ABSTRACT

MUDGAL, PRASHANT. Aggregation Mechanisms of a Whey Protein Model System at Low PH. (Under the direction of Dr. Christopher R. Daubert and Dr. E. Allen Foegeding).

Recently, there has developed increasing interest in cold-thickening whey protein ingredients for food applications where heat may not be desirable and because of their nutritional benefits over conventionally-used starches and hydrocolloids. In previous studies, a procedure allowing for the production of a cold-thickening whey protein ingredient, without any addition of salt or heat, was developed. This procedure involved pH adjustment to 3.35, thermal gelation, followed by drying and milling to a powder. Originally, this procedure was applied to whey protein isolates, but also worked with concentrates. Although, these modified powders provide certain benefits, such as instant thickening without addition of heat or salts, these ingredients were prepared at low pH and yield astringent flavors that limit use in practical applications. To effectively tailor the original modification process to expand the functionality and utility of cold-thickening modified whey protein ingredients, the basic mechanism behind the cold-thickening must be explained.

β-Lactoglobulin (β-lg) is the major component of whey protein products and was selected as a model system to investigate the cold-thickening mechanisms. Concentration effects on β-lg modification were studied at low pH using capillary and rotational viscometry, transmission electron microscopy (TEM), and high performance liquid chromatography coupled with

multi-angle laser light scattering (HPLC-MALS). From the results of capillary viscometry, a critical concentration (C_c ~ 6.4 % w/w protein) was identified below which no significant thickening functionality could be achieved. Microscopy revealed formation of flexible fibrillar network at pH 3.35 during heating at all concentrations. These flexible fibrils had a diameter of approximately 5 nm and persistence length of about 35 nm as compared to more linear and stiff fibrils formed at pH 2 and low ionic strength (*I*) conditions. Under similar heating conditions at concentrations above C_c, larger aggregates similar to microgels were observed. However, at concentrations below C_c, isolated fibrils were observed with an average contour length of about 130 nm.

In further investigations, concentration effects were studied together with ionic strength ($CaCl_2$) effects on the β-lg cold-thickening mechanism using light scattering, microscopy, and rotational viscometry. A slight increase in *I* (up to 20 mM) with β-lg concentrations above the critical concentration resulted in an increased thickening function from modified powders. The network characteristics remained fine stranded with small addition of $CaCl_2$, and increased aggregation was observed at all concentrations. The aggregates formed during heating persisted in dried powders; however, freeze concentration effects during freezing further enhanced thickening function as obtained from the reconstituted modified powders.

The size of the aggregates formed during the modification process was found to be dependent on the initial protein concentration from kinetic studies using light scattering and rheology. A

nucleation and growth mechanism best fit the aggregation at pH 3.35, and nucleation was found to be a rate limiting step below the critical concentration. Above the critical concentration, nucleation occurred rapidly leading to a higher degree of aggregation and formation of large aggregates. By manipulating concentration and heating times, it seemed feasible to control the degree of aggregation and the size of aggregates between 10^6 to 10^8 Da.

According to electrophoretic studies, disulfide linked aggregates were formed upon heating during the modification procedure. However, disulfide interactions alone could not explain the observed concentration dependent thickening differences. Some acid hydrolysis occurred at pH 3.35 and with additional hydrolysis of β-lg with the enzyme pepsin, solution viscosities increased by about two logs. Likely, formation of pre-aggregates during hydrolysis may explain increased aggregation and higher viscosities.

Aggregation Mechanisms of a Whey Protein Model System at Low PH

by
Prashant Mudgal

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DEDICATION

I dedicate this dissertation to my parents: Mr. Umesh Mudgal and Mrs. Geeta Mudgal. Without their sacrifice, their love, their understanding, their patience, their trust in me, and their teaching it would not have been possible for me to achieve a doctoral degree.

I only wish to make them proud and happy in whatever I do.

BIOGRAPHY

Prashant Mudgal was born on 10th September, 1980 in Bharatpur district of Rajasthan State, India to Umesh and Geeta Mudgal. He finished his middle school education in Bharatpur, and enjoyed his childhood playing cricket with his brothers, cousins, family and friends in home backyard. Then, Prashant left for Jaipur leaving his parents in Bharatpur for his high school education and it was the first time when he realized how much he missed home. Prashant's early interest in mathematics led to his desire of becoming a successful engineer and to get admitted in the prestigious Indian Institutes of Technology (IITs). After finishing his high school from Jaipur, he prepared and appeared for the IIT-Joint entrance examination and succeeded. Prashant was admitted to the Agricultural and Food Engineering undergraduate program at IIT-Kharagpur, India in 1998 and he graduated with a Bachelors degree in 2002.

Being interested in pursuing higher education, Prashant applied to the esteemed Food Science program at the North Carolina State University and was admitted for a Masters program in Fall, 2002. This led to his journey to the east coast of the United States. Prashant completed his M.S. degree in Fall, 2004 working under the guidance of Dr. Fred Breidt and Dr. K. P. Sandeep. During his M.S. program Prashant developed a mathematical model to predict bacteriophage-host population dynamics in sauerkraut fermentations. After finishing M.S. degree, Prashant worked on an independent project under the guidance of Dr. Brian Farkas for about a year. During this time Prashant had decided to pursue Ph.D. in Food

Science and looking to expand his horizons, he came across a challenging project and a great mentor, Dr. Christopher Daubert. With no doubts in mind, Prashant started his Ph.D. in the Spring of 2006 under the guidance of Dr. Chris Daubert. During the final year of his PhD, Prashant got married to Shikha in February, 2009 with a beginning of a new phase of life. Upon graduation, Prashant is looking forward to joining a challenging research and development position.

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First of all, I want to thank Dr. Christopher Daubert for providing me an opportunity to work on a challenging and a stimulating project and being a great mentor during these four years of trials and tribulations. I want to thank him for his continued support, patience, motivation, guidance, insights, and his earnest desire to make his students succeed in every sphere of life. His thinking outside the box, constant motivation, and ideas made this experience enjoyable and intellectually stimulating for me. His mentorship and Monday morning discussions will always be missed.

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I want to thank Dr. Foegeding for serving as a co-chair for this work. His quick insights and guidance complemented the quality of this research. His ideas allowed me to think from a different perspective and enhanced the quality of this dissertation.

Thanks to Dr. Velev for teaching me the concepts of colloidal science. I thank him for agreeing to serve on my Ph.D. committee and contributing to this project.

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Many thanks to my wife Shikha for her unconditional love, her support during the times when I needed it the most, sharing the ups and downs, and for being my life partner. I am fortunate to have you in my life.

Last but not the least, thanks to my parents (Umesh Mudgal and Geeta Mudgal), my uncles (Brijesh, Ashok, Arun, Girish, and Pradeep), my aunts (Shubhlata, Sarita, Sarika, Indu, and Suman) my brothers and cousins (Gaurav, Ankit, Abhinav, Anish, Abhay, Mohit, Monika, Dharna, Savy, Aditya, Siddharth, Archie, and Tanya), and my respected grandmother, Vidya Sharma who all have contributed to me and this work in ways difficult to describe.

I wish to thank everyone whose name is not mentioned here but have contributed to me and this work in many ways.

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CHAPTER 1.

INTRODUCTION

This chapter briefly describes the background, motivation, and rationale behind understanding the cold-thickening mechanisms of a modified whey protein ingredient. The second chapter attempts to review relevant published literature on whey proteins, whey protein cold gelation, and whey protein aggregation mechanisms especially for β-lactoglobulin (β-lg) as affected by pH, ionic strength, and thermal treatment. The next four chapters, three-six, provide an extensive account of research performed by Prashant Mudgal during 2006-2009. The purpose of this research was to investigate bio-molecular mechanisms behind cold-thickening function of a modified whey ingredient manufactured with a technology patented at NC State University. Knowing a mechanism behind the unique functionality will present processors an opportunity to manipulate manufacturing conditions to expand the functionality of whey.

1.1 BACKGROUND

Whey proteins are well known for conveying nutritional benefits and are naturally complete, containing all essential amino acids. These dairy proteins are also the richest known source of naturally occurring branched chain amino acids (BCAA), considered important for active individuals and athletes. Among all proteins, whey proteins are known to have the highest biological value, a measure of how well the protein can be absorbed and utilized in the synthesis of cells by an organism. High bio-availabilities of amino acids in whey proteins

make them a desirable choice as an ingredient in food systems. Other than nutritional benefits, whey proteins are used as emulsifiers and foaming agents in different foods, including beverages, meat products, and bakery items (Kinsella et al., 1989). Recently, there has been an increasing interest in developing whey protein-based thickeners because of their nutritional benefits over conventionally used starches and hydrocolloids in food systems (Hudson et al., 2000; 2002). In previous studies, Hudson et al. (2000) developed a procedure allowing for the production of a cold thickening, whey protein ingredient without any addition of salt or heat. This procedure involved pH adjustment to 3.35, thermal gelation, freeze drying, and finally grinding to a powder. The modified powders (Hudson et al., 2000) impart instant thickening capability upon reconstitution in aqueous environment. Originally, this procedure was applied to whey protein isolate, but also worked with whey protein concentrates (Resch and Daubert, 2002; Resch, Daubert and Foegeding, 2004). However, the thickening mechanism behind this novel dairy ingredient remains largely unknown.

1.2 MOTIVATION

Although these modified whey powders provide certain benefits such as instant thickening, these ingredients were prepared at low pH conditions and yield astringent flavors when used in different food applications. To effectively tailor the original modification process and expand the functionality and utility of cold thickening modified whey protein ingredients, the basic mechanism behind the cold thickening must be explained. The focus of this dissertation was to investigate basic mechanisms behind thickening functionality of modified whey

protein ingredients at low pH in hopes of achieving capability to mechanistically tailor functional attributes of modified whey protein ingredients, specifically whey ingredients prepared between pH 3 and 4. To this end, seven primary hypotheses were developed and investigated. The rationale behind each hypothesis and corresponding objectives are discussed as following.

Hypothesis 1: Understanding concentration effects on thermal gelation properties of β-lg at the pH of modification procedure, 3.35, can provide insight into the basic mechanism(s) governing cold thickening functionality of modified whey protein ingredients.

Other than pH, ionic strength, time-temperature of thermal treatment, and cooling/heating rate, protein concentration affect the aggregation/gelation of β-lg as well as commercial whey protein products. To investigate concentration dependent mechanisms, a β-lg model system was developed because it is a major (~ 60 % w/w) component of whey proteins and it provides a simpler system to understand first principle mechanisms. Concentration effects on β-lg and whey protein gelation have primarily been studied from a perspective of critical concentration needed to form a gel under specific conditions of pH, ionic strength, and thermal treatment. The effect of concentration on network development prior to gel formation, and its effect on functionality of modified ingredients in that regime, is less documented. Therefore, the first part of this research (Chapter 3) addresses the concentration effects on cold-thickening functionality of modified β-lg powders.

Hypothesis 2: Larger aggregates are formed after heating at higher concentrations (above a critical concentration compared to those below a critical concentration) prior to the drying step of the modification procedure. Network structures formed will be different for different concentrations and can be elucidated with transmission electron microscopy (TEM).

Investigation of hypothesis 1 prompted the determination of a critical concentration for the β-lg modification procedure. Below the critical concentration, no significant thickening function could be achieved from modified β-lg powders, irrespective of the reconstitution concentration. Therefore, the second part of Chapter 3 addresses concentration effects on the size of aggregates formed and network characteristics during β-lg thermal aggregation at pH 3.35.

Hypothesis 3: Manipulating ionic strength using $CaCl_2$ and initial protein concentration can provide desirable network characteristics and enhance thickening function from modified β-lg powders.

Ionic strength increases with the addition of salts. Since calcium is of nutritional importance, $CaCl_2$ was used to change the ionic strength of β-lg solutions. An increase in ionic strength of β-lg solutions at low pH results in a higher rate of thermal aggregation because of screening of electrostatic repulsions. Thus, the objective of the second portion of this research (Chapter 4) was to investigate the effects of supplemental $CaCl_2$ and initial protein concentration on

the size of aggregates formed during the modification process and on the cold-thickening behavior of modified β-lg powders.

Hypothesis 4: The size of aggregates formed after thermal treatment, prior to the drying step play an important role in final functionality of modified β-lg ingredients, and these aggregates persist in modified powders after the freeze drying step.

The second part of Chapter 4 addresses the effects of drying on the β-lg network characteristics during the modification process and thickening functionality.

Hypothesis 5: Disulfide interactions together with non-covalent interactions play an important role in defining thickening function from modified β-lg powders.

At neutral pH, β-lg aggregation is a result of a combination of various reactions. Upon heating above 70 °C, β-lg denatures and the thiol and hydrophobic groups become solvent accessible. Aggregates are then formed through thiol-disulfide exchange and also by thiol oxidation and non-covalent interactions. Disulfide-sulfhydryl exchange is favored at neutral to alkaline pH (Creighton, 1988). Below pH 7, the efficiency of disulfide bonding is limited; therefore the first part of Chapter 5 discusses the role of disulfide interactions at the pH of this modification process and also on the concentration dependent thickening function of modified β-lg powders.

Hypothesis 6: Partial hydrolysis of β-lg can improve β-lg aggregation properties and can be manipulated to provide a better thickening function from modified β-lg solutions.

From literature, partial hydrolysis of β-lg improved gelation properties of β-lg at neutral pH. Acid hydrolysis at acidic pH 2 and pH 3.35 was confirmed from the results of the first part of Chapter 5 with a more prominent effect at pH 2. At pH 2, acid hydrolysis was recently found to play an important role during β-lg thermal aggregation. Based on these results, the role of acid/enzymatic hydrolysis of β-lg on thickening of modified β-lg solutions was investigated in the second part of Chapter 5.

Hypothesis 7: The size of aggregates formed during β-lg thermal aggregation at pH 3.35 and low ionic strength conditions is concentration dependent. Concentration and heating time can be manipulated to obtain aggregates of desired size.

In the final part of this research, a kinetic study of β-lactoglobulin aggregation was performed at the pH of the modification procedure, 3.35 (Chapter 6). Kinetic studies were performed at varying concentrations to investigate concentration dependent effects under the low ionic strength conditions and at constant temperature. The size of aggregates formed during different treatments were investigated, and a mechanism for β-lg aggregation at pH 3.35 was explained (Chapter 6).

CHAPTER 2.

LITERATURE REVIEW

This chapter provides a discussion of general topics relevant to this research in sufficient details with respect to published literature. A brief literature review specific to research objectives of this project will be included in each research chapter (three-six). In this chapter only, figures are embedded within the text.

2.1 WHEY

Whey is approximately 94 % water and is a by-product of cheese, quark, and casein production. Whey is obtained from milk by precipitation of casein along with separation of fat from milk. Depending on the method of precipitation, there can be three different types of whey: sweet, acid, and casein. Sweet whey is recovered when the casein is precipitated by using rennet enzyme, while acid whey is recovered as a by-product when curd cheese or quark are produced by the precipitation of casein, involving addition of a lactic acid producing bacteria. When casein is produced by the direct addition of inorganic acids to milk; a by-product of this process is a special type of acid whey known as casein whey. Lactose is the major component of whey solids (~ 70 %), followed by proteins and minerals. The whey types differ in their composition and pH value. Sweet whey has the highest concentration of proteins and near neutral pH (~ 6.1), explaining why it is frequently used for the production of whey derivatives (whey protein concentrates, isolates, and hydrolysates). Acid or casein whey has a pH below 5, thus sour in taste. Whey proteins are removed from whey through a series of concentration and filtration steps, which are reviewed in the following section.

2.2 WHEY PROTEIN PRODUCTION STEPS

In the first step of whey protein production, whey is pasteurized to kill harmful and spoilage microorganisms that may be present in whey. Although pasteurization is critical for microbial safety, it may result in partial denaturation of some of the whey proteins with lower denaturation temperatures (immunoglobulin and serum albumins). After pasteurization, the liquid whey stream undergoes a clarification step to remove remaining casein fractions. Then, the liquid whey undergoes a series of membrane filtration steps to concentrate solids. Microfiltration and ultrafiltration are two common methods to concentrate whey proteins from whey. In some cases, ion exchange coupled with ultrafiltration is used to selectively increase or decrease the whey protein concentrations in milk. Ion exchange with ultrafiltration helps minimize changes in the levels of casein and milk minerals, particularly Ca and P (Oldfield et al., 2005). Other filtration techniques used to concentrate whey proteins may include reverse osmosis, nano-filtration, cross flow microfiltration, and electro-ultrafiltration. After concentration and filtration, the concentrated whey is spray dried to produce powdered whey protein ingredients for direct or indirect use in food systems. A general overview of whey protein production process from milk is shown in Figure. 2.1.

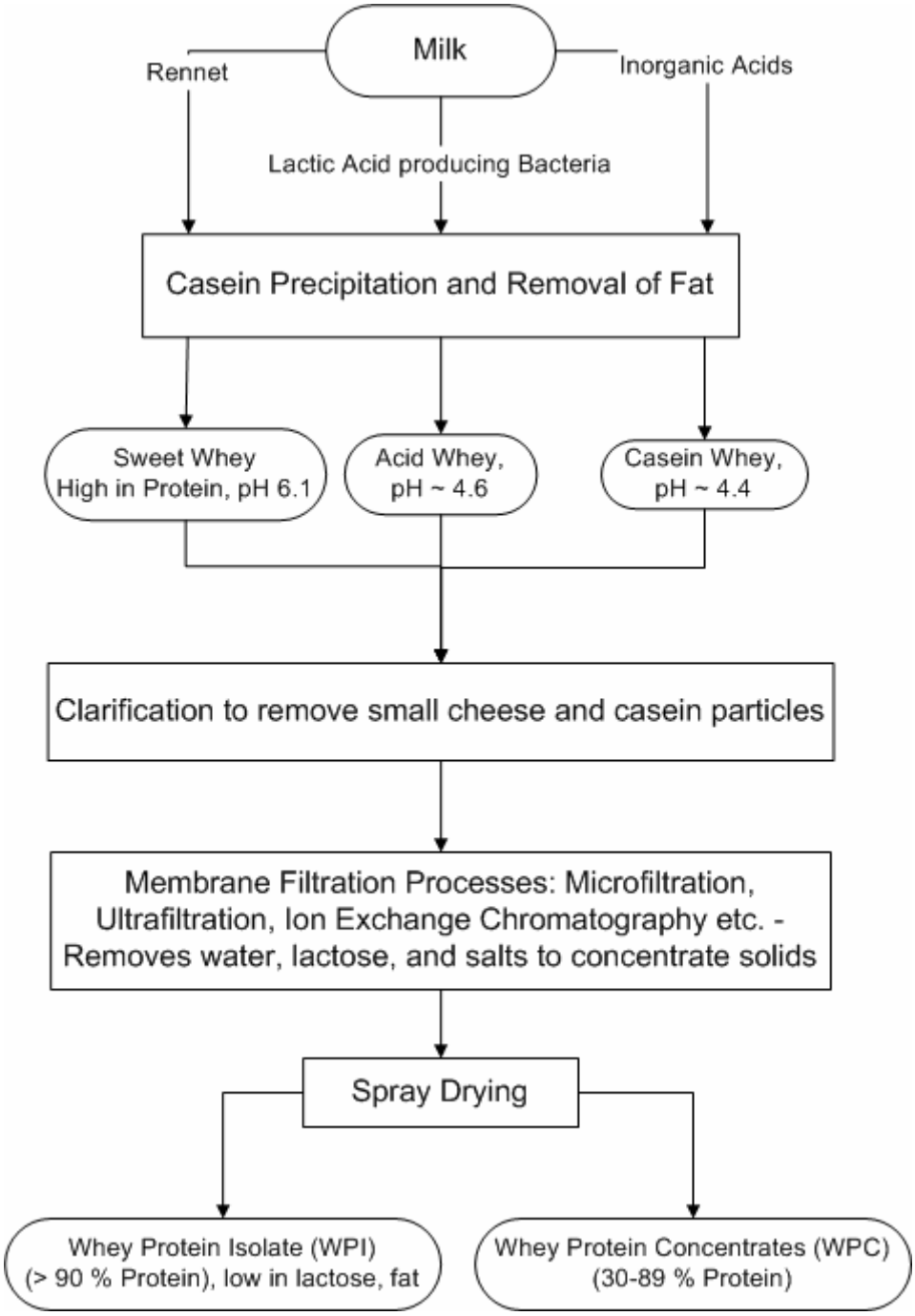

Figure 2.1. Whey protein production process from milk

2.3 WHEY PROTEINS PRODUCTS

Depending on the purity of whey proteins, derived protein products are categorized as either whey protein isolates (WPI) or whey protein concentrates (WPC). Whey protein isolate is the most pure and concentrated form of protein available (> 90 % protein), containing very low amounts of fat and lactose. Whey protein concentrates, on the other hand, may contain 30 – 89 % protein depending on the variations in extraction steps.

2.4 WHEY PROTEIN COMPOSITION

Different proteins are found in the whey fraction. The major components of whey protein are β-lactoglobulin (β-lg) (50-60 % of total whey proteins), followed by α-lactalbumin (~25 %), bovine serum albumin (~8 %), and immunoglobulins. Concentrations of different whey proteins in commercial whey protein concentrates are provided in Table 2.1. Other than these components, small amounts of lactose, lactoferrin, lactoperoxidase and different minerals such as Ca and P can be found in these commercially available whey protein products. Variations in relative proportions of different whey proteins are also present based on the processing conditions and source of whey (Wang and Lucey, 2003).

Table 2.1.

Concentration and properties of proteins in the whey protein concentrates

(Adapted from Langton and Hermansson, 1992)

Protein	Concentration	Molecular Weight	Isoelectric Point
β-lg	60 %	18600	5.13
α-lactalbumin	22 %	14160	4.2-4.5
Immunoglobulin	11 %	155000	5.5-8.3
Bovine Serum Albumin	3 %	66000	4.7-4.9
Other Proteins	4 %		

2.5 THERMAL GELATION OF WHEY PROTEINS

Protein concentration, pH, ionic strength, and time-temperature of thermal treatments affect gelation properties of whey proteins. Both covalent and non-covalent interactions play an important role during gelation of whey proteins. Different types of whey protein gels and relative roles of covalent and non-covalent interactions during whey protein gelation at different pH are discussed in subsequent sections.

2.5.1 Fine Stranded and Particulated Gels

Two types of gels are formed by whey proteins: particulated and fine-stranded. Particulated gels are formed when relatively large particles are loosely bound to each other in a random manner, resulting in poor water holding capacity. Fine stranded gels, however, are formed by

the association of strands or small diameter particles to form a network. As explained by Havea et al., 2004, the formation of a fine stranded or particulated network is determined by differences in denaturation, rate of aggregation of denatured proteins, and the relative amount of protein-protein attractive and repulsive interactions. In Havea, et al. 2004, it was concluded that when the rate of denaturation is faster than the rate of aggregation and a balance exists between protein-protein attractive and repulsive interactions, fine stranded gels are formed. In contrast, particulated gels are formed when the rate of aggregation is faster than the rate of denaturation and there is no balance between protein-protein attractive and repulsive interactions. Excessive interactions result in a randomly aggregated coagulum or precipitate, and excessive repulsion culminates in weak or non-existent aggregation. A schematic showing formation of particulated and fine-stranded β-lg gels is shown in Figure 2.2

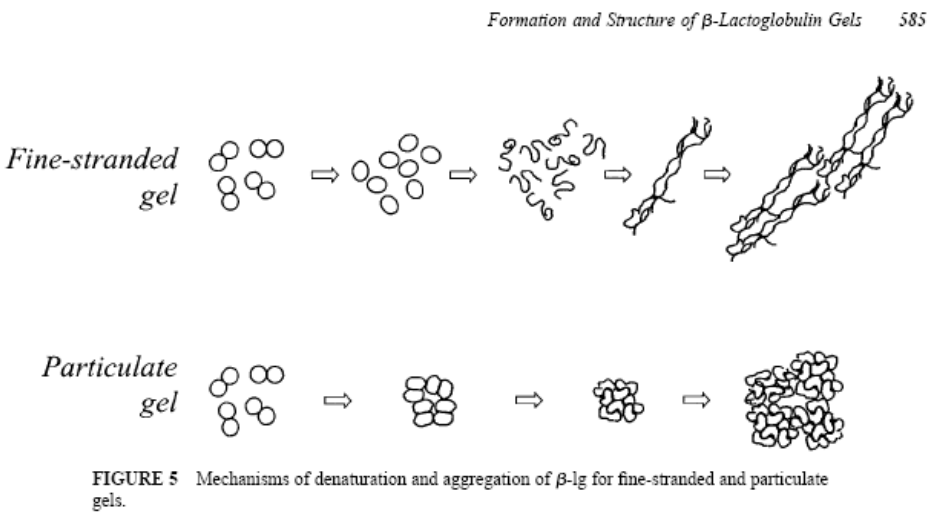

FIGURE 5 Mechanisms of denaturation and aggregation of β-lg for fine-stranded and particulate gels.

Figure 2.2. Mechanisms of denaturation and aggregation of β-lg for fine stranded and particulate gels (Adapted from Lefevre and Subirade, 2000)

2.5.2 Covalent and Non-Covalent Interactions during Thermal Gelation of Whey Proteins at Neutral pH

The formation of aggregates during gelation involves covalent (disulfide bonds) or non-covalent interactions such as hydrophobic, electrostatic, ionic and van der Waals, and each is relatively weaker than covalent bonding. Several studies have investigated the relative roles of these interactions in whey protein gelation at different pH (Havea et al., 2004; Vardhanbhuti and Foegeding, 1999; and Alting et al., 2000, 2002, 2004). Roles of these interactions in whey protein gelation vary with pH. For example, at neutral to alkaline pH, disulfide interactions are favored (Creighton, 1988). However, the relative contribution of these interactions still remains largely unknown across the broader spectrum of chemical conditions.

The relative role of disulfide bonds and other non-covalent bonds in whey protein gelation near neutral pH 7.0 have been studied using denaturing agents to alter protein conformation and reduce specific type of bonding (Vardhanabhuti and Foegeding, 1999; Havea et al., 2004). Examples of such denaturing agents are urea, dithiothreitol (DTT), sodium dodecyl sulfate (SDS) or β-mercaptoethanol. Urea breaks all non-covalent interactions and promotes unfolding, while DTT is a strong disulfide bond reducing agent which reduces both inter and intramolecular disulfide linkages. A powerful anionic detergent, SDS combines to the hydrophobic regions of the protein and carries a negative charge and denatures the protein to

a linear segment. This linear conformation allows for the formation of disulfide bonds while inhibiting other types of non-covalent interactions such as hydrogen bonds, van der Waal's and hydrophobic associations.

At pH 7.0, the role of disulfide bonds was found to dominate formation of large size aggregates after heating (Vardhanabhuti and Foegeding, 1999), as thiol-disulfide interchange is favored at neutral to alkaline pH (Creighton, 1988). When both DTT and urea were added to unheated whey protein isolate solutions, intrinsic viscosity (a measure of hydrodynamic volume and radius of gyration) increased from 5 ml/g to 18 ml/g because of more unfolding and asymmetrical structures. When control solutions were heated, intrinsic viscosity increased from 5 ml/g to 147 ml/g, while solutions containing DTT and urea had similar (18 ml/g) viscosities even after heating. However, heating in the presence of urea alone increased the intrinsic viscosity value to 170 ml/g. These results suggested that disulfide bonds were necessary at pH 7.0 to form large aggregates. Vardhanabhuti and Foegeding, (1999) suggested that at pH 7.0, polymerization of whey protein isolate was a two phase process. In the first phase, buried sulfhydryl and hydrophobic residues became solvent accessible upon heating and formed reactive structures. Then these structures reacted with each other, mainly through disulfide linkages, to form primary polymers. In the second phase, these primary polymers associated via non-covalent interactions to form final polymers.

Havea et al., (2004) studied the relative effects of both covalent and non covalent interactions in heat induced gelation of whey protein concentrates (WPC) at pH 6.9. It was concluded that

the heat induced gelation of WPC solutions was dominated by the non-covalent bonding between denatured protein molecules. Disulfide bonds appeared to give rubbery characteristics and a fine stranded type network, while non-covalent bonding gave rigidity, brittleness, and a more particulated type network (Havea et al., 2004). Gels containing SDS were mostly translucent with a fine stranded structure (~10 nm aggregates) followed by control gels, further less translucent (~100 nm aggregates) and then DTT gels which were least translucent having a particulated network (~ 200-300 nm). The SDS gels allowed only formation of disulfides and had a fine stranded structure while the DTT gels were almost totally reliant on the hydrophobic mediated association of monomeric and dimeric whey protein species. Gel rigidity was found to be a maximum in DTT gels, followed by control gels, and then SDS gels.

2.6 COLD GELLING WHEY PROTEINS

Thickening functionality of whey proteins is typically achieved with a thermal treatment. However, there has been an increasing interest in formation of cold gelling whey protein ingredients for potential application in food systems where heat may not be desirable. Typically in cold-gelation studies there are two steps, in the first step: whey proteins are heated at neutral pH and low ionic strength, above their denaturation temperature and below their critical concentration for gelation, to obtain solutions of soluble protein aggregates. These solutions are then cooled, and in the second step: cold gelation is induced with salt addition (Barbut and Foegeding, 1993; Hongsprabhas and Barbut, 1997; and Bryant and

McClements, 2000) or by lowering the pH (Alting et al., 2000; 2002; 2003; and 2004). In a different approach, β-lg solutions were heated at pH 2.0 to form soluble fibrillar aggregates, and then following cooling, the pH was adjusted to 7 or 8, and finally $CaCl_2$ was applied for network formation (Veerman et al., 2003). Since fibrils can increase viscosity on a weight efficient basis because of their high aspect ratio (length: diameter); this process resulted in a critical concentration for gelation that was an order of magnitude lower than traditional cold-gelation methods. Cold gelling ingredients at a slightly alkaline pH have also been described in powdered form which gels upon redispersion in a salt containing solution (Thomsen, 1994). This powdered ingredient was produced by heat treatment during homogenization of a whey protein concentrate followed by immediate drying. This ingredient gelled upon redispersion in a salt containing solution. Elofsson et al. (1997) found that this ingredient (Thomson et al., 1994) consisted of micron-sized aggregates that dissolved slowly over many hours at pH far away from isoelectric point and low ionic strength conditions leading to a solution of disulfide bridged aggregates of about 20-30 nm size.

2.6.1 Acid Induced Cold Gelation Mechanism

In the second step of acid induced cold gelation as explained in previous section, gelation is induced by slow acidification instead of using salts (Alting et al., 2000). Glucono-δ-lactone (GDL) slowly hydrolyzes to gluconic acid causing a gradual reduction in pH and forming a regular gel (Alting et al., 2000). Alting et al. (2000) studied the role of non-covalent

interactions versus disulfide interactions in the second stage of the cold-gelation process by the chemical modification of reactive thiol groups on the surface of reactive protein polymers or aggregates formed after the first step. Alting et al. (2000) used three different thiol blocking agents (N-ethylmaleimide (NEM), iodoacetamide (IAA), and p-chloromercuribenzoic acid (PCMB)) to exclude specific effects of agents itself. This work found that both the microstructure (from permeability measurements and confocal scanning laser microscopy (CSLM) and the initial kinetics (from turbidity measurements with time) of gelation were not perturbed by modifying the free thiol groups on the surface of the aggregates, and thus concluded that the initial morphology of the network was governed by non-covalent interactions. However, Alting et al. (2000) reported that disulfide bonds were formed during the second stage of cold-gelation, leading to larger covalently linked aggregates. Because, formation of disulfide bonds is slowed down by lowering the pH, disulfide bond formation was attributed to an increase in the effective concentration of aggregates. As a result of non-covalent interactions, the free thiol groups and disulfide bonds were reported to be very close, leading to the formation of disulfide bonds. Formation of additional disulfide bonds increased molecular weight of aggregates formed during gelation and stabilized the network, with a concomitant increase in gel hardness (Alting et al., 2000). Prior to Alting et al. (2000), disulfide bonds were reported to mainly increase average molecular weight of protein polymers during first step of cold-gelation (Hongsprabhas and Barbut, 1997). A schematic illustrating the mechanism explained above is shown in Figure 2.3 (adapted from Alting et al., 2000).

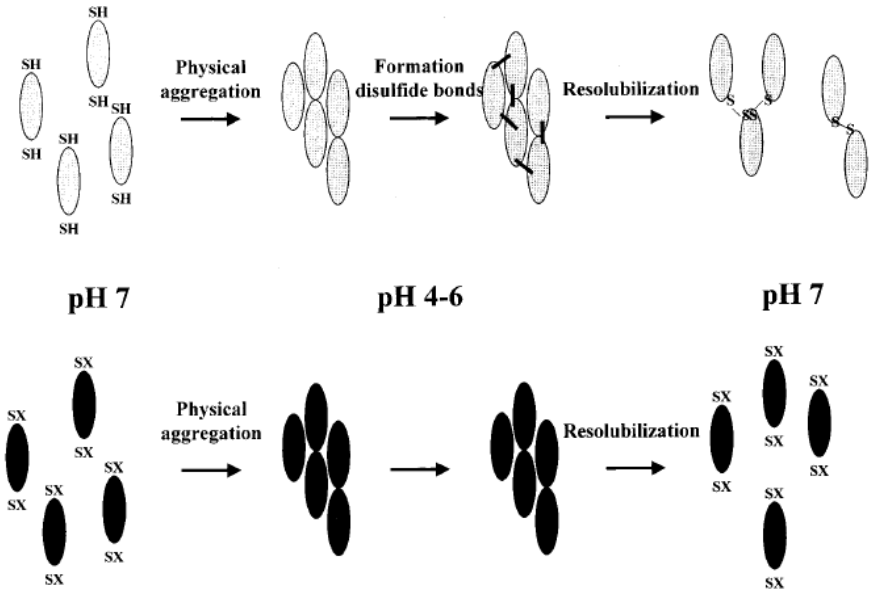

Figure 4. Model for the formation of intermolecular disulfide bridges and their role during the acid-induced cold gelation of heat-treated whey proteins. The oval shapes represent the WPI aggregates after heating (size ~80 nm). The upper part describes the gelation process of unblocked aggregates. In this case the free thiol groups (SH) can form disulfide bonds after noncovalent chemical aggregation, and resolubilization yields relatively large aggregates. The lower part indicates that when the thiol groups are blocked (SX), no disulfides can be formed. After resolubilization, the size of these aggregates is not affected by the gelation process.

Figure 2.3. Model for the formation of intermolecular disulfide bridges and their role during acid-induced cold gelation of heat-treated whey proteins. (Adapted from Alting et al., 2000)

According to Alting et al. (2004) in the second step of the cold gelation process, when the pH was lowered, electrostatic repulsions between WPI aggregates decreased, imposing formation of organized clusters. Finally, the strengthening of the gel took place through formation of intermolecular disulfide bonds. The effect of electrostatic interactions between β-lg aggregates during the second step of cold-gelation process was studied by modifying net charge of the aggregates through succinylation (Alting et al., 2002). Succinylation was brought about by reaction of the primary amino groups (lysine-residues) present on the

aggregates with the reagent succinic anhydride. This reaction resulted in the inversion of the positively charged amino groups into negatively charged carboxylic acid groups, creating a modification of the net charge value of the aggregates. Succinylation of the aggregates at neutral pH increased the net negative charge on aggregates thereby altering the isoelectric point of the aggregates formed after the first step of the cold-gelation process. Succinylation resulted in lowering of the gelation pH of aggregates from 5 to 2.5. At pH 2.5, no formation of disulfide bonds between aggregates occurred and gels showed spontaneous fracture comparable to those formed by thiol blocked WPI aggregates. Alting et al. (2002) also decreased the overall negative charge on β-lg aggregates by methylation of the carboxylic groups, which resulted in an increased gelation pH of about 9. Alting et al. (2002) demonstrated the possibility of controlling mechanical properties of cold-set gels by chemical modifications at the molecular level.

