

Modulation of Functional Properties of Whey Proteins by Microparticulation

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*To my beloved parents, Rohan, Sanjitha and
Yuthmini*

I. Abstract

Heat-induced destabilisation (denaturation) of whey proteins (WP) is one of the major problems limiting their application in food products. As a means of modification, and, thereby improvement of the WP functionality, microparticulation of WP using combined heat and high pressure shearing achieved by microfluidization (MFZ) has been proposed and assessed in this thesis.

Initially, different commercially available whey protein concentrate (WPC) powders were analysed to gain a general understanding of the interrelationship between their chemical composition and functional properties. Proximate analysis, protein type using SDS-PAGE, particle size (HPLC and particle size analysis), heat induced denaturation (DSC) and analysis of selected functional properties confirmed that they have substantial differences in composition and functionality.

In the second study, microparticulated whey protein (MWP) powders were produced from commercial cheese whey retentate, standardized to 10% (w/w) protein and subjected to MFZ at 140 MPa either with or without prior heat-induced denaturation, followed by spray-drying at neutral pH. The effect of high pressure shearing on functional properties including gelling characteristics of heat denatured or undenatured WP was assessed at pH 7 using MWP powders. DSC, SE-HPLC and SDS-PAGE indicated the creation of relatively higher as well as smaller molecular weight aggregates from completely denatured WP. The assessment of functional properties indicated that denatured MWP were better emulsifiers compared to native WP. MWP powders produced from unheated WP dispersions created firm gels upon heating, whereas those produced from denatured WP were capable of creating only cold-set gels. Covalent and non-covalent protein-protein interactions were involved during both heat- and cold-set gelation. Combined heat and high pressure shearing substantially increased the heat stability of MWP. However, the size of the particles was comparatively larger (~ 10 μm) than native WP affecting colloidal properties.

The third study focussed on the reaction kinetics of WP denaturation upon heating as a function of pH (at 4, 5 or ~ 6) and protein concentration (10, 17.5 and ~ 25 %

protein, w/w) as a foundation for devising a more feasible pathway to produce MWP powders with comparatively reduced particle size. WP dispersions were subjected to a heat treatment at 140°C for kinetic studies, thermal analysis with DSC, rheological studies and heat-induced gelation. The denaturation of β -Lg appeared to follow the first order reaction kinetics. Also, WP were more stable against heat induced denaturation at acidic pH. The heat induced gels formed at low pH possessed weak and brittle characteristics likely resulting from overall positive charge of WP at acidic pH with enhanced inter-molecular repulsive electrostatic interactions and the inhibition or suppression of thiol group activity.

Based on the kinetic studies and the results of fundamental WP interactions using chemical blockers, MWP powders were produced at pH 3, acidified with either citric or lactic acid to create a WP gel network with relatively weaker molecular linkages which would be easily dispersed by mechanical forces used during MFZ. As expected, the particle size of denatured MWP was substantially reduced to an average \sim 100 nm, producing nano-particles together with micro particles which was a 100 fold reduction compared to the average particle size of MWP produced at neutral pH. In addition, the heat stability of these powders was also substantially increased as revealed by heat coagulation time with > 3 min. These powders also had different physical functionalities from native WP including higher viscosity, improved surface and colloidal properties. Additionally, the effect of high pressure shearing on different functional properties of MWP varied with acidulant selection, depending on their relative thermal stability at low pH. At low pH, combination of heat and high pressure shearing produced WP micro-aggregates with improved colloidal stability which was not achieved by microparticulation of denatured WP at neutral pH, in addition to substantially enhanced heat stability of these powders.

Finally, the effect of simultaneous heating (from 20 – 90°C for 35 min; holding at 90°C for 20 min and cooling from 90 – 25°C in \sim 15 min) and shearing on WP aggregation was examined with four different protein concentrations (5, 10, 17.5 or \sim 25% w/w protein) and three pH (3, 5 or 7) at different shear rates (100, 500 or 1000 s^{-1}). FTIR spectroscopy revealed that the secondary structure of WP was most conserved at pH 3, followed by that at pH 5. The application of shearing has reduced the formation of molecular associations including covalent bonds at pH 7 which was

even revealed by reduced turbidity of the samples. Further, turbidity, gel colour, PAGE, surface hydrophobicity and viscosity measurements also indicated that the shear forces may change the direction and extent of heat-induced WP denaturation and aggregation.

In conclusion, microparticulation of WP using heat and high pressure shearing appeared to be a very useful and feasible approach in modulating physical functionality of WP especially heat stability. Thus, this approach, if scaled up and applied at an industrial level, would result in substantial diversified applicability of this nutritionally precious dairy ingredient.

II. Declaration

“I, Muditha Dissanayake, declare that the PhD thesis entitled “Modulation of Functional Properties of Whey Proteins by Microparticulation” is no more than 100,000 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:

Muditha Dissanayake

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CHAPTER 1

1 Introduction

1.1 Background

Whey proteins (WP) comprise one of the two major protein components of cow's milk (20%), the other component being the caseins (80%). They are not aggregating upon acidification or renneting of milk and are soluble in their native form almost independent of pH (Fox and McSweeney, 2003). WP are primarily a mixture of beta lactoglobulin (β -Lg), alpha lactalbumin (α -La), bovine serum albumin (BSA) and immunoglobulins (Ig) (Verheul et al., 1998). They can be isolated from whey, the natural by-product of cheese and casein manufacture and in their native form they exist as compact, globular proteins. Commercially different types of WP powders are available. The most important products are whey protein concentrates (WPC) and whey protein isolates (WPI). WPC contains more than 35% protein, low levels of fat and cholesterol and typically a greater amount of bioactive compounds and lactose. WPI contains more than 90% of protein and have lower levels of fat and lactose and bioactive compounds as well (Fox and McSweeney, 2003; Hettiarachchy and Ziegler, 1994; Huppertz et al., 2005).

WP impart extreme importance in digestibility, availability, amino acid pattern and biological value, sensory characteristics and multiple functionalities (McIntosh et al., 1998). Based on the amino acid composition and the rate of peptide and amino acid release in the small intestine, they present an exclusive nutritional and physiologically functional supplement, better than any other dietary protein. Some of the physiologically active peptides obtained from WP carry numerous biological benefits including opiate, antithrombotic, antioxidative activity, immunomodulation and enhancement of mineral absorption (Vasiljevic and Shah, 2007). WP are widely used in the food industry. The common applications are sport beverages and liquid meat replacements, baked products

and processed meats, salad dressings, artificial coffee creams, soups and various other dairy products (Onwalata and Tomasula, 2004; Fitzsimons, et al., 2007).

High solubility of WP over a wide range of pH (Zhu and Damodaran, 1994) is an added advantage for the exploitation of their other functionalities such as foaming, emulsifying, gelling, heat coagulation and water binding over various types of food products. In addition, WP, being excellent foaming and emulsifying agents, have an ability to form cohesive and viscoelastic films by polymerization mainly via disulfide bonds and hydrophobic interactions (Monahan et al., 1993; Lee et al., 1992; Bouaouina et al., 2006). Also, ability of WP to form heat- and cold-set stable gels to perform as a matrix for holding other components of a food is very useful in food formulations and product development (Matsudomi et al., 1993; Resch et al., 2004). This is in addition to the superior nutritional benefits obtain from WP compared to pre-gelatinized starch and hydrocolloids, which also do water holding and increasing viscosity (Resch et al., 2005).

Although WP is a unique nutritional and functional protein source, the major challenge in applicability is their heat-induced destabilization (Onwulata et al., 2004). In industrial practice, inevitable heat treatment during whey protein concentration changes the native state and affects their stability and applicability. In addition, compulsory heat treatments during processing of some food products may cause whey protein denaturation, aggregation and flocculation resulting phase separation, destabilization of emulsions or protein precipitation. For example, sedimentation may take place in production or storage of fruit juices and other liquid food preparations, having interactions with heat labile WP and other food components such as pectin even at pH below 3.5, or at high concentrations, they may form viscoelastic gels upon heating over 65°C (Iordache and Jelen, 2003). Native WP have high solubility due to the large ratio of surface hydrophilic residues in their native state. However, in the presence of denaturing agents such as heat, pressure and urea, these globular proteins get unfolded and subsequently aggregated mainly due to the exposure of previously buried apolar groups and occurrence of sulfhydryl/disulfide exchange chain reactions via activated thiol

groups (Lee et al., 1992). The rates and pathways of these physicochemical reactions are determined by the factors intrinsic to protein and extrinsic environmental factors such as protein concentration, pH, temperature, ionic strength and solvent condition (Brandenberg et al., 1992; Iordache and Jelen, 2003; Marangoni et al., 2000). Thus, in order to fully utilize their functional properties in different food systems they need to be recovered in their native, undenatured state and subsequently incorporated in a food matrix. Therefore, the knowledge of WP behaviour, particularly, the mechanisms of heat-induced unfolding, aggregation and gelation in different environmental conditions is important in conducting successful alternations which lead to grasp the maximum advantage of this excellent food ingredient. (Verheul et al., 1998; Onwalata et al., 2004; Matsudomi et al., 1993; Iordache and Jelen, 2003). Unfortunately, existing extraction methods are also poorly up-scaled limiting the application of whey proteins. But it is rather imperative to the industry in improving functionality of whey proteins by applying different conventional approaches in order to avoid heat instabilities. On the other hand, whey proteins may be stabilized by primary denaturation followed by colloidalization (Onwulata et al., 2004). The physicochemical properties of individual proteins present in WP as well as the intrinsic (food properties) and extrinsic (environment) conditions, including pH, temperature, ionic strength, solvent polarity and type, govern the overall whey protein functionality and influence the formation of primary particles, affecting the final particle size, morphology, surface properties and different interactions between individual protein particles as well as contacting biopolymers, thus contributing the applicability in various food systems.

High pressure is an appealing treatment in processing of food that often may have negative side effects on sensory characteristics such as flavour and colour (Considine et al., 2007; Huppertz et al., 2005). Microparticulation of WP is a new approach that uses high pressure shearing achieved by, for example, microfluidization without or with heating. Microparticulation may produce micro-aggregates with improved heat and colloidal properties from heat-induced WP

gels leading to modulated functionalities. Also, by controlling the extent of shear as well as kinetics of denaturation and aggregation of WP using different environmental conditions may likely lead to more heat stable and functionally more applicable microparticulated whey protein (MWP) preparations.

1.2 Research objectives

The main objective of this study is to establish a novel processing method that may lead into production of WP based ingredients with different functionalities. The proposed process involves the combined application of the heat and high pressure shearing, all of which should induce conformational changes of heat unstable WP.

The current research is more specifically aimed at:

1. Assessing the effects of complete protein denaturation and the extent of high pressure shearing on colloidal, surface and rheological properties of newly formed MWP;
2. Examining the reaction kinetics of whey protein denaturation as a function of pH and protein concentration;
3. Investigating the effects of adjusted environmental conditions, *i.e.* pH, protein concentration and acidulant selection on functional properties of MWP preparations obtained by complete heat denaturation and high shear treatment;
4. Examining the role of shear during heat induced WP aggregation as affected by different protein concentrations and pH conditions.

1.3 Thesis outline

The Chapter 1 provides background, research objectives and outline of the Thesis. Chapter 2 presents a review of literature explaining more detailed information of WP, physiological and functional importance of WP, the kinetics and factors affecting WP denaturation and aggregation, and finally, attempts at the modification of WP functionality. The compositional and functional variability of commercially available WPC on the Australian market is revealed in Chapter 3. Chapter 4 reports on a study on the functional properties of WP as affected by heat treatment and hydrodynamic high pressure shearing. Chapter 5 describes a study on reaction kinetics of WP as a function of pH and protein concentration. A study on the physical functionality of MWP at low pH is presented in Chapter 6. Chapter 7 explains the role of shear during heat-induced WP aggregation under different protein concentrations, pH and shear rates. The conclusions of the overall study and the scope for the future work are delivered in Chapter 8.

CHAPTER 2

2 Literature Review

2.1 Milk and milk proteins

Milk nourishes people of all ages and lands from newborn infant to the old and infirm. It is a secretion of mammalian females, providing all the nutrients to the young. Milk is a very complex liquid containing numerous types of molecules. It is mainly an aqueous solution of lactose, inorganic and organic salts, and dispersed colloidal particles of milk proteins and larger emulsified lipid globules. Proteins and peptides, for example, immunoglobulins, metal-binding proteins and enzymes, growth factors, hormones and antibacterial agents and some oligosaccharides present in milk deliver important physiological and protective functions (Fox and McSweeney, 2003; Thompson, et al., 2009). Bovine milk and the dairy products play an important role in human nutrition. The average composition of main components of bovine milk is: 87.1% water, 4.6% lactose, 3.3% milk proteins and 4% milk fat (Walstra et al., 2006).

Proteins are the major constituent of human diet for supporting their growth and well being. Among dietary proteins, milk proteins are most likely the best researched and described (Fox and McSweeney, 2003). Milk proteins can generally be classified into two types as caseins and whey proteins based on their solubility at pH 4.6 (Huppertz et al., 2005), where caseins are insoluble and, consequently coagulated, and the whey proteins remain soluble. Out of total milk protein content, caseins present about 80% and remaining 20% are the whey proteins.

There are four main types of caseins (α_{s1} -, α_{s2} -, β - and κ - casein) which exist in milk as large colloidal complexes or micelles composed of about several thousand molecules with the molecular mass of approximately 10^8 Da. On the other hand, the whey proteins most probably exist as monomers or as small quaternary structures. In comparison to caseins, which are extremely heat-stable and hence

not be coagulated when heated at 100°C for 24 hours or at 140°C for up to 20 – 25 min (Fox and McSweeney, 2003), whey proteins are very heat sensitive. Caseins are phosphorylated and the degree of phosphorylation varies among the individual caseins imparting them molecular charges and thereby hydration, solubility, heat stability and metal binding, especially in this instance, Ca ions. As a result, high levels of calcium phosphate are available in milk in a soluble form. Additionally, under natural conditions and in a stable colloidal suspension of surrounding water-based liquid, the casein micelles are not aggregating as a result of an inter-micellar steric and electrostatic repulsion provided by the protruding polyelectrolyte region of κ -casein from the micellar surface. Although whey proteins are not phosphorelated, they are richer in sulphur content (1.7%) compared to caseins with \sim 0.8% sulphur. Caseins lack α - and β - sheets, which is related to their higher levels of proline content compared to more structured whey proteins (Fox and McSweeney, 2003).

Cheese is a milk protein, fat and calcium enriched food product that has been made in most parts of the world from ancient times (Walther et al., 2010). During cheese manufacturing, the pH of milk is lowered to the isoelectric point of caseins (pH \sim 4.6) where a curd is formed due to casein aggregation. The coagulation of milk is accomplished by the addition of starter cultures, mostly via lactic acid bacteria, or rennet to achieve clotting by enzymatic induction, or otherwise, addition of food grade acids and/or acidogens (*e.g.* glucono- δ -lactone) to milk to induce coagulation artificially at a temperature of 20 – 40°C (Fox and McSweeney, 2003; Thompson et al., 2009, Walstra et al., 2006). In addition, the industrial casein production is carried out on a large scale, worldwide, by acid or rennet coagulation of caseins.

Table 2.1 Physical and chemical properties of caseins and whey proteins
(Adapted from Fox and McSweeney, 2003)

Property	Caseins	Whey proteins
Protein type	α -casein β -casein κ -casein	α -lactalbumin (α -La) β -lactoglobulin (β -Lg) Bovine serum albumin (BSA) Immunoglobulins Proteose peptones
Physical state in milk	Large colloidal aggregates as micelles	Monomers or small quaternary structures
Molecular weight	Casein micelle - $\sim 10^8$ Da	α -La - ~ 14 kDa β -Lg - ~ 18 kDa BSA - ~ 66 kDa
Isoelectric point	pH ~ 4.6	pH ~ 5
Solubility at pH ~ 4.6	Insoluble	Soluble
Heat stability	Very heat stable; withstand heat at 140°C up to 20-25 min	Completely denatured at 90°C for 10 min
Amino acid composition	Contain high levels of proline imparting lack of α - and β structures; Phosphorylated resulting in high solubility, hydration, heat stability, carry high Ca^{2+} ; Low in sulphur (0.8%) and mainly contained by methionine residues, little cysteine/cystine, lack intramolecular disulfide bonds	Highly structured Not phosphorylated Rich in sulphur (1.7%) and contained by cysteine and/or cystine, Number of intra molecular disulfide bonds and thiol (SH) groups respectively: α -La - 4, no SH groups β -Lg - 2 and 1 SH group BSA - 17 and 1 SH group

Whey is the liquid remaining after almost complete casein removal from milk, thus it is the natural by-product of cheese and casein manufacture. The estimated world's whey protein production exceeds 0.5 million tonnes annually (Thompson, et al., 2009). The composition of whey usually varies depending on the method of casein separation. The whey resulting from rennet coagulated casein or rennet type Cheddar or Swiss cheese manufacturing is known as sweet whey (pH > 5.6). It contains a higher lactose content than acid whey which is obtained from the manufacture of acid casein brought about by addition of either lactic or mineral acid. It also contains a higher mineral/ash content comparatively. In general, whey is a dilute, greenish yellow, translucent liquid with average of 93% water, 0.6% proteins termed as whey proteins, ~ 5% lactose, ~ 0.02% fat and ~ 0.6% minerals, vitamins and some other several constituents at trace levels (Foegeding et al., 2002; Fox and McSweeney, 2003). Whey has now been recognized as a valuable source of essential amino acids, however, until recently the disposal of whey was one of the major environmental problems.

2.2 Whey proteins

Whey proteins (WP) are mainly a mixture of β -lactoglobulin (β -Lg), α -lactalbumin (α -La), bovine serum albumin (BSA), immunoglobulins (Ig), proteose peptones and some other minor proteins including lactoperoxidase, lysosome and lactoferrin (Verheul et al., 1998; Fitzsimons et al., 2006; Anfinsen, 1967).

Some of the important physical and chemical properties of the main whey proteins are listed below.

2.2.1 β -lactoglobulin

β -Lg is the most abundant whey protein with the molecular weight of ~ 18.3 kDa with the primary sequence composed of 162 amino acids. The amino acid

sequence of β -Lg is as follows. Asp (10), Asn (5), Thr (8), Ser (7), Glu (16), Gln (9), Pro (8), Gly (4), Ala (15), Cys (5), Val (9), Met (4), Ile (10), Leu (22), Tyr (4), Phe (4), Lys (15), His (2), Trp (2) and Arg (3) (Morr and Ha, 1993). Its isoelectric point is \sim pH 5.2. It represents \sim 50% of total whey proteins and also \sim 12% of total milk proteins. There are ten genetic variants of bovine β -Lg known so far (Fox and McSweeney, 2003), however, the two common variants with equal frequency are β -Lg -A and -B which differ from each other only in two amino acids (A/B sequence differences are Asp₆₄/Gly₆₄ and Val₁₁₈/Ala₁₁₈) (de Wit, 2009; Hinrichs and Rademacher, 2004). But this slight difference has given them a significant difference in their solubility (de Wit, 2009). The next important variant is the variant C, recognizes in Jersey cows. Depending on the positioning of different amino acids and salt bridges on the protein, the heat stability of these β -Lg genetic variants differs. For example, at low temperatures, variant A is less stable than variant C as opposed to stability at higher temperatures, at which variant A is more stable than variants B and C due to its better hydrophobic packing (de la Fuente et al., 2002).

As X-ray crystallographic and NMR studies (Croguennec, et al., 2004; Thompson, et al., 2009) revealed, β -Lg has well established primary, secondary, ternary and quaternary structures with a number of β -strands forming a β -barrel structure which contains ideal sites for hydrophobic ligands and also a single α -helix area at the C-terminal end of the protein. Also, refined crystal structures have shown (Croguennec, et al., 2004) that the major features of secondary structure of β -Lg are two anti-parallel β -sheets formed by 9 strands labelled A to I, and eight of them form somewhat flattened β -barrel, which covers the thiol group of Cys₁₂₁ with the help of α -helix that is situated parallel to strands A, F, G and H. Furthermore, one side of both the β -sheets are hydrophobic and the hydrophobic sides face each other forming a highly hydrophobic cavity (Considine et al., 2007). The stability of the ternary structure of β -Lg is strongly depending on the two disulfide bonds positioned between Cys₆₆-Cys₁₆₀ and Cys₁₀₆-Cys₁₁₉ (Thompson, et al., 2009; Manderson et al., 1998). They are available as dimers

between pH ~3 and ~7.5 (Manderson et al., 1998) and, as a result, at natural pH they exist as stable non-covalent dimers at room temperature but beyond that temperature they dissociate to monomers (Croguennec, et al., 2004).

2.2.2 α -lactalbumin

α -La represents ~ 20% of total whey proteins and ~ 3.5% of total milk proteins. It is composed of 123 amino acid residues with the molecular weight of ~14 kDa and the isoelectric point of ~ pH 4.8. The amino acid sequence of α -La has been identified as, Asp (9), Asn (12), Thr (7), Ser (7), Glu (8), Gln (5), Pro (2), Gly (6), Ala (3), Cys (8), Val (6), Met (1), Ile (8), Leu (13), Tyr (4), Phe (4), Lys (12), His (3), Trp (4) and Arg (1) (Morr and Ha, 1993). Two genetic variants of α -La have been identified as A- and B- (Fox and McSweeney, 2003). This globular molecule has split into two as an α -lobe, which contains three α -helices (about 26%) and two short helices and a β -lobe that contains small three stranded β -sheets (about 14%) and a short helical structure, however 60% of the protein is unordered (Thompson, et al., 2009; Fox and McSweeney, 2003). In the native form, α -La is a monomer with 4 disulfide bridges between the amino acid residues of 6 and 120, 28 and 111, 61 and 77, and 73 and 91. However, the disulfide bond between Cys₆ and Cys₁₂₀ is more sensitive to be affected than the other three due to its lower inherent stability (Considine, et al., 2007). Meanwhile, the absence of thiol groups in the molecule makes it somewhat heat stable. (Monahan et al., 1993; Paulsson and Dejmek, 1989; Morr and Ha, 1999). It has a stable configuration between pH 5.4 and 9 (Fox and McSweeney, 2003). In addition, it has a Ca²⁺ binding site located in the cleft of two lobes via Asp residues and Ca also promotes the unfolding of α -La, which means, again the promotion of heat stability and also recovery of the native conformation. α -La is the most heat stable of the main WP (Fox and McSweeney, 2003). As confirmed by X-ray crystallographic structures of apo- and holo- (calcium bound structure) forms of α -La, Ca²⁺ ions stabilize the native structure by allowing the protein to form correct disulfide bonds, reduce

separation of lobes, assist positioning a buried solvent molecule near the Ca-binding site and affect inter- and intra-lobe hydrogen bonding, facilitating unfolding (Chrysin, 2000). However, the Ca affinity of α -La decreases at pH $< \sim 5$ since the Asp residues get protonated which results in low heat stability or denaturation at lower temperatures and inability to re-nature on cooling as well (Fox and McSweeney, 2003).

2.2.3 Bovine serum albumin

BSA consists of 582 amino acid residues with a molecular weight of ~ 66 Da (Morr and Ha, 1993). The amino acid sequence of BSA is, Asp (39), Asn (12), Asx (3), Thr (34), Ser (28), Glu (59), Gln (19), Glx (1), Pro (28), Gly (16), Ala (46), Cys (35), Val (36), Met (4), Ile (14), Leu (61), Tyr (19), Phe (27), Lys (59), His (17), Trp (2) and Arg (23) (Morr and Ha, 1993). The isoelectric pH of BSA is ~ 5.3 (Damodaran et al., 2008). It has a multi-domain (three) structure with complex ligand binding ability and specified by an oblate shape. The domains are stabilized by a number of disulfide bonds (Considine et al., 2007). It has 17 intramolecular disulfide bonds and one free thiol group at Cys₃₄ residue (Thompson, et al., 2009). It does not contain any β -sheet, instead it is composed of mainly helix, turn and extended chains (Considine et al., 2007). The C-terminal region of the molecule is more compact than the N-terminal region and the different domains show differences in hydrophobicity, net charge and ligand-binding properties (Morr and Ha, 1993). BSA is a quite recognizable transport protein for insoluble fatty acids in the circulatory system (Juliette, et al., 1995).

2.2.4 Physiological importance of whey proteins

Naturally, WP exist as compact, globular proteins with high solubility due to a large number of surface hydrophilic residues (Lee et al., 1992). Being high quality proteins they bring an extreme importance in digestibility or bioavailability,

amino acid pattern and biological value, sensory characteristics and multiple functionality (McIntosh et al., 1998). Based on the amino acid composition and the rate of peptide and amino acid release in the small intestine, WP present an exclusive nutritional and physiologically functional supplement, better than any other known dietary protein. The solubility over wide range of pH enables them to pass through the small intestine without precipitating in an acidic environment in the stomach, to hydrolyse in the small intestine and to deliver their amino acids and peptides quickly in comparison to caseins. Moreover, the bioactive peptides present in WP may bring physiological benefits improving health and reducing risks of diseases and this has become one of the main focuses of ongoing research. For example glutathione is an antioxidant that protects the body against free radical damage and can detoxify possible carcinogens. WP help to increase the glutathione concentration by providing a substrate for glutathione synthesis and also probably exerting its effect on carcinogenesis by increasing glutathione concentration (Parodi, 2007; Bounous et al, 1991). Also, WP are rich in angiotensin converting enzyme (ACE) inhibitory peptides influencing blood pressure (Richard et al., 2004) and possibly lipogenesis (Mathai et al., 2008). The branched chain amino acids (BCAA) such as leucine present in WP may participate in acceleration of protein synthesis and also act as a signal or a substrate, in activating the translational machinery in pancreatic cells (Sans et al., 2006). The WP provide immunological protection to the neonates mainly via immunoglobulins present in colostrum. In addition, these proteins extend their function by the initiation of mucosal immunity in the gut providing an optimal environment to the beneficial microflora, beyond their activity of suppression of pathogen load (Thompson, et al., 2009). Consumption of protein-rich foods, such as whey proteins, brings a number of other health benefits including stimulation of fast satiation, resulting in reduction of food intake (Manski et al., 2007) and also opioid activity and enhancement of mineral absorption (Pihlanto-Leppala, 2001).

2.2.5 Whey proteins in food systems as a functional ingredient

Along with physiological benefits, WP possess inherent excellent functional properties and desirable sensory characteristics enabling them to be used in numerous food applications including sport beverages, liquid meat replacements, baked products and processed meats, pasta, salad dressings, spreads and dips, artificial coffee creams, soups, ice cream, confectionary, infant foods and various other dairy products (Onwalata and Tomasula, 2004; Fitzsimons et al., 2006). In order for them to be used in these applications, WP should be extracted from whey using different fractionation methods, mainly membrane processing.

2.3 Commercial whey protein products

Commercial whey protein products are widely used in food industry in number of formulated food products and incorporation of these products in food systems as functional ingredients is the most practical manner of whey protein consumption (Morr and Foegeding, 1990). There are different types of WP powders available on the market as concentrates, isolates and hydrolysates. The annual worldwide production of whey protein products is about 600,000 metric tons (Damodaran et al., 2008). WPC contains up to ~ 85% proteins, low levels of fat and cholesterol and typically a higher amount of bioactive compounds and lactose. However, the fat and lactose content present in WPC may exert detrimental effects on some functional properties and the overall protein quality. Fat content may affect the foamability and raised lactose reacts with protein to impart non-enzymatic browning or affect the texture of some frozen products by crystallisation (Morr and Ha, 1993). In comparison, WPI contains more than 90% of proteins with lower levels of fat, lactose and bioactive compounds. Therefore they are relatively high quality protein powders with enhanced functionality. Whey protein hydrolysates are partially hydrolysed, pre-digested products enabling easy absorption in the gut, especially used in infant formulae (Fox and McSweeney, 2003; Hettiarachchy and Ziegler, 1994; Huppertz et al., 2005).

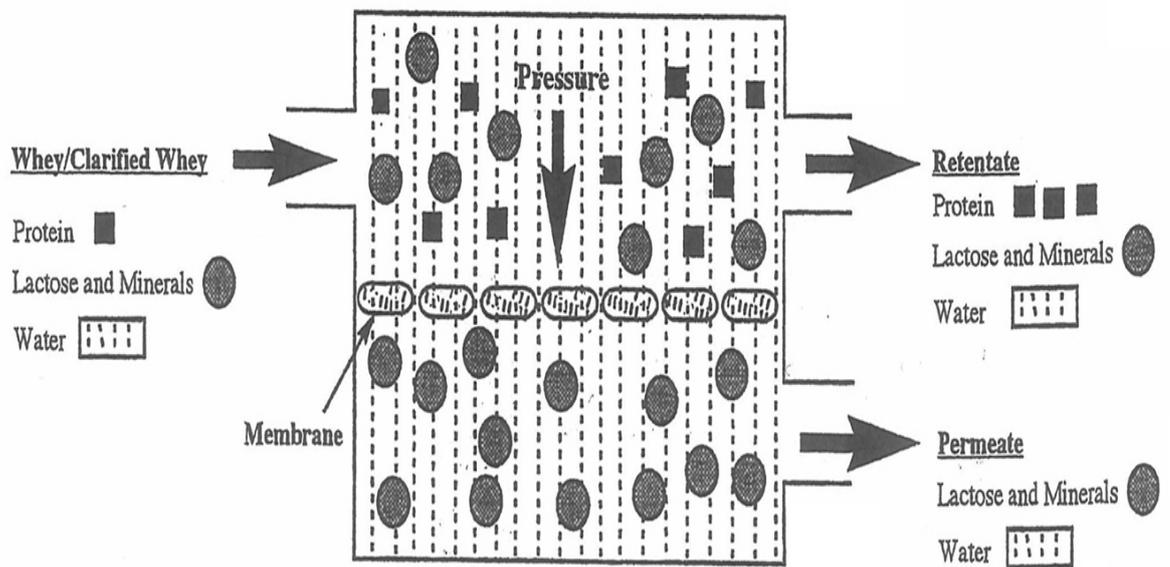


Figure 2.1 Principle of Ultrafiltration (Adapted from Fox and McSweeney, 2003)

WPC is prepared by means of ultrafiltration/diafiltration of whey to remove mainly water, lactose and minerals as a result of selective separation of whey proteins under mild processing conditions (temperature and pH). Ultrafiltration of whey usually results in retentates of ~ 24% total solids and diafiltration gives retentates of a higher total solid content ~ 28% with comparatively higher protein to total solids ratio (Fox and McSweeney, 2003). In WPI production, additional ion exchange chromatographic or microfiltration methods are employed. After further concentration, retentates of these whey proteins are spray dried to produce WPC or WPI powders (Fox and McSweeney, 2003; Morr and Ha, 1993). The membrane technology, including ultrafiltration, diafiltration and ion-exchange adsorption used in these processes has become very useful in the production of different whey protein products with varied composition and excellent functionalities (Damodaran et al., 2008).

However, there are limitations in the application of these products in food systems due to their inherent compositional variability and different processing conditions used in cheese making and in manufacturing of WPC and subsequent storage conditions as well. This may ultimately result in inconsistency in product quality and complexity of the reactivity of WP during processing of foods containing whey proteins (Morr and Foegeding, 1990; Morr and Ha, 1993; Jovanovic et al., 2005). A study carried out by Onwulata et al., (2004) using different commercial whey protein concentrate products (WPC₈₀) revealed that there were significant variations in physico-chemical properties of these samples, for example, proximate composition and particle size distribution pattern, which was mostly consistent with the variations, observed in different functional properties. The properties such as heat stability and gel firmness of most of the products appeared to have a link with calcium content. In addition, Rham and Chanton (1984) reported that different Ca chelating agents such as citrates and phosphates may enhance heat stability of WP. Also, Veith and Reynolds (2004) have reported the chelation of Ca in WPC by polyphosphate. The other common setback regarding the WPC products is the development of off-flavours with time (Javidipour and

Qian, 2008). This is mostly a result of lipid oxidation. The free radicals and numerous other organic volatile substances released from lipid oxidation may further damage the quality of the powders, by catalysing the secondary Maillard browning reaction between proteins and lactose (Morr and Ha, 1993). Further, the inconsistency in flavour of WP products and also the influence of off flavours on the finished product may limit whey ingredient applications in dairy and non-dairy foods (Whetstine et al., 2003).

2.4 Functional behaviour of whey proteins

The functionality of WP depends on the physicochemical properties of their three-dimensional protein structure including, size, shape, amino acid composition and sequence, net charge, ratio of hydrophobicity to hydrophilicity, molecular flexibility and rigidity which is on the other hand governed by the external environmental factors, such as protein concentration, pH, temperature, ionic strength, the type of ions present and the influence of other available food components (Damodaran et al., 2008; de la Fuente et al., 2002). Although, WP functionality mainly depends on the behaviour of β -Lg, the most abundant protein in whey (Verheul et al., 1998), the overall functionality entirely depends on the combination of the properties of all other components present in the whey protein product. For instance, β -Lg has excellent gelling, foaming and emulsifying properties while α -La exhibits good emulsifying properties, but its gelling ability is poor (Pearce and Kinsella, 1978).

2.4.1 Solubility and protein water interactions

Many of the important functional properties of proteins, such as, solubility, swelling, viscosity, water holding capacity, gelation, coagulation and surface properties depend on water-protein interactions (Huang and Kinsella, 1986). On

the other hand, the high aqueous solubility of whey proteins is an added advantage as a prerequisite for fully exploiting their other physical functionalities. The water molecules bind to different groups in proteins including peptide, amide, hydroxyl and other charged groups and non polar residues as well (Damodaran et al., 2008). The solubility depends not only on water-binding capacity but also other factors such as water holding capacity. For the food applications, water-holding capacity, which is the capability of a protein to absorb water and hold it against gravitational force within a protein matrix, is probably more important than water binding ability although the two properties are positively related. However, these characteristics of protein ultimately contribute to the desirable textural attributes to most food applications such as bakery products, desserts and meat substitutes. The temperature is negatively correlated with protein's water binding ability since hydrogen bonds are not stable at raised temperatures (Damodaran et al., 2008).

The solubility of a protein depends on a thermodynamic equilibrium between protein-protein and protein-solvent interactions (Hui, 2006). The intrinsic properties of proteins such as surface hydrophilicity and hydrophobicity greatly affect the protein solubility. The hydrophobic interactions promote protein-protein affinity decreasing solubility and ionic interactions promote protein-water interactions increasing solubility. The extrinsic parameters such as pH, temperature and ionic strength determine the protein solubility as well. Due to lack of electrostatic repulsion, proteins show minimum solubility around isoelectric pH (Damodaran et al., 2008). However, whey proteins are highly soluble even at their isoelectric points due to a large ratio of surface hydrophilic to hydrophobic residues. Moreover, they are soluble in the pH range of 2 - 9 which makes them suitable for incorporation into various food products such as carbonated beverages at acidic pH. Generally at low ionic strengths salts screen surface charges and it increases the solubility of globular proteins, but at higher ionic strength salts have ion specific nature on solubility (Resch et al., 2004; Damodaran et al., 2008).

2.4.2 Viscosity

The viscosity of a substance relates to its resistance to flow under an applied force (Fox and McSweeney, 2003). This property is also related to the consumer perception of some liquid and semi-solid type foods such as beverages, gravies, soups and sauces. The thickening ability of WP is a very useful functional property regarding these food products and the awareness of the viscous behaviour is practically important in relation to processing, designing and development of food products as well as processing conditions (Huang and Kinsella, 1986). The very dilute solutions of whey proteins behave as Newtonian fluids, while the flow is pseudoplastic at higher concentrations. For example, β -Lg increases the viscosity with shearing time and at very high concentrations ($\sim 40\%$, w/w) this protein shows time-dependent shear thinning behaviour probably due to partial denaturation of proteins (Fox and McSweeney, 2003). The molecular properties of proteins including the size and shape as well as protein-solvent interactions, hydrodynamic volume and the molecular flexibility in the hydrated state affect the viscosity (Genovese et al., 2007). The denatured dispersions of randomly coiled molecules show greater viscosity than do solutions of compact folded globular molecules of the same molecular weight (Damodaran et al., 2008).

2.4.3 Foaming and emulsifying

WP are efficient foaming and emulsifying agents. The amount of sulfhydryl groups, molecular flexibility, hydrophobicity and surface activity determine their ability to form stable emulsions and foams (Lee et al., 1992). The optimum foaming and emulsifying characteristics of the proteins depend on how efficiently they diffuse to the newly formed interface, get unfolded and reorient to lower interfacial tension and form cohesive and viscoelastic films by polymerisation mainly via disulfide bonds and hydrophobic interactions (Monahan et al., 1993; Lee et al., 1992; Bouaouina et al., 2006). By increasing the ratio of β -Lg to α -La,

the foam yield stress of whey protein products can be modified (Foegeding et al., 2002). Sulfhydryl groups, which are more active at pH 7, contribute to molecular flexibility while disulfide bridges contribute to rigidity. Molecular flexibility enhances emulsion and foam formation by increasing the rate of unfolding at the interface and allowing more favourable alignment of polar and non polar groups in their preferred phase (Klemaszewski and Kinsella, 1991). At pH 7, whey proteins have a net negative charge, but at pH 8 possibly due to oxidation of thiol group, they easily form disulfide bonds. Proteins at an appropriate pH with increased net negative charge repulse each other creating a barrier to the close approach of droplets, thereby retarding the rate of coalescence and resulting in more stable emulsions (Klemaszewski and Kinsella, 1991). Previous studies have revealed that there is an improved overrun and increased stability of foams at pH 7 when heated to 55°C (Phillips et al., 1990). At higher temperatures, however, foaming and emulsifying characteristics may be impaired due to protein aggregation, which decreases the availability of proteins to form films and emulsions (Phillips et al., 1990). Furthermore, since WP are even soluble at their isoelectric points (pH ~ 5), they exhibit superior foaming properties having better protein-protein interactions to form stable foams around that pH. In addition, the β -Lg B variant appeared to adsorb more rapidly at interfaces than variant A although β -Lg A and B variants vary by only two amino acids (Foegeding et al., 2002).

2.4.4 Gelation

A gel is an intermediate state between a solid and a liquid which is also defined as ‘a substantially diluted system that exhibits no steady state flow’ (Damodaran et al., 2008). The capability of undenatured WP to form stable heat induced gels is also very important in food formulations and product development as these gels can act as a basic medium to hold other components such as water, lipids, sugars, flavour in heterogeneous food systems providing textural and thickening attributes

(Matsudomi et al., 1993; Resch et al., 2004). Compared to pre-gelatinized starches and hydrocolloids, which also have the capacity of water holding and increasing viscosity, whey proteins have added superior nutritional benefits (Resch et al., 2005). Thermal gelation involves initial unfolding and subsequent aggregation of proteins depending on the balance of attractive and repulsive forces (Hudson et al., 2000). The irreversible unfolding of whey proteins occur at temperatures $> 60^{\circ}\text{C}$ (Considine et al., 2007). The process involves a series of physicochemical reactions such as dissociation, denaturation and exposure of hydrophobic residues and resulting polymerization via formation of intermolecular disulfide bonds, hydrophobic, hydrogen and ionic bonds (Brandenberg et al., 1992).

A proper balance of protein-protein as well as protein-water interactions fundamentally controls gelation mechanism and the gel appearance. If the protein-protein interactions are much greater than the protein-water interactions mostly a precipitate is formed. On the contrary, if protein-solvent interactions predominate the system would not gel. Another vital factor for the formation of a self-stranded gel network is the protein concentration. The least concentration end point (LCE) is the minimum protein concentration required to form such a network at given conditions (Gosal and Ross-Murphy, 2000; Damodaran et al., 2008). The physical characteristics of these gels can be altered by manipulating protein concentration, ionic strength, heating time and temperature, solvent condition and quality and addition of other macromolecules or filler particles (Verheul et al., 1998).

When there are large electrostatic repulsions between the proteins, *i.e.*, at low ionic strength and far from the isoelectric point (pI) of the proteins, transparent, fine stranded gel structures are formed. At $\text{pH} > \text{pI}$ fine stranded, strong elastic gels are formed and at $\text{pH} < \text{pI}$ fine stranded weak brittle gels are formed. At high ionic strength and around isoelectric point of the proteins, turbid, milk white and particulate gel structures are formed (Verheul et al., 1998).

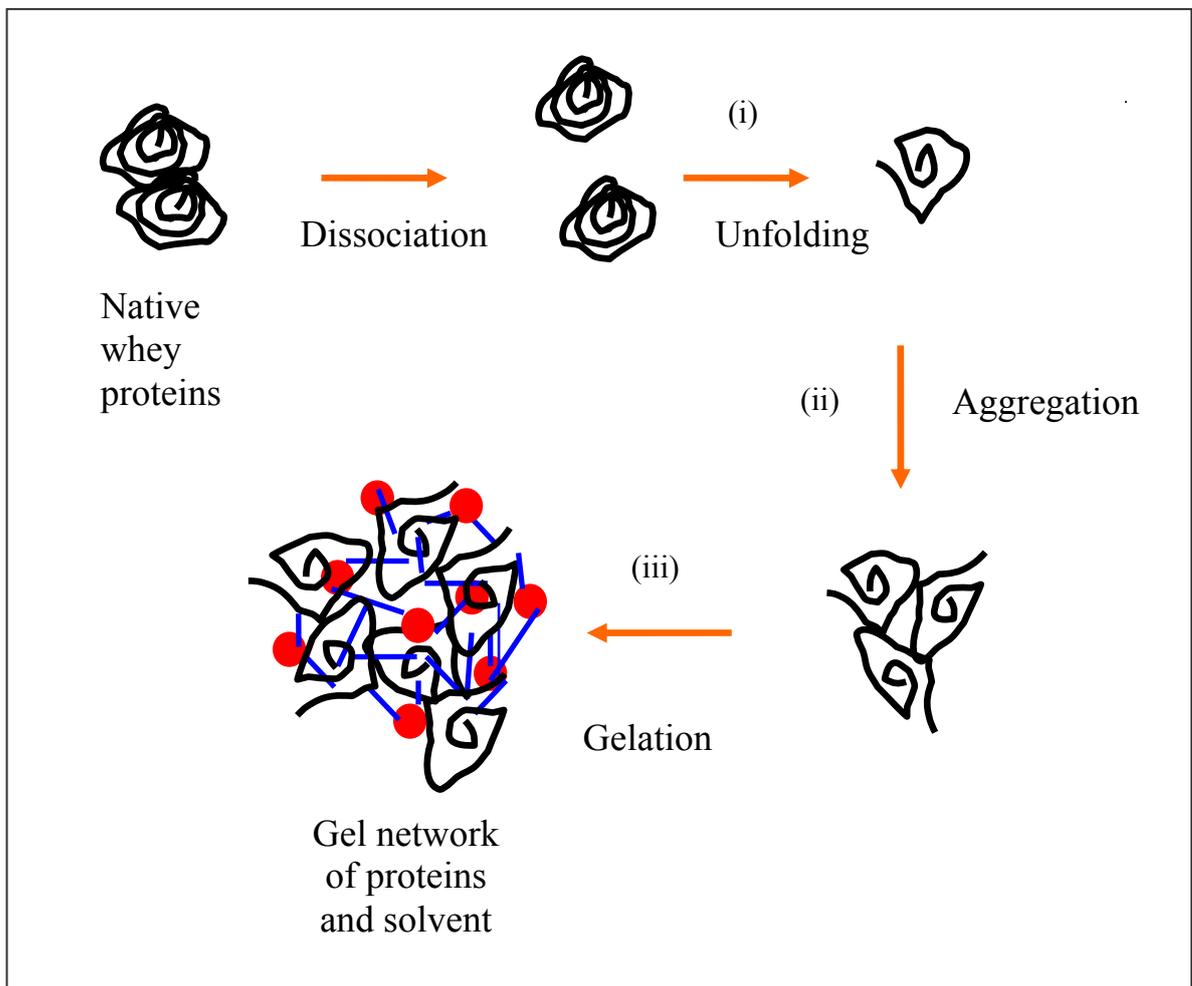


Figure 2.2 Physicochemical changes that take place during formation of viscoelastic gel network during heating of native whey proteins with simultaneous occurrence of steps (i), (ii) and (iii) (Based on Alting, 2003).

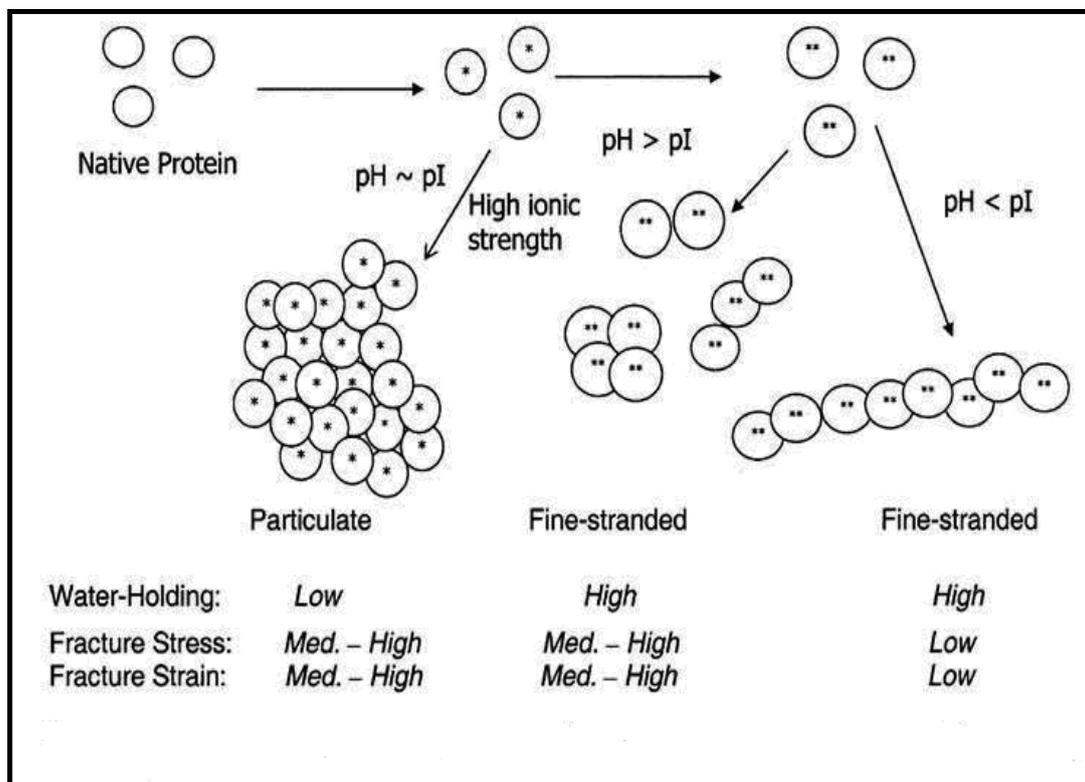
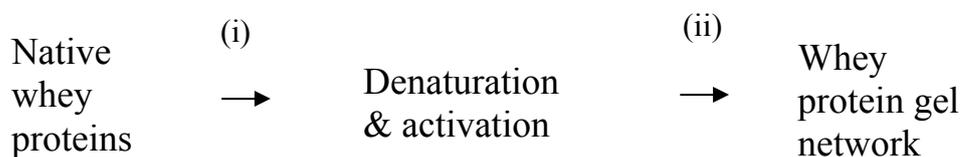


Figure 2.3 Model of globular protein gelation depicting changes from native protein to slightly unfolded (.) and more extensively unfolded (..) states, and resultant gel types. (Adapted from Foegeding, E. A., (2005), Rheology, Structure and Texture Perception in Food Protein Gels in *Food colloids: Interactions, Microstructure and Processing*).

In addition, the optimum pH for gel formation is about 7-8 for most proteins (Dickinson, 2005; Verheul et al., 1998; Damodaran et al., 2008). The translucent gels, formed primarily by hydrogen bonding, hold more water than coagulum or particulate gels and show less syneresis. In these gels, water is hydrogen bonded to carbonyl and N-H groups of the peptide bonds and water may exist as a hydrogen bonding cross linker between C=O and N-H groups of peptide segments probably restricting the flowability of water (Damodaran et al., 2008). Furthermore, a number of hydrophobic residues in the protein also determine the type of forming gel. The general trend is that the proteins with more than 31.5 mole percentage of hydrophobic residues form coagulum type gels while those with less than 31.5 mol%, usually form translucent gels in the aqueous medium (Damodaran et al., 2008).

The cold-set whey protein gelation is also useful as a novel and alternative method in formulating food products with modified functionalities (Marangoni et al., 2000). It is mostly important in preparations of heat-sensitive food products with delicate texture and flavour (Thompson et al., 2009). In comparison with heat-induced gelation, where the processes of denaturation, aggregation and gelation steps simultaneously occur, the cold gelation occurs in two separate steps as denaturation (i) and activation of proteins and gelation (ii) (Alting, 2003) as shown bellow.



Usually, the activation of protein molecules is achieved by heat-denaturation, at a protein concentration below the critical gelation concentration, and at a low ionic strength and/or far from the isoelectric point (normally at neutral pH). Consequently proteins undergo structural rearrangements, including unfolding and conversion into small soluble aggregates. Also, upon cooling, they remain soluble without gelling. Strong electrostatic repulsions between proteins and negligible salting-out effects prevent gelation of the proteins. The gelation is then accomplished at cold-set conditions by changing the solvent quality with addition of salts or adjustment of pH to screen repulsive forces or by bringing the pH towards the isoelectric point, respectively (Thompson et al, 2009; Alting et al., 2000; Marangoni et al., 2000). However, the direct addition of salt or acid does not yield a viscoelastic protein network.

The salts such as sodium chloride or calcium chloride can be added to enhance protein aggregation via screening the dispersed repulsive charges and, in the case of divalent ions, the formation of additional salt bridges between negatively charged groups on proteins (Marangoni et al., 2000). For example, when the gels are formed with CaCl_2 rather than NaCl , more rigid network is created due to ability of Ca^{2+} to more effectively screen charges than NaCl and, also, to form ion-bridges by linking negative charges on proteins (Foegeding et al., 2002). The fine stranded, transparent gels are formed by addition of relatively small amounts of salts while turbid and particulate gels are formed due to addition of relatively large amounts of salts (Alting et al., 2003).

Generally acid-induced cold-set gelation is achieved by addition of a food grade acid. Such an acidifier is for example glucono- δ -lactone (GDL) which is hydrolysed to gluconic acid upon addition to water causing a gradual lowering of pH in the medium resulting in gelling at minimum repulsion. Addition of a large amount of GDL results in quick acidification of the medium with the probability of lowering the pH value below the isoelectric point which may cause formation of weaker and brittle gels. On the other hand, the acid-induced gels, formed around the isoelectric point or kept around this pH for extended time periods,

contain larger aggregates and are likely to be stronger and more deformable (Cavallieri and Cunha, 2008). In addition, the amount of GDL required to achieve a certain pH value depends on the protein concentration (Alting et al., 2002).

2.5.1 A general description of proteins and their physicochemical characteristics

The basic structural unit of proteins is an amino acid which contains an α -carbon atom covalently attached to a hydrogen atom, an amino group, a carboxyl group and a side chain, R group. Proteins inherit acidic and basic properties due to acidic carboxyl groups and basic amino groups; as a result, at neutral pH, they act as ampholytes. In addition, physicochemical properties, the extent of hydrogen bonding and the reactivity of proteins are mostly determined by the different R groups attached to the amino acids (Petsko and Ringe, 2004).

The primary structure of protein is the linear amino acid sequence that is covalently linked through peptide bonds and one terminus of that series with the free α -amino group is known as the N-terminal and the other with the free α -COOH group is recognized as the C-terminal (Petsko and Ringe, 2004). Furthermore, the delocalization of electrons in CO-NH group creates a resonance structure which prevents protonation of N-H group, restricts rotational freedom of CO-NH bond that is further restricted by the steric hindrance of R groups of proteins, and consequently influences the backbone flexibility. This partial double bond nature also creates a partial negative charge on carbonyl oxygen atom and a partial positive charge on the H atom of the N-H group facilitating hydrogen bonds between the C=O and N-H groups of peptide backbone (Damodaran et al., 2008; Nolting, 2006). The secondary structure of proteins presents highly organized sub-structures of polypeptide chains generally existing as helical and extended sheet-like structures mostly stabilized by hydrogen bonds. The tertiary structure of protein is the three dimensional spatial arrangement of secondary

structures of single protein molecule which is folded or compacted to achieve a minimum possible level of free energy via involvement of all types of molecular interactions. For example, globular proteins are folded in such a way to bury most of the hydrophobic residues in the interior of the protein structure and away from the water environment with most of the hydrophilic residues at the protein-water interface (Damodaran et al., 2008; Nolting, 2006). However, this relocation of hydrophobic and hydrophilic residues is frequently done partially due to steric effect and the ratio of polar and apolar groups on the surface is extremely important for the physicochemical properties of the protein. It has been recognized that globular proteins generally contain a large number of hydrophobic residues and that nature is more prominent in larger globular proteins than the smaller molecules (Damodaran et al., 2008). Apart from one tertiary structure, there may be a few individual tertiary structures, found in a polypeptide chain, which are referred to as the protein domains (Damodaran et al., 2008). The quaternary structure of proteins is formed when more than one polypeptide chain are spatially arranged to a protein complex. The quaternary structures are generally called oligomers and may be homogeneous or heterogeneous dimers, trimers, tetramers or larger forms mainly created by non-covalent interactions. The proteins with more than 30% of hydrophobic groups have a greater potential to form oligomeric structures to minimize the free energy since they cannot bury all the hydrophobic residues inside their core. For example, β -Lg which contains 32 mol% of hydrophobic amino acids and exists as a monomer at pH less than 3.5 and greater than pH 7.5, a dimer between pH 5 and 7.5 and as an octomer between pH 3 and 5 (Damodaran et al., 2008; Fox and McSweeney, 2003).

There are different attractive and repulsive molecular forces involved in the stability of unique three-dimensional protein structure (Damodaran et al., 2008). They can be intrinsic van der Waals and steric forces as well as electrostatic, hydrogen bonding and hydrophobic interactions that arise with the influence of surrounding environment. Electrostatic interactions significantly affect most long range interactions of proteins and proteins with other charged molecules. van der

Waals forces are important as main contributors to the stabilization of globular proteins, followed by hydrogen bonds and thirdly, hydrophobic interactions of non-polar residues (Nolting, 2006). In order to have a stable folded protein conformation all molecular interactions have to overcompensate the destabilizing contributions from hydration of polar residues and the gain in configurational entropy upon unfolding (Nolting, 2006). Although the polypeptide chain is folded to be energetically stabilized, it always avoids the deformation of bond lengths and bond angles. The contribution of the sum of van der Waals interactions, hydrogen bonds formed between the reactive groups of the peptide chain such as, peptide groups, carboxyl groups and side chain phenolic, amide, histidine, and covalent disulfide bonds are significant to the stability of protein folding. But the hydrophobic interactions among apolar groups are the major driving force in protein folding (Damodaran et al., 2008; Fox and McSweeney, 2003).

2.5.2 Protein denaturation

The native structure of proteins is the thermodynamically most stable conformation which is formed under precise physiological conditions and presents a net product of different intra- and inter-molecular attractive and repulsive forces (Damodaran et al., 2008; Shirley, 1995). However, different environmental changes such as heat, pressure, chemicals etc. can affect the protein native structure. The major changes in the secondary, tertiary or quaternary protein structures without breaking the backbone peptide bonds are known as protein denaturation. In the case of globular proteins, the denaturation leads to consequent protein aggregation affecting their solubility as well as all other functional properties (Damodaran et al., 2008; Anema and McKenna, 1996). Usually, the degree of denaturation is established considering the proportion of protein concentration soluble at pH 7, however, it depends on other environmental factors as well (Onwulata et al., 2006).

2.6 Protein denaturation kinetics and environmental effects in whey protein aggregation

Although, the application of WP in food manufacturing is highly desirable, the major obstacle regarding the whey protein functionality and the consequent applications is their heat-induced destabilization. The industrially employed inevitable heat treatments applied during whey protein concentration, spray drying and processing of some food products containing WP in order to attain adequate safety and shelf life, change the native state of these globular proteins and affect their stability by denaturation, aggregation and flocculation resulting in phase separation, destabilization of emulsions or protein precipitation (Pearce and Kinsella, 1978; Patel and Kilara, 1990). Therefore, it is rather useful to obtain more descriptive information of the kinetics and the mechanisms of whey protein unfolding and aggregation to optimize the heat treatments and thereby extract the maximum functional, nutritional and sensory values of whey protein products.

WP show thermal transition in the temperature range of 62 – 78°C. The heat stability of major whey proteins has been recognized as in the order: α -La < BSA < immunoglobulins < β -Lg. However, denaturation of α -La appears reversible (Fox and McSweeney, 2003). The thermal transition temperature of native α -La normally occurs at < 66°C while that of β -Lg is ~ 73°C. The conformational changes of BSA are reversible between 42 and 50°C, but unfolding of the alpha helices of BSA is irreversible between 52 and 60°C (Considine et al., 2007). At natural conditions, the intramolecular disulfide bonds between Cys residues stabilize the tertiary structure of globular whey proteins along with other attractive and repulsive molecular interactions (Monahan et al., 1993; Paulsson and Dejmek, 1989). When subjected to heating, whey proteins get unfolded and consequently aggregate with the formation of different intermediate aggregates before a gel network is created (Havea et al., 2002). Sometimes, when heating, proteins acquire intermediate states between the native and unfolded conformation. An example for such an intermediate conformation is the molten globule state. The

'molten globule' state has been suggested to be unique intermediate state with a partially folded conformation, having some similarity to, yet being distinct from, both the native and fully unfolded state (Fox and McSweeney, 2003). α -La has a well characterized molten globule structure (de la Fuente et al., 2002). The protein aggregation is mainly a result of different molecular interactions such as hydrophobic via newly exposed apolar groups, electrostatic, disulfide and hydrogen bonding and van der Waals forces among proteins (Lee et al., 1992; Damodaran et al., 2008). The rates and pathways of these physicochemical reactions are determined by the factors as previously described elsewhere, temperature and heating time, the protein concentration, α -La/ β -Lg ratio, pH, ionic strength and solvent condition and the influence of other surrounding molecules (Brandenberg et al., 1992; Iordache and Jelen, 2003; Marangoni et al., 2000; Rabiey and Britten, 2009).

