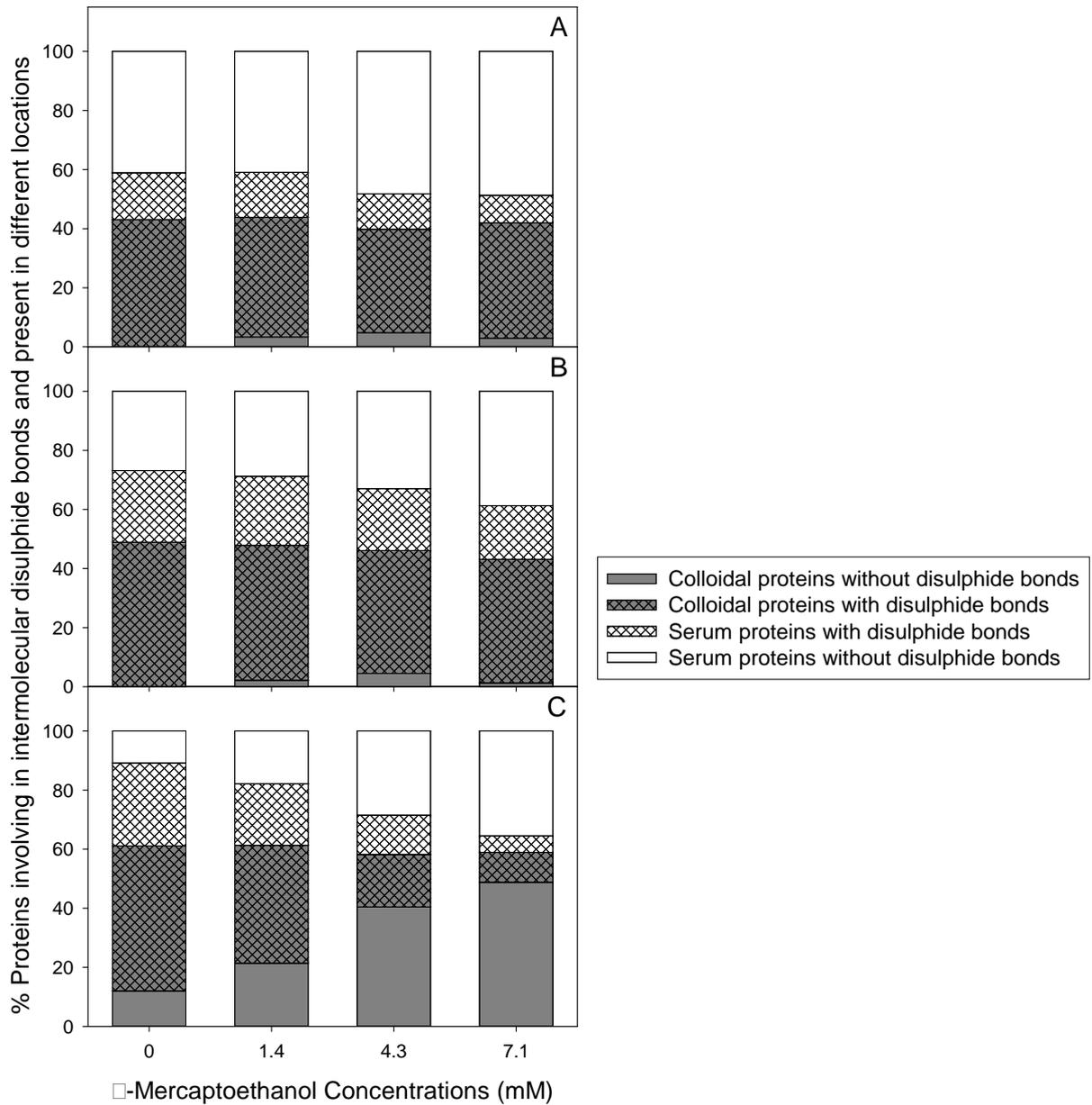


Figure 10.5: Effects of adding β -mercaptoethanol to heated skim milk on the distribution of proteins between the serum and colloidal phases and on the participation of the proteins in intermolecular disulphide bonds. A. α -lactalbumin; B. β -lactoglobulin; C. κ -casein. The data was obtained from traditional SDS-PAGE. Each data point is the mean value of two to four replicates.

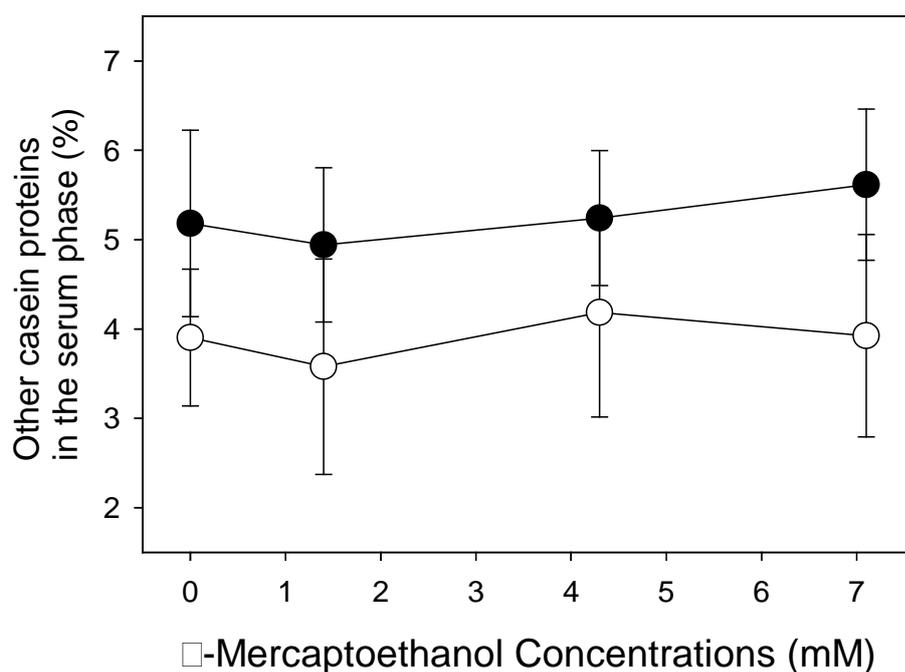


Figure 10.6: Effects of adding β -mercaptoethanol heated milk on the level of other caseins in the serum. Skim milk (●) and heated WPE skim milk (○). Other caseins refer to α_{s1} -, α_{s2} - and β -casein. Each data point is the average of two to four replicates. Error bars represent standard deviation.

In control heated WPE skim milk, the percentage of reduced α -lactalbumin and β -lactoglobulin ($10 \pm 7\%$ and $14 \pm 3\%$, respectively) was lower than that in control heated skim milk ($39 \pm 6\%$ and $27 \pm 7\%$, respectively) whereas the percentage of reduced κ -casein was comparable in both milks ($25 \pm 6\%$ and $23 \pm 5\%$, respectively). Addition of β -mercaptoethanol to heated WPE skim milk significantly increased the percentage of reduced proteins, with the increase of the percentage of monomeric κ -casein was more pronounced than that of reduced α -lactalbumin and β -lactoglobulin (Figure 10.7). As the β -mercaptoethanol concentration increased from 0 to 7.1 mM, the percentage of reduced α -lactalbumin increased from $10 \pm 7\%$ to $20 \pm 4\%$ ($p > 0.05$, Figure 10.7A), that of reduced β -lactoglobulin increased from $14 \pm 3\%$ to $25 \pm 2\%$ ($p < 0.05$, Figure 10.7B) and the percentage of monomeric κ -casein increased from $25 \pm 6\%$ to $65 \pm 2\%$ ($p < 0.05$, Figure 10.7C).

In WPE skim milk, the average percentage of serum α -lactalbumin, β -lactoglobulin, κ -casein and other caseins were $61 \pm 6\%$, $54 \pm 4\%$, $59 \pm 8\%$ and $4 \pm 1\%$ respectively (Figure 10.6 and Figure 10.7).

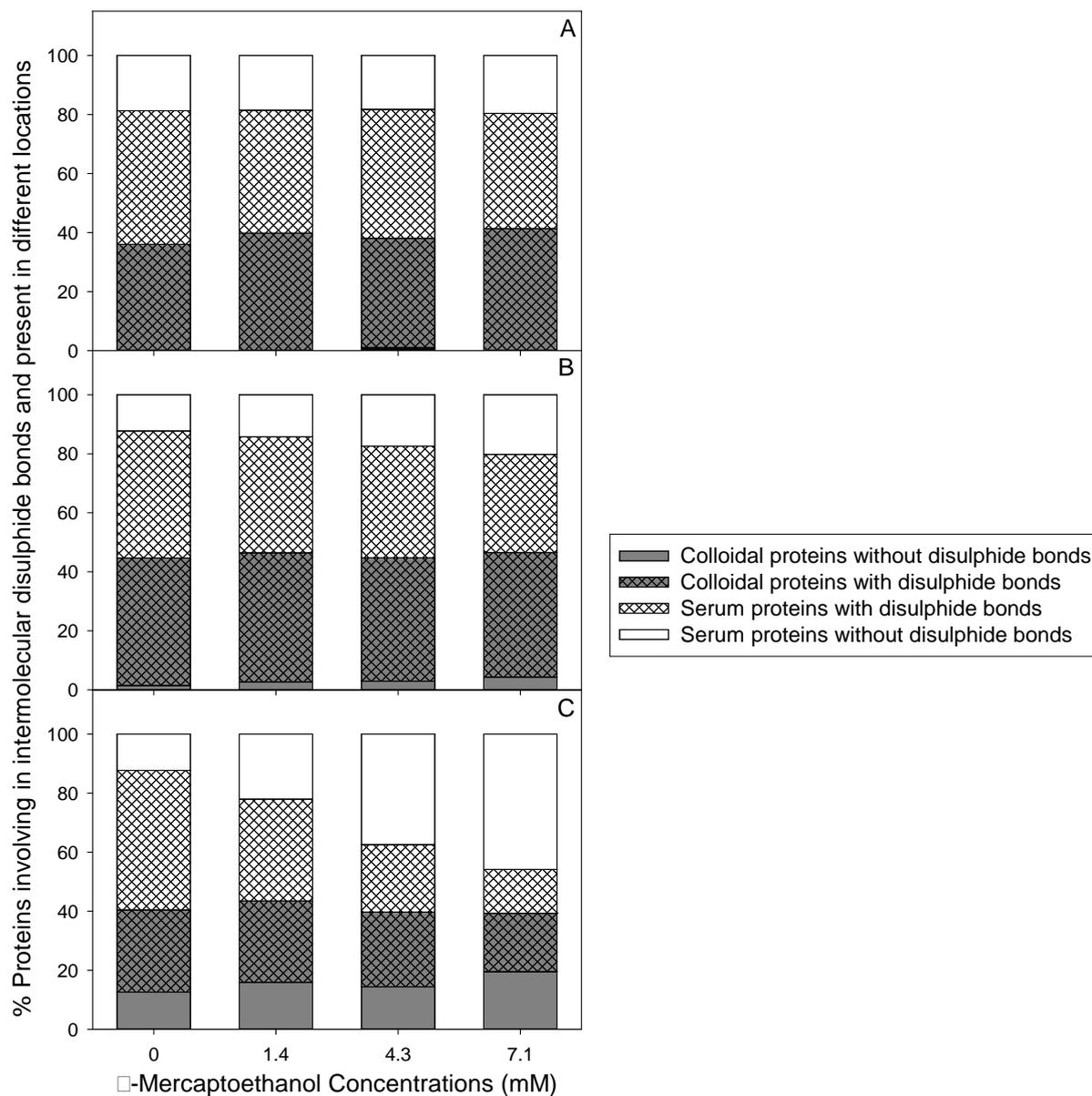


Figure 10.7: Effects of adding β -mercaptoethanol to heated WPE skim milk on the distribution of proteins between the serum and colloidal phases and on the participation of the proteins in intermolecular disulphide bonds. A. α -lactalbumin; B. β -lactoglobulin; C. κ -casein. The data was obtained from traditional SDS-PAGE. Each data point is the mean value of two to four replicates.

In summary, the electrophoretic results showed that in both milks, a higher proportion of κ -casein was reduced than α -lactalbumin or β -lactoglobulin, and this agreed with the results obtained by MF-electrophoresis (Figure 10.2). In addition, adding β -mercaptoethanol to heated milks did not affect the distribution of the proteins between the colloidal and serum phases.

The effects of β -mercaptoethanol concentrations and the reduction of the protein disulphide bonds on the size of the casein micelles is summarised in Figure 10.8. The casein micelle size in heated skim milk was not affected by adding β -mercaptoethanol and was on average 190.8 ± 1.4

nm. Similarly, the size of the casein micelles in heated WPE skim milk were, within standard deviations, the same at all β -mercaptoethanol concentrations (~ 206 nm, Figure 10.8).

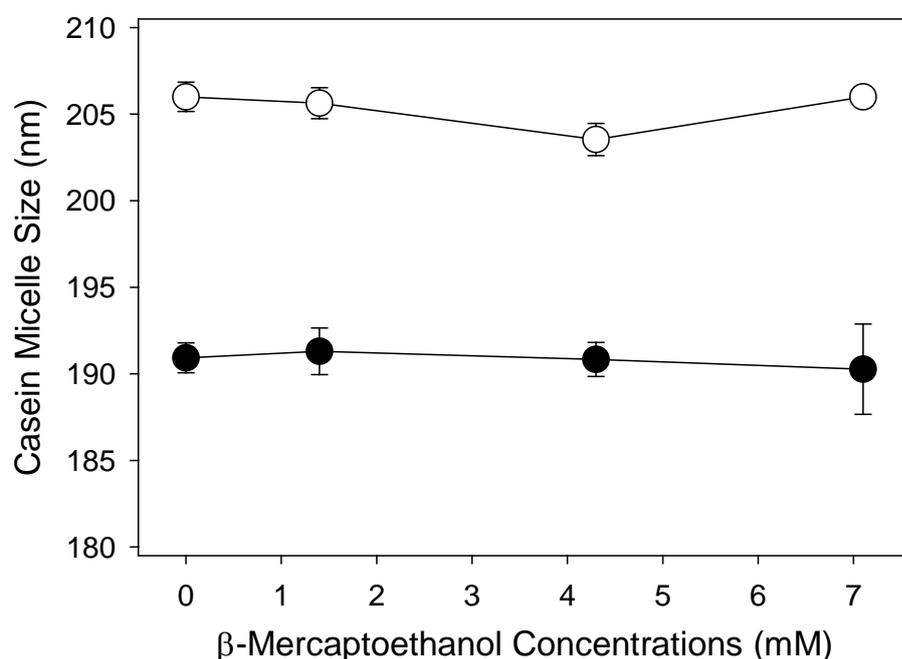


Figure 10.8: Effects of adding different concentrations of β -mercaptoethanol to heated skim milk (●) and heated WPE skim milk (○) on the size of the casein micelles. Each data point is an average of two to four replicates. Error bars represent the standard deviation.

The effects of reaction time between β -mercaptoethanol and the protein disulphide bonds on the proportion of proteins in the serum phase and the casein micelle sizes were also examined. In general, the proportion of serum proteins and the casein micelle size in heated skim milk increased with the increase of reaction time while the reaction time did not affect the proportion of serum proteins in heated WPE skim milk (Appendix 9).

Preliminary measurements of the zeta potentials of the casein micelles were performed for selected samples of heated milks containing β -mercaptoethanol. The results showed that the zeta potential values of casein micelles were not affected by the presence of β -mercaptoethanol, i.e. the reduction of the κ -casein polymers (Figure A.8, Appendix 5). Hence detailed measurements of the zeta potential were not carried out.

10.3.3 Effects of adding β -mercaptoethanol to heated milks on the rheological properties of acid gels

The effect of reaction time between β -mercaptoethanol and the milk proteins on the final G' values of the acid gels was examined. The acid gels were prepared by using GDL (2%) to acidify milks that had been heated and treated with β -mercaptoethanol for different periods of time.

The gels were made at 30 °C over a period of 3 h. The effect of reaction time on the final G' values of the acid gels was also examined and it was shown that 1 – 3 h was required to achieve the maximum effect of β -mercaptoethanol on the properties of acid gels (Figure A.12, Appendix 10). The following results showed the properties of acid gels made from heated milks that had been reacted with β -mercaptoethanol for 3 h.

The change in G' with time for acid gels made from heated skim milk and heated WPE skim milk with 7.1 mM added β -mercaptoethanol represent the typical curve of all acid gels made from the heated milks treated with β -mercaptoethanol (Figure 10.9). The change in G' of acid gels made from heated skim milks with β -mercaptoethanol had the same profile as that of control heated skim milk (Figure 10.9A). However, during the acidification of the β -mercaptoethanol treated milks, the gelation point (which was considered as the first G' value ≥ 1 Pa) occurred earlier and then the gels achieved higher G' values than in the control skim milk (Figure 10.9A). Similarly, acid gels made from heated WPE skim milk with added β -mercaptoethanol had earlier gelation pH and the gels achieved higher final G' values than gelation of heated WPE skim milk (Figure 10.9B).

