Impact of Heating and Shearing on Native Milk Proteins in Raw Milk

By

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To my beloved parents, Sanvidha and Savishka

Abstract

Processed foods are less susceptible to various biological contaminants as well as to enzymes causing spoilage as the risks associated with food borne illnesses are reduced while the shelf life is extended. In the dairy industry, thermal processing is one of the most common method used for this purpose. Treatments such as pasteurisation, strerilisation, and UHT treatment are some of the key methods that are widely applied. The complexity of these treatments impacts mainly on physico-chemical, nutritional and functional properties of the milk whereas Maillard reaction, κ-casein/β-lactoglobulin association, decrease in pH and whey protein denaturation are some of the common physico-chemical changes occurring during heating process.

In addition to heating, milk is also subjected to shear forces during diverse processing methods including pumping, homogenisation, stirring and in flow-through equipment such as heating, holding and cooling. Large velocity gradients generated by shear forces along the shear flow promote structural modifications of the milk proteins leading to unfolding via denaturation and subsequent interactions.

Thus, the both heating and shearing in combination would have a relatively greater impact on milk proteins. The simultaneous application of both heating and shearing are so common however, the concomitant effect of both heat and shear on milk proteins is less studied specially on native milk protein in raw milk. Therefore, the present study aimed at evaluating behaviour and structural modifications of native milk proteins in raw milk under various temperature and shear combinations that mimic common industrial applications.

During the first study, the structural modifications of milk proteins were investigated at temperatures 72 ºC and 140 ºC with the holding times of 15 seconds and 3 seconds,

respectively. However, caution should be exercised due to limitations of the equipment used in the experimentation, these temperatures were not reached at the same heating rate as it is usually achieved industrially. The shear rates chosen were 500 s^{-1} and 1000 s^{-1} in order to imitate common industrial applications. Raw milk samples were skimmed by centrifugation and treated using a rheometer pressure cell. Average particle size, Poly Dispersity Index (PDI) and zeta potential measurements were analysed using Zetasizer. Further, the calcium activity measurements were taken using a calcium ion selective electrode while Fourier Transform Infrared Spectroscopy was used to analyse secondary structural modifications. Surface hydrophobicity was measured using 1-anilinonaphthalene-8-sulfonic acid as the fluorescence probe and native and SDS poly acrylamide gel electrophoresis was performed under both reducing and non-reducing conditions to investigate protein interactions in detail.

Results showed that shear alone led reversible structural modifications in proteins. Simultaneous shearing at low temperature caused fragmentation of hydrophobically linked aggregates. Prominent shear-modulated aggregation occured at 140 °C mainly via sulphydryl-disulfide bridging with the increase in shear. It was confirmed that, structural changes in milk proteins were mainly influenced via magnitude of shearing while heating of milk and shear dependant molecular interactions within a milk system.

In second study, shear induced behaviour of native milk proteins in raw milk were investigated at three different temperatures (80 ºC, 100 ºC and 120 ºC) combining with three shear rates (100 s^{-1}, 500 s^{-1} and 1000 s^{-1}) along with an exposure time of one minute at each condition mimicking commonly applied conditions in commercial processing. Initially, raw milk samples were skimmed by centrifugation and initial pH measurements were taken using pH meter. These samples were then treated using rheometer pressure cell for required temperature-time-shear conditions. These treated samples were analysed for particle size distribution using zetasizer and final pH measurements were taken using pH meter. Then, the

samples were centrifuged to differentiate serum and pellet within the milk. Supernatant of each treated sample was analysed for individual protein fractions using reverse phase high performance liquid chromatography (RP-HPLC). Both supernatant and sediment were analysed using non-reducing and reducing sodium dodecyl sulphide polyacrylamide gel electrophoresis (SDS PAGE) for protein interactions.

According to results, shear has manipulated the direction and extent of heat induced milk protein interactions at different temperatures. Greater shear induced aggregation via disulphide links at 1000 s^{-1} and 80 °C with a considerable proportion of aggregates created via α_{s2}-CN and whey proteins interactions. At 100 °C, shear induced fragmentation has become the dominant mechanism at 500 s^{-1} in contrast to other two shear rates while this was also observed as the dominant mechanism at 120 °C. Surprisingly, β-LG was less involved in interactions at both 100 ºC and 120 ºC with the increase in shear probably due to unavailability of free thiol group due to structural modifications under increasing shear forces.

The present study revealed that the flow regime may lead to manipulation of heat induced casein-whey protein interactions in native milk proteins in raw milk. Depending on the extremities, shear forces would directly impact on protein denaturation, enhanced aggregation due to greater collision rates as well as fragmentation of aggregates. Therefore, the shear frequently encountered in dairy processing is a crucial parameter in determining the properties of the final product hence cannot be simply neglected.

II. Declaration

"I, Mediwaththe Gedara Anushka Thejangani Menike Mediwaththe, declare that the Masters by Research thesis entitled "Impact of Heating and Shearing of Native Milk Proteins in Raw Milk" is no more than 60,000 words in length including quotes, and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature:  Date: 31/03/2017

Mediwaththe Gedara Anushka Thejangani Menike Mediwaththe

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Chapter 1

Introduction to Thesis

1.1 Background

Depending on the type of processing, ingredients are exposed to various conditions such as high temperature, changes in pH, changes in pressure and different flow behaviours. These conditions are considered as the major importance due to their ability in manipulating characteristics of the end product. In the dairy industry, thermal processing of milk is an essential step in extending shelf life by eliminating or minimising bacterial growth. These various heating methods such as pasteurisation, in-bottle sterilisation, direct and indirect UHT processing lead to alterations of physico-chemical, functional and nutritional properties of milk up to varying degrees mainly depending on the severity of the heat, duration and composition of the milk such as total solids, pH, mineral and protein content (Oldfield, Singh, & Taylor, 2005; Xiong, Dawson, & Wan, 1993). Whey protein denaturation, κ-casein/β-lactoglobulin complexation, calcium phosphate precipitation onto casein micelles, Maillard reactions and decrease in pH are some of the physico-chemical changes that occur during heating (Singh, Creamer, & Fox, 1992). Most of these heat induced structural modifications of milk proteins have been extensively studied (Anema & McKenna, 1996; Oldfield, 1996; Singh et al., 1992; Vasbinder & de Kruif, 2003).

In addition, milk is also subjected to shear forces within some of the major unit operations such as pumping, stirring, mixing and homogenisation as well as in flow through pipes and heat exchanger throughout heating, holding and cooling during commercial processing. These shear forces acting upon molecules are a result of large velocity gradients that occur in a fluid flow and are mainly influenced by the flow behaviour of a given solution. For instance, in a surface heat exchanger, ingredients are subjected to more complex flow behaviour such as laminar and elongational flow and

vortices while in filtration, ingredients are subjected to oscillatory elongational flow. These different flow behaviours may create relatively divergent shear forces upon proteins and especially this has a great influence on coarse aggregating biopolymers such as whey proteins leading to varying degrees of denaturation and aggregation (Walkenström, Nielsen, Windhab, & Hermansson, 1999). Thus, both reversible and irreversible denaturation of proteins has been observed under various shear rates (Maa & Hsu, 1996).

Therefore, the impact of shear forces during thermal processing could result in even greater structural changes thereby leading to more pronounced denaturation and aggregation. Impact of these on aggregation behaviour of whey proteins have been studied by several authors (Steventon, 1994; Steventon, Donald, & Gladden, 1994; Taylor & Fryer, 1994; Walkenström, Windhab, & Hermansson, 1998). A study done by Steventon et al., (1994) at specific temperatures (between 75-90 °C) and fluid shear (up to 1480 s-1/ at times up to a maximum of 60 minutes) on aggregation behaviour of whey proteins using 7 % (W/W) whey protein concentrate (WPC) at pH 6.3 showed that aggregation kinetics of WPC were constant under the laminar coquette flow with a two-stage process which was initial slow rate-limiting process controlled by denaturation followed by a fast rate-limiting process dominated by the shear controlled aggregation growth and fragmentation. Further, shear is an important parameter in determining the behaviour of proteins and aggregation dimensions. In relation to this Walstra & Jenness, (1984) suggested that effects of streaming are dependent on the meeting frequency of particles which in turn strongly dependent on the size of the particles. Also, they have shown that shear effects are dependent upon streaming which may disrupt floccules and prevent them being formed. In addition, large floccules are more easily disrupted than smaller ones as they are more likely to have weak spots. Moreover, it was found that the

net growth rate and size of any protein aggregate depend upon the equilibrium between growth and shear-controlled breakage.

These studies stipulate that aggregation process can be manipulated carefully by selecting specific temperature and shear conditions while shear being an important parameter in determining aggregation dimensions.

In terms of manufacturing food products, these conditions ultimately result in numerous outcomes such as sensory perception yielding different textures, phase inversions in phase separating mixed suspensions and formation of gels due to alterations in size and morphology of aggregates (Walkenström & Hermansson, 1998; Langton, Aström, & Hermansson, 1996). In addition to that, investigation on structural modifications at various processing methods in dairy industry will aid in answering the hidden causes behind some major health concerns recently spiked among community such as asthma and food borne allergies which were believed to be due to consumption of processed food products related to dairy. Thus, investigating on impact of shear and temperature on milk proteins would benefit the industry as well as the consumers in multiple ways optimizing benefits while leaving deleterious effects to minimum.

While simultaneous application of both thermal processing and shearing is common, knowledge is lacking on their combined effects and how it would impact various interactions and structural changes of proteins, in a complex mixture such as raw milk. Therefore, the investigation of interactions among proteins under these conditions and their impact on the structural changes and behaviour of otherwise intact native proteins would allow to obtain a better understanding on how these two important factors influence the physico-chemical properties of milk and consequently the final quality of the product. Also, this would facilitate in process improvements and/or novel processing

by either minimising the changes of proteins or improving the process control and preventing or creating molecular interactions.

1.2 Research aims and objectives

The main aim of the study was to provide an understanding on how the temperature and shear in combination influence the structural changes and associated behaviour of the milk proteins in milk in their native state.

The objective of the study answers following questions;

- What are the alterations in structural attributes of milk proteins that occur during different heat treatments in combination with different shear rates?
- How these altered structural attributes relate to behaviour of milk?
- Would these interactions be manipulated via altering the temperature and shear conditions within the system?

1.3 Structure of thesis

The objective of the present study was to examine the impact on native milk proteins in raw milk upon combined heat and shear conditions commonly applied in industrial level. During the first study, temperatures selected were 72 °C and 140 °C with the holding time of 15 and 3 seconds respectively and shear rates of 500 and 1000 s^{-1} were used at each temperature. The second study was based on the shear induced behaviour of native milk proteins in raw milk at three different temperatures (80 °C, 100 °C and 120 °C) and three shear rates (100 s^{-1}, 500 s^{-1} and 1000 s^{-1}) within the range of most common industrial processing. The thesis contains following chapters;

Chapter 1 – Provides general information on background of the study, research aim, objectives and the structure of the thesis.

Chapter 2 – Presents a literature review explaining heat and shear induced structural modifications of milk proteins in detail as a background information.

Chapter 3 – Focuses on structural modifications of native milk proteins upon controlled heating and shearing

Chapter 4 – Focuses on shear induced behaviour of native milk proteins in raw milk at different temperatures

Chapter 5 - Provides a conclusion on overall chapters and discusses on future directions which can be used to optimize the processing conditions in order to obtain desired prospective of the final products thereby leaving the undesired consequences to a minimum.

Chapter 2

Literature Review

2.1 "Milk"- as an important source in human nutrition

Bovine milk is the primary source of nutrition in neonates of this species hence contains necessary nutrients in easily digestible forms. Besides meeting the nutritional requirements of its own neonate, bovine milk has also become one of the major, nutrient rich component in human diet. The milk production using domesticated cattle has begun centuries ago around in 8000 BC. The total production of cow's milk has been accounted for over 600 million tonnes per year today and USA being the largest producer which produce about 87 million tonnes per year (FAO stat, 2012). In Australia, around 30 % of dietary protein is supplied by milk and dairy products, therefore, it is regarded as a major source in nutrition.

Milk, being the highly nutritious and well-balanced diet, has become one of the major food sources in infants as a breast milk substitute. In young children, fresh milk, fermented products as well as cheese represents valuable components in their diet providing essential nutrients required for healthy growth. Functional milk proteins, essential fatty acids, vitamins and minerals like calcium and bioactive peptides in milk have favourable health effects specially on gastrointestinal, cardiovascular and immune systems preventing certain disorders while improving general health. In addition to that, emerging interest in milk components as functional foods has a major importance in many aspects such as health promoting effects, treatment and prevention of some diseases. For example, several milk-derived growth factors are used for gastrointestinal diseases and skin disorders (Pouliot & Gauthier, 2006) while phosphopeptides derived from caseins are used in some dietary and pharmaceutical supplements (Reynolds, 1999). Moreover, its potency to modulate activity via probiotics attributed to biologically active compounds which serves as therapy and prevention of certain diseases (Ferencik & Ebringer, 2003).

More research has yet to be focused on these fields hence, it is worthwhile to emphasize on further investigations on dairy aspects in order to obtain optimum benefits via this highly precious food.

2.2 Composition of milk

Bovine milk is mainly composed of fat, lactose, proteins and minerals. The average composition of milk is shown in table 2.1.

Table 2.1: Average composition of milk (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999)

Component	**Average quantity in milk (% W/W)**
Water	87.1
Fat	4.0
Protein	3.3
Casein	2.6
Whey	0.7
Lactose	4.6
Minerals	0.7
Organic acids	0.17
Miscellaneous	0.15

About 98 % of milk fat is composed of triglycerides and the remaining includes cholesterol, free fatty acids, phospholipids and cerebrosides.

Lactose is a soluble reducing disaccharide composed of glucose and galactose. It is the principal carbon source for most of the microorganisms to grow in milk and gives the milk a slightly sweet taste.

Minerals in milk occurs in either solution or associated with proteins either as undissolved salts or bound ions.

The proteins in milk, which comprise 3.3 % of milk, are constituted of two main components named caseins; which precipitate at pH 4.6 at 20 °C, and whey proteins; which remain soluble in the serum after casein precipitation. Caseins represent 80 % of the total protein content and they are present in milk mainly as spherical colloidal structures known as casein micelles.

2.2.1 Caseins

Caseins consist of four major proteins groups known as α_{s1}-, α_{s2}-, β-, κ-caseins. These four types of caseins have different genetic variants as well as they differ in primary structure (Creamer & MacGibbon, 1996). Some of the properties of these proteins are shown in table 2.2

Table 2.2: Properties of four types of caseins in milk (Walstra & Jenness, 1984)

	α_{s1}-CN	**α_{s2}-CN**	**β-CN**	**κ-CN**
Concentration (mg/100 ml)	12-15	3-4	9-11	2-4
Relative abundance (%)	38	10	36	13
Molecular weight	23600	22500	24000	19000
Phosphoseryl residues	8-9	10-13	5	1-2
Cysteine residues	0	2	0	2
Hydrophobicity	4.9	4.7	5.6	5.1

All the caseins have a high level of proline content, which results in very low content of α-helix and β-sheet structures. Therefore, these are more prone to proteolysis without prior denaturation (Fox & McSweeney, 1998). Also all these caseins are amphipathic, which have randomly distributed polar and apolar regions occurring as clusters of hydrophobic and hydrophilic regions (Creamer & MacGibbon, 1996). The most hydrophobic protein is the β-casein, while α_{s2}-casein is assumed to be the most hydrophilic. All these caseins have a strong tendency to self-associate mainly via hydrophobic interactions (Fox & McSweeney, 1998).

An interesting feature of caseins is their post translational modifications via phosphorylation, glycosylation and proteolysis. α_{s1}-, α_{s2}- and β-Caseins are phosphoproteins that undergo phosphorylation via seryl residues. The variable amount of phosphoserine residues in these caseins determine their involvement in interactions with calcium and calcium phosphate (Fox & McSweeney, 1998). Calcium binds primarily to the phosphoseryl residues with a cluster sequence of SerP-SerP-SerP-Glu-Glu (Cross, Huq, Stanton, Sum, & Reynolds, 2004). α_{s2}-Casein is the most sensitive to calcium thus precipitates at calcium concentrations less than 2 mM (Aoki, Toyooka, &

Kako, 1985; Toma & Nakai, 1973), while β-casein is the least sensitive and precipitates in a range of 8-15 mM of calcium at 37 °C (Farrell Jr & Thompson, 1988; Parker & Dalgleish, 1981; Schmidt, 1969) and no precipitation at 1°C up to a concentration of 400 mM (Farrell Jr & Thompson, 1988) . κ-Casein remains soluble at all these concentrations due to absence of phosphate centre containing three phosphoserine residues as mentioned before. Also, it is considered as the only casein known to undergo glycosylation. Three glycans consisting of galactose (Gal), *N*-acetylglucosamine (GalNAc), and *N*-acetyl neuraminic acid (NANA) have been shown to attach Thr residues of κ-casein (Huppertz, 2013).

In addition, caseins have a more chain flexibility compared to globular proteins, therefore they are more susceptible to proteolysis (Swaisgood, 1989). Flexibility of the chain and enzyme specificity of a distinct region in peptide bond of a protein leads to rapid hydrolysis eventually occurring in limited proteolysis, which produces characteristic large peptides with specific protein structures (Swaisgood, 1989; Swaisgood & Catignani, 1987).

Another post translational modification of caseins is the formation of disulphide bonds which are characteristic only to α_{s2}- and κ-caseins. The two cysteinyl residues, which are Cys_{36} and Cys_{40} of α_{s2}-casein occur in both intramolecular and intermolecular disulphide bonds (Rasmussen, Hojrup, & Petersen, 1992, 1994). κ-Casein has two cysteinyl residues in regions Cys_{11} and Cys_{88} creating a complex disulphide bonding pattern. In the native state, the disulphide linked structure of κ-casein range from monomer to multimer larger than decamer (Creamer & MacGibbon, 1996). These Cys residues of both caseins facilitate intermolecular disulphide linkages with whey proteins under certain conditions (Jang & Swaisgood, 1990; Rasmussen et al., 1992).

2.2.1.1 Structure of the casein micelle

More than 95% of caseins in milk exist as large spherical colloidal particles known as micelles. In addition to caseins, micelles consist of colloidal calcium phosphate including calcium, magnesium, phosphate and citrate and these ions are highly important in maintaining micellar integrity under certain conditions (Fox & McSweeney, 1998; Horne, 2009). Micelles are highly hydrated open structures ranging in diameter of 50 - 500nm (average ~ 150 nm). Also, these are dynamic and respond to changes in micellar environment such as temperature and pressure (Horne, 2009). At 32 °C, about 90 % caseins are within the micelles while at 4 °C, up to 50 % remains in the serum phase indicating their dynamic nature (De Kruif & Holt, 2003).