The unfolding of globular whey proteins is an endothermic heat process and can be monitored by differential scanning calorimetry (DSC) since it directly provides the enthalpy changes and temperature data associated with the unfolding transition (Paulsson and Dejmek, 1990). The endothermic peak temperature is the transition midpoint where the concentration of native and denatured state is equal and it is commonly considered as the denaturation temperature (T_d). The denaturation temperatures of proteins are mainly affected by the stability of non-covalent interactions. For example, hydrogen bonding and electrostatic interactions are exothermic in nature while hydrophobic interactions are endothermic. In addition to molecular forces, the conformational entropy, which increases as the temperature increases and thus prefers the unfolded state, also plays an important role in determining the net effect of protein denaturation (Damodaran et al., 2008). However, in an aqueous medium with a high dielectric constant, electrostatic and hydrogen bonds are not that significant in heat-denaturation of whey proteins with the majority of charged groups existing on the surface. Instead, hydrophobic interactions which favour the folded state and

conformational entropy, which favour the unfolded state, ultimately determine the denaturation temperature of proteins (Damodaran et al., 2008).

As far as aggregation of β -Lg is concerned at neutral pH, the conformational changes of the tertiary structure of β -Lg in aqueous solution vary reversibly at temperatures $< \sim 65^\circ\text{C}$ (Considine et al., 2007). However, with sufficient enough heating (at 65°C), the process of unfolding, exposure and activation of free sulfhydryl groups and the formation of related intermediate aggregates takes place due to initiation of inter- and intra-molecular thiol/disulfide exchange reactions or sulfhydryl oxidation (de la Fuente et al., 2002). Normally, this process is much more evident at relatively low temperatures (de la Fuente et al., 2002) than at higher temperatures and simultaneously, at low temperatures, the non-covalent interactions play a minor role (Considine et al., 2007). The pattern of initiation, propagation and termination of sulfhydryl/disulfide exchange chain reactions somewhat resemble the polymer radical chemistry reactions (Roefs and de Kruif, 1994).

It starts with dissociation of a β -Lg dimer with increase of thermal energy. The protein molecule is thus activated and the helix is first lifted away from the β -sheets and, as a result, a side chain can interact with nearby disulfide bond within the limits of the hydrophobic core. The exposure of previously buried hydrophobic groups and the free Cys₁₂₁ initiate aggregation via thiol-disulfide exchange which leads to further aggregation through hydrophobic associations. During this process the sulfhydryl group of Cys₁₂₁ first reacts with the disulfide bond between Cys₁₀₆–Cys₁₁₉ and forms a new disulfide bond of Cys₁₂₁–Cys₁₀₆, and a free Cys₁₁₉. The free Cys₁₁₉ then reacts with another β -Lg disulfide bond of Cys₆₆–Cys₁₆₀ resulting in a new disulfide bond between Cys₁₁₉ - Cys₆₆ and releasing a free Cys₁₆₀. The resulting Cys₁₆₀ residue position is close to the C-terminus of the β -Lg molecule, and therefore, available to interact with disulfide bonds of other protein molecules if present, with the potential of forming homogeneous or heterogeneous aggregates (Considine et al., 2007; Patel et al., 2006; Manderson et al., 1998). It has been recognized that approximately 35% of

Cys₁₆₀-Cys₆₆ disulfide bond and the free thiol in the native protein is present after the heat treatment. Also, Cys₆₆ and Cys₁₆₀ are hardly involved in the formation of disulfide bonded dimer of β -Lg molecules through a disulfide bond interchange reaction (Considine et al., 2007). In addition, ionic interactions, hydrogen bonding and calcium bridges are also equally important during protein aggregation (Brandenberg et al., 1992; Patel and Kilara, 1990; Fetahagic et al., 2002). The disulfide bonded heat induced gels have a rubbery nature which is indicated by high fracture strain (Havea, et al., 2009).

The behaviour of an individual protein during unfolding and aggregation is different when there are other proteins present in the medium. For instance, α -La has the least effect on heat-induced aggregation and sulfhydryl/disulfide interchange apparently due to absence of free thiol group, but it disappears rather more rapidly than β -Lg in whey protein mixtures (Zue and Damodaran, 1994). In the presence of either β -Lg or BSA which contain free thiol groups, α -La aggregates readily forming a mixture of homopolymer as well as heteropolymers of both proteins (Havea et al., 2001). The denatured α -La reacts with denatured β -Lg very efficiently and the rate of aggregation of α -La is higher an order of magnitude in the presence of β -Lg (Considine et al., 2007). However, in a heated whey protein mixture, the aggregation of α -La is more effectively catalysed by BSA via the activated thiol group than β -Lg since BSA starts unfolding and aggregating before β -Lg. Furthermore BSA and α -La denature at almost similar temperatures with BSA start to aggregate at a lower temperature than β -Lg. The aggregation rate of β -Lg is several times higher in the presence of BSA than alone. On the other hand, BSA appears unaffected in the presence of β -Lg (de la Fuente et al., 2002; Considine et al., 2007).

The influence of non-covalent interactions on the whey protein aggregation is highly dependent on the temperature. These interactions are especially important at higher temperatures, such as greater than 90°C (Galani and Apenten, 1999) with little contribution to aggregation at temperatures below 75°C (de la Fuente et al., 2002). In addition, the formation of non-covalent interactions decreases the

ability to form inter-molecular disulfide bonds to a certain extent since it limits the mobility of denatured molecules and thus reduces the probability of meeting each other (Havea et al., 2009). In the heated α -La/ β -Lg mixtures, α -La provides a greater contribution to non-covalent aggregation than β -Lg (de la Fuente et al., 2002). The degree of non-covalent interactions is directly proportional to the brittleness and stiffness of whey protein gels (Havea et al., 2004).

2.6.1 Effect of temperature and holding time on the extent of whey protein aggregation

The temperature and holding time play a vital role in whey protein aggregation. At lower temperatures ($\sim 67.5 - \sim 75^\circ\text{C}$), the rate governing step is unfolding of whey protein molecules, whereas, at higher temperatures ($\sim 75 - \sim 90^\circ\text{C}$) the aggregation process is rate limiting (De Wit, 1990; Considine et al., 2007). The well-ordered, stronger whey protein gels are produced at slower heating rates, however, the protein type, concentration and other conditions still play an important role in determining the overall gel characteristics (Resch et al., 2005). The size of aggregates depends on the heating temperature (Xiong et al., 1993) and at higher temperatures proteins dissociate and unfold fast with the rapid formation of reactive monomers. These activated monomers facilitate termination reactions resulting in relatively smaller aggregates. Also, firmer gels are created with increasing temperatures (Resch et al., 2005). At lower temperatures, the formation of small oligomers between trimers and pentamers is recognized as an important step in aggregation of β -Lg as these species can act as nuclei for further aggregation, hence leading to formation of relatively larger aggregates (de la Fuente et al., 2002).

2.6.2 Effect of protein concentration on whey protein aggregation

Protein concentration also has a significant effect on heat induced unfolding and aggregation of WP. While the protein unfolding appeared unaffected by protein concentration, the aggregation step appears to be highly dependent on the concentration, particularly, the monomer forms. At low protein concentrations, such as 3, 4 or 6 % (w/w) and low ionic strength, the denaturation step is slower than aggregation step (Fitzsimons, et al., 2007). By simultaneous increase of both temperature and protein concentration, the creation of multimeric species is facilitated. In addition, high protein concentrations produce higher molecular weight aggregates (de la Fuente et al., 2002).

2.6.3 Effect of pH on whey protein aggregation

Thermal stability of WP is highly affected by the pH since the kinetics of unfolding and aggregation extensively depend on electrostatic effects. At neutral pH, WP carry a net negative charge (Fox and McSweeney, 2003). Similarly to all other proteins, they are also more stable against denaturation at their isoelectric pH than at any other pH (Damodaran et al., 2008) due to minimum intra-molecular repulsions. However, at high ionic strength and low heating temperatures, the unfolding step would be a rate limiting around isoelectric point (de la Fuente et al., 2002). When pH is raised away from the isoelectric point, WP unfold more extensively due to increased electrostatic repulsion and reactivity of thiol groups (Foegeding et al., 2002) with subsequent aggregation to maintain the thermodynamic stability. At extreme pH values, the intra-molecular electrostatic repulsions result in swelling and unfolding of the protein molecule and the extent of unfolding is generally higher at extreme alkaline pH values than under extreme acidity (Damodaran et al, 2008). β -Lg undergoes a conformational change between pH 7 and 8.5, including refolding of the protein chains collectively known as Tanford transition with higher reactivity of the thiol groups (de la

Fuente et al., 2002). The reactivity and availability of the thiol groups greatly depend on the pH. For instance, at pH 8, the thiol group is readily available for further reactions and, also, in the pH range between 6.4 and 8, intermolecular disulfide bonds play an important role in protein aggregation. Moreover, the denaturation temperature of β -Lg decreases as pH is increased from 5.5 to 6.5 at low ionic strength. When pH is increased from 6 to 7.5 in the presence of 0.5 M NaCl, the rate of protein aggregation increases with simultaneous increase of G' and reduced permeability of resulting gels (Xiong et al., 1993; Foegeding et al., 2002). Furthermore, at pH 6, where non-covalent interactions are prominently involved in whey protein aggregation, the thiol/disulfide exchange reactions appear to play a part in the process. However, at very acidic pH, WP contain an overall positive charge and thiol/disulphide interchange reactions are unlikely to take place because of the impaired reactivity of the thiol groups. Under these conditions, although, unfolding readily occurs, the electrostatic interactions between monomers are totally repulsive. However the aggregation can be promoted by addition of salts to screen these charges. When whey proteins are heated at very acidic pH, large and elongated fibrillar aggregates are formed, yet, depending on the ionic strength (de la Fuente et al., 2002).

Besides pH, the thermal stability of WP is also affected by the type of acidulant. Therefore, the selection of an acidulant is important in controlling the nature of aggregation. Different acidulants, such as, hydrochloric, lactic, citric and phosphoric affect the denaturation temperature of whey proteins and have ability to produce whey protein gels with different gel characteristics (Resch et al., 2005). In addition, the calcium chelating substances may enhance the heat stability of whey proteins (de Rham and Chanton, 1984), such as, in this case, citric and phosphoric acids.

2.6.4 Effect of ionic environment on whey protein aggregation

The ionic strength and the type of ions show significant effects on the heat-induced aggregation of the WP; however, these are quite opposite effects. The increased ionic strength increases the denaturation temperature due to reduction of intra-molecular repulsions, which reduces the unfolding rate. Simultaneously, the reduction of inter-molecular repulsions facilitates the rate of aggregation (de la Fuente et al., 2002). Salts at low ionic strengths interact with proteins by non-specific electrostatic interactions which usually stabilizes protein structure. At ionic strength less than 200 mM, this effect is independent of the type of salt. However, at higher concentrations (> 1 M), salts have ion specific effects that have an effect on the structural stability of proteins. At equal ionic strength, cations increase the denaturation temperature of whey proteins greater than anions. The relative ability of various anions at equal strength to influence the structural stability of protein normally follows a series, known as, chaotropic or Hofmeister series (Damodaran et al., 2008). Additionally, the effect of salts on the structural stability of proteins depends on their ability to bind to and change hydration properties of proteins. Salts that enhance hydration of proteins and bind to proteins weakly stabilize proteins while the salts that reduce protein hydration and bind strongly destabilize proteins. The denaturing effect of salts might also be linked with destabilization of hydrophobic interactions in proteins (Damodaran et al., 2008). The divalent cations influence the protein aggregation via electrostatic shielding, ion-specific hydrophobic interactions and formation of protein-calcium-protein bridges by cross-linking adjacent anionic molecules (Havea et al., 2002). The monovalent cations affect the aggregation by reducing the extent of divalent cation bridge formation between molecules in addition to masking the electrostatic charges on proteins (Havea et al., 2002). Also, the particle size of aggregates increases as a function of increasing salt concentration (Marangoni et al., 2000). Moreover, sugars influence the thermostability of globular proteins by increasing the denaturation temperature. For example, sucrose increases the gelation temperature by thermal stabilization of the native state of the proteins

(Baier and McClements, 2001). Infact, the gelling ability of proteins decreases in the presence of sucrose at low concentrations. This may be due to an increase in continuous phase viscosity, which decreases the frequency of collision of protein molecules. As a result, the intermolecular interactions of proteins are weakened. This influence of sugar molecules occurs apparently due to prevention of the formation of non-covalent bonds rather than disulfide bonds. At high sucrose concentrations (> 10 wt%), on the other hand, the protein-protein interactions are thermodynamically preferred over protein-solvent interactions (Foegeding et al., 2002). Also, sugars protect proteins from unfolding and subsequent aggregation under pressure leading to preferential hydration of the protein (He et al., 2006).

2.6.5 Other factors affecting denaturation and aggregation of whey proteins

In the process of protein denaturation in aqueous medium, the hydration and partial penetration of water into surface cavities of proteins lead to swelling of proteins which facilitate the protein flexibility and mobility. During heating, the water molecules easily gain access to salt bridges and peptide hydrogen bonds in this more fragile structure resulting in decreasing the denaturation temperature of proteins (Fujitha and Noda, 1981). In addition, the hydration of hydrophobic residues after their exposure to water results in the decrease of partial molar volume of a protein and it affects the thermodynamic stability of a protein and thus becomes a key factor in determining protein unfolding and stability (Considine et al., 2007).

Even little conformational differences present in genetic variants of whey protein species change their physico-chemical characteristics and, as a result, they show different levels of susceptibility to heat denaturation. At pH 6.7, β -Lg A, B, and C variants have their thermostabilities in the order $C > A > B$ (de la Fuente et al., 2002). Different substitutions in amino acid residues in these variants change their hydrophobicity, charge and steric properties affecting thiol reactivity,

oligomerization, solubility and protein flexibility even to small extents which may finally influence the heat sensitivity. However, this fact again depends on protein concentration, such as that the aggregation of B variant is faster than that of A at the concentrations below 5% proteins; nevertheless, the thermal sensitivity of A becomes greater than B when the protein concentration is increased above 5%.

However, the inevitable result of protein aggregation is the loss of solubility, and consequently, protein availability and impaired functionality (Considine et al., 2007; de la Fuente et al., 2002; Lee et al., 1992). Therefore it is a critical requirement for the industry to discover novel methods to improve the functionality to avoid heat-induced undesirable effects and thereby expand the application of whey proteins over a wider range of food products.

2.7 Modification of whey protein functionality

Several attempts have been already taken to modify whey proteins and thereby improve their functionality. In reality, for the successful utilization of functional properties of whey proteins in different food systems, they should be recovered in their native, undenatured state and subsequently incorporated in heterogeneous food matrixes. Although chemical and enzymatic modifications of whey proteins are widely applied, they have certain limitations (Panyam and Kilara, 1996; Spellman et al., 2005). The application of ultrasound for short duration of time to breakdown protein aggregates formed due to preheating treatments, and to prevent reformation of aggregates and the consequent viscosity increase are also in current interest of research (Ashokkumar et al., 2009). In addition, the use of mechanical forces under isothermal and/or isobaric conditions has a significant potential to modify whey proteins (Manski et. al., 2007; Iordache and Jelen, 2003). One of such popular approaches is extrusion, where proteins are thermally denatured under high pressure conditions technically known as thermoplastic melt. The denatured proteins become fibre form as a result of the process and sudden release

of pressure evaporates water and expands the product. The extent of expansion can be controlled by adjusting the pressure and temperature (Damodaran et al., 2008). This method has been employed for years and used to produce low-moisture food products such as snacks and breakfast cereals. Modern developed extruders are used for the protein texturisation at relatively high pressures and temperatures and at moderate mechanical shear rates resulting in reversible and non-reversible effects to proteins which ultimately have an effect on the functionality. Although, extrusion helps to increase the nutritional value of food products, the structural collapse and poor expansion are the main drawbacks (Onwulata and Tomasula, 2004). The high hydrostatic pressure is an alternative method to process dairy products without the adverse effects of thermal denaturation (Patel et al., 2005). However, the static high pressure induced changes of proteins in bovine milk are still not fully exploited and it is also in the experimental level (Lopez-Fandino, 2006; Huppertz, et al., 2006).

2.7.1 Microparticulation of whey proteins

Microparticulation of WP is a robust technique that can be applied to produce novel ingredients with modulated functionalities. In this process, WP are subjected to dynamic high pressure shearing which can be achieved for example by microfluidization with or without heating. The protein based microparticulated fat replacers such as Simplese® (NutraSweet Co., Deerfield, IL) and Dairy-Lo® (Pfizer Inc., New York, NY) are also produced with the use of heat and high shear (Akoh, 1998). The microparticulated WP behave like fats, for example, they considerably improve texture and taste in low-fat food products. Therefore, this technique is used to improve most cheese types, milk desserts and yoghurts, dressings and sauces and also other fat containing food products. If simultaneous heat and shear are applied, the characteristics of the resulting aggregates depend on the shear controlled aggregate growth and shear induced aggregate break-up as well (Spiegel, 1999). Extending the value of this technique, the microfluidization

has been used to improve the solubility of denatured whey protein isolates as described by Iordache and Jelen (2003). This study focused on sedimentation behaviour of heat denatured microfluidized WP. It revealed that microfluidization disrupted the heat-induced aggregates and produced non-sedimenting WP particles. This effect was obvious when the heat denaturation was carried out at pH 3.8 and thus produced micro-particles showed complete resistance to sedimentation. Also, as Paquin et al., (1993) previously reported, microparticulation of denatured WP at pH ~ 6.7 has reduced > 80% of the particle size of aggregates below 10 μm .

2.7.2 Microfluidization

The microfluidizer (MicrofluidicsTM, Newton, Massachusetts, USA) is a special fluid processor for ultra high pressure mixing, homogenizing, uniform particle/droplet sized reduction, cell disruption and creation of nanoparticles (Iordache and Jelen, 2003). This innovative technique, the microfluidization, can be used in many applications of food/nutraceutical, pharmaceutical, biotechnology, chemical, cosmetic and energy to research, develop and improve products efficiently. The principle of operation of the microfluidizer is based on pumping a liquid product at constant high pressure of up to 275 MPa with subsequent division of the main flow into 2 smaller streams, which are then forced to collide against each other within an interaction chamber. When the product travels along the walls of micro channels of the microfluidizer, shear forces are applied to it at high velocity with an impact against the walls of the interaction chamber and the two streams colliding. In addition, the cavitations in the streams can occur by bubble forming and collapsing when they pass the different pressure zones within the interaction chamber (Barnadas-Rodriguez and Sabes, 2001).

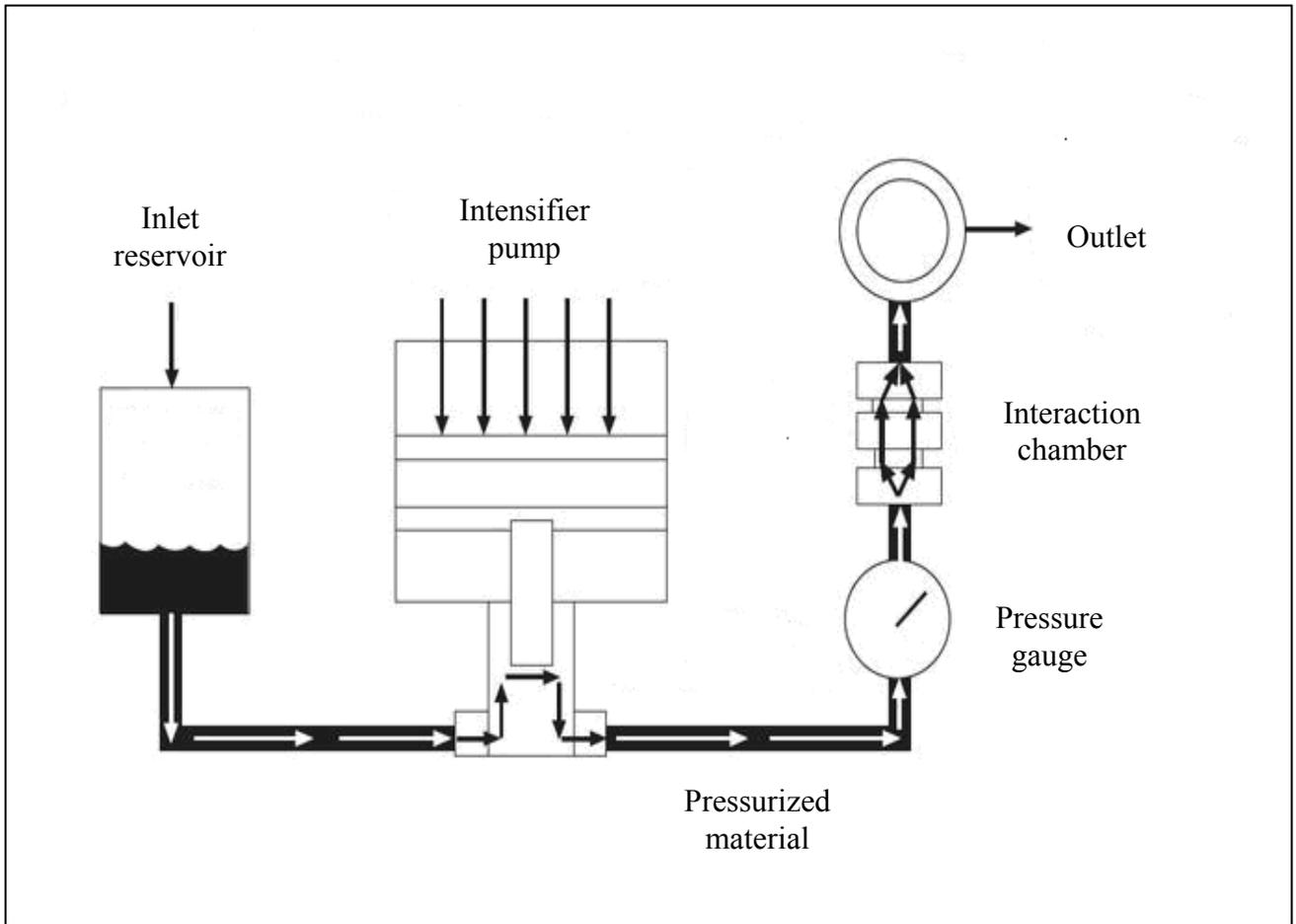


Figure 2.4 Principle of operation of a microfluidizer (Adapted from: www.equilabo.com/MICROFLUIDICS_Presentation.html and modified).

The efficiency of process primarily depends on the extent of pressure and the number of microfluidizing passes (Iordache and Jelen, 2003). The generation of heat during the processing can be minimized by efficient cooling with an iced water jacket.

Microfluidization has become known as a very effective alternative method of reducing size of fat globules compared to conventional homogenization. As an conventional homogenization method, approximately 15 MPa are used in dynamic high pressure treatments in the production of stable emulsions in dairy and other food industries (Iordache and Jelen, 2003). The nanostructure of Mozzarella cheese could be altered using microfluidization and the temperature and pressure affected the extent of modifications (McCrae, 1994). Also, compared to sonication, the microfluidization was used to produce nano-emulsions with narrower size distributions (Jafari, et al., 2006). In addition, microfluidization creates fine emulsions, which would be advantageous in ice cream like dairy desserts. As reported by Olson et al., (2003), microfluidization produced non-fat and low-fat ice-cream that had a slower meltdown without affecting sensory properties.

2.7.3 Effect of dynamic high pressure shearing on whey proteins

The application of high pressure shear on globular WP has shown particularly different physico-chemical properties from those of native proteins. During this process, the collision, compression, shearing and flowing may take place with the contraction and more importantly stretching and elongation of protein molecules leading to the conformational rearrangements. In addition, the cavitation, turbulence and temperature rise in the medium are also possible as a result of forced induced occurrences. Also, the impact time of dynamic high pressure is very short when compared to that of static high pressure (Bouaouina, et al., 2006). As a result of mechanical forces, the quaternary and tertiary structures of proteins

are more susceptible to be disrupted compared to their secondary conformation owing to the perturbation of comparatively weaker inter- and intra-molecular hydrophobic and electrostatic interactions, however, depending on the extent of applied pressure and number of microfluidizing passes, β -sheets and α -helixes are also prone to be affected via the disruption of intra-molecular hydrogen bonds (Bouaouina, et al., 2006). Although, it is impossible to rupture the already existing covalent disulfide bonds in high pressure shearing, it may enhance the formation of new covalent bonds via exposed and newly formed reactive sites. Eventually, all of these high pressure induced conformational changes may be reflected as the altered or modified functionality of the globular WP.

2.8 Shearing of whey proteins

Gaining an understanding of the behaviour of protein rich food products during processing is quite advantageous in food processing and formulation to support the increasing current interest in employing high concentration of proteins in novel foods.

The mechanical shear forces which can be achieved by shear induced mixing are known to be used to obtain alternations in the structural arrangements and solution morphology in most of biopolymer systems such as protein gels (Manski et al., 2007). The preparation procedures greatly influence the characteristics of physical gels. For example, particulate gels, which are capable of sustaining mechanical deformation at small enough strain or stresses are formed, when applying high enough shear rates (Altmann, et al., 2004). Furthermore, irreversible denaturation of proteins with the formation of spherical macro-colloidal particles is known to occur when it is combined with the high temperature and high shear (Damodaran et al., 2008). The combined effect may alter the conformational structure of whey proteins via partial denaturation of the protein, thus exposing groups that are

normally hidden in the native protein facilitating aggregation (Onwulata et al., 2006).

During the application of shear in protein gelation, the enhanced fluctuations of the system result in perturbation of intra-and inter-molecular associations which on the other hand is reflected by the changes in solution morphology expressed as storage modulus. In addition, the shear induced molecular changes and the subsequent interactions resulting in certain products may be different from their interactions and the resulting associations under temperature variations alone, since shear forces may promote or enhance the occurrence of different associations following alternative pathways (Altmann, et al., 2004). Also, different extent of shearing may produce sheared gels with distinct gel characteristics. Generally, the homogenization of a phase separating system due to shear occurs when shear rate exceeds the rate of relaxation fluctuations of the system. On the other hand, shear induced phase separation is also a possibility (Altmann, et al., 2004). This type of phase separation is basically a result of enhanced concentration differences arising due to different imposed stress levels in different regions in the sheared system (Saito et al., 1999). In general, proteins are affected by high shear processes like extrusion and mixing; however, small globular proteins (< 40 kDa) in dilute aqueous solutions appeared not affected by shear rates up to 10^5 s^{-1} (Manski et al., 2007). At higher concentrations (4 and 5.2 wt %), the effect of shear flow became more pronounced (Akkermans et al., 2008).

In addition, the globular proteins such as β -Lg show the ability to form long, thin fibrillar aggregates, when they are formed by heating at pH 2 and low ionic strength. This phenomenon is another useful approach when consider the potential use of whey proteins as functional ingredients in food products since the fibrils can give food product with a specific structure. The formation of whey protein fibrils can be achieved by prolong heating of aqueous whey protein solutions at pH 2 and low ionic strength at temperatures exceeding the denaturation temperature of the protein and the shear flow enhances fibril formation in heat

denatured β -Lg samples (Bolder et al., 2007). The fibril growth of proteins is generally associated with different periods, a lag time, a nucleation process and then the growth. The addition of already formed fibrils or seeding, continuous mixing, short shear pulses or sonication may affect the kinetics of the fibril formation. The viscosity measurements can be used to describe the nature of fibril solutions (Akkermans, et al., 2008). As exposed by X-ray fibre diffraction pattern, a β -Lg fibril is a polymeric assembly of protein molecules, based on the cross- β structural pattern held together by hydrogen bonding between the atoms of the β -strands, in which intermolecular β -sheets extend over the length of the fibril such that each β -strand within the β -sheets runs perpendicular to the fibril axis. The bonds between the proteins in the fibrils are initially weak, and become stronger with maturity (Akkermans, et al., 2008).

CHAPTER 3

3 Composition and functionality of commercial whey protein concentrates available on the Australian market

3.1 Introduction

The residual fluid of cheese and casein manufacture, generally known as whey, contains nutritionally and functionally valuable proteins such as β -lactoglobulin, α -lactalbumin, bovine serum albumin and immunoglobulins (Fitzsimons et al., 2007). These whey proteins do not precipitate at pH 4.6 at which the caseins precipitate (Fox and McSweeney, 2003) and can be concentrated by different physico-chemical methods. WP are available as concentrates with the protein content ranging from approximately 30 – 85% and isolates, containing more than 90% protein (Fox and McSweeney, 2003). Incorporation of these products in different food systems as functional ingredients by the food industry has become the best means for utilization of whey proteins (Morr and Foegeding, 1990).

The application of WPCs food ingredients is sometimes limited due to their inherent compositional variability and manufacturing history (Morr and Foegeding, 1990; Morr and Ha, 1993; Brandenberg et al., 1992). This causes significant alterations to their physicochemical and functional properties even though they may be manufactured using similar processing conditions (Patel et al., 1990; De Wit, 1990; Mangino et al., 1987). Heat treatment, membrane fractionation techniques, spray drying and other processing steps used in manufacturing as well as subsequent storage greatly influence the physico-chemical state of WPC proteins (Jovanovic et al., 2005; Morr et al., 1985). Variables such as breed of dairy cattle and the composition of the herd, cheese production method and the type of cheese manufactured also produces differences in WP functionality from manufacturer to manufacturer and from batch to batch (Morr and Ha, 1993; Onwulata et al., 2004). In addition, as reported by Regester and Smithers, 1990, seasonal variation in the relative proportions of the major

protein constituents of whey protein concentrate has important implications for the dairy industry. Seasonal changes generally included a reduction in the α -lactalbumin content of whey protein concentrate manufactured during the final three months of lactation, concomitant with a rise in the level of β -lactoglobulin.

As a result of such variations in different commercial WPCs the food industry is experiencing several fundamental problems such as inconsistency in product quality and complex behaviour of WP during manufacture of whey protein containing food products (Morr and Ha, 1993; Onwulata et al., 2004; De Wit, 1990). Therefore, it would be useful to be able to predict the functional behaviour of commercial WPCs based upon knowledge of their compositions. This would facilitate solutions to reduce inherent variation and improve optimisation during product development and formulation. The aim of this study was to examine the relationships between composition and functional properties of WPCs commercially available on the Australian market containing at least 80% proteins (WPC₈₀).

3.2 Materials and methods

3.2.1 Materials and proximate composition

The study was carried out using 8 different WPC₈₀ powders, coded as samples A to H, kindly provided by Australian and New Zealand manufacturers. The proximate analysis of WPC was conducted following AOAC methodology. Well established Kjeldahl method and a nitrogen conversion factor of 6.38 (method: 968.06, AOAC, 2000) were applied in protein determination; moisture content was determined by oven drying 1.5 g sample at 105°C till a constant weight was gain (method: 925.10, AOAC, 2000); ash content was estimated by combusting pre-solidified samples in a muffle furnace at 550°C (method: 923.03, AOAC, 2000); fat content was determined using Mojonnier method, (989.05 (modified), AOAC, 2000); calcium content was determined by atomic absorption

spectrophotometry, (method: 985.33, AOAC, 2000). Phosphorus content was determined colourimetrically using vanadomolybdophosphoric acid (Gales et al., 1966).

3.2.2 Particle size distribution

The particle size distribution pattern of WPC powders was determined by a dynamic light scattering instrument (Zetasizer-Nano ZS, Malvern instruments, Worcestershire, UK) equipped with Dispersion Technology software (version 5, Malvern instruments, Worcestershire, UK). Approximately 1% (w/w) dispersions of WPC powders were prepared and placed overnight at 4°C. The pH of the hydrated protein dispersions were corrected to 7 using 1M NaOH and diluted 1/100 with Milli-Q water before the particle size analysis was conducted. The refractive index (RI) of solvent (water) and the dispersed phase (WP) were considered as 1.33 and 1.52, respectively.

3.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS-PAGE was performed to fractionate and compare individual proteins in each WPC using either reducing or non-reducing (β -mercaptoethanol replaced by Milli-Q water) conditions following the procedure described by Ong et al., (2006) with minor modifications. About 0.01 g of each WPC powder was mixed with a reducing buffer (1 mL of 10 mM Tris, 1 mM EDTA, pH 8.0, 350 μ L of 10% SDS, 50 μ L of β -mercaptoethanol) and the solution was heated at 95°C for ~ 5 min in a water bath until the powder was completely dissolved. Protein standards of α -lactalbumin, β -lactoglobulin (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany) were prepared by dissolving 4 mg of each in 2 mL Milli-Q water. Then, all prepared WPC samples and standards were diluted with the treatment buffer

(0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M dithiothreitol, 0.02% bromophenol blue, pH 6.8). The broad range pre-stained SDS-PAGE standards (Ref. 161-0318, Bio-Rad laboratories, Hercules, CA) were used to compare the molecular weights. About 6 μ L of WPC samples, α -lactalbumin, β -lactoglobulin and 8 μ L of molecular weight standards were then loaded onto the 4 – 20 % iGels (NuSep, French Fores, Australia). The gels were run at 50 mA for ~ 50 min. The gels were then placed in de-staining solution 1 (40% methanol, 7% acetic acid) for 30 min, stained with staining solution (0.025% Coomassie Brilliant Blue R 250, 40% methanol, 7% acetic acid) for 24 h, de-stained in de-staining solution 1 for one h followed by further de-staining in de-stain solution 11 (5% methanol, 7% acetic acid) until the background became clear. A Fuji Film Intelligent Dark Box II with Fuji Film LAS – 1000 Lite V 1.3 software (Fuji Photo Film Co., Ltd., Japan) was used to obtain gel images and analyse protein bands.

3.2.4 Size exclusion high performance liquid chromatography (SE- HPLC)

The chromatographic separation of major WP in each commercial WPC powder was determined by SE-HPLC as described by Bouaouina et al., (2006) with minor modifications. WP dispersions were prepared and diluted in the mobile phase (0.05 M KH_2PO_4 , pH 6.8) to obtain the final protein concentration of around 4 mg/mL. The diluted solutions were filtered through a 0.45 μ m filter before loading into HPLC column (BioSep-SEC-S 3000, 300 x 7.8 mm, Phenomenex, Lane Cove, NSW, Australia). The eluted components were monitored by UV absorption at 280 nm (model 9050, Varian Analytical Instruments, CA, USA). The injection volume was 20 μ L and the solvent flow was maintained at 0.5 mL/min isocratically. The standard solutions of α -lactalbumin (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), β -lactoglobulin (Sigma-Aldrich), and bovine serum albumin (Sigma-Aldrich) at concentration of 10 mg/mL were used to quantify WP and resulting chromatograms were analysed using a software (Varian–Star Chromatography Workstation, version 5.31).

3.2.5 Differential scanning calorimetry (DSC)

A differential scanning calorimeter (DSC 7, Perkin Elmer, Norwalk, CT, USA) equipped with a software (Pyris Manager, v.5.0002) was used to examine the nature of thermal denaturation of WPCs. The instrument was calibrated using indium ($T_{\text{peak}}=155.87^{\circ}\text{C}$, $\Delta H=28.234$ J/g) and zinc ($T_{\text{peak}}=417.4^{\circ}\text{C}$, $\Delta H=93.337$ J/g). About 30 μl of 12% (w/w) WP dispersions at pH 7 were weighed into aluminium pans. An empty pan of equal weight was served as the reference. All pans were hermetically sealed before placing in the instrument. The samples were scanned from 25 to 100°C at the scanning rate of 10°C/min. The ΔH values and onset, endset and peak temperatures of the thermograms were recorded.

3.2.6 Functional properties of whey proteins: solubility, heat stability, emulsifying properties and foaming properties

The functional properties of WPC powders were analysed using 5% (w/w) protein dispersions at pH 7. Approximately 5% (w/w) protein dispersions were prepared by mixing the correct amount of WPC powder in Milli-Q water at room temperature. The solutions were stirred for about 2 hours using a magnetic stirrer to ensure proper dissolution. After that, the dispersions were stored overnight at 4°C before the final weight was corrected with the pH adjustment to 7 using 1M NaOH.

Protein solubility of WPCs was assessed following the method established by Morr et al., (1985) with some modifications. Ten ml portions of 5% (w/w) protein dispersions were centrifuged (Model J2HS; Beckman, Fullerton Calif., USA.) at 3,000 x g at 20°C for 20 min and the supernatants filtered through 0.45 μm filter. The protein content of the supernatant and of the original dispersion was estimated using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) with the standard curve ($r^2 = 0.957$) developed by bovine serum albumin (Sigma-Aldrich, Chemie GmbH, Steinhelm, Germany). The absorbance of the samples at 595 nm

was measured using a spectrophotometer (Pharmacia LKB; Novaspec[®] II) and the following equation was applied to estimate the percent solubility;

$$\% \text{ Solubility} = \frac{\text{protein content of supernatant (mg/mL)}}{\text{protein content of solution (mg/mL)}} \cdot 100$$

Heat stability of WP was assessed at 140°C by two different methods: a) heat coagulation time (HCT, Rattray and Jelen, 1996); and b) solubility after brief exposure to heat. HCT is defined as a time required to observe the formation of visible aggregates during exposure to excessive heating. Precisely measured 3.0 mL samples of WP dispersions were placed in glass tubes (10 mm diameter and 75 mm length), sealed, immersed and rocked in oil bath (Ratek Shaking water bath, Boronia, Australia) at 140°C. The time when the first visible aggregates appeared was recorded as HCT. For the solubility method, 3 mL samples of WP dispersions were placed in similar sealed glass tubes and exposed to same conditions for 10 s. After this time tubes were quickly removed from the oil bath, cooled instantly in an ice bath and centrifuged (Model J2HS; Beckman, Fullerton Calif., USA.) at 12,000 x g at 20°C for 20 min. Samples of original protein dispersions were also centrifuged, the supernatants of both instances were filtered through 0.45 µm filter and the protein content of the filtered supernatants was measured as before in solubility using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). Heat stability was expressed using the following equation:

$$\text{Heat stability} = \frac{\text{Protein content in supernatant after heating (mg/mL)}}{\text{Protein content in supernatant before heating (mg/mL)}} \cdot 100$$

Emulsification characteristics of WPCs were monitored using a turbidimetric method as previously described by Pears and Kinsella (1978). Emulsifying

activity index (EAI), emulsion stability and adsorbed protein of 5% (w/w) WP dispersions were measured with several modifications. The emulsions of protein dispersions were prepared by mixing canola oil and the protein solution at a ratio of 1:3 and homogenized with an Ultratarax polytron homogenizer for 3 min before microfluidizing at 20 MPa.

EAI was calculated using the following equation expressed as units of area of interface stabilized per unit weight of protein:

$$EAI = \frac{2 \cdot T}{\Phi C}$$

where T denotes turbidity; Φ is the oil volume fraction; C is the weight of protein per unit volume of aqueous phase before an emulsion is formed

Aliquots (one mL) of the emulsions were diluted serially with 0.1% sodium dodecyl sulphate (SDS) to give a final dilution of 1/3000. The absorbance of the diluted emulsions was determined in a 1.5 cm path length cuvette at 500 nm in a spectrophotometer (Pharmacia LKB; Novaspec® II). In theory, when none of the light scattered by the turbid sample reaches the photo-detector in a sample which does not adsorb light at 500 nm, turbidity is given by:

$$T = \frac{2.303 A}{l}$$

where A is the observed absorbance and l (m) is the path length of the cuvette (Pearce and Kinsella, 1978).

Aliquots (one ml) of emulsions and protein dispersions were dried separately in an IsoTemp Oven (Fisher Scientific) at 120°C to constant weight. The oil volume fraction (Φ) was calculated by the following formula:

$$\Phi = \frac{C - A - E (B - C)}{C - A + (B - C) \left[\frac{(1 + E) D_0}{(D_s - E)} \right]}$$

where A is the mass of empty pan ; B - mass of pan plus emulsion ; C - mass of pan plus dry matter of emulsion ; D_0 - density of oil ; D_s - density of protein dispersion ; E - concentration of protein in dispersion (Pearce and Kinsella, 1978).

Emulsions were held at 4°C for 24 h before analysis for emulsion stability index. ESI was calculated by the following formula:

$$ESI = \frac{(T \times \Delta t)}{\Delta T}$$

where T is the turbidity value at zero h; ΔT is change in turbidity; Δt is the time interval (Pearce and Kinsella, 1978).

Ten mL aliquots of each emulsion were centrifuged at 12,000 x g (J2HS, Beckman, Fullerton Calif., USA) at 20°C for 30 min and the protein content of the aqueous layer was measured using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) as described above. Adsorbed protein was calculated by the equation:

Adsorbed protein (mg/mL) =
protein in stock solution (mg/mL) – protein in aqueous layer of emulsion (mg/mL)

Foaming properties of 5% (w/w) protein dispersions were examined as reported previously (Phillips et al., 1990) with minor modifications. Approximately 200 mL portions of protein dispersions were poured into a bowl (3L) of a double beater Sunbeam Mixmaster Compact Pro-400 watt twin-motor stand/hand mixer (Sunbeam Corporation, Limited. CAN 000 006 771) and whipped for 20 min at ambient temperature. The beater rotational speed was adjusted to its highest speed. A sample of foam was quickly and gently filled into a 100 mL scoop with a rubber spatula taking care to avoid entrapped air pockets. Excess foam was scraped off the top of the scoop using a metal spatula to achieve a constant volume for each measurement and weight of foam was recorded. This part of the procedure was conducted within one minute. The foam overrun was calculated by the following equation:

$$\% \text{ Overrun} = \frac{(\text{wt. of } 100 \text{ mL protein}) - (\text{wt. of } 100 \text{ mL foam})}{\text{wt. of } 100 \text{ mL foam}} \cdot 100$$

Foam stability was measured by monitoring drainage at ambient temperature, (Phillips et al., 1990). To facilitate continuous measurement of drainage from foams, the stainless steel whipping bowl (3L) was modified by drilling a 0.6 cm hole in the bottom, 5.1 cm from the centre. The edge of the hole was located outside the path taken by the outside beater. The hole was sealed during whipping by adhesive tape applied to the outside of the bowl. The foams were generated from 200 mL of protein dispersion. The beaters, bowl and the protein dispersion were weighed before whipping for precisely 20 min. The bowl, beaters and foam were quickly weighed to quantify moisture loss during whipping and obtain an

accurate weight of liquid in the foam. The tape was then quickly removed, the hole cleared with a glass rod (if necessary) and a bowl placed in a ring stand at a 30° angle above a tared container on a balance pan, so the hole was at the lowest point. The beaters were oriented to avoid any disturbance of liquid flow. The drained liquid was collected in the tared container on the balance pan and the increase in weight was continuously recorded in 20 s intervals. The time to attain 50% drainage was used as an index of foam stability (Phillips et al., 1990).

3.2.7 Preparation of heat induced WPC gels

Gels were prepared following a method used by Onwulata et al., (2004) with some modifications. About 50 g of 12% (w/w) WPC dispersions were prepared using Milli-Q water and 0.03 M CaCl₂ in 100 mL plastic containers. The pH of the solutions was adjusted to 7 by 1 M NaOH. The mixtures were then kept for 15 min at room temperature; heated in a water bath at 90°C for 30 min including 10 min come up time; cooled immediately in an ice bath for 30 min and stored at 4°C for overnight before analysis.

3.2.8 Firmness of WPC gels

Firmness of heat set WPC gels was assessed by texture profile analysis and carried out using a texture analyser (TA-XT2 Plus, Stable Micro Systems Ltd., Surrey, UK) at room temperature. The gels (2.8 cm in height and 4 cm in diameter) were gently removed from the containers and compressed at 50% strain using SMS P/75 probe (Stable Micro Systems Ltd) with the crosshead speed of 1 mm s⁻¹. The resulting force-time curves were analysed by computer software (Texture Exponent 32, Stable Micro Systems). Firmness is defined as the height (g mm s⁻²) of the force peak on the first compression cycle (Bourne, 2002).

3.2.9 Rheological properties

The rheological studies were performed with a rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with supporting software (Rheoplus/32 v2.81, Anton Paar) and a double gap cylinder measuring system (DG26.7- SN7721, Anton Parr). Approximately 3.9 g of 12% (w/w) WPC dispersion was placed in the measuring unit and covered with a thin layer of low-density oil to prevent evaporation. The mixture was pre-sheared for 5 s at a shear rate of 500 s^{-1} at 20°C and held for 30 s to reach equilibrium. The flow behaviour of samples was studied first via shear rate sweep measurements over the range $0.1 - 100 \text{ s}^{-1}$ for 5 min at 20°C . This was followed by assessment of heat-induced gelation using a dynamic small amplitude oscillatory measurement (SAOM) at 1% strain and a frequency of 1 Hz. The measurements were acquired by heating the mixtures from 20 to 90°C at a heating rate of 1°C min^{-1} for about 70 min and then holding at 90°C for 10 min.

3.2.10 Water holding capacity (WHC) of gels

The water holding capacity of heat set WPC gels was determined using a method described by Purwandari et al., (2007). About 20 g of WPC gels were prepared in 50 mL falcon tubes using 12% (w/w) WPC solutions and gelation was induced as described above. The gels were stored at 4°C overnight and the weights of the gels were recorded. Subsequently they were centrifuged (Model RT7, Sorvall, DuPont, Newtown, Connecticut, USA) at $700 \times g$ at 8°C for 10 min and the whey expelled was carefully decanted and weighed. When expressed as a percentage of the initial gel weight, the weight of whey was designated as the water holding capacity. This was a measure of water released during centrifugation and re-absorbed after centrifugation.

3.2.11 Statistical analysis

All experiments were organized in a randomized block design with type of WPC as the main factor and replicates as a block. All tests were replicated at least once with subsequent sub-sampling ($n \geq 4$). Results were analysed using a General Linear Model, SAS (1996). Tukey's Studentized Range (HSD) test was used for multiple comparisons of means. The level of significance was preset at $P=0.05$.

3.3 Results and discussion

3.3.1 Proximate composition

The proximate compositions of commercial WPCs are presented in Table 3.1. Significant ($p < 0.05$) variations were noted in moisture, protein, ash, fat, phosphorous (P) and calcium (Ca) contents among the 8 samples. All WPCs contained between 73 and 85% (w/w) proteins and considerable amounts of fat, which were present in a range which is typical of industrial products (Fox and McSweeney, 2003).

Table 3.1 Proximate composition of commercial whey protein concentrates

Sample	Moisture, g.100g ⁻¹	Protein, g.100g ⁻¹	Ash, g.100g ⁻¹	Fat, g.100g ⁻¹	P, g.100g ⁻¹	Ca, g.100g ⁻¹
A	7.2±0.08 ^b	77.1±0.39 ^d	2.8±0.07 ^b	4.2±0.06 ^b	0.19±0.05 ^d	0.63±0.03 ^{bc}
B	8.2±0.22 ^a	73.6±0.32 ^e	3.0±0.02 ^b	3.6±0.15 ^c	0.24±0.03 ^{bcd}	0.72±0.06 ^b
C	6.4±0.17 ^c	82.2±1.08 ^b	2.6±0.07 ^b	4.7±0.07 ^b	0.32±0.02 ^b	0.72±0.01 ^b
D	7.2±0.04 ^b	77.3±0.25 ^d	4.1±0.07 ^a	4.3±0.14 ^b	0.50±0.05 ^a	1.11±0.04 ^a
E	7.9±0.30 ^b	79.0±0.96 ^{cd}	2.6±0.53 ^b	4.4±0.23 ^b	0.31±0.05 ^{bc}	0.48±0.09 ^{cd}
F	5.6±0.34 ^d	81.0±1.78 ^{cb}	3.0±0.08 ^b	4.4±0.43 ^b	0.23±0.02 ^{cd}	0.34±0.20 ^{de}
G	5.4±0.04 ^d	85.3±1.24 ^a	1.9±0.35 ^c	5.5±0.28 ^a	0.22±0.04 ^d	0.25±0.03 ^e
H	7.5±0.16 ^b	77.6±1.10 ^d	2.8±0.12 ^b	4.3±0.16 ^b	0.28±0.01 ^{bcd}	0.72±0.04 ^b

A, B, C, D, E, F, G, H – commercial whey protein concentrates (WPCs); P- Phosphorous; Ca - calcium. Means present the average of at least 4 independent observations (n≥4). Means with different superscript letter are significantly different ($P < 0.05$).

In addition, most of the samples contained characteristic levels (2.50 – 5.30%) of ash (Fox and McSweeney 2003) which is also consistent with that reported by others (Mangino et al., 1987; Onwulata et al., 2004). However Ca and P contents in some samples ranged over a wider range than found other studies (Mangino et al., 1987; Patel et al., 1990). Sample D contained the highest amounts of ash, Ca and P contents while sample G had the maximum protein and fat contents and the reduced ash Ca and P contents. The typical range of moisture contents of industrial WPCs ranges from 3.30 - 4.50% (Fox and McSweeney, 2003), with our samples having higher moisture contents ranging from 5.4 – 8.2%. This could have been caused by inadequate storage conditions after receipt of the samples. Furthermore, sample B contained the lowest amounts of protein and fat but maximum moisture content, which consequently could have led to underestimation of the protein content.

3.3.2 Particle size distribution, SDS PAGE and SE-HPLC analysis

Mean particle size of all WPC samples ranged from approximately 185 to 230 nm with sample G having the largest and sample D the smallest particles (Table 3.2). These values were almost comparable with those reported by Onwulata et al., (2004) for similar types of WPCs.

SDS PAGE electrophoretograms of different WPCs are given in Figure 1- A (reducing conditions) and –B (non-reducing conditions). The protein bands were identified using broad range molecular weight markers, α -La and β -Lg standards. The β -mercaptoethanol in reducing SDS PAGE has a capability to cleave disulfide bonds existing in WPC. As a result all aggregated proteins should appear in their ‘monomeric’ forms. In non-reducing environments, dissociation of non-covalent bonds takes place (Havea et al., 1998). As shown in Figure 1-A, WPC-E contained the lowest amounts of all 3 major whey proteins. Samples A, B, C, D and H had relatively reduced BSA levels. Most of the SDS PAGE observations

were in agreement with the SE-HPLC findings (Figure 3.2). Non-reducing SDS PAGE revealed that WPC- D contained the highest amounts of native like WP, followed by samples C and F. Further, the low-intensity major protein bands appeared in WPC-H (Figure 1-B) clearly indicated that these proteins were the likely those that were most affected during processing and storage. The level of denaturation mainly depends on the conditions used by different manufacturers in cheese manufacturing and whey powder processing (Onwulata et al., 2004).

3.3.3 Functional properties of WPCs: solubility, emulsifying properties and foaming behaviour

The data (Table 3.2) show that the solubility of all 5% (w/w) WPCs at pH 7 was less than 90% with significant ($p<0.05$) differences among samples and these values were relatively lower than the values obtained in some of the previous studies (Morr et al., 1985; Morr and Foegeding, 1990; Kim et al., 1989; Patel and Kilara, 1990) as well. WP in their native state are highly susceptible to heat induced denaturation (Lee et al., 1992). The reduction in solubility of these WPCs as well as variation in the values may be a consequence of partial protein denaturation due to different processing techniques related to heating and/or drying during concentration. Even partial denaturation of protein during processing may cause flocculation and precipitation during storage which may adversely affect the sensory attributes of the products (Fennema et al., 2008). In addition, the added filtration method carried out to minimise the turbidity interferences with the Bradford assay may have also removed the soluble aggregates of proteins leading to reduction in solubility. Also, WPCs may be modified during manufacturing to improve the functionality (Morr and Foegeding, 1990). The results of particle size analysis (Table 3.2) and especially, non-reducing SDS PAGE (Figure 3.1-B) are useful in understanding the extent of protein denaturation. Smaller particles hydrate more readily resulting in increased solubility (Onwulata et al., 2004; Hudson et al., 2000). The mean particle size of

sample D was significantly ($p < 0.05$) smaller than all others which was further confirmed by results from the non-reducing SDS PAGE. The major protein bands of sample D represented the highest intensity indicating least denaturation of proteins which could observe in particle size analysis as smaller particles.

Sample D also showed appreciably ($p < 0.05$) enhanced solubility. In addition, samples C and F with a higher proportion of native like proteins also exhibited significantly higher solubility and the sample G had a markedly ($p < 0.05$) larger mean particle size with comparatively low solubility. Sample H had the least solubility around 67%. This sample also showed greater heat stability with fairly high Ca content which contradicted previous reports that indicated enhanced heat sensitivity in the presence of Ca (Morr and Ha, 1993). The average particle size of this sample also was moderate indicating rather preserved native conformation. Therefore this sample was most likely modulated during manufacture in such a way aiming to improve some of the functionalities. On the other hand this modulation could have interfered with the solubility measurements of WPC using Bradford reagent. Additionally, these WPCs contained considerable amounts of fat. Bound fat may also alter the protein water interactions thus decreasing the solubility (Patel and Kilara, 1990) and bringing discrepancies among methods used in protein content determination (Morr et al., 1985).

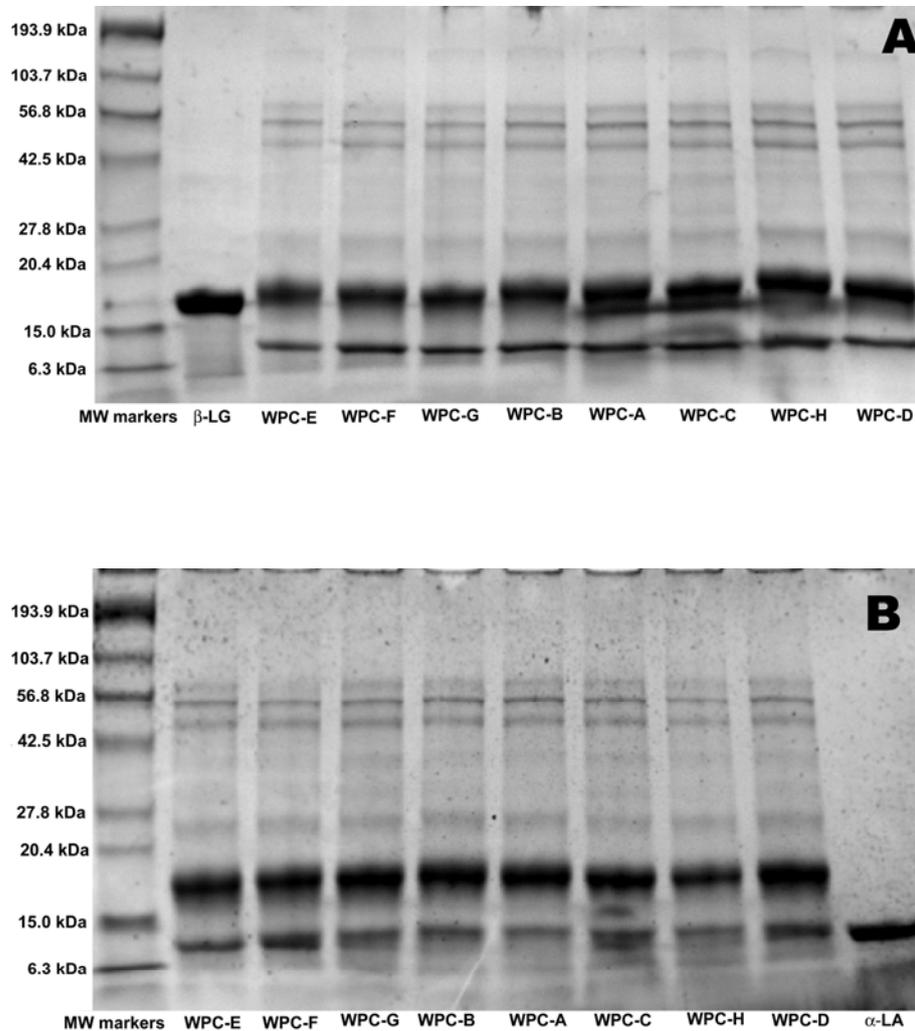


Figure 3.1 The reducing (A) and non-reducing (B) SDS-PAGE of 8 commercial WPC samples. Molecular weight markers (MW Markers); β -lactoglobulin (β - Lg); α -lactalbumin (α - Lactalbumin); 8 whey protein concentrates (WPC A - H).