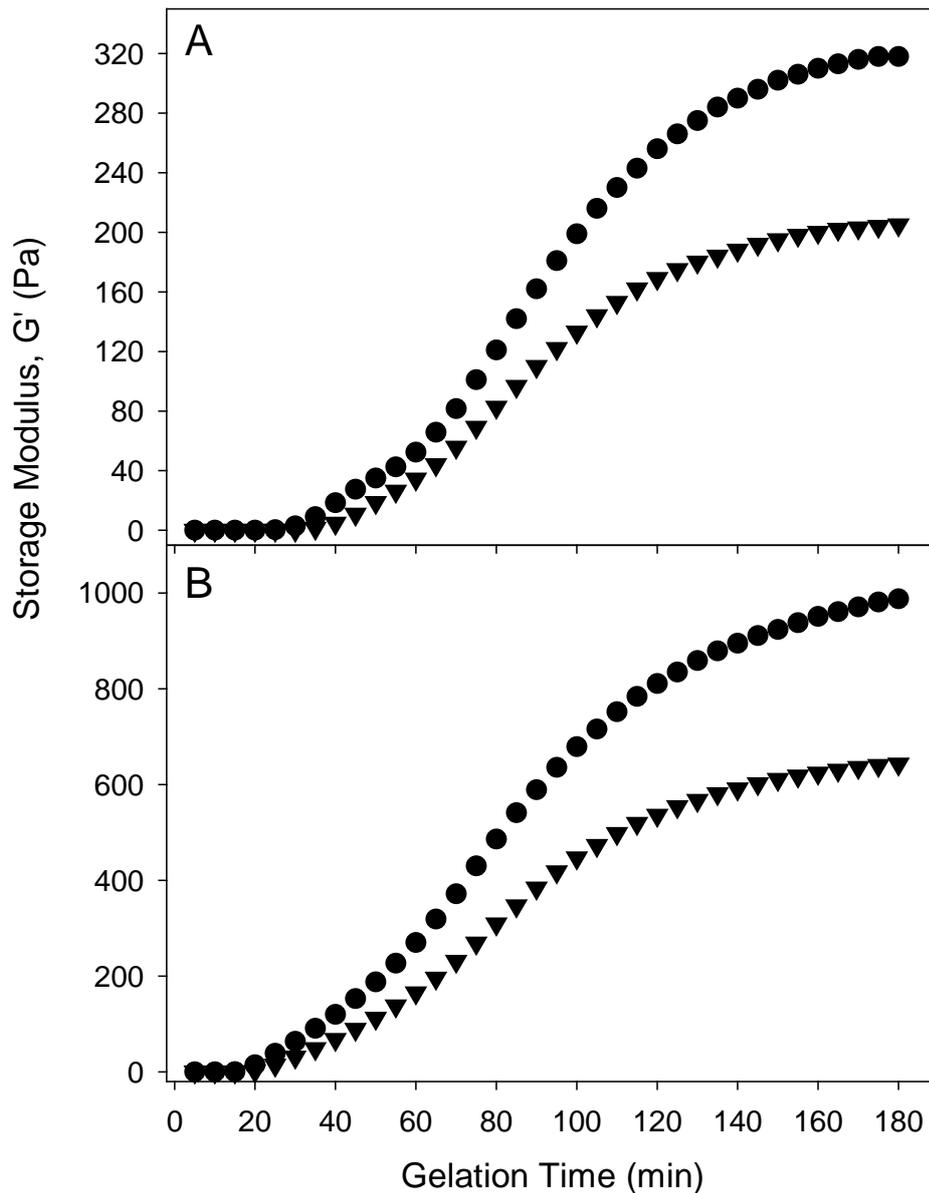


Figure 10.9: The typical change of the storage modulus during acidification of heated skim milks (A) and heated WPE skim milks (B). Heated milks were treated with 0 (▼) and 7.1 mM added β -mercaptoethanol (●) for 3 h.

The effects of β -mercaptoethanol concentrations on the gelation pH and the final G' values of acid gels made from heated skim milk and heated WPE skim milk are summarised in Figure 10.10. In heated skim milk, increasing the β -mercaptoethanol concentration from 0 to 7.1 mM caused the gelation pH to increase significantly from $\text{pH } 5.37 \pm 0.03$ to $\text{pH } 5.49 \pm 0.07$ ($p < 0.05$, Figure 10.10A). The final G' values also increased significantly from 201 ± 1 Pa to 320 ± 3 Pa as β -mercaptoethanol concentration increased from 0 to 7.1 mM ($p < 0.05$, Figure 10.10B).

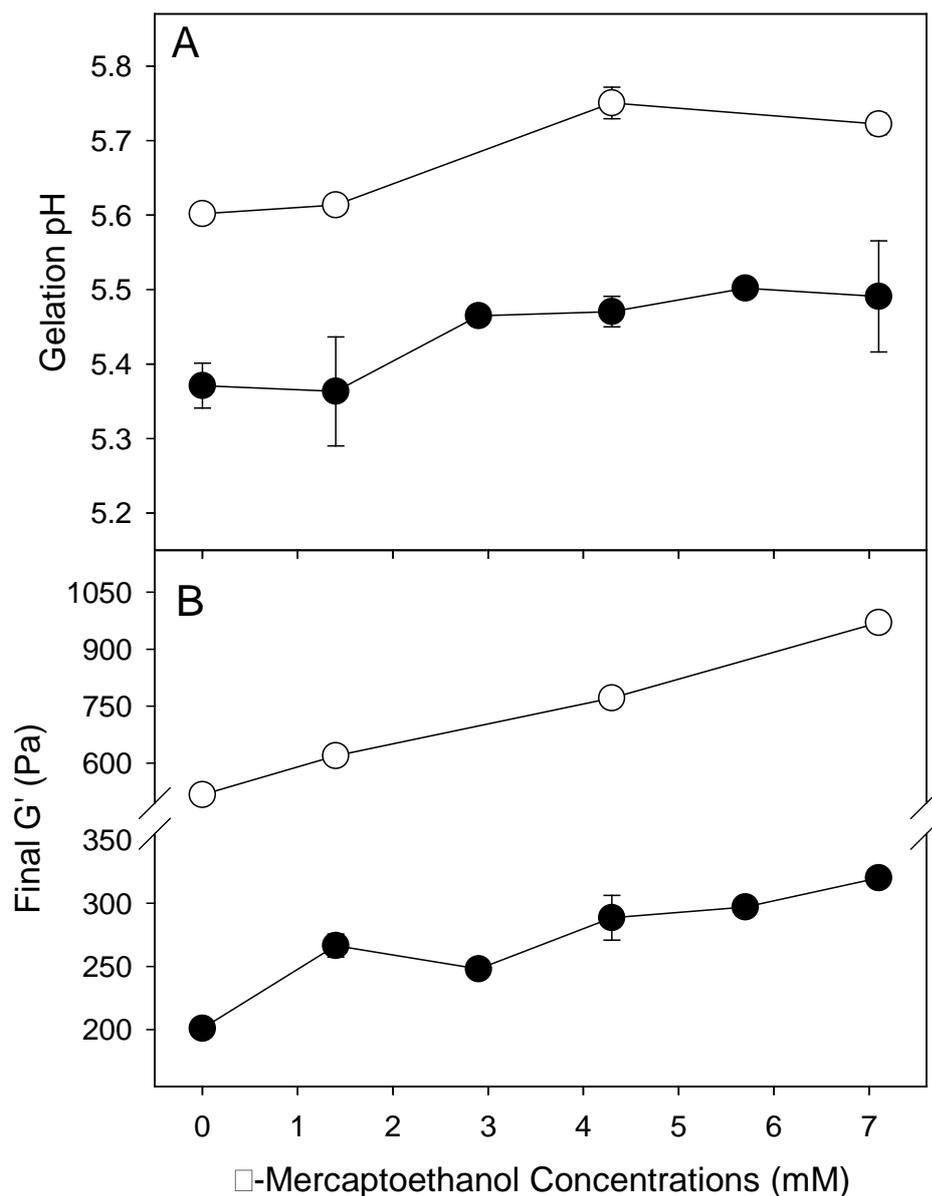


Figure 10.10: Effects of β -mercaptoethanol concentrations on the gelation pH (A) and final G' values (B) of acid gels made from skim milk (●) and WPE skim milk (○). Milks were heated before reacting with β -mercaptoethanol for 3 h. Each data point is the average of two to four replicates (except for β -mercaptoethanol of 5.7 mM which was only a single measurement). Error bars represent the standard deviation.

In heated WPE skim milk, the gelation pH was not affected and remained at $\text{pH } 5.61 \pm 0.01$ when 1.4 mM β -mercaptoethanol was added, then increased significantly to $\text{pH } 5.75 \pm 0.02$ at 4.3 mM β -mercaptoethanol ($p < 0.05$) and did not change further at 7.1 mM β -mercaptoethanol (Figure 10.10A). The final G' of the acid gels from the WPE skim milks increased linearly and significantly from 524 ± 9 Pa to 970 ± 26 Pa as the β -mercaptoethanol concentration increased from 0 to 7.1 mM (Figure 10.10B). Hence the G' values of acid gels made from heated WPE skim

milk was 1.85 fold higher than that of the control with no β -mercaptoethanol, this was compared to an increase of 1.59 fold in the G' value at 7.1mM β -mercaptoethanol in the heated skim milk (Figure 10.10B).

The $\tan \delta$ (the ratio of G'' to G') values of the acid gels made from heated skim milk and WPE skim milk with added β -mercaptoethanol were not found to be affected by the addition of β -mercaptoethanol to heated milks (Figure A.4, Appendix 2). This suggested that the increase of G' (storage modulus) values were accompanied by a proportional increase in G'' (loss modulus) values. Hence the balance between the elastic and viscous properties was maintained.

Once the acid gels were formed after 3 h, the temperature of the gels was decreased to 5°C at a rate of 1 °C min⁻¹. The G' values of the acid gels at 5°C were higher than those at 30°C, at any β -mercaptoethanol level. In heated skim milk, the G' values at 5 °C increased from 400 ± 41 Pa to 584 ± 55 Pa when β -mercaptoethanol concentration was increased from 0 to 7.1 mM (Figure 10.11A). In heated WPE skim milk, the G' values at 5 °C increased from 928 ± 28 Pa to 1680 ± 159 Pa with the increase of β -mercaptoethanol concentration from 0 to 7.1 mM (Figure 10.11A). At 5 °C, the G' values increased as a function of β -mercaptoethanol by up to 1.46 and 1.81 fold in heated skim milk and heated WPE skim milk, respectively. This was comparable with the increase of G' values of acid gels at 30 °C (1.59 and 1.85 fold increased, respectively).

The final G' values at 5 °C were plotted as a function of the final G' values at 30 °C (Figure 10.11B). The relationship between the G' at 30 °C and those at 5 °C was linear with gradients of 1.6; this was compared to a gradient of 1.7 for the gels prepared from milks heated in the presence of β -mercaptoethanol, as shown in Chapter 9. The results indicated that regardless of the β -mercaptoethanol addition level in skim milk or WPE skim milk, the G' of the acid milk gels increased by ~1.6 times when the temperature was decreased from 30 °C to 5 °C (Figure 10.11B).

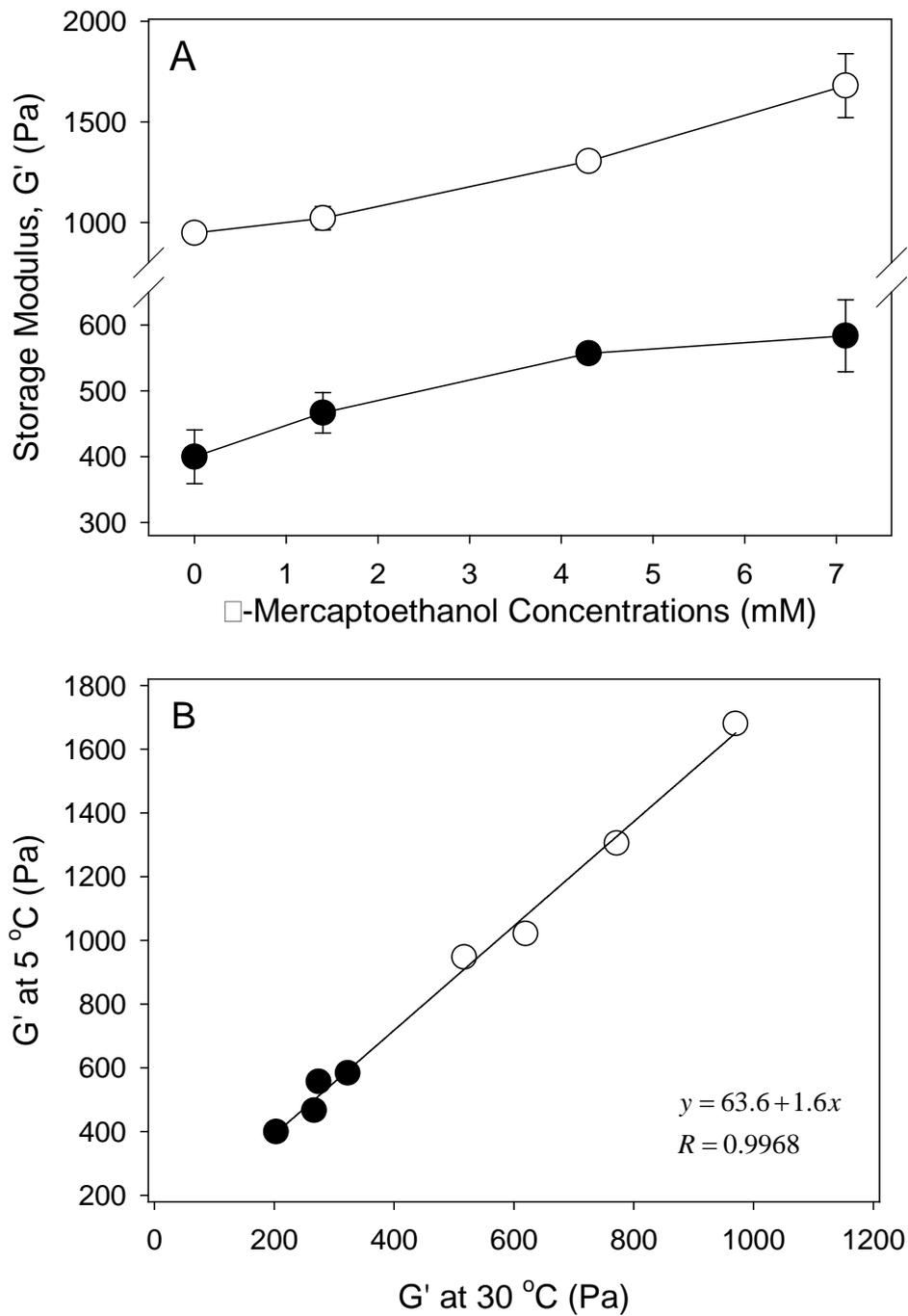


Figure 10.11: A, Effects of β -mercaptoethanol concentrations on the G' values of acid gels at 5 °C. B, The relationship between the G' values at 5 °C and the G' values at 30 °C. ●, skim milk; ○, WPE skim milk. The milks were heated before being treated with β -mercaptoethanol. Each data point is the average of two to four replicates. Error bars in A represent standard deviation.

The yield properties of the acid gels at 5 °C were examined by monitoring the change of shear stress and strain of the gels when subjected to a constant shear rate (0.005 s^{-1}). In heated skim milk, the yield strain of the acid gels prior to being broken decreased from $42 \pm 3\%$ to $35 \pm 3\%$ as the concentration of added β -mercaptoethanol increased from 0 to 7.1 mM (Figure 10.12A).

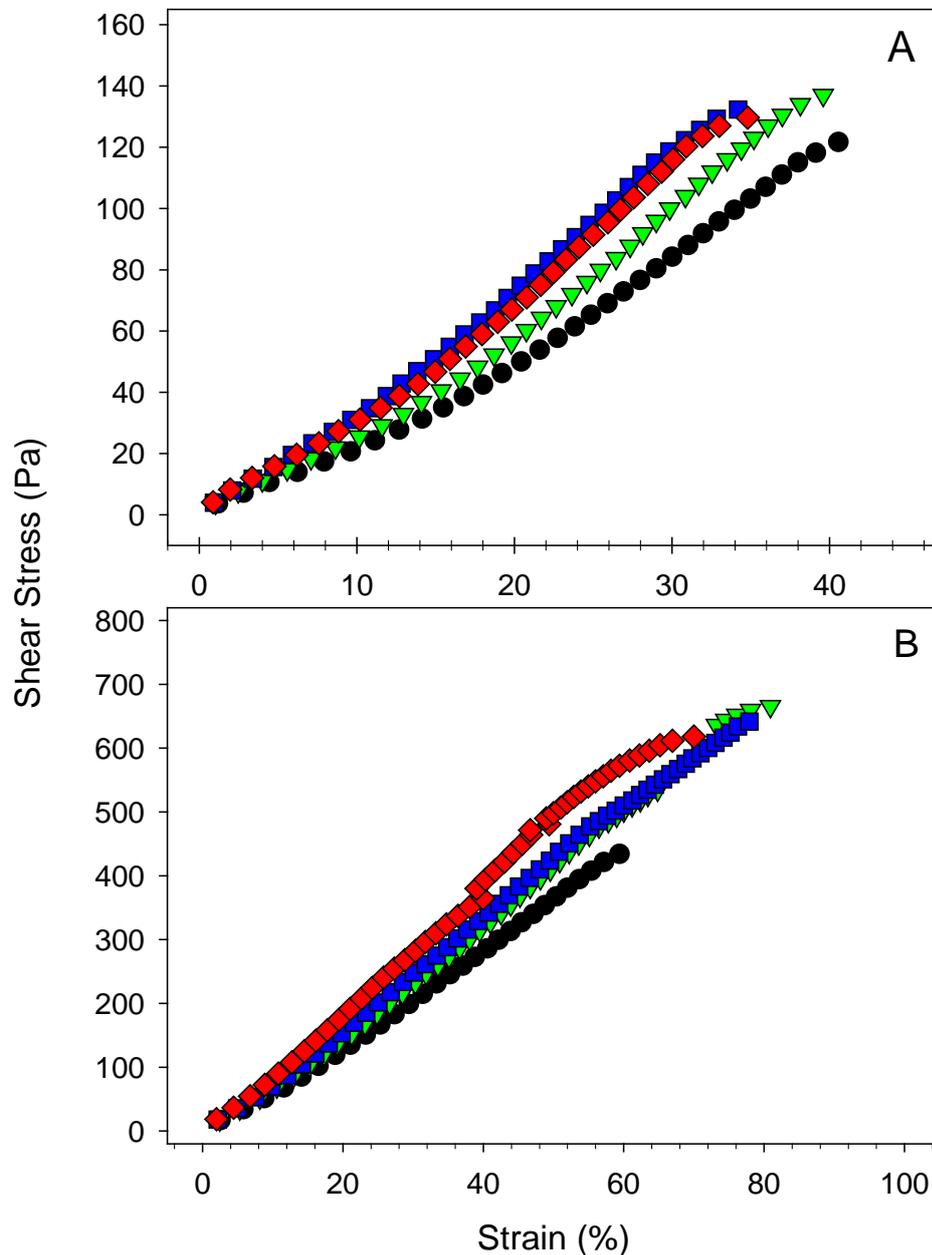


Figure 10.12: Shear stress as a function of strain at a constant shear rate (0.005 s^{-1}) for acid gels formed at $30 \text{ }^{\circ}\text{C}$ and analysed at $5 \text{ }^{\circ}\text{C}$. The acid gels were prepared from heated skim milk (A) and heated WPE skim milk (B) with different concentrations of β -mercaptoethanol. ●, 0 mM; ▼, 1.4 mM; ■, 4.3 mM; ◆, 7.1 mM β -mercaptoethanol. Each point is the average of four to six replicates. Error bars are not shown to simplify the graph.

The maximum shear stress values (yield stress) increased from 122 ± 1 Pa to 138 ± 1 Pa as β -mercaptoethanol concentration increased to 1.4 mM. The shear stress then decreased to 133 ± 2 Pa and 130 ± 3 Pa as β -mercaptoethanol increased further to 4.3 and 7.1 mM (Figure 10.12A).

In heated WPE skim milk, the yield strains and yield stresses increased from $62 \pm 4\%$ and 445 ± 8 Pa to $85 \pm 8 \%$ and 667 ± 5 Pa when β -mercaptoethanol concentrations increased from 0 to 1.4 mM (Figure 10.12B). At 7.1 mM β -mercaptoethanol, the yield strain and yield stress values decreased to $72 \pm 11\%$ and 618 ± 10 Pa, respectively. Overall, the yield strain and yield stress values of acid gels made from heated WPE skim milk with added β -mercaptoethanol were higher than those of acid gels made from control heated WPE skim milk (Figure 10.12B. See also Table 10.1 for numeric figures of yield strain and yield stress values).