Various models for the casein micelle structure have been proposed over the past and most accepted model was put forward by Holt in early 1990's and refined by Horne in late 1990's with bonding within the micelle and by Holt regarding the nature of the calcium colloidal phosphate (figure 2.1).

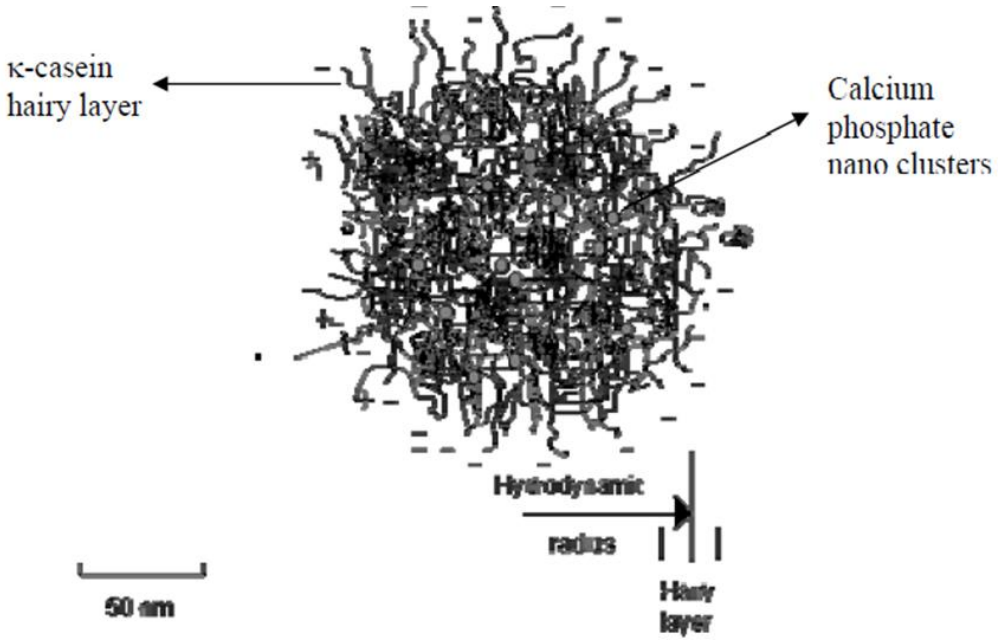

Figure 2.1: The hairy casein micelle structure (Holt & Horne, 1996)

Caseins undergo self-association in the presence of calcium, forming micro-gel structures associated through hydrophobic interactions and cross linking through calcium sensitive caseins and calcium colloidal phosphates (Holt & Horne, 1996). These structures within the casein micelles are called nanoclusters. Highly phosphorylated phosphopeptides derived from calcium sensitive caseins interact with amorphous calcium phosphate to form designated chemical complexes known as calcium phosphate nanoclusters. The nanoclusters formed have a middle amorphous calcium colloidal phosphate core surrounded by phosphopeptides arranged in a shell like structure (Holt, Timmins, Errington, & Leaver, 1998). These structures are distributed throughout the micelle more or less homogenously linked mainly via hydrophobic bonding and electrostatic forces (Patel, 2007). Micelles formed are negatively charged and hydrophilic due to hydrophilic C terminals of κ-casein projecting out in to the medium forming diffuse layer (Holt & Horne, 1996; Horne &

Davidson, 1986; Walstra, 1990). All the models proposed for casein micelle structure so far, have been reviewed in detail by many authors (De Kruif & Holt, 2003; Fox, 2003; Fox & Brodkorb, 2008; Holt et al., 1998)

2.2.2 Whey proteins

About 0.7 % of raw bovine milk is composed of whey proteins. Major whey proteins include β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA) and immunoglobulins (Ig). These are globular to ellipsoid proteins in nature and densely folded polypeptide chains, which are highly hydrophobic and heat sensitive denaturing upon 60 °C (Corredig & Dalgleish, 1996; Creamer & MacGibbon, 1996; Law & Leaver, 1997). The heat sensitivity of these proteins are in order of Ig > BSA > β-LG > α-LA (Law & Leaver, 1997). Some of the characteristics of whey proteins are shown on the table below (Table 2.3).

Table 2.3: Properties of major whey proteins in milk (Chandrapala, 2008)

Whey Protein	**MW(kDa)**	**Concentration in milk (g kg^{-1})**	**Disulphide Bonds**	**Amino Acid residues**
β-LG	18.4	3.3	2	162
α-LA	14.2	1.2	4	123
BSA	66.3	0.4	17	582
Ig	150-900	0.7	21	

2.2.2.1 β-Lactoglobulin

β-LG is the most abundant whey protein responsible for about 50 % of the total whey proteins. It is a globular protein which usually exists as a dimer of two non-covalently

bonded monomers between pH 5.5 and 7.5 (Sawyer, 2003). Several genetic variants of β-LG have been reported and among these variants A and B are the most common existing in almost equal frequency. β-LG A has Asp and Val at positions 64 and 118 which are replaced by Gly and Ala in β-LG B (Hambling, McAlpine, Sawyer, & Fox, 1992).

β-LG has 162 amino acid residues and two disulphide bonds (Cys_{66}- Cys_{160} and Cys_{106}-Cys_{119}) and a free thiol group (Cys_{121}) buried within the structure (Considine, Patel, Anema, Singh, & Creamer, 2007; Sakai, Sakurai, Sakai, Hoshino, & Goto, 2000; Sakurai, Oobatake, & Goto, 2001). It is primarily a β- sheet protein containing nine β-strands and one α-helix at the C terminal end of the molecule (Sakai et al., 2000). Reactive free thiol group is buried inside a group of hydrophobic residues between these β-strands and α-helix and around 60-65 °C, this is exposed initiating sulphydryl-disulphide interchange reactions resulting irreversible denaturation (Dalgleish, 1990; Fox & McSweeney, 1998; Law & Leaver, 1997).

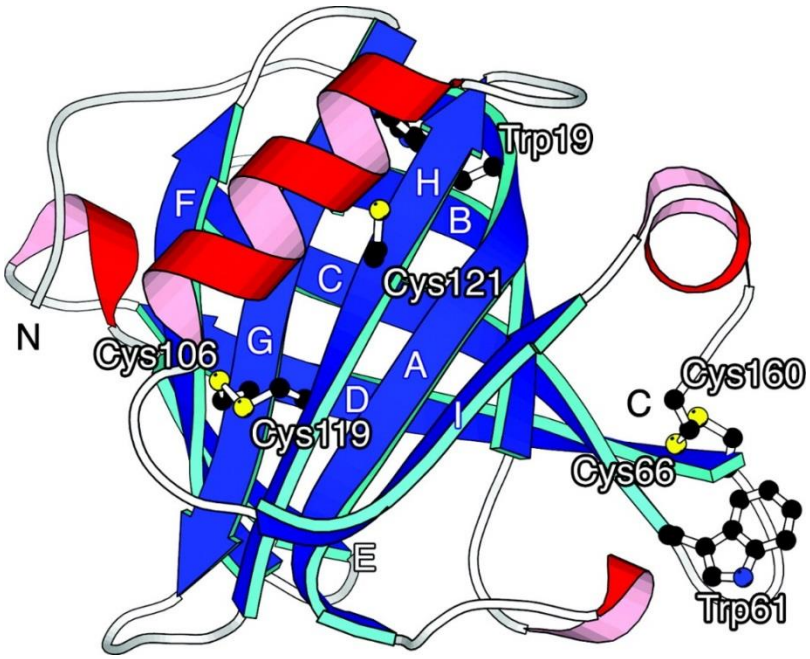

Figure 2.2: The crystalline structure of bovine β-LG indicating disulphide bonds and thiol group (Yagi, Sakurai, Kalidas, Batt, & Goto, 2003)

2.2.2.2 α-Lactalbumin

α-LA is the second major protein presenting about 20 % of the total whey proteins. It is a monomeric globular protein consisting of four disulphide bonds (Cys_6-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{61}-Cys_{77}, and Cys_{73}-Cys_{91}) (Permyakov & Berliner, 2000). Due to a lack of free thiol groups, denatured α-LA can only be incorporated in to aggregated structures via thiol-disulphide interchange reactions between thiol containing whey proteins (Dalgleish, 1990; Oldfield, Singh, Taylor, & Pearce, 2000). It exists in several environment dependant conformations; mainly apo (calcium-depleted) or holo (native, calcium-bound) form, which is dominant form in milk (Baumy & Brule, 1988).

2.2.2.3 Bovine Serum Albumin

BSA is an ellipsoid, monomeric whey protein responsible for about 10 % of the total whey proteins (Peters, 1985). It has seventeen disulphide bonds and one free thiol group

(Cys34) providing a multiloop structure to the protein (Carter & Ho, 1994). It consists of three domains and each has two large double loops and one small double loop. Each domain has characteristic charge, hydrophobicity and ligand binding capacity (Peters, 1985).

2.2.2.4 Immunoglobulins

Immunoglobulins are responsible for about 10 % of the total whey proteins and are large and complex glycoproteins that function as antibodies (Patel, 2007). They are categorised in to five major groups; IgA, IgM, IgE, IgD, and IgG and last one is subdivided into IgG_1 and IgG_2 (Farrell et al., 2004).

2.2.3 The milk salts

Milk contains several different minerals as a small fraction of milk which is about 8-9 g L^{-1} (Gaucheron, 2005). The mineral composition of each salt is shown in table 2.4.

Table 2.4: Composition of milk salts in milk ((Holt, 2004)

Constituent	Content in Milk (mM)	Diffusible Content (mM)
Calcium	29.0	10.2
Magnesium	4.9	3.4
Sodium	22.0	22.0
Potassium	38.3	38.0
Citric acid	9.5	9.4
Phosphate	20.6	12.4
Chloride	30.4	30.3
Sulphate	1.2	1.2

These minerals in milk play a significant role in structural stability of the casein micelle. They have a divergent distribution consisting of a diffusible fraction which can diffuse through a membrane (less than 15 kDa) and a non-diffusible fraction (retained by the membrane), which is believed to be associated with the casein micelles. Sodium, potassium and chloride are considered to be diffusible, while calcium, inorganic phosphate and magnesium are partially bound to caseins (Gaucheron, 2005). About 65 % of calcium and 51 % of phosphate are within the micelles while the rest remains within the serum phase (Holt, 2004). The mineral equilibrium between colloidal and aqueous phase of milk specially, calcium and phosphate equilibrium is important in maintaining micellar integrity (Aoki, Yamada, & Kako, 1990).

2.3 Whey protein denaturation

2.3.1 Mechanism of whey protein denaturation

Whey proteins in bovine milk are globular proteins with a large amount of secondary and tertiary structures. The globular structure is maintained through several different forces including hydrogen bonding, van der Waal's forces, electrostatic forces and hydrophobic bonding. When these forces are affected by either chemical or physical means, this would result in proteins to lose their native structure leading to unfolded disordered random structures (Walstra & R Jenness, 1984). This involves changes in environment such as heat, pressure or some mechanical forces which leads to disruption of bonding that maintains structural integrity. Initial step in whey protein denaturation is unfolding which involves exposing side chain reaction groups buried within the native structure, which is reversible, which is then followed by aggregation mainly via sulphydryl-disulphide interactions. Therefore, the overall whey protein denaturation can

be interpreted in two steps as unfolding and aggregation (De Wit, 1981; Mulvihill & Donovan, 1987)

2.3.2 Factors influencing whey protein denaturation

2.3.2.1 Effect of temperature

Increase in temperature results in whey protein denaturation by weakening or rupturing bonds within secondary and tertiary structures. Up to about 60 °C, proteins undergo reversible, minor structural changes along with the temperature mainly affecting hydrophobic bonding (Klarenbeek, 1984). The increase in temperature above 70 °C results in weakening of hydrophobic bonding and initiating thiol-disulphide interchange reactions (Li-Chan, 1983).

2.3.2.2 Effect of pH

pH is a main factor influencing denaturation of whey proteins. Each whey protein behaves variously at different pH conditions due to their dissimilarities within the content as well as distribution of polar residues within the polypeptide chains. For example, at pH 6.7, BSA has the highest stability while β-LG has the least. Further, it was found out β-LG denaturation decreases when pH is reduced from 7 to 4.5. In contrast, denaturation of α-LA is relatively independent of pH. (Mulvihill & Donovan, 1987).

2.3.2.3 Concentration of constituents of milk

Total solid content in milk influences differently on different whey proteins in terms of denaturation. Hillier et al., (1979) found increase in total solids resulted in decrease in denaturation of β-LG A and B while it increased the denaturation of α-LA. Moreover, in

heated whey, both BSA and Ig shows a protective mechanism when increasing the total solid content (Nielsen et al.,1973).

2.3.2.4 Content of other components

Milk salts specially Ca^{2+}, Mg^{2+} and Na^{2+} are shown to promote the denaturation and aggregation above pH of 5.5 (Varunsation et al., 1983). It was claimed that the both Ca^{2+} and Mg^{2+} bind to heat denatured whey proteins to form aggregates. Na^{2+} assumed to be mask the exposed ionic groups of thus changing electric double layer facilitating aggregation (Xiong et al., 1993).

Lactose is considered to be preventing the denaturation of whey proteins (Hilliar et al., 1979, de Wit, 1981). It was assumed that hydration of the protein molecules was increased or maintained by lactose to increase the stability against denaturation (Bernal & Jelen, 1985).

Fatty acids increase the stability of BSA. DSC studies showed that removal of naturally bound fatty acids from BSA reduces the denaturation temperature by about 7-12 ºC (Bernal & Jelen, 1985)

2.3.2.5 Effect of pressure

Degree of denaturation increases with increase in pressure or the duration in treatment (Huppertz et al., 2004; Olano, 1998a, 1998b). β-LG is the most pressure sensitive whey protein which can be denatured at pressures of 100-150 MPa. In contrary, α-LA and BSA are stable at pressures up to 400-500 MPa (Huppertz et al.,2002). The more rigid molecular structure of α-LA compared to β-LG was considered to be the main reason for increased stability against pressure induced denaturation (Gaucheron et al., 1997). Also the difference in intramolecular disulphide bonds (Gaucheron et al., 1997) as well as lack of free thiol group in α-LA (Funtenberger et al., 1997), also contribute to

stability against denaturation of α-LA. Furthermore, oligomerisation of α-LA only takes place if free -SH groups are available from other molecules thus increasing the resistance to pressure induced denaturation (Gaucheron et al., 1997). The increased stability of BSA can be explained as due to one -SH group and seventeen disulphide bonds which energy received under high pressure is not enough to break all the disulphide bonds thus maintains structural integrity (Huppertz et al., 2004).

2.3.3 Methodology for measurement of denaturation of proteins

Some of the most common methods used to measure denaturation of proteins are differential scanning calorimetry (DSC), solubility analysing technics (such as HPLC and PAGE), spectral and immunological characteristics.

DSC is used extensively to characterise mainly the denaturation of whey proteins while for other proteins up to some extent (Relkin & Launay, 1990). The endothermic heat flow caused by the protein unfolding is measured to obtain the denaturation state of a protein. This is advantageous to study the denaturation to monitor the reaction directly. However high concentration of proteins and media such as water and buffers required to measure the endothermic change and therefore cannot be used for skim milk analysis (Jelen & Rattray, 1995).

Loss of solubility due to intermolecular interactions among whey proteins due to unfolding and exposure of reactive side groups was utilised in solubility techniques to analyse denaturation. The supernatant obtained through adjusting pH to 4.6 and centrifuging at~2000 *g* for about 20 minutes at 20 ºC is used in several methods such as polyacrylamide gel analysis (PAGE), high performance liquid chromatography (HPLC) and Kjeldahl nitrogen method to analyse denaturation.

PAGE can be used for both quantitative and qualitative analysis and the separation of molecules based on the mobility across the voltage gradient as well as the molecular sieving action across the porous gel. HPLC technique is based on the separation of proteins according to their flow rates within the mobile phase depending on the distribution between mobile and stationery phases. Several techniques used to separate proteins includes reversed phase, ion-exchange, affinity and size exclusion chromatography.

Spectral methods use optical activity changes due to structural changes occurring due to unfolding of whey proteins (Harwalker, 1980a, 1980b). Two of the most common methods used are circular dichroism (CD) and optical rotary dispersion (ORD).

Whey proteins have higher ability to bind in to specific antibodies due to their high antigenicity. Denaturation leads to decrease immunological activity which in turn result in binding and this has been used as a principle to evaluate denaturation in immunological assays (Oldfield, 1998).

2.3.4 Kinetics of whey protein denaturation

Kinetics is an important aspect in quantification and modelling of denaturation of whey proteins. Conditions such as temperature, pH, protein concentration, methods and medium used directly influence the denaturation which in turn impact on kinetics. The kinetics of β-LG denaturation has been studied extensively in skim milk (Lyster, 1970; Dannenberg & Kessler, 1988; Dalgleish, 1990). Order of denaturation of whey proteins can be determined using general rate equation as follows (equation 2.1);

$$\frac{-dC}{dt} = k_n C^n \qquad (2.1)$$

C = concentration of native protein (mol l^{-1})

t = time (s)

k_n = rate constant ((mol l^{-1})$^{1-n}$$s^{-1}$)

n = reaction order

Reaction order for β-LG in skimmed milk has no specific value and differs between several studies. After establishment of order, kinetic parameters can be determined and thereby reaction can be modelled. The temperature dependence of rate constant can be defined using Arrhenius equation (equation 2.2). This is the most commonly used way of illustrating temperature dependence of the rate constant.

$$k_n = k_o exp^{\left(-\frac{E_a}{RT}\right)} \quad (2.2)$$

k_0 = frequency factor ((mol l^{-1})$^{1-n}$$s^{-1}$)

E_a = activation energy (kJ mol^{-1})

R = universal gas constant (8.314 J mol^{-1} K^{-1})

T = absolute temperature (K)

2.4 Process induced interactions of milk proteins

2.4.1 Heat induced interactions of milk proteins

Milk is a complex system which contains several protein species possibly interact with each other upon heating. Most of the milk proteins have free thiol groups and/or disulphide bonds which can initiate thiol-disulphide interchange reactions leading to aggregation. Among the whey proteins, the major protein β-LG as well as α-LA and

BSA could also participate in thiol-disulphide interactions. In addition to that, both α_{s2}-CN and κ-CN also contain disulphide bonds and therefore possible to involve in thiol-disulphide exchange reactions. Hence, these thiol-disulphide interactions could be occurred between whey proteins as well as among whey proteins and caseins.