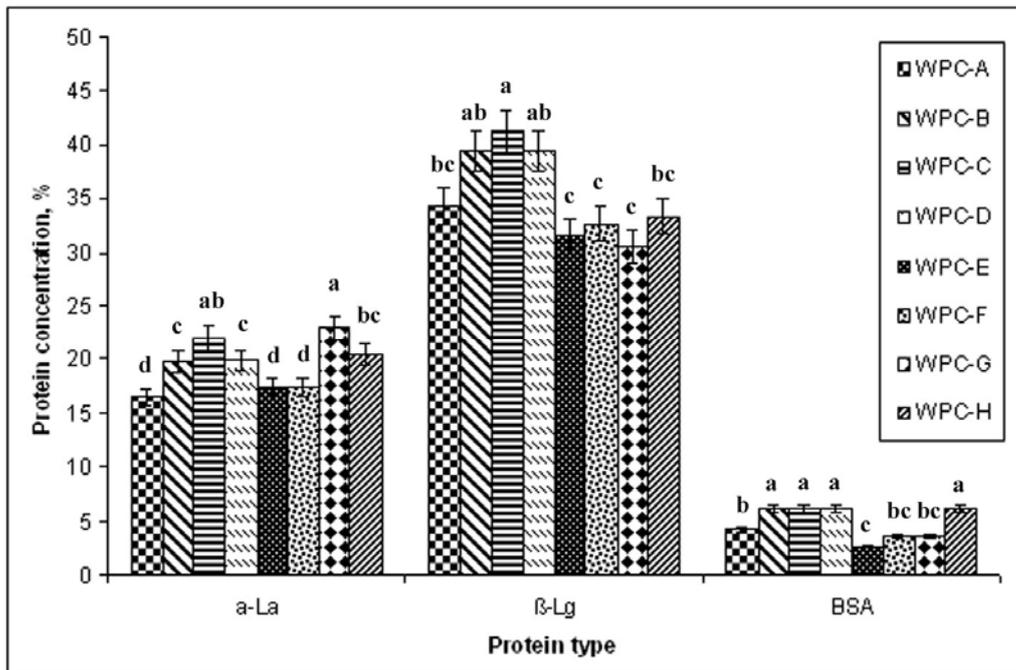


Figure 3.2 Proportion of major proteins present in 8 commercial WPC samples (WPC A - H) as detected by size exclusion HPLC. Different letters indicate significant difference ($p < 0.05$) among samples within a particular protein type.

Table 3.2 Colloidal, interfacial and gelling properties of commercial whey protein concentrates

Product	Solubility, %	Particle size, nm	EAI, m ² .g ⁻¹	ESI, h	Adsorbed protein mg.mL ⁻¹	Overrun, %	Gel firmness g mm.s ⁻²	WHC, %
A	81.0±0.8 ^{ab}	197.3±7.3 ^{cd}	6119±171 ^a	23.8±0.1 ^a	54.6±0.6 ^d	296±4.1 ^b	664±126.9 ^{cd}	98.8±0.1 ^e
B	78.7±1.7 ^{ab}	197.0±6.7 ^{cd}	4924±966 ^a	23.7±0.1 ^a	61.3±0.8 ^b	336±2.4 ^a	1296±103.0 ^c	99.5±0.1 ^{cd}
C	86.5±4.4 ^a	214.1±4.4 ^b	5136±909 ^a	23.8±0.1 ^a	55.3±2.3 ^{cd}	0 ^d	872±23.6 ^{cd}	98.9±0.2 ^e
D	86.3±2.5 ^a	185.1±4.3 ^d	5229±837 ^a	23.7±0.1 ^a	53.3±0.6 ^{de}	341±3.1 ^a	2955±507.5 ^a	99.5±0.1 ^d
E	80.6±6.8 ^{ab}	205.1±12.0 ^{bc}	6240±493 ^a	22.8±0.9 ^a	65.0±1.8 ^a	0 ^d	523±162.3 ^d	99.7±0.1 ^{bc}
F	83.2±4.7 ^a	209.8±7.5 ^b	5597±484 ^a	23.0±1.3 ^a	58.9±1.9 ^{bc}	226±5.7 ^c	240±496.7 ^{ab}	100.0±0.0 ^a
G	74.0±1.9 ^{bc}	229.6±10.6 ^a	4788±203 ^a	23.6±0.3 ^a	50.9±0.8 ^e	0 ^d	453±62.7 ^d	99.4±0.1 ^d
H	66.9±2.3 ^c	203.0±6.4 ^{bc}	5337±369 ^a	22.9±1.5 ^a	50.3±1.5 ^e	231±14.8 ^c	2043±277.0 ^b	99.9±0.1 ^{ab}

A, B, C, D, E, F, G, H – commercial whey protein concentrates (WPCs); EAI – emulsion activity index; ESI – emulsion stability index; WHC – water holding capacity. Means present the average of at least 4 independent observations (n=4). Means with different superscript letter are significantly different. ($P < 0.05$).

Emulsifying activity measures the turbidity at a single wavelength to assess the droplet size and, by implication, the interfacial area of the emulsion (Lee et al., 1992). The capacity of protein to stabilize an emulsion may be related to the interfacial area. Also, the stability of an emulsion is related to the consistency of the interfacial area (Pearce and Kinsella, 1978). The four main destabilization mechanisms related to dispersions of protein-coated oil droplets are creaming, coalescence, Ostwald ripening and flocculation. Among them, the most complicated one to control is droplet flocculation which can be delicately affected by the other factors and also it can influence directly the emulsion structure, and other stabilising mechanisms, especially creaming and coalescence. Also, the physico-chemical principles governing stability are depending on the classical electrostatic and steric stabilisation. Electrostatic stabilisation is governed by the presence of electrical charges on the surface of the droplets, and the stabilising layer adsorbed at the droplet surface. The greater the surface charge density and the lower the ionic strength of the aqueous medium, the more stable is the emulsion. Steric stabilisation arises from the presence of a polymeric or the steric obstruction at the droplet surface. More importantly, for the long-term stabilisation, the adsorbing polymer must be present at a sufficient concentration to cover the oil-water interface completely the adsorbed layer must remain permanently attached to the interface with parts of individual molecules projecting away from the surface towards the aqueous medium. Also, the steric and electrostatic contributions depend on the specific protein structure and the pH of the solution, for example, the contribution of electrostatic stabilisation is generally greater for adsorbed monolayers of compact globular proteins at pH values well away from the isoelectric point (Dickinson, 2010). In addition, net hydrophobicity of proteins, solubility and composition of WPC are correlated with emulsifying ability (Klemaszewski and Kinsella, 1991). However, as these results show all samples did not exhibit (Table 3.2) any significant difference in EAI and emulsion stability.

Proteins contribute to film formation in foams by concentrating at the interface which depends on the ability of diffusion, reducing interfacial tension and partially unfolding. The covalent disulfide bonds and secondary forces are important in reacting with neighbouring protein molecules to form continuous cohesive films. In properly homogenised protein systems, the principal attractive forces between continuous proteins are short-range hydrogen bonds, hydrophobic and electrostatic interactions and van der Waals forces as well. The ionic strength, temperature and the pH of the medium which affects the partial protein denaturation as well as protein concentration which may alter water structure with subsequent changes in hydrophobic interactions (Phillips et al., 1991; Kinsella, 1984) are equally important in forming properties. In addition, the foaming properties of WPC depend on the degree of denaturation, calcium ion concentration and lipid content (Jovanovic et al., 2005). Moreover at pH 7 whey proteins have a net negative charge which favours foam formation by enhancing protein unfolding via activated thiol groups (Lee et al., 1992). In general free fat and bound fat were negatively related with foaming properties, where as ash, calcium content positively related to (Patel and Kilara, 1990).

As shown in Table 3.2, several WPC samples (C, E & G) failed to create foam at all. Furthermore all samples exhibited low foam stability. Impaired foaming capacity implies the inability of protein to incorporate air in the liquid continuous phase and to produce strong, cohesive and stable films around air bubbles. Proximate analysis of WPCs indicated that all samples contained considerable amount of fat. Mangino et al., (1987) have shown that WPC prepared from pasteurized milk gives higher foam overrun and stability and that this is probably related to a decrease in neutral lipids during pasteurization. Lipid content in WPC can seriously impair the foaming process since surface active, polar lipids interfere with protein films by situating themselves at air/water interface (Fennema, 1996), and weaken the foaming properties by inhibiting adsorption of protein to the interphase. Furthermore, these interfering substances possess weak

cohesive and visco-elastic properties to overcome the internal pressure of air bubbles compared to whey proteins resulting rapid collapsing of bubbles.

3.3.4 Viscosity of WPC dispersion

All samples exhibited shear thinning behaviour by decreasing their apparent viscosity with increasing shear rate (Figure 3.3). This character was very apparent in samples H, A, C and lowest in sample D. In general, this property can be correlated with the particle size of the WPCs (Resch, 2004). Compared to larger particles smaller particles have greater overlapping of their electrical double layer which consists of ions with an immobile inner layer and an outer diffuse layer. When there is a strong electrical double layer overlap it imparts a higher inter-particle potential with a consequent enhanced electro-viscous effect leading to increased viscosity (Resch, 2004). The mean particle size of samples H, A and C (Table 3.2) are comparatively smaller and consistent with the apparent viscosity of their dispersions. However sample D possessed the smallest mean particle size that was difficult to correlate with its reduced apparent viscosity. Simultaneously, sample D contained the highest Ca and ash contents. The intrinsic factors of proteins such as size, volume, structure, electrical charge and symmetry and protein solvent interactions such as protein swelling and hydrodynamic hydration sphere surrounding the molecule may directly influence the apparent viscosity of a material (Fennema, 1996). Therefore the substances present in that sample may have attributed to alter the expected behaviour of apparent viscosity as shown in Figure 3.3.

3.3.5 Gelling characteristics

The viscoelastic nature of WPC gels during heating was investigated by measuring storage modulus (G') and phase angle ($\tan \delta = G''/G'$). Phase angle or

the loss tangent is the relationship between the in-phase (G') and out of phase (G'') elements of the stress-strain curves. Therefore, it is a measure of viscoelasticity at a given frequency (Rao, 1992). Additionally the physical properties of gels were examined in terms of firmness and WHC. As shown in Table 3.2, firmness and WHC among WPC gels varied significantly ($p < 0.05$). Figure 3.4 signifies the differences in phase angle during gelation of WPC samples. A smaller phase shift indicates a more solid-like nature of the gel. Sample D and F had comparatively greater solid-like character than the other samples. Sample D exhibited an interesting property around 90°C by an increased phase shift after the gel structure was formed. This sample also demonstrated the highest gel firmness and contained a high Ca and ash contents. During gelation Ca ions are involved in forming cross links between protein chains and help in the formation of networks and there is a strong correlation between Ca content and the gel strength (Paulsson and Dejmek, 1990; Brandenberg et al., 1992; Morr and Ha, 1993). However the increase in phase angle of the sample after gelling shows some evidence of brittleness. Stabilising effect of Ca bridges can be interpreted in terms of restricted mobility of unfolded peptide backbones (Paulsson and Dejmek, 1990). This fact may be the reason for the brittleness of the gel during the application of SAOM in the presence of raised Ca levels. Moreover, the gel strength (fracture stress) and firmness (G') depend on the protein concentration (Veith and Reynolds, 2004) by being tend to scale as a squared function of protein concentration. Therefore, the variation in protein content in individual samples may also affect the gel characteristics.

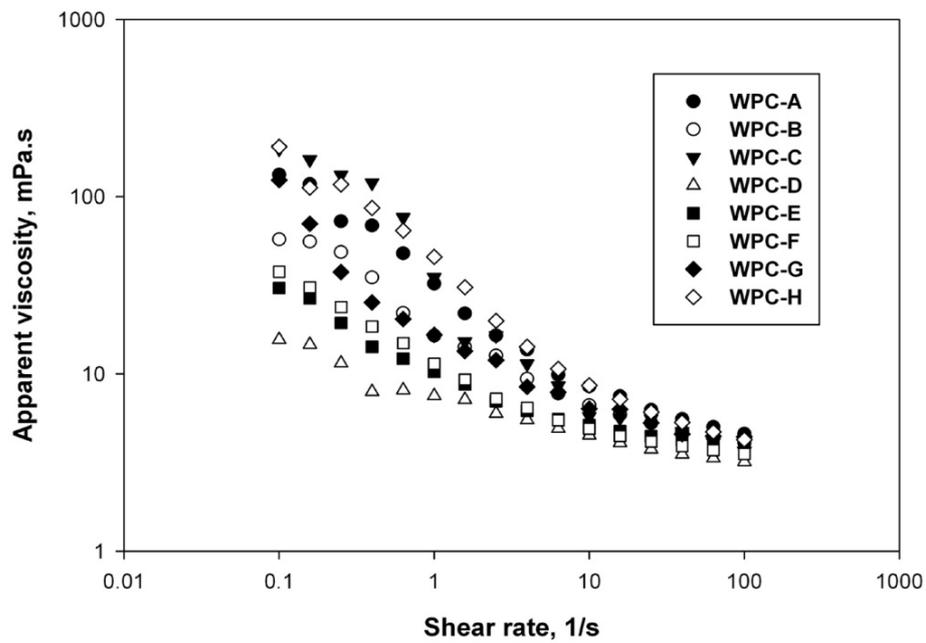


Figure 3.3 Apparent viscosity of 12% (w/w) protein dispersions at 20°C prepared from 8 commercial WPC samples. Curves present averages of at least 4 ($n \geq 4$) independent observations.

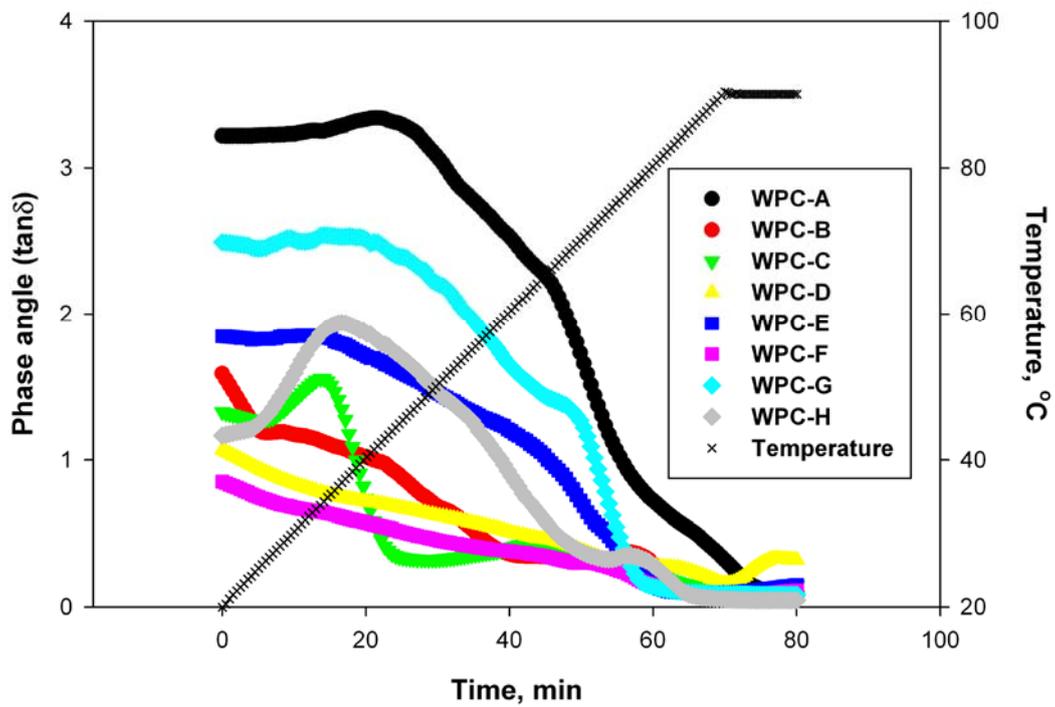


Figure 3.4 Behaviour of phase angle ($\tan \delta = G''/G'$) of commercial WPC samples during heating from 20 to 90°C at applied strain of 1% and frequency of 1 Hz. Curves present averages of at least 4 ($n \geq 4$) independent observations.

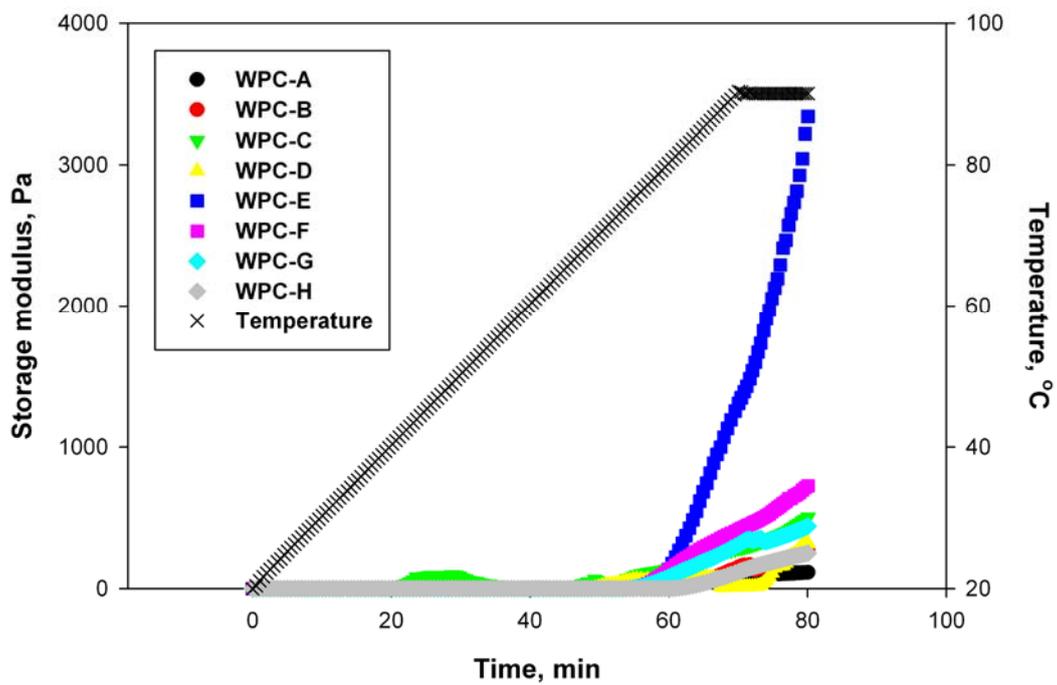


Figure 3.5 Development of storage moduli (G') of commercial WPC samples during heating from 20 to 90°C at applied strain of 1% and frequency of 1 Hz. Curves present averages of at least 4 ($n \geq 4$) independent observations.

This trend could be observed to a lesser extent in sample H as well. Samples A and G exhibited the highest phase angle initially, revealing their highly viscous (liquid) character. Sample G contained the lowest ash and Ca amounts which may have contributed to reduced partial protein denaturation during processing steps. It is difficult to correlate compositional differences of sample A with its increased initial viscous nature, but significantly reduced mean particle size with increased solubility of this sample is consistent with the observations reported by Onwulata et al., (2004).

In addition, the firmness and WHC of gels produced by sample G were significantly reduced and it can be correlated with its low ash and Ca contents. Moreover, a prominent increase in G' during gelation (Figure 3.5) and significantly reduced firmness in gels (Table 3.2) was observed with sample E. WHC can be used as a measure of pore size of the gels which allows them to release water when deformed. The larger pores should yield a lower WHC (Bowland and Foegeding, 1995). As reported by Veith and Renolds (2004), addition of polyphosphate has improved gel strength through chelation of excess Ca ions which could be remained after formation of protein-calcium-protein bridges. In addition, phosphate is also likely to increase WHC of protein gels by assisting in protein cross linking through binding to surface positive charges of proteins (Veith and Renolds, 2004). Sample A contained a significantly low P content with significantly reduced WHC. Samples F and H produced considerably firm gels together with greater WHC, and, their ash and Ca amounts also markedly high.

3.3.6 Thermal properties of WPCs

Table 3.3 summarises the thermal properties (heat stability, heat coagulation time, denaturation enthalpy, onset, endset and peak temperatures) of commercial WPCs. The results indicated that the sample D had significantly ($p<0.05$) higher heat stability and HCT.

Table 3.3 Thermal properties of whey protein concentrates

Product	Thermal stability, %	HCT, s	ΔH, J.g⁻¹	T_{Onset}, °C	T_{Endset}, °C	T_{Peak}, °C
A	68.4±4.29 ^c	14.3±0.50 ^b	1.52±0.01 ^c	62.0±0.37 ^b	79.1±0.57 ^{abc}	76.1±0.14 ^{bc}
B	68.4±3.78 ^c	14.0±1.15 ^b	1.57±0.03 ^c	61.8±0.14 ^b	79.7±0.09 ^{ab}	77.1±0.08 ^{ab}
C	64.5±2.08 ^c	13.0±1.15 ^{bc}	2.68±0.21 ^b	67.1±4.44 ^a	75.3±2.96 ^c	69.4±0.48 ^e
D	95.5±2.59 ^a	20.0±2.31 ^a	139.82±0.69 ^a	60.8±0.49 ^b	79.8±0.09 ^{ab}	68.2±0.02 ^f
E	84.5±4.41 ^b	14.5±0.58 ^b	1.65±0.27 ^c	61.4±0.13 ^b	79.5±0.35 ^{ab}	77.2±0.16 ^a
F	87.1±2.42 ^b	15.5±0.58 ^b	1.68±0.12 ^c	61.2±0.14 ^b	79.2±0.64 ^{abc}	75.8±0.21 ^{cd}
G	95.9±3.33 ^a	11.1±0.63 ^c	2.57±0.36 ^b	60.8±0.49 ^b	82.1±1.18 ^a	74.7±1.25 ^d
H	97.0±2.19 ^a	18.8±0.96 ^a	2.04±0.34 ^{bc}	61.1±0.67 ^b	75.9±3.33 ^{bc}	75.4±0.42 ^{cd}

A, B, C, D, E, F, G, H – commercial whey protein concentrates (WPCs); HCT- Heat coagulation time; ΔH - denaturation enthalpy of WP; T_{Onset}, T_{Endset}, T_{Peak} are temperatures corresponding to onset, endset and peak positions of DSC thermograms. Means present the average of at least 4 independent observations (n≥4). Means with different superscript letter are significantly different. (*P* < 0.05).

The high thermal stability of this sample was further revealed by its remarkably high denaturation enthalpy. However, the high ash and Ca contents present in this sample contradicted with the above results. Additionally, sample H also showed considerably ($p<0.05$) high heat stability and HCT and it contained moderately high ash and Ca levels as well. Addition of different Ca chelating agents may enhance heat stability of WP (de Rham and Chanton, 1984). Similarly Veith and Reynolds (2004) have reported the chelation of Ca in WPC by polyphosphate. Among 8 WPCs, sample G had significantly reduced Ca and ash levels which were consistent with its significantly high heat stability. Also, sample C had markedly ($p<0.05$) high onset temperature compared to all others demonstrating that it could withstand higher processing temperatures without major effects on protein conformation. The non-reducing SDS PAGE and SE-HPLC findings (Figure 3.1-B & Figure 3.2) also revealed that this product was less affected by the processing history.

3.4 Conclusions

Commercial whey protein concentrates showed significant variations in proximate composition, particle size distribution and several assessed functional properties. Moreover, SE-HPLC and SDS PAGE patterns revealed the differences in the amounts and properties of major WP in these powders likely incurred by differences in processing history and origin. The substantial variability in functionality was mostly consistent with the differences in physico-chemical properties of these samples. For example, calcium content had negative correlation with heat stability and coagulation time where as gel firmness positively correlated with calcium as well as phosphorous contents. Also, amount of fat had negative correlation with foaming characteristics and the mean particle size was negatively correlated with the solubility. However, in some cases it was difficult to correlate relevant functional properties with corresponding chemical composition. More importantly, regardless the proximate composition, all samples could have been successfully used as emulsifying agents. Nevertheless, this observation was not supported by one sample which contained higher ash and Ca content and subsequently had greater heat stability and formed weaker gel. The results of this study also suggest that the industry is capable of producing WPC products with targeted functionalities such as enhanced heat stability and altered viscosity.

CHAPTER 4

4 Functional properties of whey proteins affected by heat treatment and hydrodynamic high pressure shearing

4.1 Introduction

Whey proteins are unique nutritional supplements and functionally valuable food ingredients. They have important biological (digestibility, amino acid pattern, high biological value and sensory characteristics), physical and chemical functionalities (McIntosh et al., 1998). Based on their amino acid composition and rate of peptide and amino acid release in the small intestine, WP are nutritionally superior functional food supplements compared to other dietary proteins (Vasiljevic and Shah, 2007). They are extensively used in various food applications, with an increasing global demand. Commercially different types of WP powders are available. Among these the whey protein concentrates and whey protein isolates are most widely used (Fox and McSweeney, 2003). WP are highly soluble over a broad range of pH (Zhu and Damodaran, 1994), a property which is important in their application as foaming, emulsifying, gelling and water binding agents in various types of food products. WP are used in sport beverages, liquid meat replacements, baked products, processed meats, salad dressings, ice creams, artificial coffee creams, soups and various other dairy products (Fox and McSweeney, 2003; Fitzsimons et al., 2007).

WP are composed of a mixture of beta lactoglobulin, alpha lactalbumin, bovine serum albumin, immunoglobulins, proteose peptones and other minor proteins (Fitzsimons et al., 2007). In their native form they exist as compact, globular proteins (Lee et al., 1992) with high solubility due to a large proportion of surface hydrophilic residues. When subjected to denaturing agents such as heat, previously buried hydrophobic groups become exposed, and sulfhydryl/disulfide exchange chain reactions take place between exposed cysteine residues resulting in dissociation, partial unfolding and finally aggregation (Lee et al., 1992). The rates and pathways of these physicochemical reactions are determined by environmental factors such as protein concentration, pH, temperature, ionic strength and solvent condition (Brandenberg et al., 1992; Iordache and Jelen, 2003; Marangoni et al., 2000).

WP are excellent foaming and emulsifying agents. To obtain optimum foaming and emulsifying characteristics the protein must be substantially soluble, diffuse to the newly formed interface, unfold and reorient in ways that lower interfacial tension and form cohesive and viscoelastic films by polymerisation mainly via disulfide bonds and hydrophobic interactions (Lee et al., 1992; Bouaouina et al., 2006). The number of sulfhydryl groups, molecular flexibility, hydrophobicity and surface activity determine their ability to form stable emulsions and foams (Lee et al., 1992). Though the normal functionality of WP mainly depends on the behaviour of β -Lg, the most abundant protein in whey (Verheul et al., 1998), overall functionality depends on the combined properties of all WP components. While β -Lg has excellent gelling, foaming and emulsifying properties, α -La exhibits good emulsifying properties but has fairly poor gelation properties (Pearce and Kinsella, 1978). Molecular flexibility enhances emulsion and foam formation by increasing the rate of unfolding at the interface and allowing more favourable alignment of polar and non polar groups in their preferred phase (Klemaszewski and Kinsella, 1991). Sulfhydryl groups, which are more active at pH 7, contribute to molecular flexibility that influences the ability to unfold and interact with other proteins while disulfide bridges play a role in rigidity (Monahan et al., 1993). It is well known that there is an improved foaming and increased foam stability at pH 7 when WP solutions are first heated to 55°C (Phillips et al., 1990). At higher temperatures, however, foaming and emulsifying characteristics may be impaired due to protein aggregation, thereby decreasing the availability of protein to form films and stabilise emulsions (Phillips et al., 1990).

WP have significant potential use in food manufacturing but a major obstacle restricting their application is heat-induced destabilization. In industrial practice, the inevitable heat treatment employed when WP are concentrated changes the native state thus affecting their stability. In addition, compulsory heat treatments during processing of some food products containing WP may cause protein denaturation, aggregation and flocculation resulting in phase separation, destabilization of emulsions or protein precipitation (Pearce and Kinsella, 1978; Patel and Kilara, 1990). Thus, it is important for industry to improve the functionality of WP to avoid heat induced adverse effects.

Attempts to improve the functionality of heterogeneous WP preparations by chemical and enzymatic modifications have met with limited success (Spellman et al., 2005). The use of mechanical force under isothermal and/or isobaric conditions, *i.e.* by extrusion or static high-pressure treatment, may provide a more useful approach. The application of heat and high shear has been the basis for production of microparticulated products such as Simplese[®] and Dairy-Lo[®] (Akoh, 1998). Similarly, Iordache and Jelen (2003) used heat denaturation and microfluidization to improve solubility of a WP isolate. The application of heat treatment results in the perturbation of the protein size and shape and leads to exposure of hydrophobic sites, aggregation and consequently network formation (Gosal and Ross-Murphy, 2000). Under the influence of an applied shear, links between or within the network may break leading to fragmentation of clusters (Shih et al., 1990). Structural modifications of whey protein preparations by heat treatment and dynamic high-pressure shearing would possibly result in products with distinctly different functional characteristics from those of the native preparations (Considine et al., 2007; Huppertz et al., 2005).

Further, the ability of WP to form gels by immobilizing large amounts of water and other ingredients is important in processed food formulations and product development, as this phenomenon can be used to improve textural attributes (Hudson et al., 2000; Matsudomi et al., 1993; Resch et al., 2004a; Verheul et al., 1998). Also, WP possess superior nutritional attributes compared to other gel-forming agents such as pre-gelatinized starches and similar hydrocolloids (Resch et al., 2004a). However, functional properties of WP are highly dependent on their heat sensitivity as well as compositional variability (Morr and Ha, 1993), all of which greatly affect their utility in food systems. Although a number of approaches have been suggested to improve their application (Bouaouina et al., 2006; Brandenburg et al., 1992; Considine et al., 2007; Onwulata et al., 2004), much remains to be resolved before WP can gain widespread commercial importance.

Gelation is a phase transition of polymers from a liquid state with ‘disconnected monomers’ to a well connected network or gel (Rao, 1999). The sol-gel transition involves linking of the basic structural units *via* physical and/or chemical bonds, resulting in formation of a continuous network, leading to solid-like properties. Protein gelation can be induced by many extrinsic factors, such as heat, acids,

pressure, enzymes and salts (Alting et al., 2000; Bouaouina et al., 2006; Huppertz et al., 2006; Huang and Kinsella, 1986; Ju and Kilara, 1998a; Ju and Kilara, 1998b). Depending on the balance between attractive and repulsive forces among denatured protein molecules, different types of gel networks are formed (Dickinson, 2005). Protein aggregates are the basic building material for a gel network (Ju and Kilara, 1998b; Caussin et al., 2003) and the size, shape and spatial arrangement of the aggregates can have an impact on rheological behaviour, sensory quality and water-holding capacity of resulting gels. Also, depending on the macroscopic nature of the gels, they can be described as ‘true gels’, with the ability to ‘free-stand’, or ‘weak gels’, which show less clear solid nature and flow when subjected to a yield stress (Rao and Steffe, 1992).

Gelling of WP upon heating provides textural benefits in many food applications. On the other hand, cold-set WP gels are also important in preparations of heat-sensitive food products with delicate texture and flavour. This can be achieved at lower temperatures, and is thus suitable for food applications where a post-processing preservation step by heating is not required. The general pre-requisite for cold gelation process is the activation of protein molecules by denaturation, at a protein concentration below the critical gelation concentration, and at low ionic strength and/or far from the isoelectric point, followed by the addition of salts or adjustment of pH to screen repulsive forces at cold-set conditions (Alting et al., 2004; Thompson et al., 2009).

Microparticulation is a recent approach to improving or modulating WP functionality and can also be achieved by dynamic high-pressure shearing or microfluidization (MFZ) (Iordache and Jelen, 2003). The particle sizes of WP aggregates induced by heating are greatly reduced (Iordache and Jelen, 2003) by the forces of mechanical shearing. Conformational changes of native WP induced by microparticulation may thus be important in modulating their physical functionalities. Moreover, gelling properties of MWP may also be important and introduce novel ingredients in the food industry.

The general objective of this study was to assess the effects of dynamic high pressure shearing on some functional properties of native and heat-denatured whey protein preparations. Also, establish gelling behaviour of MWP induced by either heating

(heat-set gels) or changing the solvent quality (cold-set gels) in order to enhance MWP usage as functional agents in processed foods. One of the research foci was also to assess different fundamental interactions driving gelation of these MWPs.

4.2 Materials and methods

4.2.1 Materials and proximate composition

Two different sets of WP retentate containing approximately 30% total solids and their corresponding whey permeate obtained on two different occasions (in two days) were kindly provided by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). Initial compositional analysis of these samples was carried out following established AOAC methodology. Total protein was determined using a Kjeldahl method with a nitrogen conversion factor of 6.38 (method: 968.06, AOAC, 2000); moisture was determined by drying (method:925.10, AOAC, 2000); ash content was estimated by combusting pre-solidified samples (method:923.03, AOAC, 2000); fat content was determined using the Mojonnier method (method:989.05 (modified), AOAC, 2000); calcium content was determined by atomic absorption spectrophotometry (method:985.33, AOAC, 2000). Phosphorus content was determined colourimetrically using vanadomolybdophosphoric acid (Gales et al., 1966). Lactose concentration was measured by HPLC (Vasiljevic and Jelen, 2003). All chemicals used in the study were of analytical grade (Sigma-Aldrich, St. Louis, MO; BDH Chemicals, Australia Pt. Ltd., Kilsyth, Australia) and gels for polyacrylamide gel electrophoresis (PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA) and NuSep (French Fores, Australia).

4.2.2 Treatment of samples

Upon protein determination, WP retentate samples which were obtained in two different days representing replication, were adjusted to 10% (w/w) protein using corresponding whey permeates. The pH of the resulting solutions was adjusted to 7 with NaOH and then five types of samples were prepared from one replicate as follows. Two samples from one replicate were microfluidized (Model 110Y,

MicrofluidicsTM, Newton, Massachusetts, USA) using 1 and 5 passes at 140 MPa without heat treatment and two other samples were first heat denatured at 90°C for 20 min for complete WP denaturation (Fox and McSweeney, 2003) and then microfluidized using 1 and 5 passes at 140 MPa. The temperature of denatured samples during microfluidization was < 90°C. An untreated whey protein concentrate served as the control. This procedure was done twice for one original replicate and, thus, one sample was containing eight different representatives. The samples were then spray dried using a pilot scale spray dryer (SL-10 Mini-Maxi Pilot Spray Dryer, Saurin Enterprises Pty. Ltd., Melbourne, Australia). The inlet and the outlet air temperatures of spray dryer were adjusted to 180°C and 80°C respectively. All powders were placed in plastic air tight containers and stored at ambient temperature until needed for further analysis.

4.2.3 Particle size distribution

Approximately 1% (w/w) WP dispersions were prepared from the spray-dried powders and stored overnight at 4°C to achieve full hydration of proteins. These samples were diluted 1 in 100 before the analysis. The particle size distribution pattern of control and non-heat treated samples was determined by a dynamic light scattering instrument (Zetasizer-Nano ZS, Malvern instruments, Worcestershire, UK) equipped with Dispersion Technology software (version 5, Malvern instruments, Worcestershire, UK). Particle size distributions of heat- treated samples was similarly analysed by a Mastersizer (model 2000, Malvern instruments, Worcestershire, UK).

4.2.4 Size exclusion high performance liquid chromatography (SE-HPLC), sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), differential scanning calorimetry (DSC)

The degree of aggregation and denaturation of different WP preparations was observed using SE-HPLC; protein compositions of each WP preparation were also examined by SDS-PAGE under reducing and non-reducing conditions and thermal

analysis of WP preparations was carried out using a differential scanning calorimeter as previously described in Chapter 3.

4.2.5 Functional properties of treated whey proteins: solubility, heat stability, emulsifying activity index (EAI) and emulsion stability index (ESI), foam formation

Analysis of powders for various functional properties was carried out using 5 (%w/w) protein dispersions at pH 7 unless otherwise stated. The protein powder needed to make the protein dispersions was weighed into a clean dry closed beaker and Milli-Q water was added with continuous stirring for approximately 2 hrs at room temperature. The dispersion was maintained at 4°C overnight to allow for hydration of proteins. pH of the dispersions was adjusted to 7 with NaOH and the final weight was corrected before testing.

Solubility, heat stability, EAI, ESI and adsorbed protein to the oil droplets as well as foaming properties of protein dispersions were examined using the methods described in Chapter 3.

4.2.6 Preparation of heat-induced gels

Heat-induced gelation was carried out using approximately 12% (w/w) WP dispersions, following an established method (Onwulata et al., 2004) with some modifications. The dispersions were prepared with Milli-Q water and 0.03 M CaCl₂, and sample pH was adjusted to 7.0 by drop-wise addition of 1 M NaOH. The mixtures were used either for rheological studies or preparation of gels for further analysis. Gels were prepared by allowing the WP dispersions to sit for 15 min at room temperature prior to heating at 90°C for 30 min (10 min come-up time) in a water bath, followed by immediate cooling in an ice bath for 30 min. Subsequently, the gels were aged at 4°C overnight.

4.2.7 Preparation of cold-set gels

Cold-set gelation was performed to assess the rheological properties of such gels using microparticulated, heat-treated (H1 and H5) and unheated (N1 and N5) WP powders with or without glucono- δ -lactone (GDL) and NaCl. These powders were fully hydrated as 12% (w/w) WP dispersions in Milli-Q water with 0.03 M CaCl₂. These samples were divided as per MFZ treatment (1 or 5 passes) and each set contained six different samples. These included two control samples: heat treated (HT) and unheated (NHT) controls without GDL or NaCl addition, and four others mixed with GDL and/or NaCl to achieve GDL or NaCl concentrations in their final mixtures of 2% GDL, 0.1 M NaCl, 0.5 M NaCl or 2% GDL with 0.1 M NaCl.

4.2.8 Flow behaviour of microparticulated whey protein dispersions

Whey protein samples were tested by a CS/CR rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with a proprietary software (Rheoplus/32 v2.81, Anton Paar) and a double-gap-cylinder measuring system (DG26.7- SN7721, Anton Paar). About 3.9 g of 12% (w/w) WP dispersion was introduced into the measuring unit and a thin layer of low-density, low-viscosity oil was placed on top of the sample to prevent evaporation. Prior to analysis, the mixture was pre-sheared for 5 s at a controlled rate of 500 s⁻¹ at 20°C and held for 30 s to equilibrate. The flow behaviour of WP samples was investigated using shear rate sweep measurements over the range of 0.1 – 100 s⁻¹ for 5 min at the same temperature. The flow curves were fitted to Herschel-Bulkley model, presented by the equation:

Herschel-Bulkley model:
$$\sigma = \sigma_0 + k' \dot{\gamma}^{n'}$$

where k' , n' , σ , σ_0 and $\dot{\gamma}$ are consistency index (Pa.sⁿ), flow behavior index (dimensionless), shear stress (Pa), yield stress (Pa) and shear rate (1/s), respectively (Steffe, 1992).

4.2.9 Viscoelastic properties of heat and cold-set gels

In situ thermal gelation was also assessed using the same rheological system by applying dynamic small amplitude oscillatory measurements at a constant strain of 1% and frequency of 1 Hz within the viscoelastic region of the samples. During heat gelation, the samples were heated from 20 – 90°C at a heating rate of 1°C min⁻¹ for approximately 70 min and held at 90°C for 10 min, cooled from 90 to 4°C at a rate of 1°C min⁻¹ over 86 min, followed by holding at 4°C for 60 min for aging.

In situ cold-set gelation was examined using a cone-and-plate measuring system (CP 50-1, Anton Paar). This measuring system was specifically selected to avoid possible sedimentation of WP occurring in the double gap system via gravitational forces due to their larger particle size. During cold gelation, the 12% WP mixture was immediately introduced to the measuring system and the process was monitored using the same parameters as described above, at 20°C for 150 min. After this period, the frequency sweep from 1-10 Hz at 1% shear strain was used to ascertain viscoelastic properties of created gels. The pH change of the GDL-acidified WP mixtures was simultaneously recorded every 15 min using a pH meter (Model 8417, Hanna Instruments, Singapore). An additional mechanistic study assessing the prevalence of protein-protein interactions during cold-set gelation was also carried out in situ using the same cone-and-plate measuring system. Gelation was achieved as described previously (Havea et al., 1998; Havea et al., 2009) using H1 and H5 protein dispersions mixed with 2% GDL or 0.1 M NaCl in the presence of either 20 mM NEM or 1% SDS.

4.2.10 Polyacrylamide Gel Electrophoresis (PAGE)

Heat- and cold-set gels were created from the samples as described under preparation of ‘heat-induced gels’ and ‘cold-set gels’. Only the mixtures that created gels were used in PAGE analysis. All gel samples were dissolved in appropriate buffers before performing either native (with or without 0.45% polyoxyethylene sorbitan monolaurate, *i.e.*, Tween 20) or reducing / non-reducing sodium dodecyl sulphate (SDS) PAGE (Havea et al., 1998).

4.2.11 Gel permeability, water-holding capacity and colour of gels

The permeability coefficient (B) of heat-set WP gels was determined as described previously (Lee and Lucey, 2004) with slight variations. The WP mixtures, prepared for heat gelation, were placed in 50 mL Falcon tubes (Falcon, Blue Max, Becton Dickinson and company, Franklin Lakes, N.J., USA). Open-ended glass tubes (inner diameter 3.7 mm and length of 25.0 cm) were then immersed in the mixtures with the aid of rubber stoppers. The tubes were further sealed with para-film to prevent evaporation of the solvent and gelation was induced by heating at 90°C for 30 min. Afterwards, the glass tubes with the gels were removed from the Falcon tubes and the heights of the gels were measured. The glass tubes with gels were then immersed in 6% (w/w) whey solutions. Due to the osmotic pressure gradient between the top of gels and the surface of whey in the Falcon tubes, the whey diffuses through the gels and collects on the surface. The height of the whey on the gel surface was measured after two different time intervals. The experiment was conducted at 20°C and the density of the whey was 1.0033 g/mL. The permeability coefficient (B) was determined using the following equation.

$$B = - \left[\ln \frac{(h_{\infty} - h_{t2})}{(h_{\infty} - h_{t1})} \right] \cdot \frac{\eta \cdot H}{\rho \cdot g (t_2 - t_1)}$$

Where B is the permeability coefficient (m²), h_∞ is the height of the whey in the reference tube (m), and ht1 and ht2 are the heights of whey (m) in the gel tube at time t1 (s) and t2 (s) respectively. The value η is the viscosity of whey (Pa.s), ρ is the density of whey (kg/m³), g is acceleration due to gravity (ms⁻²) and H is the length of the gel (m). The reference glass tube was included with the absence of gels but supplying all other similar experimental conditions.

Water-holding capacity (WHC) of heat-set WP gels was examined using an established method (Purwandari et al., 2007). After overnight storage at 4°C, the gels were centrifuged (Model RT7, Sorvall, DuPont, Newtown, Connecticut, USA) at 700 x g at 8°C for 10 min. The supernatant (whey) was carefully decanted, and the WHC

of the gel was expressed as a percentage, taking into account the weight of gel after whey was expelled, relative to the initial weight of gel.

Colour of heat-induced gels was measured with a Minolta Chromameter (CR-300, Minolta Corporation, Ramsey, NJ, USA) using CIE 1976 (L^* - whiteness; a^* - red to green; b^* – yellow to blue) colour system. Five determinations were carried out for each gel.

4.2.12 Statistical analysis

The study was arranged as a randomized block full factorial design with the heat treatment (heat, no-heat) and a number of microfluidizing passes (1 or 5) as the major factors and the replications as blocks. All experiments were replicated at least once with subsequent sub-sampling ($n \geq 4$). Results were analysed using a General Linear Model (SAS, 1996). The level of significance was preset at $P=0.05$.

4.3 Results and discussion

4.3.1 Effect of microfluidization and heat treatment/microfluidization on whey proteins

The extent of WP denaturation and modification due to heat and high pressure shearing was examined by particle size analysis, SE-HPLC, reducing and non-reducing SDS PAGE and DSC. WP samples were of five main types: native control (C); native, microfluidized with 1 pass (N1); native, microfluidized with 5 passes (N5); heated then microfluidized with 1 pass (H1); heated then microfluidized with 5 passes (H5).

SDS PAGE electrophoregrams of different WP preparations under reducing and non-reducing conditions are presented in Figure 4.1, A to C. The broad range molecular weight markers and α -La and β -Lg standards were used to identify the protein bands. Generally, under reducing conditions in the presence of β mercaptoethanol, disulfide bonds are cleaved and consequently aggregated proteins would appear in their

monomeric forms. Therefore, as shown in Figure 4.1 -A and -B, protein bands corresponding to heat treated or non-heat treated conditions showed no substantial change in intensity. Under non-reducing conditions, dissociation of non-covalent bonds takes place (Patel et al., 2005; Havea et al., 1998); thus, WP aggregated via disulfide bonds would remain intact. As expected under non-reducing conditions corresponding main protein bands (monomeric) disappeared in the heat treated samples (Figure 4.1 -C, sample H1). However, using several microfluidizing passes resulted in reappearance of these bands (Figure 4.1 -C, H5), implying possible aggregate fragmentation.

Figure 4.2 represents the effect of complete heat denaturation and microfluidization at 140 MPa on the particle size distribution of WP powders. The average particle size of denatured/microfluidized WP (samples H1 and H5) was around 10 μm as opposed to non-heat treated samples (C, N1 and N5) with size distribution between 0.01 and 1 μm . Increasing the extent of dynamic high pressure treatment also widened the particle size distribution of non-heat treated samples (sample N5). The application of dynamic high pressure may also have influenced the conformational rearrangement of proteins (Iordache and Jelen, 2003). If the globular proteins were unfolded even to a limited extent, then aggregation of molecules via disrupted hydrophobic groups would most probably result in bigger particles as shown by the change in distribution.

Table 4.1 Compositional analysis of whey protein retentates and permeates

Component	Retentate	Permeate	*SEM
Moisture, %w/w	70.10	94.78	0.26
Protein, %w/w	24.53	0.86	0.37
Fat, %w/w	1.51	0.05	0.12
Lactose, %w/w	1.89	3.74	0.04
Ash, %w/w	1.00	0.50	0.03
Ca, mg / L	470.2	1455.0	181.6
P, mg / L	309.4	658.8	77.3

*SEM denotes a pooled standard error of the mean, $P < 0.05$. Means present the average of at least 6 independent observations (n=6).

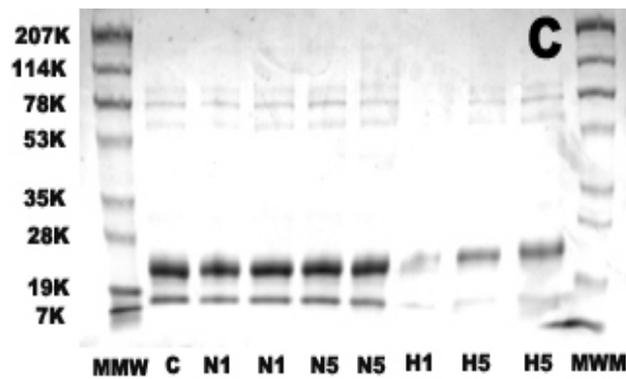
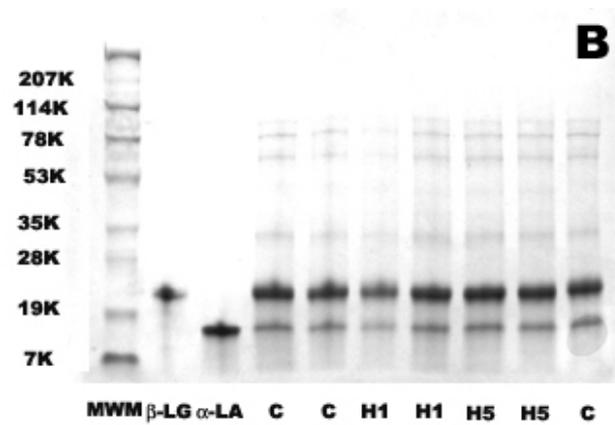
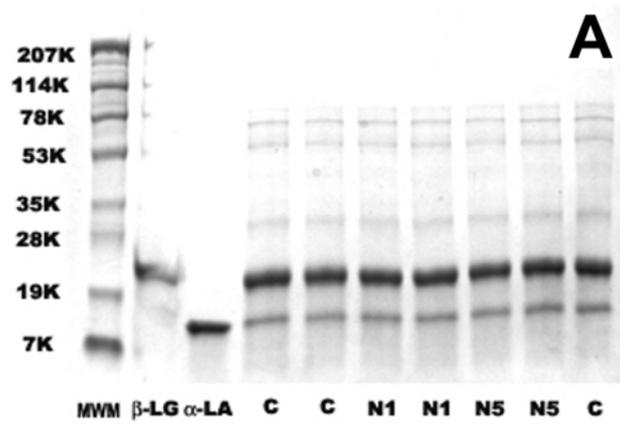


Figure 4.1 Effects of heating and microfluidization on extent of whey protein denaturation observed by reducing (A & B) and non-reducing (C) SDS-PAGE: Molecular weight standards (MWM); β -LG; α -LA; Control (C); Native, 1 pass (N1); Native, 5 passes (N5); Denatured, 1 pass (H1); Denatured, 5 passes (H5).

SE-HPLC chromatograms showed (Figure 4.3) that the control and non heat-treated/microfluidized samples had major peaks corresponding to α -La, β -Lg and BSA. Also, the major WP of these non-heat treated samples were affected by the dynamic high pressure treatment which decreased their concentrations significantly ($P < 0.05$) with increasing numbers of high pressure treatment. The concentrations of α -La, β -Lg and BSA in control WP samples were decreased from 13.5 to 12.0 mg/mL, 40.3 to 36.7 mg/mL and 3.0 to 2.2 mg/mL respectively when they were microfluidized with five passes at 140 MPa. As described by Bouaouina et al., (2006), these changes could be expected due to possible conformational rearrangements in WP resulting from dynamic high pressure treatment. Meanwhile the peaks corresponding to the major WP were absent in the SE-HPLC chromatograms of heat-treated samples. Further, some larger and smaller molecular weight species resulted from either aggregation or fragmentation of WP due to heat treatment and the dynamic high pressure treatment were observed in heat denatured samples, but their concentrations were relatively very low. In addition, the aggregates formed during treatments could not have passed the guard column to enter the column due to their size. The behaviour of WP during heating is accompanied by a change in thermal properties. These enthalpy changes associated with protein unfolding are proportional to the extent of denaturation (De Wit, 1990) and can be observed by DSC.

In Figure 4.4, all non heat-treated WP samples show one broad endothermic peak with peak height related to denaturation temperature of the major WP, β -Lg, which was around 77°C. During WP denaturation the endothermic total enthalpy is related mainly to the disruption of internal hydrogen bonds in protein and in water and to a lesser extent to the formation of protein-water bonds, excess hydrogen bonds in water around apolar groups and the disruption of van der Waals bonds between apolar groups (Paulsson and Dejmek, 1990). ΔH values obtained for the native control (C) and non-heat treated/1pass (N1) and non heat treated/5 passes (N5) samples were 2.53, 1.95 and 2.20 J/g respectively. The ΔH values have been correlated with the content of ordered secondary structure and could be used to monitor the proportion of undenatured protein (Patel et al., 1990). Decrease in denaturation enthalpy of microfluidized samples may be indication of a destruction of hydrophobic interactions caused by high pressure.

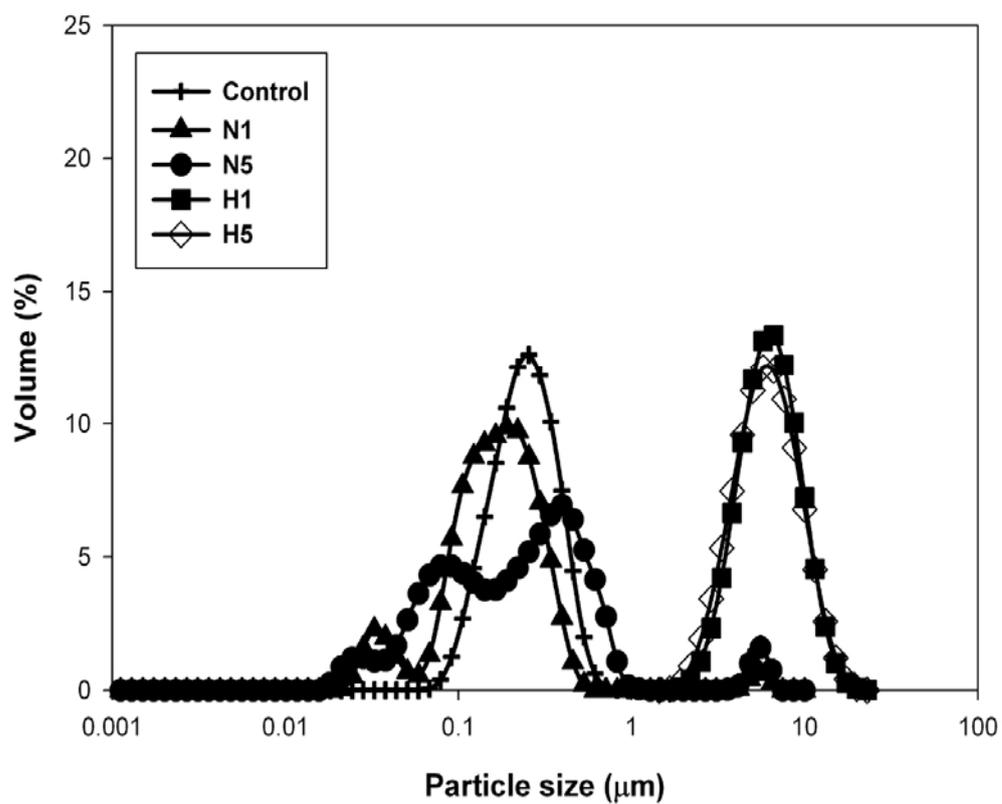


Figure 4.2 Particle size distribution pattern of WP as affected by heat treatment and microfluidization: Control; Native, 1 pass (N1); Native, 5 passes (N5); Denatured, 1 pass (H1); Denatured, 5 passes (H5).

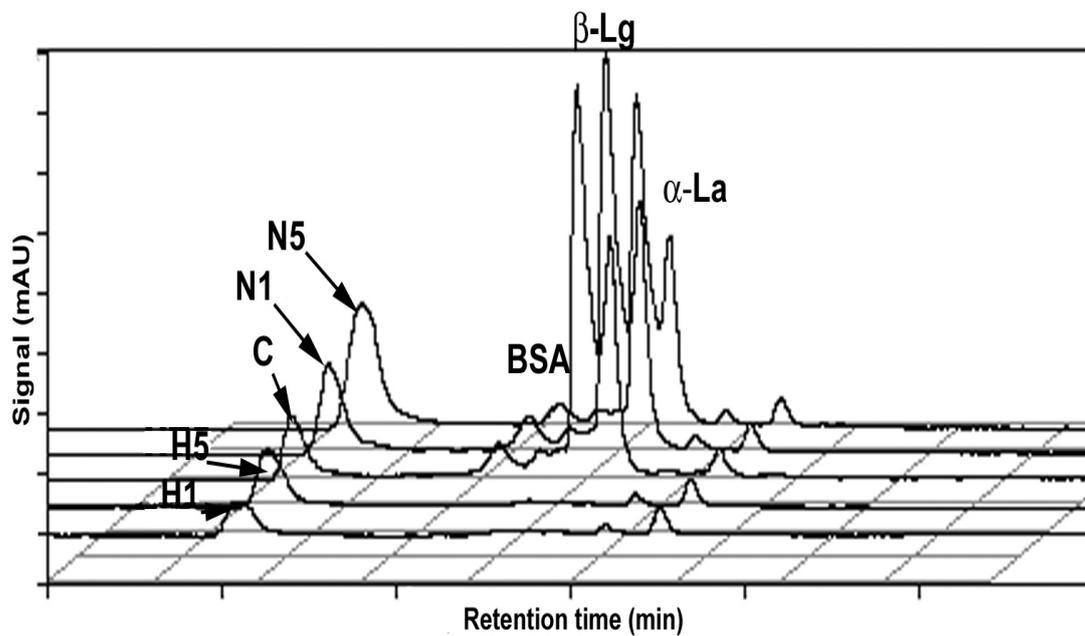


Figure 4.3 SE-HPLC profiles of native and treated whey protein preparations. Native control (C); Native 1 pass (N1); Native 5 passes (N5); Heat treated, 1 pass (H1); Heat treated, 5 passes (H5).

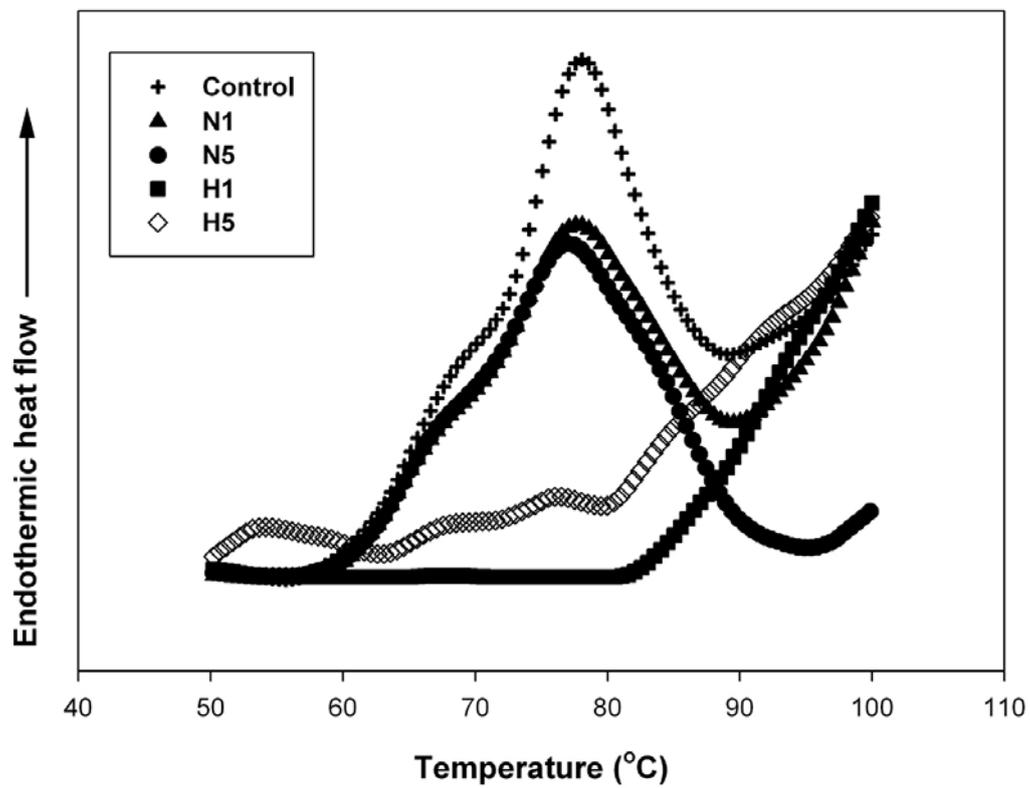


Figure 4.4 DSC thermograms of 12 (% w/w) whey protein dispersions: Control; Native, 1 pass (N1); Native, 5 passes (N5); Denatured, 1 pass (H1); Denatured, 5 passes (H5).