Table 10.1: Yield stress (Pa) and strain (%) of acid gels when subjected to constant shear rate. Acid gels were prepared from milks that had been heated before being treated with β -mercaptoethanol. The values are the mean value of two to four replicates \pm standard deviation.

β -mercaptoethanol Concentrations (mM)	Heated skim milk		Heated WPE skim milk	
	Yield Strain (%)	Yield Stress (Pa)	Yield Strain (%)	Yield Stress (Pa)
0	42 ± 3	122 ± 1	62 ± 4	445 ± 8
1.4	41 ± 3	138 ± 1	85 ± 8	667 ± 5
4.3	36 ± 4	133 ± 2	79 ± 8	643 ± 6
7.1	35 ± 3	130 ± 2	72 ± 1	618 ± 10

10.3.4 Effects of adding β -mercaptoethanol to heated milks on the microstructure of the acid gels

Confocal microscopy of the set acid gels of selected samples was carried out and the results are shown in Figure 10.13 and Figure 10.14. The network of acid gels made from heated skim milk became less dense with more open spaces (black areas) as the concentration of β -mercaptoethanol increased. The number of strands per unit area and the degree of connections between the strands in the acid gel networks decreased with the increase of β -mercaptoethanol concentrations (Figure 10.13).

The network of heated WPE skim milk acid gels was much denser, with a higher degree of connections between the proteins (Figure 10.14), compared to the network of heated skim milk acid gels (Figure 10.13). Similarly to acid heated skim milk gels, the gel network with higher concentrations of β -mercaptoethanol was less dense with more open spaces (black area in Figure 10.14). However, the effect of β -mercaptoethanol on the structure of the acid gels of WPE skim milk was not as obvious as observed in the gels prepared from skim milk only.

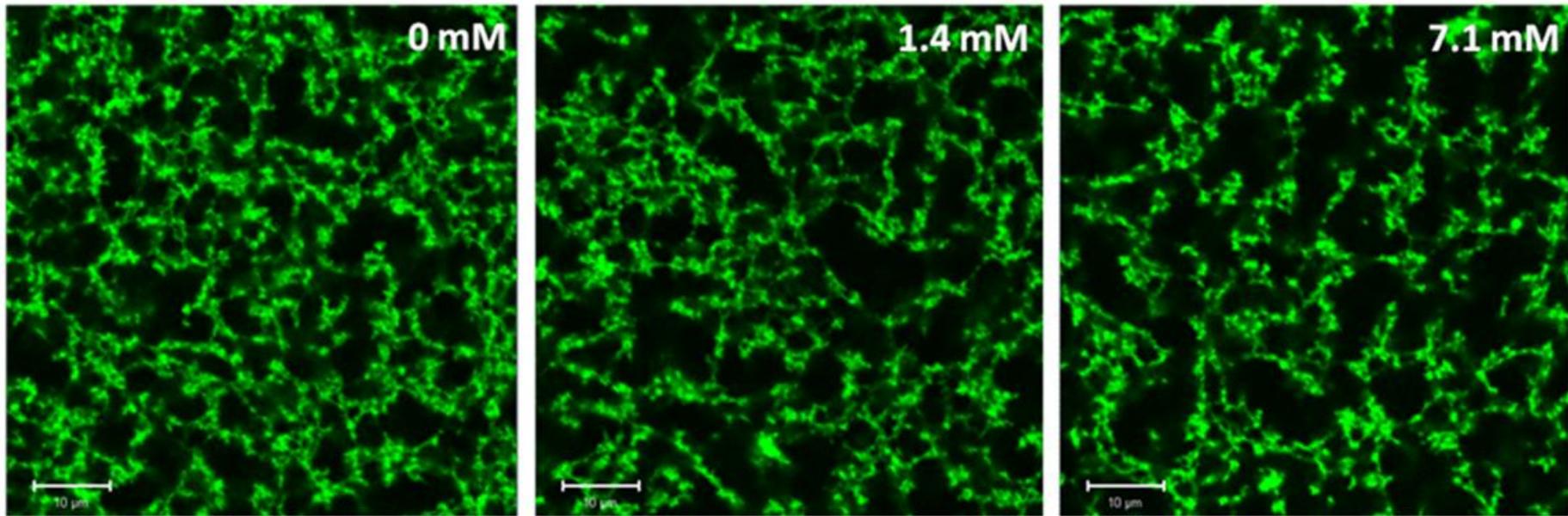


Figure 10.13: Microstructure of acid gels made from heated skim milk that had been reacted with β -mercaptoethanol ranging from 0 to 7.1 mM for 3 h. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μm .

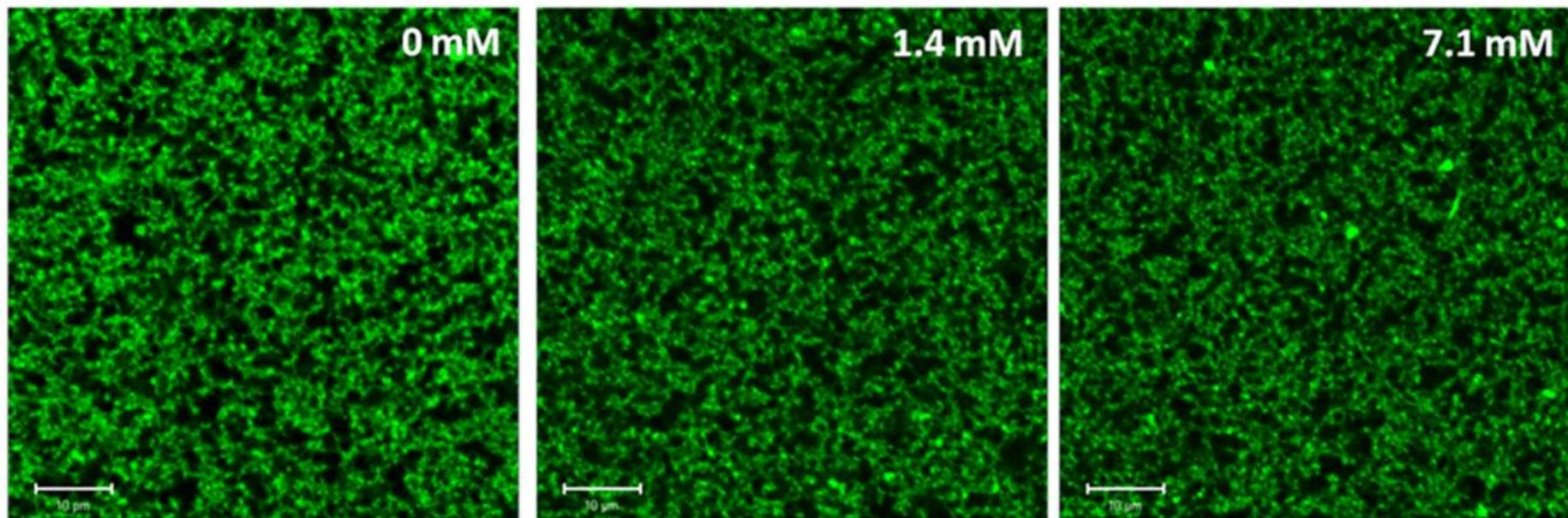


Figure 10.14: Microstructure of acid gels made from heated WPE skim milk that had been reacted with β -mercaptoethanol ranging from 0 to 7.1 mM for 3 h. The images were taken after 3 h of gelation to represent the gels at final G' point. The scale bar is 10 μ m.

10.4 Discussion

10.4.1 Effects of reducing disulphide bonds in heated milks on the protein interactions

The results on the effects of reaction time on the various properties of heated milks demonstrated that the reaction between the proteins and β -mercaptoethanol occurred over a long period of time regardless of whether the proteins were in the native states in unheated milks (Chapter 7) or in the aggregated state in heated milks (this Chapter). As the reaction time was chosen to be 3 h to be consistent between the work in unheated skim milks and in heated skim milks, the following discussion will be based on the observations and analyses made after a reaction time of 3 h.

In heated milks, the disulphide bonds of κ -casein were reduced preferentially to those of β -lactoglobulin (Figure 10.5 and Figure 10.7), regardless of the β -mercaptoethanol concentrations. Interestingly, this was also observed in unheated skim milks (Chapter 7). This indicates that in heated milks, disulphide bonds of κ -casein were more accessible to β -mercaptoethanol than those of β -lactoglobulin, despite κ -casein being part of the heat-induced aggregates involving denatured whey proteins.

A possible explanation was that κ -casein was located on the surface of the heat-induced aggregates, as already proposed previously (Guyomarc'h, Nono, Nicolai & Durand, 2009a). In the study of Guyomarc'h et al. (2009a) and other studies (Dalglish, 1990; Beaulieu et al., 1999), the size of the serum aggregates was found to decrease with increasing concentrations of κ -casein in the milk system, which supports the surface location of κ -casein. While this surface location may account for the preferential reduction of κ -casein disulphide bonds, it is worth noting that a certain number of disulphide bonds of β -lactoglobulin and α -lactalbumin belonging to the heat-induced aggregates can still be broken without releasing monomeric protein. The reduction of those disulphide bonds will be discussed in Section 10.4.2 as this can affect the properties of the resulting acid gels.

Other factors, such as differences in the microenvironment of the cysteine residues, may influence the rate of the reduction (Shaked et al., 1980; Darby & Creighton, 1995; Jensen et al., 2009), so that those involving κ -casein were reduced preferentially to those involving β -lactoglobulin. In the heat-induced aggregates, Lowe et al. (2004) found that β -lactoglobulin interacted intermolecularly via Cys⁶⁶ and Cys¹⁶⁰ while κ -casein interacted via both Cys¹¹ and Cys⁸⁸. When considering the neighbouring amino acid groups of the mentioned cysteine (Cys) amino acids, the groups next to Cys of κ -casein may make it easier for the negatively charged thiolate ion on the reducing agent to approach while the groups next to the Cys of β -lactoglobulin may

inhibit or block the approach of this thiolate ion. The reason was that the neighbouring amino acid groups in κ -casein either are positively charged (e.g. Arginine, Lysine) or have small side chains (e.g. Serine, Glutamine, Alanine); hence they did not inhibit the approach of the thiolate ion of β -mercaptoethanol. In contrast, the neighbouring amino acid groups in β -lactoglobulin are either negatively charged (e.g. Glutamic acid) or had large side chain (e.g. Histidine), thus the approaching of the thiolate ion may be inhibited.

Interestingly, in both skim milk and WPE skim milk, even though the percentage of total disulphide-linked κ -casein decreased significantly after the addition of β -mercaptoethanol, the percentage of disulphide-linked β -lactoglobulin and α -lactalbumin did not change to the same extent (Figure 10.5 and Figure 10.7). In addition, even though the disulphide bonds of the colloidal κ -casein were reduced, the distribution of the proteins between the colloidal and serum phases was not affected (Figure 10.5 and Figure 10.7). This indicated that the reduction of the disulphide bonds of the proteins, especially the disulphide bonds of κ -casein, did not affect the aggregation between the whey protein and the κ -casein and the association of the denatured whey proteins to the casein micelles. In other words, the intermolecular disulphide bonds between κ -casein and the denatured whey proteins may have been less susceptible to reduction by β -mercaptoethanol than the intermolecular disulphide bonds in polymeric κ -casein. This suggested that the heat-induced aggregates may be formed by the interactions between polymeric κ -casein and aggregated β -lactoglobulin/ α -lactalbumin via a low number of disulphide bonds.

Alternatively, the electrophoretic results suggested that the denatured whey proteins may interact with κ -casein via non-covalent interactions. Non-covalent interactions have been reported to occur during the early stage of the aggregation of denatured β -lactoglobulin and κ -casein or between denatured β -lactoglobulin proteins in solutions (Haque & Kinsella, 1988; Jang & Swaisgood, 1990). In other studies, non-covalent interactions and disulphide interactions were found to occur concurrently in heated κ -casein/ β -lactoglobulin or whey protein systems (Doi et al., 1983; Dalglish et al., 1997b; Galani & Apenten, 1999). Non-covalent interactions can also be the sole interactions between the proteins in heated milk or protein solutions when thiol/disulphide exchange reactions were prevented (Mckenzie et al., 1971; Hoffmann & van Mil, 1997). In this study, since the whey proteins were still associated with the casein micelles despite κ -casein being reduced; this indicated that non-covalent interactions may be as important as disulphide interactions in aggregation of the whey protein, especially β -lactoglobulin with κ -casein.

10.4.2 Effects of reducing disulphide bonds in heated milks on the acid gels' properties

The increase in the final G' values of the acid gels with the decrease in the total number of disulphide bonds in the milk systems demonstrated that disulphide bonds may not be necessarily required to increase the acid gel firmness. This agreed with the findings in Chapter 5 and Chapter 9. The reduction of disulphide bonds in heated milks can cause the increase in G' values of acid gels in various ways that will be described below.

In previous studies, acid gels prepared from protein or milk solutions containing free thiol groups were found to have higher G' values and hardness (measured by penetration tests) than those made from the solutions without free thiol groups due to the presence of thiol-blocking reagents (Hashizume & Sato, 1988; Lucey et al., 1998; Alting et al., 2000; Alting et al., 2003; Vasbinder et al., 2003; Lakemond & van Vliet, 2008b). It was proposed that thiol-disulphide exchange reactions occurred during acid gelation, and the newly formed disulphide bonds contributed to the high G' values and hardness of the acid gels. In this study, heated milks that were treated with β -mercaptoethanol contained higher levels of free thiol groups than control heated milks (Figure 10.5 and Figure 10.7). This implied that the thiol-disulphide exchange reactions can occur more readily during acidification of heated-treated milks than acidification of control heated milks. The exchange reactions can happen between the proteins of the same heat-induced aggregates or from different heat-induced aggregates. The increase in the level of free thiol groups, especially on the surface of the aggregates (as proposed in Section 10.4.1), may increase the chance of interactions between the proteins on different heat-induced aggregates. As a result, higher levels of disulphide interactions between the heat-induced aggregates may be formed in the acid gels made from heated-treated milks than in those made from control heated milks (Figure 10.15).

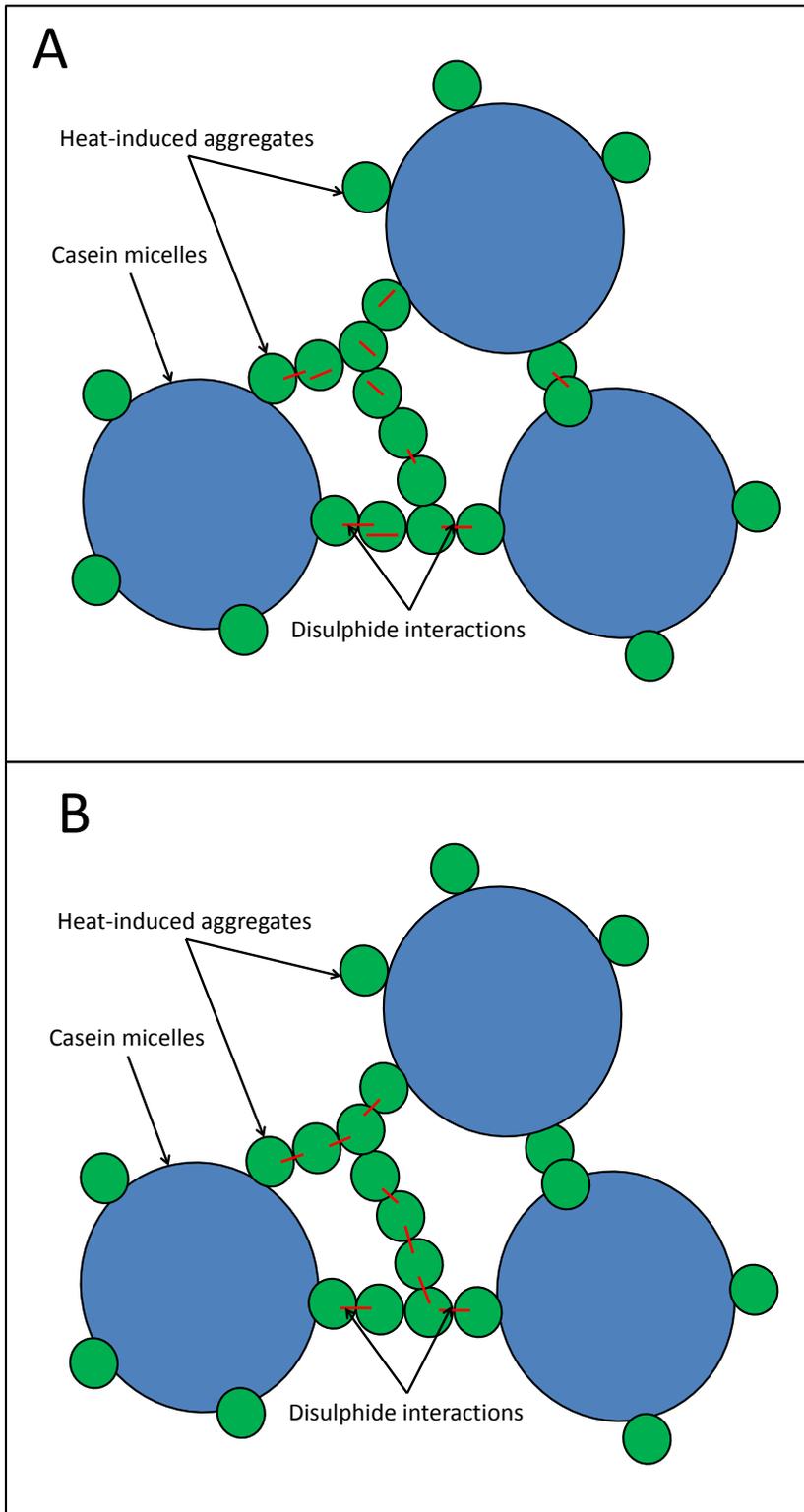


Figure 10.15: A proposed scheme of disulphide interactions between the particles in the acid gels as a result of thiol-disulphide exchange reactions during acidification. A, in control heated milks, disulphide bonds within aggregates; B, in milks heated then treated with β -mercaptoethanol, disulphide bonds between aggregates.

Alternatively, the breakage of the disulphide bonds between the proteins in the heat-induced aggregates may allow the proteins to interact non-covalently. In previous research, when thiol-disulphide bonds were inhibited by blocking the free thiol groups of β -lactoglobulin, non-covalent interactions became dominant in the heated systems (Sawyer, 1967; McKenzie et al., 1971; Xiong et al., 1993; Hoffmann & van Mil, 1997; Havea et al., 2004; Havea et al., 2009). It was proposed that without disulphide bonds, the proteins became more flexible and sites such as hydrophobic, hydrogen and ionic sites became more exposed compared to when disulphide bonds were present. Therefore, the degree of non-covalent interactions between the proteins was higher in disulphide-bond-free systems than in systems that also contained disulphide bonds (Xiong et al., 1993; Havea et al., 2009).