2.7 β-LACTOGLOBULIN

β-Lg is a major component of whey proteins (50-60 %), and the properties of β-lg play a significant role during whey protein gelation (Langton and Hermansson, 1992). This protein is comprised of 162 amino acids and has a molecular weight of 18.3 kDa. Several genetic variants of β-lg coexist, but the main ones from cow are variants A and B (Sawyer et al., 1999) differing in two amino acids Asp64Gly and Val118Ala (Sawyer et al., 1999). β-Lg has an isoelectric point (pI) around 5.2. In subsequent sections, structure, conformation, and gelation of β-lg are reviewed.

2.7.1 Structure and Conformation

β-Lg is a globular protein with two intramolecular disulfides (Cys66-Cys160 and Cys 106-Cys 119) and one free sulfhydryl group, also identified at Cys residue 121 (Sakurai et al., 2001). Its structure at different pH has been studied extensively for decades starting as early as 1934 when Palmer (Sawyer et al., 1999) studied its structure using X-ray diffraction. β-Lg can exist in different conformations, but is mostly exists as a monomer or a dimer. However, near pH 4.0 and at very low temperatures, this protein has also been found to exist as an octamer (Sawyer, 1999). Although, crystals have been obtained around pH 4, there is no definitive evidence of octamer formation. It is also known that dimerization occurs through an antiparallel β-strand interacting with the same strand in the other subunit (Sawyer, 1999). The structure of β-lg monomer is shown in Figure 2.4 below:

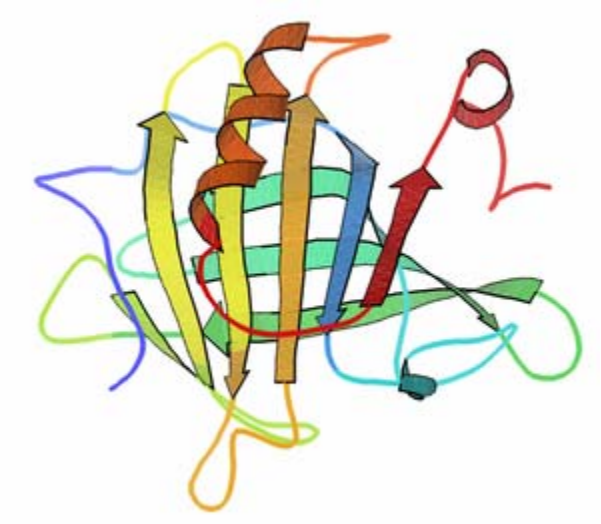

Figure 2.4. The structure of β-lg from protein data base (PDB) entry 3BLG.

The ribbons denote the secondary structure (β-sheets and α-helix). Rendered with Kinemag

2.7.2 Monomer-Dimer Equilibrium

Recent studies have found that monomer-dimer equilibrium exists at all pH levels and their relative amounts are decided by the pH, ionic strength, and temperature. At low pH, monomer-dimer equilibrium is shifted towards monomer (Aymard et al., 1996). The effect of temperature and ionic strength on monomer-dimer equilibrium was studied using static and dynamic light scattering at pH 2 (Aymard et al., 1996). At pH 2, β-lg is highly charged and excessive repulsion exists between protein molecules, especially under low ionic strength conditions. Therefore with a decrease in ionic strength equilibrium is shifted towards monomer (Aymard et al., 1996; and Renard et al., 1998). Similar results were also obtained at pH 3.0, where increased ionic strength resulted in equilibrium shift towards a dimer (Sakurai et al., 2001). Also with an increase in temperature (5 – 76 °C), dimers dissociated into monomers and equilibrium was shifted towards a monomeric structure (Aymard et al., 1996). However, the complete dissociation only took place at very low ionic strength and high temperatures (Aymard et al., 1996). As a general rule, β-lg solutions should be considered as a mixture of both monomer and dimer (Aymard et al., 1996).

2.8 β-Lg THERMAL AGGREGATION AND GELATION

Numerous studies investigated gelation properties of β-lg at different pH (Oldfield et al., 2005; Renard et al., 1992; 1998; Sagis et al., 2002; Veerman et al., 2002; 2003; Schokker et al., 2000; Aymard et al., 1996; 1999; Langton and Hermansson, 1992; Elshereef et al., 2006;

Sakurai et al., 2001; Castelletto et al., 2007; and Wada et al., 2006). However, much attention was given to pH 7.0 (Schokker et al., 2000; Ikeda and Morris, 2002) and at pH 2.0 (Veerman et al., 2002; 2003; Sagis et al., 2002; Bolder et al., 2006; 2007; Aymard et al., 1996, 1999; Ikeda and Morris, 2002; and Kavanagh, 2000, Akkermans et al., 2008a; 2008b). Very few studies have addressed β-lg gelation and microstructure between pH 3 and 4 (Langton and Hermansson, 1992). In next sections pH specific effects on β-lg aggregation are reviewed.

2.8.1 pH Effects on β-Lg Thermal Aggregation

Proteins are polymers of amino acids. Based on the respective pKa and pKb of these amino acids, proteins acquire charge depending on the pH value. Depending on the pH, β-lg forms different types of networks upon heating. Langton and Hermansson, 1992 studied the pH effect on microstructure of 12 % w/w β-lg gels using different microscopy techniques. As mentioned earlier (section 2.5.1), particulated gels are formed near the isoelectric point, and fine stranded gels are formed at pH far away from the isoelectric point. β-Lg forms a particulated network in the range pH 4-6 (Langton and Hermansson, 1992). Transparent fine stranded gels are formed below and above this region. Particulated networks are formed from the coarse aggregation of large sized spherical aggregates (200-300 nm) (Havea et al., 2004), and have a relatively open structure compared to fine stranded gels, a difference also evident from a very low critical gel concentration (1 % w/w) near the isoelectric point (Renard et al., 1992) and notable poor water holding capacities. Fine stranded gels were formed both below

pH 4.0 and above pH 6.0. However, a difference between stranded characteristics was observed, with short stiff strands at low pH and longer flexible strands at higher pH. Also a mixture of both fine stranded and particulated network was seen at pH 4.0, while at pH 6.0 either particulated or fine stranded networks were formed, indicated by a large standard deviation in fracture properties of gels at pH 6.0 (Stading and Hermansson, 1990). A regular and very dense network was observed at pH 3.5, with diameter of strands always < 5 nm. At pH 4.0, the network was observed to be more open than at pH 3.5, and certain particulated areas were seen which also resulted in a more irregular network compared to ones at pH 3.5. Thickness of strands observed at pH 4.0 was also found to be slightly higher than pH 3.5. In summary, gels formed at low pH were composed of short stiff strands with many strands joined together at one junction creating a denser network and thus a higher water holding capacity (Langton and Hermansson, 1992). Recently, several studies have focused on β-lg aggregation phenomenon at pH 2 and are reviewed in following sections.

2.8.2 β-Lg Thermal Aggregation at pH 2.0

At pH 2.0, β-lg is highly charged, carrying a positive charge of + 20 (Veerman et al., 2002). This charge distribution results in excessive repulsion and formation of fine stranded gels after heating. Several studies focused on pH 2.0 gelation of β-lg. At pH 2.0 it was shown that β-lg forms fibrillar aggregates (Veerman et al., 2002; 2003; Ikeda and Morris, 2002; and Kavanagh et al., 2000). These fibrils have diameter equivalent to that of a monomer, however some variations have been observed in the thickness of these strands (2-4 nm). Length and flexibility of these fibrils varies with pH and ionic strength as well. Flexibility of these

strands is shown to increase with increasing ionic strength; however, chain length decreases as ionic strength is increased (Veerman et al., 2002; Aymard et al., 1999; and Kavanagh et al., 2000). The length of these fibrils may be close to 1 μm. Aymard et al., 1999 determined the length to be 600 nm at pH 2.0 and ionic strength of 30 mM, compared to 38 nm at ionic strength of 0.1 M. The effect of ionic strength on flexibility/curvature of these fibrils is shown in Figures 2.5 and 2.6.

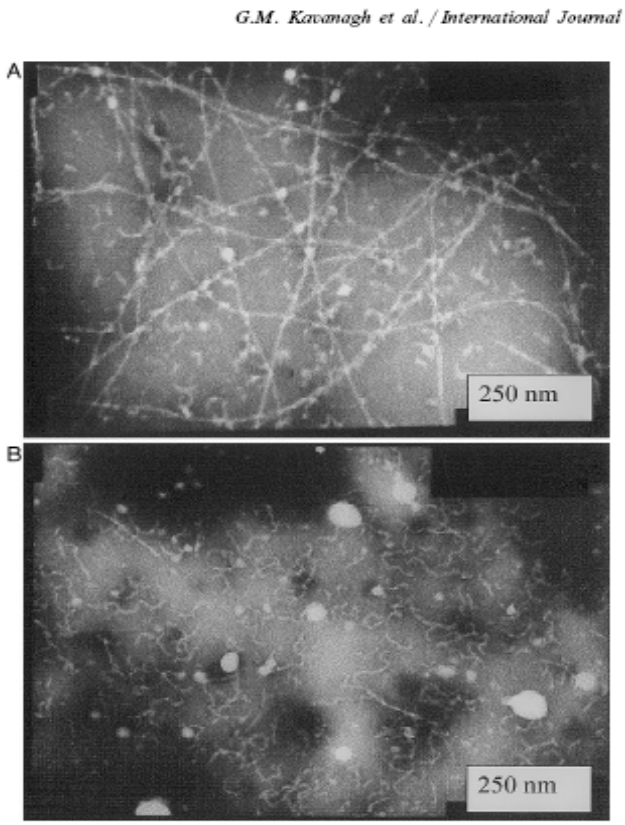

Fig. 1. (a) TEM of 3% w/w β-Lg at pH 2 after 6 h at 80°C, without added NaCl, magnification, ×48 000; b. as Fig. 1a, but in 0.05 M NaCl.

Figure 2.5. Ionic strength effects on curvature of fibrils at pH 2 (Adapted from Kavanagh et al., 2000)

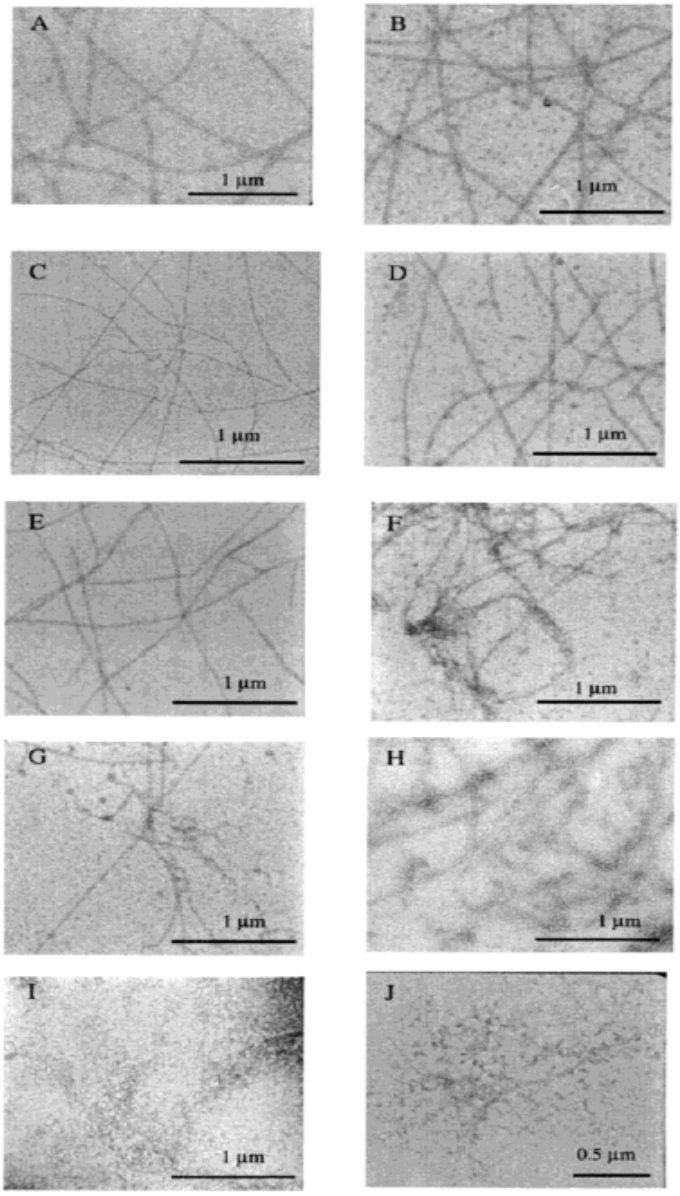

Figure 2. TEM micrographs for β-lg at pH 2 and various ionic strengths: a, 0.01 M; b, 0.02 M; c, 0.03 M; d, 0.04 M; e, 0.05 M; f, 0.06 M; g, 0.07 M; h, 0.08 M; i, 0.09 M; j, 0.10 M.

Figure 2.6. Ionic strength effects on fibrils formed at pH 2 (Adapted from Veerman et al., 2002)

2.8.3 Persistence Length

The persistence length (L_p) is a measure of the stiffness/flexibility of the polymer. The L_p is a measure of the distance over which spatial orientation of the monomers is not mutually independent or a length of polymer that persists over a certain length. Thus, the higher the persistence length, the more stiff the polymer. Persistence length was found to be decreasing with increasing ionic strength at low pH (Aymard et al., 1999), however, at pH 2.0 persistence length remained independent of ionic strength in a range of 0.01-0.08 M (Veerman et al., 2002). Similarly, Renard et al., 1992 noticed gels at pH 2.0 remained transparent over the range of ionic strength employed.

2.8.4 Persistence Length – Theory and Measurements

Formally, the persistence length is defined as the length over which the correlations in the direction of tangent are lost. It is also defined as the linear length that persists along a particular direction or how rapidly the direction of polymer changes as a function of the contour length. Persistence length of biomacromolecules plays an important role in macromolecular biophysics, and it might enable one to correlate macromolecular structure to functional properties in different systems. There are several methods and techniques frequently used to estimate the persistence length of polymer chains. Some of these are described below (from Cifra et al., 2004):

First Method, also described as the 'exact' method (Cifra et al., 2004), determines persistence length from the average projection of end-to-end distance onto the first bond angle of the chain $\vec{l}_1$. The average is taken over all possible conformations for the given thermodynamic state of the chain. This method (Eq. 1) has minimum of the approximations and thus is known as the exact method. However, this method can not be employed to determine persistence length from images where one can only obtain a particular conformation for one chain at the given thermodynamic state of the system. The persistence length should be obtained in the limit of very long/infinite chain length.

$$l_{ps} = < \sum \vec{l}_i \vec{l}_j > / < l > \quad \text{- Eq. 1}$$

Where, l_{ps} is the persistence length, and <l> is the average bond length.

The second method, or the first of the approximate methods to determine the persistence length, involves the average cosine of the bond angles in the chain. The method is described below:

$$l_{ps} = < l > /(1 - < \cos\theta >) \qquad \text{Eq. 2}$$

where θ is the angle between chain segments and <l> is the average bond length. The <cos θ> is an average correlation between the segments.

Persistence length is also calculated by the orientation correlation of bonds along the chain and an exponential decay of this correlation. Orientation correlations in chain are expressed as the scalar product of two unit bond vectors which are separated by a curvilinear distance r

along the chain. Orientation correlation are predicted as an exponential decay governed by the persistence length l_{ps} from random coil statistics (Eq. 3). In two dimensions, decay length is doubled, so Eq. 4 should be used to estimate l_{ps} (Gittes et al., 1993).

$$< \vec{u}(r)\vec{u}(0) >= \exp(-r / l_{ps}) \quad \text{Eq. 3}$$

$$< \vec{u}(r)\vec{u}(0) >= \exp(-r / 2l_{ps}) \quad \text{Eq. 4}$$

Another method to obtain persistence length is from the observed average square end to end distance and fitting data to worm like chain model (WLC) (Eq. 5).

$$< R^2 >= 2l_{ps}L_c - 2l^2{}_{ps}(1 - \exp(-L_c / l_{ps})) \quad \text{Eq. 5}$$

Where, L_c is the contour length.

These methods work best in a coil regime and tend to deviate close to coil-rod transitions. From the coil regime results of Cifra et al., 2004, it was found that Eq. 2, using the average valence angle, was more reliable than the Eq.3, using orientation correlation in the chain. The Eq. 2 estimate provided value of l_{ps} close to the best estimate, not only in theta state, but also in the good solvent in spite of its derivation using random coil statistics. The values of l_{ps} obtained from Eq. 2 were found closest to the exact method (Cifra et al., 2004). The following schematic explains the step-wise approach to determining persistence length using the bond angle method from Eq. 2 from a 2-D image.

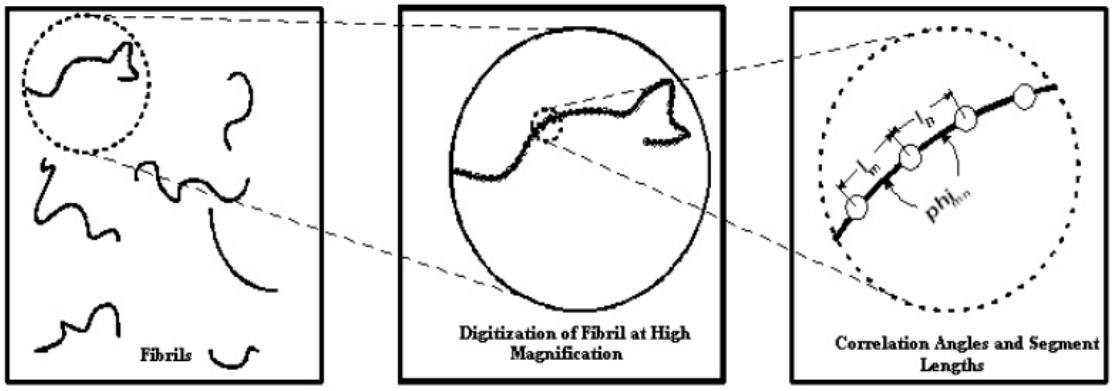

Figure 2.7. Persistence length measurements

2.9 FIBRILS FORMED AT PH 2

Fibrils can be defined as linear and semi-flexible aggregates. Several methods exist to study fibril formation, including electron microscopy, light scattering, atomic force microscopy, X-ray diffraction, rheology, gel-electrophoresis, Congo red birefringence complemented by circular dichroism and FTIR to establish the characteristic β-sheet presence (Van der Linden et al., 2007). Other less elaborate methods that are often used involve applying dyes that specifically bind to the amyloid fibrils, giving rise to fluorescence (Thioflavin T) or absorbance (Congo red).

Although fibril formation by β-lg has been extensively studied, many other proteins of animal origin can form fibrils upon heating. Examples of these proteins are ovalbumin, bovine serum albumin (BSA), and lysozyme (Akkermans et al., 2007). The formation of

fibrils can modify flow behavior, viscosity, and gelation properties in food systems on a weight efficient basis. A macromolecule, which is more linear in nature, will have a larger hydrodynamic radius in a suitable solvent; thus imparting higher viscosity to the dispersion or solution compared to a similar molecular weight macromolecule with a more branched or compact structure, illustrated in Figure 2.8.

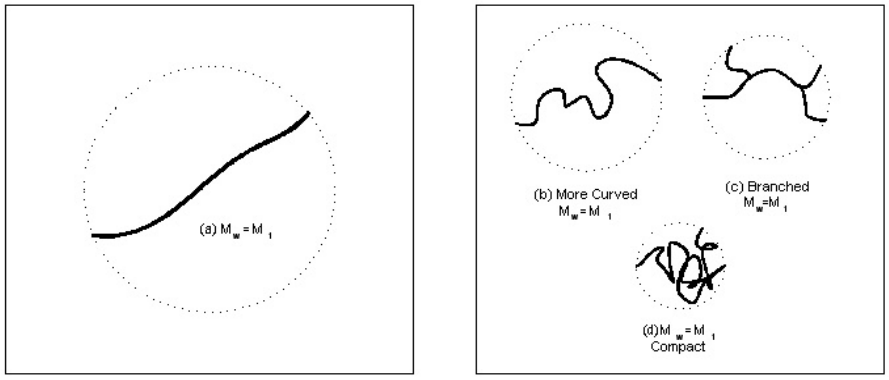

Figure 2.8. Effect of shape on hydrodynamic volume

The whey protein β-lg forms linear, rod-like fibrils at pH 2.0; characteristics of these fibrils are largely dependent on pH, ionic strength, and thermal time-temperature conditions. Recently, shearing during heating has been documented to increase fibril formation (Bolder, 2007b). Since β-lg fibrils formed at pH 2.0 have high aspect ratio (length: diameter), the gels formed are transparent. The stiffness of the fibrils is quantified by the persistence length (l_p) while contour length (l_c) denotes the curvilinear length of the fibrils as explained in sections

2.8.3 and 2.8.4. In subsequent sections, kinetic effects, shear effects, and mechanisms of β-lg fibril formation are reviewed with respect to published literature.

2.9.1 Mechanism of β-Lg Fibril Formation at pH 2 and Low Ionic Strength

At the pH values far away from the isoelectric point and at low ionic strength conditions, fine-stranded transparent gels are formed after heating at suitable β-lg concentration. Under these conditions strong electrostatic interactions exist between partly denatured β-lg molecules leading to a formation of a relatively open network structure of these transparent gels (Arnaudov et al., 2003, Schokker et al., 2000, Renard et al., 1992, Ikeda and Morris, 2002). In aqueous solutions, β-lg may undergo reversible dimerization depending on the genetic variant, concentration, pH, temperature and the screening of electrostatic repulsions (Aymard et al., 1999). Upon heating, β-lg native structure is partly altered potentially resulting in an increased exposure of buried hydrophobic residues and free thiol groups. These exposed groups may participate in aggregation, and a gel may form under suitable conditions. Thiol-disulfide exchange reaction, forming intermolecular disulfide bonds at pH 7, can not take place at pH 2.0, as thiol groups are stable at low pH. Aymard et al. (1999) studied β-lg aggregates formed upon heating at pH 2 by light scattering techniques under varied conditions of ionic strength. A slow kinetics of aggregation was reported by Aymard et al. (1999) under the conditions of low ionic strength as compared to 0.1 or 0.2 M ionic strength. Aymard et al. (1999) described these aggregates as ‘wormlike chains’ with a persistence length whose value strongly depends on ionic strength. They observed a large persistence length at low ionic strengths. Aymard et al. (1999) suggested that the values of

large persistence length at low ionic strength can not solely be attributed to electrostatic repulsions but could also involve the role of specific site binding of the proteins to each other. The presence of long linear fibrils was reported at low ionic strength conditions even when a majority of the proteins remained non-aggregated et al. (Aymard, 1999).

2.9.2 Nucleation and Growth Mechanism for Fibril Formation

A nucleation and growth mechanism was suggested for the development of the fibrils where nucleation is the rate limiting step. Once aggregates of certain size are formed, aggregation can occur rapidly (Aymard et al., 1999). It was also suggested that smaller aggregates may not be stable, and aggregation can become irreversible only when aggregates have reached a critical size of stability (Aymard et al., 1999). A nucleation and growth model was also suggested by Clark et al., 2001 for the gelation of β-lg at acidic pH. The model described gelation of β-lg at acidic pH in three main stages: an initial unfolding or dimerization step, a step of linear fibrillar aggregation via nucleation and growth and a step of random association of the fibrils. Changes in the proton secondary structure upon β-lg fibril formation at pH 2.0 have also been studied using wide angle X-ray diffraction (WAXD) and Fourier transform infrared spectroscopy (FTIR) (Kavanagh et al., 2000). It has been reported that upon fibril formation, increased amount of intermolecular hydrogen bonds and β-sheets are observed. Arnaudov et al. (2003) described formation of the fibrils at pH 2 as a multi-step process. Upon heating, β-lg molecules (native state) partially denature and form an intermediate, which can follow two routes of aggregation. In the first route, there are two steps including

(1) formation of reversible linear aggregate followed by (2) some kind of a slow consolidation step creating fibrils. After the consolidation step, no disintegration of fibrils takes place. In the second route, 'dead end species' are formed which can not participate in fibril formation. The 'dead-end' species could be either denatured monomers or small molecular weight oligomers which can not aggregate any further. They further explained that a critical concentration exists above which formation of the fibrils dominate over the formation of 'dead-end' species and vice-versa. Thus, Arnaudov et al. (2003) suggested that a critical concentration effect is evident as a result of competition between reactions of two different orders and two different rates. Above a critical concentration and during heating, more fibrils are formed, and below the critical concentration more 'dead-end' species are formed. Bolder et al. (2007b) proposed that these 'dead-end' species might be either hydrolyzed monomers of oligomers. However, recently it has been found that acid hydrolysis of β-lg at pH 2 play a crucial role in fibril formation and resulting peptides are the building blocks of these fibrils (Akkermans et al., 2008a; 2008b). Similar to the mechanism suggested by Aymard et al. (1999), Bolder et al. (2007b) suggested a nucleation and growth model for the formation of fibrils during heating at pH 2 and low ionic strength conditions.

2.9.3 Nucleation and Growth Models

Quantitative models describing the nucleation and growth models for β-lg fibril formation at low pH (2-2.5) have been reported (Bromley et al., 2005, Clark et al., 2001). In a simple model, fibril formation at pH 2.5 was modeled using a set of three ordinary differential

equations (ODE's) following some assumptions (Bromley et al., 2005). These equations described the nucleation and growth process in three steps: (1) formation of active species (2) nucleation by active species (3) fibril growth. Formation of active species was modeled as a first order reaction, while nucleation was modeled as a reaction of order n, where n is the critical number of active species needed to form a nucleus. It was assumed that the nucleation is a rate limiting process compared to the formation of active species. The growth of fibrils was also modeled as a first order reaction with respect to the non-aggregated protein material including native protein and active species. The set of mutually dependent ODE's describing the nucleation and growth model for fibril formation at low pH is described below:

$$\frac{dS}{dt} = k_1 \qquad \text{Eq. 6}$$

$$\frac{dN}{dt} = k_2 (k_1 t)^n \qquad \text{Eq. 7}$$

$$\frac{dF}{dt} = k_3 (1 - F)\left[\frac{k_2 (k_{1t})^{n+1}}{k_1 (n+1)}\right] \qquad \text{Eq. 8}$$

Where, S denotes number density of active species, N denotes number density of nuclei, F denotes number density of protein aggregates bound up in aggregates while k_1, k_2, and k_3 are rate constants for respective reactions. The analytical solution of these equations gives the following solution:

$$F = 1 - \exp\left[-k_3 \frac{k_2 (k_1 t)^{n+2}}{{k_1}^2 (n+1)(n+2)}\right] \qquad \text{Eq. 9}$$

A plot of *Ln (-Ln (1-F))* was plotted with *Ln (Time)*, and the order of the nucleation reaction was estimated to be about 4 based on the gradient of the plot for β-lg heated at 70 °C, pH 2.5.

2.9.4 Kinetics of β-Lg Fibril Formation

Kinetics of β-lg fibril formation at pH 2 has been studied in detail using different techniques (Aymard et al., 1999 and Veerman et al., 2002, and Arnaudov et al., 2003). In all studies it was found that even after prolonged heating, a significant amount of protein remains non-aggregated, and conversion values increased with increasing protein concentration at heating. Conversion notably increased with increasing heating time, but leveled off after prolonged heating times, indicating that aggregation slows down and reaches a plateau after prolonged heating times. It has also been suggested that there is no critical aggregation concentration. Aggregation occurs at all concentrations, however the conversion values increase with increasing protein concentration, and a sudden jump in conversion may be obtained above a particular concentration, suggesting a critical concentration effect (Bolder et al., 2007).

Along with the formation of fibrils, spherulites were found to coexist when WPI was heated at rest at pH 2 and low ionic strength conditions (Bolder et al., 2006). Spherulites are composed of fibrils that grow radially outward from a nucleus (Bolder et al., 2006). This nucleus may be composed of small oligomers or droplets formed by liquid-liquid phase separation (Figure 2.9). Spherulites were not found in WPI samples which were stirred during heating. A possible explanation for this observation is that stirring accelerates the

reaction kinetics for fibril formation and therefore no spherulites are formed. Bolder et al. (2007b) found that stirring increased the kinetics of fibril formation, leading to formation of more fibrils as compared to the system heated at rest. Stirring during heating was proposed to increase both the number of growing fibrils and encounters between protein monomers and between monomers and the growing fibrils.

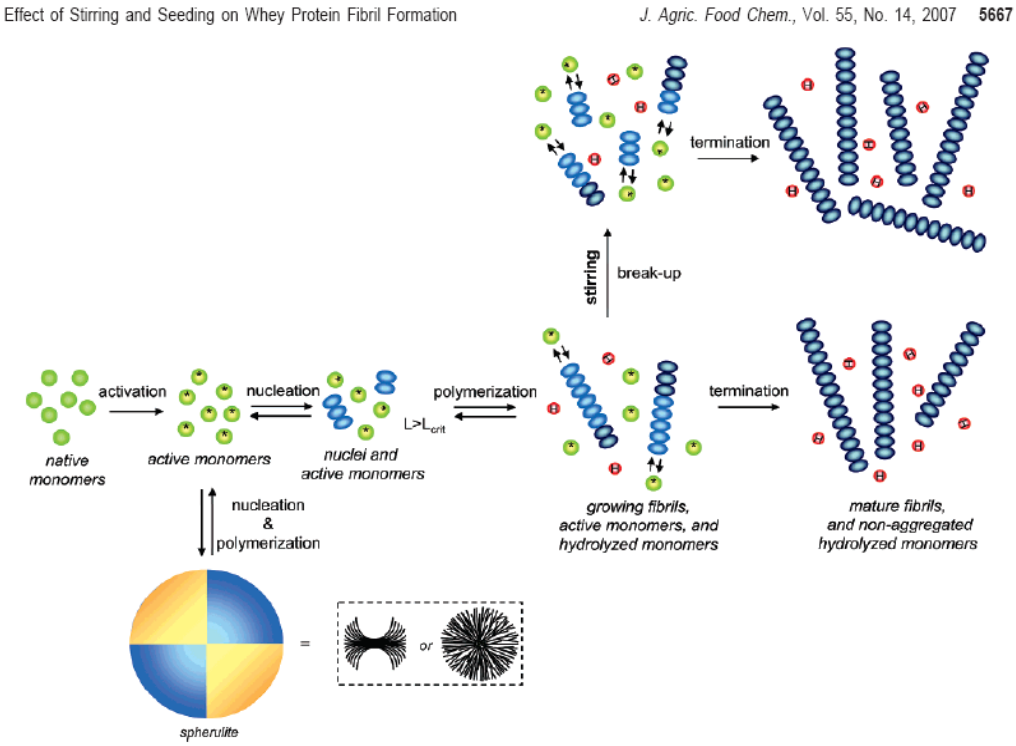

Figure 10. Schematic model for fibril formation at rest or while stirring during heating. Green circles represent protein monomers; activated protein monomers are indicated with an asterisk; reversibly aggregated monomers are represented by connected bright blue ovals; irreversibly connected monomers are represented by connected deep blue ovals; red circles with "H" indicate hydrolyzed monomers. The circle divided in quadrants represents a spherulite as observed with polarized light combined with a red compensator filter. (Note that structures are not drawn to scale.)

Figure 2.9. Schematic model for fibril formation at rest or while stirring during heating at pH 2 (Adapted from Bolder et al., 2007b)

2.10 GELATION OF β-LG VS. WHEY PROTEIN GELATION

Langton and Hermansson (1992) observed that the microstructure of whey protein concentrate gels showed similarity to β-lg gels at comparable pH. In addition, Langton and Hermansson (1992) also found that the microstructure correlated with data on fracture properties of these gels. Bolder et al. (2006) studied fibril assemblies in aqueous mixtures of whey proteins at pH 2.0 and suggested that β-lg is the only whey protein which forms fibrils at this pH. These fibrils were of the order of several micrometers with a diameter equivalent to that of a monomer as already described above. In Bolder et al., 2006, it was demonstrated that even in the presence of other whey proteins in small amounts, fibrils were formed. However, no fibril formation was observed when pure α-lactalbumin and bovine serum albumin were thermally treated at pH 2.0. In mixtures of whey proteins, spherulites (radially oriented fibrils) were found to coexist with fibrils. Changing the ratio of other whey proteins to β-lg did not change the protein concentration for gelation, purporting that β-lg was primarily responsible for gelation. In a study by Ikeda and Morris, 2002 the diameter of strands formed by β-lg at pH 2.0 was 4 nm compared to 10 nm diameter of strands formed by whey protein isolate at similar conditions. Again, this result suggested that WPI aggregates were not only composed of β-lg, but that other proteins might have changed the mechanism of aggregation. Bolder et al. (2006) reasoned that it was very unlikely at low ionic strength conditions that mixed fibrils were formed, as β-lg has a very different amino acid sequence

compared to other whey proteins. However at higher ionic strengths, aggregates may contain other proteins (Ikeda and Morris, 2002).

2.11 CRITICAL CONCENTRATION FOR GELATION

Critical concentration for gelation (C_0) is defined as the minimum concentration needed to form a gel under specific conditions (Renard et al., 1992). The pH, ionic strength, and time-temperature of heating are some factors that affect the critical concentration for gelation. Proteins acquire charge when away from their isoelectric point (pI). At the isoelectric point, the net charge on proteins is zero; above the pI, proteins acquire negative charge, and below the pI, proteins acquire positive charge. Therefore, at pH value away from the pI there is repulsion between proteins molecules. Renard and Lefebvre, 1992 developed a model to predict critical concentration for gelation at different pH values (2, 5, 6, 7, and 9) and ionic strengths (0-0.14 M NaCl). A minimum value for C_0 was found at the isoelectric point, and this value decreased with an increase in ionic strength except for pH 2.0. At this pH, electrostatic repulsion was very high and could not be screened until a certain ionic strength was achieved. Renard and Lefebvre, 1992 also found that gels formed at pH values far away from the pI were transparent. Increasing the ionic strength decreased transparency of these gels, indicating screening of double layer repulsions. Stading and Hermansson, 1992 also found a marked minimum in critical concentration for gelation near the pI for β-lg. Critical concentration for gelation increased as the pH moved away from the pI, decreasing with an increase in ionic strength.

2.12 REFERENCES

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CHAPTER 3.

COLD-SET THICKENING MECHANISM OF β-LACTOGLOBULIN AT LOW pH: CONCENTRATION EFFECTS

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3.1 ABSTRACT

There is an interest in developing protein based thickening agents for nutritional considerations. A procedure to convert whey protein concentrates or isolates into a pH modified cold thickening ingredient was developed. Concentration effects on thickening mechanism of this whey protein ingredient were studied with a β-lactoglobulin model system at the pH of the modification procedure, 3.35. In this study, concentration effects on thermal aggregation of β-lactoglobulin were studied at low pH using capillary and rotational viscometry, transmission electron microscopy (TEM), and high performance liquid chromatography coupled with multi-angle laser light scattering (HPLC-MALS). From the results of capillary viscometry, a critical concentration (C_c ~ 6.9 % w/w) was identified below which no significant thickening functionality could be achieved. Microscopy revealed formation of flexible fibrillar network at pH 3.35 during heating at all concentrations. These flexible fibrils had a diameter of about 5 nm and persistence length of about 35 nm as compared to more linear and stiff fibrils formed at pH 2 and low ionic strength conditions. Under similar heating conditions at concentration above C_c, larger aggregates similar to microgels were observed compared to the concentration below C_c, where isolated fibrils with an average contour length of about 130 nm were observed. These microgels and apparently stronger interactions between aggregates at concentrations above C_c were seemingly responsible for thickening functionality of heated β-lactoglobulin solutions and subsequently modified powders. Further investigation of β-lactoglobulin aggregation at this pH may provide capability to mechanistically tailor the functional attributes of modified ingredients.