Rupturing of hydrophobic bonds is an exothermic process (Fennema, 1996) which might lead to a decrease in ΔH of protein denaturation. The relatively slight increase in ΔH again with increasing microfluidizing passes (N5) may be due to possible reformation of intra and inter molecular hydrophobic bonds from resulting disordered protein molecules. In contrast, in heat-treated samples no such endothermic peaks were detected confirming prior irreversible denaturation of WP. It presumably reflects the heat stability of those samples which is consistent with the results of extensively longer HCT.

4.3.2 Effect of treatments on functional properties: solubility and heat stability, emulsifying activity index (EAI) and emulsion stability, foaming capacity and foam stability

Table 4.2 indicates that the heat treatment significantly ($P < 0.05$) decreased the solubility of WP (H1 and H5) compared to control. Native WP are globular with higher numbers of surface hydrophilic residues and buried hydrophobic and cysteine groups resulting high aqueous solubility (Fox and McSweeney, 2003; Zhu and Damodaran, 1994). β -Lg, α -La and BSA have 5, 8, 35 cysteine residues respectively. Naturally around neutral pH the intramolecular disulfide bonds between cysteine residues stabilize the tertiary structure of these globular proteins. β -Lg exists as dimers having 2 disulfide bridges and a buried free thiol group, α -La is a monomer with 4 disulfide bridges but no thiol groups and BSA is also a monomer having 17 disulfide bridges and 1 thiol group (Monahan et al., 1993; Paulsson and Dejmek, 1990).

Table 4.2 Colloidal and interfacial properties of different whey protein preparations produced using heat and high pressure shearing

Treatment	# of passes	Solubility, %	Heat stability, %	HCT, s	EAI, m ² g ⁻¹	ESI, h	Adsorbed protein, mg/mL	Overrun, %	Foam stability, s
Control	0	96.5 ^b	100.5 ^b	14.5 ^a	5188 ^a	23.3 ^{abc}	32.1 ^a	275 ^b	0 ^a
N	1	101.0 ^c	99.1 ^b	18.9 ^b	4654 ^a	23.2 ^{bc}	31.8 ^a	1187 ^d	23.7 ^a
	5	94.7 ^b	100.0 ^b	17.4 ^b	4666 ^a	22.3 ^c	33.4 ^b	850 ^c	301.8 ^b
H	1	28.0 ^a	93.4 ^a	87.8 ^c	8545 ^b	24.0 ^{ab}	41.2 ^c	0 ^a	0 ^a
	5	31.2 ^a	89.1 ^a	102.5 ^d	9257 ^c	24.4 ^a	41.2 ^c	0 ^a	0 ^a
*SEM		1.3	1.9	1.13	189.07	0.4	0.14	34	15.0

Treatments: N & H - non heat treated and heat treated whey proteins, respectively. HCT – heat coagulation time; EAI – emulsion activity index; ESI – emulsion stability index. *SEM: pooled standard error of the mean, P < 0.05, (n=8). The different small letter superscripts in a column indicate significant difference (P < 0.05).

During the application of dynamic high-pressure, the forced induced phenomena of cavitation, shear, turbulence and temperature rise occur simultaneously (Bouaouina et al., 2006). This may cause conformational rearrangements in quaternary and tertiary structures of proteins (Bouaouina et al., 2006) with consequent changes of some functional properties such as significantly ($P<0.05$) increased solubility (sample N1 compared to C) and the significant ($P<0.05$) decrease in solubility of sample N5 compared to N1 as observed in this experiment.

As shown in Table 4.2 heat treatment had a significant ($P<0.05$) negative effect on heat stability of WP. This comparatively low heat stability in heat treated samples (H1, H5 compared to C, N1 and N5) may be due to a possible further coagulation and precipitation of protein molecules that have already been denatured. However the combined effect of heat and number of passes significantly ($P<0.05$) increased the heat coagulation time (HCT) observed at 140°C (Table 4.2, H1 and H5). As described earlier native WP are readily denatured upon heating. But heat treated samples may withstand heat since they have been already denatured and mostly contain no active sites such as free thiol groups to initiate aggregation, which in turn retards heat coagulation. On the other hand, dynamic high pressure has a disruptive effect on intramolecular hydrophobic and electrostatic interactions which finally leads to the subsequent reformation of intra and inter molecular bonds within or between protein molecules (Bouaouina et al., 2006). Therefore microfluidization may have increased the interactions of protein molecules, reducing further availability of reactive sites, consequently increasing the heat stability.

The emulsifying activity index has been significantly ($P<0.05$) increased (Table 4.2) by heat treatment (H1, H5 compared to C, N1 and N5). The number of passes further increased it for denatured samples (H5 compared to H1). In addition, the combined effect of heat and number of passes significantly ($P<0.05$) increased the concentration of adsorbed protein on the surface of oil droplets (Table 4.2). The emulsifying activity index is a function of oil volume fraction, protein concentration and the type of equipment used to produce the emulsion (Pearce and Kinsella, 1978). Generally heat may reduce the emulsifying characteristics of proteins due to irreversible protein denaturation; however, partial protein unfolding would improve emulsifying ability (Phillips et al., 1990b). Both surface hydrophobicity which affects the affinity of the

protein for the oil-water interphase and molecular flexibility which influences the ability to unfold and interact with other proteins are important in determining emulsifying activity (Monahan et al., 1993). However the results revealed an increase in the emulsifying activity in heat denatured samples. Therefore the affinity between protein and dispersed phase might be greater than that of protein-protein and thus imparting a thermodynamically more favourable condition to form stable emulsions. This situation would have been assisted by the method used for emulsion preparation during homogenisation of emulsions using dynamic high pressure at 140 MPa. The capacity of protein to stabilize emulsions is related to the interfacial area that can be coated by proteins (Pearce and Kinsella, 1978). The exposure of buried hydrophobic groups upon heat treatment may have caused this enhanced emulsification. In addition, microfluidization may have further increased the emulsion properties of heated samples by dispersing micro aggregates and changing surface properties by unmasking hidden hydrophobic residues and repositioning them towards oil phase. All emulsions produced in this study had high emulsion stability regardless of treatments. Emulsion stability depends on the consistency of the interphase, which does not change with time. Emulsions with an appropriate pH and increased net negative charge present a barrier to the close approach of droplets thus retarding the rate of coalescence, resulting in more stable emulsions (Klemaszewski and Kinsella, 1991). In our study, the experiments were carried out at neutral pH. At this pH, WP have a net negative charge which in turn imparts greater emulsion stability by retarding coalescence through repulsion.

Results also showed (Table 4.2) that the foaming properties of WP were detrimentally affected by heating (H1, H5 compared to C, N1 and N5) while the number of passes significantly ($P < 0.05$) increased the foam overrun (N1 compared to C) and stability (N5 compared to C and N1). The extensive aggregation of WP caused by heat denaturation may have reduced the ability of proteins to produce stable films. However all non-heat treated WP samples (N1 and N5) along with controls also did not show good foaming ability. Lipids in WPC can seriously impair the foaming ability since surface active, polar lipids interfere with protein films by situating themselves at air/water interface (Fennema, 1996). Proximate analysis (Table 4.1) revealed of considerable fat content in our samples. In addition to that fact, these interfering substances possess weak cohesive and visco-elastic properties to overcome

the internal pressure of air bubbles compared to WP. As a result bubbles expand and finally collapse rapidly resulting in poor foaming. According to our results, dynamic high pressure shearing has positively affected both foam overrun and stability. The foaming properties of WP concentrates are significantly correlated with the amount of β -Lg, presenting approximately 50% of the total WP (Fitzsimons et al., 2007), and the extent of WP denaturation (Phillips et al., 1990).

4.3.3 Flow behaviour of whey protein dispersions

Table 4.3 presents the rheological parameters of 12% (w/w) WP dispersions obtained by fitting the data to the Herschel-Bulkley model. The results show that the combined effect of heat and shearing significantly ($P < 0.05$) increased the n value (H1 and H5 compared to unheated samples), while heat treatment markedly ($P < 0.05$) decreased the k value and yield stress of H1 sample compared to unheated samples. The viscosity behaviour of proteins is a result of several factors, including the size, shape and polydispersity of protein molecules and their aggregates, protein-solvent interactions, hydrodynamic volume and molecular flexibility of proteins in their hydrated state (Damodaran et al., 2008; Rao and Steffe, 1992). Figure 4.5 and Table 4.3 show the higher yield stress of N1 compared to all other samples, and also the relatively prominent shear-thinning nature of native preparations (*i.e.*, higher slopes) compared to denatured species. Shear-thinning behaviour of protein solutions can arise due to the orientation of the major axes of protein molecules in the direction of flow, as well as dissociation of weakly held dimers and oligomers into monomers (Damodaran et al., 2008). The effect of hydrodynamic pressure on globular WP is distinctly different from that of hydrostatic pressure. During application of dynamic high pressure (MFZ), the collision, shearing and flowing of molecules may occur leading to contraction and, more importantly, elongation and flowing of molecules (Akkermans et al., 2008).

In addition to quaternary and tertiary structures, the secondary conformation of WP, *i.e.* β -sheets and α -helices are also more prone to be affected as a result of dynamic high-pressure shearing due to perturbation of comparatively weaker interactions such as hydrogen bonds between protein strands (Akkermans et al., 2008).

The particle size distribution pattern, obtained in this study (Figure 4.2), showed a change in average particle size of unheated MWP. The significantly ($P < 0.05$) higher yield stress of N1 compared to the control is also indicative of the existence of greater protein-protein and protein-solvent interactions. However, extensive shearing (N5) may have caused further stretching of protein molecules, possibly leading to exposure of individual protein strands in their secondary structures. This may result in partial denaturation and likely subsequent aggregation via newly exposed reactive sites. This situation may be further explained by significantly increased n values and reduced k and τ_0 values of N5 samples and widened particle size distribution pattern of this sample (Figure 4.2). Interestingly, although viscosity values of heat-treated samples were lower at low shear rates than their unheated counterparts, it was even higher than native preparations at high shear rates (Figure 4.5). Additional conformational rearrangements of heat-treated MWP samples may have occurred at higher shear rates leading to greater particle-particle and particle-solvent attractive interactions and thus higher apparent viscosity.

Table 4.3 Rheological parameters of whey protein dispersions obtained by fitting the experimental data to Herschel-Bulkley model

Treatment	Herschel-Bulkley			
	τ_0 (Pa)	k' (mPa.s ^{n'})	n'	R
Control	2.26 ^{bc}	14 ^c	0.76 ^a	0.98
N1	5.62 ^d	12 ^{bc}	0.70 ^a	0.98
N5	2.91 ^c	9 ^{ab}	0.75 ^a	0.99
H1	0.57 ^a	7 ^a	0.89 ^b	0.99
H5	0.95 ^{ab}	9 ^{ab}	0.84 ^b	0.99
*SEM	0.53	1	0.02	0.002

Treatment – Native control; N1 – non heat treated, 1 MFZ pass; N5 - non heat treated, 5 MFZ passes; H1 - heat treated, 1 MFZ pass; H5 - heat treated, 5 MFZ passes. k' – consistency index. n' – flow behavior index. R – correlation ratio. τ_0 - yield stress. *SEM - Pooled standard error of the mean, $P < 0.05$. Means present the average of at least 8 independent observations (n=8). The different small letter superscripts in a column indicate significant difference ($P < 0.05$).

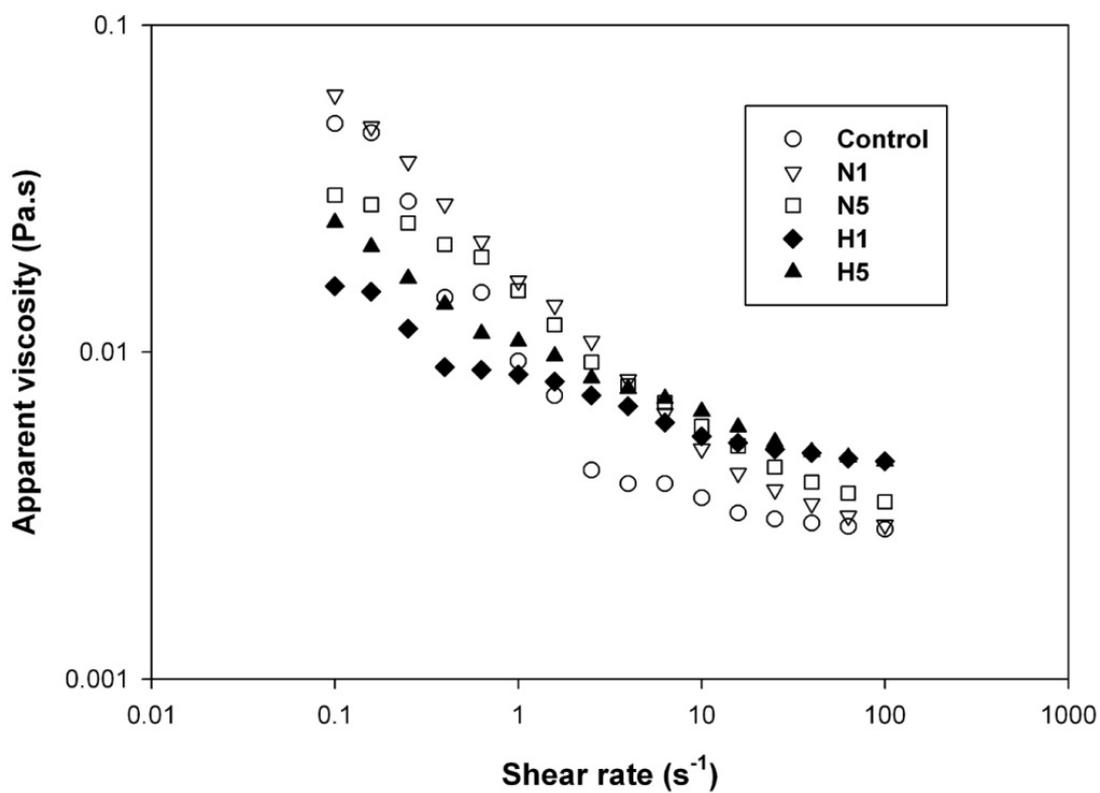


Figure 4.5 Apparent viscosity of 12% (w/w) WP dispersions at 20°C during a controlled shear rate sweep (0.1 - 100 s⁻¹). N1 = native, 1 pass; N5 = native, 5 passes; H1 = denatured, 1 pass; H5 = denatured, 5 passes.

4.3.4 Heat-induced gelation

During heat-induced gelation, conformational changes of proteins occur with protein unfolding and exposure of reactive sites such as thiol groups and hydrophobic groups followed by intermolecular aggregation and progressive development of an infinitely cross-linked and self-supporting network created from protein-protein and protein-solvent interactions (Xiong et al., 1993; Resch, 2004; Resch et al., 2004b; Matsudomi et al., 1993; Hudson et al., 2000). In addition, ionic interactions, hydrogen bonding and calcium bridges are equally important in formation of infinite viscoelastic gel network (Brandenberg et al., 1992).

Figure 4.6 shows the changes in storage modulus (G') of 12% (w/w) native and denatured WP dispersions, including an untreated control, during heating, holding, cooling and aging at constant strain and frequency. Only unheated WP preparations (N1 and N5), which could unfold and aggregate *via* various molecular interactions as described above, formed heat-induced gels. Also, the G' value increased with the number of microfluidizing passes. Formation of heat-induced gels from denatured WP (H1 and H5) is unlikely even if they were subjected to high-pressure shearing. This is mainly due to the unavailability or insufficient number of required reactive sites for cross-linking, such as thiol groups and hydrophobic groups in denatured samples.

MFZ may have a disruptive effect on protein conformation by affecting different inter- and intra-molecular interactions. Therefore, dissociation, reversible changes of the conformation and unfolding of WP molecules, and activation of the thiol group may occur under MFZ conditions, with generation of higher numbers of activated molecules available for further heat-induced aggregation, and thus a greater solid-like behaviour compared to untreated controls. Furthermore, the elasticity of the gel is directly proportional to the density of cross-linking in the network (Phillips et al., 1994). Therefore, MFZ may have changed the conformation of proteins, which in turn may have facilitated covalent chain reactions and attractive non-covalent (hydrogen, hydrophobic, van der Waals forces) protein interactions, enhancing the extent of intermolecular cross-linking (Alting et al., 2000). This was reflected by increased G' value of gels, especially, in extensively microfluidized sample (N5). During heat-induced gelation, hydrogen bonds present in native form of proteins are disrupted (Damodaran et al., 2008).

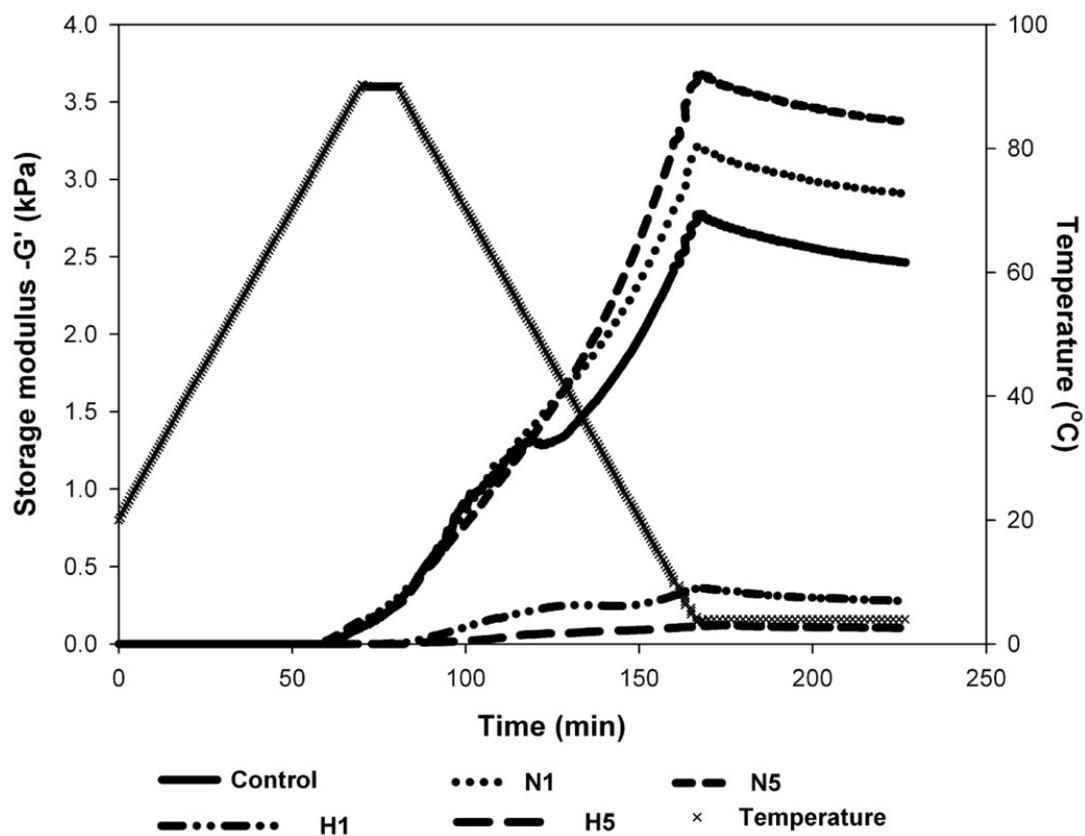


Figure 4.6 Changes in storage modulus (G') of 12% (w/w) native (N) or denatured (H) WP dispersions microparticulated by either 1 or 5 MFZ passes, during heating (20 - 90°C), holding (90°C / 10 min), cooling (90 - 4°C) and aging (4°C/approx 60 min) at constant strain (1%) and frequency (1 Hz).

However, upon cooling, these bonds may again re-establish, as observed by increased G' values of the gels. In addition, the slight decrease of G' again during gel aging may have resulted from reversibility or disruption of some hydrophobic interactions, which are generally less favoured at low temperatures (Damodaran et al., 2008).

4.3.5 Cold gelation

Figure 4.7 shows the development of storage modulus (G') during cold gelation of 12% (w/w) heat-treated WP dispersions and non-heated controls, and WP dispersions microparticulated by 1 MF pass (A) or 5 passes (B) at 20°C, at constant strain (1%) and frequency (1 Hz). The measurements were taken within the linear viscoelastic region of these gels (between 0.01 and 10% strain).

In general, cold gelation involves initial controlled thermal denaturation of proteins. In the second step, the solvent quality is changed by salt addition or acidification, inducing gelation process at low temperatures (Alting et al., 2000; Cavallieri and da Cunha, 2008). In the current study, a slightly different approach involved utilizing MWP powders for preparation of cold-set gels. In contrast to heat-induced gels, heat-treated MWP samples created cold-set gels as shown in Figure 4.7 - A and -B.

During cold gelation, even samples H1 and H5 gelled without GDL or salt addition although the gel firmness of these samples was comparatively low. It is quite possible for denatured WP at a sufficiently high concentration, such as 12% as used in this study, to form a viscoelastic gel network via intermolecular protein-protein as well as protein-water interactions through exposed reactive sites (Rao and Steffe, 1992). In comparison, unheated N1 and N5 samples did not form gels under the same conditions since they were not unfolded WP molecules for the subsequent aggregation.

In the presence of GDL or salts, H1 and H5 samples created firmer gels compared to heat-treated controls, as indicated by higher G' values. In general, increasing the number of MFZ passes increased the G' values of cold-set gels, indicating the existence of comparatively higher intermolecular interactions in the viscoelastic system. As clearly shown by Figure 4.7, firmer WP gels could be produced with 0.1 M NaCl in both instances (H1 and H5) than with 0.5 M NaCl.

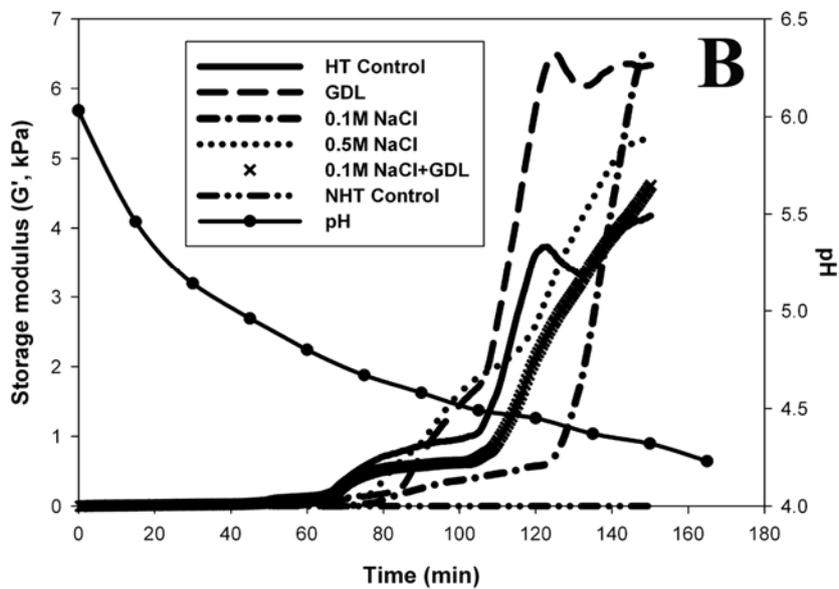
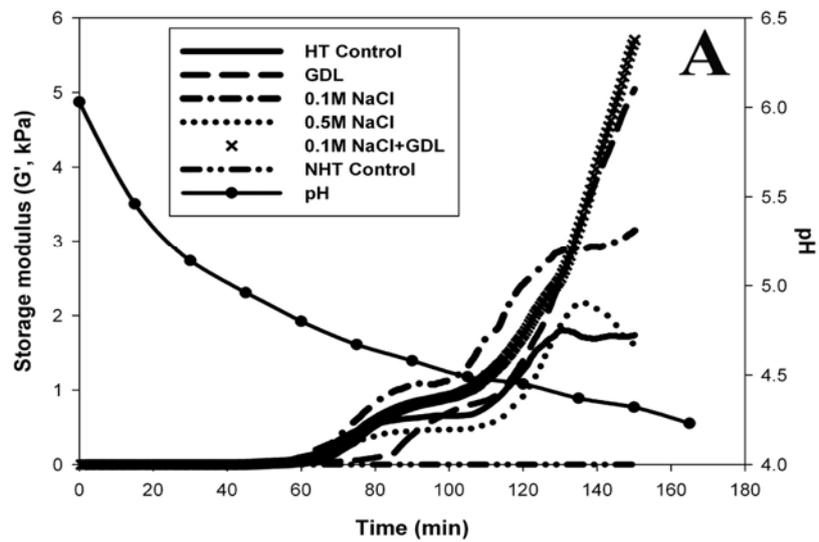


Figure 4.7 Changes of storage modulus (G') during cold (GDL, salt) gelation of 12% (w/w) denatured WP dispersions microparticulated by 1 MFZ pass (A) or 5 MFZ passes (B). Measurements were performed at 20°C at constant strain (1%) and frequency (1 Hz).

As described by Verheul and Roefs (1998a), monovalent salts primarily interact with charges on the protein at up to 0.1 M - 0.2 M NaCl, increasing intermolecular interactions, but, beyond that, charges are saturated and NaCl concentration affects the solvent properties (Brandenberg et al., 1992). Therefore, this may have happened during cold gelation of MWP at higher salt concentration (0.5 M NaCl) in this study, which had a negative effect on gel strength compared to the results obtained at lower salt concentrations. In addition, H5 samples with either 0.1 M NaCl or GDL produced the strongest gels, indicating the equal importance of shielding of charges as well as acidification in reducing repulsion between proteins during the gelation process. When both 0.1 M NaCl and GDL were present in the medium, the strength of H5 gels was lower compared to that of H1. These differences were likely due to the conformational changes of proteins obtained under varying high pressure shearing conditions, which may have affected all types of molecular interactions. In addition, the elongation of protein molecules during shearing likely played a role in the alignment of molecules, thus enhancing their interactions. The different effects of the extent of MFZ are further revealed by the G' values of H1 and H5 gels produced with GDL alone. Although the maximum G' value of H5 gel created with GDL is higher than that of H1 gel, further acidification has made the H5 gel brittle (as shown by the graph), possibly due to enhancement of repulsive positive charges on proteins. The nature of these gels is further explained by Figure 4.8 -A & -B, irrespective of their high G' values. The direct dependency of G' on frequency indicates that these gels were weaker or more brittle compared to firmer gel networks with an elastic plateau (Rao, 1999). However, if a frequency sweep over 3 to 4 orders of magnitude with much lower frequencies was used to comprise larger relaxation times it may have represented a more detailed picture of the mechanical spectrum of these protein gels.

4.3.6 Electrophoretic analysis

Immediately after thermal and cold-set gelation, WP samples were analyzed by native PAGE and SDS-PAGE to determine the nature of interactions among proteins. Figure 4.9 -A shows the results of native PAGE (with or without Tween 20) and 4.9 -B shows the SDS-PAGE (reducing or non-reducing) patterns of C (control), N1 and N5 WP samples during heat-induced gelation. The additional control sample was prepared directly from untreated, spray-dried WP powder and contained no Tween 20 in the buffer (*i.e.*, Control).

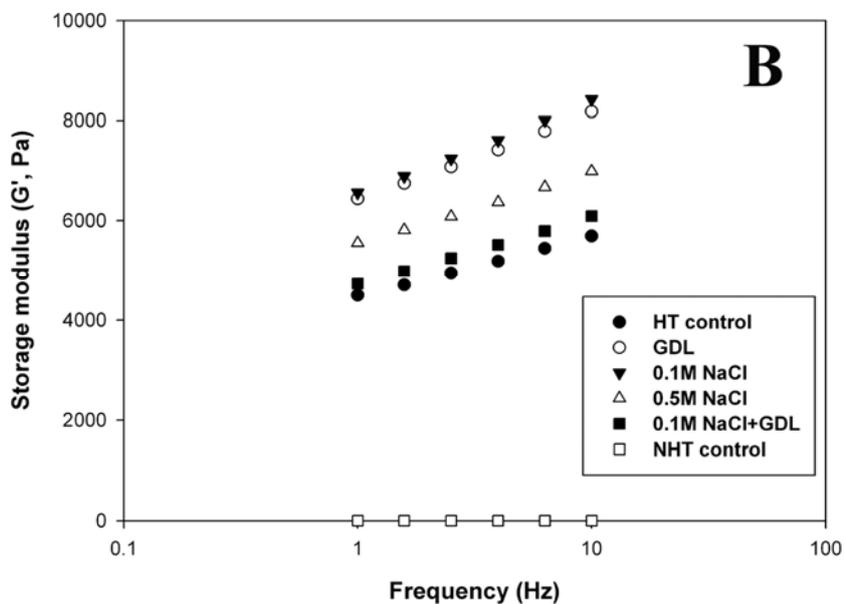
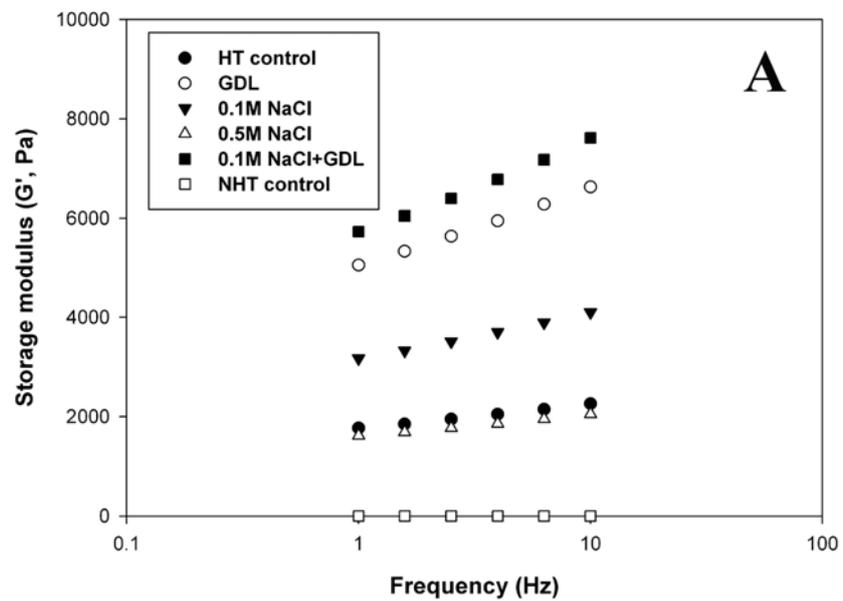


Figure 4.8 Viscoelastic properties after 150 min, expressed as storage modulus (G'), of 12% (w/w) denatured WP preparations microparticulated by 1 MFZ pass (A) or 5 MFZ passes (B) obtained by a frequency sweep (1 – 10 Hz) at a constant strain (1%) and 20°C.

As indicated by the native PAGE patterns without Tween 20 (Figure 4.9 -A), most of the protein bands originally present in the Control sample disappeared in C, N1 and N5 samples, indicating protein aggregation during heating. Very faint bands of monomeric α -LA and β -LG were still present, indicating the presence of low levels of their native forms under these conditions. The presence of these bands could also be attributed to higher lactose content in these WP preparations, which can improve the heat stability of WP (Mulsow et al., 2009).

In contrast, BSA bands were absent from this PAGE gel, which indicated their complete denaturation. These bands, however reappeared in the presence of Tween 20 (Figure 4.9 -A), indicating the importance of hydrophobic interactions during the aggregation of BSA, as Tween 20 is a weak non-ionic surfactant which affects hydrophobic interactions (Baldwin, 2009). As shown by Figure 4.9 -B, the protein bands on non-reducing SDS-PAGE are relatively less prominent than the bands on reducing PAGE, indicating that heat-induced protein aggregation has mostly occurred *via* covalent bonding (Patel et al., 2006). In addition, some disulfide-linked small aggregated proteins, possibly dimers of β -LG, were also visible without resolving under non-reducing SDS conditions.

Figure 4.10 shows the SDS-PAGE (A or B – under non-reducing or reducing conditions, respectively) patterns of WP after cold-set gelation. Protein bands of denatured WP from all types of preparations could not be observed in the native PAGE gels regardless their ability to form cold-set gels (not shown). This could again be attributed to the size of aggregates created during cold gelation and their inability to move through the electrophoretic gel. However, monomeric BSA bands again became slightly visible in the presence of Tween 20, especially in H5 samples (not shown), in contrast to SDS gels under non-reducing conditions (Figure 4.10 -A), where all protein bands were absent.

Furthermore, Figure 4.11 -A and -B show the changes in storage modulus (G') during cold gelation (either with 2% GDL or 0.1 M NaCl) of 12% (w/w) MWP preparations (H1 and H5 respectively) in the presence of SDS or NEM, and two additional samples of H1 and H5 without SDS and NEM.

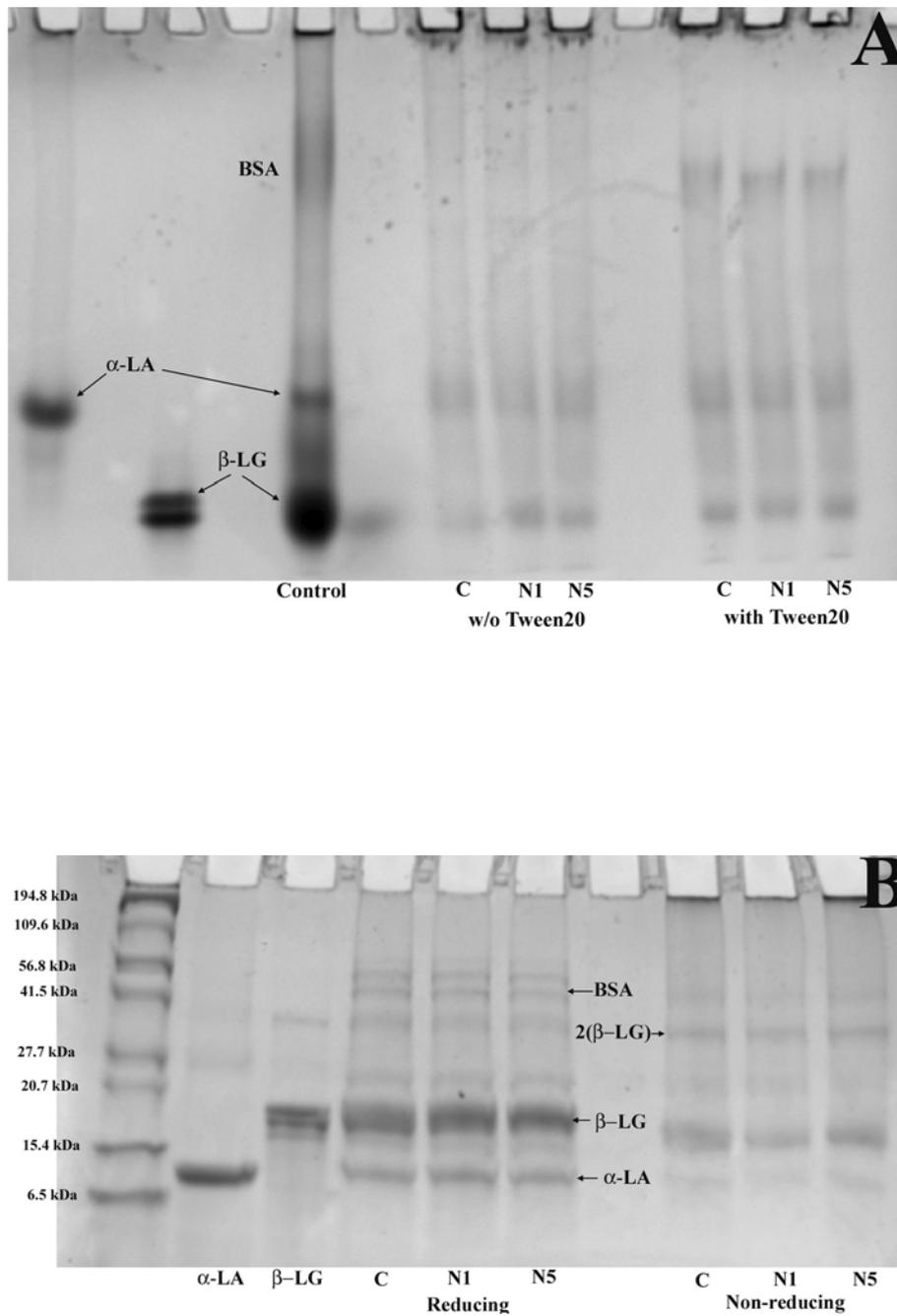


Figure 4.9 Electrophoretograms of WP dispersions analyzed by Native PAGE (A), with or without Tween 20 and SDS-PAGE (reducing or non-reducing) (B); (C - native control; N1 - native, 1 MFZ pass; N5 – native, 5 MFZ passes) after heat-induced gelation. Control was untreated native powder which was not gelled.

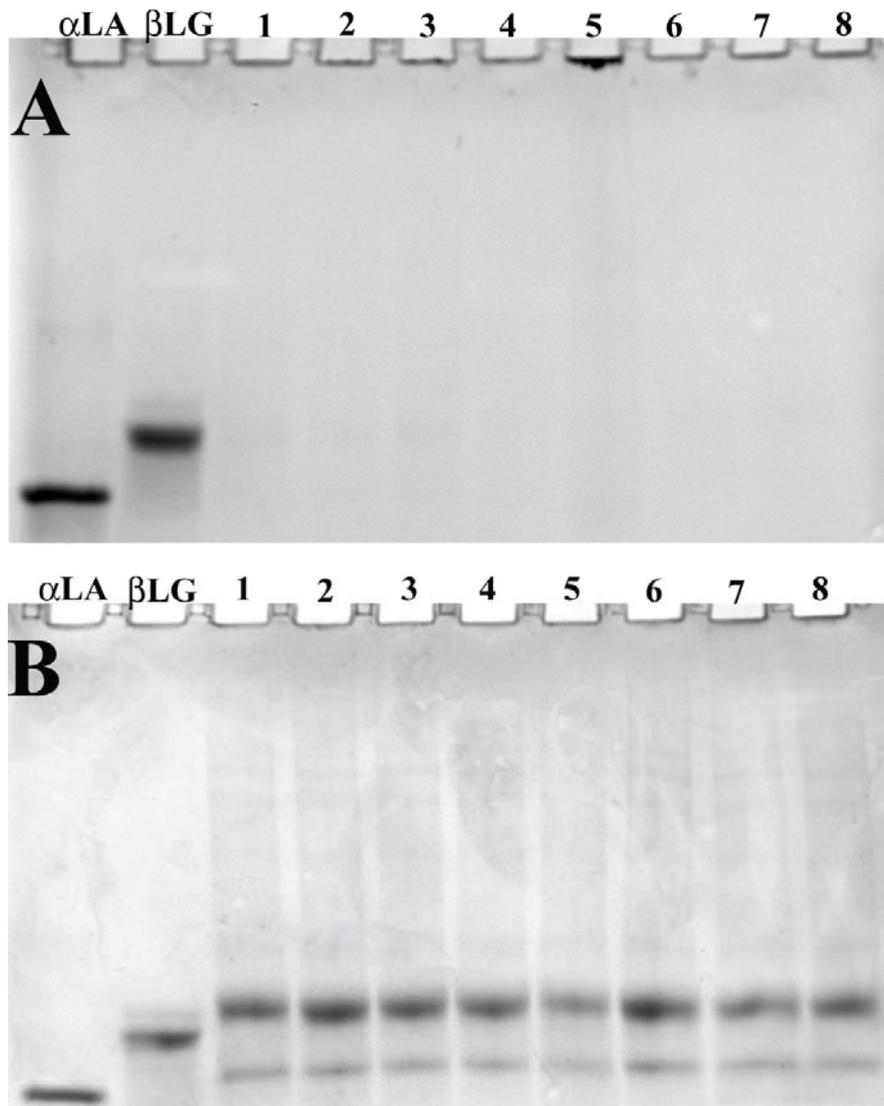


Figure 4.10 Non-reducing (A) and reducing (B) SDS-PAGE patterns of WP dispersions (H1- heat treated, 1 MFZ pass; H5 - heat treated, 5 MFZ passes) after cold-set gelation. Lanes: α LA – α -lactalbumin standard; β LG – β -lactoglobulin standard; 1 - H1 + GDL; 2 - H1 + 0.1 M NaCl; 3 - H1 + 0.5 M NaCl; 4 - H1 + GDL + 0.1 M NaCl; 5 - H5 + GDL, 6 - H5 + 0.1 M NaCl; 7 - H5 + 0.5 M NaCl; 8 - H5 + GDL + 0.1 M NaCl.

As shown in Figure 4.11 -A, GDL-acidified H1 gels (without SDS or NEM) were stronger than those produced with 0.1 M NaCl. NEM is a thiol blocker and, in its presence, formation of new covalent bonds is prevented, whereas SDS is an ionic surfactant which blocks non covalent interactions (Havea et al., 2009). However, the strength of acid or salt gels, as indicated by G' values in the presence of NEM was always higher than that of those prepared with SDS, further supporting the observations that non-covalent interactions prevail during cold gelation processes of H1 samples. In addition, as Figure 4.11 -B indicates, when acid gelation of H5 samples was carried out in the presence of either NEM or SDS, the strength of these gels was found to be comparable. This trend was also observed in salt-induced gels. This may indicate equal contribution of both covalent and non-covalent interactions during cold-gelation process of the H5 samples. Apparently, hydrodynamic high-pressure shearing altered the protein conformation and existing interactions among WP by disrupting certain associations and also promoting their reactivity by exposing buried reactive sites or creating new reactive sites which may affect both covalent and non-covalent interactions, and thus the final gel characteristics. In addition, the extent of MFZ also plays an important role, as shown by the different physical characteristics of H1 compared to H5 MWP.

4.3.7 Water-holding capacity, permeability and surface reflectance

As shown in Table 4.4, heat-set gels had almost 100% water-holding capacity (WHC). WHC quantitatively indicates water retention within a protein matrix under defined conditions and reflects the pore size of the gels (Huang and Kinsella, 1986). The extensive hydrodynamic shearing significantly ($P < 0.05$) increased the WHC of heat-set gels, which may be related to increased charge distribution on proteins, resulting in enhanced water-protein interactions (Damodaran et al., 2008). This positive effect of MFZ on the compactness of gels was further supported by permeability coefficients of heat-set gels. Permeability coefficients of heat-set gels (N1 and N5 samples) were significantly ($P < 0.05$) reduced compared to the untreated control (Table 4.4), reflecting their relative compactness.

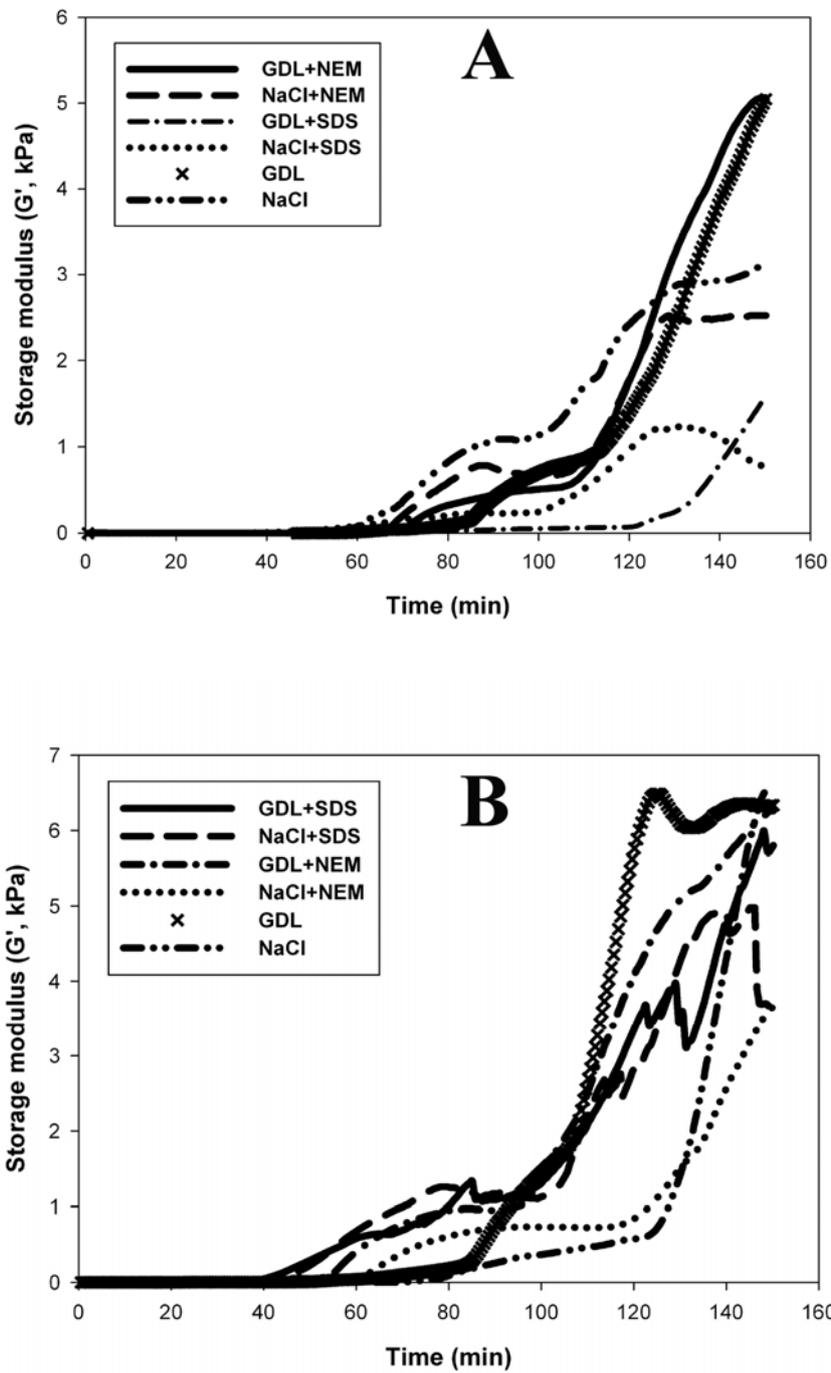


Figure 4.11 Changes in storage modulus (G') during cold gelation of 12% (w/w) denatured WP preparations of H1 (A) and H5 (B) with GDL or 0.1 M NaCl, and in the presence of SDS or NEM at constant strain (1%) and frequency (1 Hz).

Table 4.4 Physical characteristics of heat-set gels

Treatment	G' (90 °C), kPa	G' (4 °C), kPa	Permeability coefficient, m ²	WHC, %	Colour		
					L*	a*	b*
Control	2.8	2.5	6.61 10 ^{-17b}	98.8 ^a	91.1 ^a	-2.58 ^a	11.8 ^c
N1	3.2	2.9	3.32 10 ^{-17a}	99.3 ^a	92.4 ^b	-3.15 ^b	9.8 ^b
N5	3.7	3.4	3.95 10 ^{-17a}	99.0 ^a	96.0 ^c	-3.46 ^c	8.8 ^a
*SEM	0.2	0.2	5.92 10 ⁻¹⁸	1.51	0.32	0.08	0.27

Treatments - N1 – non heat treated, 1 MFZ pass; N5 - non heat treated, 5 MFZ passes; G' - Represents the average value of gel storage modulus. GDL – Glucano δ lactone. WHC – Water holding capacity. L* - Lightness. a* - Positive a* is red and negative a* is green. b* Positive b* is yellow and negative b* is blue. *SEM - Pooled standard error of the mean, $P < 0.05$. Means present the average of at least 8 independent observations (n=8). The different small letter superscripts in a column indicate significant difference ($P < 0.05$).

The permeability coefficient (B) is positively correlated with the pore size (Lee and Lucey, 2004), and lower B values of microfluidized samples are indicative of a more compact network with smaller pores. Hydrogen bonding and hydrophobic interactions are the main contributors to network formation except when multivalent ions are involved in cross-linking (Damodaran et al., 2008). In addition, formation of greater amount of disulfide bonds in MWP gels compared to untreated controls may be possible due to exposure of hidden reactive sites. This, on the other hand may contributed to the creation of a stronger network with greater cross-linking.

The appearance of heat-induced gels as indicated by color parameters, *i.e.* L* (lightness), a* (red to green) and b* (yellow to blue), is provided in Table 4.4. Microparticulation significantly ($P < 0.05$) changed the colour of heat-set gels, compared to the control gel samples as indicated by these parameters. Increased lightness of gels with extensive MFZ (5 passes) also reveals higher opacity of these gels and greater compactness. Attractive hydrophobic interactions and repulsive electrostatic interactions fundamentally control gelation mechanism and the gel appearance (Damodaran et al., 2008). Thus, hydrodynamic shearing may result in exposure of buried reactive sites which may lead not only to enhanced hydrophobic interactions and covalent bonding but also to increased ionic interactions and, finally, creation of a stronger protein-protein network and greater protein-solvent interactions, producing highly opaque gels.

4.4 Conclusions

Combined heat treatment and high pressure shearing produced micro-aggregates of WP with enhanced heat stability. However, the particle size of these aggregates was large enough to accelerate sedimentation, which in turn resulted in a poor solubility. Amount of adsorbed proteins on surface of fat droplets also increased with combined treatment, which consequently increased emulsifying activity index. High pressure shearing alone improved foaming properties of native WP. In addition, high pressure shearing fragmented denatured WP into smaller entities. Microfluidization could be a useful method for stabilising WP against heat induced changes by producing microparticulated species, which have different surface and colloidal properties.

Microparticulation of unheated or heat-treated WP produced protein powders with distinguishably different gelling behaviour. The unheated MWP produced more compact heat-set gels compared to untreated controls. Although heat-set gels were mainly created *via* covalent interactions, high-pressure shearing likely changed the protein conformation, thus affecting all other molecular interactions which may have been reflected by facilitated reactivity of proteins during heat gelation. Meanwhile heat-treated MWP created cold-set gels which were formed primarily via non-covalent associations under a reduced number of microfluidizing passes. Extensive MFZ has increased the prevalence of interactions between proteins since under these circumstances, both covalent and non-covalent interactions were found to be involved with increasing the strength of both acid and salt gels. The disruption of aggregates which were created during heating and thereby the reduction of particle size with creation of new reactive sites as well as exposure of buried reactive sites may have apparently governed the increased reactivity of MWP.

Most notably, the size of aggregates appeared to play an important role in creation of gels, as stronger gels were formed by smaller particles. Slight process modifications, including simultaneous heating and hydrodynamic high-pressure

shearing under controlled environmental conditions (pH, ionic strength) during microparticulation, may lead to further particle size reduction. For example, the average particle size of hydrated WP aggregates, which were prepared after heat-treating the protein dispersions at acidic pH and then freeze drying and grinding the formed gels, was 25.3 μm (Hudson et al., 2000). However, the average particle size of MWP produced at pH 7 in our study was also around 10 μm . Further reduction of the particle size thus appears achievable at a lower pH at which the reactivity of thiol groups is suppressed. Newly formed species would thus have small particle size and likely greater reactivity in forming gels under different conditions, all of which would greatly enhance usage of MWP in the food industry.

CHAPTER 5

5 Kinetics of whey protein denaturation as a function of pH and protein concentration

5.1 Introduction

The heat sensitivity of whey proteins is a major problem on their application in different food products regardless of their valuable nutritional benefits and excellent functionalities (De Wit, 1990). Microparticulation of whey proteins is a novel approach which was introduced to overcome negative aspects of whey proteins resulting from heat application during food processing (Iordache and Jelen 2003). As reported in Chapter 4, heat-denatured and microparticulated WP showed improved heat stability and emulsifying properties around neutral pH. Un-denatured but microfluidized whey proteins also exhibited some functional improvements including gelling, foaming and colloidal properties. However, the particle size of heat-treated MWP remained $\sim 10 \mu\text{m}$ as shown in preceding Cs and appeared to be of a fundamental importance especially if compared to sub-micron size un-denatured WP. The particle size mainly affects the colloidal properties of WP such as aqueous solubility which on the other hand has an influence on almost all other functional properties (Sava et al., 2005; Bouaouina et al., 2006). Hence, it is rather important to find other possible pathways to produce denatured MWP powders with adequate, small enough particle size. This would in turn enable them to be used successfully in different food applications where their functionalities may be utilized fully. In addition, it can be hypothesized if the heat treatment may create a WP gel network with relatively weak molecular associations the mechanical forces imposed during high pressure homogenization/microfluidization would break them easily and subsequently produce WP powders with relatively smaller particle size. Therefore the knowledge of aggregation mechanisms of WP is quite useful to avoid unnecessary stronger intermolecular interactions among WP and achieve WP gels created with desired weaker bonds in this particular instance.

The thermal transition of native WP occurs in the temperature range of 62 – 78°C. In a WP mixture, the rate of denaturation of individual proteins can be arranged in the order: α -La > BSA > immunoglobulins > β -Lg (Fox and McSweeney, 2003). When subjected to heat, the unfolding and consequent aggregation of WP take place via numerous molecular interactions such as sulfhydryl/disulfide interchange, hydrophobic, electrostatic, hydrogen bonding and van der Waals forces (Damodaran et al., 2008; Zue and Damodaran, 1994). The rates and pathways of these physicochemical reactions depend on the environmental factors including pH, protein concentration, temperature and heating time, α -La/ β -Lg ratio, ionic strength and solvent condition and the influence of other surrounding molecules (de la Fuente et al., 2002; Brandenberg et al., 1992; Marangoni et al., 2000; Rabiey and Britten, 2009; Anema and McKenna, 1996; de Wit, 2009; Le Bon et al., 1999; Verheul et al., 1998).

Thermal stability of WP is greatly affected by pH since the electrostatic effects (repulsion/attraction) prominently influence the kinetics of their unfolding and aggregation. At neutral pH, the WP have a net negative charge with facilitated reactivity of thiol groups. Consequently upon heating, the WP aggregates with strong covalent disulfide bonds are formed (de la Fuente et al., 2002). The heat induced protein gels containing disulfide bonds show a rubbery nature with high fracture strain (Havea, et al., 2009). This indicates the relative strength of molecular interactions within the gel network, which, on the other hand, creates difficulties due to their poor dispersion leading to a greater particle size upon spray drying. WP are more stable against denaturation at their isoelectric pH than at any other pH due to minimum intra-molecular repulsions (Damodaran et al., 2008). Conversely at acidic pH below their isoelectric point, WP are overall positively charged, resulting in relatively increased intramolecular repulsion facilitating the unfolding of these globular proteins. However, the involvement of thiol/disulphide interchange reactions appears unlikely because of the impaired reactivity of thiol groups under these conditions (de la Fuente et al., 2002) and WP aggregates may be mostly formed through non-covalent interactions. The WP

gels formed with non-covalent interactions characterize with low fracture strain and thus they are comparatively brittle (Havea, et al., 2009).

Temperature and protein concentration play an important role in heat induced unfolding and aggregation mechanisms of WP (Anema and McKenna, 1996). It has been identified that the protein concentration mostly affects the aggregation step rather than the unfolding of WP; for example, high protein concentrations produce higher molecular weight aggregates (Fitzsimons, et al., 2007). In addition, the extent of aggregation and the types of interaction involved in the process were apparently dependant on the protein concentration noted through the prevalence of covalent interactions among β -Lg molecules increased with the rise of WPC concentration (de la Fuente et al., 2002). Furthermore, at higher temperatures and at higher pH values, smaller WP aggregates are formed with faster aggregation rates (de la Fuente et al., 2002).

The purpose of the current study was to examine the reaction kinetics of whey protein aggregation as a function of pH and protein concentration. It is expected that this study would assist in selecting a more feasible pathway to produce MWP powders with comparatively reduced particle size.

5.2 Materials and methods

5.2.1 Materials and proximate composition

The experiment was carried out with whey protein retentates and corresponding whey permeates that were kindly provided by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). They were high protein retentates containing around 30% total solid and collected on two different days. All the chemicals used in the study were of analytical grade and Milli Q water was used to dilute all the samples and to prepare required reagents and buffers.

The compositional analysis of WP retentates and whey was conducted as reported in Chapter 4 and presented in Table 5.1. Well established Kjeldahl method and a nitrogen conversion factor of 6.38 were applied in protein determinations; moisture content was determined by oven drying samples at 105°C until a constant weight was achieved; ash content was estimated by combusting pre-solidified samples in a muffler furnace at 550°C; fat content was determined using Mojonnier method, 989.05 (modified); calcium content was determined by atomic absorption spectrophotometry. Phosphorus content was determined using a colourimetric method with vanadomolybdophosphoric acid. Lactose concentration was measured by an established HPLC method (Vasiljevic and Jelen, 2003).