In this study, the reduction of disulphide bonds in the heat-induced aggregates may break the disulphide bonds of β -lactoglobulin and α -lactalbumin in the heat-induced aggregates as well as producing monomeric κ -casein. Since almost two third of a κ -casein monomer is hydrophobic (105 hydrophobic residues in a total of 169 residues) (Walstra & Jenness, 1984) and since κ -casein may no longer be restricted in a certain arrangement once the disulphide bonds were broken, the monomeric κ -casein would have more freedom to interact non-covalently with other proteins. Regarding β -lactoglobulin and α -lactalbumin, even though reduction of disulphide bonds (especially intra-molecular disulphide bonds) did not liberate monomers, it may allow the whey proteins to rearrange and exposure other sites that may normally be inaccessible due to the disulphide bonds (Figure 10.16).

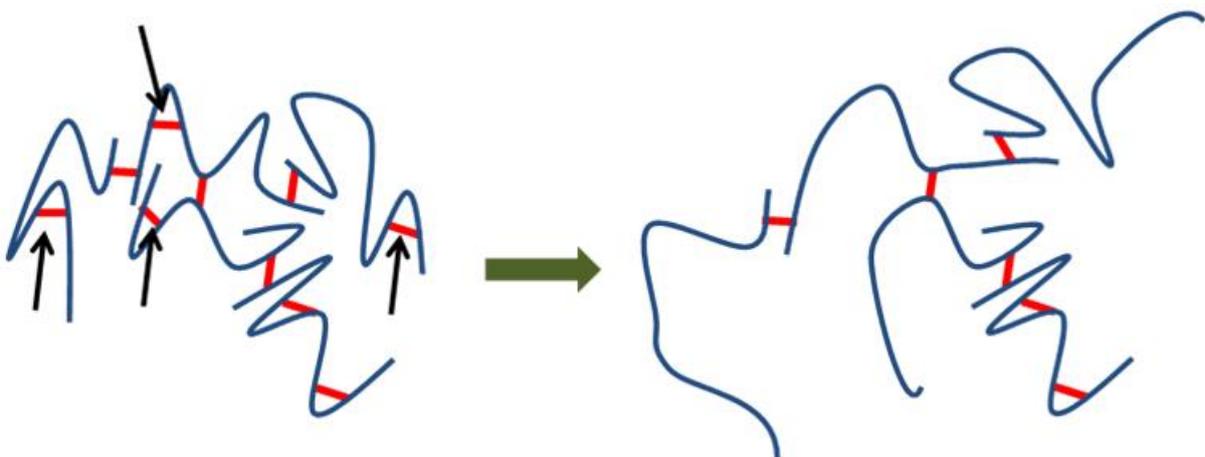


Figure 10.16: A proposed scheme of reduction of disulphide bonds in heat-induced aggregates. Left, heat-induced aggregate with intra- and inter-molecular disulphide bonds; Right, after reduction of a few disulphide bonds, the aggregate contained proteins that were more flexible and possibly exposed more sites including hydrophobic, hydrogen and ionic sites. Black arrows show disulphide bonds being reduced.

Overall, the breakage of disulphide bonds may increase the freedom for the proteins to interact via other type of bonds. Hence, the total level of interactions between the proteins in acid gels made from heated-treated milks can be higher than in acid gels of control heated milks.

Another possible explanation for the increase in G' values of acid gels with the increased concentration of β -mercaptoethanol can be that the reduction of the intermolecular disulphide bonds in the heat-induced aggregates can lead to the breakage of the heat-induced aggregates into smaller aggregates. This meant that there were higher numbers of particles that can participate in the gel network, hence increase the G' values.

In previous studies, when the pH of milk at heating was raised from 6.5 to 7.1, the levels of protein particles found in the serum were reported to increase (Anema & Klostermeyer, 1997; Anema & Li, 2000, 2003a; Vasbinder & De Kruif, 2003) and the final G' values of the subsequent acid gels also increased (Vasbinder & De Kruif, 2003; Anema et al., 2004b; Lakemond & van Vliet, 2008a). This implies that the increase in the levels of protein particles in the serum may influence the final G' of the acid gels. In this study, even though the level of serum proteins did not increase due to the addition of β -mercaptoethanol (Figure 10.5 and Figure 10.7), the reduction of intermolecular disulphide bonds of the proteins may break the heat-induced aggregates apart, producing a greater number of smaller aggregates. This would lead to a greater number of particles that can participate in the formation of the gel network during acidification. If the heat-induced aggregates can be visualised as a compact structure made up of numerous smaller aggregates, the cleavage of a number of disulphide bonds would open up the structure of the aggregates. This, together with the increase in number of smaller aggregates, may increase the number of possible arrangements and connections between the particles during gel formation. As a result, acid gels consisting of more connections would result in higher G' values.

The breakage of the initial heat-induced aggregates to smaller aggregates on the addition of β -mercaptoethanol can also explain the high gelation pH of acidified milks containing β -mercaptoethanol (Figure 10.10A). As mentioned earlier, increasing the hydrophobicity of the casein micelles and the level of whey-protein-containing aggregates can increase the gelation pH of the acidified milks (Mottar et al., 1989; Lucey et al., 1997; Lucey et al., 1998; Vasbinder et al., 2001; Puvanenthiran et al., 2002; Anema et al., 2004a; Famelart et al., 2004; Vasbinder et al., 2004; Renan et al., 2006). As proposed in Section 10.4.1 and in earlier studies, the heat-induced aggregates may consist of denatured whey protein in the interior and κ -casein on the surface. The breakage of these aggregates may result in the exposure of a higher proportion of denatured whey proteins, which had relatively higher pI and hydrophobicity than casein

micelles. Consequently, increasing the number of small aggregates with potentially exposed denatured whey proteins may initiate the gelation at higher pH (Figure 10.10A).

In summary, there were three possible reasons to explain the increase in G' values of acid gels with the increase of free thiol groups. The first reason was the enhancement in thiol-disulphide exchange reactions during acidification. The second one was the increase in freedom of interactions between the proteins. The last reason was the production of numerous smaller aggregates on the breakage of the initial heat-induced aggregates. Based on the rheological results of Chapter 6, in which blocking the thiol groups of heated milks did not affect the G' values of acid gels, the first reason may not be the main factor determining the G' values of acid gels prepared from milks heated then treated with β -mercaptoethanol. In addition, as discussed in Section 6.4.2, the gelation conditions used in this study (3 h, 30 °C and 2% GDL) may not favour the occurrence of thiol-disulphide exchange reactions during the gelation process.

The high yield stress values of acid gels containing β -mercaptoethanol compared with control gels could be caused by the three reasons mentioned above. However, the yield stress values reached a maximum then decreased with the increase of β -mercaptoethanol concentrations. This could be related to the decrease in the number of intermolecular disulphide bonds on the addition of β -mercaptoethanol. Despite the increase in the degree of connections, the lack of disulphide bonds may weaken the structure of the acid gels because without disulphide bonds, the proteins can be re-arranged more easily. As the proportion of the disulphide bonds decreased, the force required to break the gel also decreased, leading to the observation of decreasing yield stress properties (Table 10.1).

10.4.3 Proposed scenario occurring in heated milks after addition of β -mercaptoethanol

Based on the electrophoretic and rheological results, a scheme was drawn to propose what may happen to the heat-induced aggregates when β -mercaptoethanol was added to heated milks (Figure 10.17). In control heated milks, the denatured whey proteins interact with each other via disulphide bonds and non-covalent bonds to form small units that can be linked together by intermolecular disulphide bonds. Furthermore, the units can interact with κ -casein polymers via disulphide bonds. As two or more units can share the same κ -casein polymer, these units were also connected. The assembly of the units formed heat-induced aggregates (Aggregate A in Figure 10.17).

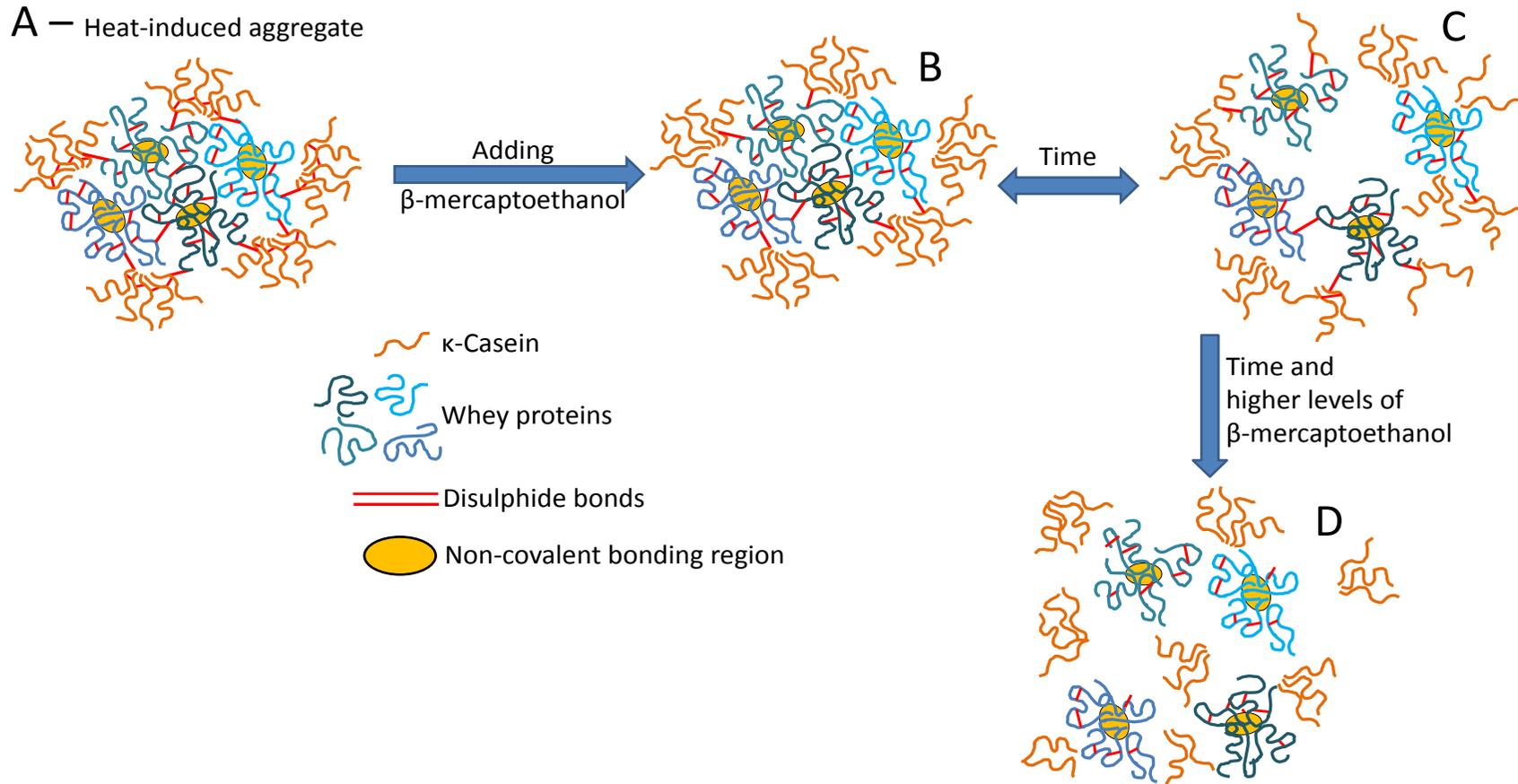


Figure 10.17: Schematic pictures showing the change of a heat-induced aggregate by the reduction of disulphide bonds. (A) heat-induced aggregates with κ -casein being located on the surface of the aggregates while the whey proteins are in the interior and all the inter-molecular disulphide bonds are intact, (B) heated-induced aggregates with the intermolecular disulphide bonds of κ -casein being reduced; hence can be broken into smaller aggregates (C); (D) shows the separated small aggregates after the disulphide bonds linking those aggregates together and to κ -casein were completely broken.

On addition of β -mercaptoethanol, the majority of κ -casein disulphide bonds were reduced (Figure 10.17B). As the κ -caseins were not disulphide-linked any more, some units were no longer connected (Figure 10.17C). When high levels of β -mercaptoethanol were added, the majority of disulphide bonds linking κ -casein to the whey-protein-aggregating-units and linking the units together would be broken. This led to the rupture of the heat-induced aggregates, releasing the smaller aggregates (Figure 10.17D).

10.4.3.1 Effects of adding β -mercaptoethanol to heated milks on the microstructure of the acid gels

In this study, acid gels with higher final G' values were more porous and seemed to consist of a lower level of connections (Figure 10.13 and Figure 10.14). This was contradictory to previous studies, in which the microstructures of acid gels with high final G' values often were found to contain a high number of connections between the proteins (Lucey et al., 1998; Guyomarc'h et al., 2009b). A possible explanation for the observation in this study is shown in Figure 10.18. In acid gels made from heated milk, the heat-induced aggregates consisting of smaller units are relatively bulky. Their connections give rise to a network that appear to contain small pores (Figure 10.18A). Upon breaking the heat-induced aggregates to smaller units, the units can spread out and can connect to each other into chains, resulting in a network that appears more porous (Figure 10.18B). Despite the porous structure, the degree of connections in the gels containing β -mercaptoethanol may be higher than that in the gels without β -mercaptoethanol as the smaller aggregates were connected by a higher number of contacting points when compared to bigger aggregates (Figure 10.18). In addition, the acid gels containing β -mercaptoethanol should have a greater degree of connections between the proteins than those without β -mercaptoethanol so that the former gels had higher G' and yield stress values than the latter ones. Unfortunately, this study did not have enough evidence to support this proposal. Further research is required to examine closely the connections in the network.

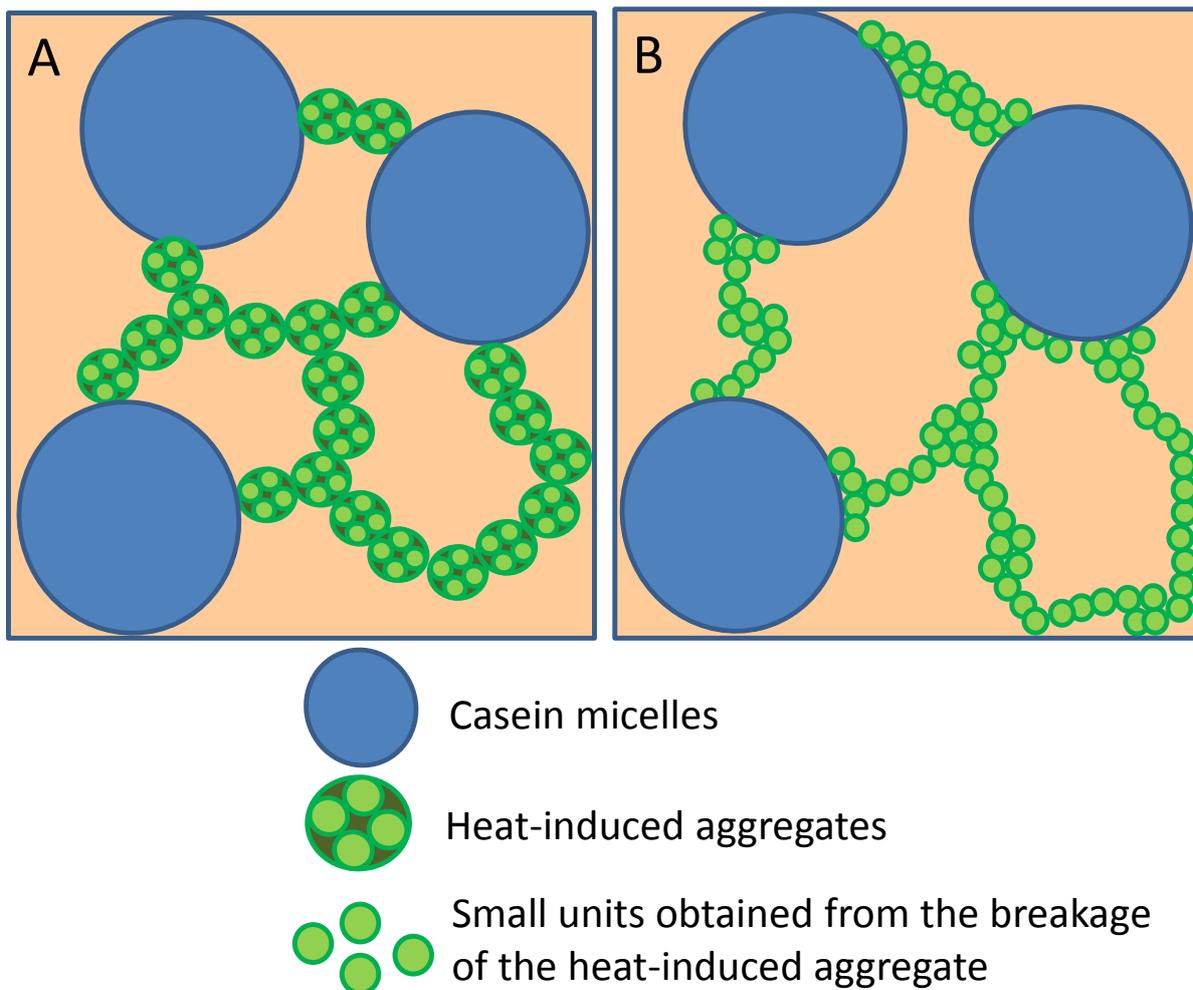


Figure 10.18: A schematic picture of a part of the acid gel network (2-dimensional) which was governed by the casein micelles and heat-induced aggregates. (A) acid gels prepared from control heated milk and (B) acid gels prepared from heated milks that had been treated with β -mercaptoethanol. Note: the number of small units is equal in both systems.

10.5 Conclusions

In heated skim milks (with and without added whey protein isolate), in which the majority of κ -casein and β -lactoglobulin were participating in intermolecular disulphide interactions, the disulphide bonds of κ -casein were still reduced in preference to those of β -lactoglobulin, as also observed in unheated skim milks. In addition, the reduction of disulphide bonds of denatured β -lactoglobulin in heated milk was as slow as that of native β -lactoglobulin in unheated milk, at any β -mercaptoethanol concentration.

Upon addition of β -mercaptoethanol to heated milks, the majority of disulphide bonds of κ -casein were reduced. A portion of the disulphide bonds of α -lactalbumin and β -lactoglobulin in the heat-induced aggregates were also broken. The combined effect may lead to the breakage of

the heat-induced aggregates to smaller aggregates. During acidification, heated milks that had been treated with β -mercaptoethanol had a higher number of aggregates that could participate in the formation of the gel network than in control heated milks. Consequently, the degree of connections in the network of gels containing β -mercaptoethanol was higher than that in the network of gel without β -mercaptoethanol. As a result, the acid gels containing β -mercaptoethanol were markedly firmer and were less prone to breakage at large deformation than the control acid gels.