3.2 INTRODUCTION

Beyond conveying nutritional benefits, whey proteins are also used as emulsifiers and foaming agents in food systems (Kinsella and Whitehead, 1989). There is an interest in developing protein based thickening agents for nutritional considerations (Hudson, Daubert and Foegeding, 2000). Thickening functionality of whey proteins is typically achieved with a thermal treatment. However, there has been an interest recently in formation of cold gelling whey protein ingredients for potential application in food systems where heat may not be desirable. Typically in cold-gelation studies, whey proteins are heated at neutral pH and low ionic strength, above their denaturation temperature and below their critical concentration for gelation, to obtain solutions of soluble protein aggregates. These solutions are then cooled, and cold gelation is induced with salt addition (Barbut and Foegeding, 1993; Hongsprabhas and Barbut, 1997; and Bryant and McClements, 2000) or by lowering the pH (Alting, Hamer, de Kruif and Visschers, 2000; Alting, de Jongh, Visschers and Simons, 2002; Alting, Hamer, de Kruif, Paques and Visschers, 2003; Alting et al., 2004). In a different approach, β-lactoglobulin solutions were heated at pH 2.0, and then following cooling, the pH was adjusted to 7 or 8 and finally $CaCl_2$ was applied for network formation (Veerman, Baptist, Sagis and van der Linden, 2003). This process resulted in a critical concentration for gelation that was an order of magnitude lower than traditional cold-gelation methods. Cold gelling ingredients at a slightly alkaline pH have also been described in powdered form which gels upon redispersion in a salt containing solution (Thomsen, 1994).

In previous studies, Hudson et al. (2000) developed a procedure allowing for the production of a cold thickening whey protein ingredient without any addition of salt or heat. This procedure involved pH adjustment to 3.35, thermal gelation, freeze drying, and finally grinding to a powder (Figure 3.1). The modified powders (Hudson et al., 2000) impart instant thickening capability upon reconstitution in water. Originally, this procedure was applied to whey protein isolate, but has also been shown to work with whey protein concentrates (Resch and Daubert, 2002; Resch, Daubert and Foegeding, 2004). The modified powders possess desirable functionality over a wide range of pH and thermal preparation conditions (Hudson and Daubert, 2002); however, the thickening mechanism remains unknown.

Mechanisms of whey protein, specifically β-lactoglobulin, aggregation have been extensively studied at pH 7 and pH 2, while limited studies have addressed aggregation mechanisms between pH 3 and pH 4 (Renard and Lefebvre, 1992; Sagis, Veerman, Ganzevles, Ramaekers, Bolder and van der Linden, 2002; Veerman, Sagis and van der Linden, 2002; Veerman et al., 2003; Schokker, Singh, Pinder and Creamer, 2000; Aymard, Durand and Nicolai, 1996; Langton and Hermansson, 1992; Elshereef, Budman, Moresoli and Legge, 2006; Aymard, Nicolai and Durand, 1999; Sakurai, Oobatake and Goto, 2001; Castelletto and Hamley, 2007; Wada, Fujita and Kitabatake, 2006; Renard, Lefebvre, Griffin and Griffin, 1998; Ikeda and Morris, 2002; Arnaudov, de Vries, Ippel and van Mierlo, 2003; and Kavanagh, Clark and Ross-Murphy, 2000).

Depending on the pH and ionic strength, different types of aggregates are formed by β-lactoglobulin which includes fibrils, flexible strands, branched and random aggregates (Bolder, Hendrickx, Sagis and van der Linden, 2006). Large random aggregates are formed at pH values close to the isoelectric point and at high ionic strength conditions (Aymard et al., 1996; Langton and Hermansson, 1992; and Sagis et al., 2002). At pH values far from the isoelectric point and at low ionic strength, strand-like aggregates are formed (Aymard et al., 1999; Kavanagh et al., 2000; and Langton and Hermansson, 1992, Stading and Hermansson, 1990). These strand-like aggregates can be rigid (fibrils), semi-flexible, or branched (Sagis et al., 2002).

At pH 2.0 and low ionic strength, long rigid strands (fibrils) are formed by β-lactoglobulin after heating (Veerman et al., 2002, 2003; Ikeda and Morris, 2002; and Kavanagh et al., 2000). At pH 2, β-lactoglobulin is highly charged (+20), forming fibrillar aggregates with diameter equivalent to that of a monomer (2-4 nm) (Veerman et al., 2002, 2003; Ikeda and Morris, 2002; and Kavanagh et al., 2000). Flexibility of these fibrils was shown to vary with ionic strength. Persistence length (length over which correlation in the direction of tangent are lost or simply the length that persists in a particular direction) is frequently used to quantify the flexibility/rigidity of polymer chains and biomacromolecules. Flexibility of fibrils increased (indicated by a decrease in persistence length) and contour length (curvilinear length of fibrils) decreased with increasing ionic strength (Veerman et al., 2002; Aymard et al., 1999; and Kavanagh et al., 2000).

The motivation for this study was to investigate basic mechanisms behind thickening functionality of cold thickening whey protein ingredients at low pH in hopes of achieving capability to mechanistically tailor functional attributes of modified whey protein ingredients. The objective of this study was to investigate concentration effects on the aggregation mechanism of β-lactoglobulin at pH 3.35 and thickening functionality of modified whey protein powders. In this study, concentration effects were investigated using capillary viscometry, rotational viscometry, transmission electron microscopy (TEM), and High performance liquid chromatography – Multi angle laser light scattering (HPLC-MALS).

3.3 MATERIALS AND METHODS

3.3.1 Protein material

β-lactoglobulin (BioPure^{R}, ~ 94 % pure, total protein ~ 98 % dry basis) was donated by Davisco Foods Inc.

3.3.2 Solution preparation

β-Lactoglobulin solutions of different concentrations (2-9 w/w %) were prepared by dissolving β-lactoglobulin in de-ionized (DI) water by continuous stirring at room temperature for 1-2 hours. Sodium azide (0.02 %) was added to all samples to prevent

microbial growth. Thereafter, solutions were adjusted to pH 3.35 using 6 N HCl. Following pH adjustment, solutions were heated at 85 °C for 3 hours in a water bath. Heated solutions were cooled and stored in a 4 °C refrigerator.

3.3.3 Shear rate sweeps

Shear rate sweeps were performed on all solutions at 25 °C using a stress controlled rheometer (ATS Rheosystems, Bordentown, NJ) to characterize flow behavior and viscosities of solutions. A smooth cylindrical cup and bob (cc 25) was used, and shear rates were varied from 10 to 100 s^{-1} using a constant rate program to minimize inertial effects on the viscosity of solutions. Depending on the number of data points desired, shear rates were varied over a time period of 600-900 s. The presence of hysteresis was diagnosed by two runs of increasing and then decreasing shear rates on β-lactoglobulin solutions. A pre-shear condition (15 s^{-1} for 30 s) was applied to all solutions to obtain uniform solutions prior to measurements. A thin film of mineral oil was applied to the sample surface to minimize sample dehydration. To characterize flow behavior power law model parameters n (power law index) and K (consistency coefficient) were determined. Equation one describes power-law model used to characterize shear thinning (pseudo-plastic) flow behavior.

$$\eta = K\dot{\gamma}^{n-1} \quad Eq.\ 1$$

3.3.4 Concentration and dilution effects on heated solutions

Heated β-lactoglobulin solutions of varying concentrations (2, 3, 4, 5, 6, 7, and 8 % w/w), pH 3.35 were prepared. After heating, an 8 % w/w β-lactoglobulin solution was diluted back to 4 % w/w and 2 % w/w to determine the effect of dilution. All solutions were then stored at 4 °C, and shear rate sweeps were performed on all samples at 25 °C. Two independent replicates were performed and power law parameters were determined.

3.3.5 Effect of longer heating at 4 % and 8 % w/w β-lactoglobulin

β-Lactoglobulin solutions at 4 % w/w were heated at 85 °C for 3 hours and 10 hours and 8 % w/w solutions were heated for 3 hours and 6 hours and shear rate sweeps were performed on all solutions at 25 °C. Two independent replicates were performed and power law parameters were determined.

3.3.6 Concentration effects using capillary viscometry

Heated solutions of β-lactoglobulin at varying concentration (2 to 9 % w/w), pH 3.35 were prepared. Cannon-Fenske capillary viscometers were used to determine specific viscosity. A plot of specific viscosity vs. protein concentration was used to identify a critical concentration, dictating a transition from dilute to a concentrated regime of β-lactoglobulin aggregates. Equations were fitted to data such that sum of square error was minimized and

coefficient of determination (R^2) was maximized. For comparison purposes, a similar plot was obtained for unheated solutions. Two independent replicates were performed of this experiment.

3.3.7 Preparation of modified β-lactoglobulin powders

Heated β-lactoglobulin solutions of different concentrations (3-9 %), pH 3.35 were prepared. The next day, sols/gels were frozen using methanol and dry ice, then freeze dried using a 4.5 L bench top freeze dryer (Labconco 73035, Kansas City, Mo), and finally ground to a powder.

3.3.8 Thickening functionality comparisons on modified powders

Heated solutions of two concentrations below (3 and 5 % w/w) and above (7 and 9 % w/w) the critical concentration (6.9 % w/w) at pH 3.35 were made using β-lactoglobulin and modified powders were prepared as described above. These powders were then hydrated in deionized water at 10 % (w/w) and 5 % (w/w). Finally, shear rate sweeps (1 to 100 s^{-1}) were performed at 25 °C to evaluate viscosity and power law model parameters.

3.3.9 Transmission electron microscopy (TEM)

Heated β-lactoglobulin solutions at pH 3.35 were prepared. All samples were diluted with DI water to a concentration of 0.04 % and pH was adjusted to 3.35 using 6 N HCl prior to TEM analysis. TEM samples were prepared by negative staining. A drop of diluted sample was deposited on a copper grid (formvar coated and further coated with evaporated carbon) and the excess sample was removed using filter paper. A drop of 2 % uranyl acetate, pH 3.5, was added for 60 s and excess sample was removed. Electron micrographs were obtained using a FEI/Phillips EM 208S Transmission electron microscope (made by Phillips, Czech Republic). For comparison purposes, a 1.5 % w/w solution of β-lactoglobulin at pH 2.0 was heated at 80 °C for 10 h to obtain linear fibrils as reported by Aymard et al. (1999) and Veerman et al. (2002).

3.3.10 Determination of persistence length and contour length

Negatives of TEM images were digitized and analyzed using Image Pro Plus v 6.0 for determination of persistence lengths (l_p) and contour lengths (l_c). Four to five fibrils were randomly selected from different quadrants of TEM micrographs. Contours of the fibrils were digitized using Image Pro Plus into several small segments such that ($l << l_c$) (Figure 3.2). Contour length (l_c) was determined by summation of all segment lengths and a persistence length was determined by the correlation of bond angles (Equation 1) along the contour (Cantor, and Schimmel, 1980; Cifra, 2004).

$$l_p = < l > /(1 - < \cos\phi >) \quad Eq.\ 2$$

Where, <l> is the average segment length and ϕ is the angle between segments. The <cos ϕ> is an average correlation between the segments. Illustration of the methodology is shown in Figure 3.2.

3.3.11 High performance liquid chromatography-Multi-angle laser light scattering (HPLC-MALS)

Heated and unheated solutions of β-lactoglobulin at concentrations (3, 4, and 8 %) were prepared in 40 mM sodium citrate/citric acid buffer at pH 3.35. Both heated and unheated samples were diluted to 5 mg/ml final concentration and filtered through a 0.45 micron filter. The Bicinchoninic acid (BCA) assay was performed prior and after filtration to determine protein loss during filtration. Two replicates were performed.

The HPLC –MALS was calibrated with 200 µl of buffer and 100 µl of BSA standard (5 mg/ml) and data were normalized based on BSA. A gel filtration column (Shodex, KW-804) was linked to a photodiode detector (Waters 2996) coupled with refractive index detector (Waters 2414) and MALS detector (Waters Technology, Dawn EOS Enhanced Optical Systems) were used to determine molar mass and concentration distribution of the samples. Twentyfive µl of heated samples and 75 µl of unheated samples at 5 mg/ml concentrations were injected onto the column.

3.4 RESULTS AND DISCUSSION

3.4.1 Concentration and dilution effects on heated solutions

β-Lactoglobulin solutions at low concentrations (2-7 % w/w) heated for 3 hours at pH 3.35 behaved like a Newtonian fluid (n ~ 1.0). However, pseudoplasticity increased rapidly with increasing concentration above 7 % as determined by the values of power law indices (n ~ 0.97 at 8 % w/w and 0.2 at 9 % w/w (data not shown to emphasize differences observed at low concentrations since viscosity of this solution was about two logs higher than 8 %) (Figure 3.3A). These results expectedly showed that solutions heated at lower concentrations (< 7%) displayed pseudoplasticity to a lesser degree and behaved Newtonian like as compared to solutions heated at higher concentrations. There was no hysteresis up to 8 % w/w. However, at 9 % w/w a very viscous solution was formed possibly due to formation of a more connected network, and some hysteresis was observed at 9 % w/w which decreased in the second run significantly indicating some breakage of network bonds formed at 9 %. Pseudoplasticity indicates decrease in viscosity with increase in shear rate which could be due to alignment of molecules/aggregates or breaking up of entanglements among aggregates or because of both. Pseudoplasticity was observed at concentrations higher than 7 %, these results suggested that at concentrations above 7 % significant interactions exist among aggregates formed, and their strength increases with concentration rapidly as inter-particle distance decreases eventually leading to formation of a continuous network or a gel. Another possible explanation is that larger aggregates are better aligned with the flow as compared to smaller aggregates and molecules. No hysteresis was observed up to 8 % which indicates that

changes in viscosity were reversible, suggesting that stronger interactions among aggregates and better alignment of large aggregates were mostly responsible for pseudoplasticity, while at 9 % w/w some breakage of network may have been responsible for hysteresis.

Apparent viscosities of heated solutions increased with concentration, however a sudden increase (~ 40 %) in apparent viscosities of samples was observed when concentration was increased from 6 to 7 % and even a more pronounced increase (~ 80 %) was observed when concentration was increased from 7 % to 8 % w/w (Figure 3.3A). These results suggested the presence of a concentration effect, with critical concentration somewhere between 6 and 7 %, which was later characterized by capillary viscometry to be 6.9 % w/w.

To determine whether the increases in apparent viscosity were reversible upon dilution, a heated 8 % solution was diluted back to 4 % and the results were compared. The viscosity of a solution heated at 8 % and diluted to 4 % was significantly greater (~ 50 %, even greater than the viscosity of a 6 % solution) than when heated directly at 4 % (Figure 3.3A). Similar results were obtained for a solution heated at 8 % and diluted to 2 % (data not shown). These results substantiated that aggregates formed upon heating were irreversible upon dilution.

3.4.2 Effect of longer heating at 4 % and 8 % w/w

To determine whether β-lactoglobulin solutions at low concentration (< 6.9 % w/w) when heated longer would attain similar viscosity compared to those heated at higher

concentration, 4 % solutions were heated for 3 hours and 10 hours respectively while 8 % solutions were heated for 3 hours and 6 hours. The apparent viscosity of a 4 % solution heated for 10 h did not catch up the viscosity of an 8 % solution heated for 3 hours (Figure 3.3B). Apparent viscosities of 4 % solutions heated for 3 and 10 hours were comparable and significantly lower than the viscosity of an 8 % solution heated for 3 hour. On the other hand, 8 % solution when heated for 6 hour had a two log increase in apparent viscosities as compared to the 8 % solution heated for 3h (Figure 3.3B). Also, pseudoplasticity of the 8 % solution increased with time of heating ($n \sim 0.3$ at 6 h of heating) indicating formation of larger aggregates and possibly stronger interactions among these aggregates. These results confirmed the presence of concentration effects. It has been reported in literature that polymers must be able to form entanglements to display strong concentration dependence (Barnes et al., 1989).

3.4.3 Identification of a critical concentration

In polymer rheology, a plot of log zero shear viscosity with log molecular weight often results in two straight lines with a slope of 1 for low molecular weights and slope of 3.4 for high molecular weights separated by a critical molecular weight (M_c) (Ferry, 1980 cited in Barnes, Hutton & Walter, 1989 and Rao, M. A., 1989). It has been reported that molecules containing few monomeric units ($M < M_c$) are indistinguishable from small molecule liquids. This sudden increase in slope values above M_c has been attributed to formation of entanglements among polymers with many monomeric units. Similarly at lower

concentrations there is no strong interaction between polymer molecules and thus in this regime physical properties change in direct proportion to the concentration and as concentration is increased there may be a change to a regime in which viscosity varies as c^3 or even higher powers (Barnes et al., 1989). However, the details of this transition depend on several factors including molecular weight distribution, chain branching, polarity and chain rigidity. It has been reported that polymers must be able to form entanglements to display strong concentration dependence (Barnes et al., 1989). A plot of log specific viscosity and log coil parameter (concentration multiplied with intrinsic viscosity) is also used for several hydrocolloids to dictate a transition from dilute to semi-dilute regime when polymer chain entanglements take place (Rao, M. A., 1999). We used a plot of log specific viscosity with log concentration to investigate concentration effects.

The plot of log specific viscosity vs. log concentration for unheated solutions was linear across all concentrations studied while two linear regimes with different slopes were observed for heated solutions (Figure 3.4). A single linear regime was obtained across all concentrations of unheated solutions, revealing a consistent increase in specific viscosity with concentration. A slope value of 1.2 (R^2 = 1) was obtained indicating a proportional increase in viscosity with concentration for unheated solutions.

For heated β-lactoglobulin solutions, two linear regimes with different slopes were observed, separated by a concentration, denoted as the critical concentration (C_c) (Figure 3.4). These results indicated the formation of aggregates upon heating, and a transition from dilute to

more concentrated regime at a critical concentration, determined to be 6.9 %. The slope of the first linear regime for heated solutions (2.0, $R^2 = 0.97$) was higher than the slope of the linear zone that encompassed all concentrations of unheated β-lactoglobulin solutions (1.2), suggesting a higher rate of viscosity increase with concentration. A plausible explanation is that during heating, denaturation takes place and some small aggregates are formed even at low protein concentrations, resulting in an increased viscosity compared with unheated solutions at similar concentrations. For concentrations higher than the identified critical concentration (~ 6.9 %), the slope value was 12.6 ($R^2 = 0.99$), indicating formation of large aggregates and stronger interactions among these aggregates because of small inter-particle distances. These large aggregates were later characterized as 'microgels' from TEM results.

3.4.4 Concentration effects on thickening functionality of modified powders

Significant differences in the functionality of modified protein powders made from protein concentrations below and above a critical concentration were observed, even when reconstituted at similar protein concentrations (Figure 3.5A). Apparent viscosities of 10 % solutions at a shear rate of ≈ 10 s^{-1} were found to be 3.3, 4.0, 32.0 and 3950 mPa-s for powders made from 3, 5, 7, and 9 % initial concentrations, respectively. These results suggested that different size aggregates might have formed at different concentrations during thermal treatment, and these aggregates persisted in powders with larger aggregates/networks contributing greater viscosity upon reconstitution at similar weight percentages. Visual inspection of these solutions showed that turbidity of reconstituted powders increased with

increasing initial protein concentration. Differences in thickening functionality of modified powders prepared below and above a critical concentration were consistently seen when reconstituted at lower concentrations (5 % w/w) (Figure 3.5B). Greater degree of pseudoplasticity (lower value of n) was observed in dispersions made from concentrations above C_c than those prepared below C_c for Figures 3.5A and 3.5B.

3.4.5 Structures of aggregates formed at different concentrations (TEM)

TEM images of heated 4 % and 8 % β-lactoglobulin solution are shown in Figures 3.6A and 6B respectively. From these images, both concentrations formed fine stranded (flexible fibrillar) networks, and the diameters of these strands were measured to be about 5 nm. At 4 % concentration (< critical concentration), smaller size aggregates (flexible fibrils) were observed as compared to the 8 % concentration (> critical concentration) where larger aggregates similar to microgels appeared (Figure 3.6B). In polymer science, these microgels are defined as intramolecularly cross-linked macromolecules and are considered as intermediate between branched and macroscopically cross-linked systems (Murray and Snowden, 1995). Microgels can also be thought of as precursors or intermediates to macroscopic gelation (Graham and Cameron, 1998; and Murray and Snowden, 1995). At 8 % concentration, microgels appeared to consist of multiple flexible strands; while at 4 %, majority of smaller aggregates appeared as individual strands with an average contour length of about 130 nm. Aymard et al. (1999) reported that at pH 2, with increasing protein concentration, larger aggregates were formed.

The average value of persistence length of fibrils formed at 4 % and 8 % were 36 +/- 12 (one standard deviation) nm and 34 +/- 9 (1 standard deviation) nm respectively. While at both concentrations similar values of persistence length were found attributed to low ionic strength in both cases, microgels were formed at higher concentration. The possible explanation is that the rate of aggregation increases at higher concentration leading to formation of network structures similar to microgels. Another possible explanation may be that at higher concentrations more flexible strands (flexible fibrils) are formed at pH 3.35. Reversible aggregation of fibrils followed by a slow strengthening step leading to irreversibility at pH 2 and low ionic strength has been suggested by Arnaudov et al. (2003) and Bolder, Sagis, Venema and van der Linden (2007). Arnaudov et al. also found from atomic force microscopy results that large aggregates were formed at with increasing protein concentration from 1 to 3 % at pH 2.0.

These results support that larger aggregates and possibly stronger colloidal interactions between these aggregates contributed to higher viscosities of solutions at concentrations above the critical concentration. Also evident from the dilution experiment (Figure 3.3B), these aggregates were not reversible when more solvent was added. In these studies, no salt was added and approximate ionic strengths were calculated based on mineral analysis to be between 15-30 mM. The values of persistence length obtained at pH 3.35 in this study indicated that a more flexible fibrillar network was formed at this pH compared to pH 2.0. From the TEM micrograph in Figure 3.6C, average persistence length of fibrils was determined to be 788 nm with contour length up to 2.5 μm. Fibrils formed at pH 2.0 are very

linear with diameters of about 4 nm (Veerman et al., 2002). The persistence length of fibrils determined at pH 2.0 fits well with those reported in literature at low ionic strength conditions. Aymard et al. (1999) reported a persistence length of 600 nm at pH 2.0 and low ionic strength from the light scattering studies, while persistence length of up to 1 μm was reported by Veerman et al. (2002) at low ionic strength conditions (< 0.05 M) at pH 2.0. Persistence length values and experimental condition from this study and literature are summarized in Table 3.1.

A fine stranded network was reported at pH 3.5 and 12 % w/w β-lactoglobulin by Langton and Hermansson (1992), who studied network characteristics of β-lactoglobulin gels formed at 12 % w/w without any added salt reported formation of fine stranded networks at pH < 4. A regular and dense network at pH 3.5 with diameter of strands < 5 nm was observed and at pH 4.0, particulated areas were observed which resulted in a more irregular network compared to that at pH 3.5 (Langton and Hermansson, 1992). As pH approaches the isoelectric point, networks tend to be more particulated in nature. At pH far away from isoelectric point, β-lactogobulin is highly charged, experiencing high repulsion under conditions of low ionic strength resulting in fine stranded networks. Flexibility and length of these strands in fine stranded networks depend on pH, ionic strength and heating conditions (Sagis et al., 2002; and Veerman et al., 2002). Flexibility of these fibrils increases as ionic strength increases at pH 2, but the mechanism behind it remains unclear. It has been suggested that large persistence length at low ionic strength may be because of both electrostatic interactions and specific binding between the proteins such as formation of

intermolecular hydrogen bonding β-sheet structures (Aymard et. al., 1999). At pH 3.35, proteins are relatively less charged, and thus encounter less electrostatic repulsion as compared to pH 2.0. Thus a more flexible type network is formed at pH 3.35 than at pH 2.0 but the mechanism remains largely unknown. Discontinuous networks were observed at both concentrations because both conditions did not gel upon heating, while aggregates similar to microgels were observed at 8 % w/w (Figure 3.6B).

3.4.6 HPLC-MALS

HPLC coupled with MALS was used to quantify the molar mass and sizes of the β-lactoglobulin aggregates formed at different concentrations during heating and compared with unheated solutions at similar concentrations. From the results of BCA assays, approximately 95 % or more of protein passed through a 0.45 μm filter for heated and unheated samples. In unheated samples, a majority of protein (Figure 3.7A and 3.7B) was found to be a mixture of monomers and dimers evident from the peak observed at 18 minutes in chromatograms also confirmed by SDS-PAGE (data not shown). This peak corresponded to a molar mass of (~ 26.4 kDa) for all unheated samples, which is intermediate between a monomer (18.6 kDa) and a dimer (~37 kDa). Aymard et al. (1996), based on light scattering studies at pH 2 and 7, suggested that β-lactoglobulin exists as a mixture of monomer and dimer at all conditions with monomer-dimer equilibrium shifting towards monomers with lowering of pH. For unheated samples (Figure 3.7A and 3.7B), it was noted that while a majority of protein was found to be in monomeric/dimeric form, small amounts of protein (<

10 %) eluted near 16 min, corresponding to a molar mass of approximately 150 kDa. A band of protein with similar molecular weight was observed from electrophoresis of unheated proteins and was absent in presence of β-mercaptoethanol (Data not Shown). From the cumulative molar mass plot (Figure 3.8A), all unheated samples displayed a similar molar mass distribution.

The concentration of monomeric-dimeric β-lactoglobulin was decreased in all heated samples and aggregate formation occurred. Heated samples at concentrations 3 and 4 % displayed similar distributions of molar masses and root mean square (RMS) radius when compared with the 8 % heated samples (Figure 3.8A and 3.8B). For 3 and 4 % heated samples, a majority of protein (~ 78 %) was found in the peak eluting near 18 min, similar to that of unheated samples (Figure 3.7A and 3.7B). While for the 8 % heated sample, approximately 55 % of the protein was found in the low molecular weight form. For all heated samples, a distribution of molar masses was obtained (Figure 3.8A and 3.8B). However, the formation of large aggregates was more evident in 8 % heated sample as can be observed from the chromatogram (Figure 3.7B) and cumulative molar mass and root mean square radius plots in Figure 3.8. From the results for the 8 % heated sample, it appeared that a majority of protein either existed in very low molecular weight form monomer-dimer or as large aggregates with corresponding high molar masses. These results are in accordance with our TEM results and critical concentration results, where formation of large aggregates was seen at 8 % compared to 4 % heated samples. Also, below the critical concentration, no significant thickening functionality could be achieved, as a majority of the protein existed in low molecular weight

form, and aggregates formed are smaller in size compared to the 8 % heated samples. Formation of large sized aggregates contributed greatly to thickening functionality of these solutions and subsequently modified powders. Apparently, conversion of monomer/dimers to aggregates during heating increased with increasing protein concentration. Increased conversion with concentration has also been previously reported at pH 2 (Veerman et al., 2002). Also, a bimodal distribution of aggregates was observed for heated samples with very small amount of intermediates (Figures 3.7 and 3.8). Similar to our results, the presence of a bimodal distribution of aggregates at pH 2 has been reported (Arnaudov et al., 2003, Schokker et al., 2000).

3.5 CONCLUSIONS

Concentration dependent differences were observed in thickening functionality of modified ingredient made from β-lactoglobulin, and a critical concentration was identified below which no significant thickening functionality (cold-gelling) could be achieved in β-lactoglobulin dispersions. It was found that at pH 3.35 flexible fibrilar networks were formed with diameter of strands ~ 5 nm and persistence length of about 35 nm. Differences in network structures below and above a critical concentration were observed through TEM, with larger aggregates similar to microgels being formed at higher concentration. From HPLC-MALS and TEM results, it was found that the conversion of monomer-dimers to large aggregates upon heating increased with increasing protein concentration, especially above the identified critical concentration in this case. Large sized aggregates or microgels are

formed at higher concentration and possibly stronger interactions exist among these aggregates because of small inter-particle distance and concentration effects which contributed greatly to the thickening functionality of heated solutions and subsequently modified powders. Further understanding of ionic strength and kinetic effects may allow us to effectively tailor the original modification process and lead to production of modified cold-thickening dairy ingredients with expanded utility and functionality.

3.6 REFERENCES

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TABLES

Table 3.1. Persistence length and contour length of fibrils formed at pH 3.35 (flexible) as compared to the fibrils formed at pH 2.0 (rigid) at low ionic strength conditions

Study	**Material**	**Conditions**	**Ionic Strength M**	**Persistence Length l_p (nm)**	**Contour Length l_c**
Aymard,* 1999	β-lactoglobulin (Sigma) L-0130	0.5-2.2 %, pH 2.0, 80 °C	0.013	600	-
Veerman, 2002	β-lactoglobulin (Sigma) L-0130	1.5 %, pH 2.0, 80 °C-10h	< 0.05	1000	> 2 μm
This Study	β-lactoglobulin (Davisco)	1.5 %, pH 2.0, 80 °C-10h	Low < 0.05	788	~ 2.5 μm
This Study	β-lactoglobulin (Davisco)	4 %, pH 3.35, 85 °C-3h	Low < 0.05	36	~ 130 nm
This Study	β-lactoglobulin (Davisco)	8 %, pH 3.35, 85 °C-3h	Low < 0.05	34	~300 nm

* Aymard, 1999 used light scattering data to determine the persistence length

FIGURES

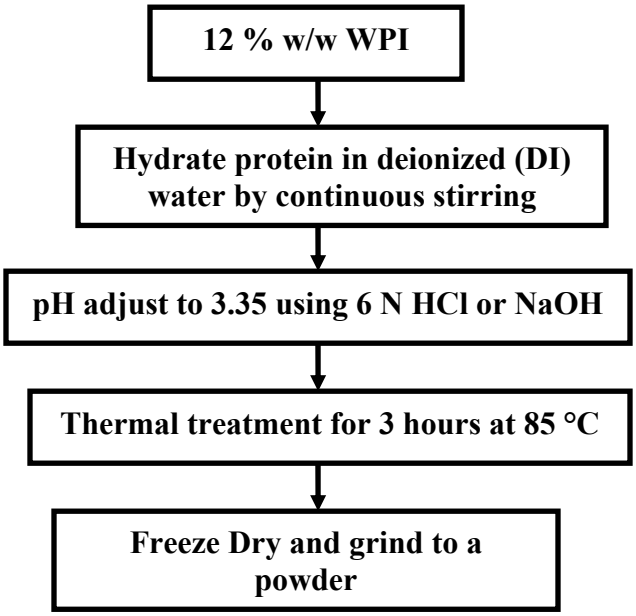

Figure 3.1. Original modification procedure according to Hudson et al., 2000

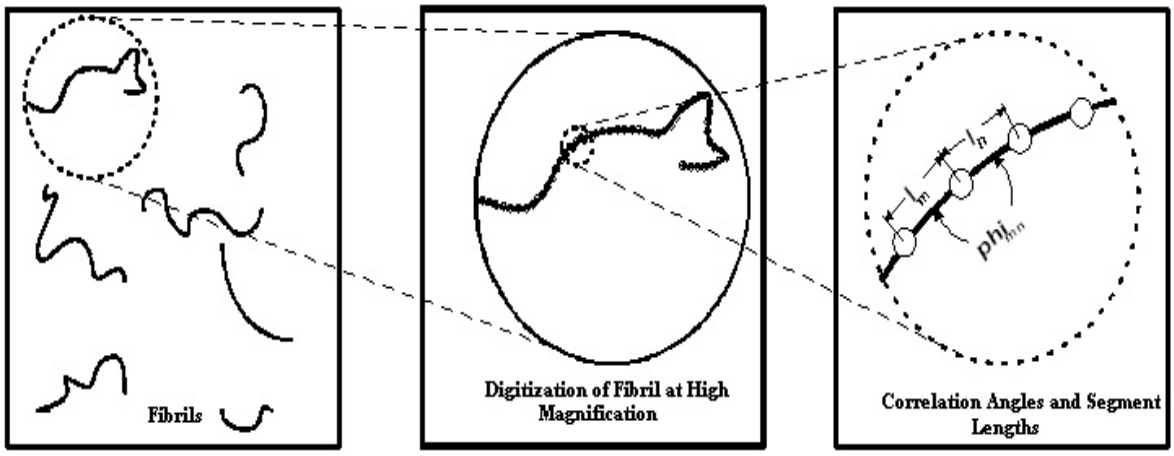

Figure 3.2. Steps in the determination of persistence length through calculations of correlation angles between segments formed by successively digitized points along the fibril contour at high magnification

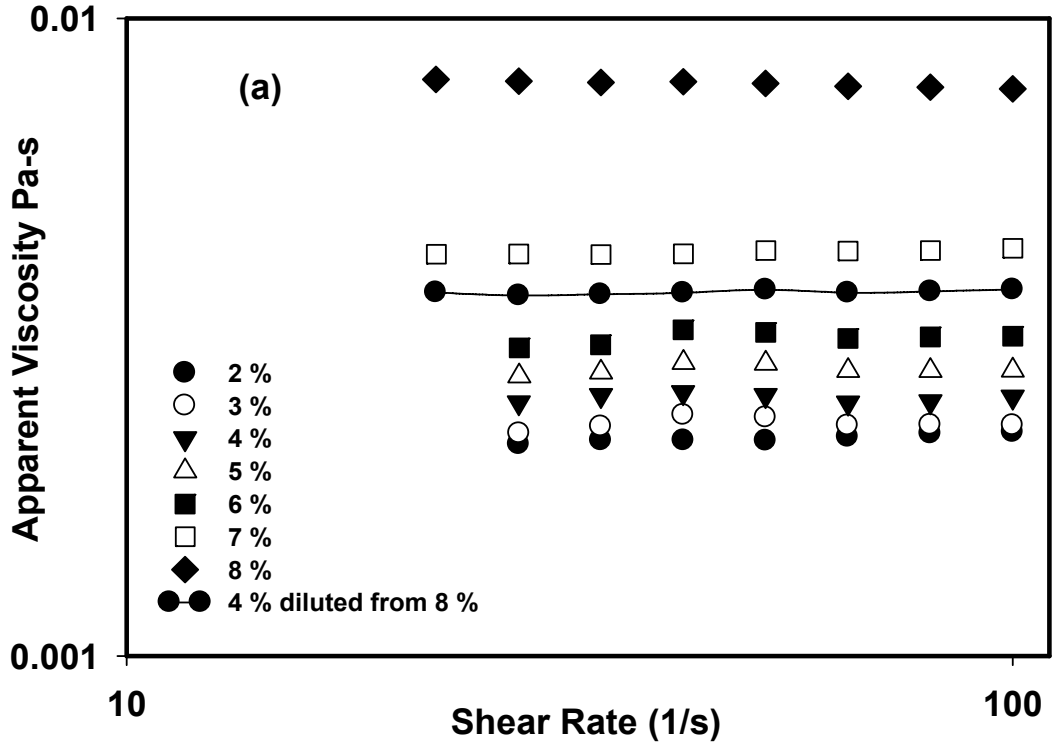

Figure 3.3A. Concentration and dilution effects on apparent viscosity of heated (85 °C-3h) β-lactoglobulin solutions, pH 3.35 at 25 °C

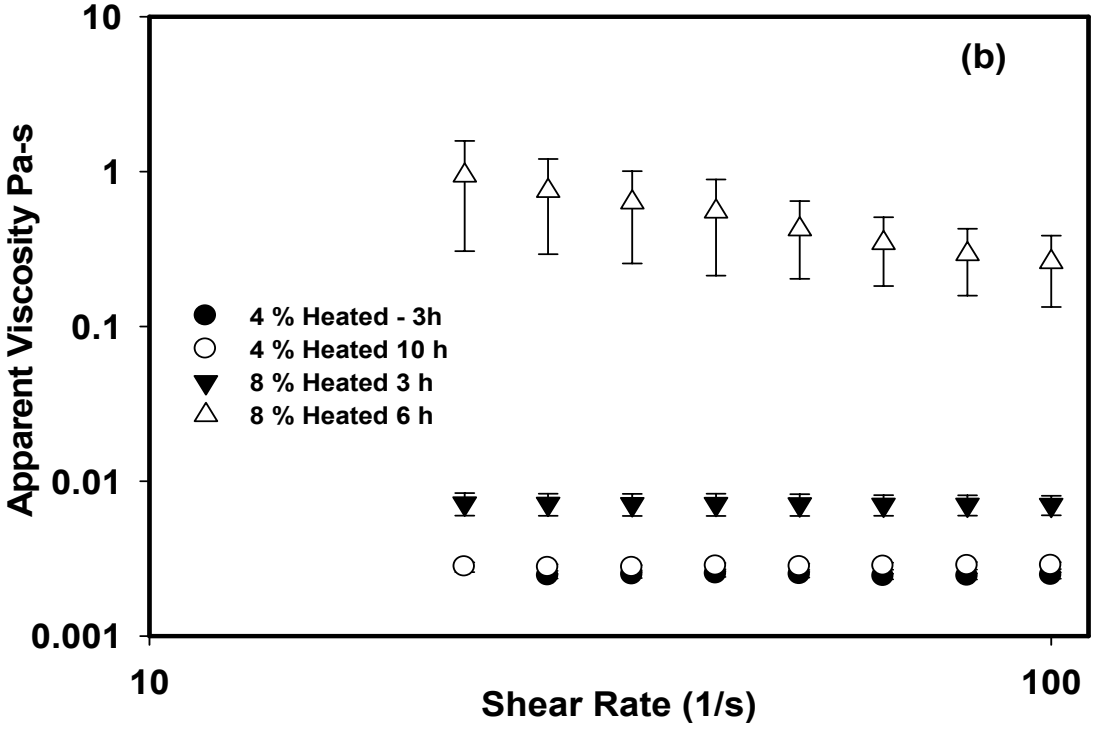

Figure 3.3B. Effects of longer heating (85 °C) above and below critical concentration (C_c) at 25 °C, pH 3.35

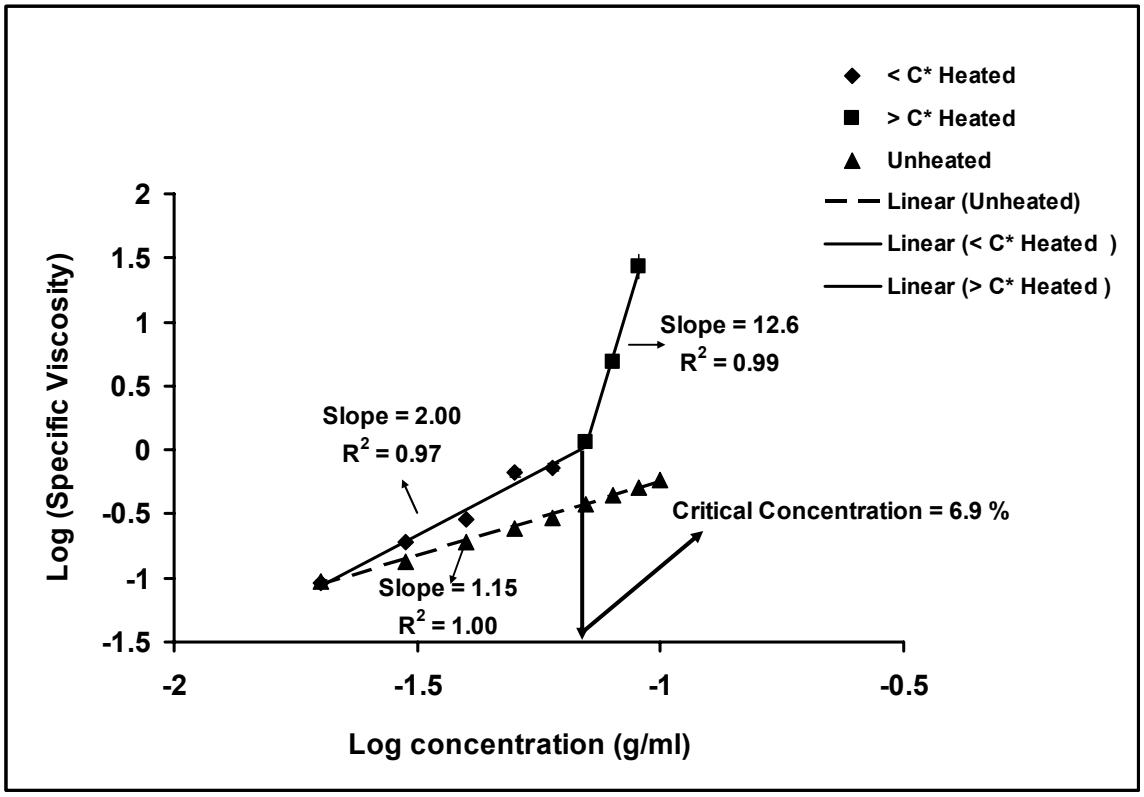

Figure 3.4. Critical concentration for heated β-lactoglobulin solutions (85°C for 3 h), pH 3.35. Dotted line represents unheated solutions at pH 3.35. Viscosity measurements were taken at 25 °C.