One of the most important interaction occurs within the milk system is the formation of irreversible β-LG/κ-CN complex via sulphydryl-disulphide and hydrophobic bonding (Jang & Swaisgood, 1990). The level of association depends on many variables such as temperature, time, individual protein concentrations, rate of heating, concentration of salts and milk pH (Anema & Li, 2000; Corredig & Dalgleish, 1996). For example, gradual increase in heating of milk above 70 °C as an indirect heating system resulted in most of the denatured β-LG and α-LA to be associated with κ-CN on the surface of the micelles while rapid heating in direct heating systems resulted in half of the denatured α-LA and β-LG to be associated with casein micelles (Corredig & Dalgleish, 1996). This was explained by Corredig & Dalgleish, (1999) as a serum ratio-dependant initial aggregation of β-LG and α-LA with the increase in temperature while subsequent association with κ-CN on the surface of the micelles with prolonged heating. Oldfield, Singh, & Taylor, (1998) suggested fast heating rates induce β-LG to form aggregates within the serum which limits degree of association with casein micelles whereas slower heating rates, smaller aggregates or monomers of β-LG may interact with the micelles allowing increased association with micelles.

The pH of milk is also an important factor in determining the degree of association of denatured whey proteins and casein micelles. This has been discussed in many review papers on heat stability of milk (O'connell & Fox, 2003; Singh, 2004; Singh et al., 1992) Heat coagulation time/pH profile of most milks show increasing heat stability of milk with the increase in pH up to about 6.7 followed by decreasing stability until 6.9

and then an increase in stability as the pH is increased further (Rose, 1961). Most recent studies show heat induced dissociation of κ-CN is pH dependant in the range 6.5-7.1 while it shows a linear increase in serum within this range and the level of serum κ-CN correlated with the denatured whey proteins. Also, it was shown that, ratio of association of denatured whey protein to κ-CN is 1:1 while it was decreased to 0.5:1 when the pH was increased to 7.1 (Anema, 2007).

Several concepts have been suggested over the sequence of interactions among whey proteins and κ-CN. Some studies propose that while heating, denatured whey proteins first interact with casein micelles and then this whey protein-κ-CN complex dissociates from the micelles (Donato & Dalgleish, 2006; Parker, Donato, & Dalgleish, 2005) and some other reports suggest that κ-CN dissociates first and then interact with denatured whey proteins in the serum or on the micelles favourably in serum phase (Anema & Li, 2000; Anema, 2007)

2.4.2 Shear induced interactions of milk proteins

Processing conditions have a direct influence on behavioural attributes of proteins in a milk system. During the processing of milk, this fluid is subjected to many processing steps such as pumping, stirring, mixing, ultrafiltration and homogenising, which in turn generate hydrodynamic shear stress on the proteins resulting in destabilisation of native structures leading to denaturation and aggregation (Bekard, Asimakis, Bertolini, & Dunstan, 2011; Chandrapala, Martin, Zisu, Kentish, & Ashokkumar, 2012; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011). Typical shear rates encountered in dairy processing can be summarized as follows;

Table 2.5: Predicted shear rates for typical food processing (Barnes, Hutton, & Walters, 1989; Sestak et al., 1983, Steffe, 1996)

Process	**Shear Rate (s^{-1})**
Mixing and stirring	10^1-10^3
Pipe flow	10^0-10^3
Spray drying	10^3-10^4

Thus, understanding of the effects of various shear flow effects during processing would allow for the most appropriate design and conditions which would yield the highest quality products and innovations which would benefit dairy industry.

In shear studies, usually two common flow fields are applied known as extensional flow and simple shear flow. A homogenous elongational flow is distinguished by a linear velocity gradient along the direction of flow while the simple shear flow is specified by a flow field that has a perpendicular velocity gradient acting upon it. The fluid mechanical shear is commonly measured as a shear rate which is also known as velocity gradient in simple shear or sometimes as a shear stress (Thomas & Geer, 2011). For Newtonian liquids, the mathematical correlation between shear stress (τ) and velocity gradient ($\dot{\gamma}$) can be expressed as $\tau = \eta * \dot{\gamma}$ where the tangential stress is called shearing which occurs when very thin layers of fluid slips on each other in a laminar flow (Bekard, Asimakis, Bertolini & Dunstan, 2011).

The equipment used to apply shear in shear studies should provide uniform velocity gradient throughout the protein solution in order to obtain the best accuracy in readings. Therefore, a uniform laminar shear flow which maintains a constant velocity gradient

throughout the entire flow field should be achieved (Logan, 2012). The transport of particles in a flow field under a laminar flow can be characterised by a single value of shear rate (Benjamin & Lawler, 2013). Flow devices used in experiments are categorised in to two main components based on the type of flow known as capillary and rotational devices. In capillary flow, velocity profile is poiseuille type where the fluid is enforced through a conduit of known dimensions by applying a pressure difference between inlet and outlet. This has a non-homogenous shear flow with the maximum shear rate at boundary of the fluid vessel and minimum at the centre with a gradual decrease towards centre. Most common rotational flow devices used in shear studies are cone and plate, parallel plate and concentric cylinder type viscometers whereas the last type was used in the present study with a pressure cell in order to prevent volatilization under high temperatures (Figure 2.3). The main advantage of this type over capillary type is, it can maintain a constant shear rate throughout the solution which allows studying time dependant behaviour over an extended period of time.


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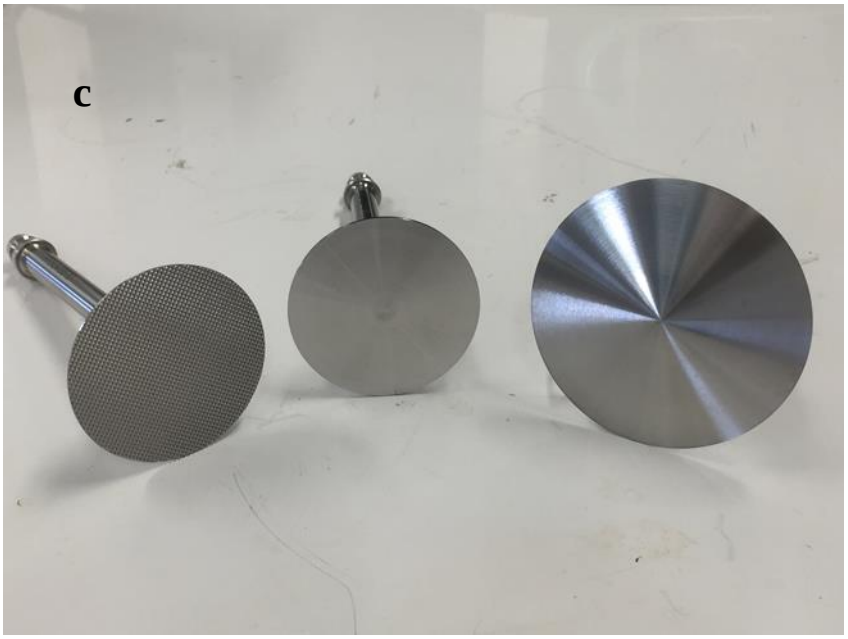


Figure 2.3: Rotational flow devices commonly used for shear studies **a.** Rheometer (Physica MCR 301 series: Anton Paar GmbH) **b**. narrow gap coaxial cylinder pressure cell used in rheometer **c**. cone and plate and parallel plate viscometers

Shear induced changes of proteins were originally studied by Charm and Wong in 1970. Catalase and carboxypeptidase solutions were subjected to shearing in a narrow gap coaxial cylinder, in which breakage of tertiary structure was observed due to shear forces. Charm and Lai (1971) found that catalase can be inactivated during circulation through ultrafiltration devices while rennet was not damaged. They suggested that, shear rate and exposure time have great importance in terms of loss of activity. Further, Tirrell and Middleman (1975) studied urea hydrolysis by urease under shear and observed both temporary and permenant inactivation. Moreover, flow based study done by Charm and Wong (1981) showed comparatively turbulent flow leads to greater loss in enzymatic activiy than streamline flow.

Later, some other studies showed that shear induced interface effect has also been responsible for structural modifications in both proteins and enzymes (Thomas &

Dunnill, 1979; Narendranathan & Dunnill, 1982; Donaldson et al., 1980; Lee & Choo; 1989). These studies led to a conclusion that gas-liquid interface is probably needed for the shear induced changes to most of the globular proteins during processsing. There is a possibilty that proteins undergo denaturation at an interface and mix back to the solution while interface is continually renewed (Thomas & Geer, 2011). These interfaces contain hydrophobic character and it is believed that the partially denatured monomers of proteins minimise surface energy by interacting with each other thereby shielding exposed hydrophobic moieties from the aqueous environment at interface. This was better explained by a study done by Sluzky et al., (1992) based on kinetics of the aggregation of insulin in the presence of air-liquid interfaces. They noticed that, insulin could be stabilised by the addition of surface active agents such as non-ionic sugar based detergents. Further, Mahler et al., (2005) found that Tween 80 (a non-ionic surfactant) seemed to stabilise immunoglobulin-G1 preventing further aggregation at air-liquid interface during agitation (Thomas & Geer, 2011).

Further, it was found that viscosity of a protein solution could also be manipulated via mechanical shear. Results showed that apparent viscosity of β-LG solutions under shear led to rheopectic behaviour for 10-30 % concentration, pseudoplastic behaviour for 10-40 % and thixotropic for 40 % concentration. It was claimed that, apparent viscosity was changed due to variations in shear rate and timing which eventually attributted to aggregation or alteration of β-LG molecules (Pradipasena & Rha, 1977).

In addition, shear could be used to manipulate the final properties of a product which becomes an added advantage in a product manufacturing. In relation to this, Walkenström and Hermansson (1998) found out that it was possible to change the characteristics of both pure and mixed particulate whey protein gels with gelatin using controlled shear. A controlled constant shear of 2 s^{-1} for 20 seconds governed the

formation of gels showed a storage modulus twice as high as unsheared gel resulting inhomogenous network with different pore sizes at various dimension levels. By applying the same shear methodology to fine stranded whey protein gels at pH 7.5 revealed minor modifications (Walkenström, 1996).

These studies reveal that shear plays a significant role in aggregation behaviour of a polymer and dimensions of aggregates. Related to this, Walstra and Jenness (1984) suggested that effects of streaming were dependant on the meeting frequency of particles (J_g) which was strongly depend on the particle size. J_g was defined in an equation shown below (equation 2.3);

$$J_g = 2/3d^3N^2G \qquad (2.3)$$

Where d is the diameter of the particle, N is the number of particles for unit volume and G is the velocity gradient (s^{-1}) (Walstra & Jenness, 1984). According to the equation, number of collisions among particles is highly dependent on a particle size. Also, they showed that shear effects are dependent upon streaming which may disrupt floccules and prevent them being formed. In addition, large floccules are more easily disrupted than smaller ones as they are more likely to have weak spots. Further, it was found that the net growth rate and size of any protein aggregate depends upon the equilibrium between growth and shear-controlled breakage.

Rate of aggregation in proteins is determined using mass transfer and reaction of components in a solution. At hydrodynamic shear conditions, mass transfer controlled aggregation proceeds as a two-stage process; diffusion controlled perikinetic growth due to random displacement of particles in Brownian motion as a consequence of thermal energy followed by the hydrodynamic shear controlled (orhtokinetic) growth. Initial aggregation rate of proteins determined by diffusion controlled collisions to formation

of primary aggregates and when these are large enough for fluid motion to become important in influencing on collisions eventually results in orthokinetic aggregation (Taylor &Fryer, 1994; Gregory, 2013). The hydrodynamic shear induced aggregates are known to be stronger than aggregates formed via Brownian motion which are easily disrupted by shearing (Oyegbile, Ay, & Narra, 2016).

The ultimate size as well as growth rate of any protein aggregates under shear determined by the equilibrium between growth and shear controlled fragmentation (Virkar, Hoare, Chan, & Dunnill, 1982; Hoare, 1982). The fragmentation mechanism proposed by Parker et al, (1979) can be stated as follows; (i) distortion and rupture due to changing dynamic pressure (ii) erosion of primary aggregates from the surface of aggregates due to aggregate and solid surface collisions and by fluid motion generated shear stress (iii) fragmentation of aggregates.

Studies so far observed that shear can cause denaturation at high shear rates, increase aggregation by promoting collisions and enhance fragmentation of aggregates (Taylor & Fryer, 1994; Xie, Qiao, & Dunstan, 2016). This concludes the impact of fluid mechanical shear on protein structures should be considered as a major importance in any processing system to avoid undesirable consequences while obtaining the maximum yield.

2.4.3 Combined heat and shear induced interactions of milk proteins

Based on the observations so far, it could be hypothesized that combined effect of heat and shear could impose more aggravated denaturation and aggregation in proteins. The impact of both shear and temperature on the aggregation behaviour of whey proteins have been studied by several authors (Steventon, 1994; Steventon, Donald, & Gladden, 1994; Taylor & Fryer, 1994; Walkenström, Windhab, & Hermansson, 1998). Steventon

et al., (1994) studied the effects of temperature (between 75-90 °C) and fluid shear (up to 1480 s^{-1}/ at times up to a maximum of 60 minutes) on aggregation behaviour of whey proteins using 7 % (W/W) whey protein concentrate (WPC) at pH 6.3. Results showed aggregation kinetics of WPC were constant under the laminar coquette flow with a two-stage process; initial slow rate-limiting process controlled by denaturation followed by a fast rate-limiting process dominated by the shear controlled aggregation growth and fragmentation.

Walkenström et al. (1998a, b) studied the effects of shear during heating from 20 ºC up to different stages of whey protein aggregation at pH 5.4. At about 50 ºC, shear induced aggregates appeared in the solution indicating shear influenced aggregation below denaturation. These aggregates were characteristic when compared to the ones formed under static conditions while different in size and compactness with strength of shear flow and temperature (Walkenström et al., 1998b)

Another study done by Spiegel and Kessler, (1998) found that heating whey protein concentrate to 80 ºC and subsequent cooling under high shear resulted in aggregates of about 10 µm. Further it was found out these aggregates could be introduced in to ice cream (Koxholtet al., 1999), soft cheese (Steffl et al., 1997) or semi hard cheese (Schreiber et al., 1998) to improve the texture, creaminess as well as increase in yield of the cheese. In addition to their textural role and increase in yield, their ability to act as fat mimetics has been proposed recently. The enhanced fat perception could be obtained via the smooth and soft small particles which would be able to lessen the friction between oral surfaces (Farrés, Douaire, & Norton, 2013). In this regard, the formation of shear induced fluid gels has attracted much attention due to relatively feasible modulation of properties resulting by modifying two parameters, shear and temperature (Moakes, Sullo & Norton, 2015). Apart from that, these aggregates are important in

stabiliser in foams as well as functional ingredient for structural and viscosity modifications in many food products (Çakir-Fuller, 2015).

Moreover, a study based on whey protein aggregation over simultaneous heating (from 20 ºC – 90 ºC for 35 min; holding at 90 ºC for 20 min and cooling from 90 – 25 ºC within 15 min) and shearing (100, 500 and 1000 s^{-1} shear rates) with four different protein concentrations (5, 10, 17.5 and 25% w/w) showed that application of shearing has decreased formation of molecular bonding at pH 7. Gel colour, turbidity, surface hydrophobicity, PAGE and viscosity measurements showed that extent and direction of heat induced whey protein denaturation and aggregation has been changed by shear forces (Dissanayake & Vasiljevic, 2010). Moreover, their studies indicated that microparticulation of whey proteins using heating and high pressure shearing could modulate physical functionality specially heat stability of whey proteins.

The influence of simultaneous heating and shearing on proteins was further evident in a study based on whey proteins at 80 ºC (Wolz, Mersch, & Kulozik, 2016). In this study, the aggregate size was found to be regulated by applying different shear rates at different protein concentrations. At a concentration of 5%, size of aggregates was initially increased due to increased number of collisions and decreased subsequently due to increase in shear stress. A decrease in aggregate size was observed at high concentrations along with the shear rate due to increase in shear stress.

In addition to whey proteins, fluid drag associated with shear could also destabilise casein micelles resulting in structural modifications such as elongation, resulting complex behavioural attributes. It was found, these can act as soft spheres that deform and align along the flow direction at high shear rates. The effective radius of the casein micelles was hypothesized to be decreased due to hydrodynamic forces on the κ-casein layer (Olivares, Berli, and Zorilla, 2013). Moreover, it is evident that, process shear

results in modifications in outer hydration sphere of casein micelles modifying stabilising energy provided by the preferential hydration (Stephen et al, 2008).

Apart from that, several studies were focused upon influence of shear on proteins in the presence of some of the other milk components. Spiegel (1999) studied on impact of lactose and heating temperature on whey protein aggregation under shear conditions. Within this study, different concentrations of whey proteins were heat and shear treated with different lactose contents in a surface heat exchanger at various temperatures. In the presence of high lactose content and temperatures below 85 ºC, protein unfolding was slowed down while loose, porous aggregate structures were observed. Over 100 ºC, aggregation was observed as the rate limiting step and very dense and compact aggregate structures were observed.

Within another study (Spiegel & Huss, 2001) based upon the shear influence on whey proteins at different pH and removal of calcium, it was observed that the denaturation rate of β-Lactoglobulin at 80 ºC was considerably retarded when the pH was reduced from 6.7 to 4.5. when calcium concentration was reduced, heat and shear treatment resulted in a gritty structure with rubber like particles within the whey protein solutions.

Therefore it can be seen that, shear could manipulate the extent and direction of heat induced interactions of milk proteins and the other components within the system has a major influence on the ultimate product. .

Chapter 3

Structural Changes of Raw Milk Proteins Subjected to Controlled Shearing and Heating

3.1 Introduction

Milk is a very perishable food item that without proper processing and handling may lose its quality attributes rapidly or even become unsafe. For these reasons, one of the major roles of food processing is to minimise the risks associated with the presence of various biological contaminants including microorganisms and enzymes. In the dairy industry, thermal processing is one of the most commonly used methods to achieve this goal with pasteurisation, sterilisation and UHT treatments as key methods being widely used. However, these treatments lead to alterations of physico-chemical, nutritional and functional properties of milk depending on the severity of the treatment. Whey protein denaturation, κ-casein/β-LG complexation, calcium phosphate precipitation onto casein micelles, Maillard reaction and decrease in pH are some of the physico-chemical changes that take place during heating (Considine et al., 2007; Huppertz, 2016).