Chapter 11 - General discussion

In this thesis, the extent of the heat-induced inter-molecular disulphide bonds in skim milk and WPE skim milk were modified by using **low** concentrations of thiol blocking reagent (NEM) and a disulphide-bond reducing agent (β -mercaptoethanol). The thiol blocking reagent was used to inhibit the thiol-disulphide exchange reactions between the proteins. Whereas, when added in low concentrations (i.e. lower than the total disulphide bonds existing in the milk systems), disulphide-bond reducing agent can enhance the exchange reactions between the proteins. The thiol reagents (NEM and β -mercaptoethanol) were added to unheated milks (Chapters 4 and 7), to unheated milks followed by heat treatment (Chapters 5 and 9) and to heated milks (Chapters 6 and 10). The interactions between the proteins and the rheological properties of acid gels prepared from milks with modified protein interactions were investigated. β -Mercaptoethanol was also added to protein solutions (β -lactoglobulin/ κ -casein or mixture of the two proteins) and the reduction of each protein was examined. Various hypotheses were proposed for the relationship between the type of protein interactions and subsequent properties of the acid gels and these were tested. The primary objective of this research was to ultimately be able to link the various protein interactions that had been affected by the inhibition or enhancement of thiol-disulphide exchange reactions to functional properties observed in the resulting acid gels. Hence the key findings of this research are discussed.

11.1 The susceptibility of the disulphide bonds of κ -casein to reduction

When an excess level of disulphide-bond reducing agent was added to skim milk, all of the existing disulphide bonds in the milk can be reduced into free thiol groups (Purkayastha et al., 1967; Sawyer, 1967). In addition, interactions between the disulphide-bond reducing agent and disulphide bonds in the proteins were often performed at temperatures $> 70\text{ }^{\circ}\text{C}$ (Goddard, 1996 as examples; Havea et al., 1998; Wada & Kitabatake, 2001). In skim milk, there are several proteins that contain disulphide bonds (e.g. α_{s2} -casein, κ -casein, α -lactalbumin and β -lactoglobulin). However it is not known if the disulphide-bond reducing agent interacted equally with all of the disulphide bonds of different milk proteins and if heat was required for the reduction reactions. This research found that when low concentrations of β -mercaptoethanol were added to skim milk and WPE skim milk, the disulphide bonds of polymeric κ -casein were reduced in preference to those of β -lactoglobulin, regardless of whether the proteins were in their native states in unheated milks/protein solutions (Chapter 7 and 8) or involved in the heat-induced aggregates in heated milks (Chapter 10). In addition, the

reactions between β -mercaptoethanol and protein disulphide bonds can occur at temperatures as low as 20 °C with the reaction rate increasing with the increase in temperature (Chapter 7, 8 and 10).

11.2 The relative importance of disulphide and non-covalent interactions in the formation of heat-induced aggregates

Heat-induced aggregates are formed when κ -casein and denatured whey proteins interact during the heating of milk at temperatures > 70 °C (Jang & Swaisgood, 1990; Corredig & Dalgleish, 1999; Lowe et al., 2004). It is generally accepted that denatured β -lactoglobulin interacts with denatured α -lactalbumin first, before the combined aggregates interact with κ -casein (Dalgleish et al., 1997a; Corredig & Dalgleish, 1999). The interactions between the proteins can be through disulphide bonds (Lowe et al., 2004) and/or non-covalent interactions (such as hydrogen, hydrophobic and ionic bonds) (Mckenzie et al., 1971; Haque & Kinsella, 1988). However, the relative importance of each type of interaction in the formation of heat-induced aggregates is not been clear. In this research, this was evaluated by inhibiting or enhancing thiol-disulphide exchange reactions between the proteins by adding NEM or β -mercaptoethanol to milks prior to heating, respectively (Chapter 5 and 9).

The formation of the heat-induced aggregates in skim milk and WPE skim milk was found to occur with denatured β -lactoglobulin interacting non-covalently with κ -casein, independently from the disulphide interactions between the proteins. This was observed when denatured β -lactoglobulin still associated with the casein micelles even though very low levels of β -lactoglobulin were involved in intermolecular disulphide bonds, due to the blocking of the free thiol groups (Chapter 5). Conversely, the proportion of β -lactoglobulin participating in heat-induced intermolecular disulphide bonds could be increased when more free thiol groups were present as a result of reducing some of the disulphide bonds produced in unheated milks prior to heating (Chapter 9).

Unlike β -lactoglobulin that can interact via both non-covalent and disulphide bonds with κ -casein, α -lactalbumin appeared to interact with other proteins mainly via disulphide bonds. When thiol-disulphide exchange reactions were inhibited, α -lactalbumin underwent reversible denaturation instead of interacting with other proteins via non-covalent interactions, as was observed for β -lactoglobulin (Chapter 5). This also shows that without disulphide interactions with β -lactoglobulin, α -lactalbumin cannot interact with κ -casein, as was observed previously (Dalgleish et al., 1997a; Oldfield et al., 1998a). When the thiol-disulphide exchange reactions were enhanced by using low levels of β -mercaptoethanol prior to heating, the proportion of α -

lactalbumin involved in intermolecular disulphide bonds was increased dramatically (Chapter 9).

In addition, a part of this research demonstrated that thiol-disulphide exchange reactions between the proteins in milk occurred only when the whey proteins, especially β -lactoglobulin were denatured (Chapter 7 and 8). It has been established that when milk is heated at temperatures $> 70\text{ }^{\circ}\text{C}$, β -lactoglobulin becomes denatured and exposes the free thiol groups that can subsequently initiate thiol-disulphide exchange reactions with other proteins (Corredig & Dalgleish, 1999). Adding of low levels of a disulphide-bond reducing agent to unheated skim milk and pure milk protein solutions (Chapters 7 and 8) was expected to introduce free thiol groups to milk proteins, hence enhance thiol-disulphide exchange reactions between the proteins without the denaturation of β -lactoglobulin. However, the absence of aggregates between the milk proteins confirmed that thiol-disulphide exchange reactions did not occur when whey proteins were native. Hence denaturation of β -lactoglobulin is considered to be required as a mean to firstly induce the non-covalent interactions between the proteins before disulphide interactions can occur. This finding supports the proposal in previous studies that non-covalent interactions are formed at an early stage in the formation of aggregates, and this is followed by the formation of disulphide bonds (Haque & Kinsella, 1988; Galani & Apenten, 1999).

When some of the intermolecular disulphide bonds between the milk proteins were reduced by adding low levels of β -mercaptoethanol to heated milks, denatured whey proteins were still found to be associated with the casein micelles (Chapter 10). This suggested that in heat-induced aggregates, denatured whey proteins were bonded to κ -casein via both disulphide and non-covalent interactions.

In conclusion, it was found that both non-covalent interactions and disulphide bonds between the milk proteins actively participate in the formation of the heat-induced aggregates. However, non-covalent bonds are probably necessary for the formation of protein aggregates while disulphide bonds are absent. This statement is supported by the findings of Havea et al. (2004; 2009). In these studies, the heat-induced gels made from whey protein concentrate (WPC) solution that contained only disulphide bonds had markedly lower G' values than those made from WPC solution that consisted of only non-covalent interactions (Havea et al., 2004; Havea et al., 2009). The disulphide bonds may specifically act in strengthening the bonding between the proteins in the aggregates and contribute to the functional properties of milk, which will be discussed in the next section.

11.3 The relative importance of disulphide bonds and non-covalent interactions between the proteins on the properties of acid gels

11.3.1 The firmness (G' values) of the acid gels

The importance of disulphide and non-covalent bonds between the proteins on the rheological properties of acid gels was evaluated by two different studies. The first study examined the relative importance of each interaction type present in the heat-induced aggregates on the properties of acid gels. In this study, low levels of thiol blocking or disulphide-bond reducing agents were added to milks before heating to modify the interactions between the proteins in the heat-induced aggregates (Chapters 5 and 9). The second study investigated the role of the bonds that may form during the acid gelation of the milk on the properties of the acid gels. This study involved adding low concentrations of thiol blocking or disulphide-bond reducing agents to unheated milks (Chapters 4 and 7) or to heated milks (Chapters 6 and 10) prior to acidification.

In the literature, the denaturation of whey proteins and the interactions between denatured whey proteins and κ -casein, particularly disulphide bonds, were reported to increase the storage modulus of acid gels (Dannenberg & Kessler, 1988c; Lucey et al., 1997; Lucey et al., 1998; Anema et al., 2004b). Experiments carried out and the results presented from this work reassessed the relative importance of each type of interaction on the rheological properties of acid gels.

Disulphide bonds in milk systems were found to be responsible for the production of acid gels with high G' values. Firstly, acid gels prepared from heated milks had markedly higher G' values compared to gels made from unheated milks. The elimination of intermolecular disulphide bonds through the addition of NEM in skim milk caused the acid gels to have lower G' values than those made from control heated skim milk (Chapter 5). The G' values of acid gels made from WPE skim milk heated in the presence of β -mercaptoethanol started to decrease at 7.1 mM β -mercaptoethanol (Chapter 9). In addition, even though acid gels prepared from WPE skim milk heated in the presence of thiol reagents (either NEM or β -mercaptoethanol) had higher G' values than those prepared from control heated milks, the G' values were higher in acid gels prepared from β -mercaptoethanol-treated WPE milk (Chapter 9) than in acid gels prepared from NEM-treated WPE milk (Chapter 5). The main difference between two systems was that milks treated with low levels of β -mercaptoethanol had a higher percentage of proteins participating in intermolecular disulphide bonds than milks treated with low level of NEM did. In NEM-treated milks, the proteins were mostly interacted via non-covalent bonds (Chapter 5).

This demonstrated that disulphide bonds were required in order to produce acid gels with increased G' values.

The disulphide bonds between the denatured whey proteins and those between whey proteins and κ -casein were considered to be more important than those between κ -casein molecules alone in heated milks in order to produce firm acid gels. When skim milk and WPE skim milk were heated in the presence of β -mercaptoethanol, the proportion of whey proteins involved in intermolecular disulphide bonds increased while the majority of κ -casein was not disulphide-bonded but the resulting acid gels had higher G' values than those prepared from control heated milks (Chapter 9). Similarly, when heated skim milk and WPE skim milk were subsequently treated with β -mercaptoethanol, the majority of the intermolecular disulphide bonds left in the system were between denatured whey proteins and the acid gels prepared from these milks had higher G' values than those prepared from control heated milks (Chapter 10).

Non-covalent interactions between the proteins in milks were found to be both important in the formation of firm acid gels. This was shown by the high G' values of acid gels prepared from WPE skim milk heated in the presence of NEM (Chapter 5), in which non-covalent bonds between the denatured β -lactoglobulin and κ -casein were proposed to be the predominant bonding interactions. In another study, it was proposed that disulphide interactions between κ -casein and β -lactoglobulin were broken on addition of β -mercaptoethanol to heated milks and any remaining interactions between these proteins were therefore non-covalent. Interestingly, the G' values of acid gels made from these treated milks were higher than those made from control heated milks (Chapter 10). Despite the decrease in the total level of disulphide bonds between the proteins in milks, the G' values of the resulting acid gels still increased (Chapter 9). This demonstrated that non-covalent interactions between the proteins alone can be sufficient for the formation of acid gels with high firmness.

Overall, both non-covalent and disulphide interactions between the proteins in milks were important in influencing the G' values of the resulting acid gels with the higher the level of connections between the proteins, the higher the G' values.

11.3.2 The yield properties of the acid gels

When the acid gels are subjected to a constant shear rate, the strain and shear stress values of the acid gels increase progressively until the acid gels cannot withstand the shear any longer and break. The yield stress is the maximum shear stress value before the breakage of the gels and the yield strain is the strain at the point the gel breaks.

The factors determining the yield stress values of the gels include the number of bonds between the proteins per cross-sectional area in the gel network and the strength of the bonds (van Vliet et al., 1991; van Vliet & Walstra, 1995; Lakemond & van Vliet, 2008b). In this study, the yield stress values of acid gels appeared to depend mainly on the disulphide bonds between the proteins. The yield stress values of the acid gels decreased with a decreasing proportion of total proteins being involved in intermolecular disulphide bonds (Chapters 5 and 9). Contrary to expectations, acid gels made from heated milks that were subsequently treated with β -mercaptoethanol (Chapter 10) had higher yield stress values than those made from control heated milks and heated milks treated with NEM (Chapter 6) despite the fact that β -mercaptoethanol-treated milks had a lower percentage of disulphide-linked proteins. This may be explained by the thiol-disulphide exchange reactions between the proteins during acidification.

In previous studies, it was proposed that thiol-disulphide exchange reactions can occur between the proteins during acidification as the acid gels that were prepared from heated milks or whey protein solutions in which all of the thiol groups were blocked had lower G' values than those made from control heated samples (Lucey et al., 1998; Alting et al., 2000; Alting et al., 2003; Vasbinder et al., 2003). The findings in this research demonstrated that the exchange reactions may occur during acid gelation but to a lesser extent when compared to those in previous studies. When the thiol groups in unheated milks and heated milks were blocked by NEM, the yield stress values of acid gels decreased markedly with the increase of NEM concentrations (Chapter 4 and 6). Whereas, the yield stress values of acid gels increased with the increase of free thiol groups in heated milks (Chapter 10). This suggested that thiol-disulphide exchange reactions did occur during acidification and that new disulphide bonds were formed between particles in the gel network. These newly formed disulphide bonds may assist the gels to withstand large deformations. Therefore, acid gels made from heated milks that were treated with NEM had lower yield stress values than those made from control heated milks, that in turn had lower yield points than those made from heated milks with added β -mercaptoethanol (Chapters 6 and 10). The yield stress values decreased with the decrease in level of free thiol groups in heated milks before acidification.

The yield strain values were dependent on factors such as the degree of curvature of the strands as the strands within the gel network first need to be straightened and then stretched until the strands or the bonds within the strands are ruptured. The yield strain is found to increase with the increase in strand curvature (van Vliet & Walstra, 1995; Lakemond & van Vliet, 2008b). In this study, the yield strain values increased with the increase of yield stress (Chapter 10) or decreased with the decrease of yield stress (Chapters 5, 6 and 9). This suggested that the strand

curvature was decreased when the total proportion of intermolecular disulphide bonds was decreased or the formation of new disulphide bonds during acid gelation was inhibited. On the other hand, the strand curvature can be increased by enhancing formation of disulphide bonds during gelation. Nevertheless, further research is required to confirm the change in the curvature of strands in the gel network as an effect of modifying the interactions between the proteins.

In conclusion, the yield stress values of the acid gels can be increased by increasing the proportion of disulphide bonds in the milk systems or by enhancing the thiol-disulphide exchange reactions between the proteins during the acid gelation, hence new inter-particle disulphide bonds can be formed between the protein particles in the gel network.

11.3.3 The effects of whey protein concentrations on the rheological properties of the acid gels

The acid gels prepared from control heated WPE skim milk had higher G' and yield stress properties than those made from heated skim milk (Chapters 5, 6, 9 and 10), as has been observed previously (Lucey et al., 1999b; Vasbinder et al., 2004). This is due to the higher concentrations of proteins that can participate in the formation of the gel network and the higher proportion of protein interactions, both disulphide and non-covalent bonds, in heated WPE skim milk than in heated skim milk. The effects of inhibiting or enhancing the thiol-disulphide exchange reactions on the protein interactions were similar in both WPE skim milk and in skim milk. For example, the decrease of the proportion of disulphide-linked whey proteins due to blocking the thiol groups of β -lactoglobulin can be observed in both skim milk and WPE skim milk that were heated in the presence of NEM (Chapter 5). When β -mercaptoethanol was added to the milk systems before heat treatment, it was observed that the proportion of disulphide-linked whey proteins increased in both skim milk and WPE skim milk at low concentrations of β -mercaptoethanol then decreased as the β -mercaptoethanol concentration increased further (Chapter 9).

In most cases, modifying the interactions between proteins had the same effects on the rheological properties of skim milk acid gels compared with those of WPE skim milk gels. This was evidenced by a) the increase in the G' values of both skim milk and WPE skim milk gels due to the enhancement of thiol-disulphide exchange reactions between the proteins during and after heat treatment (Chapters 9 and 10) and b) the decrease/increase of yield stress values of both skim milk and WPE skim milk gels due to the inhibition/enhancement of thiol-disulphide exchange reactions during acidification (Chapters 4, 6 and 10). However, the effect on the yield properties was greater in WPE skim milk gels than in skim milk gels (Chapters 4, 5, 6, 9 and 10).

Yield properties were greatly influenced by the disulphide bonds being formed between neighbouring particles during acid gelation (Chapter 10). Thiol-disulphide exchange reactions between the proteins can occur to a greater extent in WPE skim milk than in skim milk because of the high concentration of whey proteins. Firstly, the exchange reactions were first order with respect to the disulphide bond and the thiol group (Fava, 1957; Whitesides et al., 1977). Secondly, the proteins in the WPE skim milk solution or gels are more closely packed than those in skim milk solution/gels, as shown in the microstructures of the gels (Chapters 5, 6, 9 and 10). Hence the reactions can occur more readily during acid gelation of WPE skim milk compared with skim milk. As a result, modifying the interactions between the proteins by either enhancing or inhibiting the thiol-disulphide exchange reactions affected the yield properties of WPE skim milk gels to a greater extent than those of skim milk gels.

An exception was the acid gels prepared from milks heated in the presence of NEM: WPE skim milk gels showed an increase in G' values with the increase of NEM concentrations while skim milk gels showed a slight decrease in G' values with an increase of NEM concentrations (Chapter 5). It was proposed that non-covalent interactions amongst denatured β -lactoglobulin and between denatured β -lactoglobulin and κ -casein were dominant in WPE skim milk that was heated in the presence of NEM (Chapter 5). The rheological results demonstrated that even though milks containing only non-covalent interactions between the proteins can produce acid gels with high G' , there should be a sufficient proportion of proteins participating in the non-covalent interactions in order to influence the G' values. This can be achieved by adding whey proteins into the system as whey proteins can readily interact non-covalently to each other (Hoffmann & van Mil, 1997; Havea et al., 2009). A preliminary experiment was carried out to support this proposal. In this experiment, skim milk was enriched with whey protein so the protein concentration was increased to $\sim 4\%$ (Skim milk had $\sim 3\%$ protein and WPE skim milk had $\sim 4.5\%$ protein). The final G' values of acid gels prepared from 4%-protein-skim milk that had been heated in the presence of NEM were found to increase with the increase of NEM concentrations. However, this increase was lower than the increase found in WPE skim milk gels (Appendix 11 and Chapter 5). This test reinforced the claim that adding whey protein to skim milk can enhance the non-covalent interactions between the proteins when the formation of intermolecular disulphide bonds was eliminated and the high proportion of non-covalent interactions can increase the G' values of the resulting acid gels.