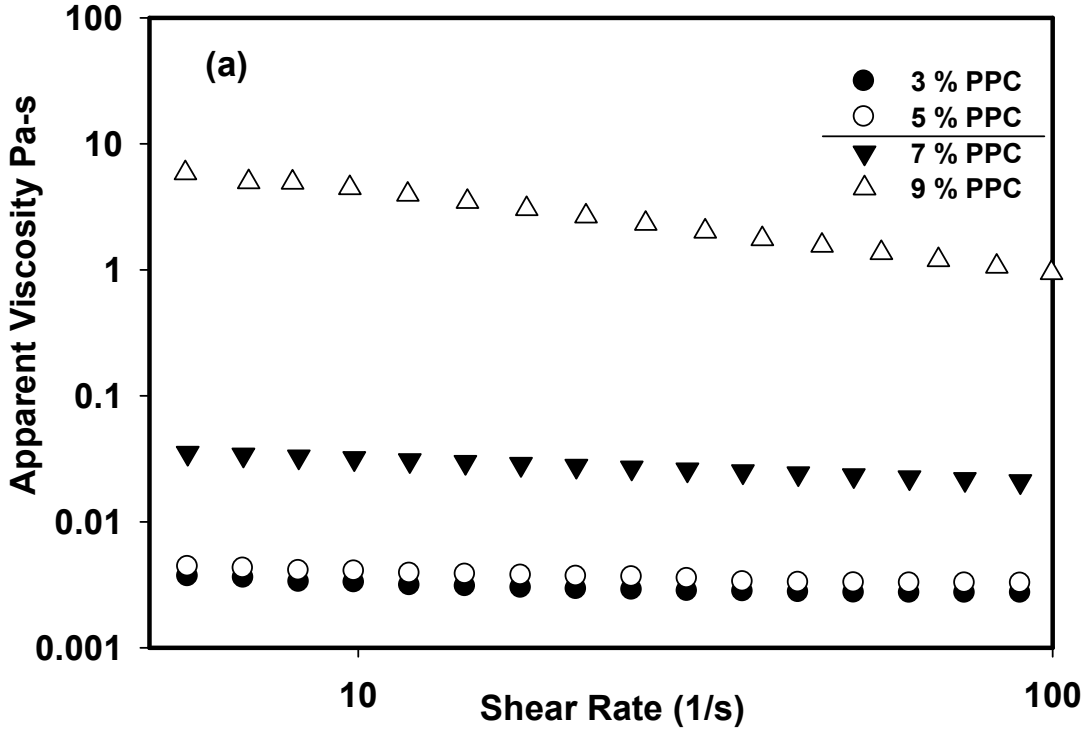

Figure 3.5A. Effect of preliminary protein concentration (PPC) on apparent viscosities of reconstituted modified powders at 10 % w/w at 25 °C

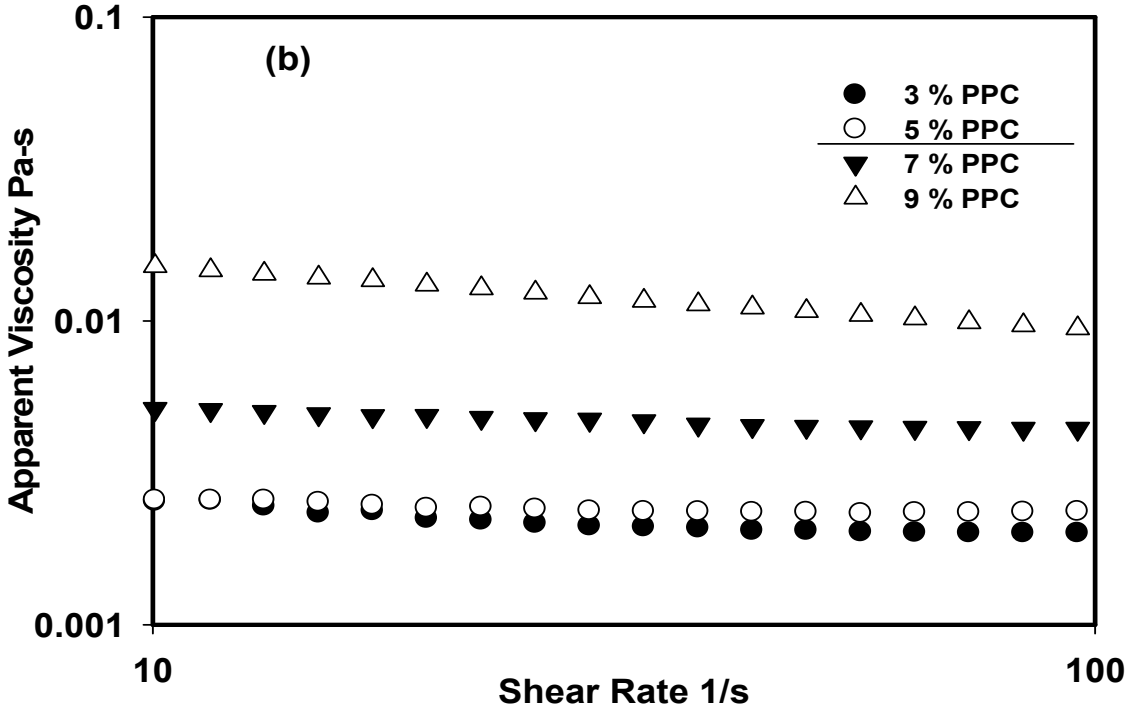

Figure 3.5B. Effect of preliminary protein concentration (PPC) on apparent viscosities of reconstituted modified powders at 5 % w/w at 25 °C.

Figure 3.6A. TEM micrograph of heated (85 °C -3h) 4 % w/w β-lactoglobulin solution at pH 3.35. Magnification bar represents 300 nm**.**

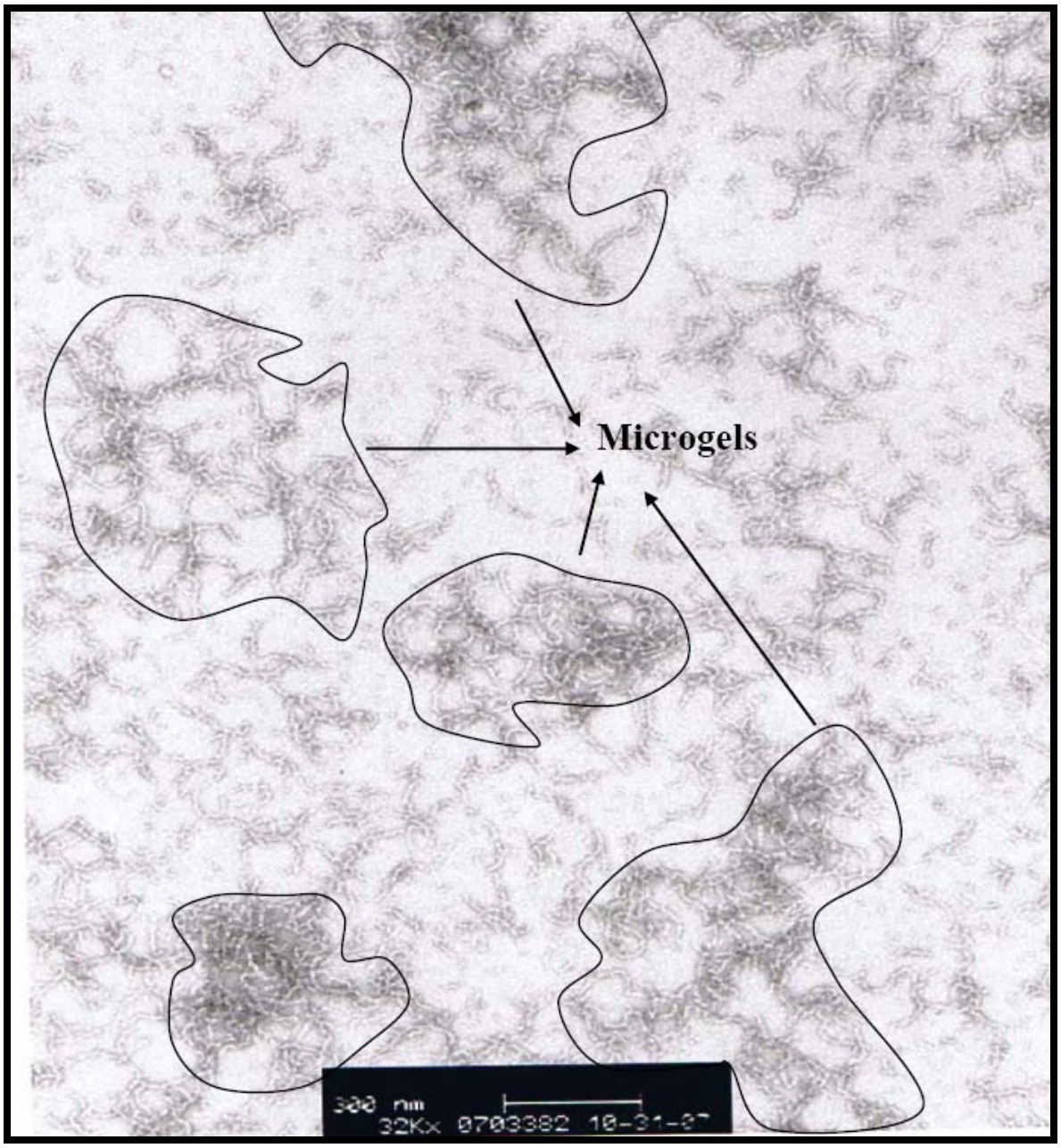

Figure 3.6B. TEM micrograph of a heated (85 °C – 3h) 8 % w/w β-lactoglobulin solution at pH 3.35. Magnification bar represents 300 nm.

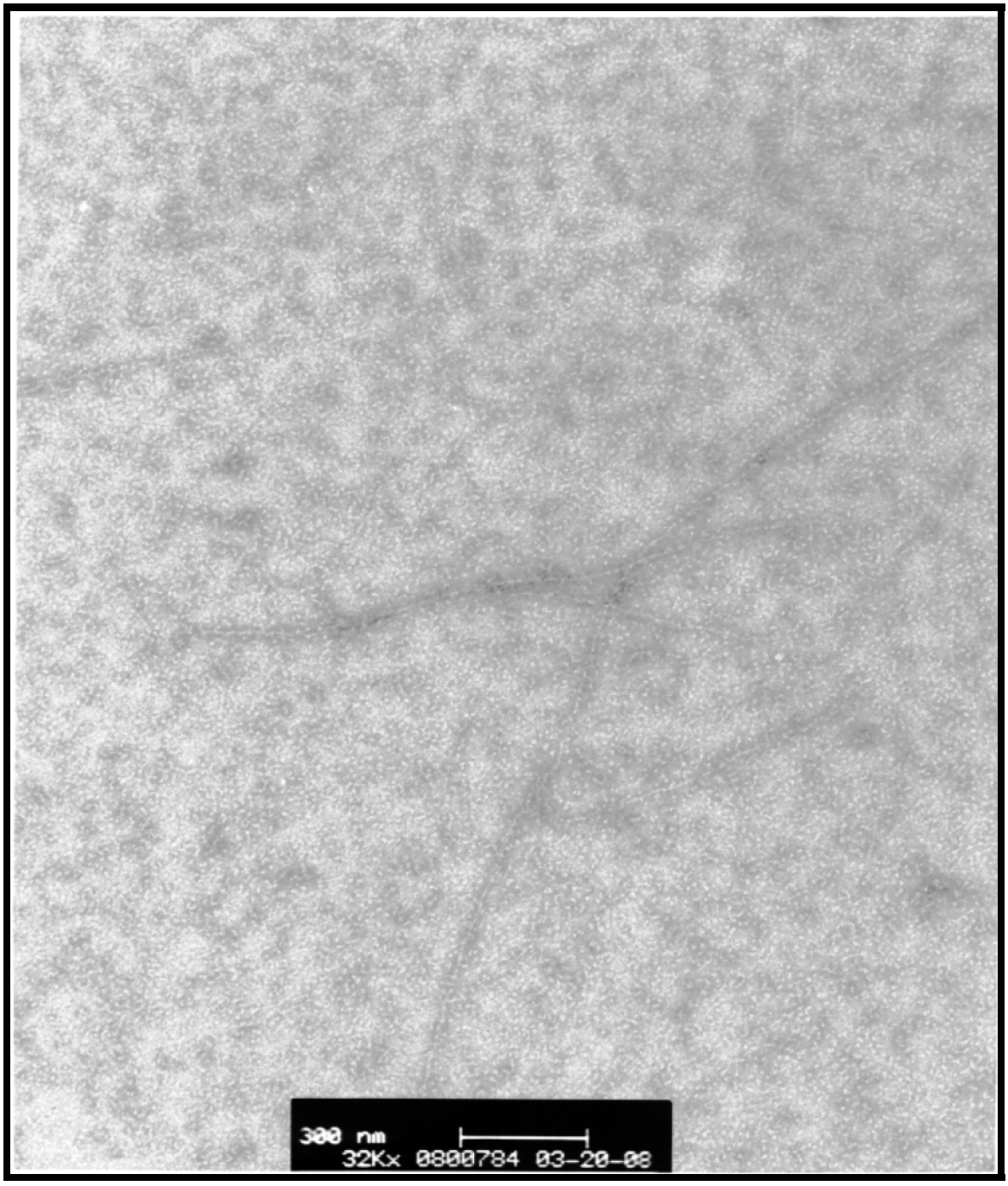

Figure 3.6C. TEM micrograph of a heated (80 °C – 10h) 1.5 % w/w β-lactoglobulin solution at pH 2.0. Magnification bar represents 300 nm.

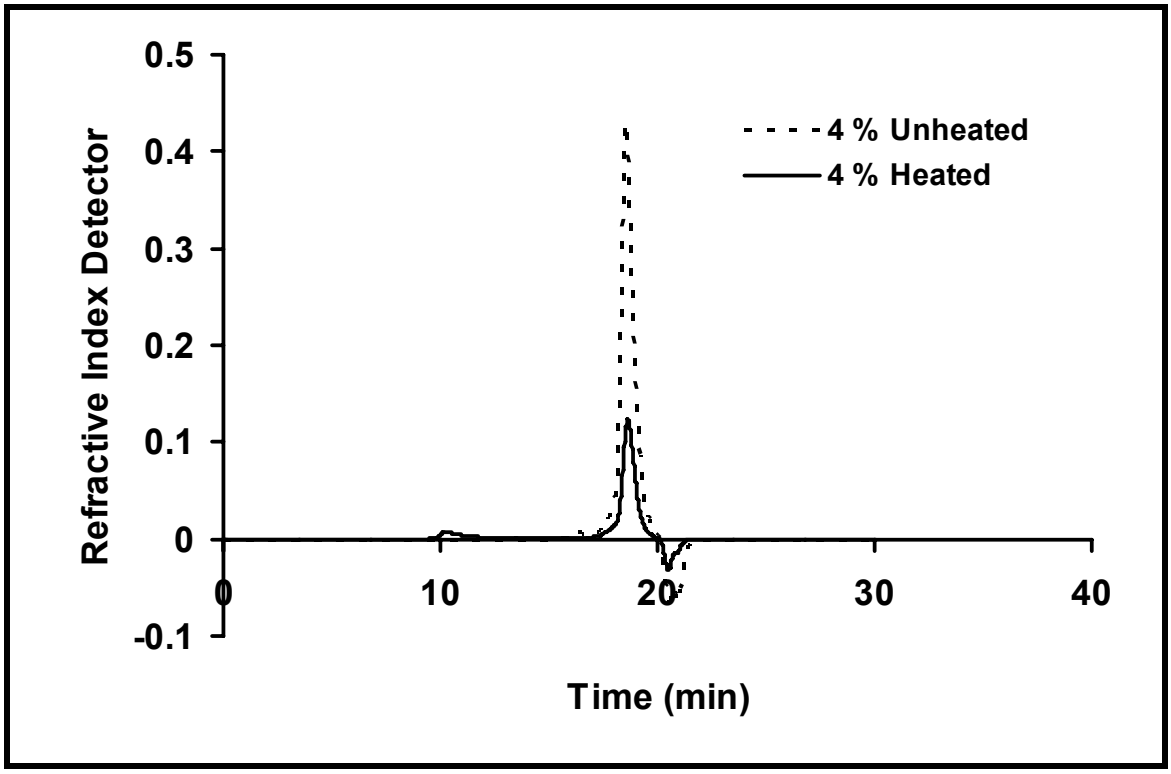

Figure 3.7A. Chromatographs of 4 % heated and Unheated, pH 3.35 (Refractive index concentration detector is shown. As all β-lactoglobulin solutions were prepared based on same total mass concentration, UV280 (not shown) and RI detector both represented protein concentration)

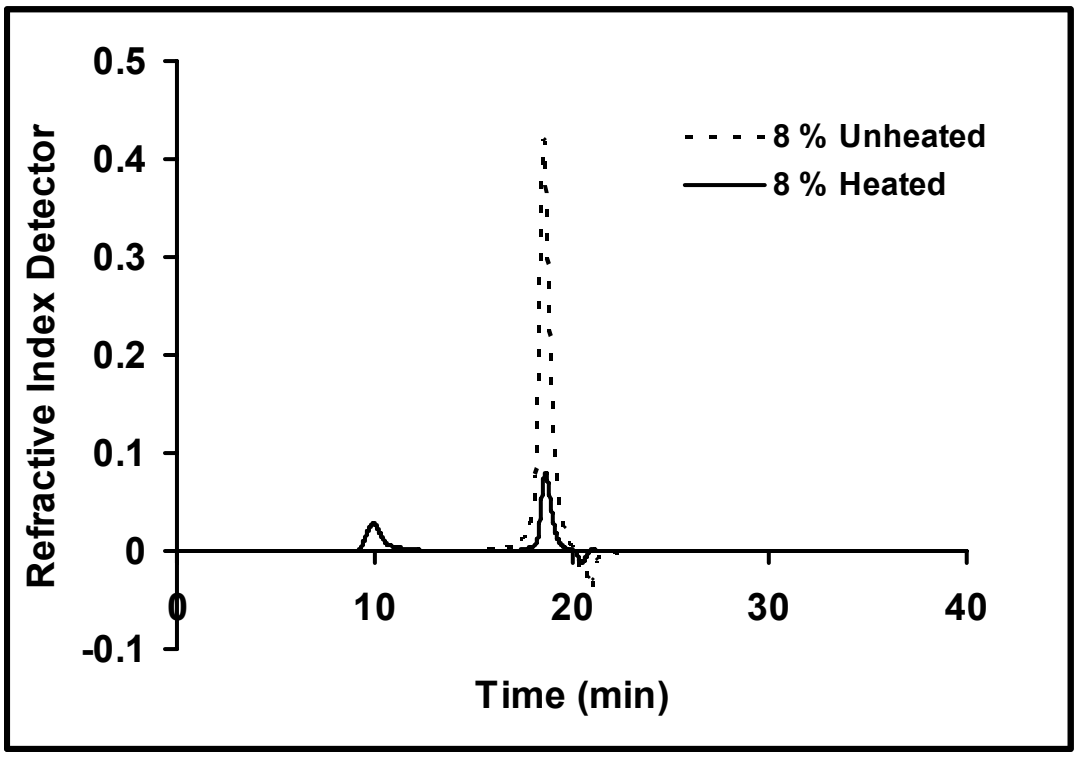

Figure 3.7B. Chromatographs of 8 % heated, and unheated pH 3.35 (Refractive index concentration detector is shown. As all β-lactoglobulin solutions were prepared based on same total mass concentration, UV280 (not shown) and RI detector both represented protein concentration)

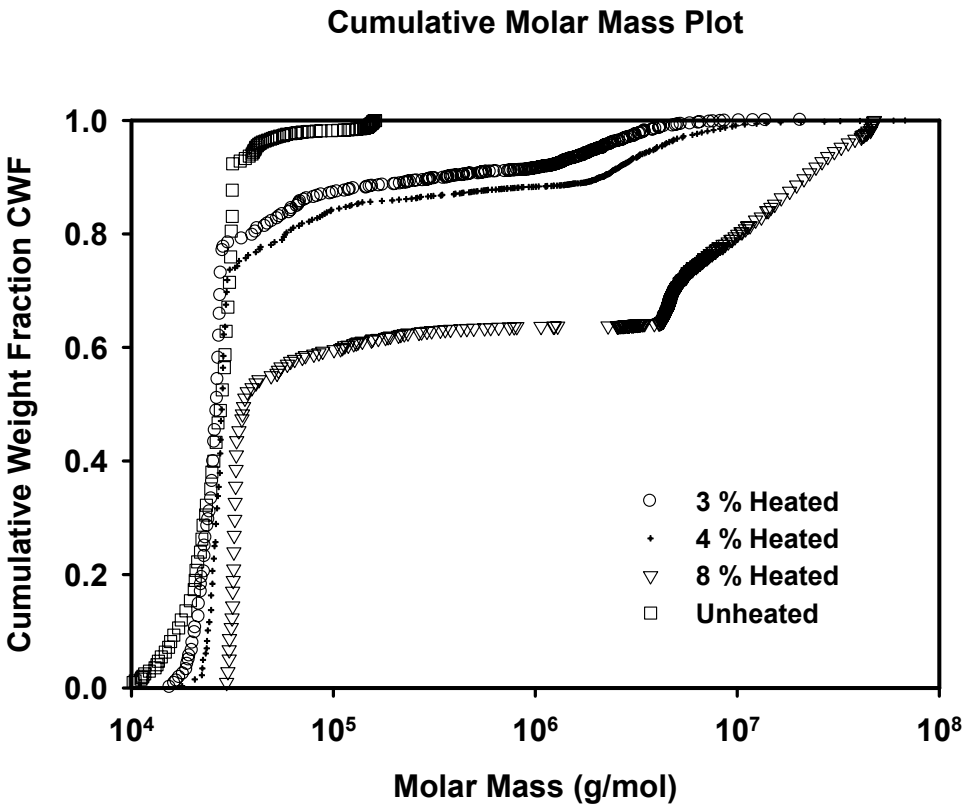

Figure 3.8A. Cumulative molar mass plot for both heated and unheated samples

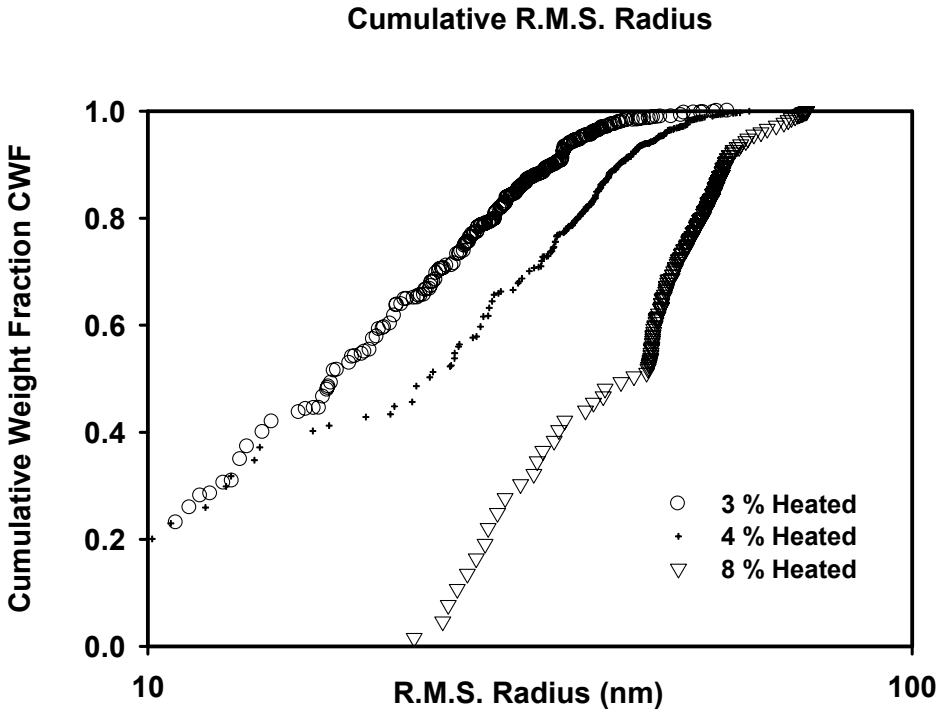

Figure 3.8B. Cumulative R.M.S. radius plots for both heated and unheated samples

CHAPTER 4.

CONCENTRATION AND IONIC STRENGTH EFFECTS ON COLD-SET THICKENING MECHANISM OF β-LACTOGLOBULIN AT LOW pH

To be submitted to: The International Dairy Journal

4.1 ABSTRACT

Concentration and ionic strength (*I*) effects specific to additional $CaCl_2$ on the cold-thickening mechanism of a modified β-lactoglobulin (β-lg) ingredient were studied using light scattering, transmission electron microscopy, and rotational viscometry at low pH. Flexible fibrillar networks were formed at pH 3.35, and more branching was observed with an increase in *I* ($CaCl_2$) below 60 mM. Additional *I* up to 60 mM increased conversion of monomers to large aggregates (> 10^6 Da), especially at concentrations above 6.9 % w/w, the critical concentration (C_c) for this modified protein system. A more connected flexible fibrillar network was observed following freeze drying with greater thickening function from reconstituted modified powders. A slight increase to *I* at concentrations greater than C_c resulted in improved thickening with similar network characteristics and thus provided an option to manipulate concentration and ionic strength to obtain improved thickening behavior.

4.2 INTRODUCTION

Nutritional benefits of whey proteins help make them desirable food ingredients. Conventionally, cold-gelation of whey proteins has been accomplished in two steps: initially, whey proteins are heated at neutral pH and low ionic strength conditions below their critical concentration for gelation. The temperature is above the denaturation temperature, however, no gelation occurs because of high repulsion conditions, and a solution of high molecular

weight whey protein aggregates is formed (Vardhanabhuti et al., 2001). Then, these solutions are cooled, and cold gelation is induced by adding salts (Barbut & Foegeding, 1993; Hongsprabhas & Barbut, 1997; Bryant & McClements, 2000; and Ju & Kilara, 1998) or by slow acidification (Alting et al., 2002; Alting et al. 2004) to screen electrostatic repulsion.

In a different approach, a procedure to convert whey protein isolates or concentrates into a pH (3.35) modified, cold-thickening ingredient (mWPI or mWPC) was developed (Hudson et al., 2000; Resch & Daubert, 2002; and Resch et al., 2004). These modified powders (mWPI and mWPC) provide desirable thickening functionality at ambient temperatures without addition of salt or heat (Hudson & Daubert, 2002; & Resch and Daubert, 2002). Clare et al. (2007) reported increased thickening capacity of mWPC in the presence of supplemental calcium during rehydration. Mudgal et al. (2009) investigated concentration effects on the cold-thickening mechanism of this ingredient using a β-lg model system, reporting a critical concentration (C*) (6.9 % w/w solids, 94 % pure) below which no significant thickening functionality could be achieved from modified β-lg powders (mβlg). Flexible, fibrillar networks were formed during heating of β-lg solutions at pH 3.35 at all concentrations, and 'microgel' structures were observed at concentrations higher than the critical concentration (C* ~ 6.9 % w/w, Mudgal et. al., 2009) using transmission electron microscopy. These 'microgels' formed during heating of β-lg solutions at pH 3.35, along with concentration dependent colloidal interactions, are seemingly responsible for cold-thickening functionality of modified powders (Mudgal et al., 2009).

β-Lg can form different types of aggregates during heating, including fibrils (rigid), flexible strands, branched or particulate aggregates depending on pH and ionic strength (Bolder et al., 2006; Mudgal et al., 2009, Krebs et al., 2007, 2009). Fine stranded networks are formed away from the isoelectric point below pH 4 and above pH 6 as reported by Langton and Hermansson, (1992). Under conditions of extreme repulsion, pH 2 and low ionic strength, β-lg forms long and linear fibrils (Veerman et al., 2002, 2003; Bolder et al., 2006, 2007a, 2007b). Application of shear resulted in enhanced fibril formation because of accelerated kinetics at pH 2 and low ionic strengths (Akkermans et al., 2008; Bolder et al., 2007b). At pH 3.35, β-lg forms flexible fibrils with persistence lengths of approximately 35 nm (Mudgal et al., 2009). Persistence length is used to quantify flexibility/rigidity of biopolymers and it increases with the linearity of fibrils or biopolymers (Cifra et al., 2004). With an increase in ionic strength of solutions, electrostatic repulsions between β-lg molecules at low pH are screened, leading to a decrease in Debye length - the major estimator of the length of electrostatic interactions, and is inversely proportional to the square root of ionic strength.

Consequently, at the same temperature and protein concentration, the kinetics of aggregation increase with an increase in ionic strength, and network formation shifts towards a particulated nature (Schokker, 2000). Addition of a high concentration of salts, such as NaCl or $CaCl_2$, results in the formation of particulated networks (Elofsson et al., 1997). Monovalent and divalent salt ions both screen electrostatic repulsion; however, divalent cations such as Ca^{2+} can form salt bridges, resulting in the cross-linking of negatively charged carboxylic group and thus are more effective (Bryant and McClements, 2000; Clare

et al., 2007). The linearity of strands decreases with an increase in ionic strength at pH 2, measured by a decrease in persistence length (Veerman, 2002; Aymard, 1999; and Kavanagh, 2000). At pH 2, Veerman, 2002 observed no significant difference in persistence length of fibrils formed after heating in the ionic strength range of 0-80 mM at 1.5 % w/w β-lg. Changes in ionic strength of β-lg solutions at low pH alter conditions of electrostatic repulsions, thereby influencing the physical characteristics (linearity, curvature, branching, and size) of aggregates formed during heating.

The objective of this study was to investigate ionic strength effects on thickening functionality of β-lg powders at the pH of the established modification procedure, 3.35. Ionic strength was varied by adding $CaCl_2$ (< 80 mM additional *I*, depending on the protein concentration). Calcium chloride was added during prior to the pH adjustment of β-lg solutions during the modification procedure (Mudgal et al., 2009). Ionic strength effects specific to $CaCl_2$ were studied at varying β-lg concentrations using multi-angle laser light scattering, transmission electron microscopy, and rotational viscometry to further elucidate the cold-thickening mechanism of this pH modified ingredient in hopes of achieving the capacity to mechanistically tailor the modification procedure while expanding functionality of resulting dairy ingredients.

4.3 MATERIAL AND METHODS

4.3.1 Protein Material

β-lactoglobulin (BioPure^{R}, ~ 94 % pure, total protein 98 % dry basis) was donated by Davisco Foods, Inc. The wet basis sample protein content was determined by micro-Kjeldahl (AOAC, 1984) to be 92.81 %. Inductively-coupled plasma atomic emission spectroscopy was used to establish the mineral content of the β-lg sample as 569 ppm phosphorus, 269 ppm calcium, 39 ppm magnesium, 106 ppm potassium, and 8079 ppm sodium.