5.2.2 Sample preparation

The kinetics of WP denaturation or aggregation was studied with respect to the concentration and pH. Samples were prepared as three different concentrations and each concentration was adjusted to three different pH values. Two samples were prepared by diluting WP retentates with corresponding whey permeates to 10, 17.5% (w/w) protein and the original retentate sample (protein content at ~ 25% w/w) was used as the third sample without diluting. Then each sample was adjusted to pH 4 and 5 with concentrated NaOH or HCl before correcting their final volumes. One sample was kept at the original pH ~ 6 as a control. The samples collected in two different days served as two replicates. These preparations were subjected to a heat treatment for further kinetic studies, thermal analysis with differential scanning calorimetry (DSC), rheological studies and heat-induced gelation.

Table 5.1 Proximate composition of WP retentates and permeates

Component	Retentate	Permeate
Total solids, %	31.33±0.21	3.74±0.17
Protein, % w/w	26.96±0.25	0.23±0.02
Fat, % w/w	1.09±0.17	0.05±0.02
Lactose, % w/w	2.26±0.32	4.14±0.57
Ash, % w/w	0.93±0.02	0.5±0.02
Ca, mg / L	0.15±0.02	0.05±0.01
P, mg / L	0.1±0.01	0.04±0.00

The data reported represent the mean of at least 6 independent observations (n=6).

5.2.3 Heat treatment and analysis of physical properties

Each preparation was heat-treated for 5, 10, 15 or 20 s separately at 140°C in an oil bath (Ratek, Boronia, Australia) followed by immediate cooling in an ice-water bath. The non-heated preparations served as controls. The extent of protein aggregation in each sample was examined using solubility and turbidity methods, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and capillary electrophoresis as described below.

The heated samples and unheated controls were immediately diluted with Milli-Q water to 0.1% w/w protein and the absorbance measurements were obtained at 20°C using a spectrophotometer (Pharmacia LKB; Novaspec[®] II) with 1.0 cm path-length cuvettes. The turbidity of the samples was expressed as the apparent optical density at 420 nm (Ju and Kilara, 1998).

The treated samples were diluted to 10% (w/w) protein concentration and centrifuged for 20 min at 3000 \times g. The supernatants were filtered through 0.45 μ m filters and the solubility was determined as explained in Chapter 4 using the Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA) with a standard curve ($r^2 = 0.9913$) developed with bovine serum albumin (Sigma-Aldrich, Chemie GmbH, Steinheim, Germany).

All samples were adjusted to 10% (w/w) protein and centrifuged at 14,000 \times g for 20 min and the supernatants were used to estimate the extent of denaturation using reducing SDS PAGE as previously described in Chapter 4.

The thermal behaviour of WP preparations was assessed following the procedure previously reported in the fourth Chapter using a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwalk, CT, USA) equipped with a software (Pyris Manager, v.5.0002). The temperature scanning was performed within the temperature interval from 25 to 140°C at 10°C / min. The ΔH values, on-set and end-set temperatures and the temperatures at peak height were recorded.

5.2.4 Flow and viscoelastic behaviour of WP dispersions

Flow behavior of WP dispersions was studied using a CS/CR rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with a software (Rheoplus/32 v2.81, Anton Paar). About 3.9 g of each WP dispersion was introduced into the double gap cylinder measuring system (DG26.7- SN7721, Anton Parr) and a thin layer of low-density oil was spread on top of the sample to minimize moisture loss. The mixture was pre-sheared for 5 s at a constant shear rate of 500 s^{-1} at 20°C and held for 30 s to equilibrate before data collection. Apparent viscosity of WP samples was recorded as shear rates were ramped over the range of $0.1 - 100 \text{ s}^{-1}$ for 5 min at 20°C . A rheological model, *viz.*, Power Law (Ostwald model); $\sigma = k\dot{\gamma}^n$, which is applied in predicting flow characteristics of foods (Rao, 1999), was used to explain the behaviour of flow curves; where, k , n , σ , and $\dot{\gamma}$ as consistency coefficient ($\text{Pa}\cdot\text{s}^n$), flow behavior index (dimensionless), shear stress (Pa), and shear rate ($1/\text{s}$) respectively.

Heat gelation of WP dispersions was carried out using the same double-gap geometry. The protein dispersions adjusted to their protein concentrations of 10, 17.5 and $\sim 25\%$ (w/w) were introduced to the system described as above and change in viscoelastic properties during gelation were investigated applying constant 1% strain and frequency of 1 Hz. The gelation was carried out by heating the samples from $20 - 90^\circ\text{C}$ at heating rate of 1°C min^{-1} for approximately 70 min and held at 90°C for 10 min, cooled from 90 to 4°C at the rate of 1°C min^{-1} for almost 40 min followed by holding at 4°C for about 4 hrs for aging.

5.2.5 Capillary electrophoresis

After the heat treatment, all concentrated samples were diluted with Milli-Q water to adjust their protein content at 10% (w/w) and centrifuged at $14,000 \times g$ for 20 min and supernatants were diluted 10 fold prior to use in capillary electrophoresis. Capillary electrophoresis was performed as described by Donkor et al. (2007)

using a CE Beckman (P/ACE model 5010) equipped with a Beckman P/ACE Station software version 1.0 and a coated capillary of 67 cm total length, 50 cm effective length and 50 μm I.D. (Beckman Instruments Inc., Fullerton, California, USA). Samples were loaded on to the column under ‘high pressure’ for 10 s. The separations were carried out at 14 kV and at 20°C using a sample buffer prepared with sodium borate and sodium phosphate as 30 mM and 17 mM respectively in the final mixture, which was at pH 8.2, and the peaks were detected at 214 nm for 30 min. The capillary was washed between analysis with 1 M NaOH, Milli-Q water and finally with the buffer.

The denaturation rate of WP was assessed via disappearance of β -Lg is related to the reaction order by the following equation:

$$-\frac{dC}{dt} = k \cdot C^n$$

This equation is then rearranged into an integrate to give

$$\int \frac{1}{C^n} \cdot dC = k \cdot \int dt$$

The solution of this integral is

$$\frac{1}{C_t^{n-1}} = \frac{1}{C_0^{n-1}} + (n-1) \cdot k \cdot t \quad 1$$

where t (s) is the reaction time, C_t (gL^{-1}) is the concentration of β -Lg after time t and 0, respectively, k ($\text{g}^{1-n} \text{L}^{n-1} \text{s}^{-1}$) is the rate constant of the reaction and n is the rate of the reaction limited to $n > 1$ (Atkins, 1990). The data were also fitted to the first order decay equation

$$C_t = C_0 \cdot e^{-k \cdot t} \quad 2$$

5.2.6 Statistical analysis

All experiments were arranged as a randomized block full factorial design. The results were analysed with the General Linear Model of SAS statistical programme (SAS, 1996). The concentration at three levels (10%, 17.5% and 25%) and pH also at three levels (4, 5 and 6) were used as the main factors and the replication as a block. All experiments were replicated at least once following sub-sampling ($n \geq 4$) and the level of significance was set at $P = 0.05$.

5.3 Results and discussion

5.3.1 Heat-induced changes in solubility of WP dispersions

Figure 5.1 -A, -B and -C show heat-induced changes in solubility of WP dispersions at different protein concentrations of 10%, 17.5%, or ~ 25% (w/w) respectively, and at pH 4, 5 or ~ 6 during heating at 140°C. This temperature was selected in the study to simulate the industrial conditions of high temperature – short time combinations. As shown, regardless of protein concentration, the general trend was the decrease of solubility of WP with prolongation of heating time. Extent of reduction of solubility reflects the extent of WP denaturation (De Wit, 1990). Hence, this property provides a reliable estimate for assessing an extent of WP denaturation, which in turn depends on the experimental conditions such as pH and heating time (Morr and Ha, 1993).

At concentration of 10% protein (Figure 5.1-A), a rapid decrease in solubility in all three samples was observed during the first 5 s, signifying the high rate of WP unfolding and aggregation. In addition, after 5 s of heating, there was no significant ($P > 0.05$) difference in solubility in any of the samples. Generally, at higher temperatures ($> 75^\circ\text{C}$), the unfolding of globular proteins may be fast and the aggregation reaction would determine the reaction rate (de la Fuente et al., 2002; Considine et al., 2007). Since these WP dispersions were heated at a very

high temperature (140°C), the intra-molecular electrostatic effects exerted due to pH differences may be negligible. Therefore, it may result in a higher rate of unfolding as well as approximately equal rate of aggregation resulting in similar aqueous solubility for all the samples. After 5 s, the solubility of pH 5 and 6 dispersions declined at a lower rate towards 20 s.

In addition, although there was a possibility of forming different intermediate aggregates under different pH conditions, the effects of these individual species could not be monitored under the experimental conditions used in the study, such as filtration through 0.45µm filters before the Bradford assay was carried out. According to de Wit, 2009, some residual secondary structures remain during heat treatments at temperatures above 80°C. For example, there were two different peaks around 75°C and 125°C were observed in DSC thermograms when β-Lg was heated upto 160°C in aqueous solutions as a function of pH. Also, pH values above 6.5, there was an increasing reduction of the peak near 75°C, compensated by an increasing peak size above 125°C. The presence of endothermic peaks above 125°C are not caused by a chemical breakdown of proteins, instead, the heat effects between 125°C and 140°C may present unfolding of a residual native or reformed secondary structures remained at 80°C. This unfolding may be initiated by the breakdown of disulfide bonds above 125°C.

The solubility of pH 4 sample remained almost constant until 10 s and then started to decline. Significant ($P < 0.05$) differences were determined in solubility between samples at pH 4 and 6 around 10 s and 20 s. It can be assumed that around 5 s, most of the WP molecules have been partially unfolded and formed aggregates which were large enough to sediment during the centrifugation part of solubility test at this 10% protein concentration. But, further gradual aggregation or the growth of aggregates of WP would occur (Verheul et al., 1998b) with increasing heating time. Also, at pH 4, WP possess an overall positive charge and hence enhanced inter-molecular repulsive electrostatic interactions between the ionizing carboxyl and amino groups (Morr and Ha, 1993) may have retarded the WP

aggregation resulting in relatively higher solubility. However, increasing kinetic energy of protein molecules gained by heating enhance the probability of collision that would enable further aggregation. Generally, at pH 5, which is around isoelectric point of WP, comparatively minimum electrostatic repulsion and hence, the maximum attraction between proteins (Damodaran et al., 2008) should have been expected. Nevertheless, significantly ($P < 0.05$) impaired solubility at pH ~ 6 rather than pH 5 may be consequence of facilitated aggregation of WP *via* additional thiol induced covalent disulfide bond formation at pH 6 in conjunction with all non-covalent attractive interactions.

When the WP dispersions at 17.5% protein were heated for 5 s at 140°C, the initial trend of rapid solubility drop observed at 10% protein dispersions was also observed but with a greater decline (Figure 5.1 –B) indicating relatively higher level of protein aggregation than in the case of less concentrated sample. The greater protein denaturation can be attributed to the increased collision frequency of protein molecules at this raised concentration.

In addition, there was a significantly ($P < 0.05$) greater decrease in solubility of pH ~ 6 than that of pH 4 around 5 s in these samples compared to those at 10% protein concentration. The solubility of pH 5 and pH 6 samples did not show significant ($P > 0.05$) differences in their solubility until 15 s at this concentration. The longer heating time (20 s) differentiated their mechanisms of aggregation clearly conveying them significantly ($P < 0.05$) different solubility values. Furthermore, the different behaviour of pH 4 samples shown by maintaining a substantially higher solubility level indicates the reduced rate of aggregation likely as a result of repulsion between molecules and possibly improved hydration of folded WP molecules via electrostatic charges at low pH (Damodaran et al., 2008).

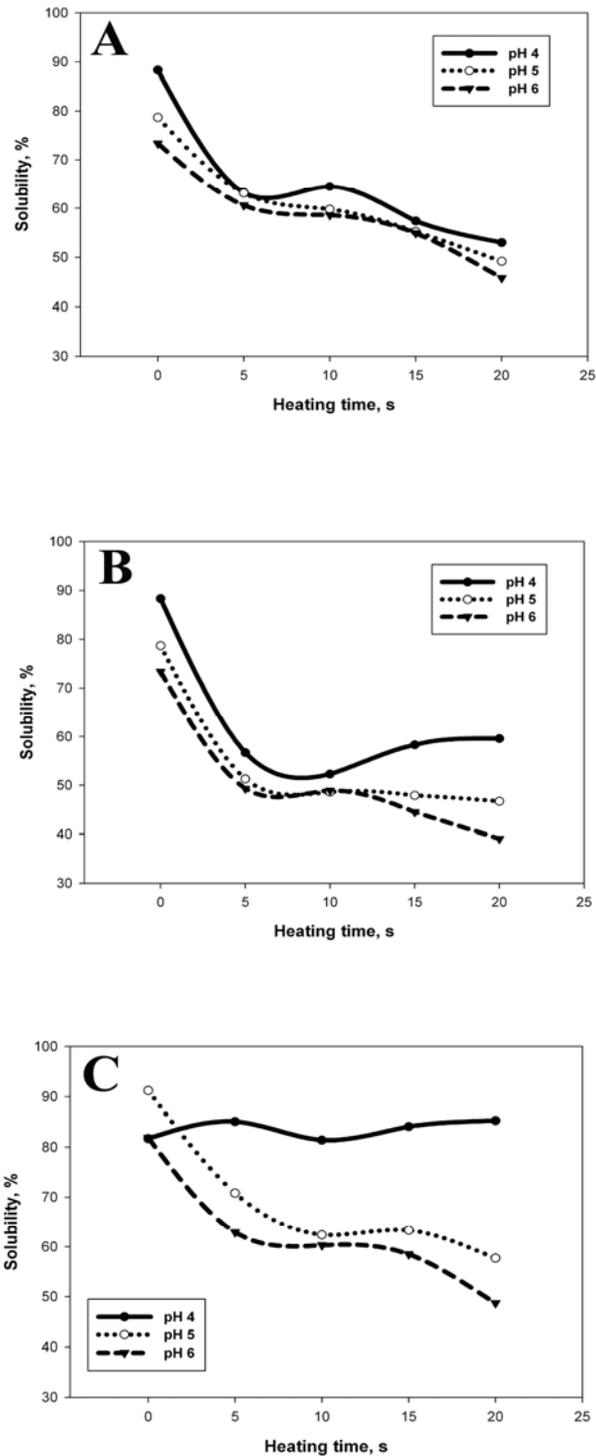


Figure 5.1 Heat-induced changes in solubility of 10% (A), 17.5% (B) or 25% (C) (w/w protein) WP dispersions at pH 4, 5 or 6, heated at 140°C for up to 20 sec.

Even at very high (~25%) protein concentrations (Figure 5.1–C), the rapid WP solubility decline was observed in pH 5 and 6 samples. In contrast to the lower concentrations, the overall solubility of WP dispersions at this concentration remained comparably higher during the heat-treatment revealing less protein denaturation. Interestingly, the solubility of pH 4 sample remained almost unchanged during heating indicating that the aggregation was inhibited. Since the solubility of a protein is a thermodynamic equilibrium between protein-protein and protein-solvent interactions (Damodaran et al., 2008), the significantly ($P < 0.05$) higher solubility of this sample in comparison to others may be a result of highly repulsive forces among protein molecules which has prevented aggregation and also increased hydration through ionic interactions which has promoted protein-water interactions (Huang and Kinsella, 1986).

In addition, the solubility test was carried out after centrifugation and filtration, the size and the density of aggregates formed under different experimental conditions also play an important role in determining solubility, because, according to Stoke's Law, the sedimentation rate of solid particles in the gravity field is mainly a function of the particle size, the density difference and the viscosity of the suspension (Atkins, 1990).

5.3.2 Heat-induced changes in turbidity of WP dispersions

Besides the solubility test, the change in turbidity of WP dispersions, determined by measuring optical density at 420 nm, was also used to monitor the extent of heat induced WP aggregation. As shown by the turbidity measurements in Figure 5.2 -A, -B and -C, the increase in turbidity was observed with increasing heating time at 140°C. The increase in turbidity of a colloidal suspension is an indicative of increase in size of the protein particles (Croguennec et al., 2004; Ju and Kilara, 1997; Banon and Hardy, 1991). Therefore it measures the efficiency of different

aggregation mechanisms involved in WP aggregation because more efficient aggregation mechanisms would produce larger particles.

Notably, the initial turbidity of pH 4 samples at all three concentrations before heating was significantly ($P < 0.05$) higher than the corresponding turbidity values of samples at pH 5 and 6. The WP at pH values away from the iso-electric point possess a net charge (Fox and McSweeney, 2003), and the increased intramolecular repulsion of proteins possibly lead to a partial unfolding. These partially unfolded molecules may form associations of a protein-water-protein type leading to a greater apparent particle size and consequently the turbidity. However, these associations may be fairly soluble or the particles are dispersed easily during the application of centrifugal forces used in solubility test as the solubility of these samples was also high. Additionally, the samples ~ pH 6 did not exhibit such an increase in initial turbidity indicating a lower extent of their denaturation. At 10% WP concentration, the samples with adjusted pH showed significant ($P < 0.05$) differences in turbidity after heating for 5 s (Figure 5.2 –A). A rapid and significant ($P < 0.05$) increase in turbidity was observed for all the samples at pH 5 upon prolonged heating (Figure 5.2 -A, -B, -C). The contribution of non-covalent interactions to the overall aggregation mechanism of WP becomes important over 75°C with hydrophobic interactions being prominently involved at elevated temperatures (Caussin et al., 2003; de la Fuente et al., 2002; Galani and Apenten, 1999).

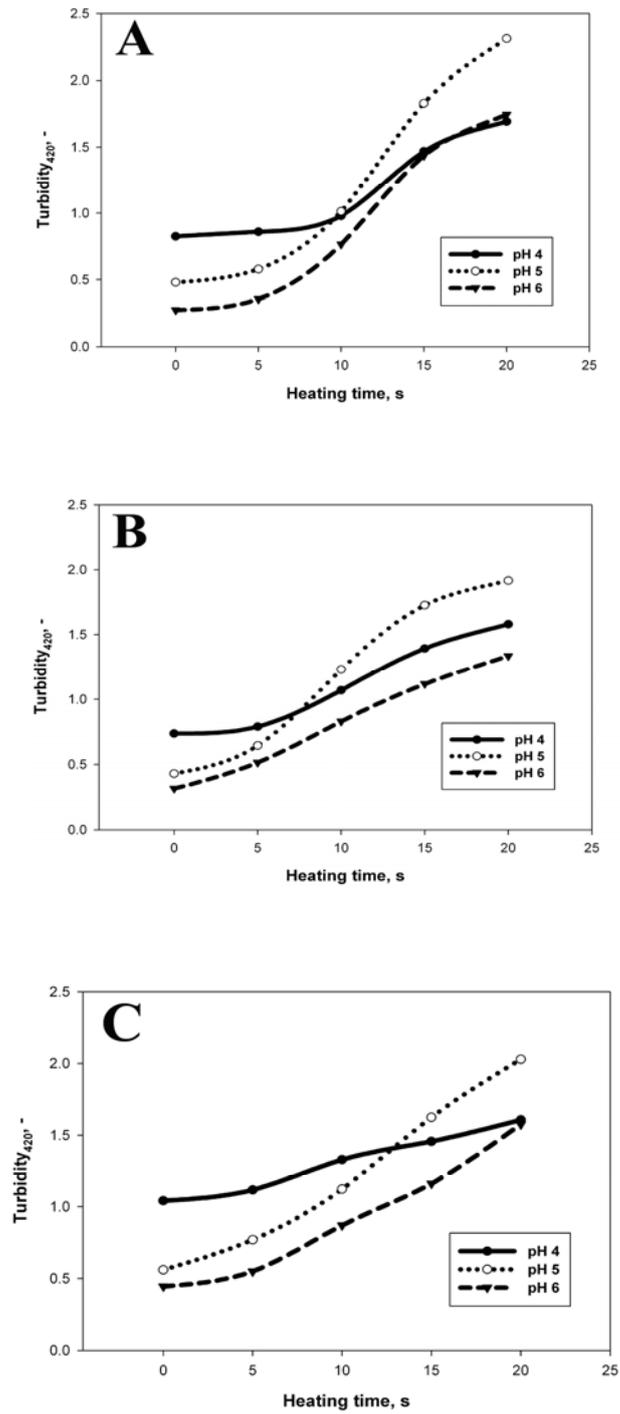


Figure 5.2 Heat-induced changes in turbidity of 10% (A), 17.5% (B) and 25% (C) (w/w %protein) WP dispersions at pH 4, 5 or 6 and heated at 140°C for up to 20 sec.

Therefore, the larger aggregates produced at pH 5 in the current study may be a result of protein aggregation *via* maximum ionic interactions between molecules under a minimum electrostatic repulsion in addition to hydrophobic and even possible disulfide covalent interactions (Verheul et al., 1998b).

Nevertheless, the solubility of this pH 5 sample was significantly ($P < 0.05$) higher than pH 6 sample particularly at higher protein concentrations. It indicates that around the isoelectric point of WP and at higher temperatures, formation of a few large aggregates would be more likely compared to that of higher number of relatively smaller aggregates created with greater protein denaturation around pH 6, where thiol-induced covalent aggregation pathway is more prominent. As described by de la Fuente et al., 2002, at higher temperatures, the formation of reactive monomers with thiol groups would be fast with fast termination reactions of thiol-thiol oxidation resulting in formation of smaller aggregates. In addition, very low increase in turbidity of pH 4 sample with increasing heating time is in agreement with its greater solubility at the concentration of ~ 25% protein which also confirms its reduced denaturation.

5.3.3 Kinetics of WP denaturation

The kinetics of β -Lg denaturation was examined using capillary electrophoresis (Figure 5.4). This protein was chosen since it is the principal WP, representing > 50% of total WP in bovine milk and thus dominates the nature of WP denaturation (Verheul et al., 1998b; de la Fuente et al., 2002). In the preliminary assessment, the data were fitted to the general reaction model and the best fit produced $n = 1.1$; however the coefficient of correlation was inferior to more commonly used expression - the first order decay. The integrated form of this expression was used to describe the kinetics of β -Lg denaturation.

$$\ln C_t = \ln C_0 - k \cdot t$$

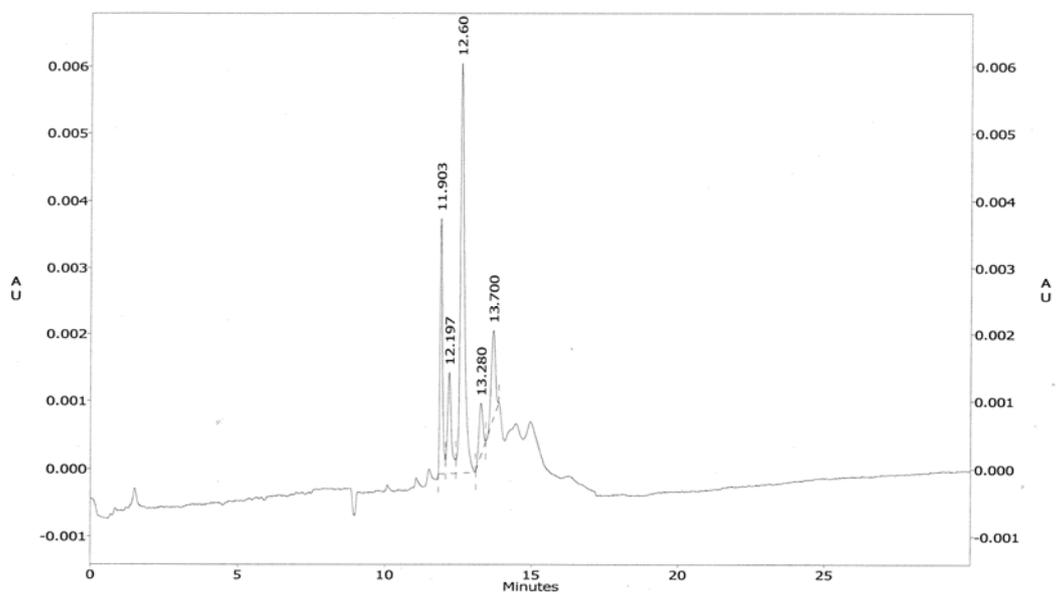


Figure 5.3. A representative CE chromatogram showing major whey proteins; Peaks with the migration times of 11.9 min, 12.6 min and 13.7 min representing α -La, β -Lg and BSA respectively.

where, C_t is the remaining β -Lg concentration without aggregating after time t and C_0 is the initial β -Lg concentration which was detected from untreated controls when the reaction time was considered as zero.

As shown in Figure 5.4 -A , -B, and -C, linear relationships were obtained for denaturation kinetics of β - Lg in different pH conditions (pH 4, 5 and \sim 6), when $\ln(C_t)$ was plotted against t . Also, they were in an acceptable accuracy as given by the high correlation coefficient (R^2) values (in the range of 0.86 - 0.97) and the denaturation of β -Lg appeared to obey the first order reaction kinetics. The rate constant, k , was obtained from the slope of the equation and the values at each pH for the three concentrations are depicted in Figure 5.5.

As clearly indicated by k values in Figure 5.5, the most striking observation was the distinctly higher rate of denaturation when 10% w/w WP dispersion was heated at pH 5. This observation was in agreement with the higher rate of turbidity increase with the highest final turbidity value (Figure 5. 2-A) and also the lowest intensities of major protein bands in SDS PAGE gels (Figure 5.6 -B) of this sample. These observations re-confirm the important role of electrostatic interactions in aggregation around iso-electric pH of WP, especially at raised temperatures. Moreover, at pH 5, although non-covalent interactions are prominent, covalent disulfide bond formation is also possible (Verheul et al., 1998b). However, at higher concentrations, (17.5% and 25% protein), the rate of aggregation at pH 5 was not that prominent relative to that at 10% protein revealing the effect of concentration on aggregation kinetics. At high concentrations such as 17.5% or 25% protein, the reaction rate may have depended on the unfolding step of WP denaturation.

According to Fitzsimons, et al. (2007) and de la Fuente et al. (2002) protein unfolding is generally independent on concentration at higher temperatures. However, thus mentioned concentrations were relatively lower than the concentrations used in this experiment. Additionally, there is a noticeable decline in the k values of pH 4 sample than that of pH 6 sample in 10% protein dispersions (Figure 5.5),

which is also supported by the significantly ($P < 0.05$) increased solubility of pH 4 sample (Figure 5.1 -A). More importantly, denaturation temperature of pH 4 sample was markedly ($P < 0.05$) higher than that of pH 6 sample as indicated by the DSC thermograms (Table 5.2).

It is useful to discuss and compare other studies on kinetics of heat induced aggregation of β -Lg under different conditions. As described by Verheul et al (1998), β -Lg at neutral pH and low ionic strength in the temperature range of 60 to 70°C created transparent dispersions containing relatively small polymeric protein particles. These aggregates have been formed *via* thiol/disulfide bond exchange propagation reactions and thiol/thiol termination reactions. When a model was developed using the data by applying steady state principle, the reaction order was calculated as 1.5. Also, they have shown that the order of reaction varied between 1 and 1.7 with varying initial protein concentration, ionic strength of the medium and pH. The initial reaction order increased with pH and decreased with ionic strength. Additionally, Le Bon et al (1999) found that β -Lg at pH 7 and 0.1 M ammonium acetate followed the reaction order of 1.5 at 67°C where as a model based on free radical polymerisation proposed by Roefs and de Kruif (1994) observed that the reaction order of β -Lg denaturation was 1.5.

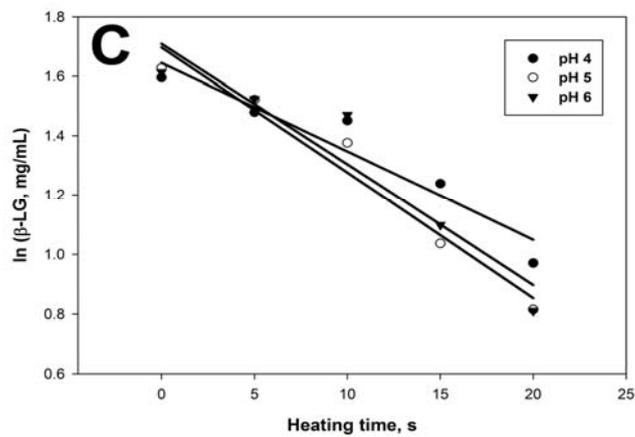
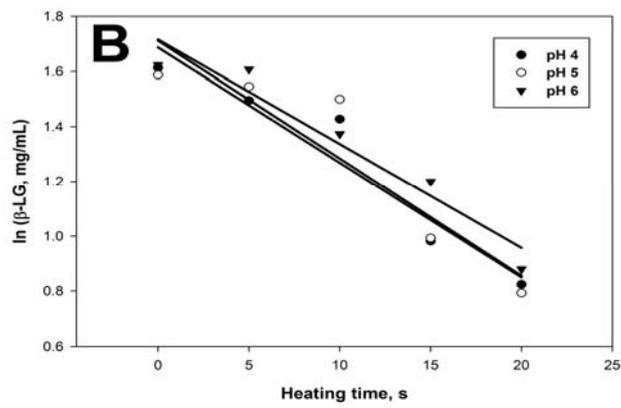
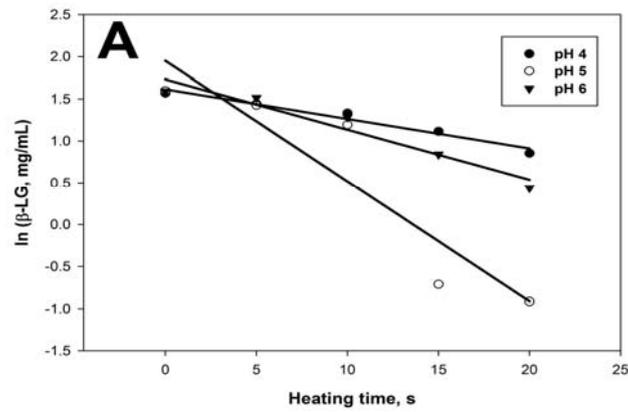


Figure 5.4 Kinetics of heat-induced β -Lg denaturation at 140°C of 10% (A), 17.5% (B) and 25% (C) (w/w %protein) WP dispersions at pH 4, 5 or 6 heated for up to 20 sec at 140°C.

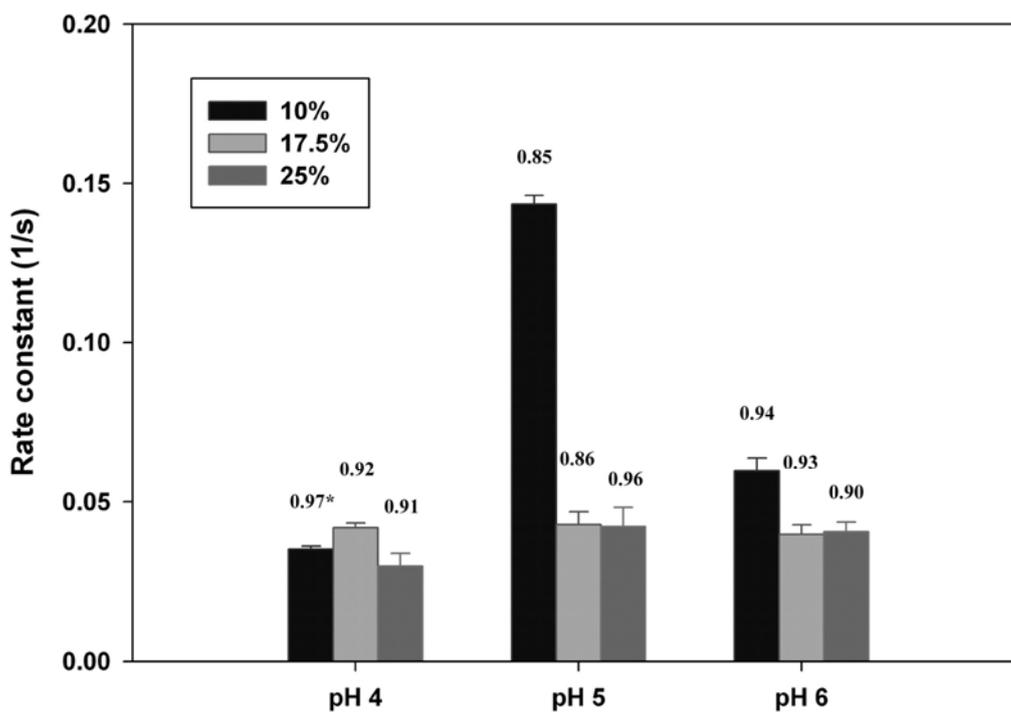


Figure 5.5 The rate constant (1/s) obtained by data fitting to the first order decay model as affected by protein concentration (10, 17.5 or 25% w/w) and pH during heat-induced β -Lg denaturation. The values above the bars* indicate the correlation coefficients obtained by data fitting to the first order decay model.

In fact, the heat-induced denaturation of proteins would be a product of kinetic and thermodynamic equilibrium (Anema and McKenna, 1996). Therefore, attractive and repulsive molecular forces, which depend on the intrinsic and extrinsic factors of the protein always govern the effectiveness of unfolding and aggregation finally affecting the rate of denaturation. For example, at low pH, the intra-molecular repulsion facilitates unfolding and the intra-molecular disulfide bonds with impaired thiol group activity probably slow down the rate of unfolding (Havea et al., 2009), and the net positive charge on proteins retards the aggregation process. In contrast, at 10% protein, the increased reaction rate of pH 6 dispersion compared to that of pH 4 is undoubtedly the result of reactivity of the activated thiol group. However, the k at pH 6 was always smaller than that at pH 5, which specifies a slower rate of aggregation since the disulfide bond formation between denatured proteins is a relatively slow reaction due to limited number of reactive sites per protein molecule (Havea et al., 2009).

Furthermore, several studies have shown that the rate of aggregation and heat induced gelation of WP, although predominated by properties of β -Lg, were also influenced by BSA (Tobitani and Ross-Murphy, 1997; Hines and Foegeding, 1993). The presence of BSA accelerated the formation of the heat set gels (Tobitani and Ross-Murphy, 1997). At certain conditions (increased ionic strength and a specific β -Lg/BSA ratio) the rate of β -Lg denaturation was also enhanced (Hines and Foegeding, 1993). For these reasons, it could be assumed that the kinetics of WP denaturation (or aggregation) would be better described by n-th order reaction kinetics (Eq. 1). However, considering that correlation coefficients obtained upon data fitting to the first order decay were greater than after applying the general kinetic model, it could be assumed that the denaturation of β -Lg (and likely aggregation) followed the first order reaction kinetics under steady state conditions.

5.3.4 Thermal behaviour of WP dispersions as determined by DSC

The DSC results revealed that the denaturation (peak) temperatures, T_d , of WP dispersions (Table 5.2) were independent of concentration having a similar trend for all three concentrations, but significantly dependent on pH of the medium. DSC thermograms were not obtained isothermally although the results obtained from DSC would be quite useful to understand the unfolding and aggregation kinetics of WP.

Differential scanning calorimetry can be used to detect the thermal transition temperatures of proteins and also to quantify the enthalpy of the conformational transition (Donovan, 1984). Thermal transition temperatures are determined from peaks in the heat capacity curve or endothermic events of the thermal profile caused by denaturing of proteins. Also, the enthalpy of the transition calculated using the area under each peak in the endotherm, is positively associated with the exposure of buried hydrophobic amino acids to solvent and negatively associated with the disruption of hydrogen bonds of secondary and tertiary structures (Privalov and Gill, 1988). Further, the protein denaturation is primarily an endothermic process whereas the aggregation associated with intermolecular interactions is an exothermic reaction which may partially mask the unfolding endotherm (Privalov, 1979).

The significantly high ($P < 0.05$) peak and end-set temperatures of pH 4 samples are consistent with reduced values of rate constants, likely due to high electrostatic repulsions (Figure 5.5). Also, the peak temperature of pH 6 sample has decreased significantly ($P < 0.05$) and coincided with its significantly ($P < 0.05$) decreased end-set temperatures which was more obvious at 10% protein concentration. This behaviour of WP at pH 6 may be correlated with the enhanced reactivity of sulfhydryl/disulfide interchange reactions which may be preferentially involved in unfolding and aggregation of WP. The onset temperatures of these samples were also relatively low indicating the denaturation process was facilitated.

Table 5.2 Thermal properties of WP samples with adjusted protein concentration and pH

Concentration, % w/w	pH	Onset temperature, °C	Endset temperature, °C	Peak temperature (T_d), °C	ΔH , J/g
10	4	79.7 ^c	90.8 ^{cd}	85.1 ^d	3.9 ^a
	5	76.0 ^c	88.0 ^b	81.4 ^c	4.6 ^{ab}
	6	70.2 ^b	85.9 ^a	78.7 ^a	4.6 ^{ab}
17.5	4	77.5 ^c	90.9 ^{cd}	84.2 ^d	4.9 ^{ab}
	5	61.7 ^a	89.5 ^{bc}	81.3 ^c	6.9 ^d
	6	68.3 ^b	86.6 ^{ab}	78.8 ^a	5.8 ^{bc}
25	4	77.2 ^c	91.7 ^d	84.6 ^d	5.0 ^{abc}
	5	68.9 ^b	89.0 ^b	81.2 ^c	5.6 ^{bc}
	6	68.5 ^b	89.0 ^b	79.8 ^b	6.2 ^{cd}
*SEM		1.3	0.6	0.3	0.4

ΔH - enthalpy change; *SEM denotes a pooled standard error of the mean, $P < 0.05$. Means present the average of at least 4 independent observations ($n=4$). The different small letter superscripts in a column indicate significant difference ($P < 0.05$).

In a mixture of WP, α -La which has a lower denaturation temperature than β -Lg (Thompson, et al., 2009) may start to denature first driven by the reactivity of the activated thiol group of β -Lg or BSA such as at pH 6 in the current study shown by acquired low onset temperature. However, the significantly ($P < 0.05$) low onset value at pH 5 at 17.5% contradicts this observation and further testing is required to further address this phenomenon.

The enthalpy change (ΔH) of WP denaturation and aggregation may be a sum of formation and disengagement of various inter- and intra-molecular interactions prevailing in WP (Paulsson and Dejmek, 1990). The conformational entropy of the system increases with increasing temperature and therefore prefers unfolded state of globular proteins (Damodaran et al., 2008). Moreover, electrostatic and hydrogen interactions would be suppressed under these conditions since WP were in an aqueous medium with a high dielectric constant. Hydrophobic interactions, which favour the folded state, and conformational entropy, which favours the unfolded state, eventually determine the denaturation temperature of proteins during heating (Damodaran et al., 2008). Therefore depending on the conformational stability of WP under different environmental conditions, the extent of thermal changes varies as indicated by different endothermic peaks in DSC thermograms. For example, there was a significant ($P < 0.05$) increase in the ΔH value for 17.5% WP dispersion at pH 5. It is a reflection of a relatively conformationally stable state which required increase in entropy to induce unfolding of WP around isoelectric point (Fox and McSweeney, 2003). This energy was also used for disrupting strengthened hydrophobic interactions under minimum intra-molecular repulsion.

The overall DSC data, except T_d , conveyed information about the concentration dependence of the WP concentrates during denaturation process. The rate and the mechanism of unfolding and aggregation of WP depends greatly on temperature and holding time (de la Fuente et al., 2002), making it fairly difficult to compare with other studies such as this one with the experimental DSC data obtained at 140°C.

5.3.5 Electrophoretic analysis

The SDS PAGE analysis under reducing conditions was carried out using the supernatants of WP dispersions which were obtained after heating at 140°C. Figure 5.6 represents the SDS PAGE patterns of 10% (A), 17.5% (B) or ~25% proteins (C) at pH 4 (i), pH 5 (ii), or pH ~ 6 (iii) WP concentrate samples.

As depicted by Figure 5.6 –A, clear differences among the protein bands of three PAGE gels were observed. The highest intensity in major WP bands was observed at pH 4, indicating the least aggregation of WP of this sample compared to other two at this concentration and also compared to all the other samples at higher concentrations, which can be correlated with the smallest value of the rate constant (k) of this sample. In addition, a noticeable decrease in intensity of two major protein bands, corresponding to α -La and β -Lg of this sample, was observed after heating for 20 s (lane 5). Apparently from this Figure, α -La was disappearing faster than β -Lg. However the increasing turbidity and decreasing solubility values corresponding to this sample signify the commencement of protein aggregation before this time point. Therefore, even if the WP aggregation occurred at this pH, these would rather be small oligomers with weaker intermolecular interactions due to the repulsive electrostatic forces.

The prevailing electrostatic charges may have also facilitated protein hydration and their increased aqueous solubility leading to their prominent appearance in SDS PAGE gels. Most obviously, the highest aggregation of proteins out of all the combinations of experimental parameters was depicted by Figure 5.6 -A (pH 5) gel with the least intensity of its major protein bands. This was rather expected under these, for aggregation, favourable, attractive electrostatic interactions. The protein bands at pH 6 of 10% protein (Figure 5.6 -A) gel show gradual and an intermediate level of protein denaturation compared to the other two samples at this concentration which is well in agreement with slow thiol mediated WP aggregation.

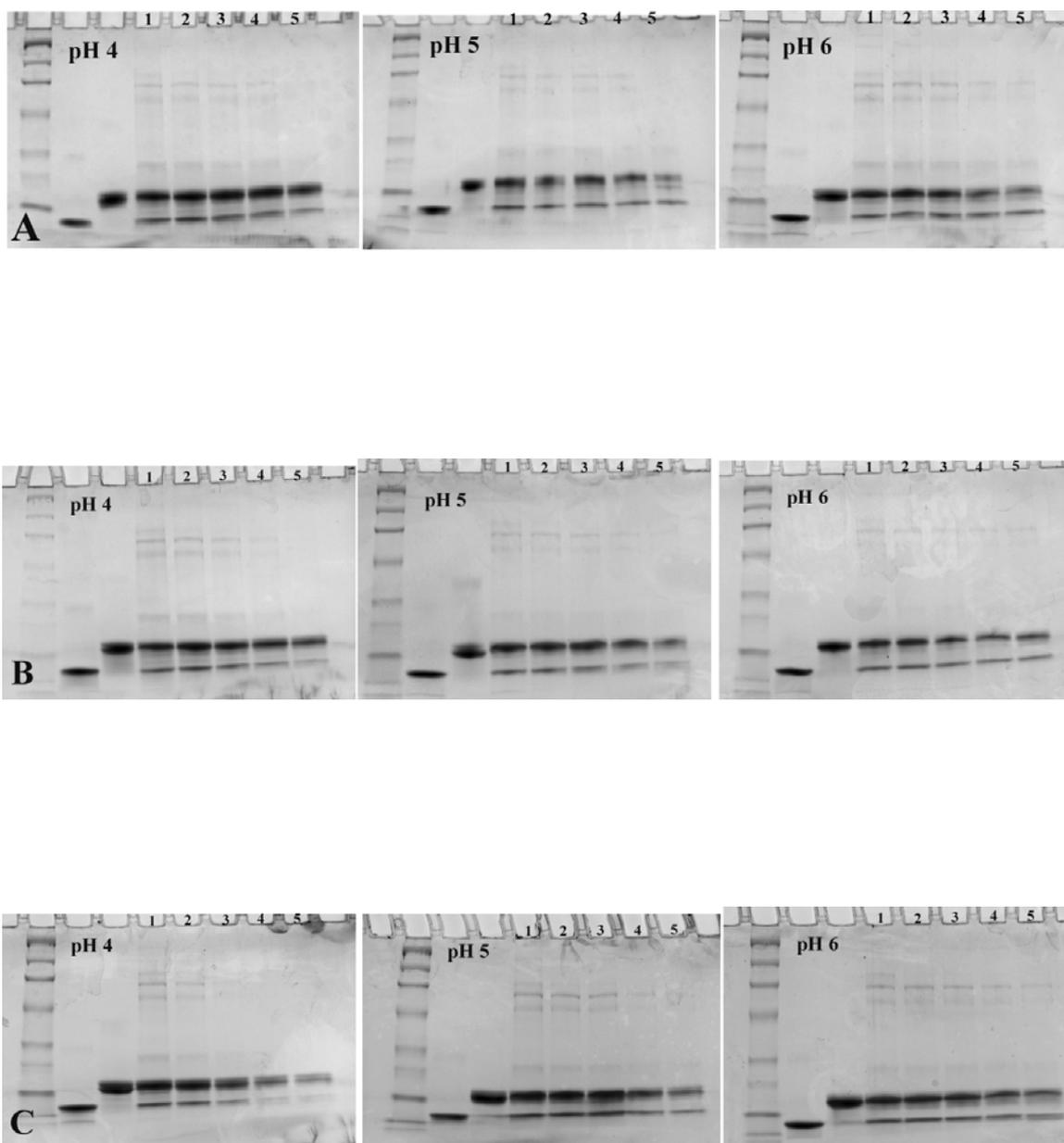


Figure 5.6 Reducing SDS-PAGE patterns of remaining WP after heat treatment of 10% (A), 17.5% (B) and 25% (C) (w/w %protein) WP dispersions at 140° C. The pH of these dispersions was adjusted to pH 4, 5 or 6 prior to heating. Lanes from left to right: molecular weight marker; α -lactalbumin standard; β -lactoglobulin standard; 1 – 5, dispersions heated for 0, 5, 10, 15 or 20 s respectively.

The appearance of principal WP bands with greater intensity in PAGE gel in Figure 5.6 -B (concentration at 17.5% w/w protein) at pH 4 signifies the least thermal denaturation of WP compared to the proteins at other two pH levels at this same concentration. This again exemplifies the stability of WP at low pH against denaturation and subsequently aggregation. Even after 20 s of heating at 140°C, considerable amount of un-denatured β -Lg was present in this sample. On the other hand denaturation of α -La was almost completed by this time indicating comparatively greater denaturation rates of α -La. In heated mixtures of WP, the contribution of non-covalent bonding to aggregation was greater for α -La than β -Lg (de la Fuente et al., 2002). This is also confirmed by the relatively fast disappearance rates of α -La bands in Figures 5.6 -B (at pH 4 and 5) and 5.6 -C (at pH 4), under which conditions non-covalent interactions were very likely. The major protein bands in gels corresponding to 17.5% and 25% protein concentrations (Figure 5.6 -B and -C) are representative of a rapid disappearance of these proteins after 15 s of heating at pH 5. This trend confirms the conformational stability of WP against unfolding around isoelectric point; but, once unfolded, aggregation proceeds faster due to lowered inter-molecular repulsions as shown by faint protein bands obtained after 10 s of extensive heating. In comparison to aggregation behaviour at pH 5, a gradual denaturation of proteins occurred at pH 6 at both 17.5 and 25 % concentrations (Figure 5.6 -B, pH 6 and Figure 5.6 -C, pH 6) which was more prominent after 5 s. The disappearance of α -La and β -Lg bands at equal intensities indicate the nature of thiol induced WP aggregation at very high temperatures such as in this study, which may be different from that mechanism governing the denaturation at lower temperatures. For instance, at such a high temperature, the rate of oxidation of reactive monomers is faster (de la Fuente et al., 2002) and the WP denaturation would only be affected by the rate of formation of reactive monomers. In such a situation, the effect of inherent reactivity of an individual protein may be negligible. Otherwise, compared to β -Lg, α -La reacts rather more rapidly at relatively lower temperatures (Considine et al., 2007) due to possession of a greater number of intra-molecular disulphide bonds, inherently greater reactivity

and its lower thermal transition temperature (de la Fuente et al., 2002). Another controversy was observed for the sample containing 25% w/w protein at pH 4 (Figure 5.6 -C). As presented, comparatively greater aggregation of major WP could be identified compared to samples of the same concentration at pH 5 or 6, respectively.

5.3.6 Flow behaviour of WP samples

Figure 5.7 –A, -B and –C and Table 5.3 present the rheological parameters of WP dispersions, including apparent viscosity, consistency coefficient, k ($\text{Pa}\cdot\text{s}^n$), and flow behaviour index, n (dimensionless), obtained by the model fitting, which used to characterise the flow behaviour of the WP samples (10, 17.5 and 25%, respectively) at pH 4, 5 and ~ 6 . The k and n values were obtained with high correlation coefficients by fitted to the Power Law model. As Figure 5.7 –A, -B and –C demonstrate the apparent viscosity of all WP dispersions has decreased with increasing shear rate, thus they could be referred to as non-Newtonian fluids with shear thinning behaviour (Rao, 1999). The statistics also showed that pH alone has a significant ($P < 0.05$) effect on k values where as the interaction between pH and concentration has significantly ($P < 0.05$) affected both the k and n values of WP dispersions. The molecular properties of WP always influence their flow behaviour as described in Chapter 4. Whey proteins, especially β -Lg, show a Newtonian behaviour up to 5% w/w protein, but shear thinning at higher concentrations (Fox and McSweeney, 2003). The pseudo-plastic (shear thinning) character of these dispersions may have resulted from the disruption of weak inter-particle linkages at sufficiently high enough hydrodynamic forces which were generated when shearing (Rao, 1999) and also the affinity of protein molecules to align with the direction of flow (Damodaran et al., 2008). According to Figure 5.7 -A, pH did not show considerable effect in changing the shear thinning behaviour of WP at comparatively diluted 10% concentration as shown by almost mirrored flow curves.

Table 5.3 Rheological parameters of whey protein dispersions, containing 10, 17.5 or 25% (w/w) proteins, obtained by fitting the experimental data to Power Law model

Concentration, % w/w	pH	k, mPa.s ⁿ	n	R ²
10	4	19.5 ^a	0.76 ^b	0.98
	5	16.5 ^a	0.73 ^a	0.97
	~ 6	27.8 ^a	0.64 ^a	0.97
17.5	4	59.8 ^a	0.87 ^{bc}	0.99
	5	31.0 ^a	0.82 ^b	0.99
	~ 6	27.8 ^a	0.78 ^b	0.99
25	4	911.3 ^c	0.75 ^b	0.98
	5	237.0 ^b	0.77 ^b	0.94
	~ 6	55.3 ^a	0.94 ^c	0.99
*SEM		45.0	0.03	0.02

k– consistency coefficient; n – flow behavior index. R²– correlation coefficient; *SEM - Pooled standard error of the mean, $P < 0.05$. Means present the average of at least 4 independent observations (n=4). The different small letter superscripts in a column indicate significant difference ($P < 0.05$).

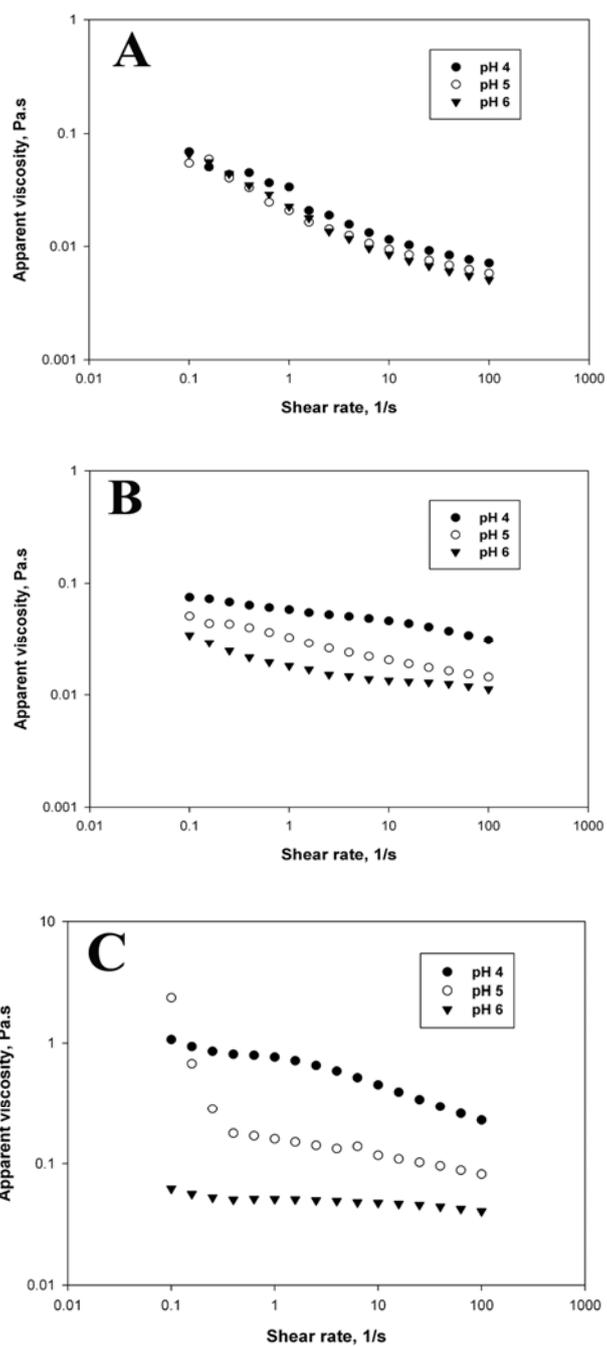


Figure 5.7 Apparent viscosity of 10% (A), 17.5% (B) and ~25% (C) (%w/w protein) WP dispersions at pH 4, 5 or 6 during a controlled shear rate sweep ($0.1 - 100 \text{ s}^{-1}$) at 20°C .

In diluted solutions, the polymer molecules behave independently, and the viscosity depends on the dimensions of the polymer such as hydrodynamic volume and the radius of gyration (Rao, 1999). As results indicate, the WP, particularly as compact globular proteins, may not have any substantial difference in their dimensions in the presence of vast amount of solvent although the pH is changed.

However, when the concentration was raised up to 17.5% protein, the sample at pH 4 showed the highest initial viscosity (yield stress) relative to the samples at other two pH values. It also exhibited a comparatively higher viscosity values through out the shear rate ramp. The WP dispersion at pH 5 possessed an intermediate effect on apparent viscosity and the sample ~ pH 6 showed the lowest viscosity during the shear rate sweep. The greater viscosity of pH 4 sample indicates its higher resistance to induced flow relative to other two samples. Due to WP charged nature, their rheological behaviour may also be influenced by pH of the medium. At acidic pH, the degree of hydration of proteins may become greater due to greater net positive charge on protein molecules leading to a greater affinity of water molecules towards proteins. In addition, the intra-molecular repulsion present in WP under an acidic pH may facilitate partial unfolding of proteins in conjunction with hydrodynamic forces which may finally end up in increased viscosity of the dispersion. Solutions of randomly coiled molecules frequently display greater viscosity than solutions of compactly folded macromolecules of same molecular weight (Damodaran et al., 2008). Similarly the lowest apparent viscosity of pH 6 dispersion may have resulted from enhanced repulsive forces predominating at lower shear rates and likely weak interactions among protein aggregates easily disrupted under increased shear.

The change of apparent viscosity during shearing of WP dispersions containing ~ 25% protein (Figure 5.7 -C) was fairly different from the other two concentrations since the highest initial yield point was observed for pH 5 sample. However, with the application of shear force, the apparent viscosity declined sharply indicating brittleness of this system. Such a high initial viscosity of this sample may be

attributed to elevated protein-protein attractive interactions through hydrophobic and ionic surface patches present in WP under a minimum electrostatic repulsion. In addition, the protein-protein attraction may have been facilitated *via* increased volume fraction of dispersed particles at a lower water content minimizing the inter particle distances (Patocka et al., 2006). Moreover, the pH 4 sample maintained the overall maximum viscosity similar to what was observed for the sample with 17.5% proteins.

Several important observations can be made from Table 5.3. The comparatively larger k values were obtained at lower pH at higher protein concentrations. The k value or consistency index is directly proportional to the resistance of a material to flow (Rao, 1999). On the other hand, the flow behaviour indexes (n) had a similar pattern for samples containing 10 or 17.5% proteins, deviating more from Newtonian flow with elevation of pH. This was however reversed for the concentrated sample (~25%) with the highest n value obtained at pH 6 which could indicate enhanced flow due to greater intra-molecular repulsions.

5.3.7 Viscoelastic properties of whey protein dispersions during heat-induced gelation

Figure 5.8 presents the change in storage modulus (G') of 10%, 17.5% and ~ 25% w/w WP dispersions during heat-induced gelation. G' is a measure of the elastic character of viscoelastic gel systems (Rao and Steffe, 1992). As indicated by the figures, storage modulus of each WP dispersion has increased during heating and cooling. During aging the storage moduli of these dispersions would reach a plateau with more or less similarities. In addition, the storage moduli of all type of gels have tremendously increased with the rise in protein concentration which emphasises the importance of protein concentration when controlling gel characteristics, in this case, the firmness of gels.