During commercial processing, in addition to heat milk is also subjected to shear forces during various processing steps such as pumping, stirring, mixing and homogenisation, and even flow through heat exchangers including heating, holding and cooling. Depending on the extent and complexity of flow, structural modifications of milk proteins may take place to a varying degree upon exposure to these hydrodynamic shear forces (Chandrapala et al., 2011; 2012). Large velocity gradients created by shear forces during fluid flow may incur conformational transitions or even unfolding and exposure of hydrophobic amino acids, which could eventually lead to various interactions within and among protein structures and consequently aggregation (Stasio & Cristofaro, 2010). Both irreversible and reversible denaturation of proteins has been observed depending on the shear rate applied (Maa & Hsu, 1996). Folded conformations of proteins present

a fine balance between the stability and instability of native proteins governed by a great number of rather small but cooperative contributions and compensations of attractive and repulsive forces. Most studies on the effects of shear focused on enzymes since they lose their activity rapidly upon shear exposure. However, shear alone does not act as a primary driver of protein denaturation as Jaspe and Hagen (2006) demonstrated no detectable unfolding of the horse cytochrome c protein at 25 ºC exposed to shear rates of up to 200,000 s-1 for several millliseconds. Several requirements need to be satisfied for a protein unfolding to take place. Molecular weight of a protein or the viscosity (or shear stress) of the solution needs to be sufficiently high to induce unfolding (Jaspe & Hagen 2006). For example, the unfolding of Immunoglobulin-G1 monoclonal antibody in a solution with a viscosity of 3 mPa.s and sheared at 20,000 s-1 (<0.06 pico Newtons) was found to be far less in comparison to exerted shear (~ 140 pN) due to surface tensions at the air-water interface (Bee et al, 2009). Therefore, adsorption to solid surfaces (with a possible shear synergy) or pump cavitation stresses would be much more important causes of unfolding and aggregation than a simple flow shear.

Combined effects of temperature and shear forces during processing could impose even greater structural changes thereby leading to more pronounced denaturation and aggregation. During heating, globular proteins unfold and engage in various interactions governed by a collision frequency of unfolded monomers (Taboada-Serrano et al, 2005). In general, the extent of interactions and thus aggregation is driven by Brownian motion, termed perikinetic aggregation, and differential settling. However, with the elevated flow rates under hydrodynamic shear conditions, the collision of particles results in orthokinetic aggregation (Meyer & Deglon, 2011; Walstra, 2001). This may ultimately result in numerous outcomes such as varying sensory perception yielding different food textures, phase inversions in phase separating mixed suspensions and

formation of gels due to alterations in size and morphology of aggregates (Walkenström & Hermansson, 1998; Langton et al., 1996). Although simultaneous application of both thermal processing and shearing is common, studies focused on their combined effects and how it would impact various interactions and structural changes of proteins are limited, especially starting with the raw milk. Therefore, the investigation of interactions among proteins under these conditions and their impact on the structural changes of otherwise intact native proteins would provide some valuable information on how these two important processing factors influence the physico-chemical properties of milk and consequently the final quality of the product. For this reason, some conditions applied in the study resembled pasteurisation (HTST) and ultra-high temperature (UHT) processing at commercial level exhibiting temperatures 72 °C and 140 °C with the holding time of 15 and 3 seconds respectively. However, caution should be exercised as due to limitations of the equipment used in the experimentation; these temperatures were not reached at the same heating rate as it is usually achieved industrially. Furthermore, the selected shear rates of 500 and 1000 s-1 were chosen in order to mimic those usually observed at an industrial level.

3.2 Materials and Methods

3.2.1 Materials

Fresh whole raw milk was obtained from Warrnambool Cheese and Butter Factory (Warrnambool, Australia) and was skimmed by centrifugation (Avanti J-26SXPI, Beckman Coulter, Harbor Boulevard, CA, USA) at 12000 *g* for 20 minutes at 20 ºC. The raw skim milk composition was analysed as described by Chandrapala (2008). The Australian Standard method 2300.1.5 (1988) was used to determine the ash content and contained 0.75% (w/w) ash content in raw milk. The AOAC official modified

Mojonnier ether extraction method 989.05 was used for the fat determination and 0.087% (w/w) fat was detected in raw milk. Kjeldahl method and nitrogen conversion factor of 6.38 (989.05, AOAC, 2000) were applied in protein determination and contained 3.30% (w/w) total proteins, out of which 2.30% (w/w) caseins, 0.98% (w/w) whey proteins and 0.01% (w/w) non-protein nitrogen. Lactose content was calculated as 4.80% (w/w) by subtracting the protein, fat and ash contents of the sample from the total solid content. .All the chemicals used for analysis were obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia) and ultrapure water (Milli-Q water, Merck Millipore, Bayswater, Vic, Australia) was used at all times.

3.2.2 Treatment of samples

Raw skim milk was subjected to three different shear rates (0, 500, or 1000 s^{-1}) and three time-temperature combinations of 20 °C for 19 seconds, 72 °C for 15 seconds and 140 °C for 3 seconds in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301, Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a constant pressure of 250 kPa following the method of Liyanaarachchi et al., (2015). The intact samples not subjected to shear at 20 °C were used as the controls. The samples subjected to heating at 72 °C were heated at a rate of 3.2 ºC min^{-1} to the required temperature, held there for 15 seconds, and cooled down to 20 ºC at the same rate. All the samples treated to 140 °C were heated at a rate of 5 °C min^{-1}, held at 140 °C for 3 seconds and then cooled at the same rate down to 20 ºC. Shearing was maintained throughout the entire process of heating, holding and cooling periods.

3.2.3 Particle size and zeta potential measurements

Average particle size, polydispersity index (PDI) and zeta potential were acquired using a Zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) (Liyanaarachchi et al., 2015). Three readings were obtained from each sample and averages were calculated for comparative analysis. Treated samples were diluted 1000 times using skim milk ultra-filtrate (SMUF) prior to measurements. The refractive index of milk and SMUF used in the calculations were 1.338 and 1.334 respectively.

3.2.4 Fourier transform infrared spectroscopy analysis

Changes in the secondary structure of proteins were assessed immediately after each treatment using a FTIR spectrometer (PerkinElmer Frontier FTIR Spectrometer, MA, USA). FTIR spectra were obtained at room temperature with an average of 16 scans with a resolution of 4 cm^{-1} after background subtraction (Liyanaarachchi et al., 2015). Second derivative of all FTIR spectra was obtained using an associated software (Perkin Elmer) to enhance the resolution.

3.2.5 Calcium activity measurements

The Ca^{2+} activity was measured immediately after treatment at 25 °C using a Calcium Ion Selective electrode connected to a pH meter (Metrohm, 713 pH Meter, Herisau, Switzerland) fitted with a reference Ag/AgCl electrode (Chandrapala et al., 2010). Regular calibrations were carried out with the $CaCl_2$ solutions in the range of 0.5 mM – 20 mM with the ionic strength of 80 mM, adjusted with KOH. Activity coefficient of Ca^{2+} used for the calculations was 0.425 as given by Debye-Huckel approximation (MacInnes 1961).

3.2.6 Surface hydrophobicity measurements

Surface hydrophobicity of the treated milk samples was analysed using 1-anilinonaphthalene-8-sulfonic acid (ANS; Sigma Aldrich, St Louis, MO, USA) as the fluorescence probe as described by (Alizadeh-Pasdar & Li-Chan, 2000) with minor modifications. All milk samples were diluted to obtain a series of dilutions within 0.02-0.12% (w/w) with pH 7.0 phosphate buffer. A 20 µL aliquot of 8×10^{-3} M ANS solution was added to 4 mL of sample and the mixture was vortexed. These vortexed samples were kept in dark for 15 minutes and the relative fluorescence intensity (RFI) measurements were taken using Schimadzu spectrofluorometer (5301-PC, Shimadzu Corp., Kyoto, Japan) at excitation and emission wavelengths of 390 nm and 470 nm respectively. The RFI of each solution was measured starting from buffer blank and then the lowest to highest protein concentration. The RFI of each dilution blank was subtracted from each of the corresponding protein solution with ANS to obtain the net RFI. The initial slope of the net RFI versus protein concentration plot was calculated using linear regression analysis and used as an index for protein surface hydrophobicity.

3.2.7 Polyacrylamide gel electrophoresis

For native PAGE, 50 µL of sample was mixed with 950 µL of native sample buffer. 40 µL of sample was mixed with 1 mL of SDS sample buffer for SDS PAGE under non-reducing conditions, while 20 µL of 2-mercaptoethanol was added and heated in boiling water bath for 4 minutes for reduced conditions. Stock solutions of 0.006% (w/w) α Lactalbumin, β-Lactoglobulin and BSA were prepared using MilliQ water and diluted by 10 fold with sample buffer and used as standards. Casein standard was prepared using Sodium Caseinate 0.056% stock solution prepared using MilliQ water and by

diluting with sample buffer (× 14 times). Pre-stained protein standard (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular marker.

Native and SDS resolving and stacking gels were prepared and casted between two glass plates as described by (Grewal et al., 2016) with some minor modifications. 10 µL from each sample, standards and molecular marker were loaded in to gels for analysis.

The gels were placed in a Bio-Rad Protean II xi cell filled with Tris glycine electrode buffer. Electrophoresis was performed at 70mA, 210V, and 6.5W. The resultant gels were stained using Coomassie Blue for one hour and destained using a mixture of 0.1% Isopropanol, and 0.1% Glacial Acetic Acid in water overnight. Gel Images were taken using ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories).

Relative band intensities (RBI) of native gel proteins were calculated for comparison. The RBI was defined as

$$RBI = \frac{Band\ Intensity}{Total\ lane\ Intensity} \qquad (3.1)$$

3.2.8 Statistical Analysis

The experiments were designed as a randomized, blocked split-plot design with temperature as the main plot and shear as the subplot. This block was replicated with 3 sub samplings. The results of the various determinations were analysed using the GLM procedure of SAS (SAS Institute, 1996), establishing effects of main factors (temperature and shear) and their interactions. The level of significance was set at $P \leq 0.05$.

3.3 Results and Discussion

3.3.1 Shear induced changes of milk proteins at 20° C

Even at room temperature, applied shear had an effect on certain properties of milk proteins. The control sample had an average particle size of ~147 nm and this was shifted towards significantly higher values ($p<0.05$) under applied shear to ~158 nm at 500 s^{-1} along with an increase in PDI of ~13%. Further increase in shear up to 1000 s^{-1} has not resulted in a noticeable change in average particle size or in PDI (Table 3.1).

Table 3.1: Surface potential, particle size (Z-average), polydispersity Index (PDI), hydrophobicity and calcium activity of milk treated at 20, 72 or 140 °C with simultaneous shearing (0, 500 or 1000 s^{-1}).

Temperature (°C)	Shear (s^{-1})	Zeta potential (mV)	Average particle Size (d. nm)]	Polydispersity Index (PDI)	Surface hydrophobicity	Calcium activity (mM)
20	0	-24.5^{a}	146.6^{a}	0.099^{a}	52.5^{a}	1.01^{a}
	500	-23.1^{ab}	158.6^{b}	0.112^{a}	48.4^{b}	1.12^{a}
	1000	-23.2^{ab}	158.5^{b}	0.108^{a}	47.8^{b}	1.12^{a}
72	0	-22.4^{ab}	146.8^{c}	0.104^{b}	65.4^{c}	0.89^{b}
	500	-22.7^{b}	147.7^{c}	0.111^{b}	77.0^{d}	0.85^{b}
	1000	-21.6^{b}	148.6^{c}	0.167^{c}	74.7^{d}	0.85^{b}
140	0	-24.2^{a}	156.2^{d}	0.137^{d}	101.1^{e}	0.75^{c}
	500	-24.9^{a}	163.7^{e}	0.134^{d}	100.9^{e}	0.71^{c}
	1000	-23.4^{a}	165.6^{e}	0.138^{d}	101.6^{e}	0.72^{c}
SEM*		-0.6	1.5	0.007	1.3	0.03

*Pooled standard error of the mean; The means indicated with different superscripts are significantly different ($p<0.05$)

However, at both 500 s^{-1} and 1000 s^{-1}, shear induced aggregation was not observed as indicated by the native PAGE data (Table 3.2).

Table 3.2: Relative Band Intensity (RBI) of proteins subjected to different treatments resolved by under native electrophoretic conditions and quantified using a ChemiDoc imager.

Temperature (ºC)	Shear (s^{-1})	α_s-casein + β-casein	κ-casein	β-LG	α-LA
20	0	39.2^{a}	26.3^{a}	16.1^{a}	4.4^{a}
	500	38.4^{a}	26.2^{a}	15.7^{a}	4.1^{a}
	1000	36.7^{b}	26.9^{a}	17.7^{b}	3.9^{a}
72	0	37.1^{c}	26.7^{b}	10.6^{c}	3.2^{b}
	500	42.3^{d}	29.3^{c}	14.2^{d}	4.1^{c}
	1000	34.2^{e}	29.7^{c}	13.4^{d}	3.8^{b}
140	0	44.5^{f}	30.8^{d}	0.9^{e}	1.9^{d}
	500	45.3^{f}	30.8^{d}	0.6^{e}	1.4^{d}
	1000	45.5^{g}	30.5^{d}	0.1^{e}	1.2^{e}
SEM*		0.3	0.7	0.4	0.2

*Pooled standard error of the mean; The means indicated with different superscripts are significantly different ($p<0.05$).

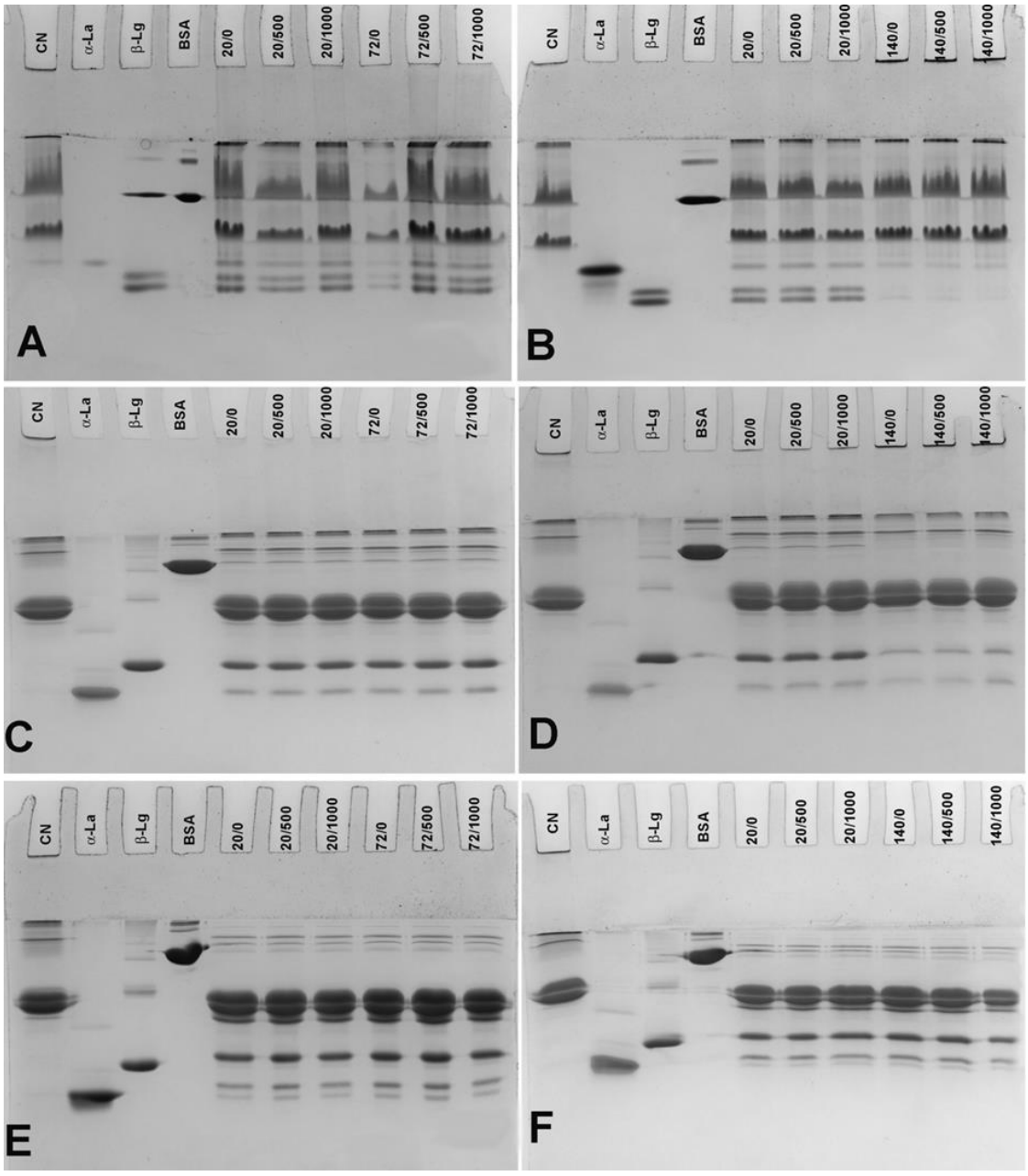


Figure 3.1: Native (A and B), non-reducing (C and D) and reducing SDS (E and F) polyacrylamide gel analysis. (For all gels, lane 5 (L5) – 20 °C/0 s-1, L6 – 20 °C/500 s-1, L7 – 20 °C/1000 s-1, for gels A, C and E, L8 - 72 °C/0s-1, L9 - 72 °C/500 s-1, L10 - 72 °C/1000 s-1, and for gels B, D and F, L8 - 100 °C/0 s-1, L9 - 100 °C/500 s-1, L10 - 100 °C/1000 s-1 respectively).

Therefore, the increase in average particle size as well as in PDI would be expected due to either elongation or molecular unfolding or as a consequence of both.

Polymers like proteins subjected to a simple shear flow as in the current study are expected to undergo a combination of rotational and deformational velocities of equal intensity in the flow direction (Bekard & Dunstan, 2009). Within the rotational flow field, these molecules undergo whole body rotation in the direction of flow with zero shear strain. However, in the deformation cycle, molecules are exposed to stretch and compressive (tumbling) events of equal magnitude. Molecules experience hydrodynamic drag forces (stretching) in the deformational flow field, which would overcome cohesive stabilising forces, including the intramolecular hydrogen bonds, stabilising the helical structures resulting structural destabilisations leading to molecular unfolding. Since individual molecules randomly access both compression and stretching events, periodic elongation, relaxation and tumbling result as temporal fluctuations leading to complex behavioural attributes (Bekard & Dunstan 2009).