4.3.2 Solution preparation

β-Lg solutions of different concentrations (2, 4 and 8 % w/w solids) and different ionic strengths (0 - 80 mM) were prepared by adding appropriate amount of $CaCl_2$ and dissolving β-lg in de-ionized (DI) water by continuous stirring at room temperature for 1-2 hours. Commercial whey protein products and ingredients contain trace amounts of salts. Based on the conductivity measurements (linear relation with ionic strength from $CaCl_2$) of the β-lg solutions of varying concentrations and added $CaCl_2$, ionic strengths of β-lg solutions without any added salts were approximated between 10-40 mM, depending on protein concentration (2-8 % w/w). Then, $CaCl_2$ was added in increments of 20 mM at (2 and 4 % w/w solids) or10 mM at (8 % w/w solids), such that maximum ionic strength of solutions did

not exceed 0.1 M and no gelation occurred. Sodium azide (0.02 %) was added to all samples to prevent microbial growth. Thereafter, solutions were adjusted to pH 3.35 using 6 N HCl. Following pH adjustment, solutions were heated at 85 °C for 3 hours in a water bath. Then, these modified β-lg solutions were cooled and stored in a 4 °C refrigerator for 24 h.

4.3.3 Shear rate sweeps

Shear rate sweeps were performed on all solutions at 25 °C using a stress controlled rheometer (ATS Rheosystems, Bordentown, NJ) to characterize flow behavior and viscosities of solutions (2, 4, 8 and 10 % w/w). A smooth, 25 mm concentric cylindrical geometry was used, and shear rates were varied from 1 to 100 s^{-1} using a constant rate program to minimize inertial effects. Depending on the number of data points desired, shear rates were varied over a time period of 600-900 s. The presence of hysteresis was diagnosed by two runs of increasing and then decreasing shear rates on β-lg solutions. A pre-shear condition (15 s^{-1} for 30 s) was applied to all solutions to obtain uniform solutions prior to measurements with a consistent baseline shear history. A thin film of mineral oil was applied to the sample surface to minimize sample dehydration. To characterize flow behavior, power law model parameters were determined. Equation 2 presents the power-law model to characterize flow behavior. A minimum of two independent replicates were performed for all solutions.

$$\eta = K\dot{\gamma}^{n-1} \quad Eq.\ 2$$

4.3.4 Preparation of modified β-lactoglobulin powders for microscopy and viscometry

Heated β-lg solutions of desired concentration and ionic strength at pH 3.35 were prepared and stored at 4 °C for 24 h. The next day, sols/gels were frozen using methanol and dry ice, then freeze dried using a 4.5 L bench top freeze dryer (Labconco 73035, Kansas City, Mo), and finally ground to a powder.

4.3.5 High performance liquid chromatography-Multi-angle laser light scattering (HPLC-MALS)

To characterize distribution of molar mass of aggregates formed at different concentrations and ionic strength, light scattering studies were performed. Heated solutions of β-lg at various concentrations (2, 4, and 8 %) and ionic strength (0-80 mM) were prepared in 40 mM sodium citrate/citric acid buffer at pH 3.35. Then samples were diluted to 5 mg/ml final concentration and filtered through a 0.45 micron filter. The Bicinchoninic acid (BCA) assay (Methodology developed by Thermo Scientific Inc., Rockford, IL) was performed prior and after filtration to determine protein loss during filtration. Two independent replicates were performed.

The HPLC–MALS was calibrated with 200 µl of buffer and 100 µl of BSA (Bovine serum albumin) standard (5 mg/ml), and data were normalized based on BSA. A gel filtration

column (Shodex, KW-804) was linked to a photodiode detector (Waters 2996) coupled with refractive index detector (Waters 2414) and MALS detector (Waters Technology, Dawn EOS Enhanced Optical Systems) were used to determine molar mass and concentration distribution of the samples. Twentyfive μl of samples at 5 mg/ml concentrations were injected onto the column.

4.3.6 Transmission electron microscopy (TEM)

Heated β-lg solutions at different concentration (4 and 8 % w/w) and ionic strength (0-0.08 M) were prepared at pH 3.35. All samples were diluted with deionized water to a concentration of 0.04 % and pH was adjusted to 3.35 using 6 N HCl acid prior to TEM analysis. The TEM samples were prepared by negative staining, and a drop of diluted sample was deposited on a copper grid (formvar coated and further coated with evaporated carbon) and the excess sample was removed using filter paper. A drop of 2 % uranyl acetate, pH 3.5, was added for 60 s and excess reagent was removed. Digital electron micrographs were obtained using a FEI/Phillips EM 208S Transmission electron microscope (made by Phillips, Czech Republic).

4.4 RESULTS AND DISCUSSION

Modified β-lactoglobulin solutions

4.4.1 HPLC-MALS

A distribution of molar mass versus cumulative weight fraction (CWF) was obtained from light scattering data of heated β-lg dispersions prepared at different concentrations (2, 4 and 8 % w/w) and ionic strengths at pH 3.35 (Figure 4.1). At all concentrations, the percentage of monomers/dimers (CWF corresponding to molar mass < 36 kDa) aggregating into β-lg polymers (conversion) increased with increasing ionic strength. At the same temperature and protein concentration, increased conversion values with increasing ionic strength were reported at pH 2.5 using size exclusion chromatography (SEC)-MALS (Schokker et al., 2000).

However, this increase in conversion with ionic strength at pH 3.35 was more prominent at higher protein concentrations. At 2 % w/w, more than 77 % of protein remained non-aggregated under all ionic strength conditions studied (Figure 4.1A), and conversion increased from 3 % at 0 mM additional *I* to 23 % at 60 and 80 mM *I* No significant increase in conversion was obtained when *I* was increased from 60 to 80 mM at 2 % w/w concentration, indicating that no noticeable increase in aggregation kinetics resulted after 60 mM at 2 % w/w (Figure 4.1A). Identical trends were obtained from two independent replicates of this experiment. Similarly at 4 % w/w, conversion increased from 17 % at 0 mM to 31 % at 60 mM additional *I* (Figure 4.1B). While for 8 % dispersions, conversion

increased from 40 % to 60 % with an increase of 10 mM *I* (Figure 4.1B). Also, at 8 % w/w, β-lg aggregates (~ 10^8 Da) were formed as compared to ~10^7 Da size aggregates formed at *I* (0-60 mM additional) studied at 4 % w/w (Figure 4.1B). These results showed that at lower protein concentrations, the maximum size of aggregates did not increase with increasing ionic strength (0-80 mM), which can be attributed to larger inter-particle distances and in resulting weaker aggregation at lower concentrations (Mudgal et al., 2009). These results were validated by the cumulative weight fractions (CWF) vs. root mean square (R.M.S.) radius plots (data not shown), where maximum size (R.M.S. radius) of aggregates determined at 8 % was greater than 80 nm compared to less than 50 nm at lower protein concentrations.

Distribution of β-lg aggregates of varying molar masses was plotted as a function of *I* at all concentrations (Figure 4.2). Four categories were described which were monomers/oligomers (< 10^5 Da), intermediates (10^5 – 10^6 Da), small aggregates (10^6-10^7 Da), and large aggregates (> 10^7 Da). As shown in Figure 4.2A, a majority of heated 2 % dispersions were less than 10^5 Da in molar mass. The percentage of 'small aggregates' (10^6-10^7 Da) increased with increasing ionic strength, while the percentage of intermediates remained small and relatively unchanged, indicating a bimodal distribution of the aggregates. This bimodal distribution at low pH has previously been reported (Mudgal et al., 2009; Arnaudov et al., 2003; and Schokker et al., 2000). As observed in Figure 4.1A, no 'large aggregates' (> 10^7 Da) were formed at 2 % w/w up to 40 mM ionic strength, with less than 1 % measured at 60 and 80

mM *I*. Similar results were obtained at 4 % w/w (Figure 4.2B), and very few aggregates > 10^7 Da were observed, with a maximum of about 6 % at 60 mM *I*.

At 8 % w/w a relatively high percentage (> 15 %) of 'large aggregates' (> 10^7 Da) was formed (Figure 4.2C), and the maximum molar mass of these aggregates was ~10^8 Da (Figure 4.1B). Similar trends were observed for two independent replicates of this experiment. With addition of 20 mM *I* to 8 % dispersions of β-lg, a significant percentage (> 25 %) of very large aggregates (> 0.45 μm in size) was formed during heating at pH 3.35, which did not pass through a 0.45 μm membrane, and therefore could not be analyzed by HPLC-MALS.

Based on the HPLC-MALS data, the loss of monomeric/oligomeric fraction (< 10^5 Da) with increase in ionic strength was determined at varying concentrations (Figure 4.2D). It was found from the slopes that the rate of decrease of monomers/oligomers with increasing ionic strength was comparable at lower protein concentrations (-0.29 at 2 % w/w and -0.32 at 4 % w/w). Rate on decrease of monomers/oligomers was linear with increase in ionic strength up to 60 mM as observed from the high R^2 values of linear fits for 2 and 4 % w/w concentration. While at 8 % w/w, this rate was higher ~ -0.45 as determined by the two data points available at this concentration (Figure 4.2D). These results indicated that with the increase in ionic strength, the rate of aggregation was significantly higher at 8 % w/w as compared to lower protein concentrations (2 and 4 % w/w).

4.4.2 Viscometry

Shear rate sweeps were performed on heated β-lg dispersions at pH 3.35 without any added salt and with maximum additional *I* (80 mM at 2 %, 60 mM at 4 % and 20 mM at 8 % w/w) (Figure 4.3). No significant increase in apparent viscosities was observed at 2 % w/w with increasing *I*, while a small increase (~ 15 % from 0 to 60 mM *I*) was observed at 4 % dispersions (Figure 4.3). For 8 % dispersions, apparent viscosities increased by more than one log with an addition of 20 mM *I* (6.67 mM $CaCl_2$) (Figure 4.3). All dispersions at 2 and 4 % w/w displayed Newtonian flow behavior (n ~ 1). However, dispersions heated at 8 % w/w were shear thinning, and pseudoplasticity increased from 0 mM *I* (n ~ 0.97) to 20 mM *I* (n ~ 0.6). Higher pseudoplasticity at 8 %, 20 mM *I* suggested formation of larger aggregates, which may have aligned better with shear flow. The changes in apparent viscosities with shear were reversible, indicating no permanent breakage of the network or aggregates with shear. Significant percentage (> 15 %) of large aggregates (> 10^7 Da) and stronger colloidal interactions seem responsible for the high viscosities at higher concentrations.

4.4.3 TEM

To characterize and obtain visual images of the aggregates formed during heating, TEM was performed. Flexible fibrils were observed in heated β-lg dispersions at 4 % w/w with 25 mM and 50 mM additional *I* ($CaCl_2$) at pH 3.35. At 25 mM *I* (Figure 4.4A), isolated fibrils were observed, similar to those observed at 4 % w/w heated β-lg solutions without any added salt,

pH 3.35 (Mudgal et al., 2009). While at 50 mM additional *I*, few large aggregates with more branching were visible (Figure 4.4B). An increase in branching with ionic strength at pH 2 was previously documented with addition of NaCl (Aymard et al., 1999; Kavanagh et al., 2000; and Veerman et al., 2002). These results are consistent with the light scattering results (HPLC-MALS), where an increase in large aggregate percentage was observed with increasing ionic strength. These results showed that the addition of up to 50 mM additional *I* resulted in the formation of flexible, fibrillar networks, while increased branching was observed at a higher *I*. At 8 % w/w also, flexible fibrillar networks were observed with larger (compared to 4 % w/w) aggregates ('microgels') also reported previously (Mudgal et al., 2009) (Figure 4.5). With 20 mM additional *I*, aggregates with a more connected network were observed (Figure 4.5B) which were deemed responsible for increased thickening function of the heated dispersions. These results correlate well with the light scattering and rheological data.

Rehydrated modified β-lactoglobulin powders:

4.4.4 Thickening functionality of modified powders

Thickening behavior of modified β-lg powders was determined by performing shear rate sweeps on rehydrated modified powders. Heated β-lg solutions at different concentrations (4, 8 and 10 % w/w) and ionic strengths at pH 3.35 were freeze dried and ground to form modified β-lg powders. These powders were then reconstituted in water at 10 % (w/w) and 5 % (w/w) solids, and the apparent viscosities were measured. Consistent with the previous

reports (Mudgal et al. 2009), powders prepared from 4 % w/w did not increase viscosity (Figure 4.6). While inclusion of 20 mM *I* (6.67 mM $CaCl_2$) to 8 % solutions significantly improved thickening functionality of modified powders (by ~ 1 log) (Figure 4.6A and 4.6B). The modified powders prepared from 10 % dispersions provided even greater thickening benefit once reconstituted at both 10 and 5 % w/w, respectively (Figure 4.6A and 4.6B). Similar to the flow profiles obtained prior to freeze drying, modified powders prepared from 4 % w/w displayed Newtonian flow (n ~ 1), while other dispersion at 8 % or higher displayed pseudoplasticity. Pseudoplasticity of modified powders increased from n ~ 0.94 (8 % w/w) to n ~ 0.74 (8 % w/w + 20 mM *I*) when rehydrated at 5 % w/w. Rehydrated modified powders prepared from 10 % modified β-lg solutions displayed even higher degree of pseudoplasticity (n ~ 0.49) when reconstituted at 5 % w/w. These results indicated that formation of larger aggregates resulted in higher degree of pseudoplasticity as discussed in previous sections. Rehydrated modified powders displayed greater shear thinning when reconstituted at a higher concentration of 10 % w/w, and similar trends were observed. These results suggested that the colloidal interactions among these large aggregates were responsible for increased viscosity, and could be diminished with increasing shear. Modified powders prepared from 4 % and 8 % with no added salt displayed no hysteresis. Small hysteresis was observed at low shear rates for 8 % with 20 mM *I* and 10 % w/w dispersions when reconstituted at 10 % w/w indicating a formation of a weak network, which was likely broken with shear and was not completely reversible (Figure 4.6).

These results suggest that larger aggregates formed during heating of β-lg dispersions at pH 3.35 persist in modified powders, providing the thickening benefit. Mudgal et al., (2009)

reported that formation of continuous fibrillar aggregates (microgels) was essential to obtaining cold-thickening function from modified powders. To further investigate network characteristics of rehydrated modified powders, TEM was performed.

4.4.5 TEM on rehydrated modified powders

Flexible fibrillar type networks were also observed in rehydrated modified powders. These networks were similar to those obtained prior to freeze drying of heated solutions. At lower protein concentrations (4 % w/w), isolated fibrillar strands appeared similar to fibrillar networks formed prior to freeze drying (Figure 4.7A), while at 8 %, the microgels appeared to aggregate further, and thus a more connected network was visible (Figure 4.7B). The micrograph of 8 % solutions at 20 mM *I* showed a more closely connected network with larger aggregates (Figure 4.7C). These results suggested that network characteristics remained similar after freeze drying, but it appeared that freeze concentration effects during freezing led to further aggregation of microgels, resulting with formation of even larger aggregates and improved thickening function. These observations correlate well with the light scattering and viscometry data and may help explain a mechanism for the thickening function of modified powders at pH 3.35.

4.4.6 Cold-set thickening mechanism:

β-Lg exists primarily as monomers or dimers at neutral pH. With decreasing pH, monomer-dimer equilibrium is shifted towards monomer (Aymard, 1996, Sakurai, 2001). With an

increase to ionic strength, more dimer formation was observed (Renard et al., 1998; Aymard et al., 1996). The effect of temperature and ionic strength on monomer-dimer equilibrium was studied using static and dynamic light scattering at pH 2 (Aymard, 1996). With an increase in temperature (5 – 76 °C), dimers dissociated into monomers and equilibrium was shifted towards a monomeric structure (Aymard, 1996).

A schematic of the suggested cold-set thickening mechanism is shown in Figure 4.8. Initially, solutions of β-lg at neutral pH are shown to be a mixture of monomers and dimers. During pH adjustment to 3.35, the dimers dissociate into monomers and there are fewer dimers, which further dissociate into monomers upon heating at 85 °C. As a result of heating, monomers are denatured exposing hidden hydrophobic residues and aggregate into oligomers and polymers depending on protein concentration, ionic strength, and the time-temperature relationship of heating. Heating of β-lg solutions below the critical concentration (C*) for this protein system resulted in formation of isolated flexible fibrils at pH 3.35 (Mudgal et al., 2009). The maximum molar mass of these fibrils was approximately 10^7 Da. Because of larger inter-particle distances at lower concentrations, there were possibly weaker colloidal interactions that resulted in lower conversion of monomers to aggregates and low viscosities of heated solutions. With a small increase in *I* at low protein concentrations < C*, more isolated flexible fibrils were formed, and a higher conversion of monomers to aggregates was obtained. However, the maximum size of aggregates remained roughly the same (~ 10^7 Da) and due to the presence of weaker interactions there was no significant improvement to viscosity. When these dispersions were freeze dried and ground to a powder, network characteristics persisted, and no thickening function was obtained. At concentrations greater

than C*, larger aggregates were formed (microgels) as shown in Figure 4.8. With addition of small *I*, network characteristics still remained fine stranded, but even larger aggregates were formed. Smaller inter-particle distances and stronger colloidal interactions resulted in formation of these microgels, and higher viscosities of these dispersions were observed (Mudgal et al, 2009). During freeze drying, freeze concentration effects likely caused further aggregation of microgels and resulting in more compact aggregates and highly viscous solutions upon rehydration. At low pH and low ionic strength, β-lg thermal aggregation seemed to result from a subtle balance between electrostatic repulsion and hydrophobic attractions. This balance will nevertheless change with ionic strength, pH, and temperature of heating, thus affecting the resulting network characteristics. Addition of salts in higher amounts may diminish electrostatic interactions significantly, thus disrupting this balance, and network characteristics may shift towards a particulated type as observed previously (Schokker et al., 2000).

4.5 CONCLUSIONS

While long and linear fibrils are formed at pH 2 and low ionic strength, fine stranded networks with much flexible strands were formed at pH 3.35 with ionic strength up to 0.1 M. At lower protein concentrations, additional *I* did not increase viscosities significantly, attributed to large inter-particle distances and weaker colloidal interactions. While at higher protein concentrations (C > C*), aggregate sizes increased with a small increase in *I* and improved thickening was observed. Freeze concentration effects seemed to further aggregate

microgels, resulting in a more compact network upon rehydration and thus a better thickening function. Improved thickening from modified powders was reported with a small addition of $CaCl_2$ at concentrations > C*, and overall, a potential for improving the original process was discovered, leading to expanded functionality of modified powders at low pH.

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Figures:

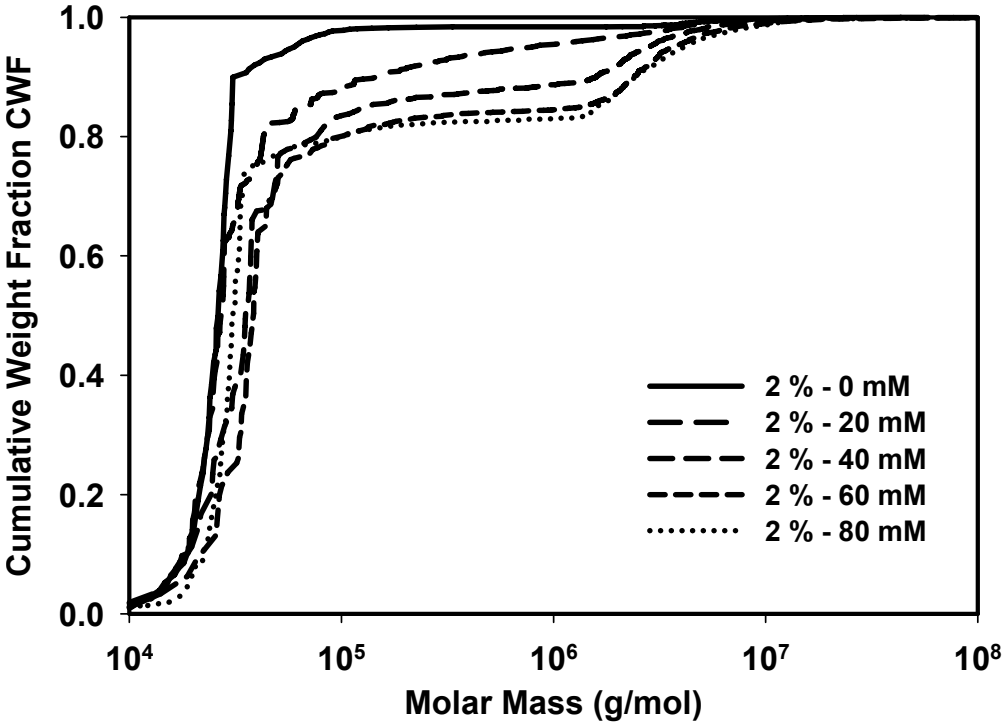

Figure 4.1A. Ionic strength effects on molar mass distribution of aggregates formed during heating of 2% w/w β-lactoglobulin solutions at pH 3.35

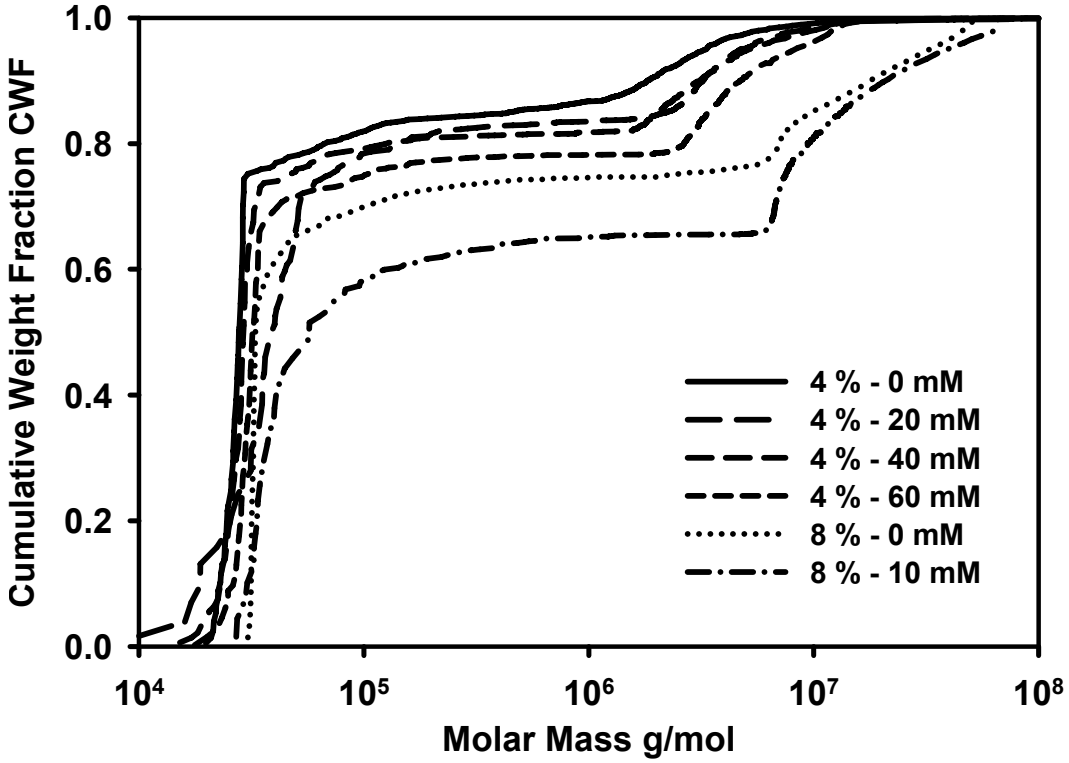

Figure 4.1B. Ionic strength effects on molar mass distribution of aggregates formed during heating of 4 % w/w and 8 % w/w β-lactoglobulin solutions at pH 3.35

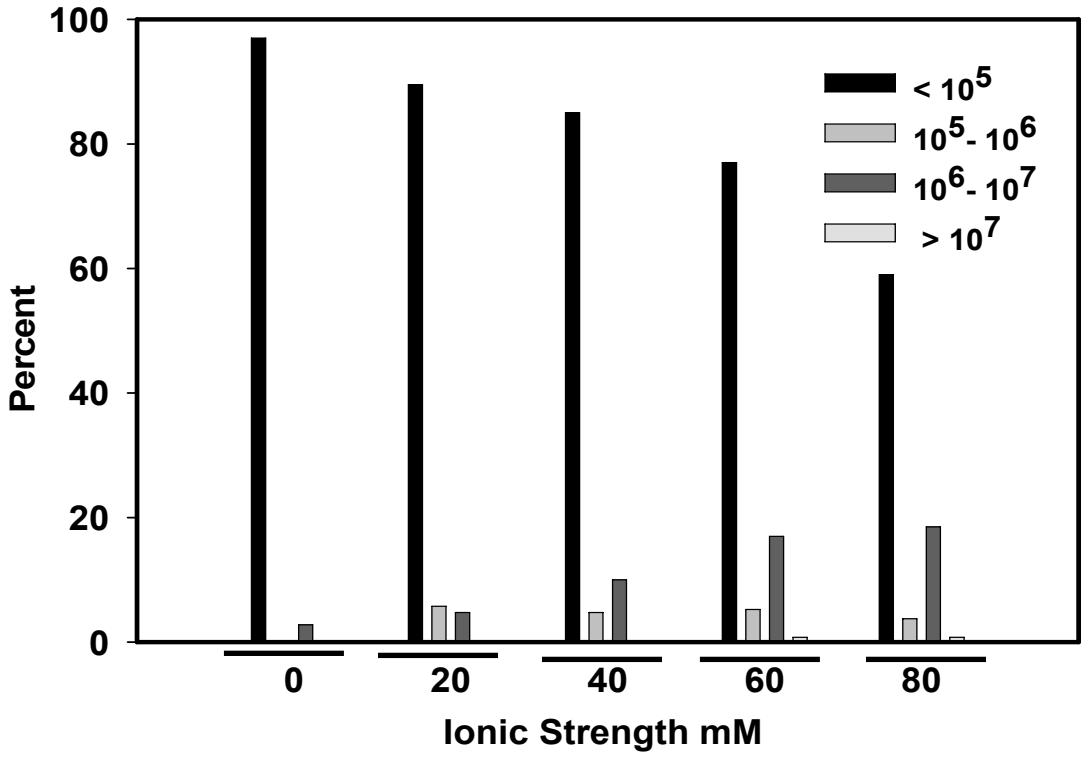

Figure 4.2A. Concentration distribution for range of aggregates formed during heating of 2 % w/w β-lactoglobulin solutions at pH 3.35 as a function of ionic strength

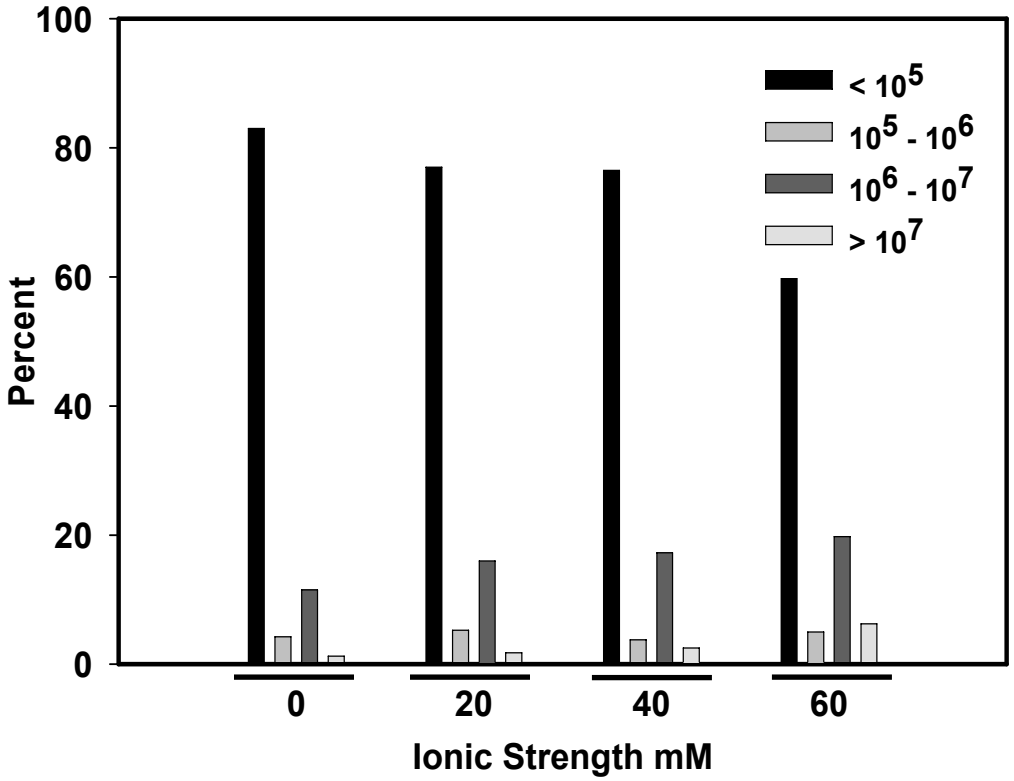

Figure 4.2B. Concentration distribution for range of aggregates formed during heating of 4 % w/w β-lactoglobulin solutions at pH 3.35 as a function of ionic strength

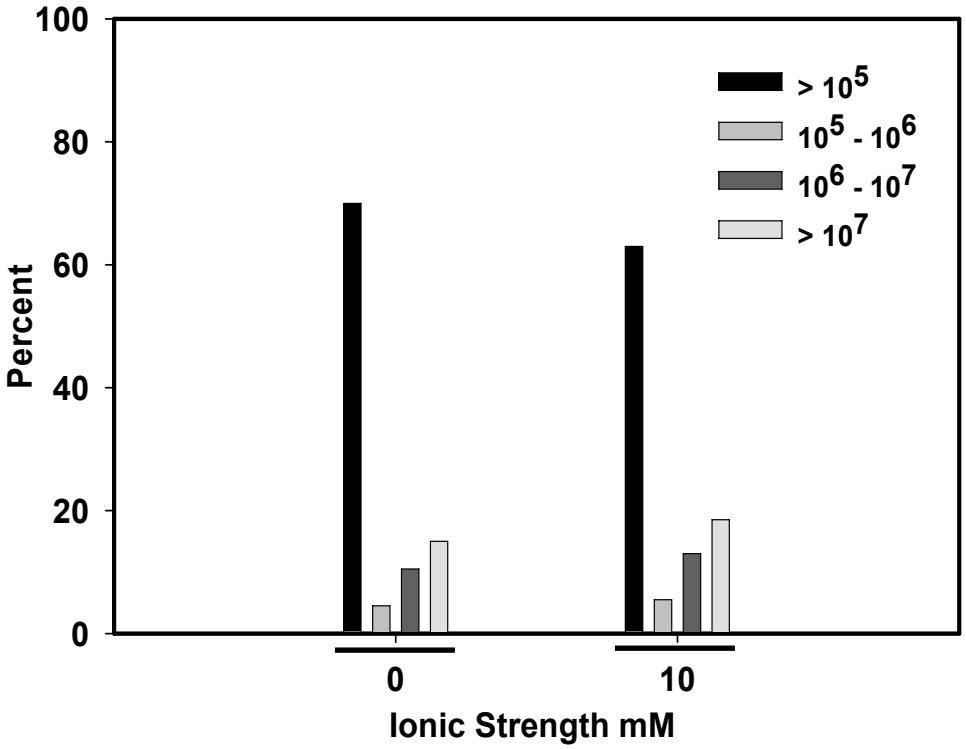

Figure 4.2C. Concentration distribution for range of aggregates formed during heating of 8 % w/w β-lactoglobulin solutions at pH 3.35 as a function of ionic strength

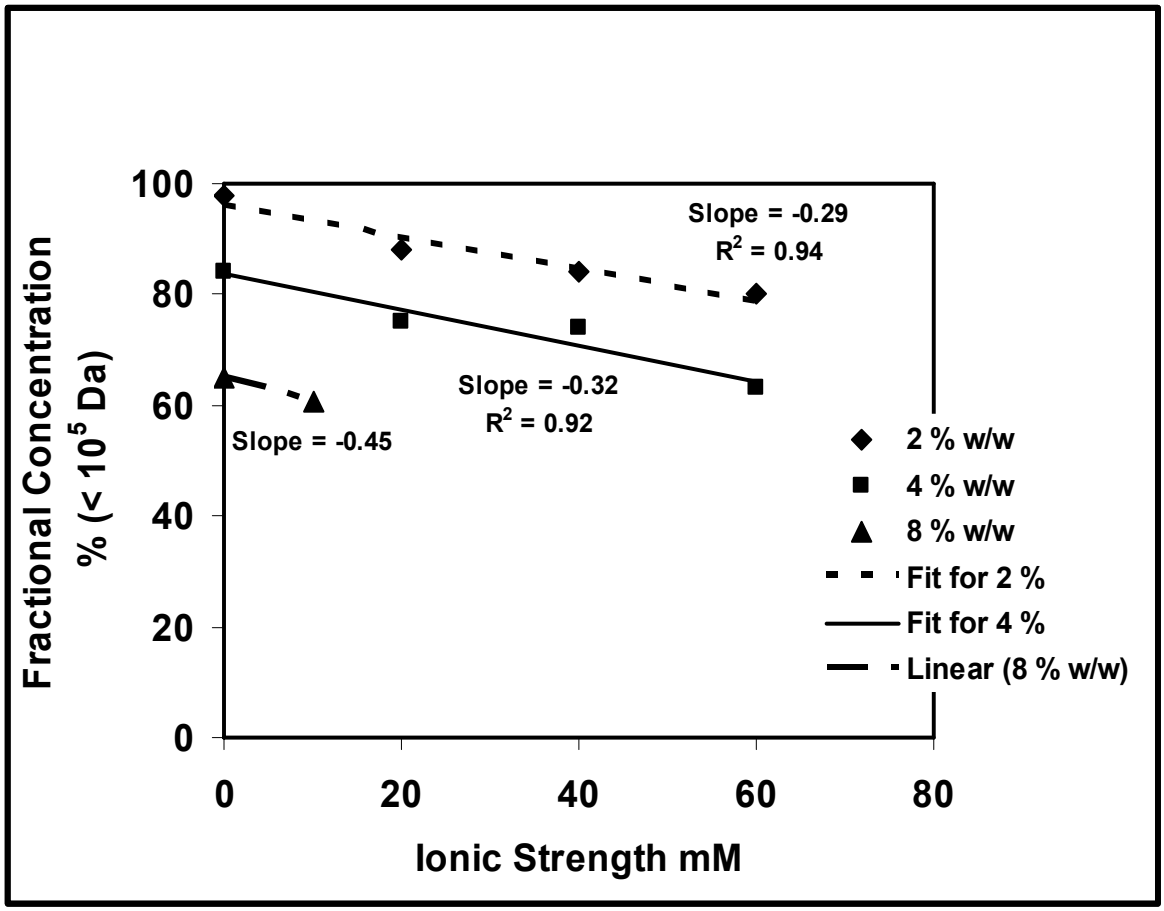

Figure 4.2D. Loss of monomers/oligomers (< 10^5 Da) with increase in ionic strength at varying β-lg concentrations

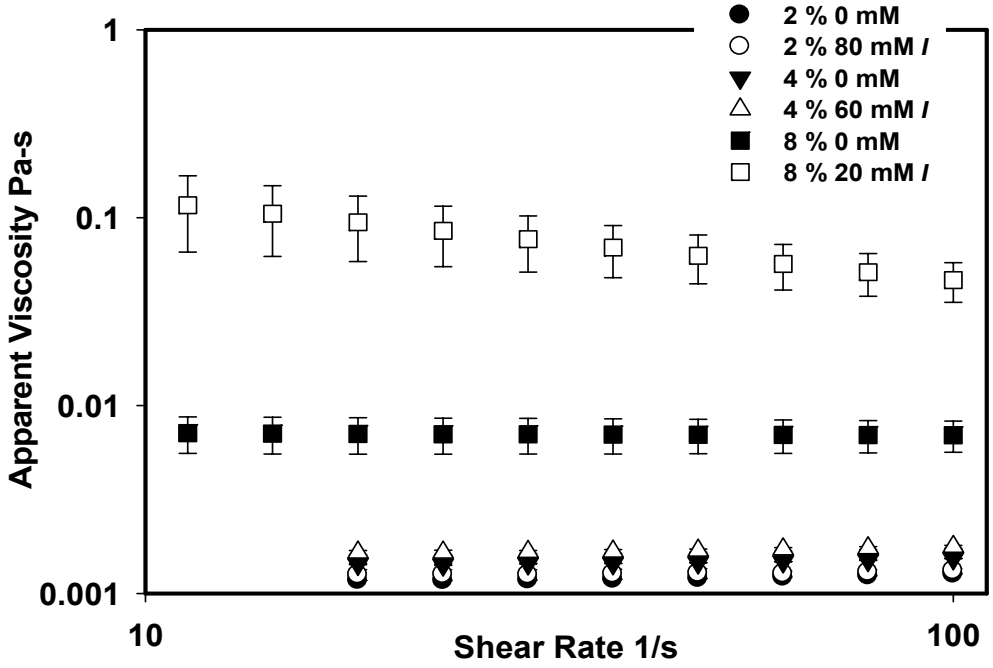

Figure 4.3. Ionic strength and concentration effects on apparent viscosities of heated β-lactoglobulin solutions at pH 3.35. Error bars denote one standard deviation.