11.4 General remarks

The findings of this research demonstrated that the thiol-disulphide exchange reactions between the proteins can be systematically enhanced or inhibited by adding low concentrations of a disulphide-bond reducing agent or a thiol blocking reagent to the milk systems. Hence it is

possible to manipulate the proportion of disulphide bonds and non-covalent interactions as well as the composition of the heat-induced aggregates in the milk systems. The knowledge gained from this research allows the production of milk products with desired properties. For example, it is possible to produce an acid milk gel that had both G' and yield stress at high values or an acid gel that had high G' value but low yield stress. The possibility of controlling the functional properties of the milk products is highly desirable in the dairy industry.

The results from this research emphasised the importance of non-covalent interactions between the proteins during the formation of heat-induced aggregates. This piece of information can contribute to the overall understanding of the mechanism of interactions between the proteins to form heat-induced aggregates in heated milk systems, a topic that has been extensively studied but has not been fully understood.

Furthermore, the interactions between the whey proteins in milk have been shown to be as important as those between β -lactoglobulin and κ -casein in the heat-induced aggregation and the functional properties of milk products. Whey protein concentration in milk can influence the properties of the heat-induced aggregates as whey proteins are considered to make up the interior of those aggregates with κ -casein on the surface. In addition, an increase in the level of whey protein in milk facilitates the interactions between the proteins during the heating milk and formation of acid gels. Therefore, the concentration of whey protein can be adjusted to generate more protein interactions, and hence tailor the properties of heated milk and milk products.

Chapter 12 - Conclusions and recommendations

12.1 Conclusions

A series of studies involving the modification of the interactions between the proteins in milks by enhancing or inhibiting the thiol-disulphide exchange reactions between the proteins showed that non-covalent interactions between denatured β -lactoglobulin and κ -casein may occur first and are required for the formation of heat-induced aggregates. After the initial aggregation due to non-covalent bonding, intermolecular disulphide bonds between β -lactoglobulin, κ -casein and α -lactalbumin can then be formed. The formation of these disulphide bonds can be enhanced by systematically enhancing the thiol-disulphide exchange reactions between milk proteins during heating. The intermolecular disulphide bonds between β -lactoglobulin and α -lactalbumin were important in the association of α -lactalbumin with the heat-induced aggregates whereas intermolecular disulphide bonds between β -lactoglobulin and κ -casein can be absent in the aggregates.

The same studies revealed that both disulphide bonds and non-covalent interactions between proteins in the heat-induced aggregates played an important role in the formation of acid gels with high G' values. In some cases, non-covalent interactions between the proteins in milks were sufficient to produce high G' acid gels. On the other hand, the yield properties of the acid gels were dependent mostly on the disulphide bonds in the gel network. The increase in the total proportion of disulphide-linked proteins in milk before acidification can increase the yield stress values of the gels. In addition, the enhancement of thiol-disulphide exchange reactions between the proteins during acid gelation can increase the connections between the protein particles in the gel network, hence strengthening the acid gels against the large deformation.

The findings of this research have shown that the functional properties of milk products can be tailored by modifying the proportion of disulphide interactions in relative to the non-covalent interactions between the proteins in milk systems.

12.2 Recommendations for future work

This piece of research has shown that introducing free thiol groups to milk systems enhanced the thiol-disulphide exchange reactions between the milk proteins. The mechanism of formation of heat-induced aggregates in the treated milks was also proposed based on the existing findings (Chapter 9 and 10). Further research is necessary to determine which proteins were capable of initiating the disulphide exchange reactions apart from the natural initiator (β -lactoglobulin) when that protein was induced with free thiol groups. The finding will be able to

verify the proposed mechanisms in this research regarding the interactions between the proteins in milks treated with β -mercaptoethanol.

In order to verify which protein can initiate the thiol-disulphide exchange reactions, reduction of κ -casein, α -lactalbumin and β -lactoglobulin should be carried out separately. This means that each protein should be reduced, one at a time, to contain free thiol groups. Preliminary experiments were attempted during this research. However due to time constraints, the experiments were not pursued further. The conditions and methods can be found in Appendix 12.

It will be worthwhile to investigate the reduction of α -lactalbumin and β -lactoglobulin disulphide bonds on addition of the disulphide reducing agents to milks. Differential Scanning Calorimetry can be a technique to be employed to monitor the conformational change of the proteins. As the intra-molecular disulphide bonds of the whey proteins contribute to the determination of the tertiary structure of the protein, the breakage of those disulphide bonds will lead to a structural change. Monitoring the structural change of the whey protein may provide information on the degree of reduction that takes place and the changes to protein structure. This experiment may also provide information on the relative accessibility of the disulphide bonds of α -lactalbumin and β -lactoglobulin towards the disulphide bond reducing agents.

Throughout the research, it was thought that the composition and size of the heat-induced aggregates were affected by modifying the interactions between the proteins. These proposals could be verified by examining soluble aggregates using size exclusion column chromatography (SEC), SEC with Multi-angle laser light scatter (SECMALLS) and the aggregates on the casein micelles using atomic force microscopy (AFM). The SEC/SECMALLS techniques will provide information on the composition of the aggregates and an estimation of the molecular weight of the aggregates, hence an estimation of the aggregate size. The AFM technique can provide information on the structure and the size of the aggregates. Hence a finding may provide information on the importance of size and composition of the heat-induced aggregates on the properties of the resulting acid gels.

Chapter 13 - References

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Chapter 14 - Appendices

Appendix 1: Characteristics of casein micelles

The summary of the characteristics of the casein micelles that was mentioned in Section 2.3.3 (p. 2-10) is presented in the appendix.

Table A.1: Average characteristics of casein micelles (Fox & Brodkorb, 2008).

Diameter	200 nm (range: ~ 50 - 500 nm)
Surface area	$8 \times 10^{-10} \text{ cm}^2$
Volume	$2.1 \times 10^{-15} \text{ cm}^3$
Density (hydrated)	1.0632 g cm^{-3}
Mass	$2.2 \times 10^{-15} \text{ g}$
Water content	63 %
Hydration	$3.7 \text{ g H}_2\text{O g}^{-1} \text{ protein}$
Voluminosity	$44 \text{ cm}^3\text{g}^{-1}$
Molecular mass (hydrated)	$1.3 \times 10^9 \text{ Da}$
Molecular mass (dehydrated)	$5 \times 10^8 \text{ Da}$
Number of peptide chains	5×10^3
Number of particles per mL milk	$10^{14} - 10^{16}$
Surface of micelles per mL milk	$5 \times 10^4 \text{ cm}^3$
Mean free distance	240 nm

Appendix 2: The $\tan \delta$ values of acid gels

The change of $\tan \delta$ values (G''/G') during the acid gelation of unheated milks with added NEM, which was mentioned in Sections 4.3.3 (p/4-77), is presented in Figure A.1. The presence of NEM did not affect the $\tan \delta$ values of the acidified milks.

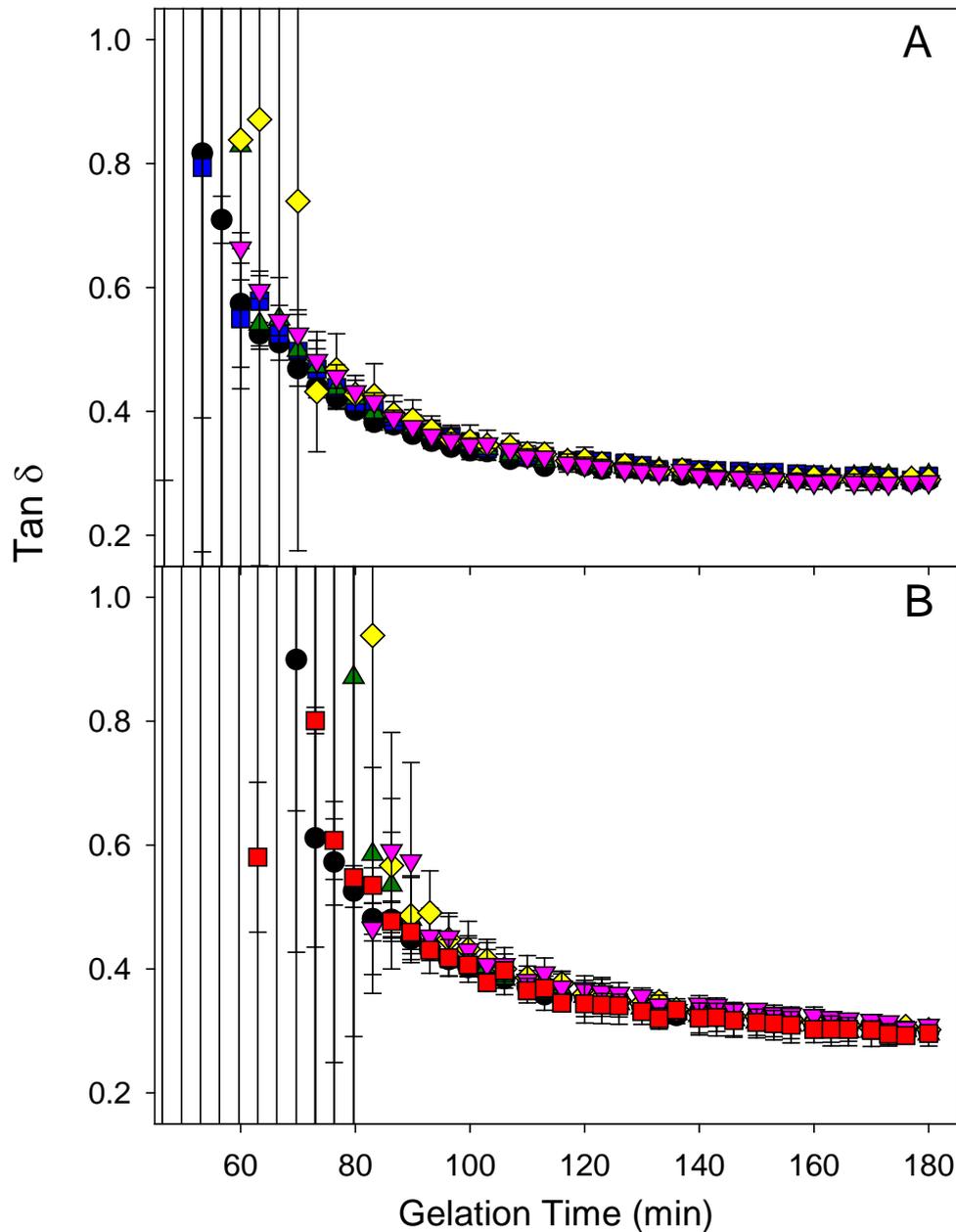


Figure A.1: The change of $\tan \delta$ during acidification of (A) unheated skim milk and (B) unheated WPE skim milk with different concentrations of NEM. ●, 0 mM; ■, 0.08 mM; ▲, 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM; ■, 0.8 mM NEM. Each data point is the average of two to five replicates. Error bars represent the standard deviation.

The effect of NEM concentrations on the change of $\tan \delta$ values during acid gelation of heated skim milk and heated WPE skim milk that was mentioned in Section 6.3.2 (p/6-129) is summarised in Figure A.2.

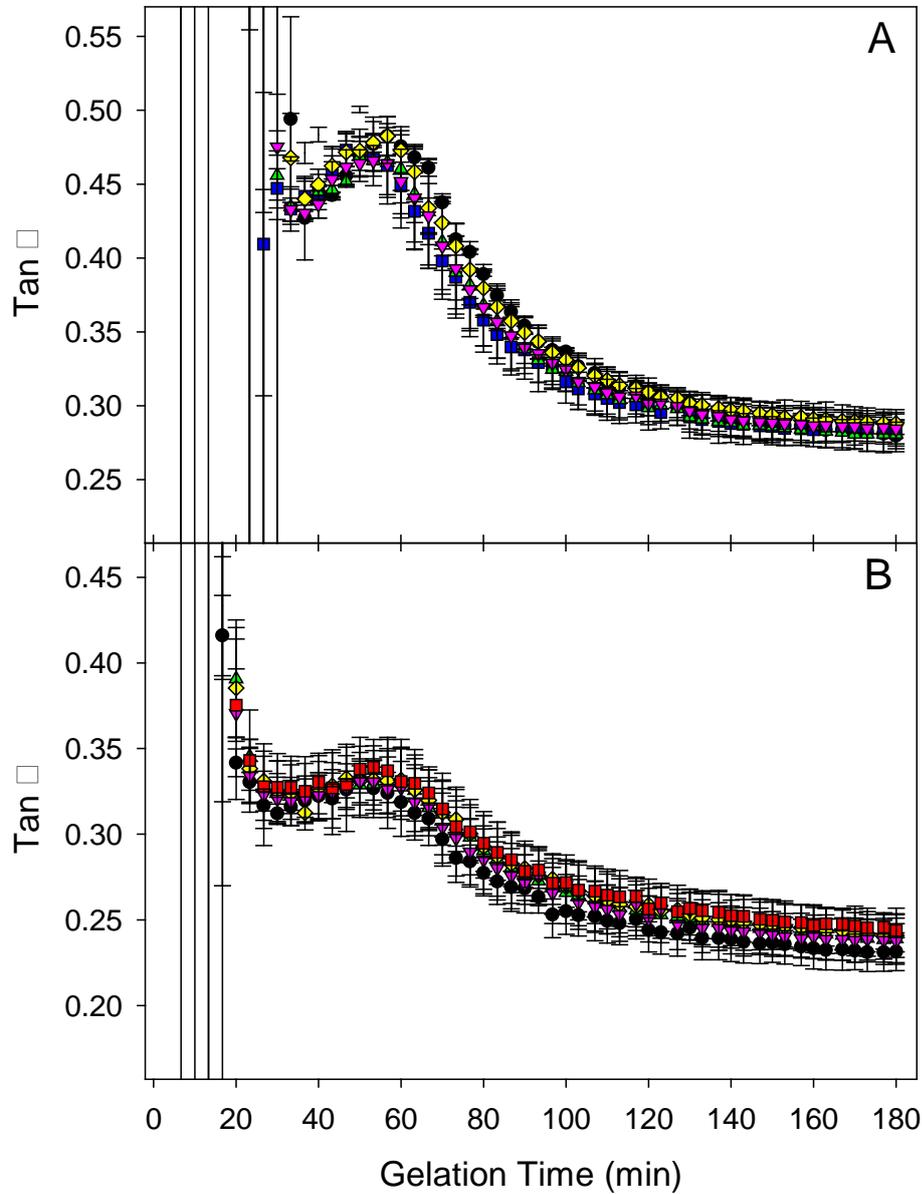


Figure A.2: Effects of adding NEM to heated skim milk (A) and heated WPE skim milk (B) on the change of $\tan \delta$ during acidification. ●, 0 mM ; ■, 0.08 mM; ▲, 0.24 mM; ◆, 0.4 mM; ▼, 0.6 mM and ■, 0.8 mM. Each point is the average of four to six replicates. Error bars represent the standard deviation.

Results of the effects of β -mercaptoethanol on the change of $\tan \delta$ values during the acid gelation of unheated milks were mentioned in Section 7.3.4 (p/7-157) and are present in Figure A.3.

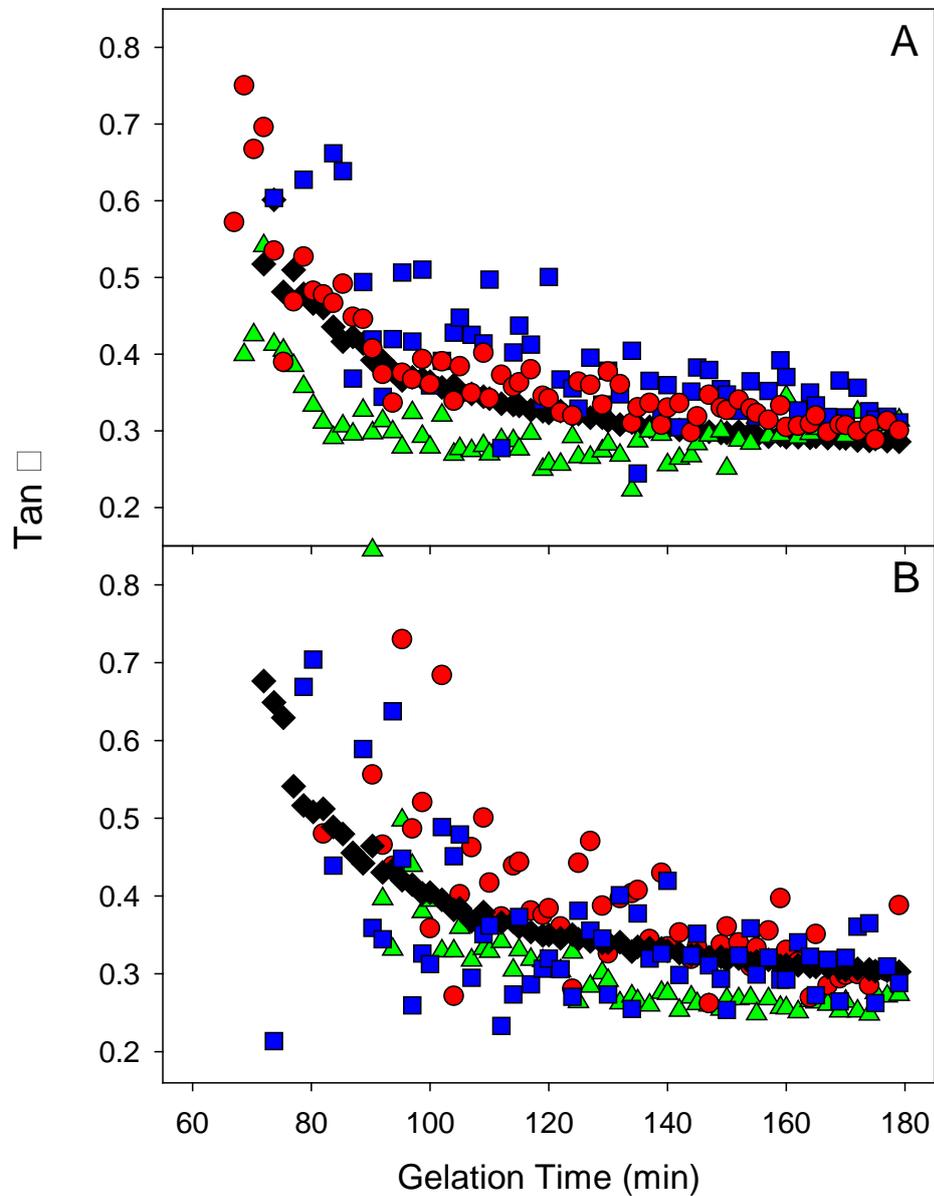


Figure A.3: The change of $\tan \delta$ during the acid gelation of unheated skim milk (A) and unheated WPE skim milk (B) that had been treated with zero mM (\blacklozenge), 1.4 mM (\blacktriangle), 4.3 mM (\blacksquare) and 7.1 mM (\bullet) β -mercaptoethanol for 3 h. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

Effects of β -mercaptoethanol on the change of $\tan \delta$ values during acidification of heated milks were mentioned in Section 10.3.3 (p/10-237) and are present in Figure A.4.