Changes in the secondary structure were analysed using the second-derivative of the FTIR spectra to obtain the enhanced resolution of hidden peaks and to clearly separate overlapping spectra (Byler et al., 1995; Rieppo et al., 2012).

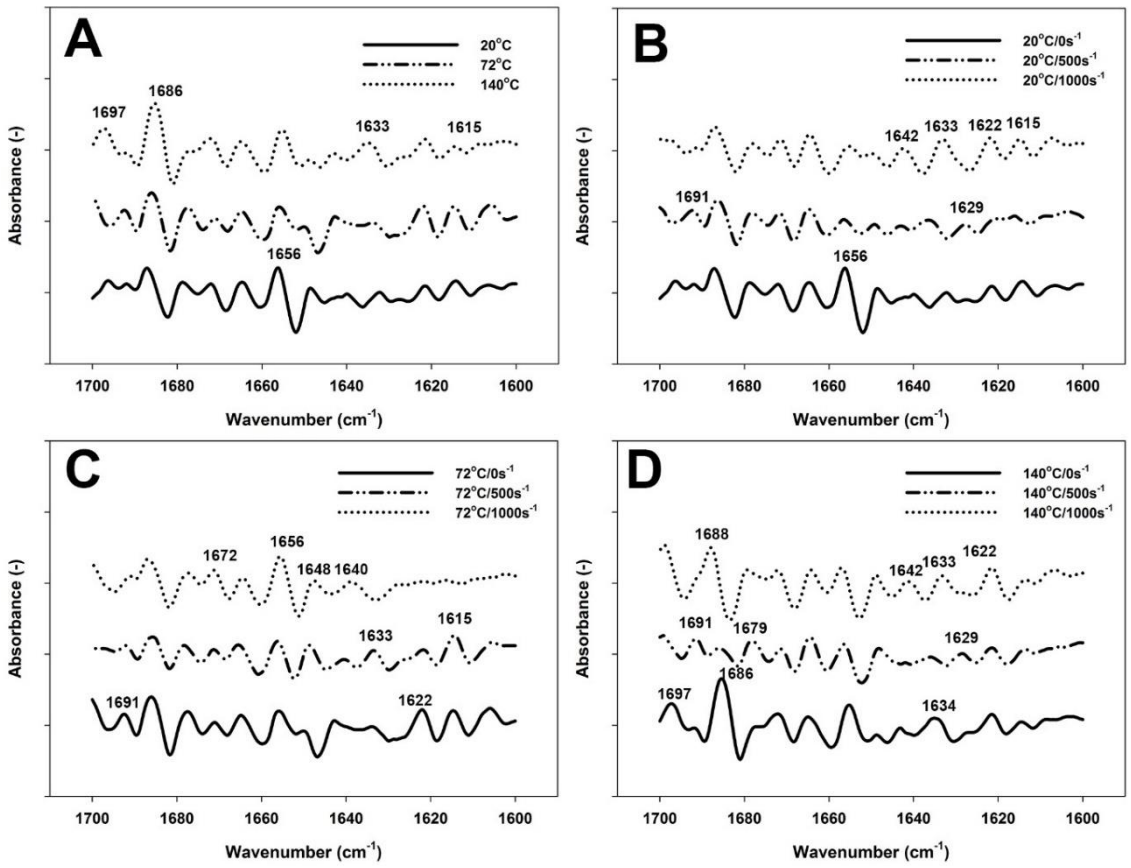


Figure 3.2: Second derivative FTIR spectra of the Amide I region (1600 – 1700 cm $^{-}$1) obtained for milk processed at 20 °C, 72 °C and 140 °C at no shear (A), 20 °C at 0 s^{-1}, 500 s^{-1} and 1000 s^{-1} (B), 72 °C at 0 s^{-1}, 500 s^{-1}, 1000 s^{-1} (C) and 140 °C at 0 s^{-1}, 500 s^{-1}, 1000 s^{-1} shear rates (D).

In the absence of shear at 20 °C, the peaks were prominent at ~1656 cm^{-1} denoting the native α-helical structure, and at ~ 1622 and 1633 cm^{-1} due to intermolecular anti-parallel β-sheets between two β-LG monomers in their dimeric form (Lefevre & Subirade, 1999). With the increase in shear up to 500 s^{-1}, the intensity of the peak at 1656 cm^{-1} was significantly reduced indicating changes in the native α-helical structure. In addition, the disappearance of both peaks at ~ 1622 and 1633 cm^{-1} signifies dissociation of β-LG dimeric forms, recognised as the initial step of β-LG denaturation

(Liyanaarachchi et al., 2015). This was further supported by the appearance of a peak at ~ 1629 cm^{-1} indicating exposure of a β-sheet denoting partial unfolding (Lefevre & Subirade, 1999). The distinguishable peak at ~1691 cm^{-1} as well as disappearance of the one at ~ 1615 cm^{-1} which are assigned to β-sheet structures reveal unfolding of molecules into more random structures (Kong & Yu, 2007). Further increase in shear up to 1000 s^{-1} resulted in either retention or reformation of many of the β-sheet structures. However, the more distinguishable peaks at ~1622, 1633, 1615 and 1642 cm^{-1} compared to those in the absence of shear depict some form of rearrangement in the β-sheet structures. Overall, the increase in shear in the absence of heat have likely resulted in minimal secondary structural modifications including reversible denaturation and unfolding.

While surface potential appeared unaffected by applied shear, the measured surface hydrophobicity declined significantly ($p<0.05$) as the shear was enhanced (Table 3.1). In their native state, whey proteins adopt a more thermodynamically stable arrangement by having polar amino acids facing the exterior with non-polar amino acids buried in the interior minimising surface tensions due to hydrophobic repulsion. In the event of protein unfolding, these nonpolar residues would be exposed, which would increase surface hydrophobicity. When compared with the control, affinity of the ANS probe and tightness of surface hydrophobic bindings within the molecules were significantly reduced. This likely indicate that the surface hydrophobic sites within the molecules were either spread or fragmented with the increase in shear forces resulting in a decrease in surface hydrophobicity (Ÿuksel & Erdem, 2004). Also, it appears that hydrophobic calyx within the β-LG appears to be not affected as observed in the FTIR data. This signifies that both shear rates induced changes on the particle surface, especially involving hydrophobic sites on the secondary structure.

3.3.2 Shear induced changes of milk proteins at 72° C

Almost no change in particle size was observed with the increase in shear rates during heating at 72 °C (Table 3.1). However, PDI shows a significant (~61 %) increase at 1000 s^{-1} suggesting a major degree of non-uniformity between particles. Native PAGE data (Table 3.2) depicts an increase in both β-LG (>30 %) and α-LA (>25 %) at both shear rates indicating a shear-driven fragmentation of hydrophobically linked aggregates in which these proteins were involved. The increase in temperature alone up to 72 °C has resulted in protein unfolding and aggregation via hydrophobic bonding. This was evident in the FTIR data as it shows reductions in peaks at ~1633 and 1656 cm^{-1} denoting decline in β-sheet and native α-helical structures respectively along with the increase in peaks at ~ 1615, 1622 and 1693 cm^{-1} depicting incline in β-sheet driven aggregation. Shear stress generated in the deformational flow field would be sufficiently great to have separated these loosely bound, hydrophobically linked aggregates. This is evident in surface hydrophobicity data as it shows a significant difference ($p<0.05$) at both shear rates compared to the sample not subjected to shearing, indicating shear induced structural modifications. Surface hydrophobicity data acquired at 72 °C indicated about 18% increase at 500 s^{-1} demonstrating a greater level of exposed hydrophobic sites due to fragmentation of hydrophobically linked aggregates at this stage in comparison to 20 ºC. Fragmentation of hydrophobic aggregates was confirmed by the FTIR analysis with a reduction of peaks at ~1622 and 1691 cm^{-1}, which denotes β-sheets, at 500 s^{-1}. Apart from that, appearance of a more prominent peak at ~ 1648 cm^{-1} denoting an unordered structure depicts a certain level of aggregation (Lefevre & Subirade, 1999). At 1000 s^{-1}, further reductions in peaks at ~1622, ~1633 and 1691 cm^{-1} were observed indicating enhanced fragmentation (Figure 3.1B). In addition, increased peaks at ~ 1640 and 1672 cm^{-1} denoting β-sheets and β-turns, respectively, indicate

further protein aggregation. The pressure from compression of molecules in the deformational flow field would have facilitated a rapid association of fragmented molecules leading to aggregation (Bekard & Dunstan, 2009). On the other hand, increasing shear to 1000 s^{-1}, no apparent difference in surface hydrophobicity was observed.

3.3.3 Shear induced changes of milk proteins at 140° C

In comparison to the samples treated at 72 °C, the samples treated at 140 °C underwent significant heat and shear induced changes. First of all, the treatment resulted in an increase in the average particle size upon enhanced shearing (Table 3.1). The FTIR data show more prominent peaks at both ~ 1686 cm^{-1}, corresponding to intermolecular β-sheet aggregates, and at ~ 1697 cm^{-1} due to antiparallel β-sheet driven aggregation (Lefèvre & Subirade, 1999), in comparison to those at 72 ºC, indicating a greater extent of aggregation. In addition, the peak at ~1656 cm^{-1} corresponding to native intramolecular α helix structures (Rahaman et al., 2015) completely diminished. All these observations again indicate aggregation due to changes in the secondary structure of whey proteins at elevated temperatures. In contrast to an increase in surface hydrophobicity due to rise in temperature, no significant change in the surface potential of these particles caused by shearing was observed (Table 3.1). However, elevation of hydrophobicity at 140 °C appears contradictory with the protein aggregation that was evident from the increase in particle size since aggregation can mask the hydrophobic sites on β-LG molecules reducing overall surface hydrophobicity (Rahaman et al., 2015). It is possible that denatured whey protein monomers have undergone sulphydryl disulphide interactions with the cysteine residues of κ-caseins and α_{s2}-caseins increasing the size of the particles as observed from the particle size distribution (Law & Leaver,

1997; Oldfield et al., 2000). In addition, denatured whey proteins can complex with other whey proteins or free κ-casein in the serum phase (Singh & Waungana, 2001). As seen in Table 3.2, the intensities of α-LA and β-LG bands were the weakest in milk processed at 140 °C in comparison to those at 20 °C or 72 °C. In addition, a clear thick band at the entry of the stacking gel corresponding to high molecular weight aggregates (MW > 250 kDa), which were not able to penetrate through the gel, was more prominent at 140 °C as compared to 72 °C further indicated extensive aggregation. These aggregates were significantly reduced in the presence of SDS as this thick band at the top of the stacking gel was minimized in the non-reducing SDS-PAGE (Figure 3.2D) suggesting substantial involvement of non-covalent interactions in the aggregation process. This was followed with a simultaneous rise in the band intensities of β-LG and α-LA. Under reducing conditions, the high MW aggregates at the top of the gel completely disappeared, which means that only disulphide linked aggregates were present and no other covalent bonding was involved in the aggregation.

Table 3.3: Concentration (mg /ml) of individual proteins subjected to different treatments resolved by under non-reducing (NR) or reducing (R) electrophoretic conditions and quantified using a ChemiDoc imager

Temperature °C	Shear s^{-1}	BSA		α_s-casein		β-casein		κ-casein		β-LG		α-LA	
		NR	R	NR	R	NR	R	NR	R	NR	R	NR	R
20	0	0.042	0.048	0.63	0.68	0.55	0.64	0.05	0.21	0.32	0.38	0.11	0.12
	500	0.036	0.053	0.70	0.71	0.66	0.69	0.05	0.24	0.34	0.37	0.11	0.13
	1000	0.036	0.051	0.72	0.74	0.67	0.76	0.05	0.19	0.35	0.36	0.12	0.11
72	0	0.036	0.047	0.68	0. 77	0.67	0.76	0.06	0.25	0.34	0.41	0.11	0.14
	500	0.042	0.053	0.71	0.86	0.67	0.71	0.06	0.18	0.37	0.44	0.12	0.13
	1000	0.054	0.055	0.73	0.84	0.68	0.77	0.07	0.21	0.41	0.45	0.09	0.13
140	0	0.012	0.043	0.78	0.77	0.70	0.73	0.08	0.22	0.09	0.35	0.09	0.11
	500	0.006	0.051	0.80	0.83	0.68	0.78	0.07	0.20	0.06	0.35	0.07	0.12
	1000	0.008	0.047	0.81	0.87	0.69	0.70	0.07	0.19	0.07	0.38	0.07	0.12

According to the protein concentration data obtained from the gels (Table 3.3) at 140 ºC, it can be seen that most of β-LG (~0.26 mg/ml) was involved in sulphydryl-disulfide bonding which also included BSA (~0.03 mg/ml) and a small fraction of α-LA (~ 0.02 mg/ml). Strong covalent interactions prevailed at 140 °C via denatured whey proteins and the free caseins present in the serum phase. These free serum caseins and colloidal caseins are in an equilibrium that depends on pH and temperature. Structure of the casein micelle is maintained by various hydrophobic, electrostatic and van der Waal interactions along with the calcium colloidal phosphate. Any disturbance in these interactions by temperature and pH can lead to disintegration of the casein micelles (Anema & Klostermeyer, 1997). A decrease in the calcium activity was evident (Table 3.1), which was further pronounced at 140 °C as compared to 72 °C likely caused by precipitation of calcium phosphate onto the surface of the equipment. This contradicts to a previous report (Zhang & Aoki, 1996), which noted that approximately 60% of calcium and 40% of phosphate from the serum phase transferred to the colloidal phase on heating to 90°C for 40 min. The decrease in calcium activity is reversible upon cooling depending on the severity of heating (Geerts et al., 1983). It is possible that transfer of calcium and serum phosphate onto casein micelles can screen the negative charges on the micelles while reducing zeta potential and electrostatic repulsions among caseins (Singh, 2004). Shearing however had no apparent effect on calcium activity (Table 3.1).

With introduction of shear at 140 ºC, shifting of the average particle size to slightly greater values were evident (Table 3.1) when the samples were subjected to shearing at 500 s^{-1} possibly due to aggregation. However further enhancement of shear had no observable effect on the average particle size. Complementarily, at 500 s^{-1} the FTIR data show a more prominent peak at ~ 1679 cm^{-1} denoting intermolecular β-sheet crosslinking and at ~1629 cm^{-1} corresponding to the presence of β-turns (Lefèvre & Subirade, 1999). Further increase in shear resulted in prominent peaks occurring at ~1622 and 1633 cm^{-1} probably due to re-

association of monomers into dimers. In addition, more distinguishable peaks at ~1688 and 1697 cm^{-1} attest to intermolecular and antiparallel β-sheet driven aggregation, respectively. At the same time, random coils were intensified as indicated by a peak at ~1642 cm^{-1} (Kong & Yu, 2007). The data in Table 3.1 indicates that shearing did not affect the calcium equilibrium and any impact on the casein micelle was brought about the application of heating. This was supported by relevant SDS PAGE electropherograms (Table 3.3), which showed no apparent changes in the band intensities of the caseins as a function of shear rate. While Table 3.1 indicated increase in average particle size, this was not followed by changes in either hydrophobicity or surface potential. From the data, whey proteins appear, as expected, the most affected by this particular treatment. Native PAGE analysis of the samples treated at 140 °C showed a more prominent depletion of β-LG and α-LA irrespective of shear in contrast to these at 72 °C (Table 3.2). In addition, the presence of high molecular weight aggregates at the arrival to the stacking gel under all three shear rates indicates aggregation with the involvement of both of these proteins.

According to non-reducing SDS-PAGE, the reduced band intensities of these two whey proteins and the reduced thickness of the high molecular weight aggregates on the stacking gel under all shear rates when compared to native gel stipulate the involvement of some non-covalent interactions. The disappearance of high molecular weight aggregates at the entrance of the stacking gel of the reducing SDS gel confirms that aggregation was mainly due to sulphydryl-disulphide bonding among the proteins at 140 °C under all shear rates. BSA appears to be the most affected by shearing at this temperature since its concentration in non-reducing SDS-PAGE was substantially reduced (by 50%) with introduction of shear (Table 3.3). BSA concentration was significantly reduced further when milk was heated to 140 ºC due to almost complete denaturation/aggregation due to a high number of reactive -SH groups. This could be a reason why shear did not have any obvious effect. At this

temperature, β-LG and α-LA appeared to be slightly affected in comparison to BSA (Table 3.3). The main casein involved in these interactions was κ-casein, which appeared unaffected by shear variations.

3.4 Conclusion

Shear alone at 20 ºC has induced subtle structural modifications resulting in reversible unfolding of native proteins at 500 s^{-1} while inducing a structural reformation at 1000 s^{-1}. Fragmentation of hydrophobically linked whey protein aggregates was prominent at 72 ºC upon shearing at 500 s^{-1} with no significant structural impact at 1000 s^{-1} in comparison to those at 140 °C. Both non- and covalent interactions were involved in aggregate formation as indicated by the PAGE analysis. Mineral data signifies no disruption of casein micelles at any given condition while whey proteins and κ-casein were mainly involved in aggregate formation at 140 °C. The impact of shear thus appears to be temperature dependent. At low temperatures, below denaturation temperature of major whey proteins, fragmentation of weakly held hydrophobic aggregates was prominent. At higher temperatures aggregation predominated over fragmentation due to establishment of covalent bonds among proteins, which cannot be easily cleaved via shear forces. Further studies involving controlled heating and shearing of various dairy systems would be useful in terms of improvements in emulsion stability, flavour, textural properties such as gelation as well as minimising detrimental consequences such as scaling and precipitation of proteins in the production lines.

Chapter 4

Shear Induced Behaviour of Native Milk Proteins of Raw Milk at Different Temperatures

4.1 Introduction

Milk is processed to minimize the risks associated with the presence of harmful microbial contaminants and enzymes thus extending the shelf-life by ensuring safety and stability. Heat treatment is one of the major dairy processing techniques, which involves some of the unit operations such as flow through pumps and piping, heat transfer in heat exchangers and homogenisation. Application of these techniques results in a number of physicochemical changes in milk, including change in mineral balance and pH decline, dissociation of the casein micelle and denaturation of whey proteins and their interactions with dissociated caseins (Huppertz, 2016). Decrease in pH is mainly impacted by precipitation of the tertiary calcium phosphate and formation of organic acids due to degradation of lactose through isomerization/degradation and the Maillard reaction. Casein dissociation, especially κ-casein, from the micelle takes place followed by its interactions with whey proteins, especially β-lactoglobulin. The rate of this process is affected by the temperature of heat treatment and pH of milk. These phenomena have been extensively studied (Roefs & de Kruif, 1994; Verheul et al., 1998; Oldfield, Singh, & Taylor, 2005). However, relatively little is known about the impact of mechanical forces accompanying the heat treatment, although milk is subjected to shear forces under some of the major unit operations of commercial milk processing such as pumping, stirring, or homogenisation (Chandrapala et al, 2011; 2012).