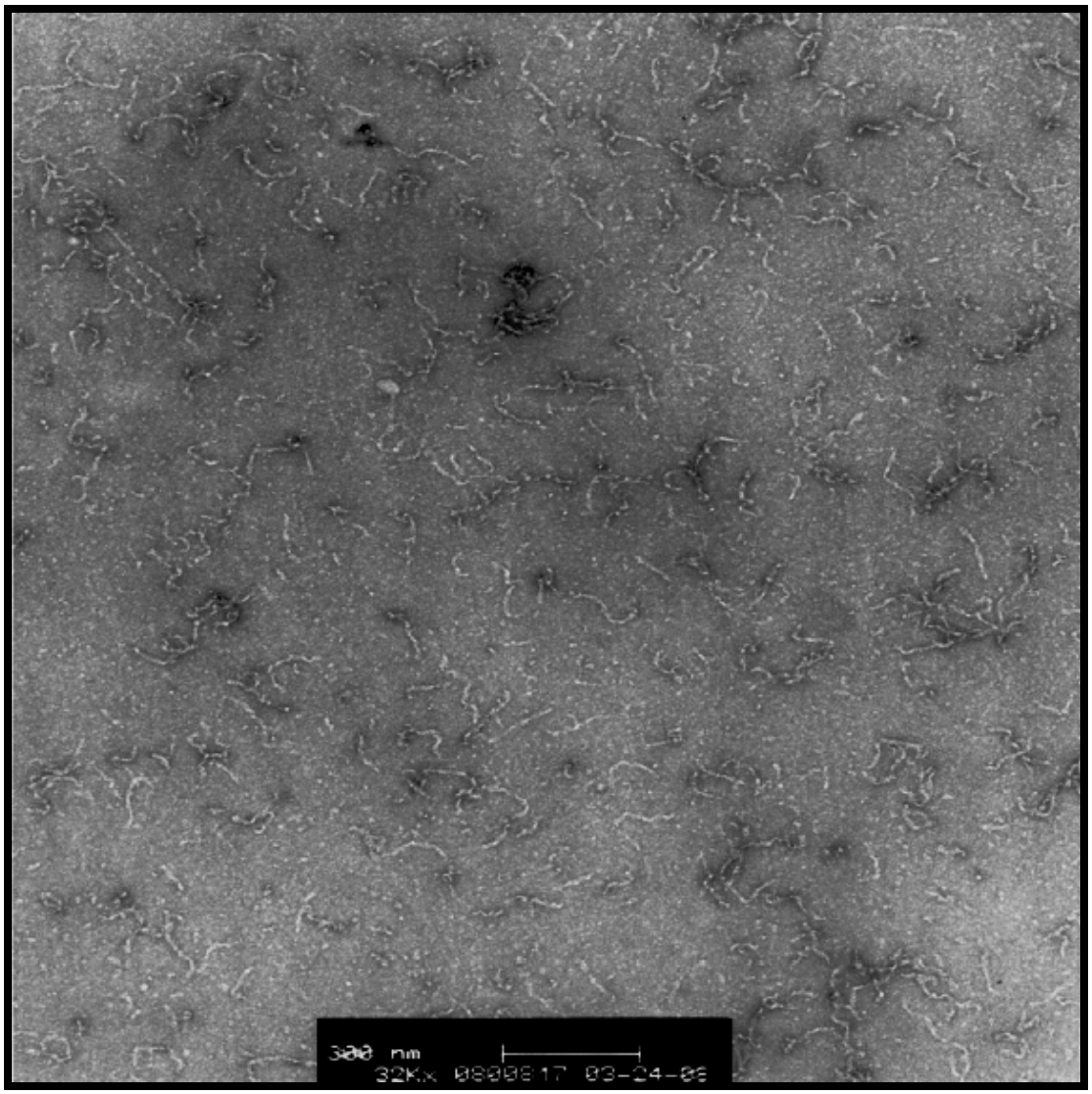

Figure 4.4A. TEM micrographs of heated β-lactoglobulin solutions at 4 %, 25 mM added ionic strength

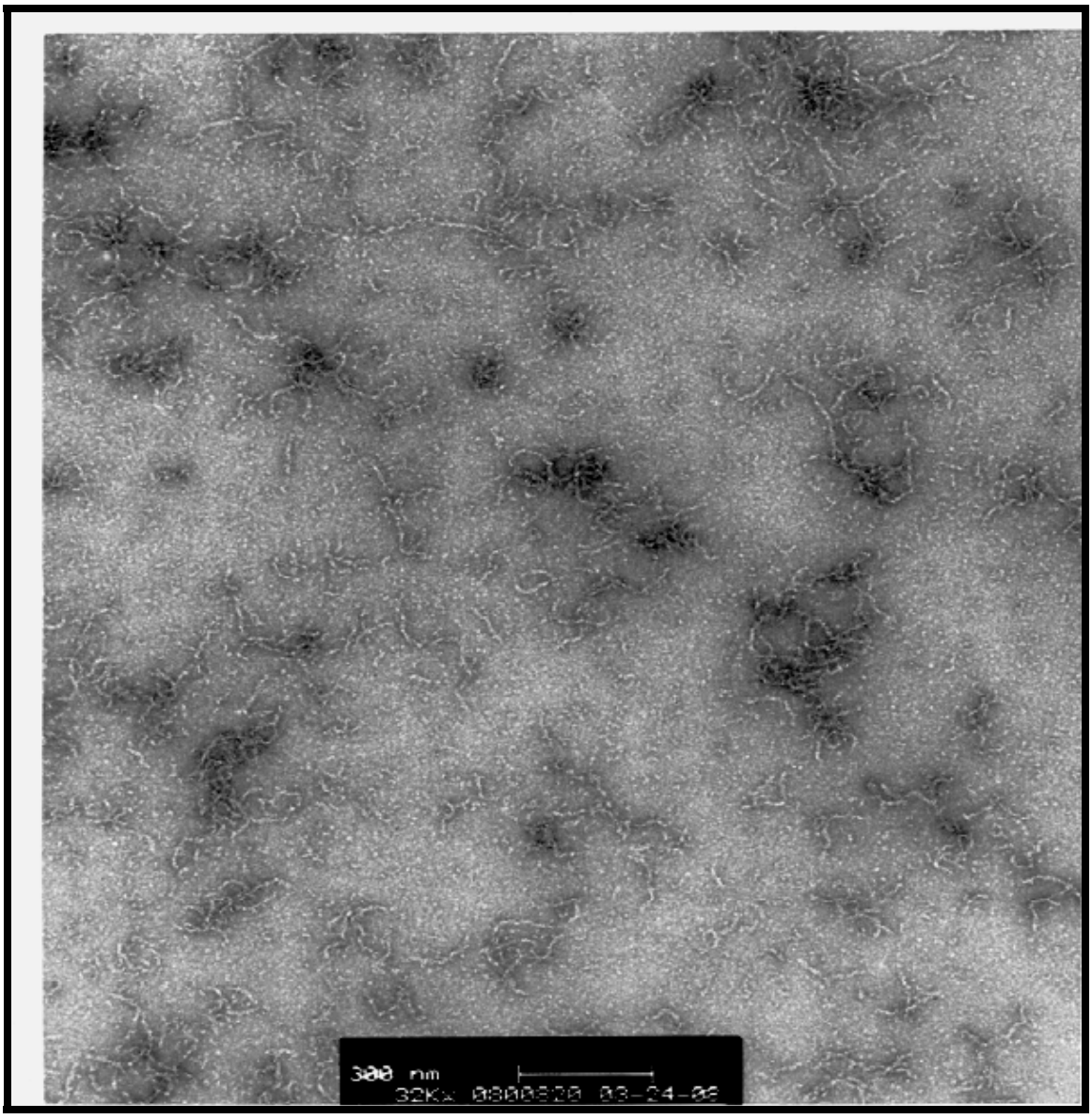

Figure 4.4B. TEM micrographs of heated β-lactoglobulin solutions at 4 %, 50 mM additional ionic strength

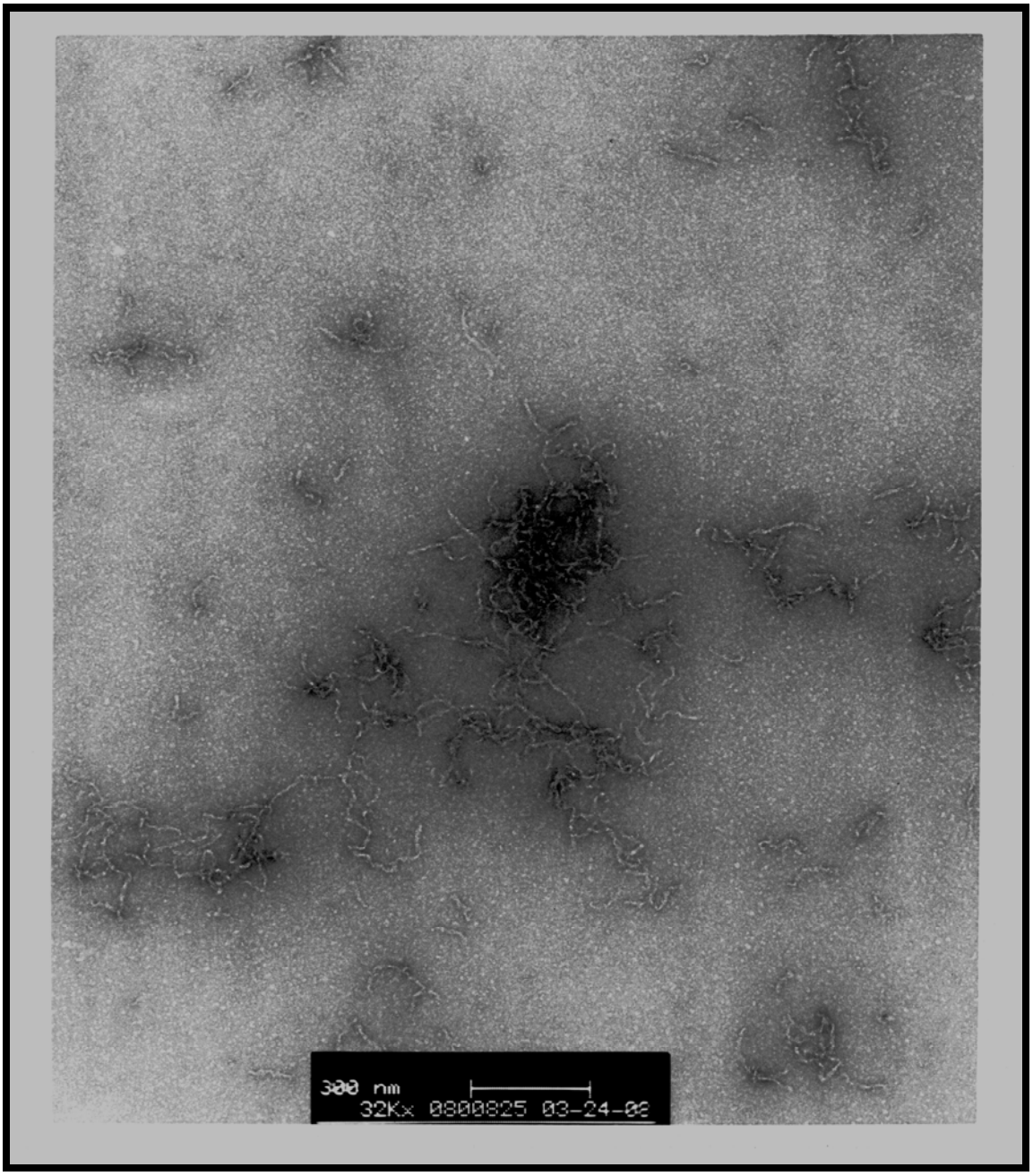

Figure 4.5A. TEM micrograph of 8 % w/w β-lactoglobulin at pH 3.35 with no additional ionic strength.

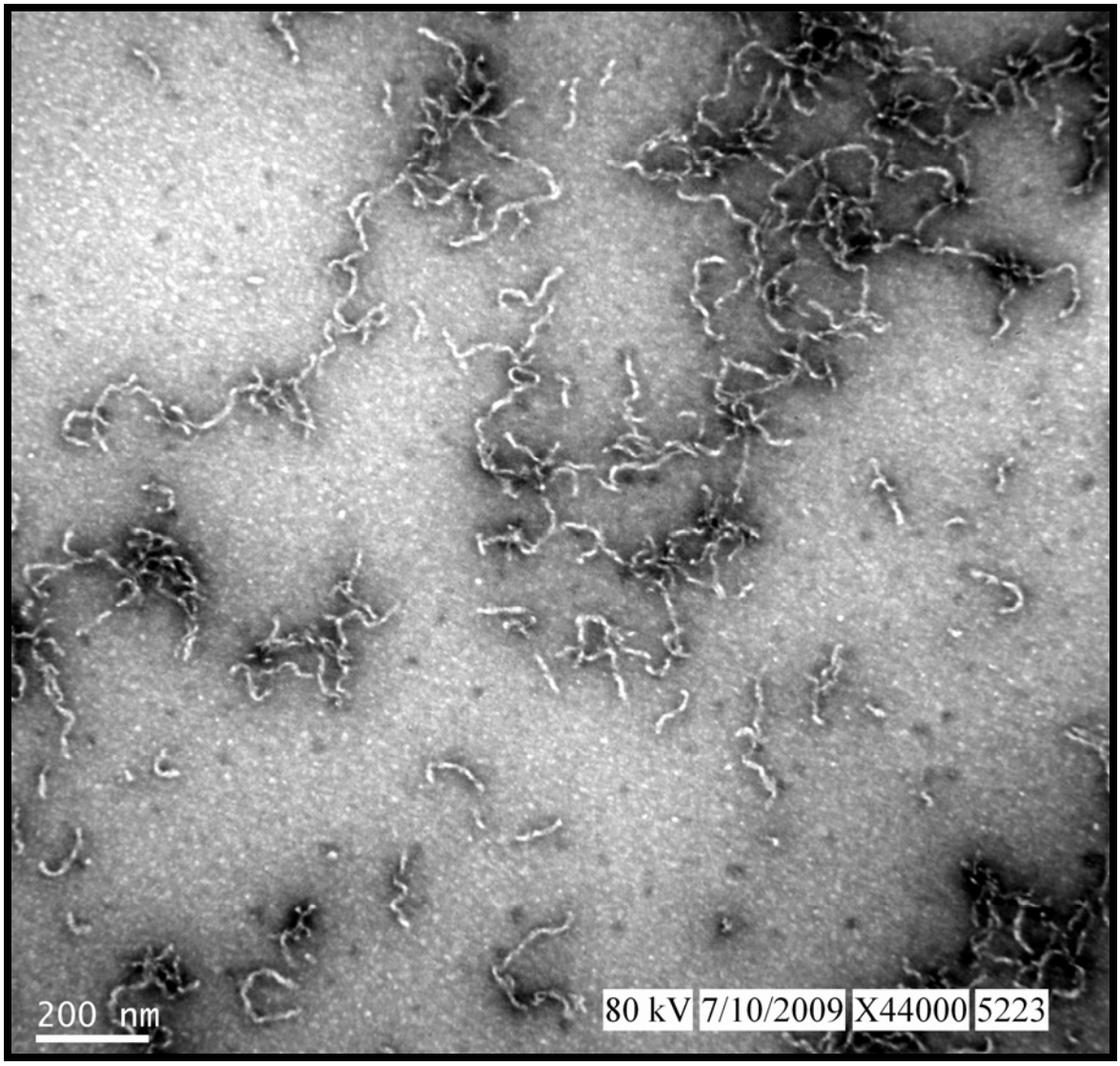

Figure 4.5B. TEM micrograph of 8 % w/w β-lactoglobulin at pH 3.35 with 20 mM ionic strength.

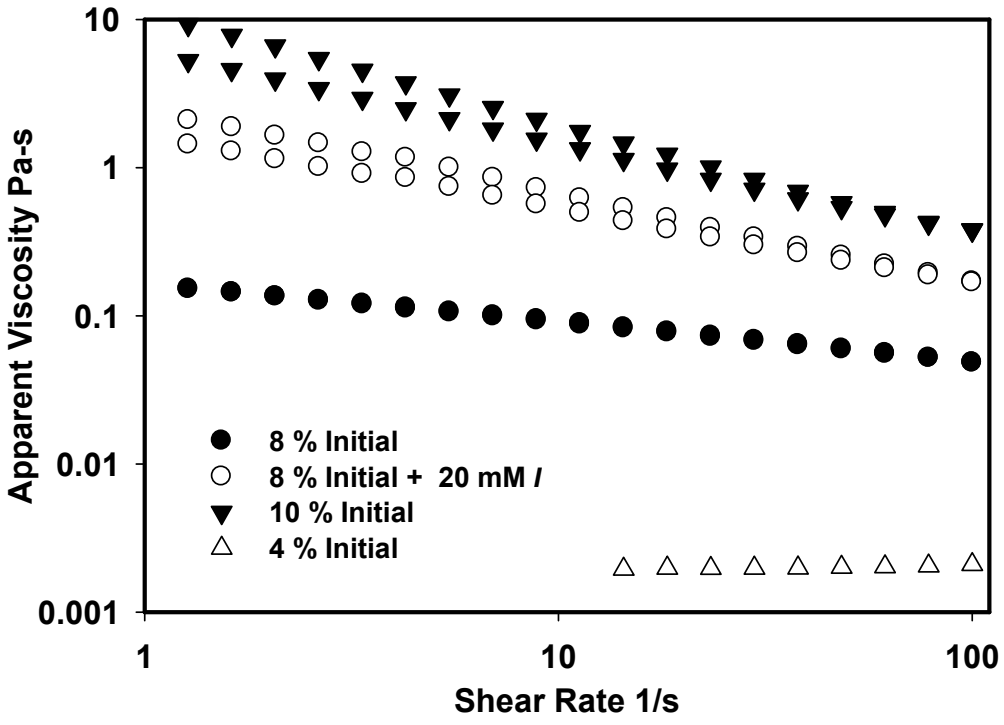

Figure 4.6A. Apparent viscosities of modified β-lactoglobulin powders reconstituted at 10 % w/w

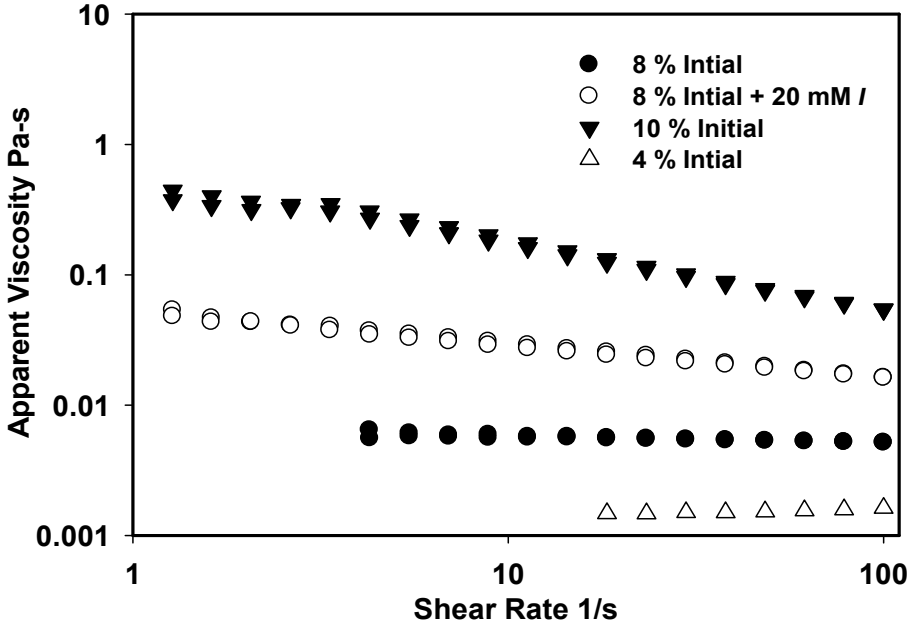

Figure 4.6B. Apparent viscosities of modified β-lactoglobulin powders reconstituted at 5 % w/w

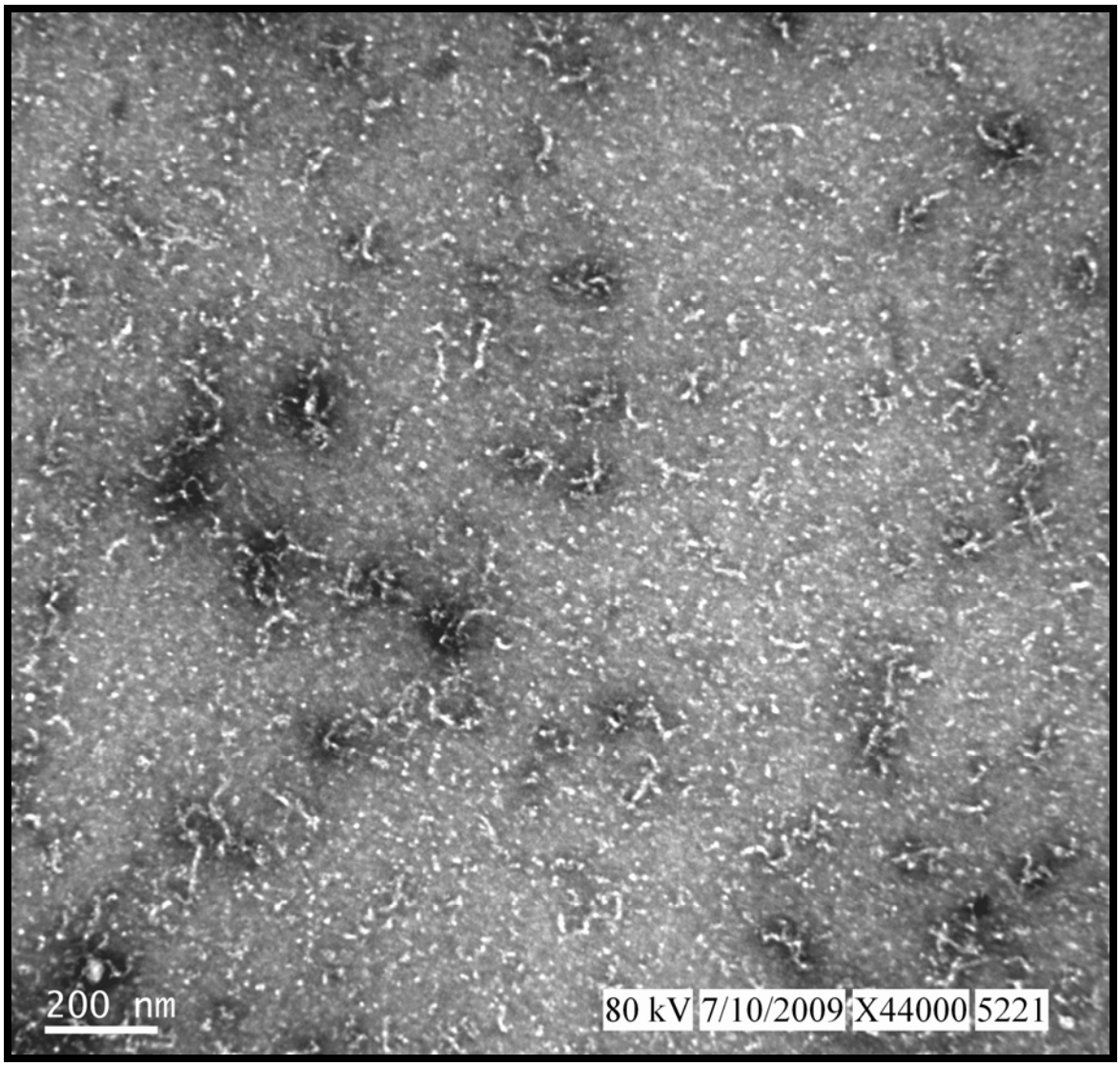

Figure 4.7A. TEM micrograph of reconstituted mβlg with 4 % PC and no additional ionic strength

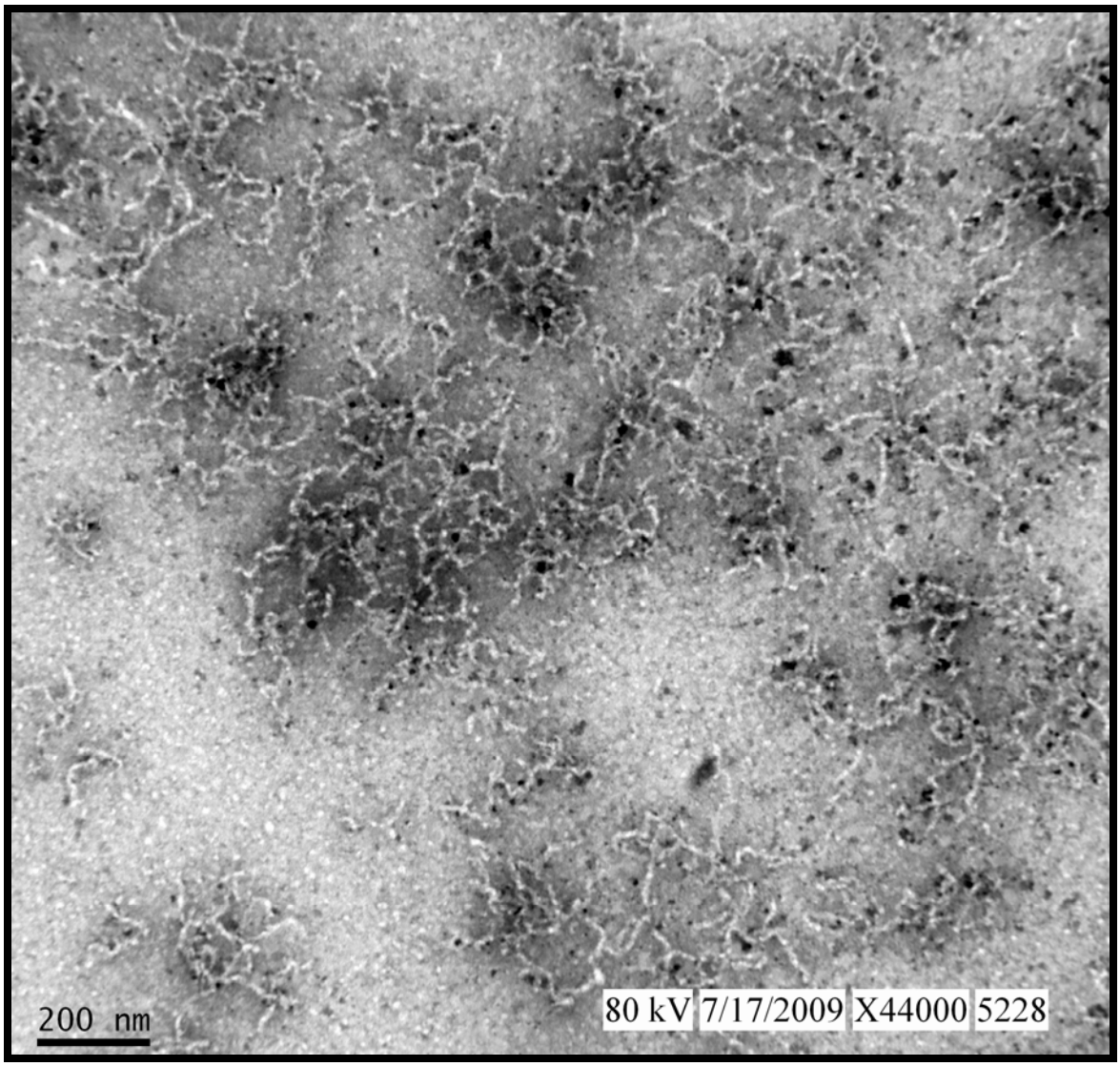

Figure 4.7B. TEM micrograph of reconstituted mβlg with 8 % PC and no additional ionic strength**.**

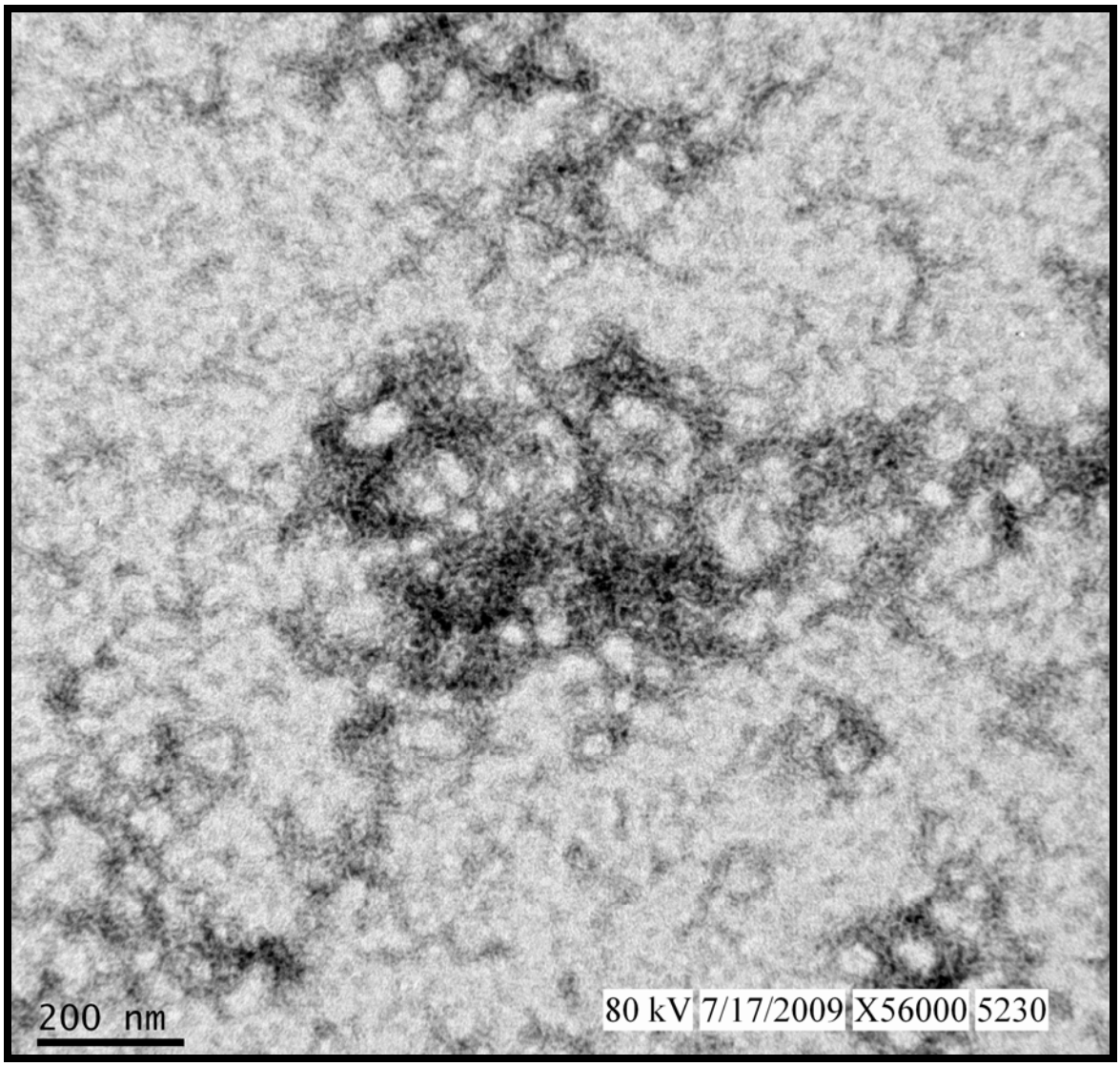

Figure 4.7C. TEM micrograph of reconstituted mβlg with 8 % PC and 20 mM additional ionic strength

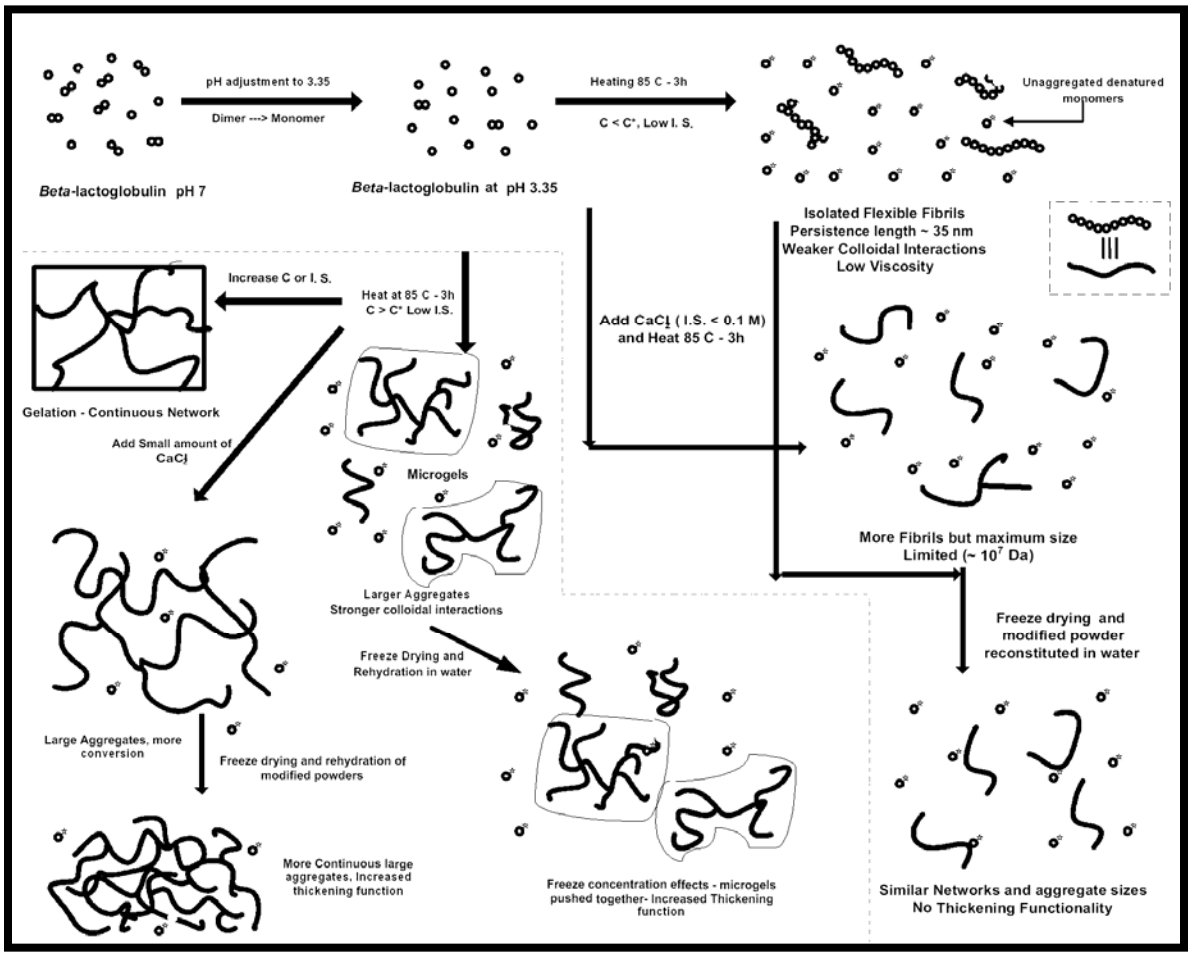

Figure 4.8. Schematic representation cold-thickening mechanism of β-lactoglobulin at pH 3.35: Concentration and ionic strength effects

CHAPTER 5.