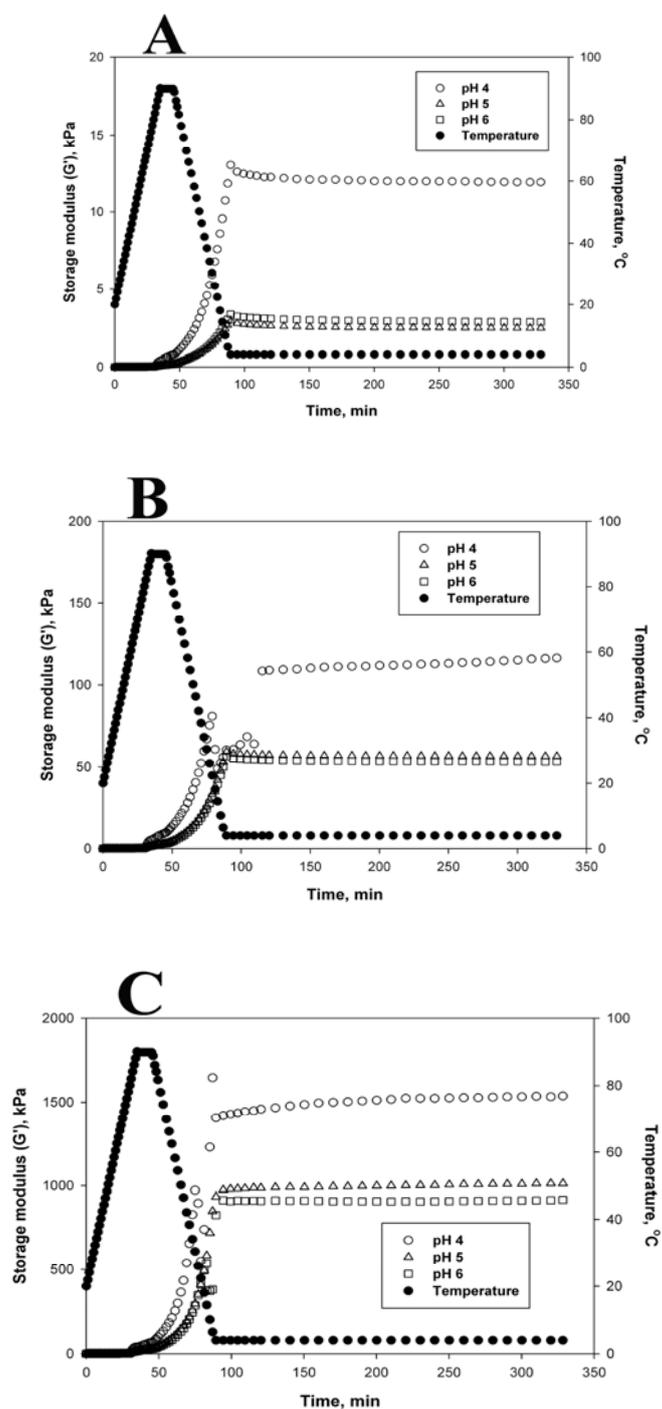


Figure 5.8 Storage modulus (G') of 10% (A), 17.5% (B) and 25% (C) (%w/w protein) WP dispersions at pH 4, 5 or 6 during heating (20 - 90°C), holding (90°C / 10 min), cooling (90 - 4°C) and aging (4°C/approx 4 hr) at a constant strain (1%) and frequency (1 Hz).

Regardless of concentration, the other most significant observation in the results is the greatly increased G' values of pH 4 gels in comparison to those formed at pH 5 or 6. The WP dispersions at pH 4 showed greater structure forming capability even during heating period compared to others. It further increased immensely during cooling, as clearly given by the relatively increased G' values at each concentration.

As explained earlier in this chapter, the intra-molecular repulsions of WP at pH 4 always favours the rapid unfolding during heating which may also be enhanced by the subsequent protein-water interactions under a net positive charge forming a cohesive viscoelastic network mainly *via* exposed hydrophobic groups and also with other molecular interactions to form a viscoelastic gel network. Since the thiol groups are inactive at pH 4, the disulfide bond formation is unlikely (de la Fuente et al., 2002). Therefore, the facilitated non-covalent interactions were mainly responsible for creating firmer WP gels at this pH. These findings were in agreement with the observations of Havea et al., (2009), which described the characteristics of firmer (higher storage modulus) WP gels formed through non-covalent interactions.

The prominently increased G' of these gels during cooling may have resulted from relatively facilitated hydrogen bond formation and electrostatic interactions which are stable at lower temperatures (Damodaran et al, 2008). Also, according to Dickinson (2005), at pH below the isoelectric point, fine stranded, weak, less firm gels are formed. The figures (5.9 –A, -B and –C) show that the firmness of these gels increased with the rise in protein concentration.

When compared to the gels formed at pH 4, those formed at pH 5 or 6 possessed comparatively lower storage moduli indicative of creation of softer but elastic gels. At pH values above the isoelectric point, activated thiol groups form covalent disulfide bonds and these gels are relatively elastic (Dickinson, 2005; Havea, et al., 2009). In addition, the net negative charge present on protein molecules provides them with adequate time for a proper alignment before they

associate *via* different interactions thus resulting in elastic gels (Morr and Ha, 1993). At both of these pH values, WP gels had almost equal gel strength. However, the slightly increased G' of pH 6 sample at 10% protein may have resulted from the enhanced covalent bond formation compared to pH 5 sample. The comparatively stronger gel behaviour of pH 5 gels at each concentration may be a result of minimum molecular repulsion during aggregation, therefore stronger non-covalent interactions along with possible contribution of covalent bonds (Verheul et al., 1998b).

5.4 Conclusions

The denaturation of β -Lg has apparently followed the first order reaction kinetics under the conditions applied in the current study. A substantial increase in the rate of thermal denaturation of WP around their isoelectric point was observed at relatively low 10% protein concentration, which was also further in agreement with the PAGE analysis, greater turbidity and gelling characteristics of the sample. The occurrence of maximum aggregation with larger aggregates around the isoelectric point was likely a result of protein aggregation *via* maximum molecular interactions under a minimum electrostatic repulsion. Heat induced changes reduced the solubility of WP regardless of pH at relatively low protein concentrations. At higher protein concentrations and low pH, WP maintained substantially greater solubility than at any other pH indicating the reduced rate of aggregation. On the other hand, relatively most reduced solubility at pH \sim 6 was indicative of facilitated WP aggregation. Furthermore, denaturation temperatures of WP dispersions were independent of protein concentration but greatly depended on pH of the medium. In addition, all WP dispersions showed shear thinning behaviour around the ambient temperature.

The lowest rate of WP denaturation was observed at acidic pH which was confirmed by the significantly increased solubility and low turbidity at this pH.

However, the initially high turbidity of these samples, irrespective of concentration, indicates that these proteins may be partially unfolded and aggregated as well as at the same time highly hydrated. This fact is further supported by increased yield stress of these samples. These aggregates, however, appeared weak as well as greatly dependant on extrinsic factors since the solubility of these samples was also high. Furthermore, highly cross-linked, harder gels were formed at acidic pH, but were weak and brittle. The brittleness of these gels increased with the rise in protein concentration. The overall results indicate that WP are more stable against heat induced denaturation at acidic pH and the heat induced gels formed at low pH possess weak and brittle characteristics. This information may be very useful with regard to defining conditions (pH, protein concentration, temperature, ionic strength) that are required to produce microparticulated whey proteins of desired functional characteristics. Thus it may be postulated that microparticulation of whey proteins at low pH and greater protein concentration may produce WP ingredients that are heat stable but with other physical functionalities resembling native WP.

CHAPTER 6

6 Physical functionalities of microparticulated whey proteins obtained at low pH adjusted by selected acidulants

6.1 Introduction

Microparticulation of WP using combined heat and high pressure shearing (microfluidization) could successfully modulate and stabilize WP against heat at neutral pH. Apart from enhanced heat stability, denatured MWP exhibited different functionalities from those of native WP such as improved emulsifying activity and gelling properties as shown in Chapter 4. Although microparticulation produced micro-aggregates by dispersing heat denatured WP aggregates, the particle size of these aggregates was not small enough to prevent sedimentation. This may potentially adversely affect all other WP functionalities restricting their successful application in various heterogeneous food systems.

As previously explained in detail and also mainly recognized by the study of kinetics of heat induced WP denaturation of this research, the WP at acidic pH were more stable against heat induced unfolding and aggregation. Consequently they formed heat-set gels that were relatively brittle and weak. In comparison to these gels, those formed at neutral pH exhibit strong and elastic gel properties. The weak nature of acid gels primarily arises due to the possession of overall positive charge of WP at acidic pH with enhanced inter-molecular repulsive electrostatic interactions and the inhibition or suppression of thiol group activity (de la Fuente et al., 2002; Damodaran et al., 2008; Havea, et al., 2009; Morr and Ha, 1993; Resch et al., 2005).

In addition, most WP products contain considerable amount of calcium which is associated with the creation of strong gel network due to additional cross-linking by formation of calcium bridges between protein molecules (Brandenberg et al.,

1992; Patel and Kilara, 1990; Fetahagic et al, 2002). Moreover, addition of calcium salts decreases β -Lg aggregation temperature, particularly at low ionic strength, but further addition of salts brings negative effects to the gel network after the optimal gel conditions were achieved (Xiong et al., 1993). Addition of a calcium chelating agents such as citrate ions may produce relatively weaker WP gels and also impart them enhanced heat stability (de Rham and Chanton, 1984). Further, the awareness of the relative influence of different acidulants on WP gelation would also be beneficial for manufacturing of MWP at low pH. The variations in gel quality due to different acidulants effects may be related to the specific anion effects such as size of the ion and the charge density which may determine their ability to perturb the solvent, water, structure (Resch et al, 2005a). For example, the large ions with low surface charge density generally exert the greatest disruption in the hydrogen bonded structure of water while smaller ions with greater charge density tend to have the smallest effect on the disorder of the solvent finally influencing hydrophobic interactions within the protein molecule in solution (Resch et al, 2005).

The principal aim of the current study was to produce heat stable MWP powders with improved colloidal stability through the reduction of particle size. The study was carried out at low pH in order to create a WP gel network with relatively weaker molecular linkages which would be easily dispersed by mechanical forces used in high pressure homogenization/microfluidization. In addition, the influence of change in the ionic environment was also assessed by using different acidulants.

6.2 Materials and Methods

6.2.1 Materials, proximate composition and sample preparation

Whey protein retentate samples used in the study was a gift from Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). They contained

approximately 30% total solids and were collected in two different occasions. Initial gross composition of the retentates was reported in Chapter 5. All the chemicals used in the study were of analytical grade.

The study was composed of two separate phases. The fundamental WP interactions responsible for the protein aggregation at low pH during heating at 140°C for 30 s were examined in the first phase using a turbidity method, differential scanning calorimetry (DSC) and polyacrylamide gel electrophoresis (PAGE). In the second phase, different functional properties of MWP powders produced at low pH were assessed.

During the first part of the study, all samples were adjusted to 7% (w/w) protein by either diluting with Milli-Q water without changing the pH (pH ~ 6) or adjusted to pH ~ 3 using citric (VWR international, Leicestershire, U.K.) or lactic acid (VWR international, Leicestershire, U.K.). Various molecular interactions were assessed by addition of different chemical bond blocker: hydrophobic interactions were assessed in the presence of 0.5% polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma, St. Louis, USA); non-covalent interactions were determined in the presence of 1% sodium-dodecylsulphate (SDS) (Merck, KGaA, Darmstadt, Germany); new covalent bond formation was prevented by addition of 20 mM *N*-ethylmaleimide (NEM) (Sigma, St. Louis, USA) and 10 mM dithiothreitol (DTT). In addition, DTT reduces existing covalent disulfide bonds present in proteins. (Havea et al., 2009). The one control at pH ~ 6 and the other two at pH 3 for each acidulant did not contain any chemical, thus, representing five types of samples for each solvent; i.e. water or for 2 acidulants. The protein concentration was adjusted to 7% (w/w) intentionally since this was the highest WP concentration that could have been treated using microfluidization. Also, the Milli-Q water was purposely used to dilute the samples to minimise the effect of lactose since whey permeate contained considerable amount of lactose (~ 4.15%, w/w).

6.2.2 Heat treatment of WP retentates

Heat treatment was carried out according to the method described in Chapter 3, section 3.2.6. Approximately 3.0 mL samples of WP dispersions were measured into small glass tubes (10 mm in diameter and 75 mm in length), sealed with rubber stoppers, immersed and shaken in an oil bath (Ratek, Boronia, Australia) at 140°C for 30 s followed by immediate cooling in an ice bath before analysis. After the heat treatment, WP samples were homogenized by slightly vortexing before the analysis of physical, chemical and thermal properties of heat treated WP concentrates as described below.

6.2.3 Sample analysis

The electrophoretic analysis of heat-treated samples were performed as native or reducing/non-reducing SDS (sodium dodecyl sulphate) PAGE as described elsewhere by Havea et al, (1998) with some minor modifications. About 0.2 g of protein samples were dissolved in appropriate buffers and loaded onto either 4 – 15% Tris-HCl Ready gels (Bio- Rad, Hercules, CA) for native PAGE or 4 – 20% iGels (NuSep, French Fores, Australia) for SDS PAGE (reducing / non reducing) using a cell (Bio-Rad Protean[®] II xi) filled with relevant tank buffer. Native PAGE was carried out at 25 mA or SDS PAGE was at 50 mA. After the de-staining process images were taken using a Fuji Film Intelligent Dark Box II with Fuji Film LAS – 1000 Lite V1.3 software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Turbidity of heat treated samples was determined by immediately diluting them to 0.1% (w/w) protein and measuring absorbance at 420 nm using a spectrophotometer (Novaspec 11, Pharmacia LKB, Norfolk, UK). The apparent optical density at 420 nm was used to present the turbidity (Ju and Kilara, 1998).

Thermal analysis of WP preparations was performed using a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwak, CT) and software (Pyris Manager,

v.5.0002) as described in Chapter 4. The temperature scan was carried out covering the region of 25 to 140°C at a scanning rate of 10°C/min.

6.2.4 Production of MWP powders and sample preparation for analysis

Four types of MWP powders were prepared from 7% (w/w) WP dispersions at pH 3 with citric or lactic acid by microfluidizing at 140 MPa with 5 microfluidizing passes without or with heating them at 90°C for 20 min followed by spray drying. Spray drying was conducted using a pilot scale spray dryer (SL-10 Mini-Maxi Pilot Spray Dryer, Saurin Enterprises Pty. Ltd., Melbourne, Australia). The inlet and the outlet air temperatures of spray dryer were adjusted to 180°C and 80°C respectively. The flow rate was automatically adjusted by itself maintaining the outlet temperature a constant. Three control samples at the same protein concentration were prepared either by diluting with Milli-Q water without changing pH (pH ~ 6) or by adjusting pH approximately to 3 with citric or lactic acid and then spray drying without microfluidization. Approximately 5% protein (w/w) dispersions were prepared from spray dried powders using Milli-Q water. The protein powders required to prepare the dispersions were added portion wise to beakers containing almost correct amount of Milli-Q water with continuous stirring for about 2 hours. When the samples were properly dissolved, they were maintained at 4 °C for overnight without adjusting the pH. After overnight hydration the concentration of dispersions were corrected with Milli-Q water and the analysis of physical, chemical and thermal properties of the dispersions were conducted as follows.

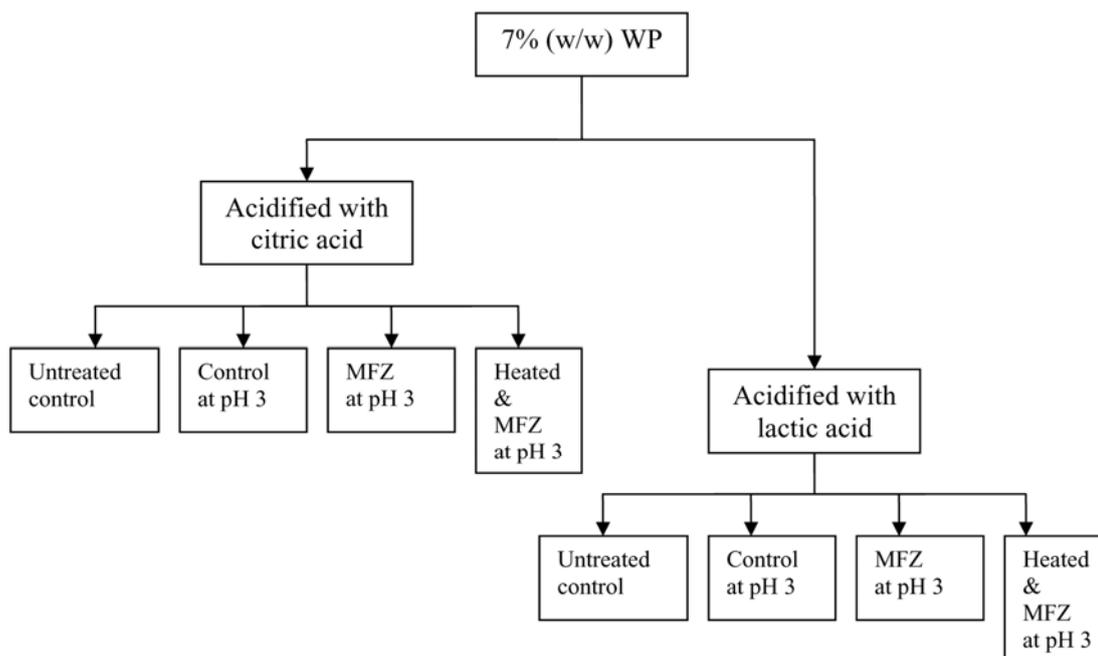


Figure 6.1 Experimental design for obtaining MWP at low pH (MFZ – microfluidization).

6.2.5 Sample analysis

Electrophoretic analysis of the powders as reducing and non-reducing SDS PAGE and native PAGE as well as thermal analysis of 12% (w/w) WP dispersions using a differential scanning calorimeter (DSC 7, Perkin Elmer, Norwak, CT) and the software (Pyris Manager, v.5.0002) in the range of 25 - 140°C were carried out as previously described in this thesis.

Approximately 1% (w/w) whey protein dispersions were hydrated overnight and diluted 1/100 with Milli-Q water. The particle size distribution pattern was obtained using a laser scattering Zetasizer-Nano ZS (Malvern instruments, Worcestershire, UK), equipped with Malvern Zetasizer software (version 6.01, Malvern instruments, Worcestershire, UK). The refractive index (RI) of solvent (water) and the dispersed phase (WP) were considered as 1.33 and 1.52, respectively.

The functional properties of 5% (w/w) WP dispersions, *viz*, solubility, heat stability, heat coagulation time, emulsifying activity, emulsion stability and adsorbed protein were measured using the methods previously described in Chapter 4. However, in the current study, the measurements of protein content in solubility and adsorbed protein were obtained using Kjeldahl method. The protein content of samples needed for heat stability expressed by a solubility method was evaluated using the Bradford reagent as used in previous studies due to insufficient sample size for protein analysis using the Kjeldahl method.

The flow behaviour of approximately 12% (w/w) WP dispersions was examined, using a CS/CR rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with a proprietary software (Rheoplus/32 v2.81, Anton Paar) and a double-gap-cylinder measuring system (DG26.7- SN7721, Anton Paar) as previously described in Chapter 4 of this thesis. The pH of the dispersions was corrected to 7 prior to introducing the rheometer.

In situ heat gelation was also assessed using the same rheological system with the application of dynamic small amplitude oscillatory measurements at a constant strain of 1% and frequency of 1 Hz. The final pH of all preparations was corrected to 7 before introducing them into the rheometer. The rheological measurements were obtained when the samples were heated from 20 – 90°C at a heating rate of 1°C min⁻¹ for approximately 70 min and held at 90°C for 10 min.

In situ cold-set acid and salt gelation was examined using 2% w/w glucono- δ -lactone (GDL) powder and 0.1 M NaCl respectively and a bob and cup measuring system (CC 27 – SN8767, Anton Paar) of the rheometer following the same procedure described in the Chapter 4. The final pH of whey protein preparations was adjusted to 7 before they were introduced into the rheometer.

6.2.6 Statistical analysis

The study on the fundamental protein interactions during heating in the presence of different chemical blockers was arranged as a randomised block full factorial design with the acidulant at two levels (citric or lactic acid) and chemicals (Tween 20, SDS, NEM or DTT excluding the control) as the major factors and replicates as blocks.

The study of functionalities of MWP powders was also organized as a randomized full factorial design with acidulant, microfluidization and heat-treatment as the major factors and replicates as blocks.

Replication of all experiments was carried out twice with subsequent sub-sampling (n=4). Results were analysed using a General Linear Model of SAS statistical programme (SAS 1996) and the level of significance was preset at $P=0.05$. Tukey's Studentized Range (HSD) test was used for multiple comparisons of means of functional and colloidal properties.

6.3 Results and discussion

6.3.1 Electrophoretic analysis of heated WP in the presence of chemical blockers

The Figure 6.2 –A, -B and –C present the native PAGE patterns of 7% (w/w) WP dispersions heated at 140°C for 30 s in water at pH ~ 6 and under acidic conditions adjusted by either citric or lactic acid to pH 3, in the presence of Tween 20, SDS, NEM or DTT. In addition, one sample (lane 1 of Figures 6.2 -A, -B and -C) in each medium served as a control without any bond blocker addition. These chemicals affect protein aggregation by preventing formation of different molecular associations or resolving aggregates by breaking the formed bonds. Hydrophobic interactions are affected by Tween 20 while SDS affects all types of non covalent interactions. NEM prevents formation of new covalent associations due to blockage of thiol groups. In the presence of DTT all existing intramolecular disulfide linkages are reduced and formation of new disulfide bonds during heating is prevented restricting protein aggregation only to non-covalent linkages (Baldwin, 2009; Havea et al., 2009). Native PAGE patterns of heat-treated WP in the presence of different chemical blockers in water around pH 6 indicate that most of the native WP were denatured. The only exception was when they were heated in the presence of SDS and the aggregated materials could be seen on top of the stacking gel. This clearly reveals that most of the proteins have been aggregated through non-covalent associations since SDS prevents formation of all types of non-covalent associations in proteins during heating. In addition, the absence of almost all of the monomeric protein bands in the PAGE gel when DTT was present in the medium confirms it (Figure 6.2 – A, lane 5). The temperature of the medium plays an important role governing the aggregation pathways of proteins with non-covalent interactions prevailing at higher temperatures (> 90°C) (de la Fuente et al., 2002). Also, except the SDS containing sample, the faint protein bands relevant to smaller aggregated materials were present in the Lanes 1, 4 and 5. These small aggregates (a) may have been formed

via hydrophobically driven associations since they were absent in lanes 2 and 3 with Tween 20 and SDS which can prevent formation of hydrophobic interactions. The band corresponding to (b) as well as the two bands present in lane 4 (with NEM) corresponding to even smaller protein aggregates may be smaller non-covalent WP aggregates.

As indicated by the native PAGE pattern of WP in an acidic medium (Figure 6.2 – B and -C) most of the monomeric protein bands were visible regardless of the acidulant type, indicating increased stability of WP against heat-induced denaturation at acidic pH compared to a neutral pH. However, some aggregated materials remaining on top the stacking gel is an indicative of a certain extent of protein aggregation. Also, the more intense protein bands present in lane 5 (Figure 6.2 -B & -C), relative to others corresponding to WP with DTT, are an indication of further obstruction of protein aggregation.

This observation was more pronounced with citric acid compared to lactic acid. It emphasizes that either WP samples already contained covalently aggregated materials before heating or formation of other non-covalent associations have been facilitated by existing covalent associations although covalent bond mediated WP aggregation was very unlikely at pH 3.

It is generally expected that about 8 – 10% denatured and aggregated proteins may be present in fresh commercial WPC products and some of them may have been formed through covalent aggregation (Havea et al., 2009). It was not observed in our samples as indicated by native PAGE (Figure 6.2). The opposite results obtained for the sample in the presence of SDS, indicating increased aggregation, may be a consequence of possible complexation of β -Lg with SDS at low pH (Jung et al., 2008).

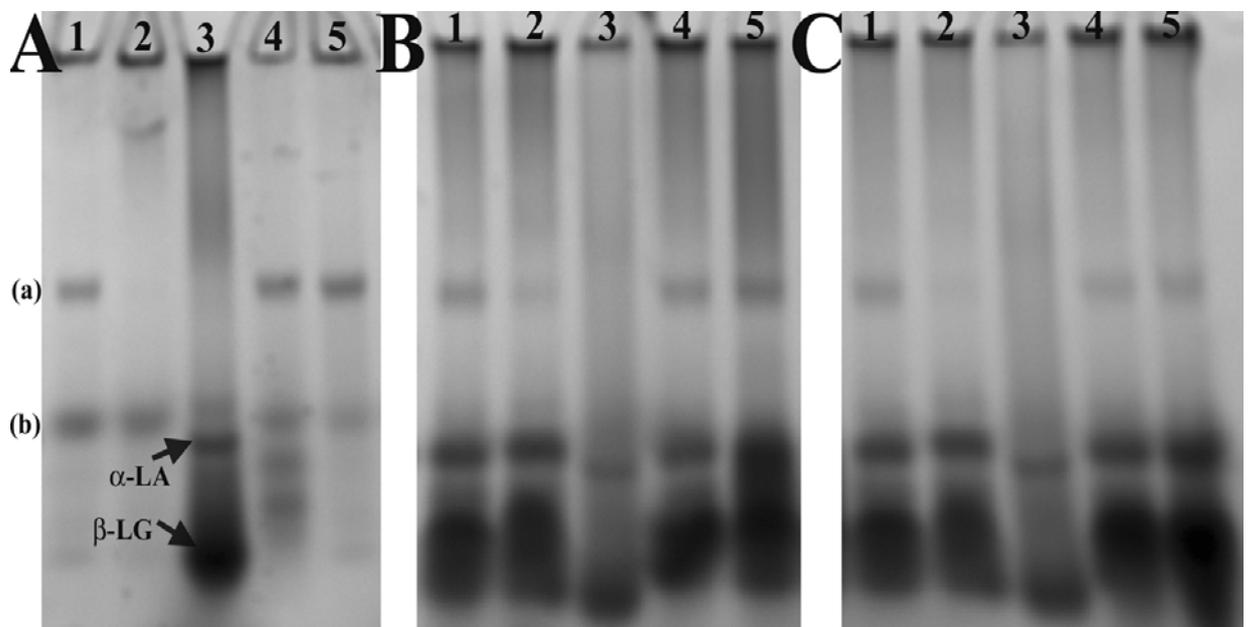


Figure 6.2 Native PAGE patterns of WP (7% protein w/w) in water (pH not adjusted) (A), citric acid (B) or lactic acid at pH 3 (C) after heating at 140°C for 30 s. Lanes from left to right: Lane 1 – WP dispersion with no chemical, lane 2–5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively.

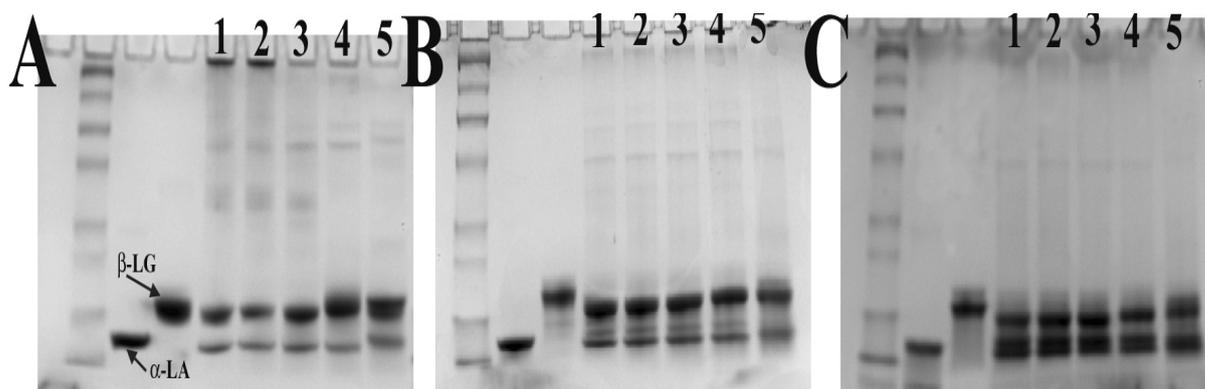


Figure 6.3 Non-reducing SDS PAGE patterns of WP (7% protein w/w) in water (pH not adjusted) (A), citric acid (B) or lactic acid at pH 3 (C) after heating at 140°C for 30 s. Lanes from left to right: Lane 1 – WP dispersion with no chemical, lane 2– 5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively.

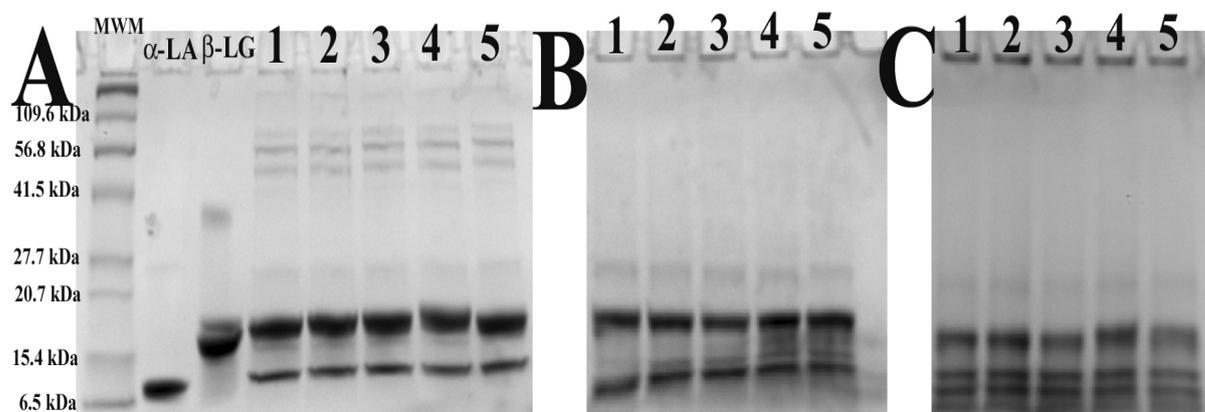


Figure 6.4 Reducing SDS PAGE patterns of WP (7% protein w/w) in water (pH not adjusted) (A), citric acid (B) or lactic acid at pH 3 (C) after heating at 140°C for 30 s. Lanes from left to right: Lane 1 – WP dispersion with no chemical, lane 2– 5, WP dispersions with Tween 20, SDS, NEM and DTT, respectively.

SDS non-reducing PAGE patterns of 7% (w/w) heated WP at ~ pH 6 or pH 3 adjusted with citric or lactic acid, respectively, in the presence of different chemical blocking agents are presented by Figures 6.3 –A, -B and –C. Figure 6.3 –A, lane 1 & 2, corresponding to the control and the sample with Tween 20, shows the presence of some aggregated materials on top of the stacking gel as well as resolved protein bands attributed to α -La and β -Lg, which were absent in the native gels. This indicates that the WP aggregation has occurred *via* both covalent and non-covalent interactions in the control sample as well as in the presence of Tween 20 where only the hydrophobic interactions were prevented. This is further confirmed by the presence of strong β -Lg bands in lanes 4 and 5 of this figure related to the samples with NEM and DTT which could prevent additional covalently driven aggregate formation. The sample with SDS, in which non-covalent aggregations were restricted, also confirmed the occurrence of certain degree of covalent aggregation at this pH as the intensity of monomeric β -Lg band was less pronounced (Figure 6.3 –A, lane 3) compared to those in lanes 4 and 5. In addition, faint smears of protein bands attributed to small aggregates present in lanes 1, 2 and 3 verified that they were newly formed covalent associations due to the absence of these bands in the samples with NEM and DTT. However, at low pH with citric or lactic acid, the bands related to monomeric proteins were present with high as well as almost similar intensities (Figure 6.3 – B and -C) indicating even if little aggregation of proteins has occurred under acidic conditions, as shown by native PAGE gels, these aggregates may have been formed via non-covalent interactions.

Reducing SDS PAGE patterns of heat treated WP specify that in the presence of β -mercaptoethanol (Figure 6.4 –A, -B and -C) WP show some aggregate formation when the medium was acidic in contrast to normal protein behaviour around neutral pH where only monomeric protein bands can be visible. This was more apparent with lactic acid due to the presence of such aggregated materials on top of the stacking gel. This observation was even confirmed by the absence of most of the higher molecular weight monomers in these PAGE gels. However, the

appearance of two bands under acidic pH corresponding to α -La may be its two genetic variants (Fox and McSweeney, 2003).

6.3.2 Turbidity of heated WP in the presence of chemical blockers

Figure 6.5 presents heat-induced changes in turbidity of WP dispersions in water (at pH \sim 6) or in citric or lactic acid (at pH 3) in the presence of Tween 20, SDS, NEM and DTT and of the control samples after heat-treatment at 140° C for 30 s. As previously described in Chapter 5, turbidity of a colloidal dispersion increases with the aggregate growth. The figure shows that the turbidity of the samples under acidic conditions (except WP with SDS) was significantly ($P < 0.05$) lower than those of at neutral pH, implying greatly reduced WP aggregation confirming the PAGE results. Furthermore, turbidity of WP samples was not significantly ($P > 0.05$) affected by the type of acidulant. However, significant ($P < 0.05$) differences of turbidity could be observed when WP were heated around neutral pH. It was significantly ($P < 0.05$) lower when WP were heated in the presence of SDS at this pH as opposed to acidic pH. The markedly low turbidity around neutral pH in the presence of SDS reconfirms that the WP aggregation has occurred mainly via non-covalent associations. However, at acidic pH SDS has enhanced the aggregate formation. The extent of aggregate formation was not significantly ($P > 0.05$) influenced by Tween 20 compared to the control at pH \sim 6. This emphasises that WP aggregation under these particular experimental conditions was mainly driven by other molecular interactions such as electrostatic, covalent and van der Waals forces after unfolding. In addition, the turbidity of the sample containing NEM was equivalent to the control, and the turbidity with DTT was significantly greater than control or NEM. Therefore, it concludes that whey protein aggregation *via* non covalent interactions has been facilitated when DTT was present in the medium compared to NEM.

In fact, the DTT containing sample showed the greatest aggregate formation at neutral pH, consistent with the results shown by native PAGE gels. This may be a

result of efficient unfolding of WP due to cleavage of intra-molecular disulfide covalent linkages by DTT ultimately facilitating consequent aggregation *via* non-covalent interactions with elevated turbidity levels. The significantly low ($P<0.05$) turbidity of the WP sample with SDS at pH around 6 further confirms the importance of non-covalent interactions during WP aggregation.

6.3.3 Thermal analysis of WP dispersions

Thermal behaviour of WP preparations under described experimental conditions and in the presence of chemical blockers was analysed using a DSC. The results are reported in Table 6.1. The DSC thermograms showed that the peak/denaturation temperatures (T_d) of samples in acidic medium significantly ($P<0.05$) higher confirming the previous observations of enhanced stability of WP at low pH. The unexpected effect exerted by SDS was again proven by significantly ($P<0.05$) elevated peak temperatures of WP samples with lactic acid. On the other hand, significantly ($P<0.05$) increased onset, endset and peak temperatures as well as enthalpy values of the samples containing SDS were observed when they were treated at pH adjusted with lactic acid. The enthalpy of samples with SDS in citric acid and water was comparatively lower than those were in lactic acid which again confirms the different thermal behaviour of these samples.

In addition, with citric acid, there was a significant ($P<0.05$) reduction in onset temperature of WP when NEM and DTT were used. Also, when Tween 20 was present in the medium at neutral pH, the onset temperature of WP denaturation has significantly ($P<0.05$) decreased, all of which are indicative of facilitated denaturation of WP likely mediated by enhanced unfolding *via* affected intra-molecular hydrophobic, covalent or other interactions.

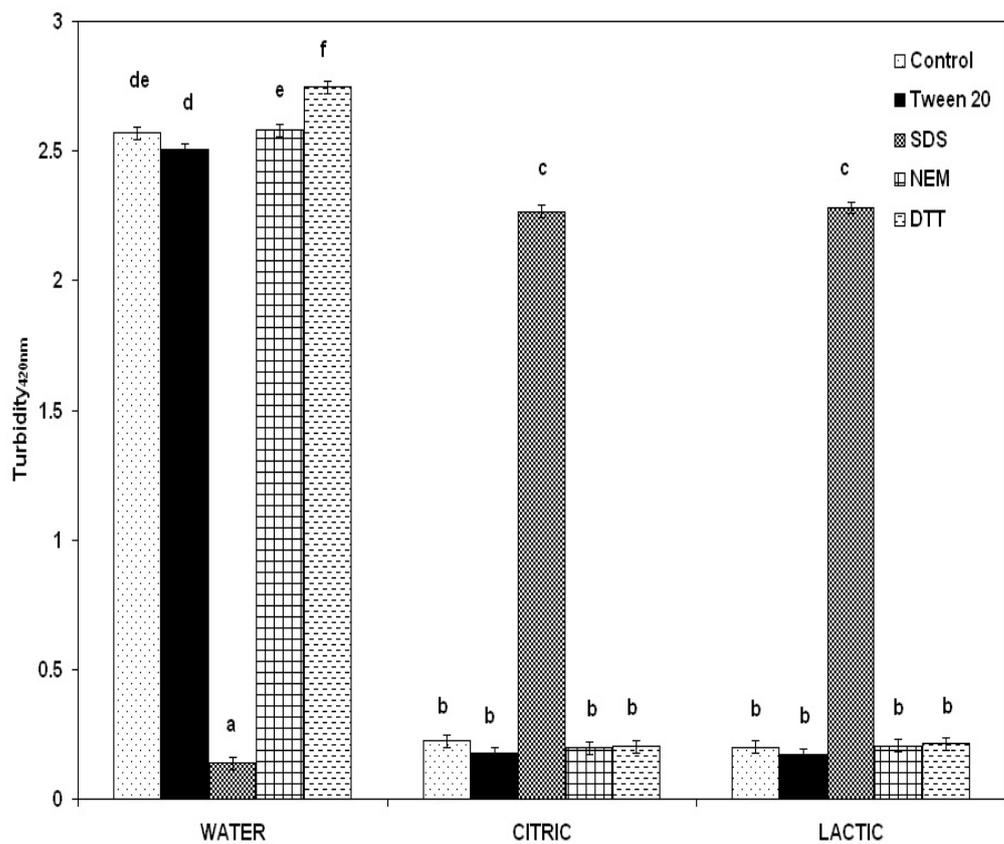


Figure 6.5 Heat-induced changes in turbidity of 7% (w/w) WP dispersions at pH ~ 6 in water or at pH 3 in citric acid or lactic acid without a chemical blocker (Control), or in the presence of Tween 20, SDS, NEM and DTT after heat-treating at 140° C for 30 s.

Table 6.1 Thermal analysis of WP dispersions containing 7% (w/w protein) at pH ~ 6 (not adjusted, aqueous medium) or pH 3 adjusted with citric or lactic acid and in the presence of chemical blockers

Medium (pH)	Chemical	Onset temperature, °C	Endset temperature, °C	Peak (T_d) temperature, °C	ΔH , J/g
Water (pH ~ 6)	Control	66.2 ^b	81.4 ^a	74.2 ^a	3.3 ^a
	Tween 20	60.7 ^a	82.8 ^a	76.8 ^a	3.6 ^a
	SDS	64.4 ^b	83.3 ^a	80.7 ^b	0.6 ^a
	NEM	64.5 ^b	80.9 ^a	73.3 ^a	2.3 ^a
	DTT	65.9 ^b	81.2 ^a	74.5 ^a	3.3 ^a
Citric acid (pH 3)	Control	72.9 ^{cd}	91.2 ^{bc}	81.5 ^b	4.3 ^a
	Tween 20	72.1 ^c	92.7 ^{bc}	83.2 ^b	6.0 ^a
	SDS	73.4 ^{cd}	88.4 ^b	83.5 ^b	1.2 ^a
	NEM	66.8 ^b	90.7 ^{bc}	80.3 ^b	7.1 ^a
	DTT	67.3 ^b	96.6 ^c	82.9 ^b	10.0 ^b
Lactic acid (pH 3)	Control	69.8 ^{bc}	94.8 ^c	81.4 ^b	4.7 ^a
	Tween 20	69.4 ^{bc}	90.4 ^{bc}	82.1 ^b	5.2 ^a
	SDS	77.7 ^d	111.7 ^d	95.8 ^c	14.5 ^c
	NEM	65.6 ^b	86.7 ^b	78.8 ^b	5.1 ^a
	DTT	71.5 ^c	88.6 ^b	80.3 ^b	5.1 ^a
*SEM		1.71	1.82	1.40	1.26

ΔH and T_d - denaturation enthalpy and denaturation temperature of WP, respectively. *SEM – Pooled standard error of the mean. Means present the average of at least 4 independent observations ($n \geq 4$). Means with different superscripts in a column indicate significant difference ($P < 0.05$).

After studying the fundamental WP interactions responsible for heat-induced WP denaturation and aggregation at low pH, different types of MWP powders were produced at low pH using two acidulants, citric and lactic acids, followed by spray drying these (7% w/w) protein dispersions. The extent of WP denaturation and modification of microparticulated powders due to heat and high pressure shearing under low pH (at pH 3) and also the different acidulant effects between citric and lactic acids were examined by native PAGE analysis, DSC and particle size distribution pattern. The WP dispersions which were used to prepare the powders were of seven main types: Native control (Control); WP in citric acid (Control-CA); WP in citric acid and microfluidized at 140 MPa/5 passes through the microfluidizer (M-CA); WP in citric acid, heated at 90°C/20 min then microfluidized at 140 MPa/5 passes (HTM-CA); WP in lactic acid (Control-LA); WP in lactic acid and microfluidized at 140 MPa/5 passes (L-CA); WP in lactic acid, heated at 90°C/20 min then microfluidized at 140 MPa/5 passes (HTM-LA).

6.3.4 Electrophoretic analysis of MWP at low pH

As depicted by Figure 6.6 -A, in native PAGE gel, protein bands related to major WP, β -Lg and α -La were clearly visible (lanes 1, 2, 3 and 5, 6, 7) while very small amount of aggregated proteins was also present on top of the stacking gel. However, most importantly, monomeric WP bands of heat treated WP were absent (lanes 4 and 8) regardless of acidulant type, confirming the complete WP denaturation upon heating. In contrast, as Figure 6.2 shows when WP were heated at 140°C, for a short time of 30 s, all monomeric protein bands became visible. Although both samples were at pH 3 where intermolecular repulsion was very high, the longer time interval used in former case has allowed aggregation of proteins leading to absent of bands in native PAGE.

The presence of monomeric protein bands in SDS non-reducing PAGE gel of HTM-CA sample (Figure 6.6 – B, Lane 4) indicates that non-covalent interactions

were responsible for the aggregation. However, in both types of SDS PAGE gels with lactic acid, such bands related to HTM-LA (Figure 6.6 –B & -C, lanes 8 & 8 correspondingly) were very faint.

This could be due to a possible ion specific aggregation of lactate ion with reducing agents, although it was expected to observe more resolved bands, especially, under SDS reducing conditions. Furthermore, the monomeric β -Lg band of M-LA sample under SDS non-reducing conditions (Figure 6.6 –B, lane 7) showed comparatively greater intensity than other samples. However the interpretation of these observations is rather difficult since the influence of SDS on cleaving WP aggregates is different at low pH. The reducing SDS PAGE gels, particularly with citric acid clearly display two separate bands corresponding to α -La and this was also observed previously in the first part of this study. It can be suggested that these bands may be two genetic variants of α -La.

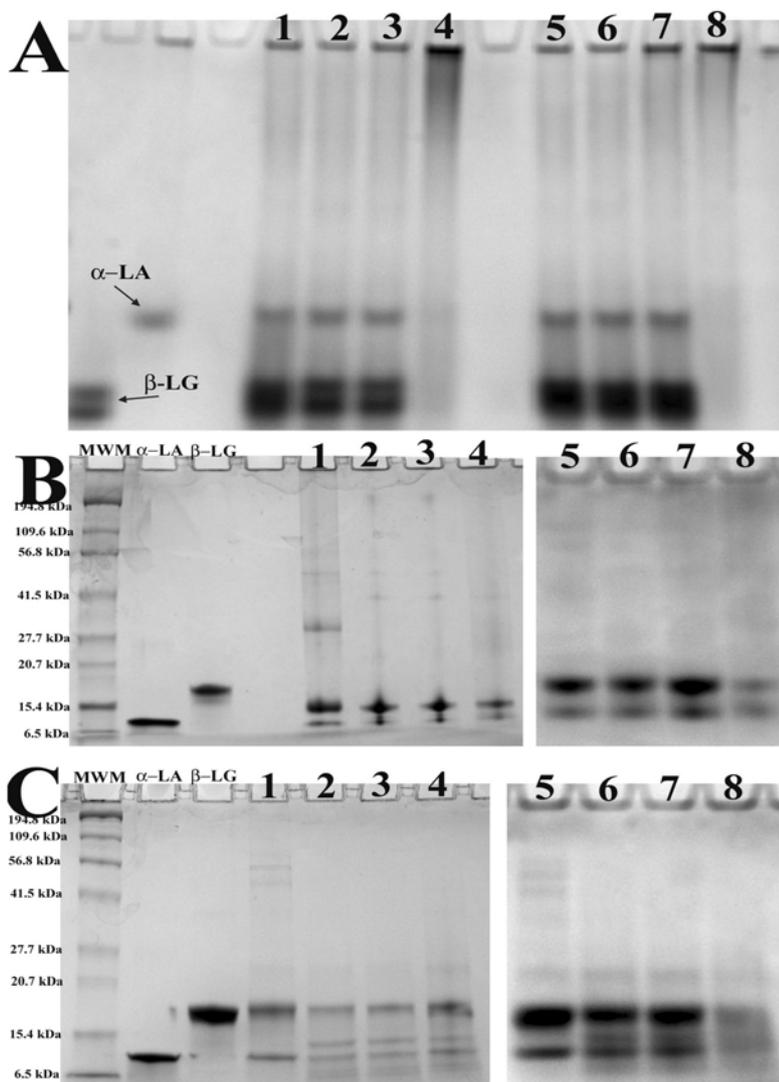


Figure 6.6 Native (A), SDS non-reducing (B) and SDS-reducing PAGE (C) patterns of WP powders produced by spray drying of 7% (w/w) WP dispersions in citric or lactic acid at pH 3 after subjecting to different treatments as follows. Lanes from left to right: β -Lg standard, α -La standard, Lanes: 1 - untreated control (Control); 2 - citric acid acidified control (Control-CA); 3 - citric acid acidified and microfluidized (M-CA); 4 - citric acid acidified, heated and microfluidized (HTM-CA); 5 - untreated control (Control); 6 - lactic acid acidified control (Control-LA); 7 - lactic acid acidified and microfluidized (M-LA); 8 - lactic acid acidified, heated and microfluidized (HTM-LA) respectively.

6.3.5 Thermal analysis of MWP at low pH

The thermograms shown in Figure 6.7 describe thermal behaviour of different WP samples prepared from MWP at low pH and corresponding controls. DSC analysis mainly assists in understanding the extent of prior WP denaturation as well as the heat withstanding ability of proteins depending on the shifts in their denaturation temperatures. The extent of protein denaturation is related to the enthalpy change associated with protein unfolding. Also, the unfolding of globular WP is an endothermic heat process (Paulsson and Dejmek, 1990; De Wit, 1990). The DSC thermograms of WP samples obtained in the current study clearly indicate that the denatured MWP sample did not contain endothermic peaks confirming the enhanced heat stability of the WP imparted by irreversible denaturation caused by heating process. In contrast, the undenatured WP samples possessed broad endothermic peaks. There was a broad endothermic peak with peak height located around 77°C related to denaturation temperature of the major WP, β -Lg, at native conditions which is consistent with the literature reports (Fitzsimons, et al., 2007; Patel et al., 1990; De Wit, 1990). Similarly, the occurrence of two other broad peaks with peak temperature around 82°C belongs to undenatured samples acidified with citric acid to pH 3 (Control-CA, M-CA). In addition, the broad peaks in DSC thermograms of the samples acidified with lactic acid (control-LA, M-LA) were visible with their peak height corresponding to denaturation of globular protein around 88°C. The higher denaturation temperatures of WP at low pH confirmed their enhanced heat stability. This occurrence may be a consequence of prevalence of stronger hydrogen bonding at acidic pH (Resch et al., 2005), inhibition of thiol group activity and purely repulsive electrostatic interactions between monomers upon protein unfolding (de la Fuente et al., 2002), all of which created unfavourable conditions for subsequent protein aggregation.

The endothermic total enthalpy of protein denaturation is primarily linked to the disruption of different endothermic and exothermic molecular associations (Damodaran et al., 2008). Therefore, decrease in denaturation enthalpy of M-CA

sample compared to control-CA sample as well as increase in denaturation enthalpy of M-LA samples compared to control-LA sample may be a consequence of applied high pressure shearing on these WP. Further, the different peak temperatures and enthalpy values obtained for thermograms of WP in citric and lactic acids showed the variable effects of acidulant. Hence, the specific influence of lactate ions on heat stability of WP apparently conquered the effect that citrates impart by chelating calcium.

6.3.6 Particle size distribution pattern of MWP at low pH

Figure 6.8 and Table 6.2 present the particle size distribution patterns of MWP powders produced at low pH. As the figure indicates, the most significant observation was the confinement of average particle size of all WP samples to a sub-micron level. In addition, the average particle size of denatured MWP was significantly ($P < 0.05$) smaller than that of the control at neutral pH (Table 6.2). This is a greater reduction of particle size of denatured WP when compared to that of MWP obtained at neutral pH as described in Chapter 4 which had their average particle size around 10 μm . In addition, both types of MWP derived from lactic acid samples had significantly ($P < 0.05$) reduced particle size compared to that of lactic acid control. Microparticulation alone also had a significantly ($P < 0.05$) positive effect reducing the particle size of the WP prepared using citric acid; on the other hand, the relative particle size of heat treated MWP (HTM-CA) was not different ($P > 0.05$) from the control citric acid samples. In general, these findings indicate that microparticulation has a direct impact on particle size reduction of WP gels particularly created at low pH conditions.

As previously described in this thesis, the application of high pressure shearing during microfluidization may have affected WP in different ways. The process would result in simultaneous collision, compression, shearing and flowing with the contraction and more importantly stretching and elongation of protein

molecules (Bouaouina, et al., 2006; Altmann, et al., 2004; Akkermans, et al., 2008). These impacts may lead to induced alternations in WP particles such as conformational rearrangements which promote or enhance the existence of different molecular associations (Altmann, et al., 2004). In addition, the WP gels prepared under acidic conditions possess weak brittle characteristics mainly acquired by relatively weaker linkages which may be confined only to non-covalent interactions under highly repulsive conditions. Therefore, high pressure shearing on the other hand may have easily disrupted these weak linkages and involved in possible rearrangements in molecules finally generating smaller WP particles. In addition, the results suggest that there was no significant ($P>0.05$) effect *via* calcium chelating ability of citrate ions on gel formation. Although it was expected that masking of calcium ions may lead to further reduction of particle size of WP by creating a weaker gel network, the instability of citrate ions at extremely low pH may have prevented chelation of calcium.

6.3.7 Effects of microparticulation at low pH on selected functional properties (solubility, heat stability, emulsifying capacity) of MWP powders

Table 6.2 summarises the major findings in regard to changes of selected physical functionalities as affected by microparticulation of WP at low pH adjusted two acidulants – citric or lactic acid. The functional properties assessed included solubility, heat stability and emulsifying capacity of MWP.

Solubility is usually a key indicator of a protein's ability to exhibit other functional properties. Meanwhile, the solution conditions such as pH, ionic environment and temperature are essentially important in controlling the solubility as the solubility is affected by the protein-water and protein-protein interactions (Hui, 2006). The results in Table 6.2 indicate that applied treatment (microparticulation, heating, acidification) significantly ($P<0.05$) reduced solubility of resulting MWP in comparison to control. On the other hand, solubility among treated samples was fairly similar ($P>0.05$).

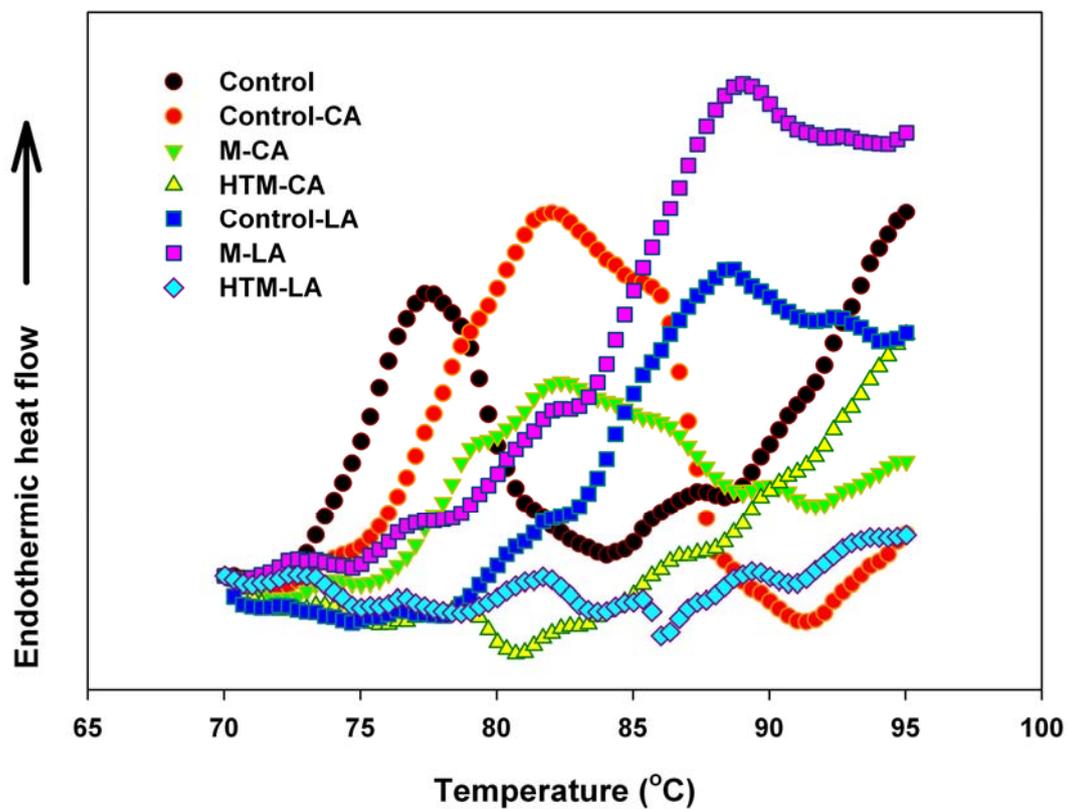


Figure 6.7 DSC thermograms of 12 (% w/w) whey protein dispersions prepared from MWP and the controls. Legend: untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA).

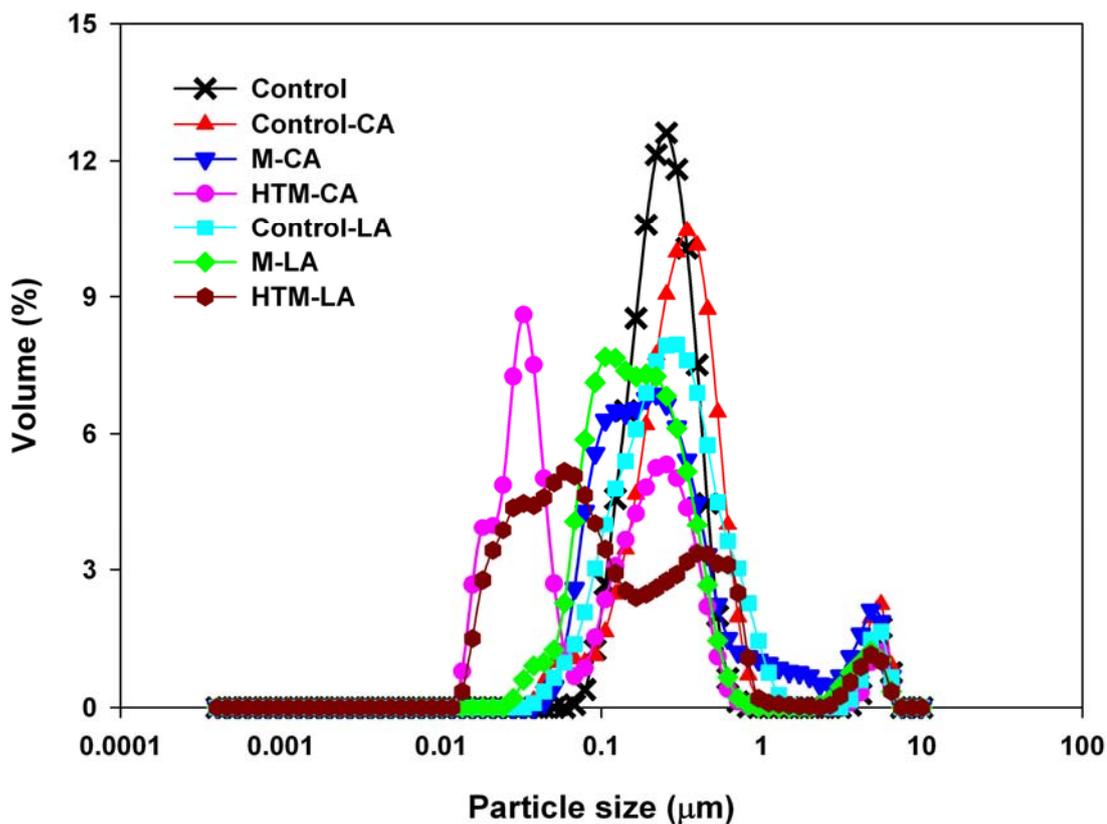


Figure 6.8 Particle size distribution pattern of MWP obtained by different treatment at low pH. Legend: untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA).