Flow is broadly classified into extensional and shear flow. A homogeneous extensional flow is distinguished by a linear velocity gradient of the form $V_y = \dot{\gamma} y$, where V_y presents linear velocity, $\dot{\gamma}$ is velocity gradient and y is a distance between centre of the polymer and flow axis. In this particular case, the strain rate, $\dot{\gamma} = \frac{\partial x_y}{\partial y}$ is considered constant (Perkins, Smith & Chu, 1997; Bekard, Asimakis, Bertolini & Dunstan, 2011). On the other hand, a simple shear flow, such as in the current study, is characterised by a velocity gradient perpendicular to the

flow field and distinguished by a linear superposition of rotational flow with a vorticity ω, and elongational (deformational) flow with a strain rate depicted as $\dot{\gamma} = \frac{\partial v_x}{\partial y}$. In a rotational flow field, protein molecules experience whole body rotation with no hydrodynamic strain and therefore their structural integrity should not have affected substantially. In the elongational flow field under shear, protein molecules are subjected to stretching and compression events of equal magnitude. Due to random exposure of individual molecules during these two events, temporal fluctuations such as periodic elongation, relaxation and tumbling can take place resulting in a complex behaviour within molecular structures (Bekard, Barnham, White & Dunstan, 2011). Stretching events expose structural element to a hydrodynamic shear stress, which could overwhelm the stabilising cohesive forces such as intramolecular hydrogen bonds, resulting in unfolding. Molecular extension is further amplified with increasing shear rate as a result of stronger hydrodynamic drag in the flow field (Bekard et al, 2011). This was observed previously with at applied shear at 20 ºC resulted in reversible unfolding of whey proteins in raw milk by substantially reducing intensity of native α-helical and β-sheet structures (Chapter 3). Apart from that, this fluid drag associated with the shear flow could even destabilise the casein micelle resulting in structural transformations into more elongated shapes leading to a complex behaviour. For example, at high concentrations, casein micelles act as soft spheres that deform and align along with the flow direction at high shear rates (Olivares, Berli & Zorilla, 2013).

Moreover, process shear may impact on the outer hydration sphere of proteins modifying the stabilising energy provided by the preferential hydration. This results in exposure of inner, non-polar residues facilitating aggregation with increase in shear (Stephen et al., 2008). The hydration sphere formed by the hydrophilic glycomacropeptide of k-casein, which projects out into the aqueous phase of milk, has an equal negative charge, which repels individual micelles from each other thus remaining solubilised in the colloidal form without

precipitation (Spreer, 1998). The impact of shear could result in alterations of these hydration spheres resulting in changes of the overall charge distribution in the surrounding leading to flocculation. Flocculation of milk proteins is a time dependant process, which largely depends on a number and efficiency of molecular collisions. In many systems including milk, the collisions between particles occur at a rate dependant on the Brownian motion known as perikinetic aggregation; however, in a system subjected to elevated flow rates under hydrodynamic shear conditions, particles usually follow orthokinetic aggregation (Walstra, 2001). Under these conditions, net growth rate and size of any protein complexes depend on the equilibrium between shear-induced growth and shear-controlled breakage (Steventon et al., 1998). Therefore, the heat induced casein-whey protein interactions can be controlled by governing the extent of shearing.

However, most of the studies related to shear and temperature induced changes are confined to specific proteins (mainly whey) in aqueous solutions under specified conditions (Steventon, Donald, & Gladden, 1994; Walkenström & Hermansson,1998). The knowledge is lacking in terms of whey protein and casein interactions subjected to both processing conditions - shear and heat - in a complex protein mixture such as raw skim milk. Understanding these changes may facilitate process improvements in dairy products such as textural properties and/or novel processing in order to either minimize the changes of proteins or improve the process control and creation or prevention of molecular interactions. Therefore, the present study has investigated the shear and heat associated changes of milk proteins in raw cow's milk at three different temperatures (80, 100 or 120 °C) and three shear rates (100, 500 or 1000 s^{-1}), which are within the range of most common industrial processing of milk such as in the preheating stage at milk powder manufacture. However, caution should be exercised as due to limitations of the equipment used in the experimentation, these temperatures were not reached at the same heating rate as it is usually achieved industrially

4.2 Materials and Methods

Fresh whole raw milk, obtained from Murray Goulburn Co-operative Co. Ltd (Laverton, Australia), was skimmed by centrifugation (Avanti J-26SXPI, Beckman Coulter, Harbor Boulevard, CA, USA) at 12,000 *g* for 20 minutes at 20 °C. The composition of the raw skim milk was 0.75% (w/w) ash, 0.085% (w/w) fat, 4.8 % (w/w) lactose and 3.28% (w/w) total proteins, out of which 2.29 % (w/w) caseins, 0.98 % (w/w) whey proteins and 0.01 % (w/w) non-protein nitrogen (Chapter 3). Lactose content was calculated as 4.8 % (w/w) by subtracting the protein, fat and ash contents of the sample from the total solid content. All the chemicals used for analysis were obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia) and ultrapure water (Milli-Q water, Merck Millipore, Bayswater, Vic, Australia) was used at all times.

4.2.1 Treatment of samples

Raw skim milk was subjected to three different shear rates (100, 500, or 1000 s^{-1}) for one minute at three temperatures (80, 100 or 120 °C) in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series: Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with a constant pressure of 250 kPa following the method of Liyanaarachchi, Ramchandran, & Vasiljevic (2015). Samples not subjected to shear at 20 °C were used as the controls. The samples subjected to heating under all three temperatures were heated at a rate of 5 ºC/min to the required temperature, held there for 1 minute and cooled at 5 °C/min to 20 ºC. Shear stress and viscosity were assessed for each treatment condition and Reynolds Number (*Re*) was calculated using the equation 4.1 (Childs, 2011),

$$Re = \frac{\rho \omega a(b-a)}{\mu} \qquad (4.1)$$

Angular speed (rad.s^{-1}) was calculated using equation 4.2

$$\omega = \dot{\gamma} \frac{(b-a)}{\frac{b+a}{2}} \qquad (4.2)$$

Density of milk was estimated at a given temperature from equation 3 (McCarthy & Singh, 2009)

$$\rho = -0.2307\times10^{-2}T^2 - 0.2655T + 1040.51 - F(-0.478\times10^{-4}T^2 + 0.969\times10^{-2}T + 0.967) \qquad (4.3)$$

Where ρ is density of milk in $kg.m^{-3}$, ω is angular speed in $rad.s^{-1}$, a is radius of a bob used in the determination (m), b is the radios of the cup used in the determination (m), μ is dynamic viscosity (Pa.s), $\dot{\gamma}$ is shear rate in s^{-1}, F is fat content in the samples ($w.w^{-1}$) and T is the temperature of determination (°C).

Applying above equations, the flow was characterised as laminar under all treatment conditions. The pH of each treated sample was measured immediately after treatment using a pH meter (WTW Inolab pH 720, Weilheim, Germany) and the first stable endpoint was recorded. Another portion of the treated sample was immediately centrifuged (Beckman Optima L-70 Ultracentrifuge, USA) at 100,000 g for 1 hour at 20 °C. The supernatant was carefully removed and used for RP-HPLC and PAGE analysis, while the pellet was used for the PAGE analysis.

4.2.2 Particle size measurements

Particle size measurements were performed straight after treatment using a Zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) as described by Liyanaarachchi et al. (2015). Treated samples were diluted 1000 times using skim milk ultra-filtrate (SMUF)

prior to measurements. The SMUF was obtained by ultrafiltration of skim milk used in the experiments at 15 ºC with a SEPA CF membrane module and polyethersulfone (PES) membrane (190x140 mm) with a molecular cut-off of 10 KDa, acquired from Sterlitech Corporation (Kent, WA, USA). The refractive indexes of 1.338 and 1.334 for milk and UF, respectively, were used in the calculations.

4.2.3 Reverse phase high performance liquid chromatography (RP-HPLC) analysis

Supernatant of each treated skim milk sample was analysed for the content of individual protein fractions (αs-CN, β-CN, κ-CN, α-LA and β-LG) by RP-HPLC using the Zorbax 300SB-C8 RP-HPLC column (silica-based packing, 3.5 micron, 300A, Agilent Technologies Inc., USA) as the stationary phase. Water (mobile phase A) and acetonitrile (mobile phase B) solutions both containing 0.1% v/v trifluoroacetic acid (TFA) were used as mobile phases. A gradient elution programme was run at a constant flow rate of 0.8 $mL.min^{-1}$ and followed 0-40 min linear gradient from 30% B to 50% B; 40-42 min linear gradient from 50% B to 100% B; 42-43 min isocratic elution 100% B; 43-46 min linear gradient from 100% B to 30% B followed by a 5 min isocratic elution at the initial conditions using detector wavelength of 215 nm as described by Aprianita et al., (2015).

0.4 mL of skim milk supernatant was diluted with 1.6 mL of denaturing urea solution (8M urea, 165 mM Tris, 44mM sodium citrate and 0.3% v/v β-mercaptoethanol) and filtered through 0.45 µm pore cellulose membrane before analysis. Standard solutions were prepared as described by Bonizzi, Buffoni & Feligini, (2009). 249.5 mg of purified α_s-CN (70% purity, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia), 200.5 g of purified β-CN (98% purity, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia), 51.2 mg of purified κ-CN (98% purity, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia), 201.4 mg of β-LG (90% purity, Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia), and 100.0 mg of α-LA (85% purity, Sigma-

Aldrich Pty Ltd, Castle Hill, NSW, Australia) were dissolved respectively in 10 mL of denaturing urea solution to prepare single fraction mother solutions. Then, 1 mL of each single solution was mixed with 2 mL of denaturing urea solution to prepare the mixed standard solution. After that, a set of four mixed concentration standard solutions were prepared by adding 0.4 mL, 0.8 mL, 1.2 mL and 1.6 mL of mixed mother solutions to 1.6 mL, 1.2 mL, 0.8 mL and 0.4 mL of denaturing urea solution respectively. These standard solutions were used to obtain calibration curves for α_s-CN, β-CN, κ-CN, β-LG and α-LA proteins.

4.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis

SDS PAGE under reducing and non-reducing conditions was carried out for both the supernatants and the pellets. The pellet was dissolved in an SDS sample buffer prepared in milliQ water using 12.5% (v/v) 0.5M Tris/HCl, 10% Glycerol, and 20% sodium dodecyl sulphate (10% (w/v) SDS) and stirred continuously for 48 hours at 5 °C after which 2.5% bromophenol blue stock (0.05% w/v) was added. The dissolved pellet sample was heated at 40 °C for 15 minutes and was used for non-reducing PAGE analysis, while a pellet sample heated at 95 °C for 15 minutes after the addition of 2-mercaptoethanol was resolved through the reducing SDS PAGE. Similarly, 40 µL aliquot of supernatant was mixed with 1 mL of the SDS sample buffer for SDS PAGE under non-reducing conditions, while 20 µL of 2-mercaptoethanol was added and the mixture was heated in a boiling water bath for 4 minutes to reduce the samples.

Stock solutions of 0.006% (w/w) α-LA and β-LG were prepared using MilliQ water and diluted 10 fold with the sample buffer and used as standards. The casein standard was prepared using 0.056% (w/w) sodium caseinate stock solution in MilliQ water diluted 14 fold

with the sample buffer. SDS resolving and stacking gels were prepared and casted between two glass plates as described by Grewal, Chandrapala, Donkor, Apostolopoulos, & Vasiljevic (2017). Exactly 10 µL from each sample and standards were loaded in to gels for analysis and the electrophoresis was performed at 70mA, 210V, and 6.5W. The resultant gels were stained using Coomassie Blue for one hour and destained using a mixture of 0.1% isopropanol, and 0.1% glacial acetic acid in water overnight. Gel Images were taken using ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories).

4.2.5 Statistical analysis

Statistical analysis for the obtained results followed the same procedure as described in chapter 3 in section 3.2.8.

4.3 Results

4.3.1 Treatment of raw skim milk at 80 °C

Particle size measurements obtained after each treatment at this temperature showed that shear and temperature had a significant impact on the particle size distribution. For example the control, untreated sample, had an average particle size of ~164 nm, which this was shifted to significantly lower values ($P<0.05$) upon heating at 80°C with applied shear of 100 s^{-1} (~ 123 nm) and comparatively slightly ($P>0.05$) lower at 500 s^{-1} (~142 nm) (Table 4.1) .

Table 4.1: Initial and final pH and average particle sizes (nm) of milk samples treated at a different temperature (80, 100 or 120°C) and shear rate (100, 500 or 1000 s^{-1}). Unheated raw skim milk served as a control.

Temp (ºC)	Shear (s^{-1})	pH (-)	Average Particle Size (nm)
Control		6.74	164
	100	6.65^{aA}	123^{aA}
80	500	6.70^{bA}	142^{bA}
	1000	6.69^{aA}	170^{cA}
	100	6.59^{aB}	162^{aB}
100	500	6.62^{aB}	155^{bB}
	1000	6.63^{aB}	163^{aB}
	100	6.49^{aC}	164^{aB}
120	500	6.56^{bC}	163^{aC}
	1000	6.58^{bC}	190^{bC}
SEM*		0.01	2.14

SEM* - Pooled standard error of the mean; the means indicated with different superscripts are significantly different ($p<0.05$). Different capital letter superscripts indicate significant difference ($p<0.05$) influenced by temperature at a particular shear rate and different small letter superscripts indicate significant difference ($p<0.05$) influenced by a shear rate at a particular temperature.

In contrast, at 1000 s^{-1}, a likely shear induced denaturation and aggregation of proteins moved the average particle size towards higher values (~170 nm) compared to the control (Fig 1A).

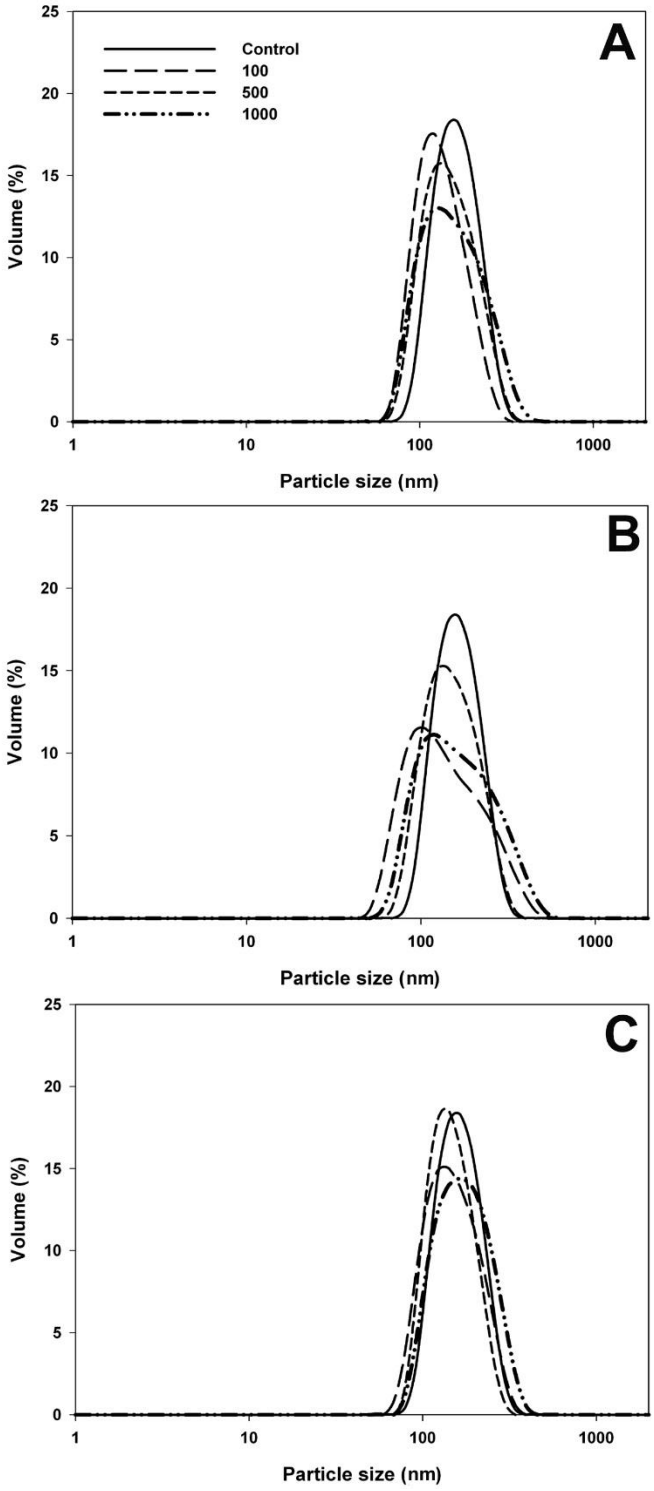
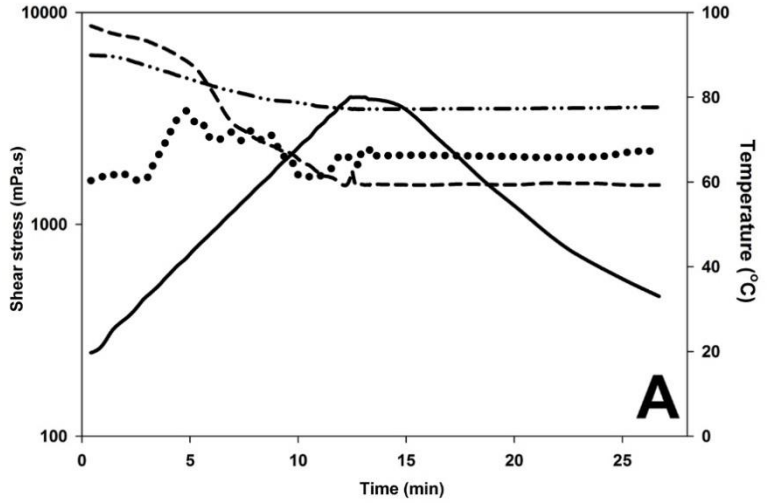
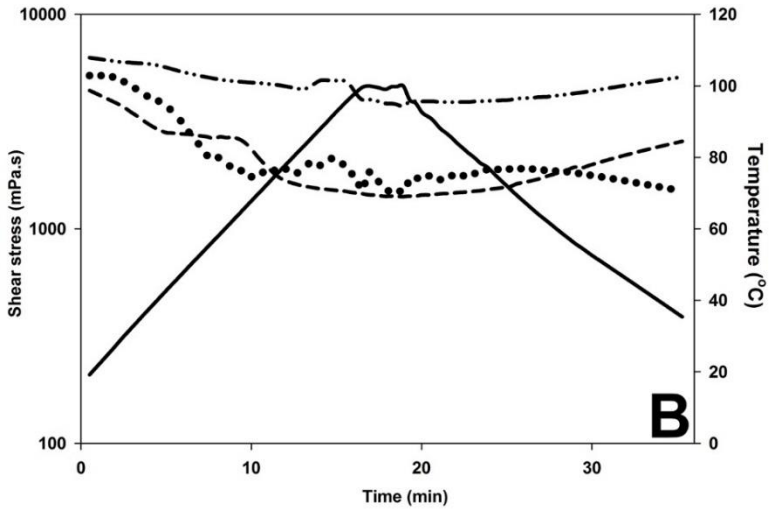


Figure 4.1: Particle size distribution of raw skim milk processed at 80 °C (A), 100 °C (B) and 120 °C (C) at different shear rates (100 - — —, 500 - – – – or 1000 s^{-1} - —··) for 1 minute. Unsheared milk (——) served as a control.