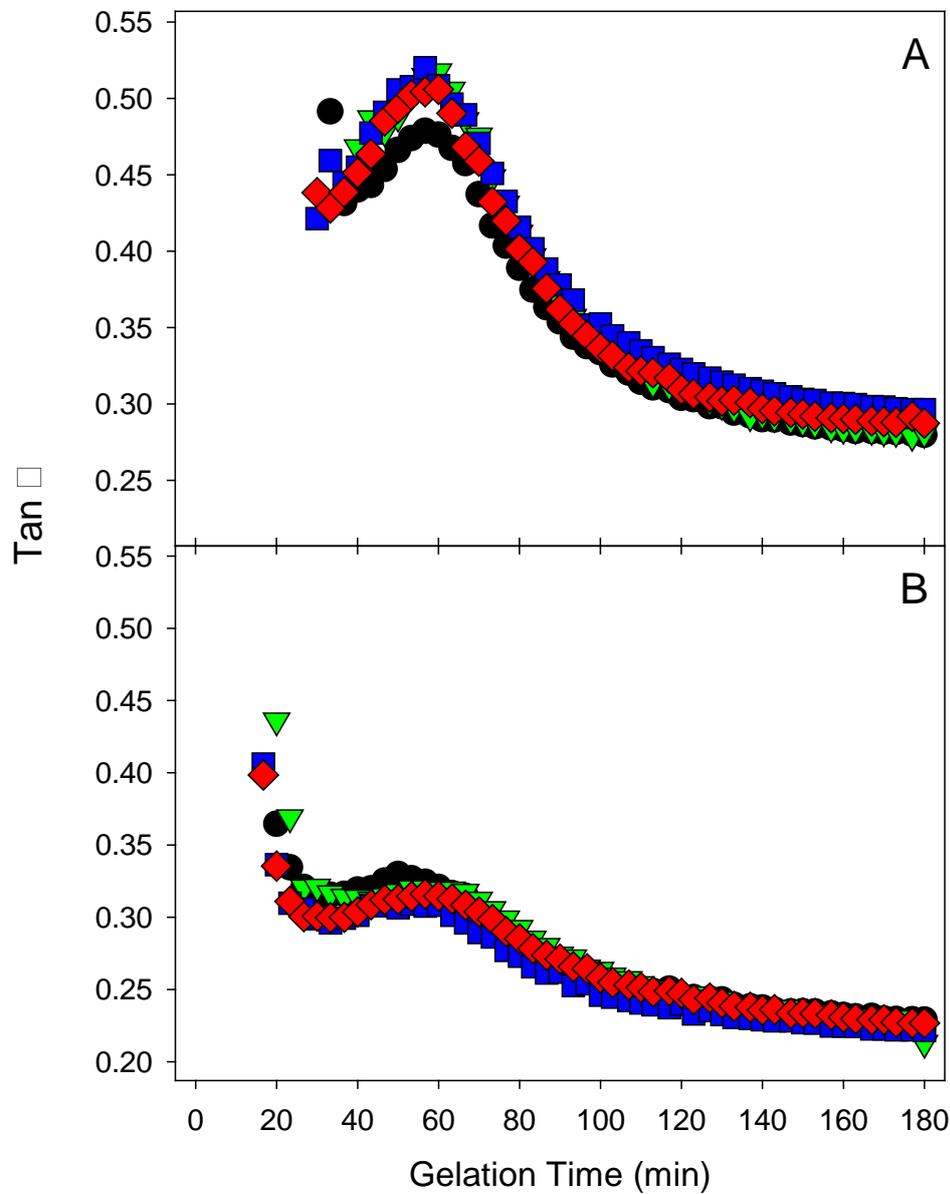


Figure A.4: The change of $\tan \delta$ values during the acid gelation of skim milk (A) and WPE skim milk (B). Milks were heated (80 °C, 30 min) before reacting with β -mercaptoethanol. ●, 0 mM; ▼, 1.4 mM; ■, 4.3 mM; ◆, 7.1 mM β -mercaptoethanol. Each point is the average of four to six replicates. Error bars represent the standard deviation.

Appendix 3: Effect of cone and plate geometry on the acid gelation of NEM-treated WPE skim milk

Results were mentioned in Section 5.2, p/5-89.

The acid gelation of WPE skim milk that was heated in the presence of 0.8 mM NEM was initially measured using a cone and plate geometry. After the milks gelled, the G' values increased progressively and suddenly decreased. The G' values then either continued to decrease or increased again. This indicated that the structure of the forming acid gels were probably broken.

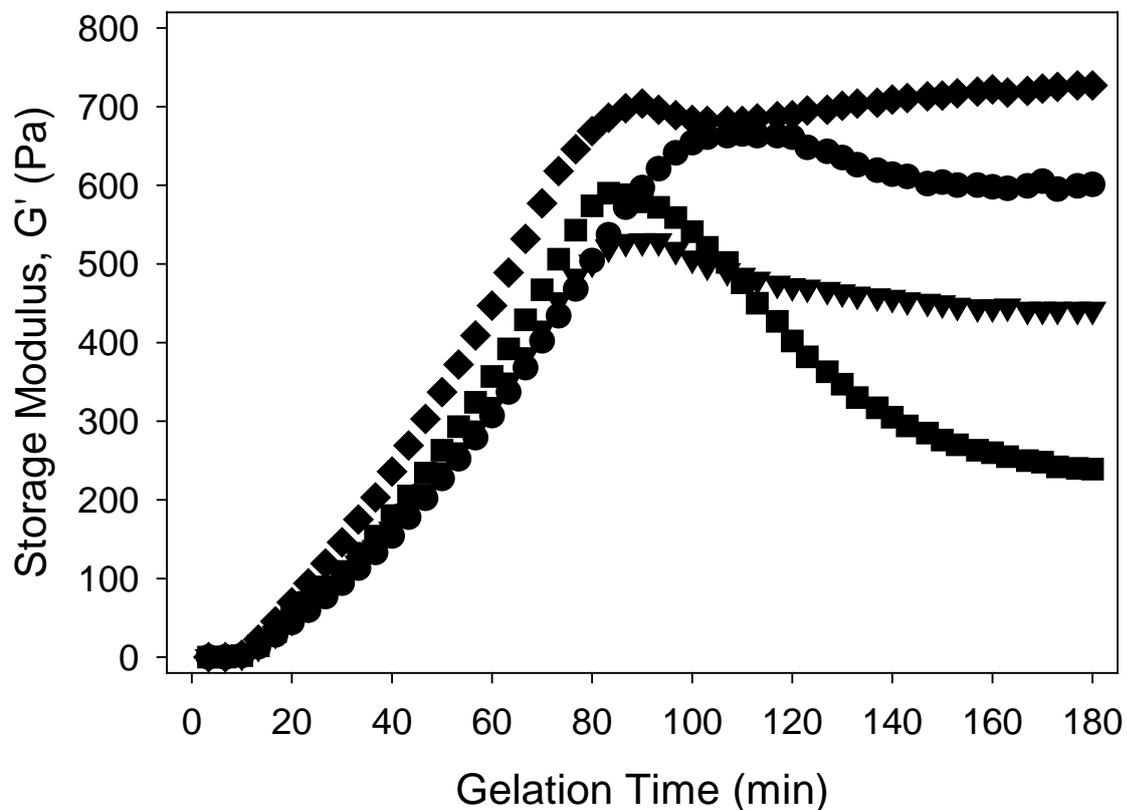


Figure A.5: The change of G' values during the acid gelation of WPE skim milk that was heated in the presence of 0.8 mM NEM. \blacklozenge \blacksquare \blacktriangledown \bullet , independent measurements carried out using a cone and plate geometry.

Appendix 4: Effects of reaction time and β -mercaptoethanol concentrations on the properties of unheated skim milk

Results were mentioned in Section 7.3.1.1 (p/7-145).

Figure A.6 and Figure A.7 demonstrate the effects of reaction time after addition of β -mercaptoethanol to unheated skim milk prior to analysis on the size of the casein micelles in skim milk and the final G' values of the resulting acid gels.

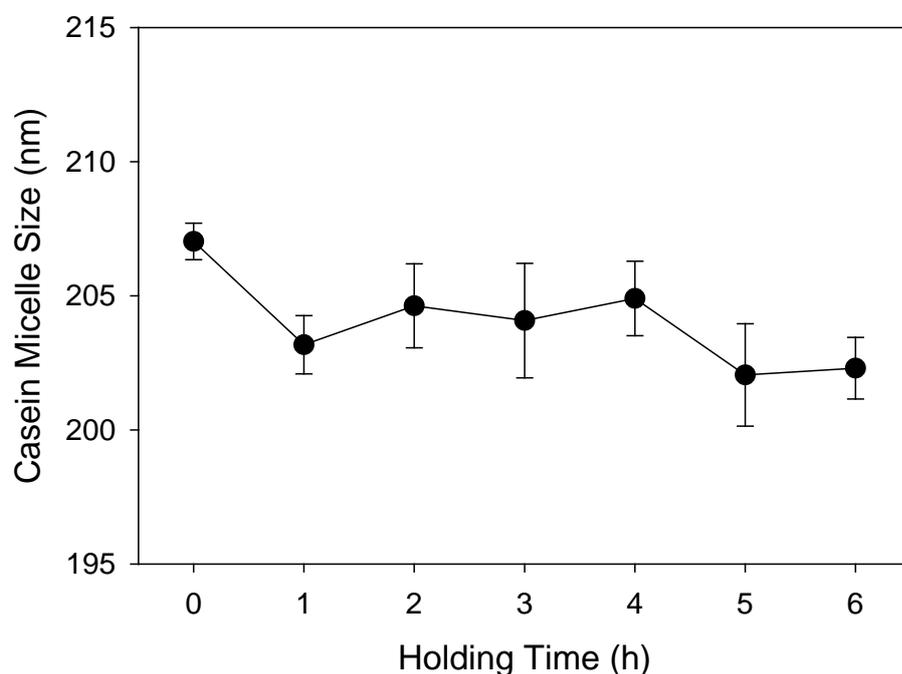


Figure A.6: The effect of reaction time on the size of the casein micelles in skim milk treated with 7.1 mM β -mercaptoethanol. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

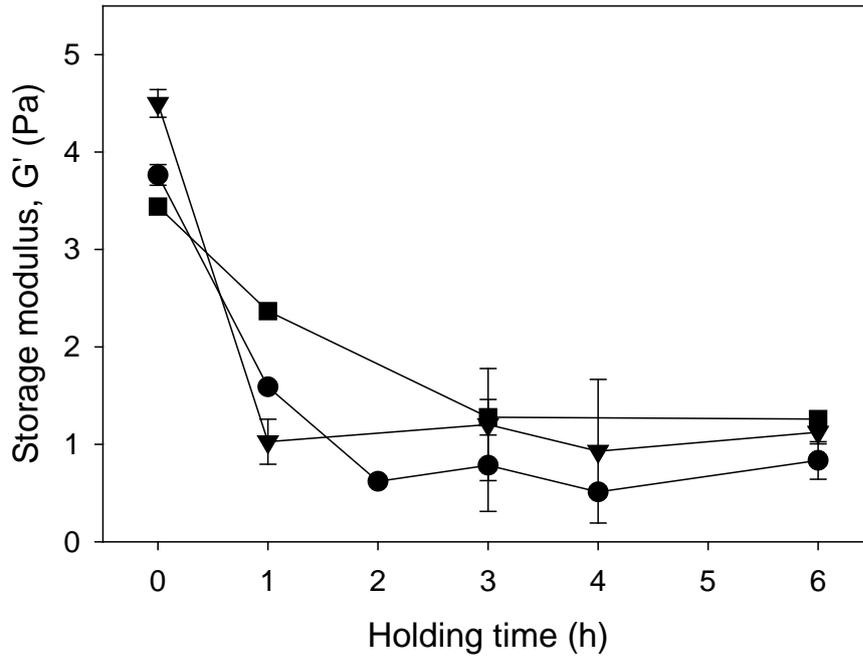


Figure A.7: The effect of reaction time on the final G' of acid gels made from skim milk treated with varying β -ME concentrations: 1.4 (■), 4.3 (●) and 7.1 (▼) mM. Each data point is the average of two to four replicates. Error bars represent the standard deviation.

Appendix 5: Effects of β -mercaptoethanol concentrations on the zeta potential of casein micelles in skim milk

Results were mentioned in Section 7.3.3 (p/7-155) and Section 10.3.2 (p/10-233).

Figure A.8 shows that the zeta potential of casein micelles was not affected by the addition of β -mercaptoethanol to skim milk and WPE skim milk, regardless of whether β -mercaptoethanol was added into unheated milk or heated milk or unheated milk followed by heat treatment.

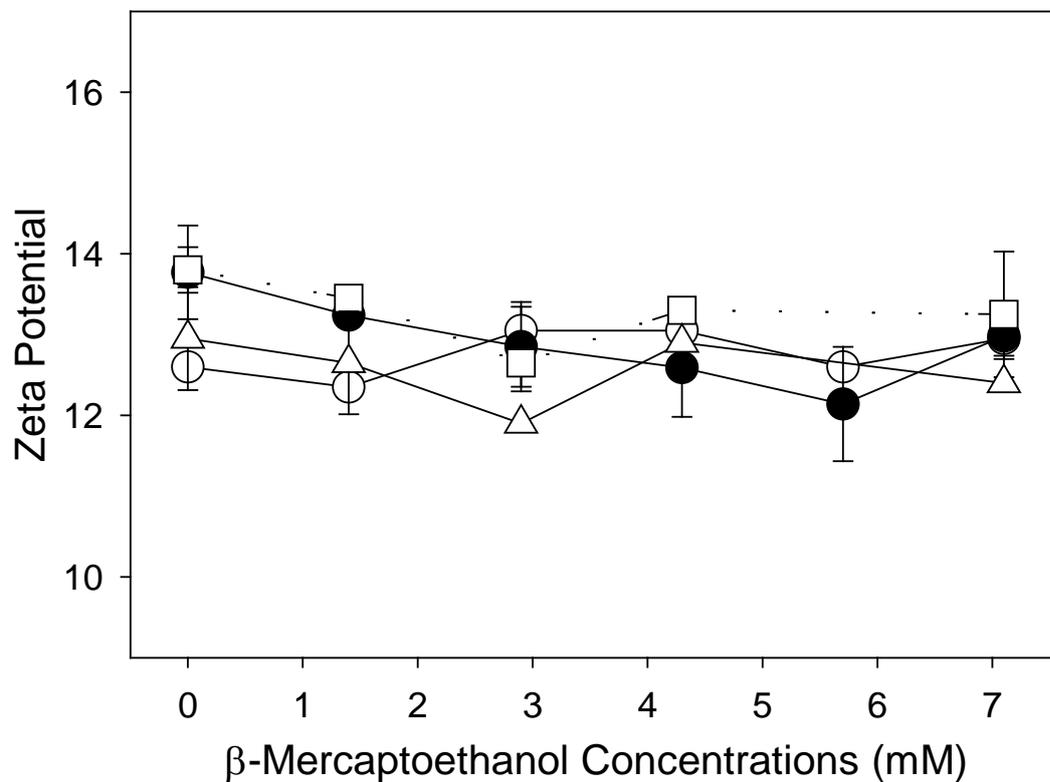


Figure A.8: The effects of β -mercaptoethanol concentrations on the zeta potential of the casein micelles in milks. □, unheated skim milk; △, heated skim milks; ○, skim milk heated in the presence of β -mercaptoethanol; ●, WPE skim milk heated in the presence of β -mercaptoethanol.

Appendix 6: Effects of β -mercaptoethanol concentrations, temperatures and time of reaction on the reduction of κ -casein and β -lactoglobulin

This was mentioned in Section 8.3.4 (p/181).

MF-electropherograms in Figure A.9 show the typical change of the level of reduced β -lactoglobulin and monomeric κ -casein peaks when β -mercaptoethanol reacted with the proteins at different temperatures for different period of time.

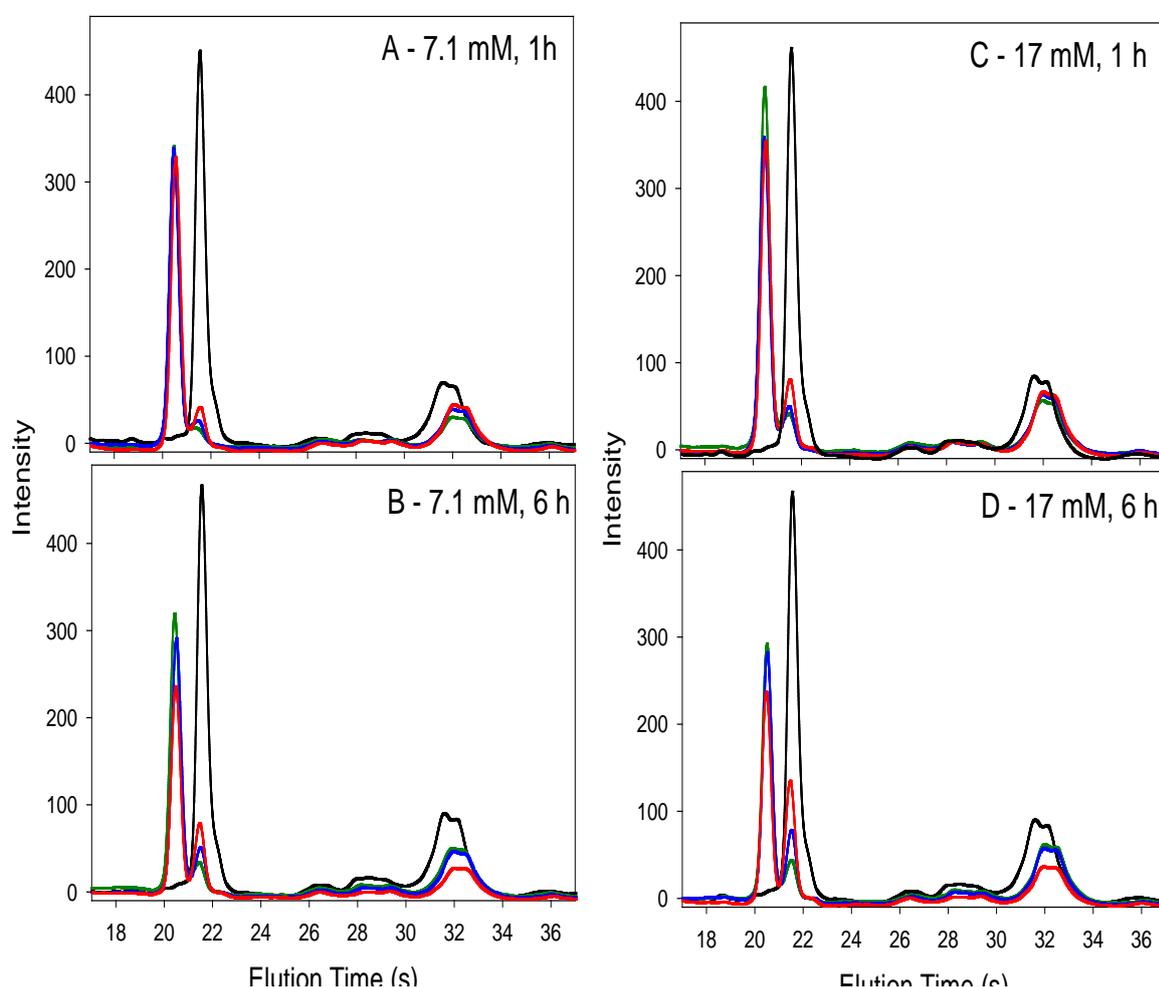


Figure A.9: MF-electropherograms showing the typical change in the peak intensity of non-reduced and reduced β -lactoglobulin and κ -casein in the mixed-protein solution that had been treated with 7.1 mM and 17 mM β -mercaptoethanol for 1 and 6 h at 20, 30 and 40 °C. Green line, 20 °C; Blue line, 30 °C and Red line, 40 °C. Black line represents the total amount of β -lactoglobulin and κ -casein present in the studied solution.