Table 6.2 Colloidal and interfacial properties of microparticulated WP powders produced at pH 3 using citric and lactic acids

Treatment	Solubility, %	Heat stability, %	HCT, s	EAI, $\text{m}^2 \cdot \text{g}^{-1}$	ESI, h	Adsorbed protein, mg/mL	Average particle size, nm
Control	86.4±2.9 ^a	26.0±1.5 ^c	~ 9	6521±651 ^c	25.8±0.2 ^a	7.5±0.9 ^c	131±6.1 ^a
Control-CA	67.4±2.7 ^b	34.2±0.5 ^b	~ 90	9062±2011 ^b	25.2±0.1 ^b	14.9±1.3 ^a	112±11.5 ^{abc}
M-CA	70.4±0.4 ^b	35.2±2.2 ^a	~ 90	5625±628 ^c	25.3±0.1 ^b	12.8±0.4 ^b	84±4 ^d
HTM-CA	67.3±0. ^b	19.7±1.6 ^d	> 180	6566±806 ^c	26.0±0.1 ^a	15.0±0.4 ^a	101±19.3 ^{bcd}
Control-LA	70.0±1.2 ^b	17.6±1.1 ^f	~ 90	9366±1530 ^a	25.2±0.1 ^b	14.1±1.0 ^{ab}	123±5.7 ^{ab}
M-LA	68.8±3.5 ^b	19.0±0.6 ^e	~ 90	8183±1378 ^{abc}	24.4±0.3 ^c	12.2±0.6 ^b	91±1.8 ^{cd}
HTM-LA	68.9±1.0 ^b	10.9±1.0 ^g	> 180	7546±191 ^{abc}	25.9±0.3 ^a	15.0±1.0 ^a	100±6.3 ^{cd}

CA- citric acid; M-CA - microparticulated, citric acid; HTM-CA- heat-treated, microparticulated, citric acid; LA- lactic acid; M-LA - microparticulated, lactic acid; HTM-LA- heat-treated, microparticulated, lactic acid; HCT – heat coagulation time; EAI – emulsion activity index; ESI – emulsion stability index. The different small letter superscripts in a column indicate significant difference ($P < 0.05$).

WP are highly soluble over a wide range of pH (Damodaran et al., 2008). However, at very low pH, due to high intra molecular repulsions, some of WP molecules may partially unfold or deviate from their existing conformation, which may lead into aggregation and subsequent partial loss of solubility. However this is not in agreement with reduced average particle size of these WP at low pH. Therefore, this loss of solubility could be due to different hydration properties and acidulant effects on WP solubility compared to the native control. Heat induced protein aggregation generally reduces aqueous solubility of protein resulting in impaired solubility (Considine et al., 2007; de la Fuente et al., 2002). Although low in comparison to the control, solubility of denatured MWP powders obtained at low pH was actually higher than those of denatured MWP produced at neutral pH as mentioned in Chapter 4 and presents a substantial improvement. This stems directly from a combination of high pressure shearing with change in processing conditions, which has rendered the improved colloidal properties by reducing the particle size of WP micro aggregates and thus the comparable solubility.

In addition to the improved colloidal stability denatured MWP at low pH possessed high heat withstanding ability, with a heat coagulation time (HCT) > 3 min for both the samples, compared to the undenatured MWP at acidic pH which showed the visible aggregates after around 1.5 min. Also, the HCT of native control was ~ 9 sec. These high HCT values are consistent with the outcome of DSC analysis for these samples which also showed either absence of endothermic peaks or greater denaturation temperatures compared to native controls. However, the reduced % heat stability of all samples is somewhat contradictory and may have been resulted from specific acidulant effects.

Emulsifying properties of MWP powders were assessed by two well established parameters: emulsion activity index (EAI) and emulsion stability index (ESI). The EAI values of samples prepared from control citric and lactic acid powders were significantly ($P < 0.05$) higher than that of the untreated control. The EAI depends on oil volume fraction, protein concentration and the type of equipment used to produce the emulsion (Pearce and Kinsella, 1978). In addition, the molecular

flexibility, which determines the degree of unfolding of proteins, the extent of interactions of proteins with other molecules, and surface hydrophobicity, which influences the affinity of proteins for the oil-water interphase, are impotent factors controlling emulsification (Monahan et al., 1993). Therefore, markedly increased emulsification activity of most of acidic samples may be a result of increased flexibility of proteins at low pH resulting from higher intra-molecular repulsion, compared to that of proteins at neutral conditions. As revealed by the statistical analysis, high pressure shearing alone as well as combined heat and high pressure shearing significantly ($P < 0.05$) reduced the EAI of samples prepared from powders derived from heated samples after acidified with citric acid compared to their citric acid control. On the other hand, microparticulation did not considerably affect EAI of the WP originated from lactic acid, relative to that of the acidic control. The variability in EAI in MWP prepared by different acidulants may have resulted from specific anion effects. These effects mainly involve perturbation of the solvent structure, which may consequently lead to conformational changes in the protein structure, including hydrophobic interactions, finally imparting alternations in functional attributes of the proteins (Resch et al, 2005).

Further, significantly ($P < 0.05$) more stable emulsions were created from denatured and microparticulated samples of both the acidulants and they also showed significantly ($P < 0.05$) greater ability to adsorb to the surfaces of oil droplets compared to other acidic samples. Additionally, the emulsion stability of native controls was also comparable to denatured samples although they had significantly ($P < 0.05$) lower adsorbing properties to oil droplet surfaces. High pressure shearing only had a significantly ($P < 0.05$) negative effect on the emulsion stability of WP derived from lactic acid gels (M-LA). In general, the WP in all samples prepared from acidic derivatives had significantly ($P < 0.05$) higher capability to adsorb onto the oil droplets compared to that ability of native control. Usually, heat reduces the emulsifying characteristics of proteins owing to protein aggregation. Nevertheless, as shown by particle size distribution pattern

and the outcome of emulsifying properties, it is evident that microparticulation of denatured WP have not only reduced the size of the particles but also changed the protein structure by exposing previously buried favourable reactive sites such as hydrophobic groups to the interphase. The exposure of such groups may cause proper alignment of hydrophilic and hydrophobic residues, consequently, the improvements in interfacial properties since the emulsifying capacity depends on the interfacial area that can be coated by proteins (Pearce and Kinsella, 1978). Also, the emulsion stability depends on the steadiness of the interphase which is on the other hand governed mostly by different molecular properties. One such property is a net charge of the protein molecule, which presents a barrier to the close approach of oil droplets and thereby preventing coalescence (Klemaszewski and Kinsella, 1991). Also, they form coherent monolayers by the involvement of intermolecular disulfide bonds (Damodaran et al., 2008). Both these situations may be possible in native controls where the proteins contained a net negative charge as well as reactive thiol groups under neutral pH. The increased emulsion stability of denatured microparticulated samples may be a result of exposure of hydrophobic sites during denaturation and also likely modification of protein secondary conformation due to high pressure shearing with elevated concentration of adsorbed protein on oil droplet surfaces.

6.3.8 Flow behaviour of WP dispersions

The Figure 6.9 presents the apparent viscosity of 12% (w/w) WP preparations derived from MWP powders as well as untreated and acidified controls adjusted to pH 7 and at 20°C. As indicated by the figure, all dispersions exhibited non-Newtonian and shear thinning behaviour. This may arise due to favourable orientation of protein molecules to the major axes of the direction of flow with the possible dissociation of weakly held oligomers into their smaller species (Damodaran et al., 2008). More importantly, the denatured MWP samples had comparatively higher yield point and they maintained higher viscosity levels

throughout the shear rate ramp. The bulk rheology of a colloidal dispersion is determined by a combination of various forces which are mainly dependant on the particle size of dispersion. The average particle size of these WP varied around 100 nm. Therefore, the flow behaviour depends on hydrodynamic forces, which arise from the relative motion of particles to the surrounding fluid, Brownian motions, which are ever-present thermal randomizing forces and also on the elastic interparticle forces (Genovese et al., 2007). Also, particle shape, size and size distribution, particle deformability and liquid polarity could affect the flow behaviour; for example, non-spherical particles cause increase in viscosity due to extra energy dissipation during flow. Furthermore, for a given particle concentration, the viscosity decreases with increasing polydispersity indicated by particle size distribution width (Genovese et al., 2007). In addition, heated WP have a greater ability to be hydrated than native proteins (Fox and McSweeney, 2003). The increase in apparent viscosity of denatured MWP may have resulted from much higher water holding capacity, especially under a net positive charge where hydrogen bond formation is most likely as well as possible partial protein unfolding through elevated intra-molecular repulsion and consequent formation of physical cross links (Resch, 2004). In addition, high pressure shearing may have caused further conformational rearrangements in denatured MWP affecting protein-protein and protein-solvent interactions leading to viscosity increments. Also, particle size distribution pattern of these WP preparations revealed that the average particle size of MWP was significantly ($P < 0.05$) smaller than that of the native control. Therefore, apart from other possibilities, the greater secondary electroviscous effects, which can arise due to increased overlapping of the electrical double layer (Resch, 2004), and raised interparticle potential of smaller particles resulted from HTM-LA and HTM-CA compared to larger counterparts may also have imparted greater viscosity. Additionally, high pressure shearing has not imposed a noticeable effect on the apparent viscosity of undenatured WP samples except the dispersion prepared as LA acidified control. It exhibited higher viscosity than the other samples during shear rate ramp, which may be a consequence of an anion specific effect.

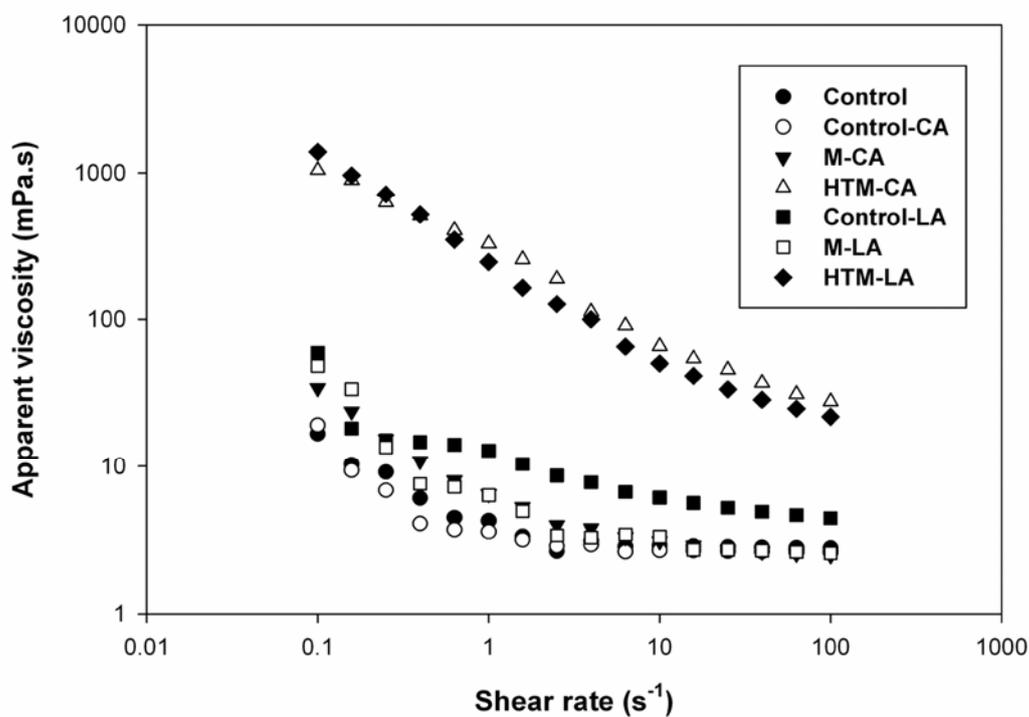


Figure 6.9 Apparent viscosity of 12% (w/w) WP dispersions prepared by addition of untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA) to water and adjusting to pH 7. A shear rate control sweep ($0.1 - 100 \text{ s}^{-1}$) was conducted at 20°C .

The findings of Resch et al. (2005) also previously revealed that the β -Lg dispersions derived from powders prepared with lactic acid had the greater viscosity with higher water holding capacity compared to those prepared with citric acid. Also, the powders prepared with citric acid even exhibited extreme difficulties in redispersing in water which is consistent with the findings of the current work in this thesis.

6.3.9 Gelling properties of MWP

Heat-induced gelation of MWP produced at low pH was studied with small amplitude oscillatory rheology. Figure 6.10 presents the changes in storage modulus (G') during heat-induced gelation of 12% (w/w) unheated and heat-treated MWP samples and the controls prepared from powders produced at pH \sim 6, and at pH 3 with citric and lactic acids during heating, holding, cooling and aging at constant strain and frequency. The final pH of all preparations was corrected to 7 before introducing them into the rheometer. The evolution of storage modulus (G') as a function of time was used to determine the extent of gelation. Results suggest that all previously unheated WP samples gelled upon heating with the native control having the highest G' , which is indicative of creation of relatively more cross-linked viscoelastic gel network compared to acidic samples. During heat-induced gelation initial unfolding and subsequent aggregation of proteins occur and the nature of viscoelastic gel network is governed by the extent of attractive and repulsive forces between protein-protein and protein-water (Hudson et al., 2000). In general, at pH $>$ pI fine stranded, strong elastic gels are formed as opposed to pH $<$ pI, at which fine stranded weak brittle gels are created (Dickinson, 2005). Compared to the firmer network structure of untreated control with higher G' value, the acidic controls possessed relatively weak gel networks.

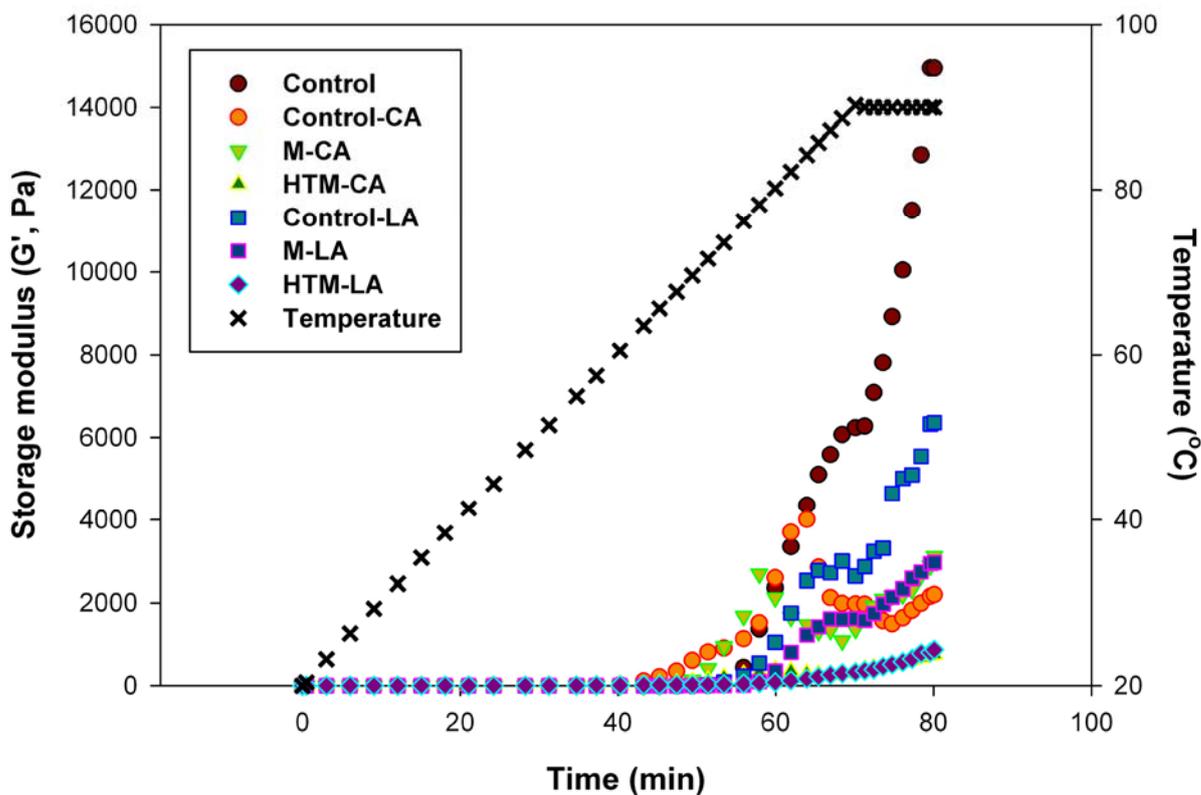


Figure 6.10 Changes in storage modulus (G') of 12% (w/w) WP dispersions during heating (20 - 90°C) and holding (90°C / 10 min) at constant strain (1%) and frequency (1 Hz). Legend: untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA).

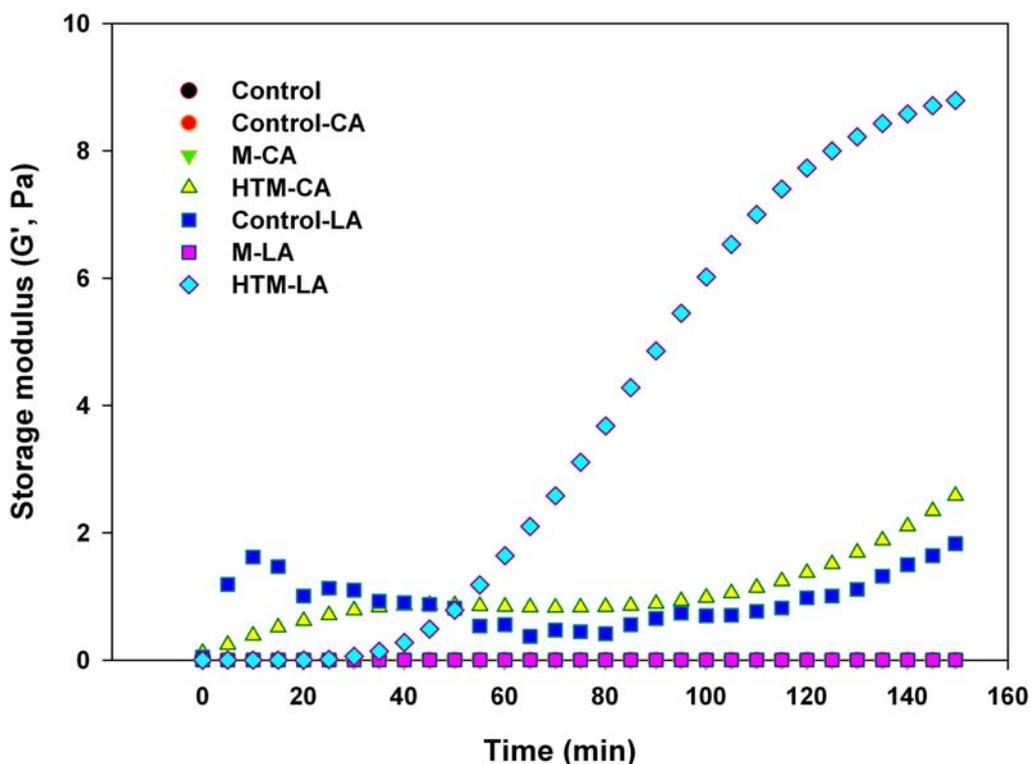


Figure 6.11 Changes of storage modulus (G') during acid induced gelation of 12% (w/w) WP dispersions by addition with 2% GDL. Legend: untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA). Measurements were performed at 20°C at constant strain (1%) and frequency (1 Hz). (The graphs of Control, Control-CA, M-CA and M-LA have been overlapped in the Figure).

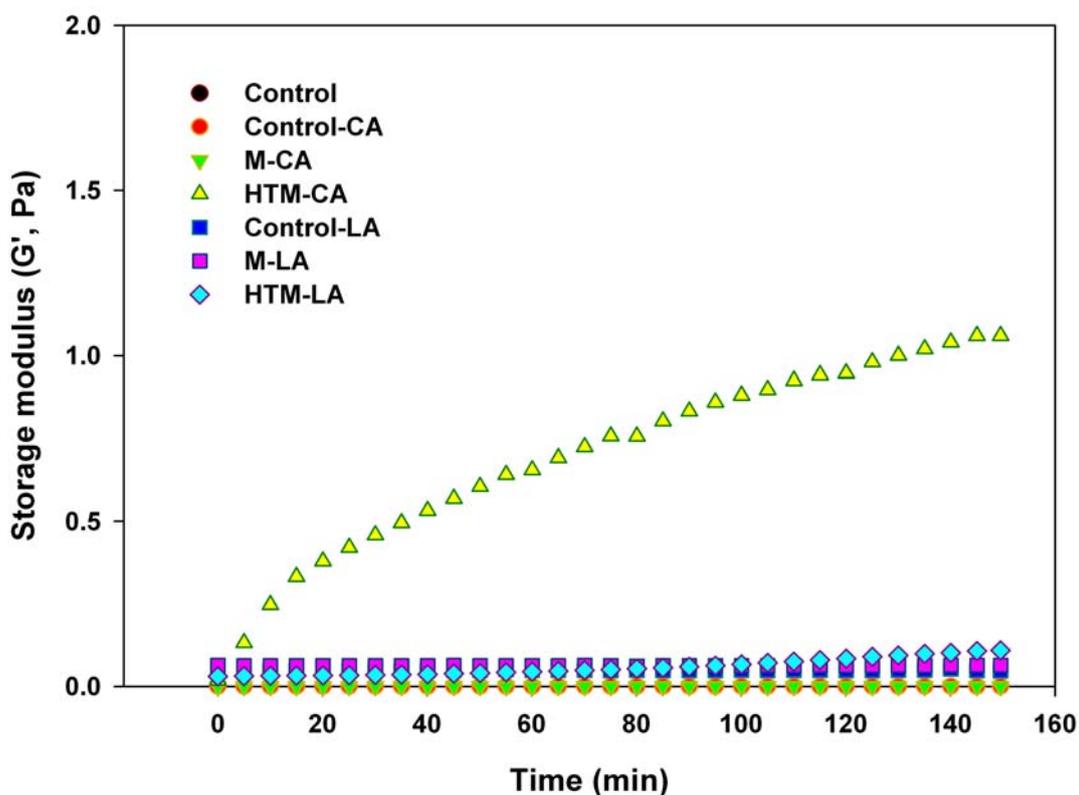


Figure 6.12 Changes of storage modulus (G') during salt induced gelation of 12% (w/w) WP dispersions with 0.1 M NaCl. Legend: untreated control (Control); citric acid acidified control (Control-CA); citric acid acidified and microfluidized (M-CA); citric acid acidified, heated and microfluidized (HTM-CA); lactic acid acidified control (Control-LA); lactic acid acidified and microfluidized (M-LA); lactic acid acidified, heated and microfluidized (HTM-LA). Measurements were performed at 20°C at constant strain (1%) and frequency (1 Hz).

Although, the pH of the whey protein systems was adjusted to 7 before acquiring rheological measurements, these findings indicate of a possible influence of citrate and lactate ions together with other ions present in the medium, in structure formation during heating. As previously described in this thesis the ionic strength after a certain level can create a repulsive molecular environment leading to a weak viscoelastic network. In addition, among acidic samples, lactic acid control had the highest G' value, but high pressure shearing has imparted a negative effect on the gel strength as shown by progress of G' of M-LA sample during heating. These results are consistent with the flow behaviour of these lactic acid samples. Meanwhile, microfluidization has imparted greater gelling ability to treated samples with higher G' for citric acid samples compared to the citric acid control.

As indicated by thermal analysis, citric acid samples started gelling before lactic acid samples. The elevated T_d of WP with lactic acid presents an additional explanation for the weak gelling behaviour and limited instant thickening ability (Resch et al., 2005). These results are in agreement with previously reported results for gelling behaviour of derivatized β -Lg powders produced with citric acid and lactic acid (Resch et al., 2005). It is explained by citric acid acidified sample rapidly undergoing gelation at 80°C and forming large disordered aggregates, which strongly scatter light in contrast to slower network formation of lactic acid gels with an ordered filament aggregation mechanism.

Conversely, the denatured MWP did not form gels during heating (Figure 6.10). This observation is in agreement with thermal analysis as well as previous finding reported in Chapter 4. Formation of heat-induced gels from heat-treated WP is highly unlikely primarily because of unavailability of most of required reactive sites for cross-linking, such as hydrophobic groups in denatured samples. However, the slightly increased G' of these samples may be a consequence of increased hydration of denatured WP particles or weak particle-particle interactions via possibly modified surface properties due to high pressure shearing which may lead to increase the G' measurements to some extent.

Figure 6.11 presents the development of storage modulus of MWP created at low pH and the controls during acid gelation with 2% GDL. As clearly demonstrated by the figure, only heat treated and microparticulated lactic acid sample showed a certain trend to form a weak network with the modulus around 9 Pa. But, other samples did not show considerable gel formation. As depicted by the Figure 6.12, the results of salt-induced gelation obtained for these whey protein samples also indicate absence of gel formation. The maximum G' value obtained for these samples occurred below 2 Pa.

6.4 Conclusions

The studies conducted to observe fundamental WP interactions using chemical blockers revealed that WP were more stable against heat-induced denaturation at pH \sim 3 compared to that at their natural pH. Also, there was no apparent acidulant effect on WP aggregation under the temperature/time combination used in this fundamental study. WP aggregation during heating around their natural pH was mainly driven via non-covalent interactions with concomitant creation of covalent bonds. However, covalent interactions were almost inhibited at pH 3 confining WP aggregation to non-covalent linkages, consequently increasing the denaturation temperatures of WP.

The denatured MWP have been completely denatured. Most importantly, and as required, the particle size of denatured WP as significantly reduced under the implemented experimental conditions by reducing them to around 100 nm, which was a 100 fold reduction compared to the average particle size of MWP produced at neutral pH. In addition, the particle size distribution of all types of WP was restricted to below 1 μ m. Therefore, at low pH, combination of heat and high pressure shearing produced WP micro-aggregates with improved colloidal stability which was not achieved by microparticulation of denatured WP at neutral pH, in addition to substantially enhanced heat stability of these powders.

Consequently, the solubility of denatured MWP powders created at low pH was significantly higher, with the values comparable to undenatured acidic controls, compared to that of denatured MWP produced at neutral pH. Although, EAI was not considerably affected by microparticulation, more stable emulsions were created from denatured MWP with greater adsorbing ability to oil droplet surfaces. Also, the viscosity of denatured MWP samples was greater than that of undenatured WP samples. The gelling attributes upon heating of undenatured MWP were influenced by the two acidulants depending on their relative thermal stability such as slower gelation of WP prepared with lactic acid compared to that of WP with citric acid, which was also reflected by elevated T_d of WP with lactic acid. Additionally, denatured MWP produced with lactic acid created acid induced gels with considerable firmness despite their inability in formation of salt gels with considerable strength. Further, the effect of high pressure shearing on different functional properties of MWP varied with acidulant selection mirrored by variable functional attributes of MWP. Altering pH resulted in modulation of intra- and intermolecular interactions that led to particle size reduction and improved heat stability of MWP, which fundamentally modified their physical functionalities and likely diversified applicability of this important dairy ingredient.

CHAPTER 7

7 Role of shear during heat induced whey protein aggregation

7.1 Introduction

As suggested and shown in preceding chapters, microparticulation of WP using dynamic high pressure shearing in conjunction with heating appears to be an useful approach in modulating functional properties of heat sensitive WP. The physical and chemical properties of WP are highly dependent on their processing history. Shear, which is used in microparticulation of WP, is known to cause alterations in the structural arrangement and solution morphology of colloidal systems (Altmann et al., 2004). For example, in combination with heat, different shear settings may produce aggregates with different yield and cohesive strain and stresses. The microparticulated products obtained *via* high pressure shearing have shown substantially different physical and functional characteristics such as solubility, particle size and heat stability as described in previous chapters of this thesis. In addition, the extent of shearing imposed is also important in differentiating these properties (Iordache and Jelen, 2003). Also, as described by Altmann et al., (2004), the higher the shear rate the smaller would be the particle size.

Upon application of shear, the biopolymer (protein) system gains energy which would result in enhanced fluctuations of the components of the system. The change in solution morphology is a direct result of the changes in conformation of proteins. These changes are brought about by disruption of certain existing molecular associations which may induce imbalances in the force distribution between molecules and consequently promote the occurrence of other associations. Therefore, under shear, the molecules may interact through alternative pathways which are not accessible under simple environmental variations alone (Altmann et al., 2004). Moreover, under different shearing

conditions accompanied with heating or even in the absence of heating, the conformational changes of WP resulting from the loss of native structure as well as the aggregation behaviour may be reversible or irreversible depending on the environmental conditions such as pH, protein concentration, presence of ions and other contributing factors (Bolder et al., 2007; Manski et al., 2007). For example, at low protein concentrations, unfolded WP molecules may form irreversible aggregates due to not only lower probability of molecular collisions but also a greater amount of bound liquid within the network resulting in fragile aggregates which can be deformed by a flow induced shear. On the other hand, at higher concentrations, apart from the close packing of protein molecules, induced aggregates have lower tendency to bind solvent with poor deformability under shear flow (Spigel, 1999). The changes in the environmental pH also govern the extent and direction of the molecular forces present and thereby the nature of resulting gel networks. At pH above the iso-electric point of proteins ($\text{pH} > \text{pI}$), thiol-disulphide interchange chain reactions encourage polymer cross linking, as opposed to low pH (below pI) low thiol group activity leads to less stable gel networks with a high susceptibility to rearrangements. These structure rearrangements may induce the formation of locally denser clusters finally forming more turbid gels (Rabiey and Britten, 2009).

Depending on the thermodynamic state of the protein dispersion, shear flow may cause phase transitions in near-critical concentrations with an approximate transition point around 10% protein where the viscosity increases rapidly due to the increased density of the proteins (Manski et al., 2007). As described by Macritchie and Owens (1969) and Izmailova (1979), coagulation of proteins, leading to the formation of a true gelled or continuous network, may occur under specific conditions when stress is applied to the system. In several studies it has been shown that due to compression the particles are pushed through the intermolecular repulsive barrier (Schwartz et al., 2001; Fenwick et al., 2001), which would result in a gelled state where they are held together by predominating non-covalent interactions as reported by Hellebust et al., (1993).

Therefore, for the full applicability of microparticulation it would be of great importance to understand various protein-protein and protein-solvent interactions during simultaneous high pressure shearing and heating. In-depth knowledge of these interactions as well as understanding of the strength of different molecular associations under different environmental and processing conditions may aid in optimising desirable properties of microparticulated products including colloidal solubility/stability, heat stability and particle size. This may consequently facilitate the expansion of the use of protein-rich food products in the food industry. Therefore, the aim of the current study was to examine the role of shear during heat induced WP aggregation as affected by different protein concentrations and pH conditions.

7.2 Material and Methods

7.2.1 Sample preparation and treatment

The study was conducted using the same whey protein retentates and whey permeate samples used in Kinetic studies in Chapter 5 which were kindly provided by Warrnambool Cheese and Butter Factory (Warrnambool, Victoria, Australia). The gross composition of these materials was reported in Chapter 5.

Samples were prepared by combining the retentates with corresponding whey permeates to produce WP preparation containing four different protein concentrations: 5, 10, 17.5 and ~ 25% (w/w protein). Each WP suspension was then adjusted by drop wise addition of concentrated HCl or NaOH to preselected three pH values to pH 3, pH 5 or pH 7, finally resulting in 12 different types of samples. The pH of each sample was adjusted before the final volume was corrected. All the chemicals used in the study were of analytical grade.

The sample treatment consisted of simultaneously applying heat and shear using a rheometer geometry. Simultaneously, the WP denaturation and aggregation

behaviour was studied, first, by examining the flow properties of samples using a starch cell (Physica Smart, Starch analyzer-Anton Paar) attached to a CR/CS rheometer (MCR 301, Anton Paar, GmbH, Germany) equipped with a proprietary software (Rheoplus/32 v2.81, Anton Paar). Approximately 20 mL of WP samples were introduced into the starch cell and covered with the cap to prevent evaporation. The mixture was pre-sheared for 5 s at a controlled rate of 100 s^{-1} at 20°C and held for 30 s to equilibrate. The flow behaviour of each WP sample was investigated acquiring viscosity measurements at three different shear rates of 100 s^{-1} , 500 s^{-1} or 1000 s^{-1} on separate occasions during heating from $20 - 90^\circ\text{C}$ for 35 min; holding at 90°C for 20 min and cooling from $90 - 25^\circ\text{C}$ in ~ 15 min.

7.2.2 Sample analysis

Immediately after acquiring rheological parameters, sheared and heat-treated WP samples were mixed in appropriate buffers (pH 3, 5 or 7) for further analysis. The buffer solutions of pH 3, 5 and 7 were prepared by mixing 0.1 M citric acid and 0.2 M disodium hydrogen ortho-phosphate (Na_2HPO_4) as previously described by Dawson et al. (1969). The 0.1 M citric acid and 0.2 M (Na_2HPO_4) solutions were first prepared and mixed in the volume ratios of 79.45:20.55, 48.5:51.5, or 17.65:82.35 to obtain diluted working buffers of pH 3, 5 and 7, respectively.

The heat and shear influenced conformational and structural changes of WP were examined using different techniques including turbidity, gel colour, PAGE, surface hydrophobicity and the protein band assignment in IR spectrum by Fourier transform infra red spectroscopy (FTIR).

The turbidity of all heat-treated and sheared WP samples was assessed by immediately diluting samples to 0.05% (w/w) protein with corresponding buffers and measuring the absorbance at 420 nm (Ju and Kilara, 1998b). The turbidity of the samples was expressed as the apparent optical density.

The colour of sheared and heat treated gels was measured using a Minolta Chromameter (CR-300, Minolta Corporation, Ramsey, NJ) as described in Chapter 4.

The surface hydrophobicity of the WP suspensions was assessed spectrofluorometrically using 6-propionyl-2-(*N-N*-dimethylamino)-naphthalene (PRODAN) (Sigma-Aldrich, St.Louis, MO, U.S.A) as previously described by Alizadeh-Pasdar and Li-Chan (2000). The fluorescence probe PRODAN was particularly selected since the study was conducted over a broad pH range. Heated and sheared samples along with controls were immediately after treatment diluted to 0.05% (w/v) protein with corresponding buffers at pH 3, 5 or 7 prepared from the stock solutions of 0.1 M citric acid and 0.2 M (Na_2HPO_4). The final dilutions of the samples were adjusted to the range of 0.002 – 0.01% (v/v). A stock solution of the fluorescence probe PRODAN was prepared as 1.41 mM in methanol in screw capped vials. Then the vials were covered with aluminium foil; sealed caps with Para-film to avoid evaporation and stored in the freezer ($\leq 10^\circ\text{C}$) until they were used. Approximately 10 μL portions of PRODAN probe was added to 4 mL diluted protein dispersions; the mixture was homogenised by vortexing and kept in dark for 15 min before the fluorescence measurements were taken.

The relative fluorescence intensity (RFI) of protein samples were measured using Shimadzu 5301-PC (Shimadzu Corp., Kyoto, Japan) spectrofluorometer by fixing excitation and emission wave lengths at 365 nm and 465 nm respectively with the slit widths of 5 nm. The net RFI values were expressed by subtracting RFI values of buffer blank (buffer and the fluorescence probe) and the protein blank (protein solution without the probe) from corresponding protein solutions with the probe. The initial slope of the net RFI vs. protein concentration plot was calculated from linear regression analysis and used as an index for protein surface hydrophobicity.

7.2.3 Electrophoretic analysis (PAGE)

Following *in situ* treatment using the starch cell of the rheometer as described above, all the samples were diluted in appropriate buffers of reducing and non-reducing SDS-PAGE as described in Chapter 4. The WP samples for native PAGE were dispersed in a native buffer (12.5% w/w 0.5 M Tris-HCl, pH 6.8; 30% glycerol; 0.2 mgml⁻¹ bromophenol blue) prepared in Milli-Q water. Native gel samples were run at 25 mA for around 2 hrs using a Bio-Rad Protean[®] II xi cell filled with the tank buffer (0.025 M Tris, 0.192 M Glycine). The gels used in SDS-PAGE and native PAGE were 4-20%, Tris-HCl, NuSep iGels (French Fores, Australia) and 4-15% Tris-HCl gels, (Bio-Rad Laboratories, Richmond, CA) respectively. About 6 μ L of WPC samples, α -lactalbumin, β -lactoglobulin and 8 μ L of molecular weight standards were loaded onto the Gels (NuSep, French Fores, Australia). The staining and de-staining processes were carried out using the same method described in SDS PAGE procedure and the gel images were taken with a Fuji Film Intelligent Dark Box II with Fuji Film LAS – 1000 Lite V1.3 software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

7.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR measurements were carried out using a Shimadzu IRAffinity-1, Fourier transform infrared spectrofluorometer, (Shimadzu Corp., Kyoto, Japan) with the combined IRsolution FTIR software (Shimadzu corporation, Kyoto, Japan). The spectra of controls and treated WP samples were obtained in the absorbance mode using calcium fluoride semi-demountable cell window (Perkin Elmer, MA, U.S.A.). For each spectrum, an average of 16 scans was recorded at 2 cm⁻¹ resolution in the range of 1400 – 1800 cm⁻¹ after atmospheric and solvent background subtraction. The Fourier self-deconvolutions were performed with the aid of the software in order to recognize the corresponding peaks under the broad *amide I* region.

7.2.5 Statistical analysis

All experiments were organized as a randomized block split plot design with concentration at 4 levels (5%, 10%, 17.5% or 25%), pH at three levels (3, 5 or 7) and shear rate at 3 levels (100 s^{-1} , 500 s^{-1} or 1000 s^{-1}) as major factors. The concentration was used as a main plot with pH and shear rate as a subplot. Replication was used as a block. The General Linear Model of SAS statistical programme (SAS, 1996) was used to analyse the results. All experiments were replicated at least once with subsequent sub-sampling ($n \geq 4$) and the level of significance was set at $P = 0.05$.

7.3 Results and discussion

7.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a useful technique for studying the secondary structure of proteins. The amide 1 band occurs in the range of $1700 - 1600\text{ cm}^{-1}$ of IR spectra arises primarily from the characteristic C=O stretching vibrations (approximately 80%) of the amide group weakly coupled to the in-plane N-H bending ($< 20\%$) and C-N stretching modes (Jones et al., 1994; Kong and Yu, 2007). The frequencies of amide 1 band components are closely related to secondary structural elements of the proteins (Kong and Yu, 2007). Depending on unique molecular geometry as well as the nature of hydrogen bonding of each type of secondary structure of proteins, such as α -helices, β -sheets, turns and random structures, different C=O stretching frequencies are observed in amide 1 region (Jones et al., 1994; Kong and Yu, 2007). Therefore, the effect of shear on conformational changes of WP with respect to concentration and pH during heat-induced gelation was monitored using the amide 1 region of FTIR spectroscopy.

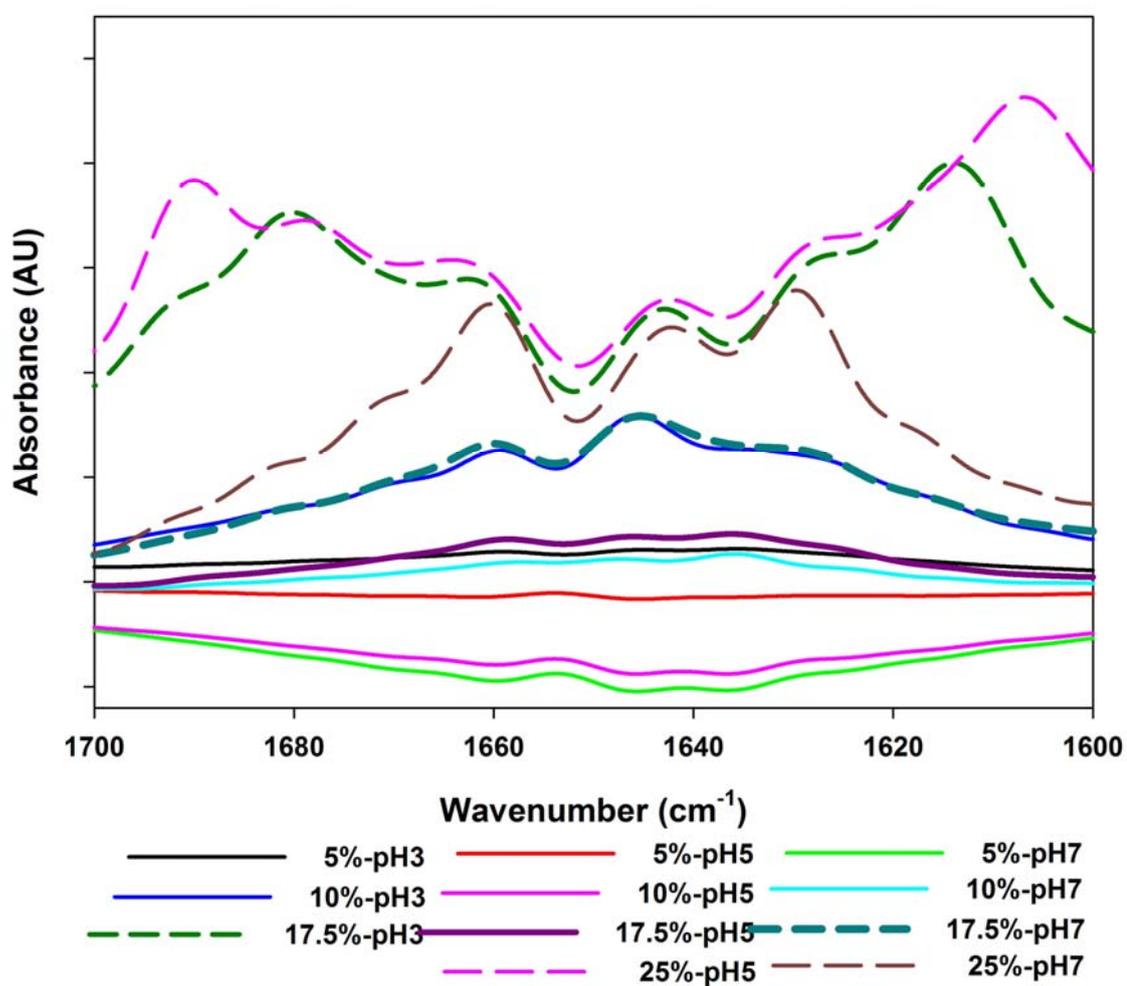


Figure 7.1 Deconvoluted IR spectra of non-treated WP control samples containing 5, 10, 17.5 or 25 % (w/w) protein at pH 3, 5 or 7.

Figure 7.1 presents the deconvoluted IR spectra of amide 1 region of WP control samples at concentrations of 5, 10, 17.5 or 25 % protein (w/w) and pH values of 3, 5 or 7. Apparently, the FTIR spectra were directly concentration dependent with higher concentrations showing greater intensities of bands. In addition, pH has also exerted a substantial effect on the absorption frequencies of this region. Moreover, the overlapping of contours of 10% at pH 3 and 17.5% at pH 7 which is normally unlikely indicates the dependence of protein conformational changes due to extrinsic factors. The characteristic bands located in amide 1 region of FTIR spectra can generally be assigned as follows (Kong and Yu, 2007; Lee et al., 2007; Ganim et al., 2008): the bands positioned around 1690 cm^{-1} , $1680\text{-}1675\text{ cm}^{-1}$ and $1642\text{-}1624\text{ cm}^{-1}$ are assigned to β -sheets; the bands located at 1680 cm^{-1} , 1666 cm^{-1} are assigned to β -turn structures; the random coil is representing the bands placed at $1655\text{-}1645\text{ cm}^{-1}$; the characteristic bands of α -helix are located between 1655 and 1650 cm^{-1} . Also, the protein aggregation occurs with inter-molecular β -sheet formation via hydrogen bonds. Therefore, the appearance of new bands in the range of $1613 - 1619\text{ cm}^{-1}$ for fine stranded gels and in $1620 - 1623\text{ cm}^{-1}$ region for particulate gels (Lefevre and Subirade, 2001) as well as a band appearing at $\sim 1682\text{ cm}^{-1}$ which is related to antiparallel inter-molecular β -sheets are representative of the protein aggregation (Remondetto and Subirade, 2003). The reason for absorbing lower wave-numbers in the IR region by fine stranded gels is due to weakening of the C=O bond of amide groups in aggregates by stronger inter-molecular hydrogen bonds compared to those in particulate gels formed around iso electric point (Remondetto and Subirade, 2003).

The control sample at pH 3 and containing 17.5% protein mainly lacks the band around 1690 cm^{-1} assigned to native β -sheet structures in addition to the band between 1655 and 1645 cm^{-1} related to α -helix/random coil. Also, there is a broad peak around 1680 cm^{-1} which may be related to the overlapping of $1680\text{-}1675\text{ cm}^{-1}$ band assigned to native β -sheet structure and the 1682 cm^{-1} band assigned to antiparallel inter-molecular β -sheets of aggregated proteins. Also, there was a prominent peak around $1613 - 1619\text{ cm}^{-1}$ region associated with aggregated

proteins. In conjunction with these observations, the presence of other peaks related to native secondary structures of proteins confirm the partial unfolding/denaturation and aggregation of pH 3 control at this concentration.

When compared to the control at pH 3, the control sample of pH 5 at 25 % protein (Figure 7.1) represents almost all secondary structural elements related to the native protein structure as identified by relevant peak intensities of FTIR spectra. However, it also did not show a peak between 1655 and 1650 cm^{-1} related to α -helical structures revealing the susceptibility of this structure to denaturation. Furthermore, the spectra did not contain aggregated components as observed in pH 3 revealing the stability of protein conformation around the isoelectric point.

The amide 1 FTIR contour of 25% protein at pH 7 untreated control (Figure 7.1) shows some of the characteristic native structures around 1630 cm^{-1} designated to β -sheet structure, 1655-1645 cm^{-1} for random coil and also the band around 1660 cm^{-1} which may have been coupled with the bands between 1655-1650 cm^{-1} attributed to β -turn and α -helical structures. But it does not show absorbance relevant to β -sheet structures around 1690 cm^{-1} and 1680 cm^{-1} may be due to partial denaturation of WP.

Therefore, the overall result of the FTIR spectrum of control WP samples reveals that the most affected native conformation was observed at pH 3 where as the most conserved native conformation was preserved in pH 5 sample.

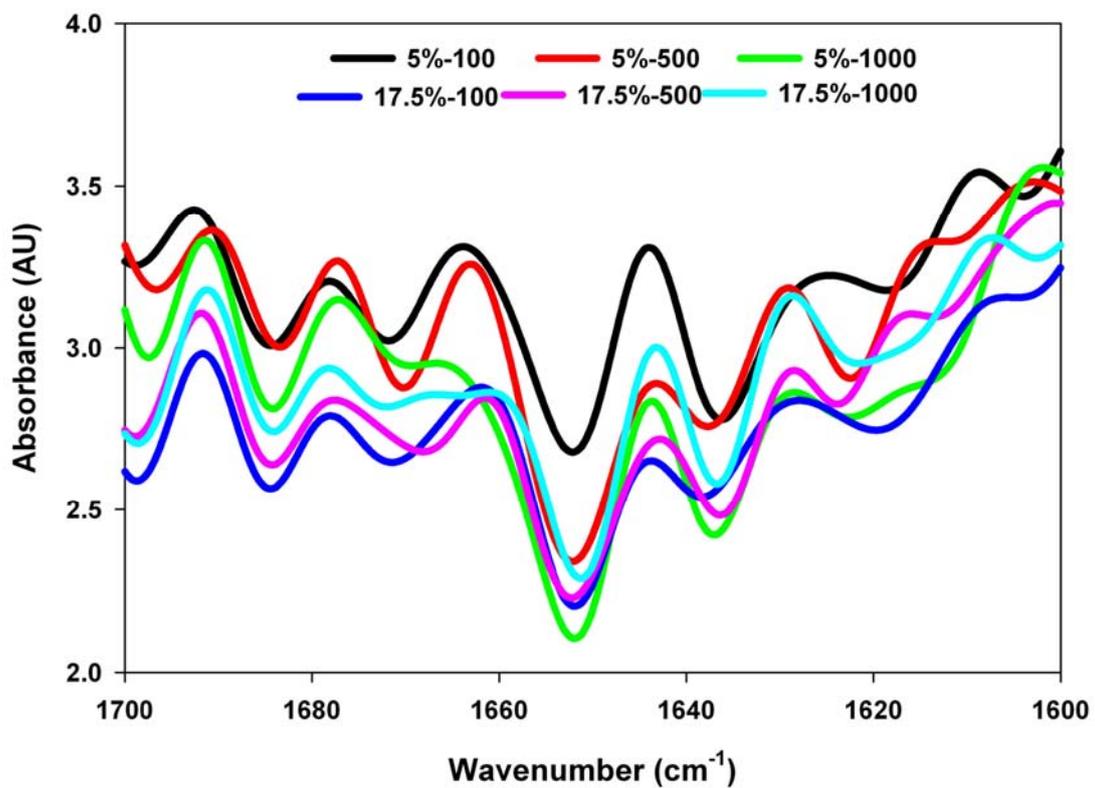


Figure 7.2 Deconvoluted IR spectra of sheared heat-induced WP gels at pH 3, containing 5 or 17.5 % (w/w) protein and sheared at the rates of 100 s^{-1} , 500 s^{-1} or 1000 s^{-1} .

The Figure 7.2 shows the FTIR spectra of sheared gels at concentrations of 5 and 17.5 % (w/w) protein at pH 3. As shown by the figure, the spectra contain most of the characteristic peaks with distinctive frequencies attributed to native conformation of WP. However, the peaks related to α -helical structures (1655-1650 cm^{-1}) were totally absent in each contour indicating their high sensitivity to heat and shear treatment. Further, when the WP samples at both concentrations were sheared at the rate of 100 s^{-1} , the bands located in the range of 1613-1619 cm^{-1} related to intermolecular β -sheet formation during protein aggregation were not observed. However, these bands reappeared when sheared at 500 s^{-1} indicating the formation of inter-molecular β -sheet structures or protein aggregation. Further increase in shear rate resulted in disappearance of these aggregated bands as well as the disappearance of bands corresponding to native β -turn structure at ~ 1660 cm^{-1} indicating the substantial effects of shearing on the structural rearrangement during heating of WP at low pH. Additionally, the absence of peaks related to α -helices in the spectra is quite possible since comparatively weak α -helices are denatured faster than more stable β -sheets (Considine et al., 2007).

Therefore, during the application of simultaneous heat and shear at pH 3, most of the native conformation of WP has been conserved although minor conformational changes could be seen. The shear forces appeared to protect or re-establish the native protein conformation during heating which was even confirmed by observing the untreated control that appeared comparatively more denatured.

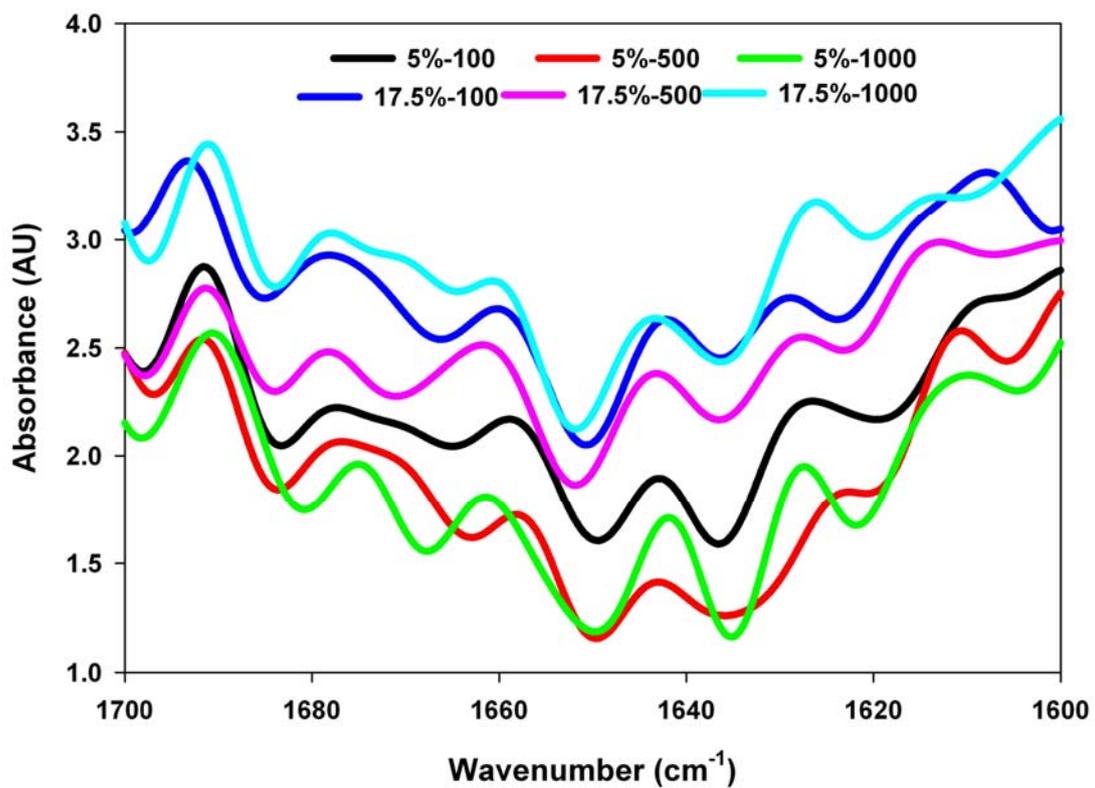


Figure 7.3 Deconvoluted IR spectra of sheared heat-induced WP gels at pH 5, containing 5 or 17.5 % (w/w) protein and sheared at the rates of 100 s⁻¹, 500 s⁻¹ or 1000 s⁻¹.

The Figure 7.3 shows the FTIR spectra of sheared heat-induced WP gels at pH 5, at concentrations of 5 and 17.5 % protein. The spectra indicate that most of the characteristic peaks corresponding to different secondary structural elements present in WP have disappeared compared to those at pH 3. For example, the intensity of bands assign to β -sheet and β -turn structures around 1680 cm^{-1} was much reduced in almost all the samples. It was very prominent in 5% protein samples sheared at 100 s^{-1} and 500 s^{-1} . However, the structure has been regained by extensive shearing at 1000 s^{-1} at the same concentration. Furthermore, when the WP samples containing 17.5% proteins were sheared at the rate of 1000 s^{-1} , the absorbance of this band has decreased compared to other two at the same concentration which confirmed that the shearing had a very profound effect on the conformation. Also, the intensity of bands around 1630 cm^{-1} assigned to β -sheet structure of samples of both concentrations was lower at low shear rates but comparatively increased when sheared at 1000 s^{-1} showing less denaturation. Most of these observations emphasise the importance of interaction of shear and concentration in addition to the effects of pH in governing the final protein conformation or the extent of aggregation as a result of the simultaneous application of shear and heat. In addition, the FTIR spectra of these samples, except the samples sheared at 100 s^{-1} , displayed peaks around 1615 cm^{-1} related to the aggregated band. However, the shearing at very high rates such as 1000 s^{-1} at low 5% protein concentration has clearly reduced the protein denaturation around the iso electric point as indicated by appearance of intense bands attributed to native structure.

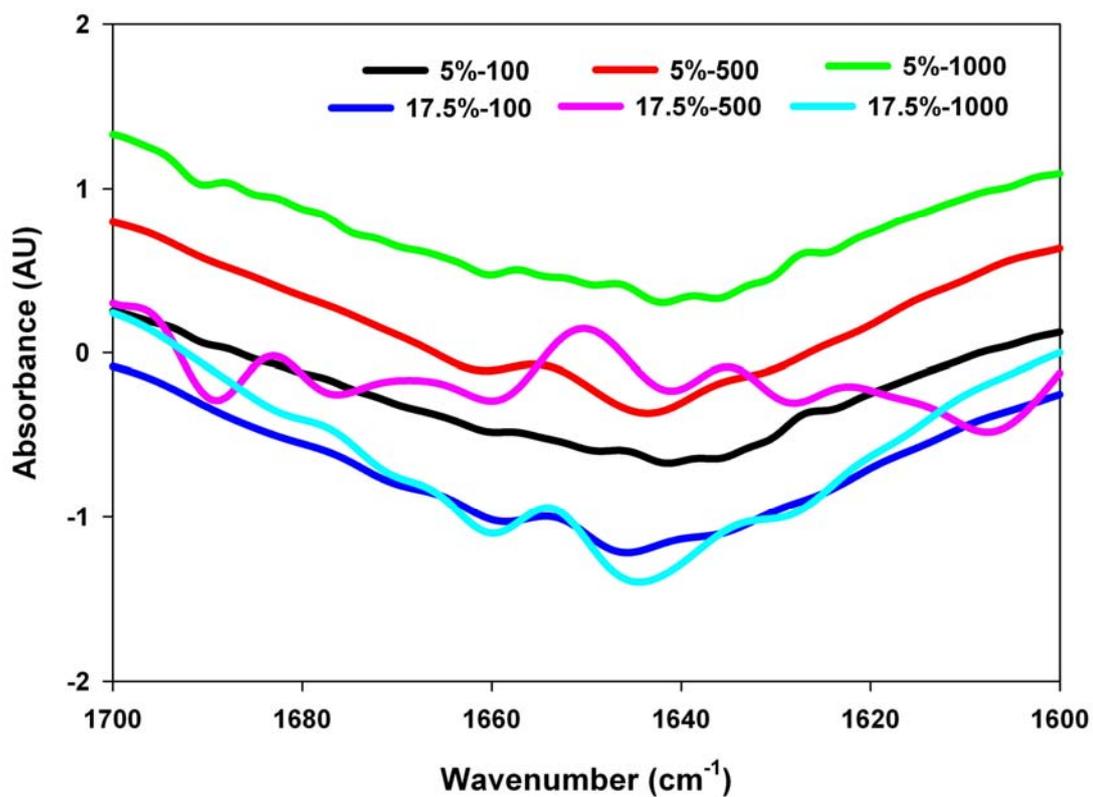


Figure 7.4 Deconvoluted IR spectra of sheared heat-induced WP gels at pH 7; containing 5 or 17.5 % (w/w) protein and sheared at the rates of 100 s^{-1} , 500 s^{-1} or 1000 s^{-1} .