DISULFIDE INTERACTIONS AND HYDROLYSIS EFFECTS ON β-LACTOGLOBULIN AGGREGATION AT LOW pH

To be submitted to: The Journal of Agricultural and Food Chemistry

5.1 ABSTRACT

Roles of sulfhydryl/disulfide interactions and acid hydrolysis on β-lg thermal aggregation at acidic pH 3.35 and 2 were studied using rheology, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), transmission electron microscopy (TEM), and western blotting. The β-lg solutions were treated with pepsin to examine the effects of enzymatic hydrolysis on the functionality of heated β-lg dispersions at pH 3.35. Pepsin promoted further hydrolysis as measured by 12 % increase in free amines beyond the acid hydrolysis at pH 3.35, resulting in an increase of approximately two logs to the viscosities of heated 8 % w/w β-lg solutions at pH 3.35. Seemingly, hydrolysis promoted thermal aggregation of β-lg, correlating well with viscosity increases. During thermal aggregation (85 °C – 3h) of β-lg at pH 3.35, limited disulfide linked aggregation was seen compared to that at pH 7, based on electrophoresis data. However, beyond the existence of limited disulfide interactions, acid hydrolysis and non-covalent interactions more likely play a crucial role in defining the functionality of acidified powdered modified whey ingredients.

5.2 INTRODUCTION

A cold-thickening, pH-modified whey protein ingredient was developed to impart thickening function upon reconstitution in water, without addition of salt or heat (Hudson et al., 2000). Cold-thickening whey ingredients have proven to be a suitable alternative to conventionally-used starches for food applications where 1) heat may not be desirable, or 2) nutritional

benefits are desired (Hudson et al., 2000, Resch et al., 2002). The concentration effects on the cold-thickening mechanism of this ingredient were studied at the pH of the modification procedure, 3.35, using a β-lg model system (Mudgal et al., 2009). A critical concentration (C_c ~ 6.4 % w/w protein, 6.9 % w/w solids) was reported for this system, below which no significant thickening function was achieved from modified powders, even when reconstituted at elevated solids concentration (10 % w/w) (Mudgal et al., 2009). During heating (85 °C – 3h) at pH 3.35, β-lg monomers formed flexible fibrillar networks with ~ 5 nm diameter strands and ~ 35 nm persistence lengths (Mudgal et al., 2009). β-Lg is the major (~ 60 %) constituent of commercially available whey protein products (Langton and Hermansson, 1992) and is believed to be the only protein responsible for fibril formation in whey products at pH 2.0 (Bolder et al., 2006). β-Lg aggregation mechanisms thus affect the functional properties of commercial whey protein products.

Depending on the pH and ionic strength, β-lg forms different types of aggregates upon heating, leading to formation of either fine stranded or particulated gels (Langton and Hermansson, 1992; Havea et al., 2004). β-Lg forms a particulated network in the pH range of 4-6. Transparent, fine stranded gels, however, are formed below and above this region of pH and at low ionic strength conditions (Langton and Hermansson, 1992). Depending on pH, β-lg molecule net charge will vary, with zero net charge at the isoelectric point, 5.1. More specifically, β-lg is a globular protein with two intramolecular disulfides (Cys66-Cys160 and Cys 106-Cys 119) and one free sulfhydryl group identified at Cys residue 121 (Sakurai et al., 2001). This protein is mostly found to exist either as a monomer or dimer, and there is a

monomer-dimer equilibrium which is affected by pH, ionic strength, and temperature (Aymard et al., 1996, Sakurai et al., 2001, Renard et al., 1998). Upon heating, β-lg denatures, and buried hydrophobic regions and thiol groups become solvent accessible (Vardhanabhuti et al., 1999, Moro et al., 2001). The formation of aggregates during gelation involves covalent (disulfide bonds) or non-covalent interactions, such as hydrophobic, electrostatic, ionic, and van der Waals, where each interaction is weaker than covalent bonding. Several studies have attempted to define the relative roles played by each type of interaction during whey protein gelation at neutral pH (Havea et al., 2004; Vardhanabhuti and Foegeding, 1999; and Alting et al., 2000, 2002). However, these interactions change with pH. For example at neutral to alkaline pH, disulfide interactions are favored (Creighton, 1988). Overall, the relative contribution of these various interactions still remains generally unknown.

At pH 7.0, β-lg aggregation is a result of a combination of various reactions. Upon heating above 70 °C, β-lg denatures and dimers dissociate into monomers, and the thiol and hydrophobic groups become solvent accessible. Then, aggregates are formed through thiol-disulfide exchange and also by thiol oxidation and non-covalent interactions (Galani et al., 1999; Hoffmann et al., 1997). Below pH 7, the existence of disulfide bonding is limited; therefore at low pH, when proteins are heated, other types of bonding become more relevant (Schokker et al., 2000). At pH 2.0, β-lg is highly charged, carrying a positive charge of + 20. At pH 2.0, it was shown that β-lg forms fibrillar aggregates with diameter ~ 4 nm and length 1-10 μm (Veerman et al., 2002, 2003; Ikeda et al., 2002; and Kavanagh et al., 2000, Bolder et al., 2007, Akkermans et al., 2008, Van der Linden et al., 2007) at low ionic strength

conditions. The length and flexibility of these fibrils vary with pH and ionic strength. Recently, it was observed that at pH 2, acid hydrolysis of β-lg takes place between aspartic acid residues and other amino acids (Akkermans et al., 2008). Peptides between 2000-8000 Da were proposed to be the building blocks for fibrils formed at pH 2 (Akkermans et al., 2008).

Effects of enzymatic hydrolysis of β-lg on gelation properties and its microstructure have been well documented, especially at pH 7 or 8 (Otte, 1997a) using different proteases such as trypsin, Glu and Asp specific protease from *Bacillus licheniformis*, *Bacillus subtilis*, Alcalase, bromelain, and papain. Limited proteolysis lowered the gel point and improved gelation properties (Otte, 1997a; Otte, 1996; Chen, 1994; Ju, 1995; Cassens et al., 1999; Doucet, 2005; Otte, 1997c, Guo, 1995). Fewer studies, however, have studied the effects of enzymatic hydrolysis on β-lg aggregation and rheology at low pH conditions (Dalgalarrondo, 1995; Akkermans, 2008b; Guo, 1995).

The first objective of this study was to investigate the role of disulfide interactions during the β-lg thermal aggregation at pH 3.35. The second objective was to determine and compare the role of acid hydrolysis versus pepsin hydrolysis on β-lg thermal aggregation at pH 3.35. Network characteristics and the mechanism of β-lg aggregation at pH 2, 3.35 and 7 were studied and compared using microscopy, SDS-PAGE, western blotting, OPA assay, and rheology. Finally, the cold-thickening mechanism at pH 3.35 was discussed with respect to disulfide interactions and partial β-lg hydrolysis.

5.3 MATERIAL AND METHODS

5.3.1 Protein material

β-Lg (BioPure^{R}, ~ 94 % pure, total protein 98 % dry basis) was donated by Davisco Foods, Inc. The wet basis protein content was determined by micro-Kjeldahl (AOAC, 1984) to be 92.81 %.

5.3.2 Solution preparation

β-Lg solutions at desired concentrations (3, 5, 7, 8 and 9 % w/w solids) were prepared by dissolving β-lg in de-ionized (DI) water by continuous stirring at room temperature for 1-2 hours. Sodium azide (0.02 %) was added to all samples to prevent microbial growth. Thereafter, solutions were adjusted to pH 3.35 using 6 N HCl. Following pH adjustment, solutions were heated at 85 °C for 3 hours in a water bath. Heated solutions were cooled and stored in a 4 °C refrigerator for 24h.

5.3.3 Preparation of modified β-lg powders

The next day, sols/gels were quickly frozen in 100-200 ml freeze drying glass bottles by immersing in a mixture of methanol and dry ice. The frozen samples were then freeze dried

using a 4.5 L bench top freeze dryer (Labconco 73035, Kansas City, Mo). The dried material was milled manually to a fine powder.

5.3.4 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Solutions of β-lg at pH 3.35, pH 2.0 and pH 7.0 (7 % w/w solids, unheated and heated for 85 °C – 3 hours) were studied. Also, reconstituted modified β-lg powders prepared at different preliminary protein concentrations (3, 5, 7, and 9 % w/w solids) were studied. β-Mercaptoethanol (β-ME) is a denaturant that reduces disulfide bonds, and all aforementioned samples were studied with both reducing (+ β-ME) and non-reducing (- β-ME) gels to examine the disulfide bond patterns. The β-lg samples, prepared at 8% protein, pH 3.35, and (+/-) pepsin hydrolysis were studied in the presence of 5.0% β-mercaptoethanol. A Bicinchoninic Acid (BCA) assay (Methodology developed by Thermo Scientific Inc., Rockford, IL) was performed on all samples prior to electrophoresis to determine the protein content. Based on BCA results, β-lg samples were diluted with distilled, de-ionized (DD) water to a uniform protein concentration of 2 mg/ml. Thereafter, diluted β-lg samples were mixed with Tricine-SDS sample buffer [(InVitrogen Inc., Carlsbad, CA); (+/-) βME, at a 1:1 v/v ratio, then heated at 100 °C for 10 minutes prior to loading on to 10-20 % Tris-Tricine polyacrylamide gels (InVitrogen Inc.)]. After electrophoresis, gels were stained using a colloidal blue staining reagent (InVitrogen Inc.).

Pepsin Hydrolysis: Native β-lg is resistant to hydrolysis by pepsin because of its compact globular structure (Guo et al, 1995, Dalgalarrondo et al., 1995). Heat treatment above the

denaturation temperature for 10-15 minutes can render it accessible to pepsin hydrolysis (Guo et al., 1995). Therefore, β-lg solutions (8 % w/w solids), pH 3.35 were heat denatured at 85 °C for 15 minutes (< critical time for gelation under these conditions) to render it accessible to pepsin hydrolysis. Then, these solutions were incubated with pepsin (Sigma Chemicals P-6887, porcine pepsin, 3276 Units/mg solids, P-6887) at 37 °C. The β-lg -pepsin mix was shaken at 130 rpm for 2, 4, 6, 24 and 48 hours. Pepsin was added at an enzyme: protein ratio of 1:50 and 3276 units of protease activity were added. Control β-lg solutions, without pepsin treatment, were processed identically for comparison purposes. At least two independent replicates were performed.

5.3.5 Western Blott

To confirm the identity of higher and lower molecular weight β-lg bands, western blotting was performed. After transfer, the membranes were blocked overnight at room temperature using 3% gelatin (BioRad, Inc.) prepared with phosphate-buffered saline containing 0.02% Tween (PBS/Tween). Polyclonal rabbit antibodies, directed against β-lg (Bethyl Laboratories, Montgomery, Texas), were diluted 1:2000 with PBS/Tween containing 1% gelatin and incubated with the membrane for a minimum of 2-3 h at room temperature. After washing 3X with PBS/Tween, the membrane was incubated with the detection antibody solution. In this case, a goat-anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Pierce, Rockford, Illinois) was diluted 1:2000 with PBS/Tween/1% gelatin and incubated for an additional 2-3 h at room temperature. Again, after rinsing 3X with PBS/Tween, positive β-lg

protein bands were visualized after addition of the HRP substrates, diaminobenzidine and hydrogen peroxide (Pierce Inc., Rockford, IL).

5.3.6 Ortho-pthalaldehyde (OPA) Analysis

Hydrolysis of β-lg results in formation of peptides and an emergence of more reactive amino groups. A degree of hydrolysis can be linearly correlated to the increase in concentration of reactive amino groups. The concentration of reactive amino groups was determined using an OPA assay. All samples were microcentrifuged (13,600 *x g*, 5 minutes, room temperature) prior to analysis, and the soluble supernatant diluted appropriately. Then, 25 µl of the sample volume was added to 2.0 ml of the OPA reagent, incubated for 5 minutes at room temperature, and the absorbance read at A340 nm using a Gilford 2600 spectrophotometer (Oberlin, Ohio). All final readings fell within an absorbancy range from [0.1 - 1.5] at A340 nm, and each data point was made in triplicate. This procedure was adapted from the method of Church and coworkers (1983).

5.3.7 Transmission electron microscopy (TEM)

Heated β-lg solutions (8 % w/w) were prepared at pH 3.35, and pH 7. All samples were diluted with DI water to a concentration of 0.04 % and the pH was adjusted to 3.35 using 6 N HCl prior to TEM analysis. TEM samples were prepared by negative staining, and a drop of

diluted sample was deposited on a copper grid (formvar coated and further coated with evaporated carbon). Excess sample was removed using filter paper. A drop of
2 % uranyl acetate or phosphotungstic acid (PTA), pH 3.5, was added for 60 s and excess reagent removed. Digital electron micrographs were obtained using a FEI/Phillips EM 208S Transmission electron microscope (made by Phillips, Czech Republic).

5.3.8 Shear rate sweeps

Shear rate sweeps were performed at 25 °C using a stress controlled rheometer (ATS Rheosystems, Bordentown, NJ) to characterize flow behavior and measure the apparent viscosity of β-lg solutions. Two concentrations (7 and 8 % w/w), prepared above the identified β-lg critical concentration for the modification process (C_c ~ 6.9 % w/w solids) were selected. A smooth, 25-mm concentric cylindrical geometry was used, and shear rates were varied from 1 to 100 s^{-1} using a constant rate program to minimize inertial effects. Shear rates were varied over a time period of 600-900 s. Hysteresis was identified with two consecutive runs of increasing and decreasing shear rates on β-lg solutions. A pre-shear condition (15 s^{-1} for 30 s) was applied to all test solutions to obtain uniformity prior to measurement. Furthermore, a thin film of mineral oil was applied to the sample surface to minimize sample dehydration. To characterize flow behavior, power law model parameters were determined. Equation one presents the power-law model used to characterize flow behavior. At least, two independent replicates were performed for all solutions.

$$\eta = K\dot{\gamma}^{n-1} \quad \textit{Eq. 1}$$

5.3.9 Charge calculation on β-lg as a function of pH

Proteins are polymers of amino acids. Based on the respective pKa and pKb of these amino acids, proteins acquire charge depending on the pH. Based on the primary structure of bovine β-lg genetic variant B (Farrell et al, 2004), the charge on β-lg was approximated at different pH values.

5.4 RESULTS AND DISCUSSION

5.4.1 pH effects on the apparent viscosity of heated β-lg dispersions

To determine pH effects on the rheology of heated β-lg dispersions shear rate sweeps were performed. The apparent viscosity of heated β-lg dispersions (85 °C -3 hours) at 7 and 8 % w/w solids (above C_c ~ 6.9 % w/w, pH 3.35) at pH 3.35, 2, and pH 7 were measured, and power law indices were calculated. A power law index (n) closer to 1 represents Newtonian flow behavior, while lower n values denote pseudoplasticity. Dispersions at pH 3.35 and 7 displayed more Newtonian-like flow behavior (n > 0.98) at both 7 and 8 % (w/w) concentrations with comparable viscosities (Figure 5.1). On the other hand, heated dispersions at pH 2 were very viscous (2-3 logs higher than at pH 3.35), showing pseudoplastic behavior (n ~ 0.33 at 7 % w/w and n ~ 0.17 at 8 % w/w) and formed a solid-like gel at 8 % solids concentration. The apparent viscosity increased with concentration (7-8 % w/w) at all pH values, with a significant increase at pH 2 (~ 1 log). The viscosity of a

solution is affected by the shape, size of aggregates and interactions among these aggregates. β-Lg is known to form long, linear fibrils at pH 2, which increase viscosities on a weight efficient basis because of their large hydrodynamic volume, resulting in a reduced critical concentration for gelation (Van der Linden et al., 2007, Akkermans et al., 2008).

5.4.2 Role of disulfide interactions during modification process at pH 3.35 and at pH 2 using (+/-) β-ME

Disulfide interactions are favored at neutral to alkaline pH and play an important role in β-lg aggregation at pH 7 (Creighton, 1988, Vardhanabhuti et al., 1999, Havea et al., 2004, Alting et al., 2002, Alting et al., 2004). To determine whether disulfide bonds result from the modification process at pH 3.35, SDS-PAGE was performed under reducing (+ βME) and non-reducing conditions (- βME). Monomers, dimers and some distinct oligomeric bands were observed in non-heated solutions, pH 3.35 (Figure 5.2A, Lane1). Western blotting confirmed that these high molecular weight bands were indeed β-lg oligomers (Figure 5.6B). These oligomers were present in the unheated samples and could not be reduced to monomers in the presence of β-ME, this suggested that either β-ME: protein ratio was low or they were held together with isopeptide bond cross-links. Increasing β-ME: protein ratio did not result in further reduction of these dimers and oligomers (data not shown), suggesting that these oligomers were held together with isopeptide bonds. Isopeptide cross-links are covalently held and thus can not be reduced with β-ME. Isopeptide bonds can form between the side chain amine of lysine and the side chain carboxyl group of either glutamate or

aspartate (Stryer, 1995). It has been reported that about 1-2 % of the lysine in milk proteins in bound in isopeptide cross-links (Lorenzen, 2007). As whey proteins undergo some degree of heat treatment during their purification, the amount of isopeptide cross-links could be more and explains β-dimers and oligomers in the starting β-lg material. While in heated sample at pH 3.35, a HMW smearing pattern (Figure 5.2A, lane 2) was observed which disappeared in the presence of β-ME as visible in the reducing gel (Figure 5.2B, lane 2), indicating that these complexes were likely linked through disulfide bonds. These results confirmed that disulfide linked aggregates were formed during the modification process (85 °C – 3 h heating at pH 3.35).

In the non-reducing gel, lane 3 (Figure 5.2A), dimers and some distinct oligomeric bands were observed in unheated β-lg at pH 2, similar to the unheated β-lg at pH 3.35 (Figure 5.2A, lane 1) further suggesting that these oligomers were present in the starting material and were held together with isopeptide cross-links. Whereas, in heated samples at pH 2 (lane 4, Figure 5.2A), no continuous smearing of high molecular weight complexes was observed, suggesting no disulfide aggregate formation. For heated samples at pH 2.0, oligomers disappeared under both reducing and non-reducing conditions (Figure 5.2A, lane 4), with some loss of dimer formation under reducing conditions (Figure 5.2B, lane 4). Several distinct bands of low molecular weight were seen, likely caused by acid hydrolysis during extensive heating (85 °C – 3h) of samples at pH 2 (Figure 5.2, lane 4). A few distinct lower molecular weight bands were also observed at pH 3.35, further suggesting that some degree of acid hydrolysis took place under these conditions (Figure 5.2B, lane 2). Although acid

hydrolysis occurred at both pH 3.35 and 2 as expected, it was more effective at pH 2. There is also recent evidence of acid hydrolysis and its role in fibril formation at pH 2 by independent investigators (Bolder et al., 2007; Akkermans et al., 2008). Furthermore, it was reported that peptides resulting from acid hydrolysis at pH 2 participated in β-lg fibril formation (Akkermans et al., 2008).

In an earlier study, a critical concentration (C_c) (using a β-lg model system, pH 3.35) was identified as 6.9 % w/w solids (Mudgal et al., 2009). No significant thickening function was achieved using modified powders prepared at starting concentrations below the C_c, regardless of reconstituting concentration (Mudgal et al., 2009). To understand these concentration dependent differences, SDS-PAGE was performed on modified β-lg powders prepared at two protein concentrations below (3, 5 % w/w solids) and two above the C_c (7 and 9 % w/w solids). High molecular weight complexes were observed in all modified β-lg samples at different starting protein concentrations, as evidenced by the banding patterns seen in the non reducing gel (Figure 5.2A, lanes 5-8). These complexes were much less prominent in the reducing gel, containing β-ME, following reduction of disulfide bonds. Similar protein band profiles were obtained for all modified samples, irrespective of the initial protein concentration from which manufactured. Previously, differences in functionality were clearly established by Mudgal et al., (2009), in which case, those results combined with these observations strongly suggest that in addition to disulfide bonds, non-covalent interactions most likely play a crucial role when defining the functionality of powdered, modified whey ingredients at pH 3.35. Lastly, SDS-PAGE results at low pH (2 and 3.35) were compared to

those at pH 7.0 under non-reducing conditions (Figure 5.2C). These patterns revealed formation of very high molecular weight complexes at pH 7.0, as compared to either pH 3.35 or pH 2.0, and may be attributed to more favorable disulfide interactions at higher pH as thiol groups are less stable at alkaline pH (Figure 5.2C).

5.4.3 Structural differences at pH 7 and 3.35 as observed through TEM

TEM was accomplished to compare network characteristics of heated β-lg dispersions at pH 7 and pH 3.35, as these systems displayed similar viscosities versus those at pH 2 (Figures 5.3A and 5.3B). At pH 7, repeating structural units were connected, and β-lg strands were visible within these structures (Figure 5.3A), while fine stranded flexible firbillar structures were observed at pH 3.35, 8 % w/w (Figure 5.3B). Langton and Hermansson (1992) reported differences in strand characteristics above pH 6 to those below pH 4.

5.4.4 Pepsin hydrolysis of β-lg and the effect on apparent viscosity of heated β-lg dispersions at pH 3.35

In recent studies, it was shown that acid hydrolysis of β-lg at pH 2 plays an important role in fibril formation (Akkermans et al., 2008a and 2008b). In the present study, hydrolysis was detected at pH 2, with lesser hydrolysis at pH 3.35 based on SDS-PAGE results. Using this

information together with viscosity data, acid hydrolysis of β-lg at low pH is hypothesized to promote thermal aggregation, resulting in higher viscosity of heated dispersions. To test this hypothesis, β-lg was hydrolyzed with pepsin at pH 3.35 to determine the effects of additional enzymatic cleavage on the apparent viscosities of heated β-lg solutions.

Preheat treatment at 85 °C – 15 minutes rendered β-lg more susceptible to pepsin hydrolysis with no significant increase in the viscosity at pH 3.35 (Figure 5.4). Pepsin-treated β-lg and the control held at (24 h, 37 °C) exhibited similar viscosities and displayed Newtonian flow. After 24 hours of enzyme treatment increased there was an increase of approximately 12 % in concentration of free amines compared to the control samples, based on OPA assay measurements (data not shown). There was no further increase in concentration of free amines after 48 hours; however, the viscosity of the pepsin-treated β-lg for 48 h was higher than the 24 h samples (Figure 5.4). This outcome may have resulted because of the formation of aggregates, generated during hydrolysis of β-lg when incubated for longer periods. Formation of aggregates after hydrolysis at pH 7 was reported in past research (Otte et al., 1997a, 1997b). The 48 h – pepsin treated sample also displayed pseudoplasticity ($n \sim 0.88$) as explained earlier. The apparent viscosity of pepsin-treated β-lg solutions increased significantly (~ two log) after heating as compared to control samples at pH 3.35. Both 24 h and 48 h pepsin-treated samples exhibited similar viscosities after heating (Figure 5.4). These results suggested that the additional hydrolysis promoted the formation of large aggregates at pH 3.35. Pepsin-treated β-lg displayed a higher degree of pseudoplasticity ($n \sim 0.42$) when heated, indicating stronger interactions and possibly formation of larger aggregates.

Improved gelation properties of β-lg after hydrolysis were previously reported at neutral pH as well (Ju et al., 1995, Chen et al., 1994, Otte et al., 1997a).

5.4.5 Structural characteristics of heated pepsin hydrolysate at pH 3.35 using TEM

To observe network characteristics of heated β-lg (pepsin hydrolysates, 24h), TEM was performed. As observed in Figure 5.5 A and B, large fine stranded aggregates comprised of flexible fibrils were formed. The formation of these larger microgels explained increased viscosities of heated pepsin hydrolysates. Furthermore, increased size of aggregates together with stronger inter-particle interactions may explain the observed higher viscosity of heated, partially-hydrolyzed β-lg solutions.

5.4.6 Molecular weight distribution of aggregates formed during heating of pepsin hydrolysates using SDS-PAGE

To confirm that additional hydrolysis of β-lg occurred with pepsin, SDS-PAGE was performed. Pepsin-treated β-lg (24 h at 37°C) revealed the generation of a new protein band exhibiting a molecular weight < 4 kDa (Figure 5.6A). Furthermore, the larger forms of the protein substrate (tri-, tetra-, and oligo-mers) significantly diminished after pepsin digestion

(Figure 5.6A), where it appeared that pepsin was more effective in cleaving larger forms of β-lg compared to monomers or dimers.

Continued incubation with pepsin for a total of 48 h at 37°C, revealed a protein staining pattern that was essentially identical to the one seen with the 24 h pepsin-treated β-lg. These results suggested no further hydrolysis as confirmed using OPA assay (data not shown). Subsequent heating of the control sample (24 h, 37°C) at 85°C for 3 h also produced a similar banding pattern, revealing the generation of smaller sized β-lg fragments; however, in this case, these products were generated via acid hydrolysis (Figure 5.6A). Again, a concomitant loss of higher molecular-sized forms of β-lg was noted. Taken together, the hydrolytic end products generated using a pepsin-treated β-lg sample represented a combination of acid/protease released fragments with a significant increase in the apparent viscosity compared to the effects of acid hydrolysis, alone, on these flow parameters.

5.4.7 Confirmation of β-lg banding patterns using Western Blotting

Western blotting analysis confirmed distinct staining of β-lg monomers, dimers, and trimers with faint detection of oligomers (Figure 5.6B). Thus, the identity of β-lg antigen, associated with each sized protein species, was confirmed although the antiserum did not bind to the smaller end products generated either by acid hydrolysis or cleavage with pepsin. Likely, the small size of the β-lg fragment was too small to elicit antibody binding activity.

5.4.8 Cold-thickening mechanism of β-lg at acidic pH

At low pH, such as 2 and 3.35, β-lg is positively charged and experiences electrostatic repulsion. During heating, conformational changes takes place, and buried hydrophobic residues become solvent accessible. Because, aggregation and thickening at low pH is observed only during heating, hydrophobic interactions appear to play an important role. At a pH value near the isoelectric point, particulated networks with random aggregates are formed, while at pH away from the isoelectric point, fine stranded networks are formed because of controlled and limited aggregation. Therefore, at low pH, aggregation seems to result from a subtle balance among electrostatic and hydrophobic interactions. Hydrolysis results in formation of peptides, which changes protein conformation; therefore, hydrophobic interactions and charge distributions impact aggregation phenomena. In Figure 5.7, the charge distribution of β-lg as a function of pH is shown based on the primary structure of bovine β-lg genetic variant B. Acid hydrolysis is favored at low pH, whereas disulfide interactions are favored at higher pH. The aggregation mechanism of β-lg is therefore a combination of all these factors, thus affecting network characteristics, particle size, inter-particle interactions, and finally the rheology of heated protein dispersions.

5.5 CONCLUSIONS

During the modification process, thermal treatment at pH 3.35 resulted in the formation of disulfide linked aggregates, confirmed by the electrophoresis data. Very limited disulfide

interactions occurred at pH 2 after heating. Acid hydrolysis resulted in the formation of distinct lower molecular weight products at both pH 2 and 3.35, with a more prominent effect at pH 2. Pepsin further cleaved β-lg at pH 3.35 which resulted in a 12 % increase in free amines which increased the viscosity of heated β-lg solutions at pH 3.35 by two logs. Beyond the presence of limited disulfide interactions, acid hydrolysis and non-covalent interactions more likely play a crucial role in defining the functionality of powdered modified whey ingredients.

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Figures:

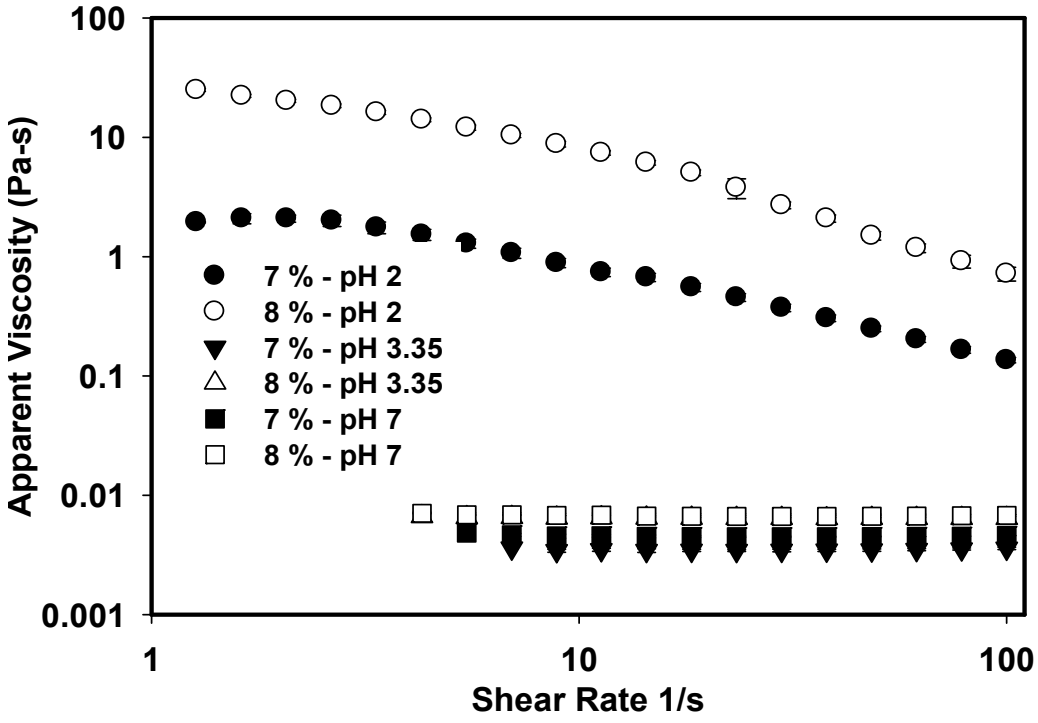
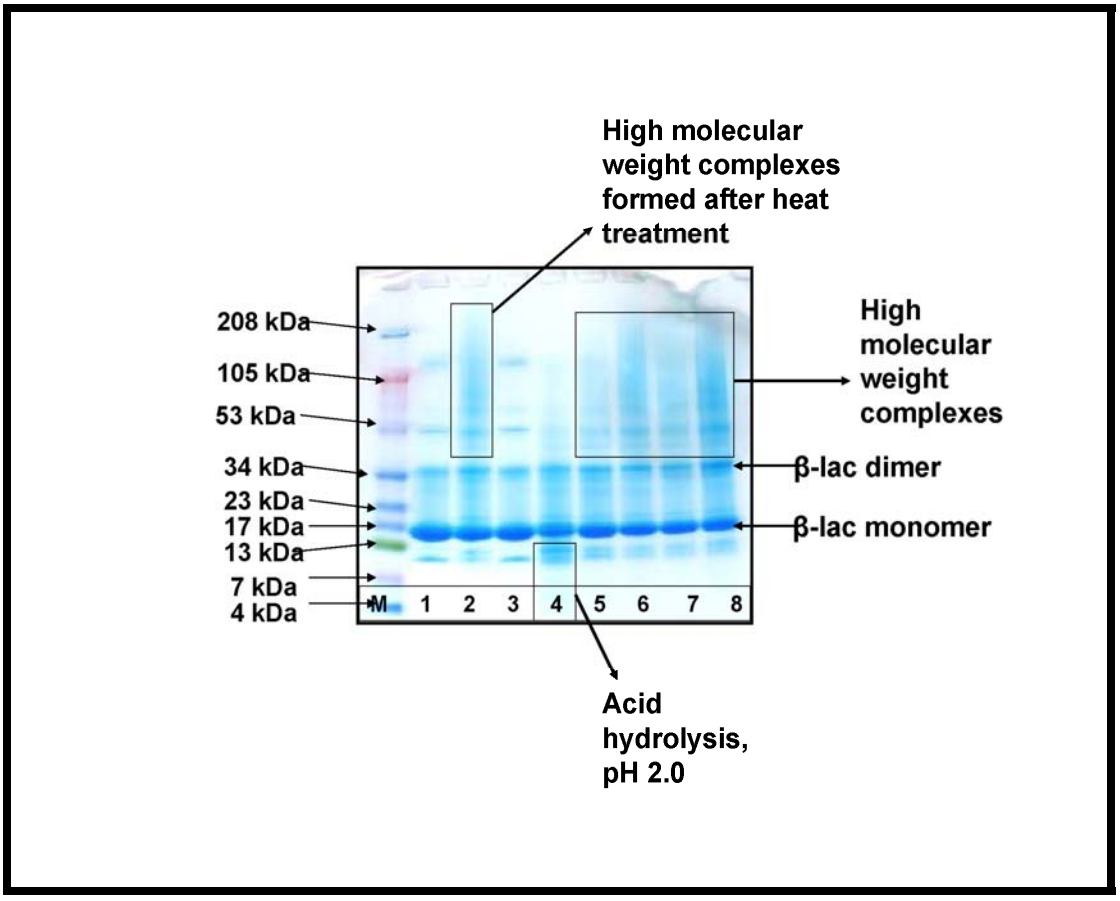


Figure 5.1. Apparent viscosity of heated β-lg dispersions at pH 2, 3.35 and 7. Error bars denote one standard deviation.



(-βME)

Figure 5.2A. SDS-PAGE banding profile of unheated and heated β-lg at pH 2 and 3.35, prepared at 7 % w/w (solids) and rehydrated modified β-lg powders prepared from 3, 5, 7 and 9 % (w/w) β-lg in the absence of β-mercaptoethanol.

Lane M: Marker, [1] pH 3.35, unheated [2] pH 3.35, heated [3] pH 2, unheated [4] pH 2, heated [5] mβlg, 3 % w/w [6] mβlg, 5 % w/w [7] mβ-lg, 7 % w/w [8] mβ-lg, 9 % w/w.