These observations could not be correlated to the extent of applied shear stress (Fig 2A) as measured shear stress at 100 s^{-1} was greater than that at 500 s^{-1}.





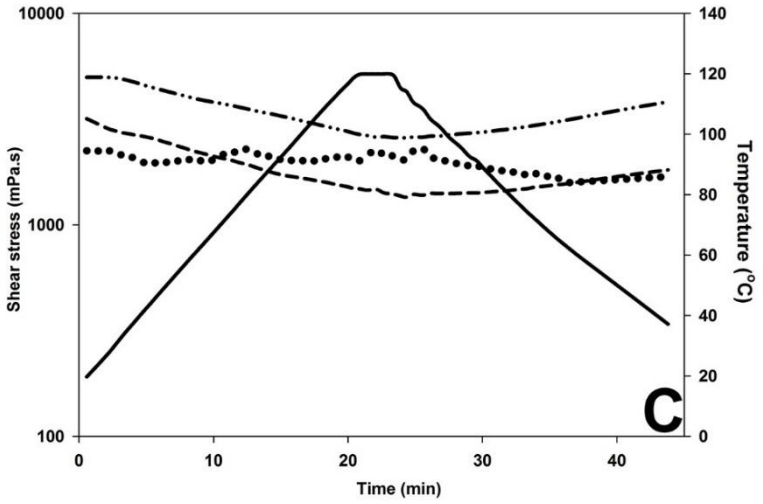


Figure 4.2: Shear stress – time – temperature profile of raw milk processed at 80 °C (A), 100 °C (B) and 120 °C (C) at different shear rates (100 - •••••, 500 - – – –, or 1000 s^{-1} - –··) for 1 minute. Solid line (——) depicts the temperature profile during treatments.

The protein behaviour during simultaneous heating and shearing was analysed in both the supernatant and sediment phase after centrifugation. At all three shear rates, disulphide linked protein complexes were observed at the entrance of the stacking gel with smaller complexes being created at 1000 s^{-1} in the serum phase according to the SDS PAGE data. As shown by reducing SDS PAGE, these mainly consisted of κ-CN, β-LG and α-LA. In addition, faded bands of α_s-CN caseins at all three shear rates compared to the control in the reducing SDS PAGE (Fig 3A) imply possible sedimentation after heat and shear induced self-aggregation or aggregation with other protein species.

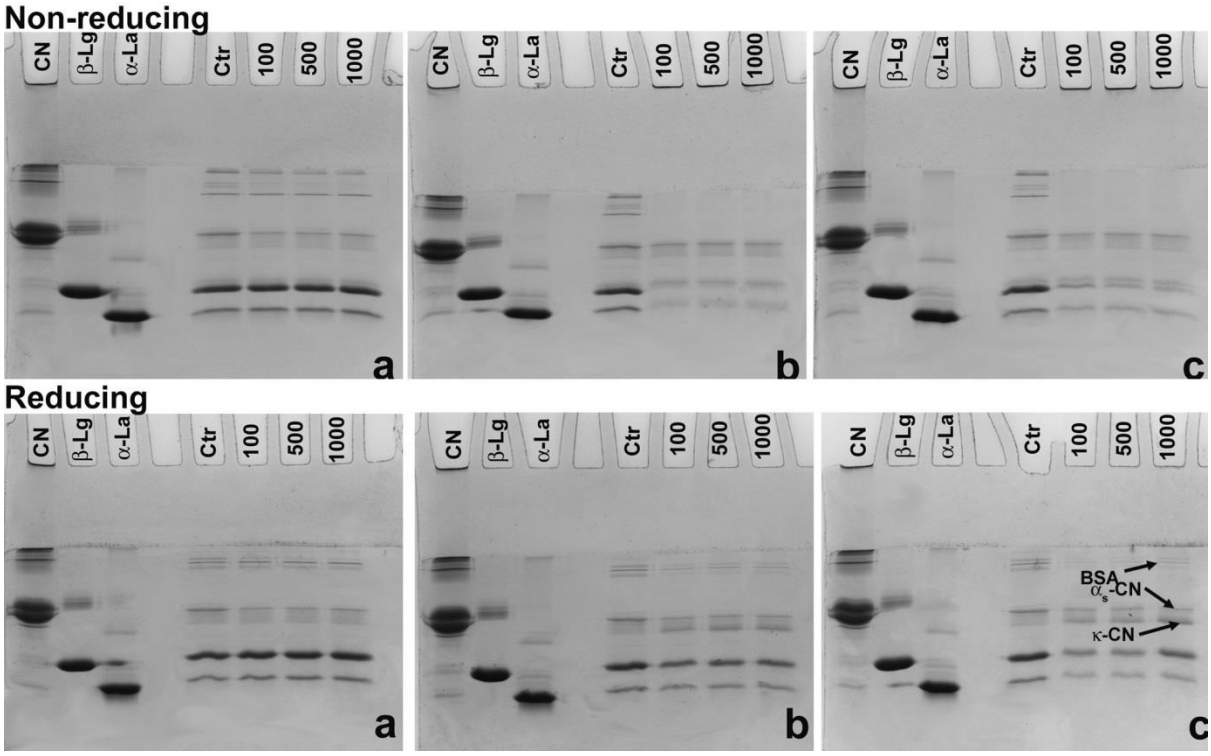


Figure 4.3: Non-reducing and reducing SDS polyacrylamide gel electrophoresis analysis of the serum phase of raw skim milk treated at 80 °C (a), 100 °C (b) and 120 °C (c) at different shear rates (100, 500 or 1000 s^{-1}) for 1 minute.

This was also evident in the results of the HPLC analysis of the serum phase (Table 4.2), which showed a 25 % reduction in α_s-CN with the increase in shear from 100 s^{-1} to 1000 s^{-1}. Further~9% decrease in both κ and β-CN and 17-18 % decrease in both β-LG and α-LA were observed in the serum, which indicates their strong interactions with the casein micelle and creation of complexes that sedimented upon centrifugation.

Table 4.2: HPLC analysis of a relative protein content of major milk proteins in the serum phase of milk subjected to different temperatures and shear rates. Relative protein content was obtained by comparing concentrations of proteins remaining in the serum after centrifugation to that of the control.

Protein	Temperature, °C									SEM*
	80			100			120			
	Shear rate, s^{-1}									
	100	500	1000	100	500	1000	100	500	1000	
κ-CN	0.91^{aA}	0.89^{aA}	0.83^{bA}	1.43^{bB}	1.73^{cB}	1.19^{aB}	1.50^{aC}	1.60^{bC}	2.40^{cC}	
αs-CN	0.87^{aA}	0.82^{bA}	0.65^{cA}	0.79^{aB}	0.78^{aA}	0.77^{aB}	0.77^{aC}	0.81^{aA}	0.96^{bC}	
β-CN	0.75^{aA}	0.89^{bA}	0.68^{cA}	0.32^{aB}	0.29^{aB}	0.29^{aB}	0.45^{aC}	0.52^{bC}	0.56^{bC}	0.02
α-LA	0.73^{aA}	0.76^{aA}	0.60^{bA}	0.72^{aA}	0.86^{bB}	0.68^{cB}	0.92^{aB}	0.98^{bC}	0.99^{bC}	
β-LG	0.94^{aA}	0.85^{bA}	0.78^{cA}	0.38^{aB}	0.46^{bB}	0.25^{cB}	0.19^{aC}	0.21^{aC}	0.26^{bB}	

SEM* - Pooled standard error of the mean; the means indicated with different superscripts are significantly different ($p<0.05$). Different capital letter superscripts indicate significant difference ($p<0.05$) influenced by temperature at a particular shear rate and different small letter superscripts indicate significant difference ($p<0.05$) influenced by a shear rate at a particular temperature.

These complexes, which were located in the sediment phase, were evident at all three shear rates (Fig 4A-reducing) at the entrance of stacking gel in the non-reducing SDS PAGE and were more pronounced at 1000 s^{-1}.

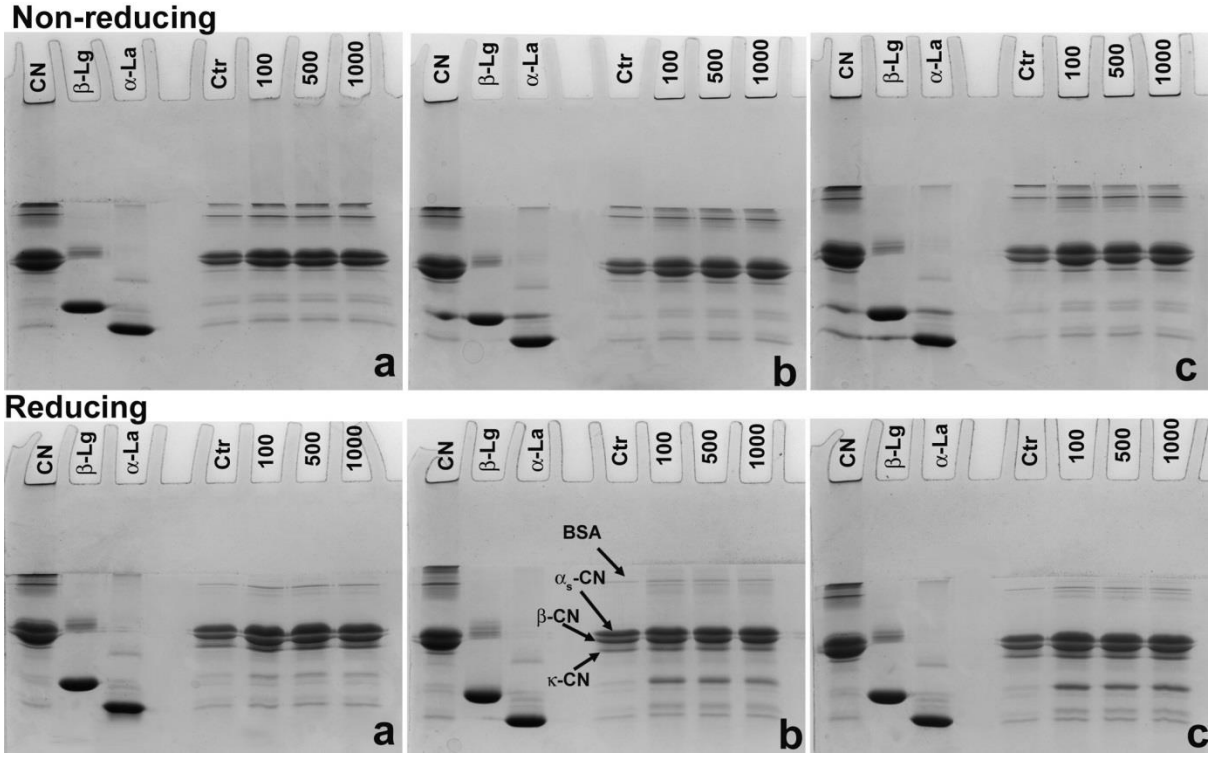


Figure 4.4: Non-reducing and reducing SDS polyacrylamide gel electrophoresis analysis of sediment phase of raw skim milk treated at 80 °C (a), 100 °C (b) and 120 °C (c) at different shear rates (100, 500 or 1000 s^{-1}) for 1 minute.

As indicated by Table 4.1, a reduction in pH was observed as a general trend at all three shear rates in comparison to the control. However, while pH notably declined (~ 0.1) at 100 s^{-1} presumably due to combined heat and shear induced changes in the mineral equilibrium of the raw milk, it recovered significantly with the rise in shear from 100 s^{-1} to 500 s^{-1}. Further increase in shear did not change the pH value significantly.

4.3.2 Treatment of raw skim milk at 100 °C

Although the average particle size appeared similar to that of the untreated control, comparatively broader particle size distribution was observed at both 100 and 1000 s^{-1} (Fig. 1B) denoting both aggregation and fragmentation of newly created protein particles.

However, at 500 s^{-1}, an average particle size has only been slightly lowered compared to the other two shear rates with a narrow particle size distribution extending more towards the below average particle size range of control suggesting more pronounced shear induced fragmentation. This again could not be related to the extent of measured shear stress (Fig 2B) as it was lower than that at 100 or 1000 s^{-1}. This observation could also be linked to sensitivity of the rheological element, which likely was not able to provide accurate measurements at a low shear rate. Complementarily, the HPLC data show an increase in the κ-CN content in the serum phase in comparison to that of the control suggesting dissociation of the micellar κ-CN upon exposure to all three shear rates. The amount of κ-CN present in the serum was the greatest at 500 s^{-1} as compared to that at 100 s^{-1} or 1000 s^{-1}. At 500 s^{-1}, β-LG content also increased by~ 21% in the serum following the same pattern as κ-CN. The increase in α-LA levels also appeared similar to that of β-LG (~ 20%) suggesting possible interactions among them in similar proportions. On the other hand, the content of both αs-CN and β-CN was not significantly different at 500 s^{-1} in comparison to other two shear rates. Increase in κ-CN, β-LG and α-LA in the serum at 500 s^{-1} was evident as disulphide linked complexes were resolved under reducing conditions (Fig 3b-reducing). These soluble complexes were identified in comparatively lower amounts at other two shear rates while being the lowest at 1000 s^{-1}. In addition, the prominent reduction in the serum β-LG (~35%) at 1000 s^{-1} suggests enhanced interactions with the casein micelle and precipitation in the sediment phase accompanied with a reduction in the serum κ-CN. Comparison between the non-reducing and reducing SDS PAGE (Fig 3b) shows that the main proteins involved in disulphide interactions were κ-CN and α_s-CN as well as whey proteins β-LG, α-LA and BSA.

As seen in Table 4.2, pH of treated samples was further lowered in comparison to those treated at 80 ºC. The increased length of the treatment time (both heating and cooling) as well as time dependent behaviour of pH during cooling would probably have contributed to this

phenomenon. Interestingly, a pattern of pH recovery was obtained similar to that at 80°C; however, in this case, a relatively lower increase was observed with the increase in shear rate compared to 80°C.

4.3.3 Treatment of raw skim milk at 120 °C

The average particle size at 1000 s^{-1} increased substantially (~190 nm) in comparison to that of the control, while other two shear rates produced particles of an average size similar to that of the control (~164 nm) (Fig. 1C). At the same time, shear stress imposed by shearing at 100 s^{-1} initially followed an expected pattern initially, being lower than that at 500 or 1000 s^{-1}. However, it appeared largely unaffected by temperature as it remained almost constant during heating and slightly declined during cooling stage. This could imply heat induced viscosity increase due to molecular interactions, although further exploration is needed as this observation could not be related to increase in particle size. While particle size distribution did not demonstrate a great extent of aggregation, protein complexes linked by disulphide bonds were evident in both, the serum (Fig 3C) and the sediment (Fig 4C) phases regardless of shear rate. These complexes were more predominant at 1000 s^{-1} involving κ-CN, α_s-CN, β-LG and α-LA. The HPLC data also show an increase in all caseins and whey proteins in the serum phase concomitant with the rise in shear. At 100 s^{-1}, the κ-CN content in the serum phase has risen by~50% compared to that of the control denoting κ-CN dissociation from the micelle while an~80% reduction of β-LG was observed along with a 10% decrease in α-LA signifying formation of disulphide linkages with the casein micelle resulting in creation of large complexes that would settle in the sediment phase. The increase in shear up to 500 s^{-1}, resulted in ~11% increase in β-LG and ~6% increase in κ-CN with a ratio of ~1.5:1 in the serum phase. Further increase in shear up to 1000 s^{-1} resulted in almost~37% increase in β-LG concentration in the serum phase along with a~60% increase in κ-CN implying the

presence of a β-LG/κ-CN complex in a relatively lower proportion (ratio of ~ 0.5:1) in the serum. In addition, around 25% increase in α_s-CN was also observed in the serum.

Further decrease in pH was also observed in comparison to the control at this temperature following the general trend as observed previously (Table 4.1). Similar to other two temperatures, recovery of pH was observed with the increase in shear however relatively at a slower rate likely due to further increase in treatment time as well as prolonged cooling in comparison to other conditions.

4.4 Discussion

From presented results, it appears that applying different flow regimes (shear rates) would create conditions during processing of milk that would govern protein interactions and their behaviour diversely. Upon heating at 80 ºC, it would be expected that most of the whey proteins would undergo reversible denaturation initiated by unfolding, which could be enhanced via hydrodynamic drag forces. Impact of shear stress at this temperature appeared more pronounced at both 500 and 1000 s^{-1} shear rates (Fig 2A), while at 100 s^{-1}, although the fluid was subjected to a laminar flow, molecular interactions were likely governed by Brownian motion and temperature as shear stress did not follow expected pattern.

In contrast to 100 s^{-1} or 500 s^{-1} shear rate, exposing raw milk to shear rate of 1000 s^{-1} has resulted in growth of particles. Enhanced unfolding due to shear and pressure imposed by compression due to rotational diffusion (tumbling end-over-end) would facilitate rapid association via hydrophobic and electrostatic interactions (Bekard & Dunstan, 2009). In addition, the periodic end-over-end tumbling in the flow field imposed by high shear rates may give rise to conformational distortions resulting in kinked states promoting slow unravelling of molecules and enhancing intermolecular hydrodynamic interactions (Bekard et al, 2011). Molecules brought together via hydrophobic interactions are likely to engage in

disulphide bridging during heating due to exposure of hidden free sulphydryl groups during unfolding (Liyanaarachchi et al., 2015). The results related to the rise in the average particle size at 1000 s^{-1} is supported by significant reductions in the content of κ-CN, α-CN and β-CN as well as both β-LG and α-LA in the serum phase suggesting their involvement in the overall aggregation as shown by the HPLC data. The significant decrease in α_s-CN (~25%) over κ-CN (~9%) in the serum suggests that α_{s2}-CN appears to be the predominant casein involved in these associations as opposed to κ-CN under applied shear. Also, nearly ~17-18 % decline in the serum content of both β-LG and α-LA at 1000 s^{-1} signifies that both whey proteins were associated with these caseins in similar proportions.