The Figure 7.4 shows the deconvoluted IR spectra of sheared heat-induced WP gels at 5 and 17.5 % protein and at pH 7 at amide I region. As shown by the figure, the relative intensities of all the peaks were reduced confirming lower absorption of each secondary structural element of protein. This may be due to the disruption of common secondary structures or a highly denatured condition during simultaneously applied heat and shear. In general, the groups with strong polarity such as OH and C=O vibrations appear strongly. Also, the vibrations that arise due to bonds with weak polarity, such as C-C or S-S stretching, appear very weakly or do not appear in IR region (Geara, 1999) which may be expected under a intermolecular disulfide bond formation. The decrease of predominant secondary structural elements with concomitant addition of intermolecular β -sheet structures provides evidences of protein aggregation. The 17.5% protein sample sheared at 500 s^{-1} is a clear indication of the inter-molecular antiparallel β -sheet structure around 1682 cm^{-1} distinctive to aggregation and a broader peak around 1620 cm^{-1} attributes to more disordered structure of the protein. Also, the absorbance of IR radiation between $1645\text{-}1655\text{ cm}^{-1}$ shown by this concentrated sample (17.5 % protein) when sheared at 500 s^{-1} and 1000 s^{-1} confirms either increase in random coil structure which may arise due to protein unfolding or preserved α -helical structures mostly attributed to more undenatured protein state. However, owing to the reduced intensity of bands it is difficult to predict the nature of actual secondary conformation of the sheared WP gels at this pH and further studies would assist to have a greater understanding of the conformational alternations of the samples.

7.3.2 Electrophoretic analysis (PAGE)

The sheared, heat-induced gels of WP samples were analysed electrophoretically using native and non-reducing SDS PAGE to elaborate on the nature of molecular interactions involved in WP aggregation. The native PAGE patterns of WP aggregates depicted by Figure 7.5 –A, -B and -C showed that most of the native WP were denatured and aggregated during treatments. At pH 3, under acidic conditions BSA and β -Lg bands completely disappeared but some faint α -La bands could still be observed. This may be due to enhanced stability of α -La when the thiol group activity of BSA and β -Lg is almost inhibited even though it was a mixture of WP (de la Fuente et al., 2002). The BSA molecule undergoes acid denaturation at pH 4 owing to intra-molecular repulsion (Morr and Ha, 1993) and if carefully examined the absence of relevant band can be identified in control C₁ sample (Figure 7.5 –D). Also, the BSA bands appearing in native gels of pH 5 and pH 7 indicate their resistance to denaturation under lower acidity. Generally, in a heated WP mixture, α -La and BSA are more susceptible to denaturation in comparison to β -Lg, especially at pH 7. However, this result may be an influence of shear forces which may have changed the action of thiol induced WP aggregation. This is further indicated by non reducing SDS PAGE (Figure 7.6 –A, -B, -C and –D).

Under non-reducing conditions, without presence of further reducing agents such as β -mercaptoethanol only the non-covalent bonds present in proteins are dissociated (Havea et al, 1998). One of the most important observations found in these non reducing PAGE patterns is more pronounced disappearance of protein bands at pH 5 samples than the corresponding bands at other two pH values. Also, the aggregated materials possibly formed via covalent bonds could be identified on top of the stacking gel. This result primarily indicates that the covalent bonds were more dominant during WP aggregation at pH 5 even more than at pH 7. This is an unusual observation compared to the behaviour of WP during heating without shearing.

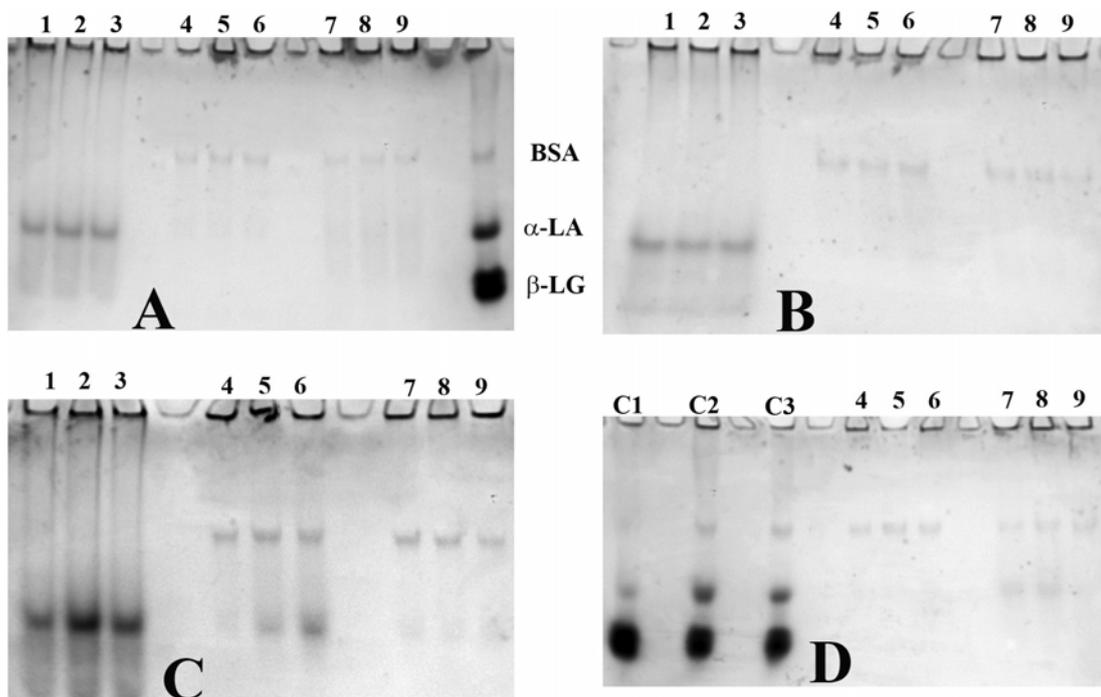


Figure 7.5 Native PAGE electrophoregrams of WP dispersions prepared at different pH and protein concentrations and simultaneously heated and sheared. Captions: pH 3 - lanes 1, 2, 3; pH 5 - lanes 4, 5, 6; pH 7 – lanes 7, 8, 9. The heating regime included heating from 20°C to 90°C, holding at 90°C and cooling from 90°C to 25°C with simultaneous shearing at 100 s⁻¹ (lanes - 1, 4, 7), 500 s⁻¹ (lanes - 2, 5, 8) or 1000 s⁻¹ (lanes – 3, 6, 9). The protein concentrations used were 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w). Lanes C₁, C₂ and C₃ in the native PAGE gel D represent the untreated controls at pH 3, 5 and 7, respectively.

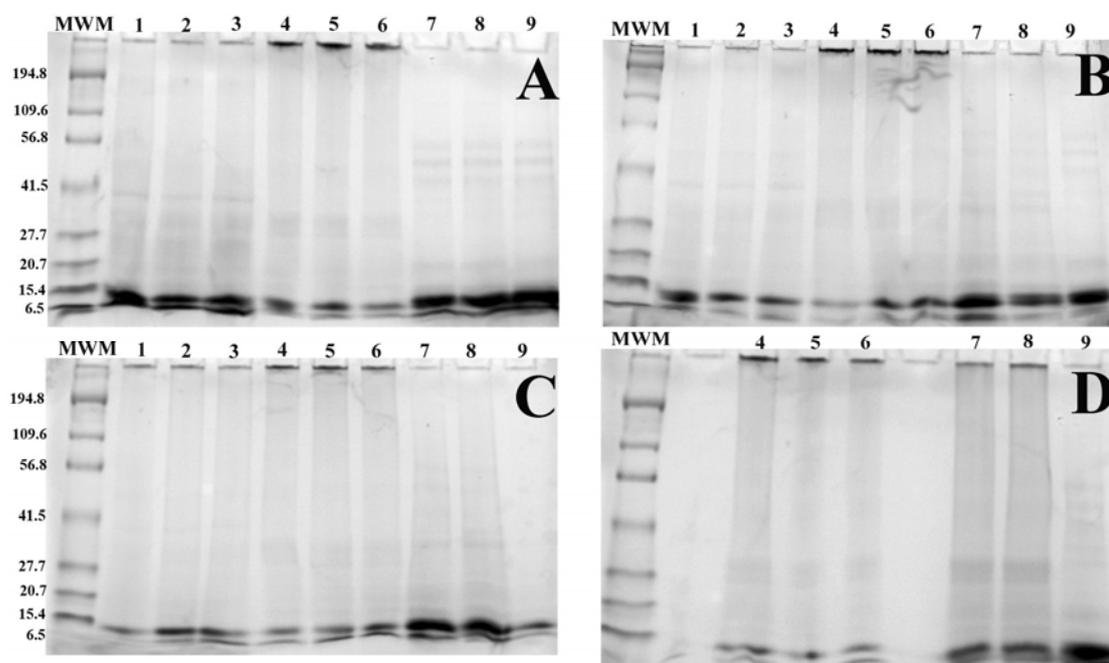


Figure 7.6 Non-reducing SDS PAGE electrophoregrams of WP dispersions prepared at different pH and protein concentrations and simultaneously heated and sheared. Captions: pH 3 - lanes 1, 2, 3; pH 5 - lanes 4, 5, 6; pH 7 – lanes 7, 8, 9. The heating regime included heating from 20°C to 90°C, holding at 90°C and cooling from 90°C to 25°C with simultaneous shearing at 100 s⁻¹ (lanes - 1, 4, 7), 500 s⁻¹ (lanes - 2, 5, 8) or 1000 s⁻¹ (lanes – 3, 6, 9). The protein concentrations used were 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w). MWM lanes present molecular weight markers with their corresponding molecular size, expressed in kDa.

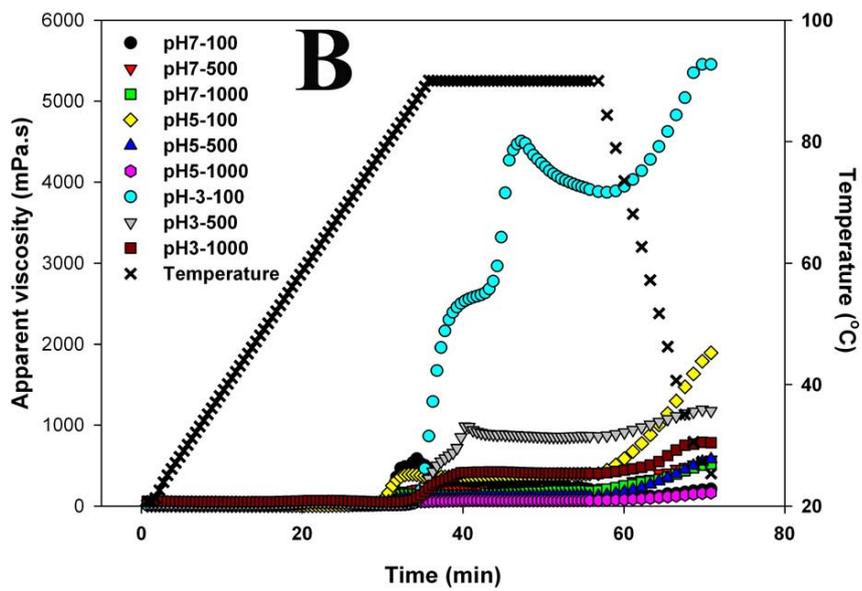
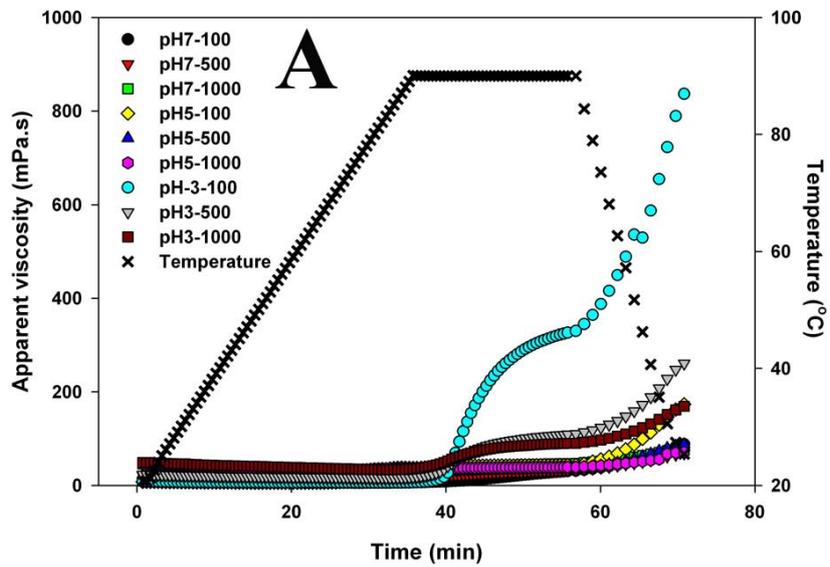
The similar pattern of results was obtained for each concentration. Normally, the covalent bond formation is most likely when thiol group activity is greatly enhanced, for instance, at pH 7. However, the disulfide covalent bond formation between denatured proteins is a relatively slow reaction due to limited number of reactive sites per protein molecule (Havea et al., 2009). Also, the shear forces may have even reduced the chance of formation of covalent bonds by continuous perturbation under a net repulsive condition at pH 7. But at pH 5, under a minimum inter-molecular repulsion and with the influence of shear the formation of covalent bonding may have been facilitated. Further, the preceding non-covalent interactions between protein molecules facilitate the thiol/disulfide exchange by creating a non-polar environment and increasing the contact time between molecules (de la Fuente et al., 2002). Therefore, increased covalent bond formation at pH 5 may be a result of accomplishment of most of the requirements needed for successful formation of disulfide bonds.

At 5% protein concentration, the SDS monomeric bands of β -Lg and α -La of pH 3 and pH 7 samples were clearly visible (Figure 7.6 –A). When the concentration was increased towards 17.5% (Figure 7.6 –B and -C), there was a greater disappearance of monomeric protein bands at pH 3 which is an indicative of aggregation of WP with relatively stronger molecular associations in sheared WP gels. The greater aggregation at pH 3 can also be attributed to the conformational instability of native WP under highly positive net charge and hence the facilitated unfolding *via* intra-molecular repulsion. Normally, the covalent disulfide bond formation is very slow and covalently associated high molecular weight aggregate formation is very unlikely around pH 3 (Shimada and Cheftel, 1989). Therefore, the different effects of shear such as stretching and elongation of protein molecules might have changed the nature of bond formation between WP molecules likely due to enhanced reactivity through exposing previously buried thiol groups or creating new reactive sites at pH 3. Also, the improved aggregation behaviour of pH 3 WP samples depicted by SDS-PAGE pattern can be correlated with the gradual increase of turbidity of these samples when the

concentration was increasing. Meanwhile, comparatively increased intensity of monomeric protein bands at pH 7 (Figure 7.6 –A, -B, -C and -D) confirms that most of these aggregates have formed *via* non-covalent associations. With the exception of samples containing 17.5% protein concentration (Figure 7.6 –C), the extensive shearing (1000 s^{-1}) have reduced formation of covalent bonds particularly at pH 7 as shown by relatively more intensive monomeric protein bands.

7.3.3 Apparent viscosity during continuous shearing and heat induced WP aggregation

The apparent viscosity of all WP samples during heat induced aggregation as expected was directly proportional to protein concentration, increasing with elevation of the protein concentration (Figure 7.7 –A, -B, -C and –D). Another important observation is that the apparent viscosity of WP dispersions at pH 3 was substantially greater than those at other pH regardless the extent of shearing. Apparently, the extent of shearing played an important role in changing the flow behaviour of heated WP dispersions by reducing the apparent viscosity with increasing the rate of shearing. The sample containing ~ 25% protein (w/w) prepared at pH 3 gelled even during the preparation likely due to high protein concentration and acidity and therefore could not be assessed for its properties. This also indicated that microparticulation of WP at this concentration and pH may not be feasible due to enhanced attractive forces under these conditions. The samples containing 5 or 10% WP at pH 3 exhibited a considerable increase in apparent viscosity during holding at 90°C , which was further enhanced during the cooling period.



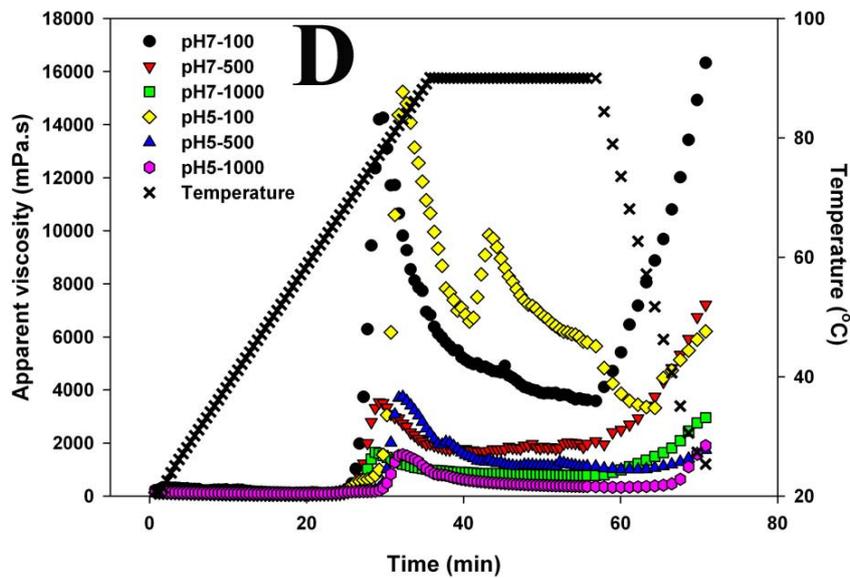
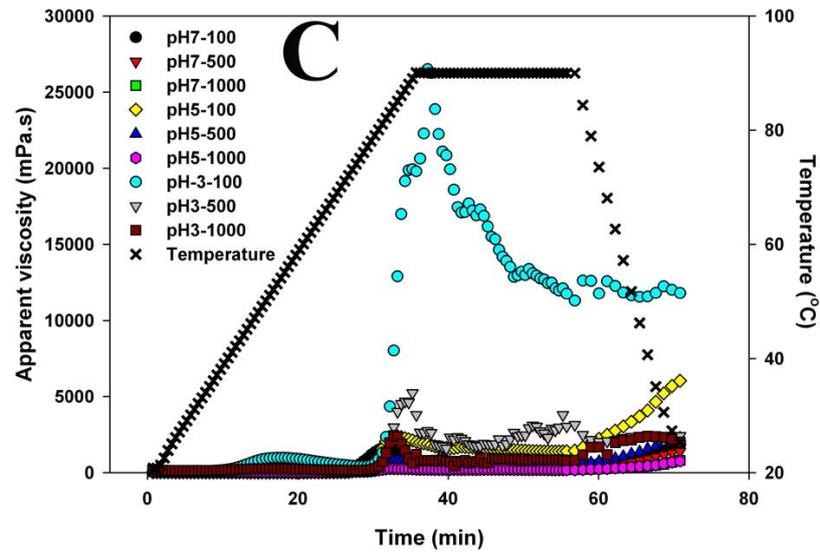


Figure 7.7 Evolution of apparent viscosity of WP dispersions prepared at 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w) proteins, respectively, and pH 3, 5 or 7 during simultaneous heating/cooling regime (from 20°C to 90°C, holding at 90°C, cooling from 90°C to 25°C) and shearing (at 100, 500 or 1000 s⁻¹).

As clearly revealed by Figure 7.7 –A, the sample at low concentrations (5% protein) and high acidity (pH 3) showed the highest apparent viscosity during shearing at 100 s^{-1} . A great increase in apparent viscosity was eventually observed during heating followed by a remarkable shear thickening behaviour during cooling period. Two vital factors that affect viscosity of proteins are the hydrodynamic shape and the size of the protein molecules (Zue and Damodaran, 1994). Therefore, the rapid increase in the viscosity of this sample may be due to significant increase in hydrodynamic size and shape of WP particles. At pH 3, the intra-molecular repulsion of WP is very high and as a result, either proteins get partially unfolded with least amount of energy required. This enhances formation of soluble aggregates if the protein concentration and other factors are satisfactory or they become highly hydrated due to increased ionization (Huang and Kinsella, 1986) and hence form a network structure with surrounding water. Shear thickening nature arises owing to increase in the size of the structural units as a result of shear (Rao, 1999).

As shown by Figure 7.7 –B, describing changes of apparent viscosity during shearing of WP dispersions at 10% protein, the sample at pH 3 followed a similar pattern to one observed at 5%; however during holding period at 90°C , a decline in apparent viscosity was observed likely due to structural collapse. Consequently the structure was recovered during cooling as indicated by the rise in apparent viscosity. This structural collapse may be a result of destruction of hydrogen bonds and electrostatic associations generated during WP aggregation since these interactions are not stable at elevated temperatures (Paulsson and Dejmek, 1990; Damodaran et al., 2008). In addition, the whole process may have been enhanced by the application of shearing. However, this was followed by an apparent shear thickening behaviour during cooling which indicates the importance of hydrogen bonding and electrostatic interactions in structuring of a gel system, especially, at low protein concentrations or high solvent level. However the sample at pH 3 containing 17.5% proteins (Figure 7.7 –C) showed a shear thinning behaviour after initial substantial rise in apparent viscosity. The dispersion of already created structural units of the gel system due to shear forces may have been further facilitated by the electrostatic repulsion between protein molecules of this sample at low pH. This can be expected especially under relatively low water content since stability of a viscoelastic gel system depends on the balance between attractive and repulsive forces (Thompson et al., 2009). Further, the initial bonds

between proteins are fairly weak (Akkermans et al., 2008) and as a result these bonds are quite possible to be dislocated with the application of shear forces. Shear thinning behaviour occurs due to the collapse of structural units in the protein system as a result of hydrodynamic forces generated during shear. When the hydrodynamic forces during shear are sufficiently high, the inter-particle linkages are broken resulting in reduction in the size of the structural units with lower resistance to flow (Rao, 1999). In addition, these sheared structural units may align with the direction of flow further reducing the apparent viscosity of the system (Damodaran et al., 2008). However, as a general observation, the increase in shear rate could decrease the apparent viscosity as clearly shown by the flow curves of all samples at each concentration.

The samples at pH 5 at protein concentrations of 5, 10 and 17.5% (w/w) also exhibited a gradual shear thickening behaviour (structural build-up) during holding and cooling periods. However, their apparent viscosity was not as high as those of pH 3 samples. The WP exhibit minimum intra-molecular repulsion around their isoelectric point, \sim pH 5 (Fox and McSweeney, 2003), which coincides with a greater resistance to unfolding around this pH. Once unfolded and in conjunction with hydrophobic associations, the attractive electrostatic interactions, hydrogen bonds and van der Waals forces strengthen the structure of new aggregates under minimum protein-protein repulsion. This was clearly shown by the increased apparent viscosity of this sample particularly at cooling phase where electrostatic interactions and hydrogen bonds are very stable. The electrostatic properties were the major factor determining fracture properties of protein gels (Foegeding et al., 2002); thus heating WP dispersions around the iso-electric point may result in formation of turbid pasty protein coagulum that is subjected to syneresis because of the strong attractive forces between the protein molecules (Morr and Ha, 1993). Furthermore, the aggregate properties result from a dynamic state of balance between shear-controlled aggregate growth and shear-induced aggregate break-up (Spigel, 1999). Therefore, the decrease in apparent viscosity of these samples at pH 5 with increasing shear rate may have resulted from dispersion of aggregates under shear and orientation of such dispersed particles in the direction of the facilitating flow. This trend was clearly observed at all three concentrations (Figures 7.7 –A, -B and –C).

However, at the highest protein concentration of 25% the apparent viscosity of WP samples during simultaneous heating and shearing exhibited a different pattern in comparison to those at lower protein concentrations. The Figure 7.7 –D presents the apparent viscosity of samples at pH 5 and 7 containing 25% protein concentration. In contrast to at relatively low protein concentrations, the pH 7 samples at this concentration displayed a different flow behaviour with prominently increased shear thinning nature during heating followed by shear thickening behaviour during cooling. At such a high concentration the probability of molecular collisions and thus interactions of protein molecules is undoubtedly high resulting in dense and compact aggregates with low immobilized serum. The solid, rigid structure provides high stability against shear-induced fragmentation enabling large aggregates to flow (Spigel, 1999) which finally leads to higher viscosity. The pH 7 sample sheared at 100 s^{-1} has shown increased apparent viscosity even during heating to 90°C , followed by structural collapse during holding at 90°C reflected in substantial viscosity decline. But, the structure has recovered during cooling period attaining apparent viscosity greater than that before holding period. According to these observations, the thiol induced covalent bond formation is highly unlikely under the application of shear force. If these bonds have been formed, a rapid viscosity decline should have not been observed especially that these strong covalent bonds may not be easily disrupted by a physical force. Also, the shear forces may have dispersed the molecular associations most possibly formed via hydrophobic interactions as a result of facilitated elongation of immature aggregates (Akkermans et al., 2008), initially conveying shear thinning property to the system. During cooling, on the other hand, with a greater extent of hydrogen and electrostatic interactions and also possible thiol-disulfide interchange chain reactions, all of which participate in creation of a further cross-linking (Rabiey and Britten, 2009), strong and elastic protein network may be reformed imparting a shear thickening nature to the system. At pH 7, as thiol groups are easily activated it would be expected for covalent bonds to be formed over the course of heating, which would aid in strengthening of the protein network. Similar trend in the evolution of apparent viscosity was observed to certain extent when the sample was sheared at 500 s^{-1} . Similar to the samples prepared at pH 7, the sample prepared at pH 5 and sheared at 100 s^{-1} also showed similar pattern in the flow curves although the final viscosity was substantially lower. The shear thinning followed by shear thickening behaviour

identified in the flow curves of these samples indicate that the system may be unstable with possible phase separation when shearing.

7.3.4 Turbidity of sheared gels

The turbidity is a measure of light scattering by particles in a suspension. The particle size distribution of the scattering particles is the main factor in altering the turbidity of the suspension. Also, the turbidity is directly proportional to the volume of the scattering particle (Gribble et al., 2010). Therefore, the difference in turbidity was correlated to express the extent of aggregation of WP during heating under shear.

Figure 7.8 –A, -B, -C and –D present the turbidity values of heated and sheared WP samples. The most obvious observation shown by the figures is significantly ($P < 0.05$) increased turbidity of pH 5 samples at each concentration compared to other two. Around iso-electric pH, WP possess maximum stability towards aggregation after unfolding, the fact, that was previously described in Chapter 5 into a greater detail. Furthermore, as depicted by Figure 7.8 –A, at pH 5, the treated samples containing 5% (w/w) proteins showed markedly ($P < 0.05$) increased turbidity compared to untreated control. Increasing the extent of shear had produced significantly ($P < 0.05$) larger WP aggregates. Therefore, in addition to the facilitated heat-induced WP aggregation at pH 5, the shear forces seemingly have further increased the aggregate growth. This trend was similar for the samples treated at 10% and 17.5% protein concentrations at the same pH condition, but at 25% protein the elevation of shear rate had a significant positive influence on the aggregate size at lower rates, but extensive shearing at 1000 s^{-1} has not imparted any noticeable effect to change the size of WP aggregates. In addition, 17.5% and 25% protein at this pH showed decrease in turbidity. It indicates that maximum unfolding and aggregation rates of WP under simultaneous heating and shearing may not be achieved with very dense protein systems.

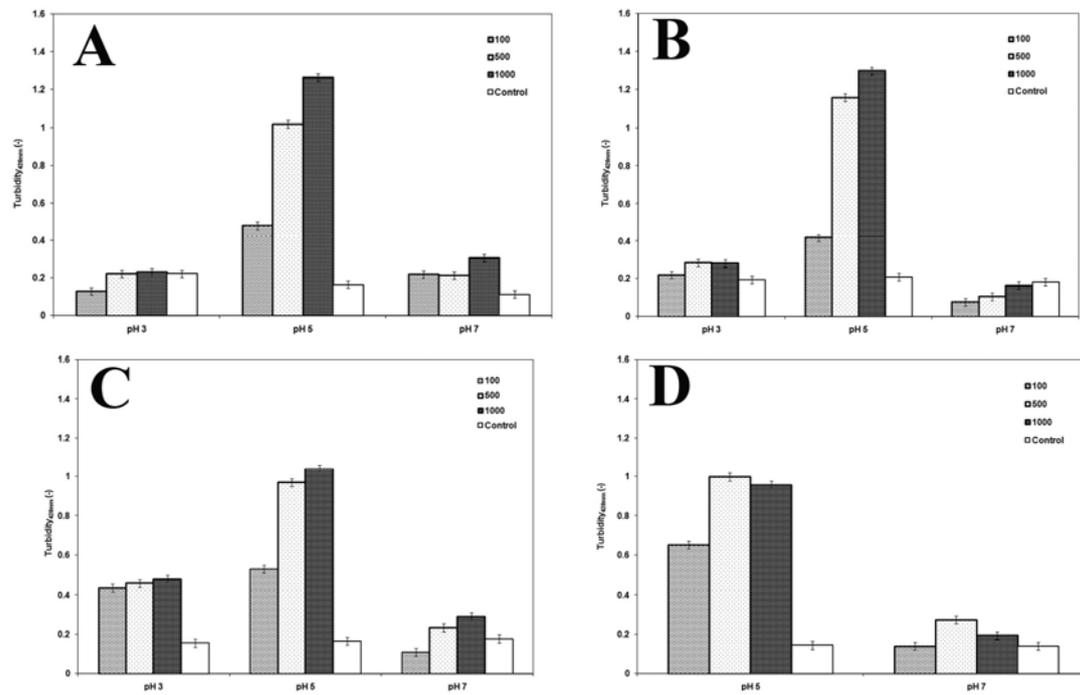


Figure 7.8 Turbidity of WP dispersions containing 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w) protein, prepared at pH 3, 5 or 7 and treated by heating from 20 to 90°C, holding at 90°C and cooling from 90 to 25°C and simultaneous shearing at 100, 500 or 1000 s⁻¹.

The turbidity of pH 3 WP sample prepared at 5% protein and sheared at the rate of 100 s^{-1} was significantly ($P < 0.05$) lower compared to the untreated control, mostly attributing to dissociation of certain molecular interactions leading to reduction of particle size. When the same sample was sheared at a greater rate (500 or 1000 s^{-1}) a significant ($P < 0.05$) increase in the particle size was achieved; however the turbidity was comparable ($P > 0.05$) to that of the control indicating the inhibition of WP protein aggregation at such low concentrations under net positive electrostatic interactions driving the repulsion. However, increase in protein concentration to 17.5% (Figure 7.8 –C) significantly ($P < 0.05$) increased the size of WP aggregates as depicted by rise in turbidity. At high protein concentrations further aggregation is no longer inhibited by electrostatic repulsion (Le Bon et al., 1999). The concentration affects the protein aggregation and the higher the protein concentration the higher would be the molecular weight of aggregates (Fitzsimons, et al., 2007). However, shear had no apparent effect ($P > 0.05$) on the size of WP aggregates at pH 3 likely controlled by the consequences of enhanced hydration of the WP molecules at low pH (Huang and Kinsella, 1986).

Surprisingly increasing the rate of shearing did not produce expected effect, *i.e.* reduction of particle size depicted as turbidity decline. For example, WP sample prepared at pH 7 containing 5% (w/w) protein showed a significantly ($P < 0.05$) greater turbidity after shearing at 1000 s^{-1} than those sheared at lower rates (Figure 7.8 –A). The unusually low turbidity obtained for WP sample with 10% proteins at pH 7, which was even below the turbidity of the control sample (Figure 7.8 –B), is somewhat controversial, but, may be attributed to phase separation, which was visually observed for this sample. Similarly, the WP sample containing 17.5% (w/w) proteins at the same pH and sheared at 100 s^{-1} also experienced the phase separation and reduced turbidity. With a phase separation the shear forces tend to be applied to a more concentrated area of the protein system and higher protein concentrations apparently protect the protein from extensive unfolding (Zue and Damodaran, 1994) which may be related to lower turbidity due to conservation of native protein structure.

Increased shear rates at pH 7 for 17.5% and 25% concentrations showed the similar pattern as those at pH 5. However, a significant ($P < 0.05$) decline in turbidity and thus

particle size was observed at 25% protein concentration after shearing at 1000 s^{-1} . Apparently extensive shearing may not accelerate the protein aggregation at very high concentration. The overall turbidity increase at pH 7 was lower compared to that at other two pH values signifying less aggregation. Although elastic gel structures are created when WP are heated at $\text{pH} \geq 6$ due to high net negative electrostatic charges between molecules that allows adequate time for them to properly align before they interact (Morr and Ha, 1993), the application of shear appeared to either inhibit or interrupt formation of strong and elastic bonds between WP molecules as shown by reduced levels of aggregation.

Additionally, by the acquisition of particle size measurements directly after shearing the WP systems, there would have valuable information for the research which may complement and confirm the viscosity and turbidity data.

7.3.5 Colour of gels

The Figure 7.9 –A, -B, -C and –D present the lightness (L^*) of sheared heat-induced WP gels. If the protein gels are more opaque, more light is reflected back and it is indicated by increased L^* values (Caussin, et al., 2003). The gel appearance depends on the gelation mechanism which is on the other hand governed by the different attractive and repulsive molecular interactions (Damodaran et al., 2008). As obtained by the outcome of statistical analysis, most importantly, the shear rate has not significantly ($P > 0.05$) affected the L^* of the WP gels. On the other hand, pH and different parameter interaction including pH and concentration, shear rate and concentration and pH and shear rate had significant ($P < 0.05$) influence on the L^* of the gels. Regardless of protein concentration, the highest L^* values were obtained for pH 5 samples confirming facilitated cross-linking between protein molecules around the iso-electric point. The L^* of the gels measured at pH 7 was greater ($P < 0.05$) than that of the gels formed at acidic pH. In the presence of a net positive charge at pH 3, the combination of proteins with water molecules is highly positive. Therefore, in addition to highly repulsive protein-protein interactions, more water imbibed gel structure would most certainly produce more transparent gels with smaller L^* values.

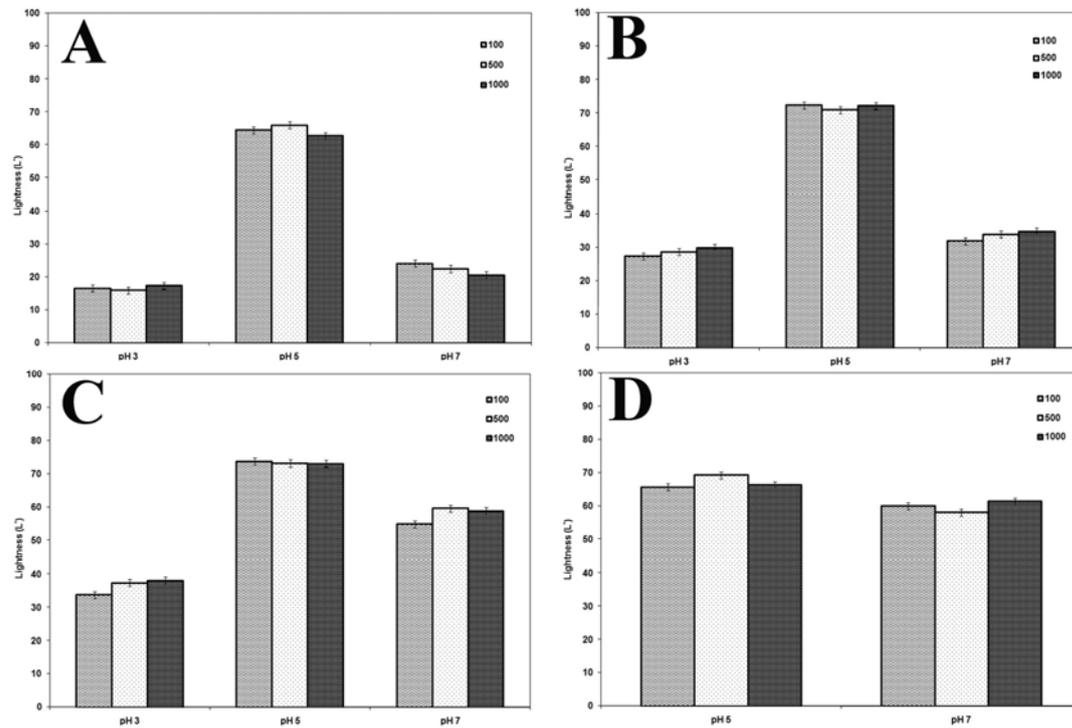


Figure 7.9 Lightness of WP dispersions containing 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w) protein, prepared at pH 3, 5 or 7 and treated by heating from 20 to 90°C, holding at 90°C and cooling from 90 to 25°C and simultaneous shearing at 100, 500 or 1000 s⁻¹.

At pH 7, the additional covalent bond formation may have increased the degree of cross linking of heated and sheared gels resulting in a considerably higher lightness of gels. Also, the increase in concentration had significantly ($P < 0.05$) increased the L^* of the gels in most cases under each pH value. This may be due to facilitated molecular associations with increase in molecular density.

7.3.6 Hydrophobicity

According to the definition of hydrophobicity it is the excess free energy of solute dissolved in, compared to that in an organic solvent under similar conditions (Damodaran et al., 2008). Hydrophobic interactions are important in maintaining the tertiary structure of globular WP and about 40-50% of non polar groups can be present in the surface area of globular proteins (Damodaran et al., 2008). During heating, WP are unfolded increasing the hydrophobicity due to exposure of buried hydrophobic groups. But, the subsequent aggregation via hydrophobic as well as other molecular interactions leads to decline in hydrophobicity. Therefore, hydrophobicity is a good measure for understanding and predicting protein-protein interactions (Sava et al., 2005). The hydrophobicity of WP may be altered depending on the experimental conditions. Therefore, a fluorescence probe (Sava et al., 2005) can be used to measure the extent of surface hydrophobicity of WP. The common anionic fluorescence probes such as ANS (1-anilinonaphthalene-8-sulfonic acid) or *cis*-parinaric acid give higher hydrophobicity values at acidic pH compared to that at alkaline pH due to the possible interaction with positive charges resulting in overestimation of hydrophobicity (Alizadeh-Pasdar and Li-Chan, 2000). The current study involved three different pH conditions. The 6-propionyl-2-(*N,N*-dimethylamino)-naphthalene (PRODAN) was an aromatic hydrophobic probe without an ionisable group (Alizadeh-Pasdar and Li-Chan, 2000). Since PRODAN molecules are uncharged, the interference of unnecessary electrostatic contributions in hydrophobicity measurements is supposed to be avoided.

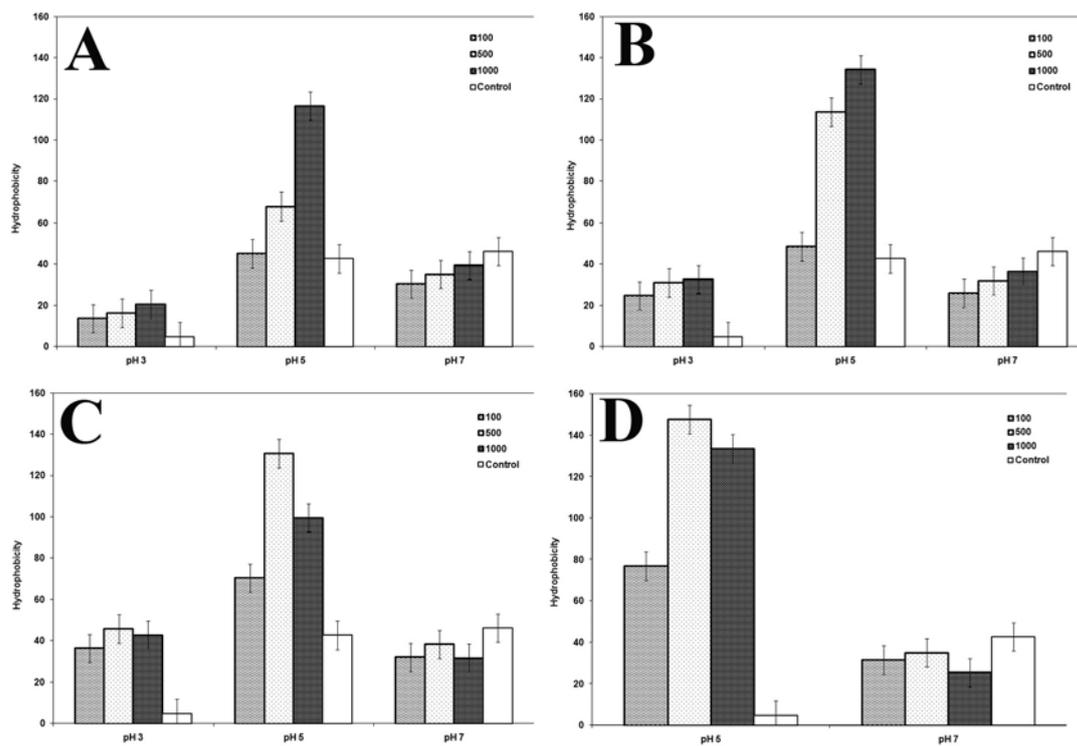


Figure 7.10 Hydrophobicity of WP dispersions containing 5 (A), 10 (B), 17.5 (C) or 25% (D) (w/w) protein, prepared at pH 3, 5 or 7 and treated by heating from 20 to 90°C, holding at 90°C and cooling from 90 to 25°C and simultaneous shearing at 100, 500 or 1000 s⁻¹.

Surface hydrophobicity of heat treated and sheared WP samples is depicted in Figure 7.10 –A, -B, -C and –D. In general, there was a significant ($P<0.05$) increase in hydrophobicity in sheared WP samples at pH 5 compared to samples at other two pH conditions, regardless of protein concentration. Furthermore, shearing apparently enhanced hydrophobicity of heat treated WP dispersion at and below pH 5, which was significantly ($P<0.05$) lower than that of the control samples. This was however opposite at neutral pH, where conformational perturbation due to shear resulted in slightly lower hydrophobicity in comparison to the controls.

At pH 3, although there was a possibility of greater protein aggregation, the application of shearing may have easily disrupted the protein associations formed under a highly repulsive condition and also induced molecular stretching exposing more non-polar sites leading to increased hydrophobicity. On the contrary, at pH 7, the effect of shear may have been insufficient to disturb the more stable covalent disulfide bonds whose formation is usually facilitated at higher protein concentration (Damodaran et al., 2008). The hydrophobicity of control samples at pH 7 was always greater than the other corresponding samples and that observation was significant ($P<0.05$) at 10% protein (sheared at 100 s^{-1}) and at 25% protein (sheared at 1000 s^{-1}). This outcome is consistent with results reported by Zue and Damodaran (1994) which described relatively higher surface hydrophobicity of β -Lg and BSA in their native state than in the heat-denatured state.

At 5% protein concentration (Figure 7.10 –A), the pH 5 samples showed a significant ($P<0.05$) increase in the hydrophobic character of the protein surface with increased shear rate. This is in addition to their markedly greater hydrophobicity compared to that at other two pH conditions. However, there was no significant ($P>0.05$) difference between the hydrophobicity of the sample sheared at 100 s^{-1} and untreated control at this pH. In general, heating promotes unfolding and aggregation and consequently reduces hydrophobicity. This may be more apparent at pH 5, where WP are the least heat stable. However, upon shearing significantly ($P<0.05$) higher hydrophobicity was achieved for this sample revealing that shearing has modified the surface characteristics exposing a greater number of hydrophobic sites. Turbidity of the sheared gels at this pH was also significantly ($P<0.05$) high. It appears that the increase in hydrophobicity is rather a consequence of the disruption of large protein

aggregates, which results in the unmasking of buried hydrophobic groups. As Bouaouina et al., (2006) previously explained the dynamic high pressure treated WP showed better stabilizing properties by great increase of their surface hydrophobicity attributed to increased exposure of hydrophobic sites resulting from large protein aggregate disruption. According to findings described in Chapter 3, microparticulated and heated WP were good emulsifiers likely due to increased surface hydrophobicity, which was confirmed in the current Chapter. Furthermore, heating and shear alter the conformational structure of the WP through partial denaturation of the protein, thereby exposing groups that are normally concealed in the native protein (Onwulata et al., 2006). It is also important to mention that individual hydrophobic residues, randomly exposed at the protein surface, do not have the ability to act as strong binding sites for fluorescence probes, for example, well defined retinol binding hydrophobic cavity of β -Lg formed by grouping of non-polar residues on the protein surface (Zue and Damodaran, 1994). Despite lower hydrophobicity of the samples at pH 3 and pH 7, increase in shear rate did not considerably affect on their surface hydrophobicity in both instances. However, hydrophobicity of pH 7 samples was higher compared to those of pH 3 at this concentration. Also, shearing WP at the same rate at pH 3 or 7 did not show any apparent effect. Therefore, at pH 3, WP may be less susceptible to protein unfolding and thus lower exposure of hydrophobic groups resulting in low hydrophobicity values. The least heat-sensitive pH range for WP lies between pH 2.5 and 3.5 where proteins retain their good solubility, and the hydrophobicity showed little or no significant change in surface after heating at pH 3 (Alizadeh-Pasdar and Li-Chan, 2000). The WP, especially β -Lg, are characterized by a tighter conformation at acidic pH compared to a more hydrophobic and flexible molecule at pH values above 7.5. As reported previously, comparatively higher hydrophobicity values for heated WP were obtained at alkaline pH conditions relative to those at pH 3 (Alizadeh-Pasdar and Li-Chan, 2000).

The samples with 10% protein concentration also exhibited more or less the similar trend as those containing 5% protein. However, in contrast to the degree of surface hydrophobicity of acidic samples at 5% protein, these corresponding samples showed significantly ($P < 0.05$) increased hydrophobicity compared to that of untreated control. As shown by Figure 7.10 -C and -D, when the protein concentration was raised to 17.5% or 25%, the most obvious difference in the hydrophobicity values was

the decrease in hydrophobicity when the samples were sheared at 1000 s^{-1} regardless of the pH value. This was in particular significant ($P < 0.05$) effect at pH 5 of 17.5% protein sample. This observation reveals that the protein surface modification depends on the shear rate as well as other environmental factors. Dense protein systems provide limitations to the geometries and shear rates that are accessible with rheometers due to their high viscoelasticity (Manski et al., 2007).

7.4 Conclusions

All the factors examined, including protein concentration, pH and shearing, had a profound effect on the properties of whey proteins examined in this study. The secondary structure of WP was most conserved at pH 3 indicating the least denaturation of these proteins. This was followed by treatment at pH 5, while the extent of denaturation at pH 7 appeared to be high as shown by FTIR spectroscopy. The considerably compactness of WP gels also signifies a greater cross linking of this gel structure formed at pH 7. However, both lightness and FTIR studies were carried out for semi-solid, sheared WP gels where further WP aggregation was quite possible *via* suitable aggregation pathways after heat and shear treatments due to the presence of highly activated molecules. On the other hand, significantly reduced hydrophobicity of samples treated at pH 7 indicate greater intramolecular compactness which was even confirmed by the reduction of covalent bond formation of these samples as shown by PAGE and lower apparent viscosity. This potentially indicated that it may be possible to treat individual molecules at this pH applying a greater shear. The greatest heat-induced protein aggregation with simultaneous shearing has occurred at pH 5 involving all types of molecular interactions. The size of thus formed aggregates appeared to be substantially greater than those formed at pH 3 and 7 as indicated by turbidity values. The application of shearing have minimized the formation of molecular linkages including covalent bonds at pH 7 which was even revealed by reduced turbidity of the samples. Furthermore, the PAGE results showing facilitated aggregation via strong molecular associations at pH 3 are somewhat contradictory. This may be a result of complexation of β -Lg with SDS at

low pH which would lead to overestimation of covalent aggregation (Jung et al., 2008).

In general, it can be concluded that the shear forces may change the direction and extent of heat-induced WP denaturation and aggregation. However, the extent of the influence of shear may be controlled and/or manipulated by the extrinsic factors such as pH and protein concentration as in this particular study, or some other factors including ionic strength, quality of ionic environment and even the rate of heating.

CHAPTER 8

8 Conclusions and scope for the future work

8.1 Conclusions

The analysis of commercial WPC powders revealed that they have substantial variations in proximate composition, particle size distribution pattern and several assessed functional properties. These variations likely occurred due to the differences in origin and their processing history. The significant variations found in functional properties of these commercial WPCs were primarily consistent with the differences in their physico-chemical properties. For instance, the calcium concentration in these samples was directly linked to heat stability and firmness of the gel. Nevertheless, in some cases the chemical composition was not correlated with a relevant functionality such as altered viscosity and enhanced heat stability even with high calcium content. This indirectly indicated that the dairy industry was capable of producing WPC products with a specific application.

Microparticulation of WP with the combination of heat treatment and high pressure shearing at neutral pH produced micro-aggregates with enhanced heat stability. However, the particle size of these aggregates was larger compared to that of native WP, which, on the other hand, adversely affected most of the other physical functionalities, particularly, the colloidal properties. Furthermore, the denatured MWP possessed improved functionalities from those of native WP, such as greater emulsifying activity due to likely a greater amount of adsorbed proteins on the surface of fat droplets. In addition, high pressure shearing alone improved foaming properties of undenatured WP. The undenatured MWP produced heat-set gels with a greater solid-like behavior than the gels produced from untreated controls. The PAGE analysis revealed that the covalent interactions were mainly involved in creation of the heat-set gels. Moreover, high-pressure shearing likely changed the protein conformation, through exposure of various hidden hydrophobic and other reactive sites finally imparting greater non-covalent interactions than the untreated controls which may help to produce stronger gels. Although, denatured MWP did not create heat-induced gels they formed considerably firmed cold-set (acid or salt) gels with the involvement of both covalent and non-covalent interactions. The prevalence of

interactions and thereby the strength of these gels were dependent on the extent of hydrodynamic high-pressure shearing.

The study of kinetics of whey protein denaturation as a function of pH and protein concentration indicated that the denaturation of β -Lg has apparently followed the first order reaction kinetics under the conditions applied in the study. In addition, the rate of thermal denaturation of WP around their isoelectric point at relatively low protein concentration has shown substantial increase, which was even confirmed by the PAGE analysis, greater turbidity and gelling characteristics of this sample. Also, the maximum levels of WP aggregation with larger aggregates formed around isoelectric point may be due to greatest molecular interactions under a minimum electrostatic repulsion. At low pH and elevated protein concentrations, WP retained significantly greater solubility than at any other pH, which could be attributed to low aggregation rates. However, at relatively low protein concentrations, solubility of WP decreased as a result of heat induced changes regardless of the pH. On the other hand, relatively most impaired solubility at pH around 6 was an indicative of facilitated WP aggregation. The protein concentration played no apparent role in regards to observed onset of the denaturation, as opposed to pH of the medium that fundamentally shifted the denaturation onset. The lowest rate of WP denaturation was observed at acidic pH which was further confirmed by substantially improved solubility and low turbidity. Prior to heating, the WP at this pH appeared comparatively more hydrated than they were at any other pH conditions and it was clearly shown by the elevated yield stress at 17.5% protein concentrations at low pH. However, at 25% protein the highest initial yield point was obtained around isoelectric pH. All WP dispersions exhibited shear thinning behaviour with increasing shear rate around the ambient temperature. Moreover, harder gels were formed at acidic pH than at any other pH. However, these gels were characterized by weak and brittle nature, with brittleness of these gels increasing proportionally with the protein concentration. In general, WP were more stable against heat induced denaturation at acidic pH.

The study on fundamental WP interactions during heating using different chemicals to block the reactive sites revealed that WP were more stable against heat-induced denaturation at pH \sim 3 in comparison to that at their natural pH. The non-covalent interactions were primarily responsible for WP aggregation during heating around

their natural pH, with creation of covalent bonds equally important during this process. Meanwhile, the covalent interactions were almost inhibited at pH 3 confining WP aggregation to non-covalent linkages, consequently increasing the denaturation temperatures of WP. Also, there was no apparent acidulant effect observed on WP aggregation under the experimental conditions used in this fundamental study.

MWP powders prepared at low pH contained completely denatured WP as indicated by the thermal and PAGE analysis. This information was very important in subsequent assessments of properties of denatured MWP. Most importantly, the average particle size of denatured MWP was significantly reduced to ~100 nm, which was a 100 fold reduction compared to the average particle size of MWP produced at neutral pH. Furthermore, the particle size distribution of all types of WP was restricted to below 1 μm . Subsequently, the solubility of these denatured MWP powders was substantially greater than that of denatured MWP produced at neutral pH. Therefore the combined heat and high pressure shearing produced WP micro-aggregates with improved colloidal stability at low pH, which was not attained by microparticulation of denatured WP at neutral pH. In addition, heat stability of these powders was substantially increased (greater than 3 min) as shown by heat coagulation time. These powders also possessed improved emulsifying properties with ability to create more stable emulsions with a greater capacity to adsorb onto the oil droplet surface. In addition, the denatured MWP samples showed comparatively higher viscosity in comparison to undenatured species. The characteristics of thermally induced gels produced from undenatured MWP were influenced by two acidulants, citric and lactic acids, which apparently governed relative thermal stability of WP at low pH. For example, slower gelation of WP prepared with lactic acid compared to that of WP with citric acid could be correlated with the elevated T_d of WP acidified with lactic acid. The denatured MWP produced with lactic acid created acid induced cold gels with considerable firmness. However, their ability to create salt induced gels was low. Further, the effect of high pressure shearing on examined functional properties of MWP varied with acidulant selection. Therefore, the production of denatured MWP at low pH apparently resulted in modulation of intra- and intermolecular interactions of WP that led to particle size reduction and improved heat stability of newly created micro aggregates, which fundamentally modified their

physical functionalities and thereby the application in food products minimising adverse effects.

The study on the role of shear on heat-induced WP aggregation revealed that the protein concentration, pH and shear rate had a significant influence on structure of WP. During the treatment application, the least denaturation of WP has occurred at pH 3 compared to that at higher pH levels as indicated by changes of the secondary conformation of treated WP. The WP at pH 7 were almost completely denatured resulting in a high cross-linked network as indicated by the greater compactness of WP gels. On the other hand, significantly reduced hydrophobicity of these samples at pH 7 indicated the greater intra-molecular compactness as confirmed by the reduction of covalent bond formation of these samples and lower apparent viscosity. However, the application of shearing have minimized the formation of molecular linkages including covalent bonds at pH 7 which was even revealed by reduced turbidity of these samples. Therefore, the effect of shear at this pH appeared to be greatly dependent on the post experimental conditions since simultaneous heating and shearing most likely activated the globular WP by revealing buried reactive sites leading to further reactivity. The greatest heat-induced protein aggregation with simultaneous shearing has occurred at pH 5 involving all types of molecular interactions forming larger aggregates compared to those formed at pH 3 and 7.

As an overall, the shear forces may change the direction and extent of heat-induced WP denaturation and aggregation and the extent of influence of shear may be controlled and/or manipulated by the extrinsic factors such as pH and protein concentration or some other factors including ionic strength, quality of ionic environment and even the temperature and rate of heating.

8.2 Scope for the future work

The findings of the current research have revealed that microparticulation of denatured WP may be a feasible industrial approach that could be successfully applied to alleviate problems associated with heat sensitivity of WP. While MWP produced under conditions in this thesis improved colloidal stability and several modulated

physical functionalities, further conformational changes of WP under defined conditions, such as ionic strength and composition of the ionic environment, extent of pressure during microparticulation, time/temperature combinations during heat-denaturation of WP may lead to production of MWP with targeted physical functionalities from those observed in this research.

In addition, such products may be used in different model and real food systems to identify the interactions between MWP and the other biopolymers present in food systems. For example, complexation with other food hydrocolloids such as pectin, carrageenan or xanthan gum with MWP powders obtained especially at low pH conditions in the aqueous solutions under controlled environmental conditions may lead to creation of more thermodynamically favourable food systems. As observed in the study, denatured MWP possessed better emulsifying capabilities and different gelling characteristics than those of untreated controls. Therefore, the complexation of these ingredients with other biopolymers *i.e.* polysaccharides, may result in novel ingredients or in food systems with improved or different textural properties. In addition, further investigations such as application of heat-treatment after complexation on such systems as well as further rheological and imaging studies would be a useful approach in understanding the behaviour of these food systems during actual processing and post processing conditions.

Moreover, further investigations should be devised to assess properties of cold-set gels (acid or salt) as affected by changing experimental conditions in order to optimise textural and sensory characteristics of these gels. These MWP may be an appealing and nutritionally better alternative to hydrocolloids for use in heat sensitive food systems. Addition of certain ions such as phosphates to the system may also result in improved water holding capacity of these gels.

The application of shear under very acidic pH conditions is known to produce fibrillar forms of proteins. Microparticulation of WP at low pH may also create such conformational entities which may have a potential for application in foods as well as health products. The recognition of structural changes of the proteins can be conducted with the help of techniques such as FTIR spectroscopy. In addition, testing of foaming properties of MWP produced at low pH with reduced particle size is important.

Further, the denatured MWP may differently act inside the gut compared to native WP. Therefore, *in vitro* and *in vivo* research on peptide release of these modified protein products would be another direction of further research to identify the beneficial or detrimental effects of these peptides. In conclusion, all of these listed recommendations would ultimately broaden the applicability of this newly created food ingredient.

CHAPTER 9

9 References

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