Appendix 7: Calculation of the increase in volume of the casein micelles in milk heated in the presence of β -mercaptoethanol.

Results were mentioned in Section 9.4.1.3 (p/9-215) regarding the increase in volume of the casein micelles in milks that were heated in the presence of β -mercaptoethanol.

$$V = \frac{4}{3} \times \pi \times r^3 \quad \text{Equation A.1}$$

Table A.2: Effects of β -mercaptoethanol concentrations on the average size of the casein micelles, their corresponding volume and the ratio of the casein micelle size in treated milks to that in control milks.

β -mercaptoethanol concentration (mM)	Skim milk			WPE skim milk		
	Average diameter (nm)	Volume (nm ³)	Ratio	Average diameter (nm)	Volume (nm ³)	Ratio
0	190.0	3.6×10^6	1	207.1	4.7×10^6	1
1.4	189.9	3.6×10^6	1	223.3	5.8×10^6	1.2
4.3	201.3	4.3×10^6	1.2	277.2	7.5×10^6	2.4
7.1	211.5	5.0×10^6	1.4	404.3	11×10^6	7.4

Appendix 8: Comparison the results obtained from MF-electrophoresis to those from SDS-PAGE.

MF-electrophoresis and SDS-PAGE gave different results on the levels of reduced β -lactoglobulin and monomeric κ -casein in heated milks that were treated with β -mercaptoethanol. Results were mentioned in Section 10.3.2 (p/10-229) and show in Table A.3.

Table A.3: The difference in the percentage of reduced β -lactoglobulin and monomeric κ -casein over the total of that protein presence in heated skim milk found by different electrophoretic methods.

Concentration of β -mercaptoethanol (mM)	β -Lactoglobulin		κ -casein	
	SDS-PAGE	MF-electrophoresis	SDS-PAGE	MF-electrophoresis
0	27 \pm 7	9 \pm 1	23 \pm 5	20 \pm 2
1.4	31 \pm 10	9 \pm 2	39 \pm 5	22 \pm 1
4.3	37 \pm 7	10 \pm 1	69 \pm 11	40 \pm 3
7.1	40 \pm 8	5 \pm 2	84 \pm 7	56 \pm 4

Appendix 9: Effects of reaction time on the levels of serum proteins and casein micelle size in heated skim milk

Results were mentioned in Section 10.3.2 (p/10-233) and are present in Figure A.10 and A.11. The figures show the effects of reaction time on the levels of serum proteins and the casein micelle size in heated skim milk after addition of 7.1 mM β -mercaptoethanol.

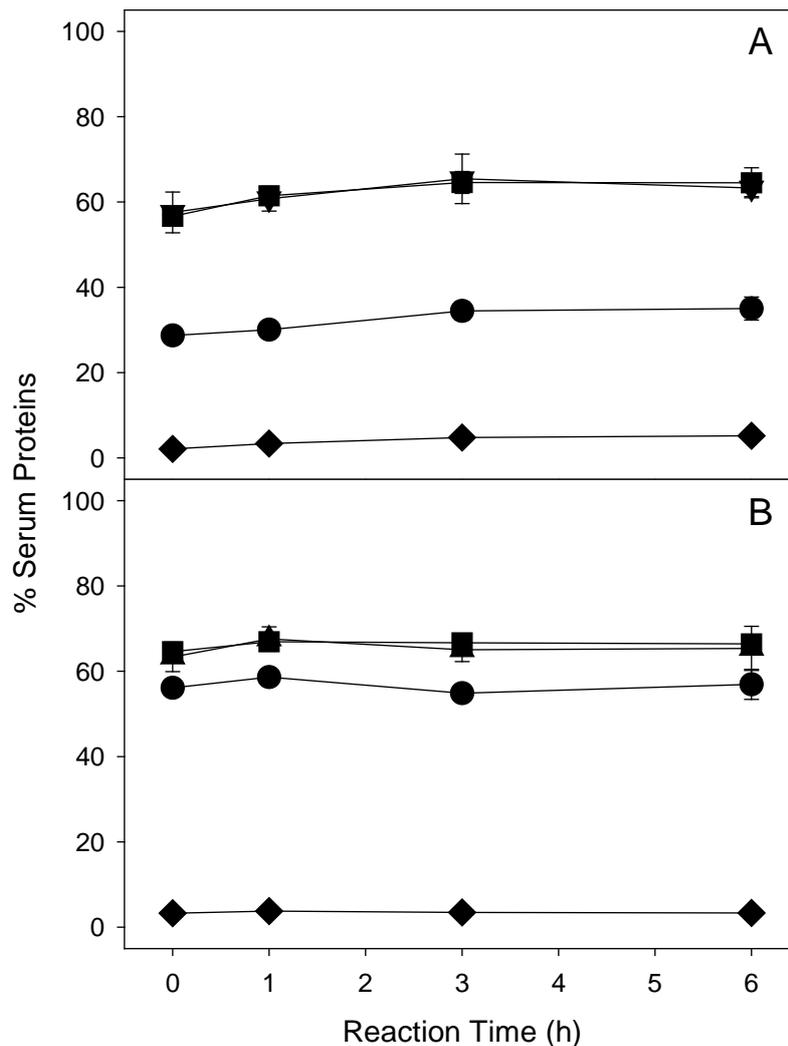


Figure A.10: Effects of reaction time on the percentage of α -lactalbumin (▲), β -lactoglobulin (■), κ -casein (●) and other caseins (◆) in the serum of heated skim milk (A) and heated WPE skim milk (B) with 7.1 mM added β -mercaptoethanol. Analysis used traditional SDS-PAGE. Each data point is the average value of two to four replicates. The error bar is the standard deviation of the replicates.

In heated skim milk, the addition 7.1 mM of β -mercaptoethanol caused the percentage of serum κ -casein to increase significantly from $29 \pm 2\%$ to $35 \pm 2\%$ over 3 h ($p < 0.05$, Figure A.10A)

whereas the percentage of other caseins in the serum increased progressively and significantly from $2.1 \pm 1.1\%$ to $5.2 \pm 1.0\%$ ($p < 0.05$) as the reaction time increased from 0 to 6 h (Figure A.10A). The percentage of serum β -lactoglobulin increased significantly from $57 \pm 2\%$ to $65 \pm 3\%$ over 3 h ($p < 0.05$). Although the percentage of serum α -lactalbumin increased from 58 ± 5 to $65 \pm 6\%$, this difference was not significant due to the higher differences in replicate analyses. In heated WPE skim milk, the percentage of serum proteins in the heated skim milk did not change further as the reaction time increased from 3 to 6 h (Figure A.10A).

In contrast, when 7.1 mM β -mercaptoethanol was added to heated WPE skim milk, the reaction time did not affect the percentage of α -lactalbumin, β -lactoglobulin and κ -casein remaining in the serum (Figure A.10B). However, the proportion of α_s - and β -casein remaining in the serum increased slightly but significantly from $3.3 \pm 0.2\%$ to $3.8 \pm 0.1\%$ ($p < 0.05$) as the reaction time increased from 0 to 1h. As the reaction time increased further, the proportion of the other casein in the serum phase decreased only slightly ($p > 0.05$, Figure A.10B).

The size increased progressively and significantly from 188 ± 2 nm to 193 ± 1 nm as the reaction time changed from zero to 12 h ($p < 0.05$, Figure A.11). This indicates that the reaction time had an effect on the size of the casein micelles, as it did on the reduction of the disulphide bonds of κ -casein and β -lactoglobulin (Figure A.11).

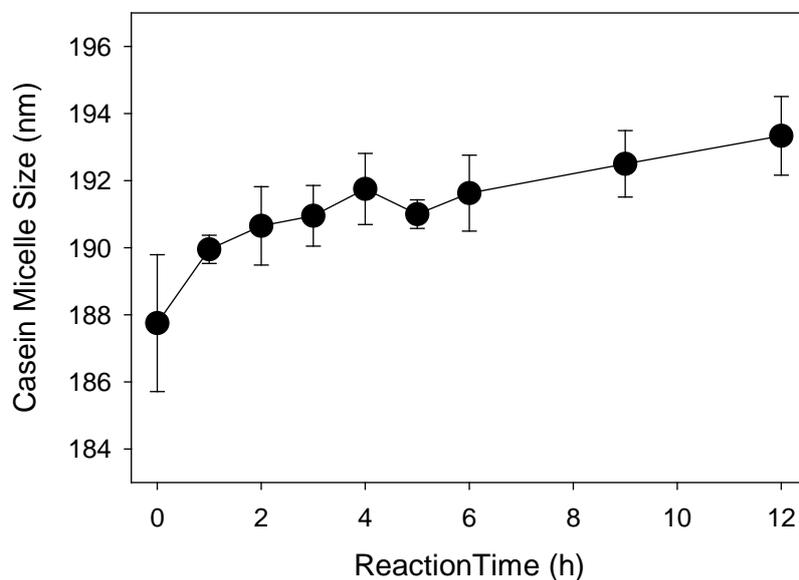


Figure A.11: Effect of reaction time on size of the casein micelle in heated skim milk that was treated with 7.1 mM β -mercaptoethanol for different periods of time. Each data point is the average of three to four replicates. Error bars represent standard deviation.

Appendix 10: Effect of reaction time on the final G' values of acid gels made from heated milks with added β -mercaptoethanol.

Results were mentioned in Section 10.3.3 (p/10-234) regarding the effect of reaction time on the final G' values of acid gels prepared from heated milks that were treated with β -mercaptoethanol.

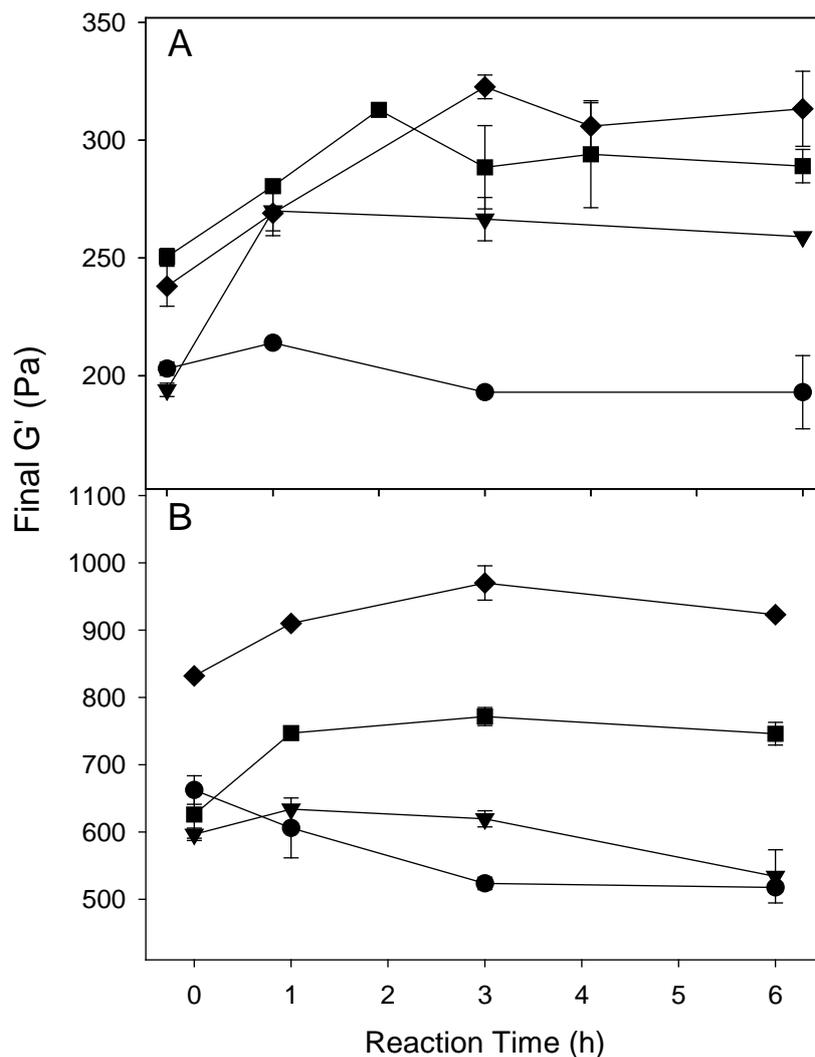


Figure A.12: Effects of reaction time and β -mercaptoethanol concentrations on the final G' values of acid gels made from heated skim milk (A) and heated WPE skim milk (B). ●, 0 mM; ▼, 1.4 mM; ■, 4.3 mM; ◆, 7.1 mM of β -mercaptoethanol as a function of reaction time. Each data point is an average of two to four replicates. Error bars represent the standard deviation.

The final G' values of acid gels prepared from heated skim milks were, within standard deviations, the same at any holding time (i.e. the time after the milk was heated and before the

milk was acidified). The final G' values of acid gels prepared from heated skim milk with 1.4, 4.3 and 7.1 mM added β -mercaptoethanol increased significantly. But a maximum final G' was reached for 1.4, 4.3 and 7.1 mM β -mercaptoethanol after 1, 2 and 3 h of reaction time with β -mercaptoethanol, respectively ($p < 0.05$, Figure A.12A). After the maximum values, the final G' values of acid gels with 1.4 and 7.1 mM added β -mercaptoethanol remained constant as the reaction time was prolonged further. In contrast, the final G' values of acid gels with 4.3 mM β -mercaptoethanol, after reaching the maximum at 2 h, decreased at 3 h of reaction time then did not change after longer reaction time (Figure A.12A).

In WPE skim milk, the final G' values of control acid gels decreased constantly as the holding time increased from zero to 3 h and remained at ~ 524 Pa as the holding time was increased to 6 h (Figure A.12B). The final G' values of acid gels with 1.4 and 7.1 mM added β -mercaptoethanol increased to reach a maximum G' after 1 h while the G' values of gels with 4.3 mM β -mercaptoethanol reached the maximum after 3 h of reaction time. After reaching the maximum, the G' values of acid gels with 1.4 mM and 7.1 mM β -mercaptoethanol decreased progressively as the reaction time was prolonged while the acid gels with 4.3 mM did not change with further reaction time (Figure A.12B).

Appendix 11: Effects of whey proteins concentration on the proportion of non-covalent interactions in milk systems

The effect of NEM on the final G' values of acid gels prepared from skim milk with ~4% protein concentration was mentioned in Section 11.3.3 (p/11-263) and the results are shown in Figure A. 13.

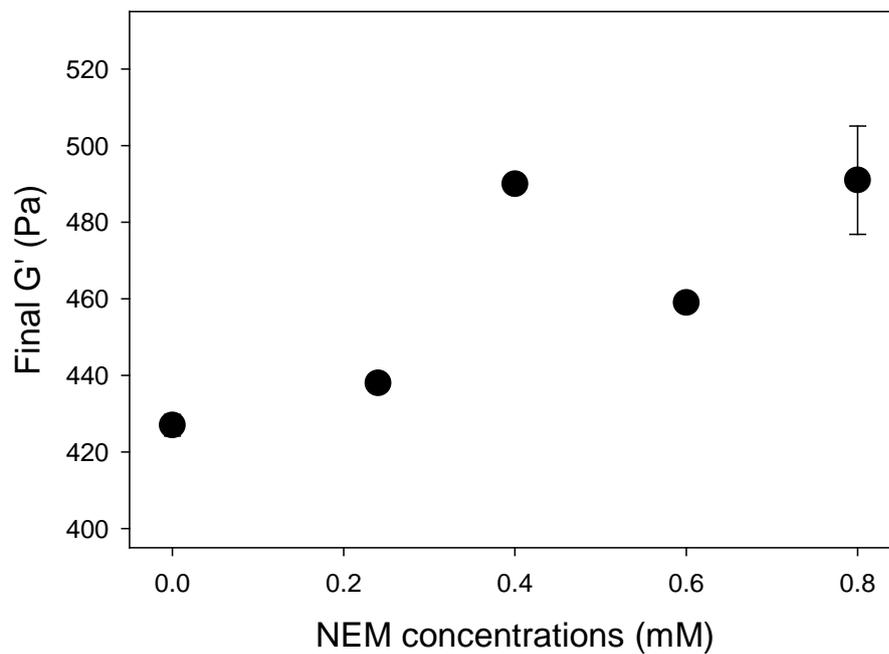


Figure A. 13: Effects of NEM concentrations on the final G' values of acid gels prepared from skim milk (with 4% protein) that was heated in the presence of NEM.

Appendix 12: Recommended experiment

Aim: To identify which milk protein can initiate the thiol-disulphide exchange reactions when that protein was induced with free thiol groups.

Approach: Reduction of κ -casein and whey protein should be carried out separately and then different milk components are recombined to the original state of milk before heat treatment.

Methods and conditions:

- Separating casein proteins from whey proteins can be carried out using a combination of ultrafiltration and microfiltration techniques.
- Once the reduction is completed, disulphide-bond reducing agent should be washed out before the milk components can be recombined. This is to limit the reduction of other milk proteins and the effect of the reagent on the protein interactions during heating milk.
- It should be noted that washing out the disulphide-bond reducing agent can lead to the re-formation of disulphide bonds. This can be eliminated by carrying out the washing step in non-oxygen environment and at temperatures $< 5\text{ }^{\circ}\text{C}$.
- When one component is reduced (e.g. casein protein component is reduced), the other component should be treated with thiol-blocking reagent (e.g. whey proteins are to react with thiol-blocking reagent). This is to ensure that only one component contains free thiol groups and is responsible for any thiol-disulphide exchange reactions to occur during heating. The thiol-blocking reagent should also be washed out from the solution before recombining the different components.
- After the recombination of different components to the initial state of milk, that milk will be heated. Analysis involved examining the percentage of proteins in the serum or colloidal phase and the percentage of proteins participating in disulphide bonds. The results will verify whether a protein that has been induced with free thiol groups can initiate thiol-disulphide exchange reactions with other proteins.