Some of these observations may be related to our previous work, in which we noted that a content of α_{s2}-CN in the serum increased upon heating at elevated temperatures (Chapter 3) likely due to precipitation of colloidal calcium phosphate which would affect integrity of the casein micelle. α_{s2}-CN contains two cystyl residues (Cys_{36} and Cys_{40}), which occur as intra- and intermolecular disulphide bonds within the protein structure (Rasmussen et al., 1994a). κ-CN also contains two Cys residues (Cys_{11} and Cys_{88}) and can be found as a heterogeneous polymeric protein connected by disulphide bonding via these two Cys groups in the monomeric protein (Rasmussen et al.,1999). These disulphide bonds are also able to engage through disulphide interchange reactions with the whey proteins containing thiol groups. During the heating of milk, κ-CN readily engages in these reactions, while α_{s2}-CN slowly interacts with denatured whey proteins mainly due to its location in the interior of the micelle. However, in the presence of shear, α_{s2}-CN appears to predominate in these interactions with the whey proteins likely due to gained access to the micellar interior via elongation of the micelle in the shear induced flow as demonstrated by viscosity decline during heating (data not shown), its possible dissociation from the micelle as it was found in the serum phase (Figure 4.3), or even conformational repositioning of the whey proteins that would somehow

access the interior of the micelle and enable interactions. Moreover, the most common whey protein, β-LG has two disulphide bonds (Cys_{66}-Cys_{160} and Cys_{106}-Cys_{119}) and a free reactive thiol group (Cys_{121}), which is important in initiating intermolecular thiol-disulphide interactions with these two caseins. β-LG usually exists in an equilibrium between dimeric and monomeric forms at room temperature and increase in temperature results in dissociation of these dimers into monomers. At low temperatures, dissociation is reversible while at higher temperatures, denaturation of β-LG along with the loss of secondary and tertiary structures results in exposure of fee reactive thiol group leading to sulphydryl-disulfide interactions (>70ºC) (Wijayanti, Banasal & Deeth, (2014). In contrast to β-LG, α-LA has no free thiol group and contains only four disulphide bonds (Cys_6-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{60}-Cys_{77} and Cys_{73}-Cys_{90}), thus it would be involved only in disulphide interchange reactions and basically having minimized its overall involvement (Livney Verespej, & Dalgleish, 2003). As a result, during heating, a larger proportion of β-LG associates with the caseins compared to α-LA. With application of greater shear, complexation involving a greater proportion of α-LA as opposed to β-LG would suggest possible extensive intermolecular disulphide interchange reactions, more likely with Cys_{40} in α_{s2}-CN, which is located more towards the exterior in the molecular structure (Corredig & Dalgleish, 1999; Lowe et al., 2004) in a region of a high charge density and low hydrophobicity (Farrel et al., 2009). Livney et al. (2003) reported that a wide range of complexes was created during heating of an α-LA/β-LG mixture through primarily thiol-disulphide interchanges, however steric effects appeared important in dictating disulfide bond formation which could have been overcome by application of shear.

In addition, a similar proportion of β-CN is also involved in aggregation as κ-CN and this further confirms that shear induced greater involvement of these proteins in the process. The significant decrease of β-CN in the serum at 1000 s^{-1} reveals possible self-association via

hydrophobic linkages due to presence of distinct polar and hydrophobic domains, which would probably manifest itself in the shear dependent association due to increased number of collisions (Walstra, 2001). Similarly, κ-CN dissociation was not observed with the increase in shear as evident by comparatively less amount of κ-CN against the control.

At 100 ºC, shear induced aggregation has become dominant at both 100 s^{-1} and 1000 s^{-1} shear rates, whereas at 500 s^{-1}, shear controlled fragmentation appears to govern the equilibrium as observed in the particle size distribution data. Aggregation between denatured whey proteins and the casein micelle is a multi-stage process dependant on inter-particle motions, which are largely governed by three basic mechanisms – fluid motion (orthokinetic coagulation), Brownian diffusion (perikinetic coagulation) and buoyancy (gravitational sedimentation) (Kim and Kramer, 2006). Fluid motion also exerts shear induced stresses that cause fracture of created aggregates (Kim and Kramer, 2006). However, the measured shear stress was the lowest at 500 s^{-1} at the beginning and most of the time throughout the cycle (Fig 2B), which is contradictory to expectations that it would be clearly shear rate and temperature dependant. While sensitivity of the instrument at low shear rate should be questioned, the Brownian motion should not be neglected as the molecules would move faster at elevated temperatures due to greater kinetic energy that may even enable them to move between streamlines. More importantly, at this temperature aggregation was clearly shear dependant as increase in shear from 500 s^{-1} to 1000 s^{-1} was accompanied with protein associations into casein-whey protein complexes.

The HPLC data shows that the amount of dissociated κ-CN present in the serum was highest at 500 s^{-1} compared to 100 or 1000 s^{-1} suggesting enhanced dissociation of this protein from the micelle. Dissociation of individual caseins from the casein micelle with the increase in temperature has been studied extensively and has been attributed to either change in charge of

caseins or change in state and composition of calcium colloidal phosphate (CCP), which maintains the micellar structure (O'Mahony and Fox, 2013; De Kruif, 1999). It has been reported that dissociation of κ-CN occurs in milk when the temperature is raised above that of the ambient at the initial pH of ~6.8 and increases proportionally with temperature rise up to 90 ºC (Anema & Klostermeyer, 1997; Anema, 1998). Therefore, a temperature dependant dissociation of κ-CN would be expected at 100 ºC while the increase in dissociated κ-CN at 500 s^{-1} can be explained likely by prominent shear-induced fragmentation of created complexes. Furthermore, relatively great pH recovery upon shearing may not be related to reformation of CCP, as this process would likely have reinforced the micellar integrity. The dissociated levels of κ-CN in the serum phase can also be correlated with a level of whey proteins in the serum during a heat induced dissociation of κ-CN depending on the pH of the system (Anema, 2007). With increase in shear up to 500 s^{-1}, serum β-LG experienced~21% increase similar to κ-CN suggesting their interactions in similar proportions (~ 1:1) in formation of β-LG-κ-CN complexes and their solubilisation in the serum phase. The increase in α-LA levels also appeared similar to that of β-LG (~ 20%), which may indicate that these proteins were involved in interactions in similar proportions. This appears slightly contradictory to studies that assessed properties of these proteins during heating without shear, which reported that α-LA participated in a smaller proportion compared to β-LG (Oldfield, 1996). This also suggests a likelihood of either a conformational rearrangement of β-LG preventing further bond formation probably due to poorer accessibility to its free thiol group (Cys_{121}), which would limit thiol-disulphide interchange reactions, or conformational rearrangement of α-LA minimizing steric repulsions and greater reactivity (Livney et al., 2003).

At 1000 s^{-1}, the reduction in the serum β-LG compared to that at other two shear rates suggest possible interactions with the micelle, which appears to also affect concentration of κ-CN in

the serum at 100 ºC. This is an interesting observation and may only be explained by a creation of a α_{s2}-CN/β-LG/κ-CN complex as β-LG would link these two caseins via disulphide interchange due to presence of Cys residues in their primary structures (Huppertz, 2016). Formation of such a complex has been previously reported during UHTST treatment of milk (Snoeren & van der Spek, 1977). High shear would lead to structural transformations in the casein micelle such as either elongations along with the fluid drag (Olivares et al., 2013) or swellings which would potentially improve the association of β-LG with the micelles resulting in a notable decline of its concentration in the serum phase. However, concentration of α_s- and β-CN in the serum appeared un-affected by shear at this temperature indicating that they remained integral part of the micelle. Moreover, change in temperature from 80 to 100°C reduced the content of β-CN in the serum by almost 50% stipulating increased hydrophobic interactions with other caseins in the casein micelle (Huppertz, 2016).

At 120ºC, shear controlled fragmentation has dominated over the aggregation with the increase in shear although the bulk property shows a slight increase in the average particle size at 1000 s^{-1}. Along with the greater proportion of unfolded and aggregated particles due to increase in temperature, a relatively considerable proportion of protein complexes would be subjected to increased hydrodynamic shear stress leading to a break-up of complexes loosely associated via hydrophobic and electrostatic interactions. In general, these complexes can fragment by pressure fluctuation in fluid flow, abrasion of primary complexes from newly created particle surface or particle fragmentation from large complexes (Steventon, 1992; Taylor and Fryer, 1994).

As shown by the HPLC data, the serum levels of β-LG have decreased by ~ 80% at 100 s^{-1} obviously due to covalent interactions with the casein micelle. Apart from interactions with the micellar κ-CN, part of the β-LG has been associated with α_{s2}-CN within the κ-CN

depleted micelles via thiol-disulfide interchange as evident in the PAGE data. This reveals disulphide linked aggregation and creation of an α_{s2}-CN/β-LG/κ-CN complex in the sediment phase associated with the micelles. Increase in temperature along with low shear (100 s^{-1}) probably led to creation of a more porous, κ-CN depleted, micelle, which allowed β-LG to access interior and form disulphide bonds with α_{s2}-CN. Further increase in shear, up to 500 s^{-1}, resulted first in ~10% increase of β-LG in the serum phase, which further rose to ~37% following accretion of shear rate up to 1000 s^{-1}. In contrast, κ-CN concentration has risen far more than that of β-LG with nearly a 60% increase noted at 1000 s^{-1} denoting its dissociation from the micellar form. Moreover, 25% increase in α_s-CN was also observed in the serum. This may signify that shear controlled fragmentation has dominated over the aggregation process with the increase in shear resulting in soluble complexes of α_{s2}-CN/β-LG/κ-CN in the serum. Interestingly, the analysis of the SDS data (Fig 3C-reducing) showed that BSA was also involved in disulphide bridging and formation of these soluble complexes at 1000 s^{-1} at 120°C. Fig 3C (reducing) also shows appearance of two bands likely belonging to BSA and lactoferrin, which were part of larger but soluble complexes that remained at the top of the gel. Apart from that, the increase in the serum casein levels including β-CN (25%) may also demonstrate partial dissolution of CCP within the micelles resulting in liberation of caseins located in the interior at both 500 and 1000 s^{-1} shear rates. This was very significant ($p<0.05$) at 1000 s^{-1}. The casein micelle is a network of primary casein particles that are linked via calcium phosphate nanoclusters. Changes in the structural organisation of the micelles occur due to either altered casein-casein interactions, changes in casein-nanocluster interactions or changes in nanoclusters.

Moreover, a notable decrease in the final pH was observed with rise in temperature at 100 s^{-1}, clearly indicating a very pronounced effect of heating. However, this pH would recover initially significantly and then slightly as shear was increased to 500 and 1000 s^{-1},

respectively. The rapid reduction in pH during first few minutes of heating is mainly due to heat induced precipitation of tertiary calcium phosphate, which lowers the buffering capacity of milk. Subsequent continuation of heating, especially to 120°C, leads to a gradual pH reduction due to formation of organic acids via degradation of lactose through isomerization/degradation and Maillard reactions (Huppertz, 2016). The Maillard reaction involves the interaction of lactose with ε-amino group of lysine to produce lactulosyllysine, which can be subsequently degraded into formic acid and galactose. Lactosylation was evident at all temperatures, but more at elevated shear as seen on the PAGE data (Figure 4.3 and 4.4). Moreover, heat induced dephosphorylation of caseins with the subsequent precipitation of the released phosphate as tertiary calcium phosphate is also considered to reduce pH in milk (Deeth & Lewis, 2016). Shear induced fragmentation of the casein micelle/whey protein complexes may have exposed negatively charged amino acid residues that counteracted an increase in hydrogen ion concentration and eventually increased the pH of the system.

4.5 Conclusion

Interactions between the casein micelle and the whey proteins during heating of raw milk at different temperatures are dependent on the flow regime. At 80 ºC, shear induced aggregation appears as the dominant mechanism. It was the greatest at 1000 s^{-1} with a considerable proportion of complexes involving interactions between α_{s2}-CN and whey proteins. Participation of both β-LG and α-LA in similar proportions in the aggregate formation may imply that shear has promoted disulphide interchange reactions between α_{s2}-CN and α-LA in addition to α_{s2}-CN/β-LG interactions with the casein micelle. At 100 ºC, fragmentation of complexes appeared to be a dominant mechanism at 500 s^{-1}, which then shifted towards enhanced aggregation at 1000 s^{-1} accompanied with β-LG/κ-CN complexation in the

sediment phase via shear induced structural deformation of the casein micelle. Shear controlled fragmentation was observed as the governing mechanism at 120 ºC with the increase in levels of α_{s2}-CN/β-LG/κ-CN soluble complex in the serum phase. Aggregate fragmentation and dissolution of soluble protein complexes likely counteracted a partial dissolution of CCP and creation of formic acid during heating by neutralizing liberated hydrogen ions manifested in a significant partial recovery of pH.

As observed so far, milk proteins behaved differently under various shear rates at different temperatures subsequently undergoing substantial conformational changes. Understanding such a complex behaviour of intermolecular bonding under these conditions would be useful for process improvements as in case of protein precipitation on surfaces or improving textural characteristics of dairy products. Therefore, the shear, encountered very frequently in dairy processing cannot be simply disregarded as it has a major impact especially on milk proteins. Hence, application of a specific flow regime at a particular temperature may be required to produce a product of required properties along with more efficient processing.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

In addition to heat, shear forces are frequently encountered in some major unit operations in milk processing such as in flow through pumping, homogenising and stirring. These in combination would impose substantial impact on milk protein structures during processing. In most of the commercial processing methods, the simultaneous application of heat and shear are common however, limited study has focused on combined effect on structural changes, interactions among proteins and kinetics specially related to native milk proteins in raw milk. Hence, the main objective of the proposed research was to find out the native protein behavioural attributes in raw milk in terms of structural changes and kinetics within the range of most common industrial processing steps. In this regard, an integration of quantitative and qualitative structural analysing techniques was employed to investigate modifications *in situ* and in real time. However, due to limitations of the equipment used in the experimentation, these temperatures were not reached at the same heating rate as it is usually achieved industrially and relatively longer heating and cooling rates were used compared to industrial level.

As observed in first study, shear alone resulted in reversible structural modifications while increase in temperature up to 72 ºC, resulted in shear driven fragmentation of hydrophobically linked aggregates. At high temperatures (140 ºC), orthokinetic shear-modulated aggregation was observed. This reveals at low temperatures, usually the temperatures below denaturation of major whey proteins, fragmentation of weakly held aggregates becomes dominant while at higher temperatures, aggregation predominates over fragmentation due to establishment of covalent bonding which cannot be easily cleaved through shear forces.

To obtain additional insight in to the influence of fluid shear on proteins, further studies on protein behaviour in raw milk at three temperatures (80, 100, 120 ºC) and shear rates (100, 500, 1000 s^{-1}) which resembled the preheating stages in cooperated during the production of milk powder was investigated. Shear induced aggregation was observed as the predominant mechanism at 80 ºC with the increase in shear. Interestingly, a substantial amount of aggregates was observed involving interactions between α_{s2}-CN and whey proteins while similar participation of β-LG and α-LA in aggregation implied that shear forces have assisted in some disulphide interchange reactions between α_{s2}-CN and α-LA in addition to α_{s2}-CN/β-LG interactions with the casein micelle. Apart from that, relatively less participation of κ-CN in aggregate formation was observed and this was presumed to be due to shear induced structural preservation hindering further reactions while providing access possibly via elongated micelles as evident by viscosity decline at this stage for further reactions with the caseins located interior. Shear induced fragmentation was observed as the dominant mechanism at 500 s^{-1} which then shifted towards prominent aggregation at 1000 s^{-1} with an increased β-LG/κ-CN complexation along with shear induced structural deformations in micelles at 100 ºC. At 120 ºC, shear, controlled fragmentation was observed as the governing mechanism in 120 ºC with the increase in levels of α_{s2}-CN/β-LG/κ-CN soluble complex in the serum phase. With the increase in shear, significant recovery of pH was observed likely due to restrained partial dissolution of CCP and formation of formic acid via aggregate fragmentation and dissolution of soluble protein complexes. Lactosylation was evident at all three temperatures with more elevated levels at high shear rates indicating the occurrence of Maillard reactions probably due to longer heating and cooling rates at experimentation.

Overall, this work highlights flow regime may alter the direction and extent of the heat-induced whey protein and casein micelle interactions in native milk proteins. Hence, shear forces encountered in dairy processing is an important parameter to be considered in the quest

to unravel the conditions that promote protein denaturation, aggregation as well as fragmentation within the thermal processing and cannot be simply neglected. Specific flow regime at a temperature required to be applied in order to minimise complications encountered in processing and to enhance the efficiency. In addition, establishment of proper conditions would suitably utilise to achieve desired properties within food products within various manufacturing processes.

5.2 Future directions

This study has revealed a major area that needs to be investigated further. Present study has broadened the knowledge on impact of combination of selected shear rates and temperatures on milk proteins in raw milk that are usually applied in commercial processing. Based on the findings, it was observed that processing techniques such as heat treatment and homogenisation may directly influence milk protein structures resulting in alterations of their functionalities. This can also induce immunogenic and antigenic potential thus modulating human immune system in many ways. Minor proteins such as immunoglobulins may be impacted by the different processing regimes resulting in altered immunomodulatory properties in such a way preventing occurrence of allergies. Thereby investigation on impact of these processing methods in relation to immunomodulation is an important tool in understanding the potential risks as well as benefits associated with various processing techniques with regards to human consumption.

The information would assist in identifying relationship between immunoregulation leading to allergic reactions of milk proteins and their state impacted by different processing conditions which may lead to proposing modifications in processing regimes that would

improve positive immunomodulatory potential of these proteins and consequently improve the health status of the population.

Apart from that, the knowledge obtained could suitably utilise in product improvements in terms of textural and sensory aspects via gaining insight further in to the emulsion stability and gelation properties. Moreover, understanding on these aspects would be useful in process improvements by minimising detrimental effects such as scaling and protein precipitation on surfaces in production lines.

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