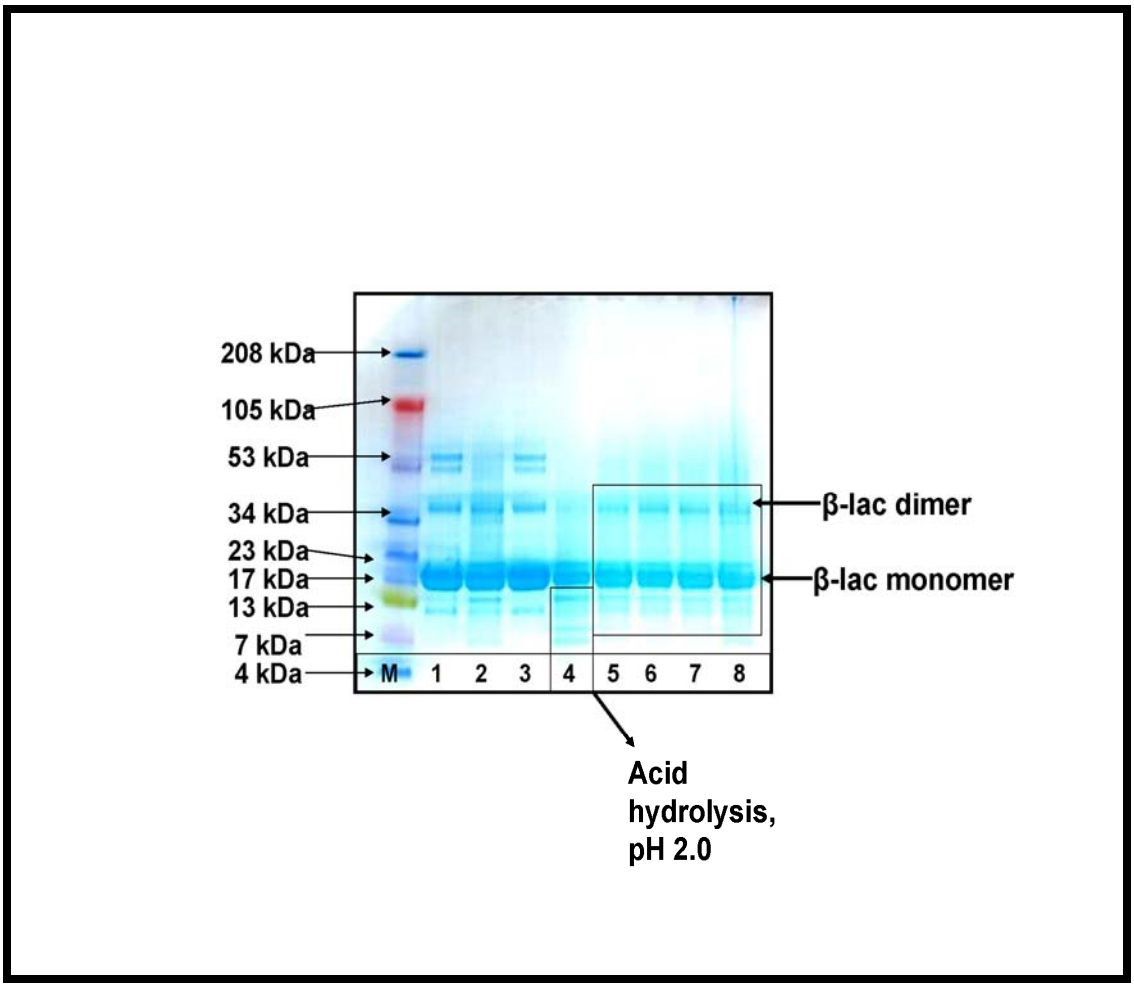
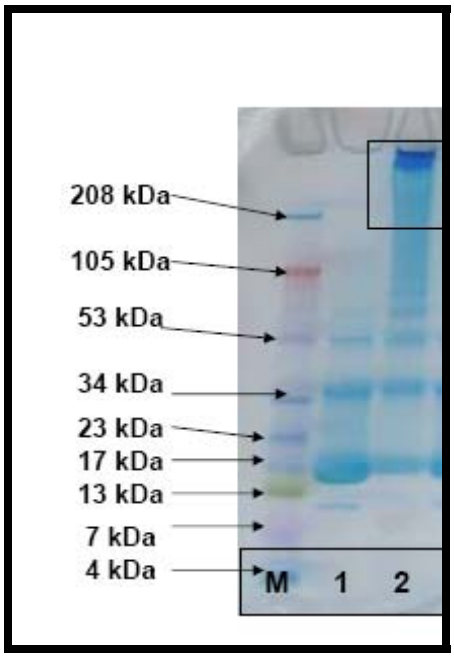


Figure 5.2B. SDS-PAGE banding profile of unheated and heated β-lg at pH 2 and 3.35, prepared at 7 % w/w (solids) and rehydrated modified β-lg powders prepared from 3, 5, 7 and 9 % (w/w) β-lg in the presence of β-mercaptoethanol.

Lane M: Marker, [1] pH 3.35, unheated [2] pH 3.35, heated [3] pH 2, unheated [4] pH 2, heated [5] mβlg, 3 % w/w [6] mβlg, 5 % w/w [7] mβ-lg, 7 % w/w [8] mβ-lg, 9 % w/w.



(-) βME

Figure 2(C)

Figure 5.2C. SDS-PAGE banding profile of unheated and heated β-lg at pH 7. Panel (C), Lane M: Marker, [1] β-lg, unheated, pH 7, (-) βME [2] β-lg, heated, pH 7, and (-) βME.

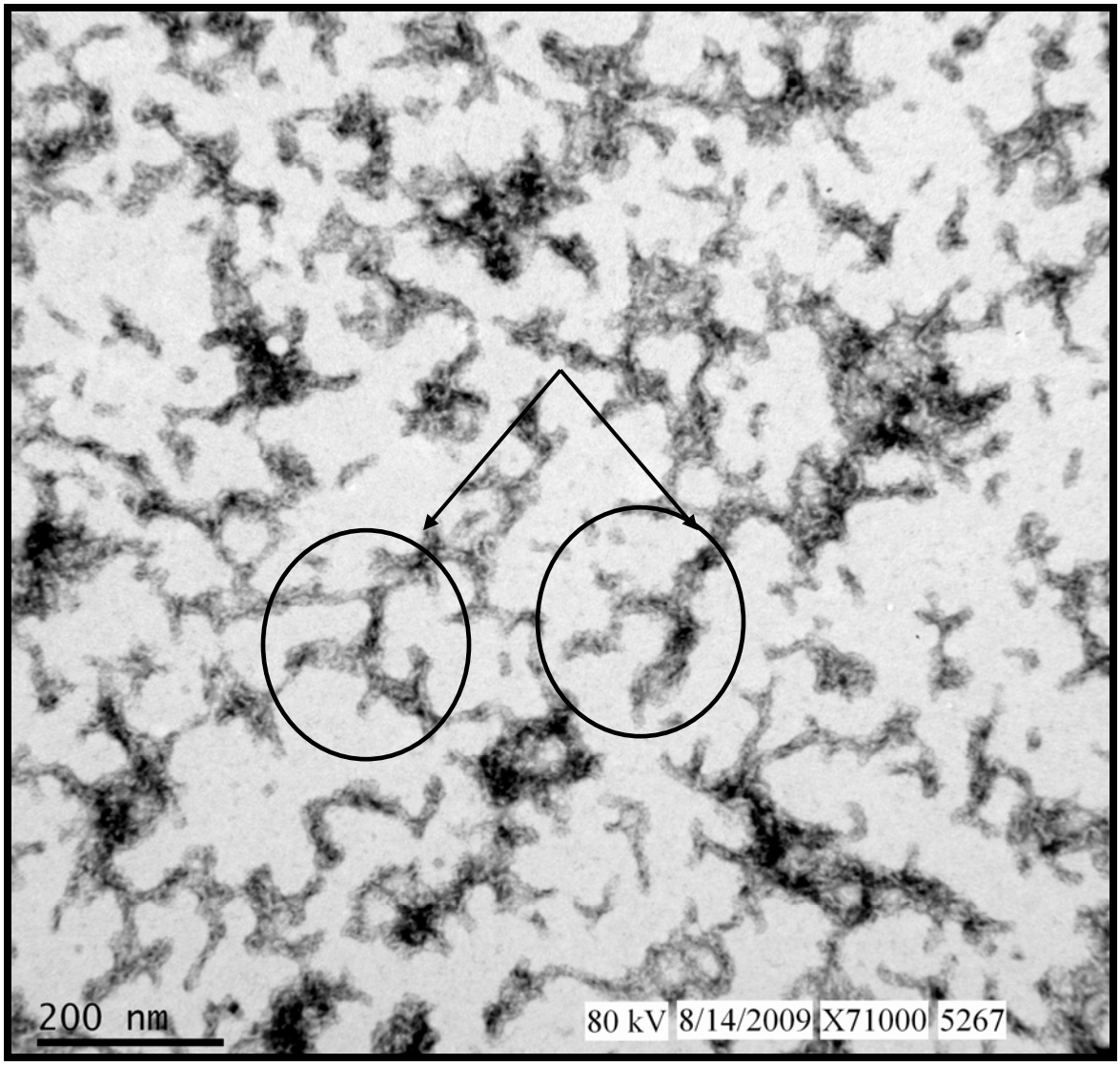


Figure 5.3A. TEM micrographs of heated β-lg dispersions at pH 7, heated at 8 % w/w, 85 °C for 3 h.

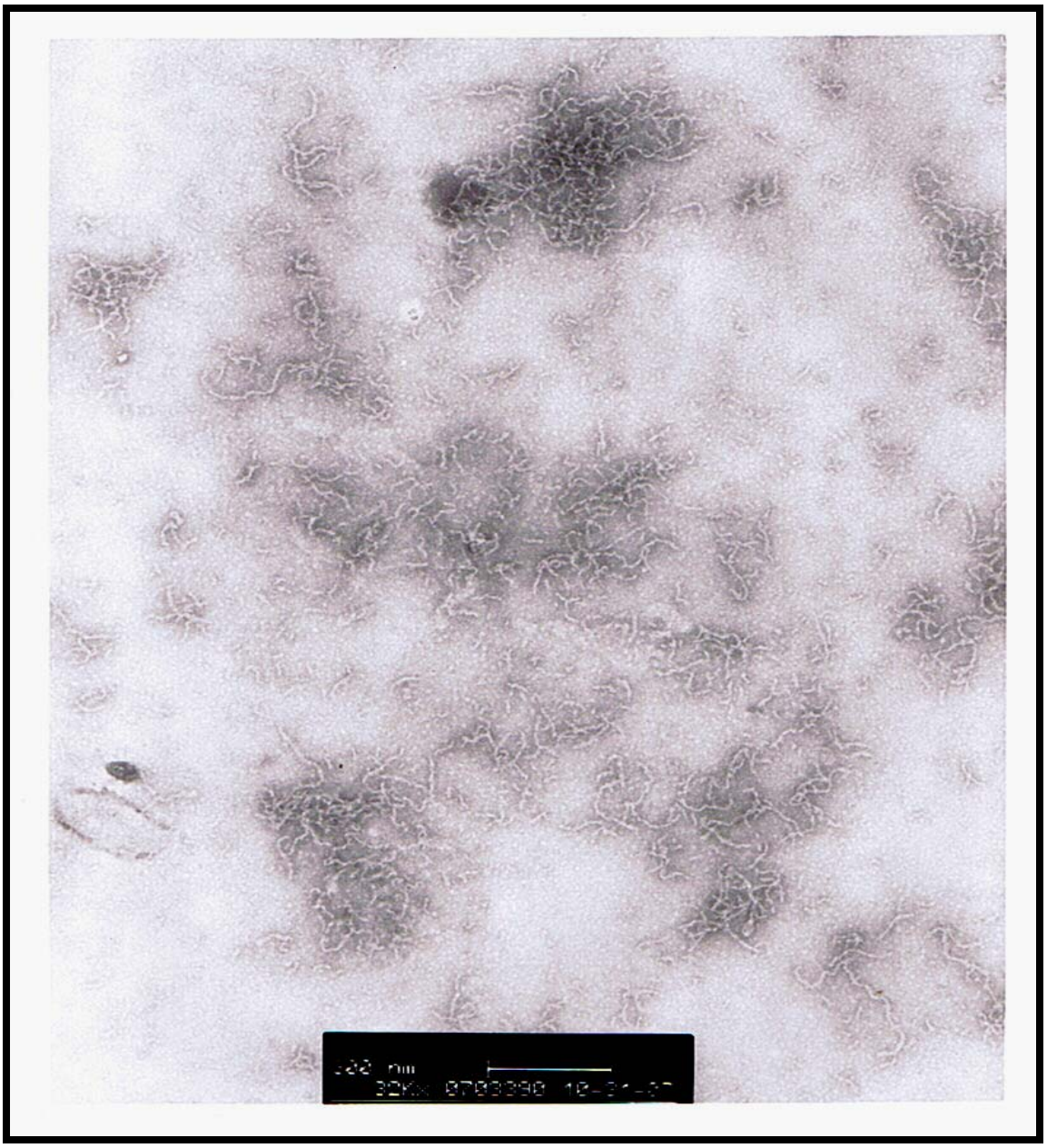


Figure 5.3B. TEM micrographs of heated β-lg dispersions at 3.35, heated at 8 % w/w, 85 °C for 3 h. Scale bar represents 300 nm.

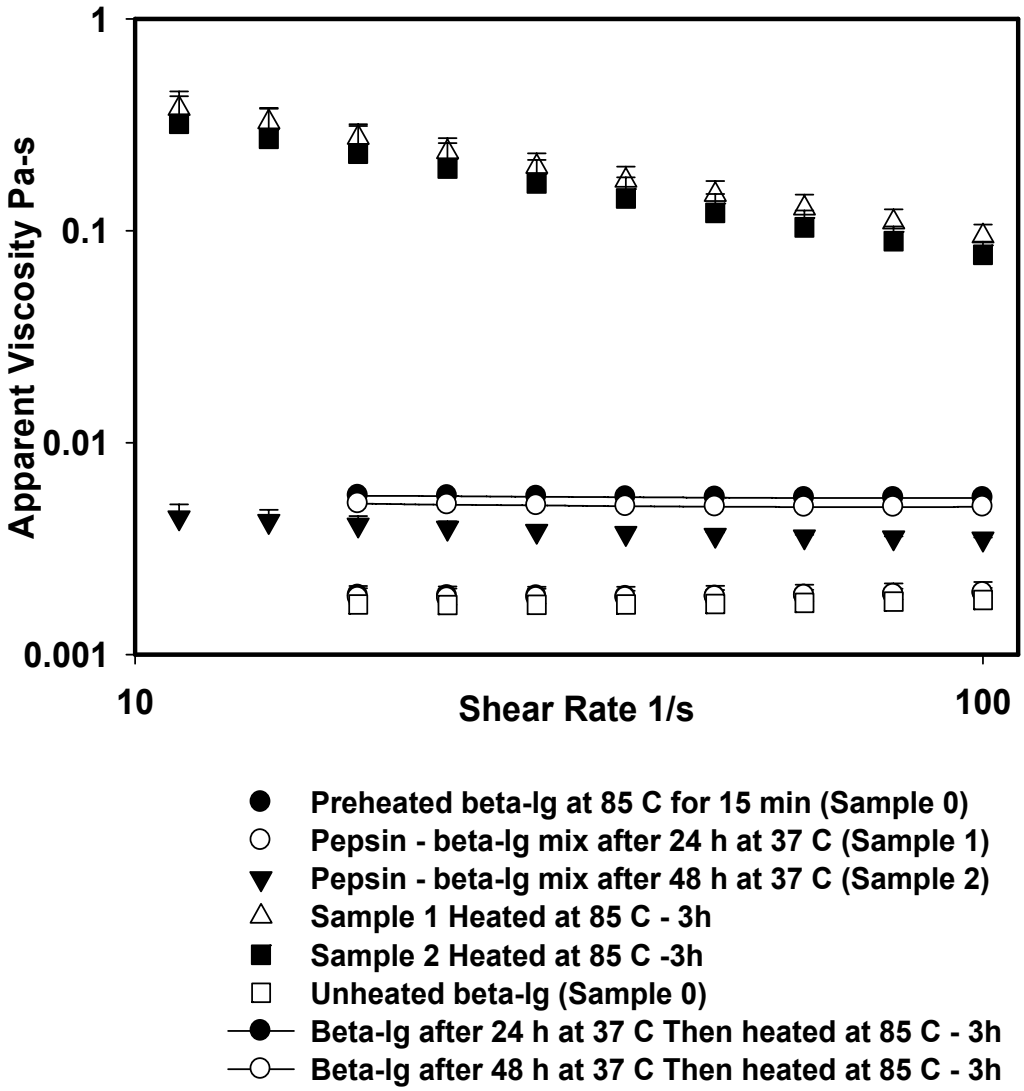


Figure 5.4. Apparent viscosities of pepsin hydrolyzed β-lg, compared with control with or without extended heated at 85 °C for 3h, pH 3.35. Error bars denote one standard deviation.

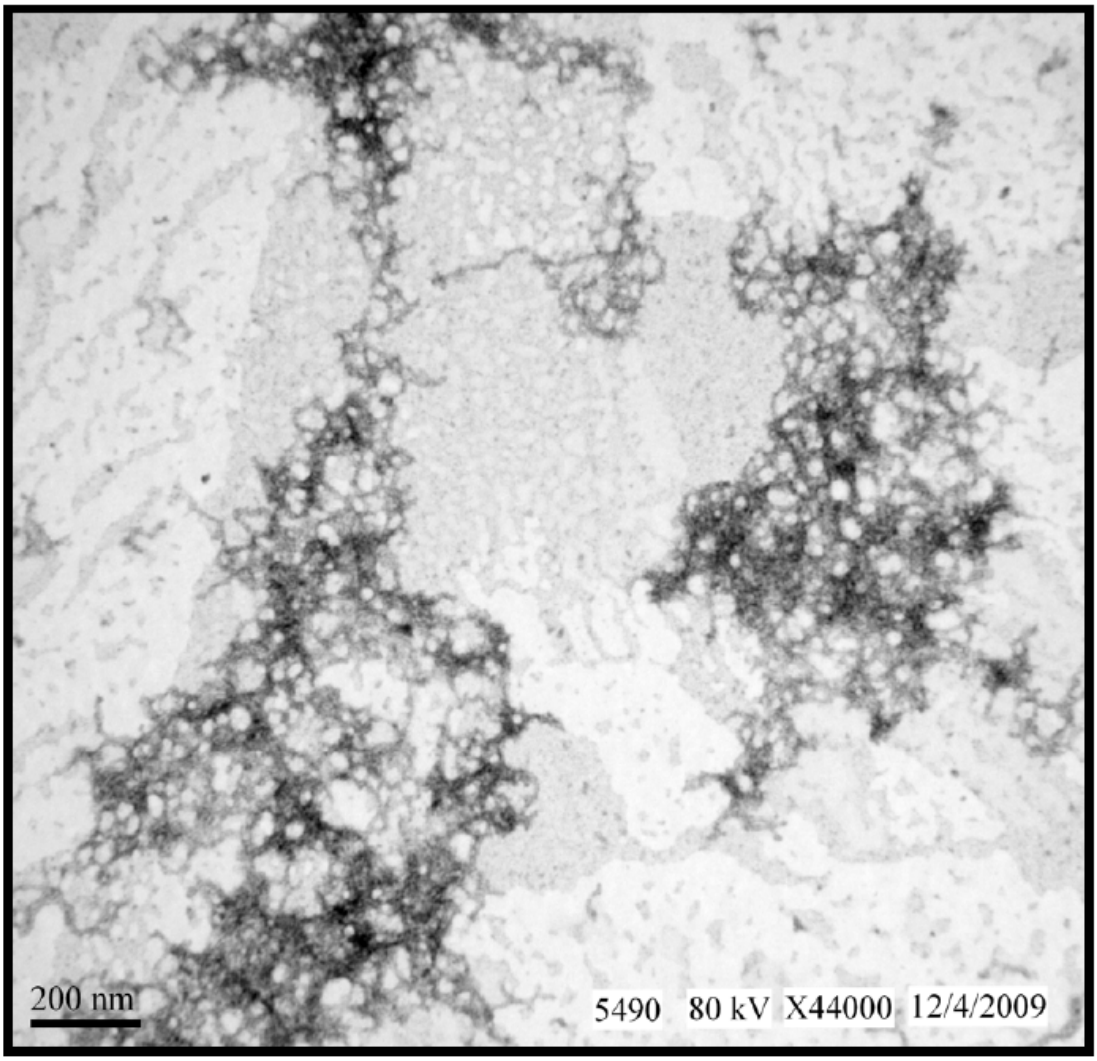


Figure 5.5A. TEM micrograph of pepsin hydrolyzed β-lg (24 h – 37 °C) (8 % w/w) heated for 85 °C for 3 h, pH 3.35, replicate 1

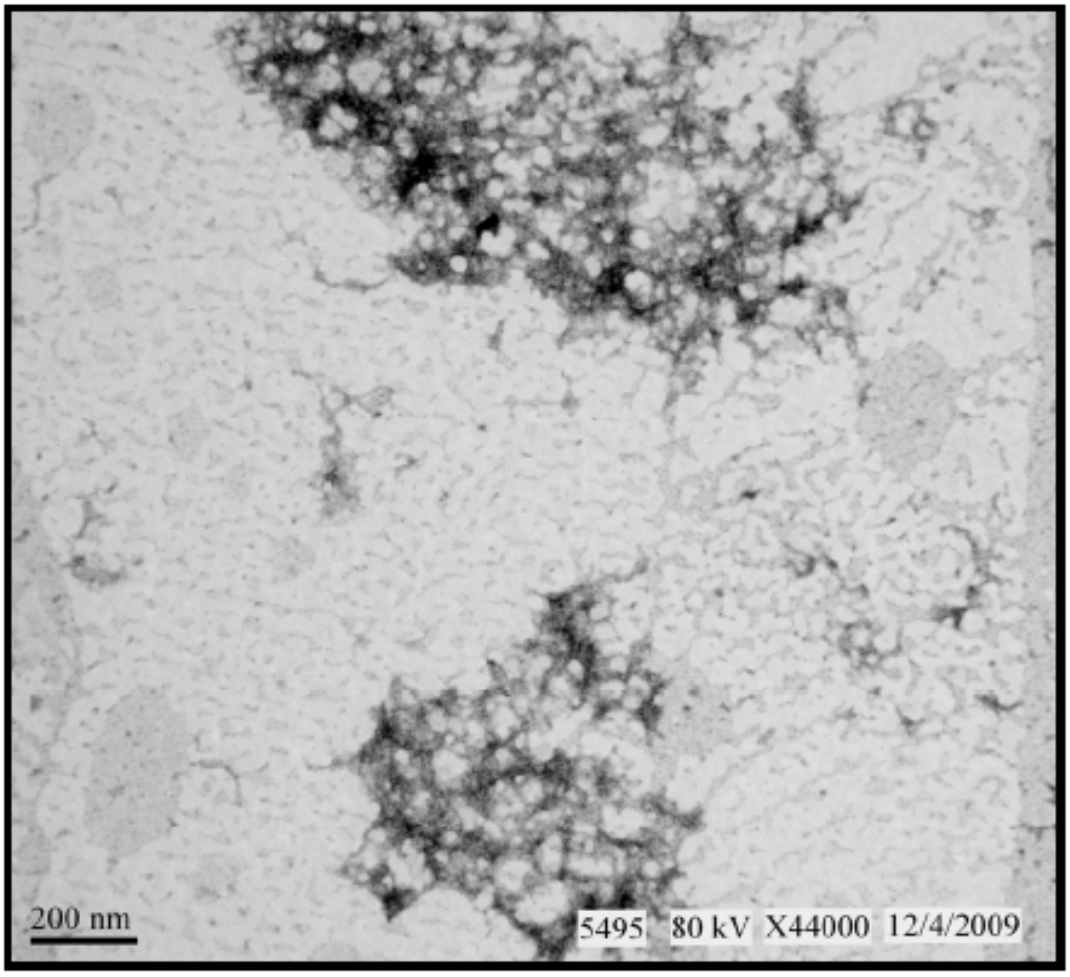


Figure 5.5B. TEM micrograph of pepsin hydrolyzed β-lg (24 h – 37 °C) (8 % w/w) heated for 85 °C for 3 h, pH 3.35, replicate 2

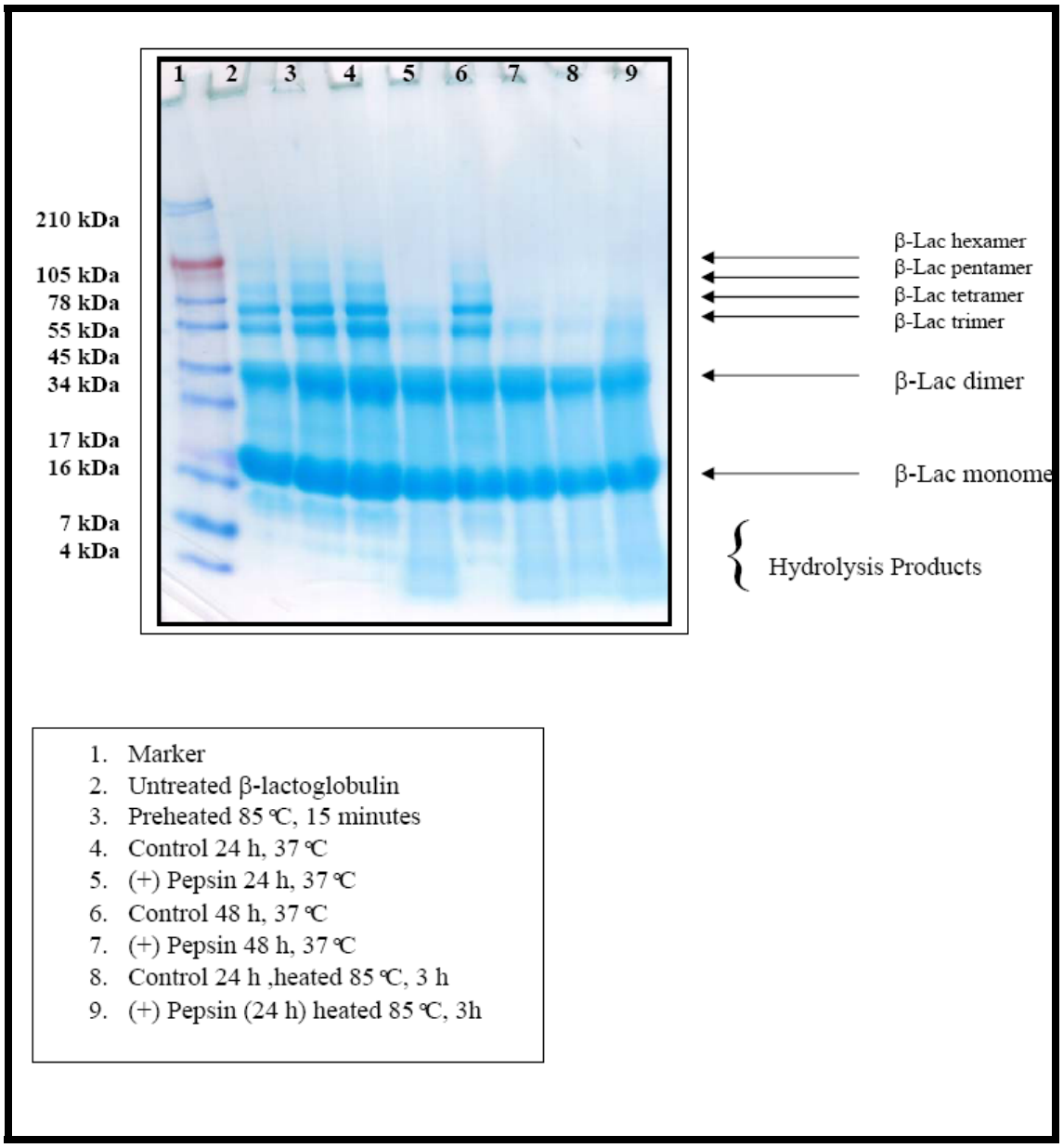


Figure 5.6A. SDS-PAGE - Pepsin hydrolysis of β-lg

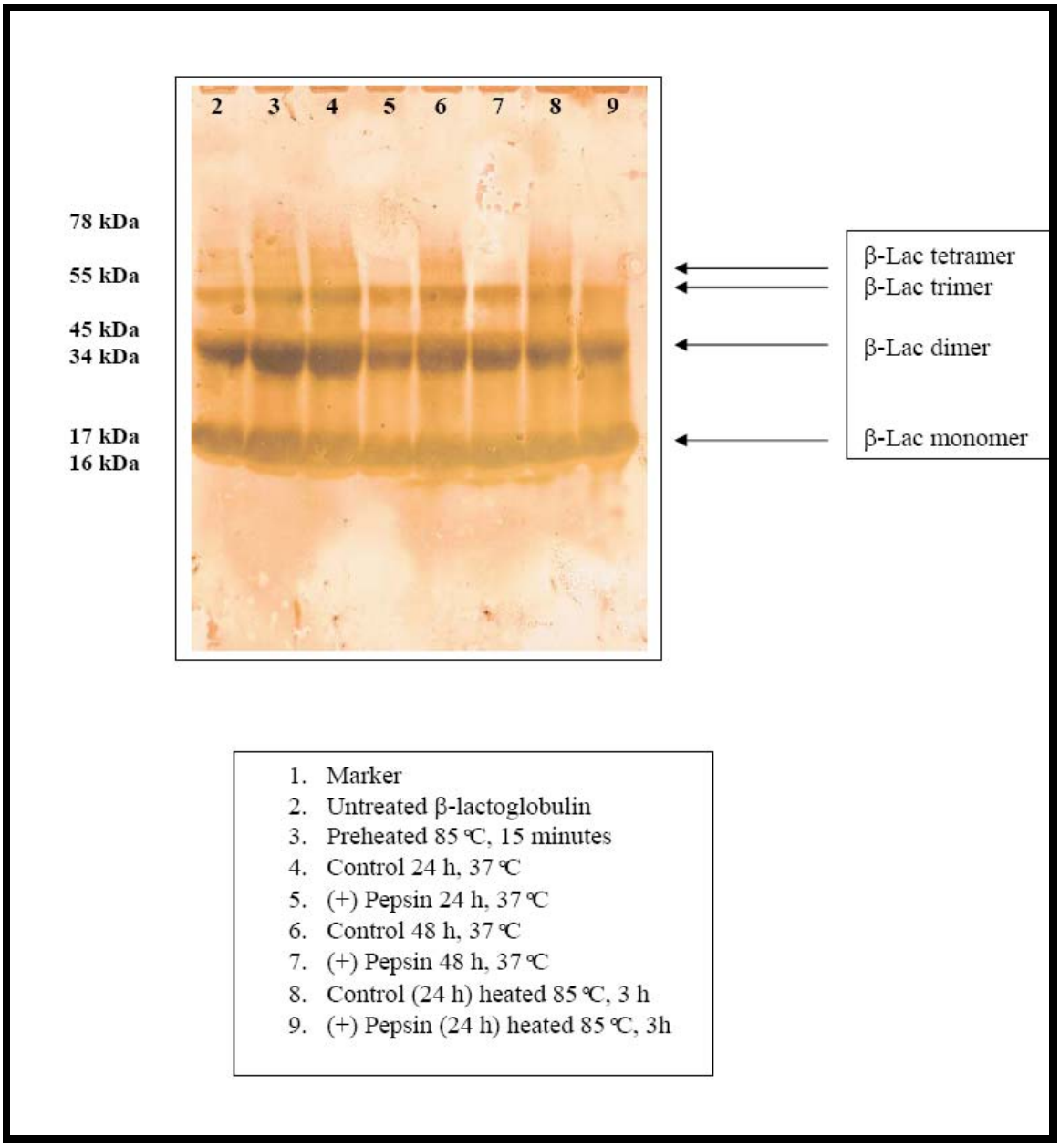


Figure 5.6B. Western blotting – Pepsin hydrolysis of β-lg

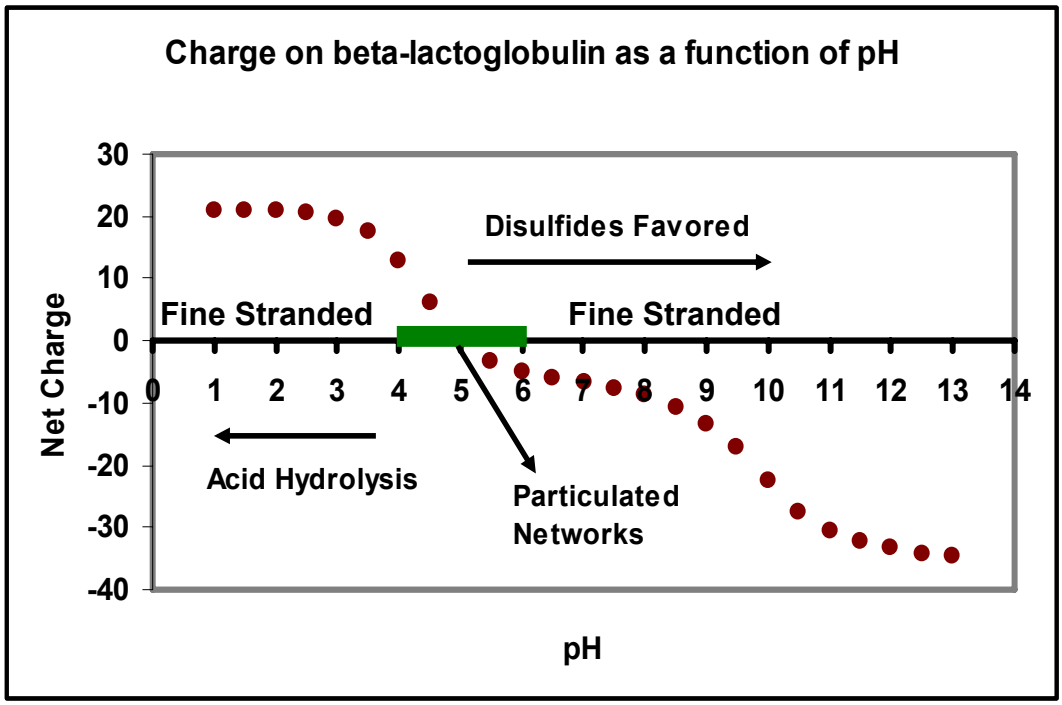


Figure 5.7. pH Effects on net charge of β-lg, disulfide interactions and acid hydrolysis during β-lg thermal aggregation

CHAPTER 6.

KINETIC STUDY OF β-LACTOGLOBULIN THERMAL AGGREGATION AT pH 3.35 AND LOW IONIC STRENGTH

To be submitted to: The Journal of Food Science

6.1 ABSTRACT

Kinetics of β-lactoglobulin (β-lg) thermal aggregation at pH 3.35, 85 °C, and low ionic strength conditions was studied using high performance liquid chromatography (HPLC) coupled with multi-angle laser light scattering (MALS) and rheology. Rate of β-lg aggregation was found to be of first order with respect to the initial protein concentration. Conversion of native-like β-lg monomers/dimers (< 36 kDa) to aggregates increased with initial concentration and heating times. The size of the aggregates formed during heating was dependent on the initial protein concentration. A simple nucleation and growth model was described for the β-lg aggregation at pH 3.35, where nucleation was found to be a rate limiting step below the previously identified critical concentration, C_c ~ 6.4 % protein. Above the C_c, nucleation occurred quickly and was not rate limiting. Critical size of the nucleus varied with the protein concentration with larger critical size needed at lower protein concentrations.

6.2 INTRODUCTION

Cold-thickening whey protein ingredients can be used as a suitable alternative to conventionally used starches because of their nutritional benefits (Hudson et al., 2002). A process to convert whey protein ingredients into a pH (3.35) modified cold-thickening ingredient was developed and originally applied to whey protein isolates and concentrates (Hudson et al., 2000; Resch et al, 2002). The cold-thickening mechanism of this ingredient was studied using a β-lg model system, and a critical concentration (~ 6.4 % w/w protein, 6.9

% solids) was identified below which no thickening function could be achieved from modified β-lg powders (Mudgal et al., 2009). Formation of microgels composed of flexible fibrillar strands and stronger colloidal interactions among these aggregates were likely responsible for the thickening function when manufactured above the β-lg critical concentration (Mudgal et al., 2009).

Upon heating above 70 °C, β-lg denatures and conformational changes to its native structure take place (Verheul et al., 1998; Schokker et al., 2000). During denaturation, buried thiol group at Cys-121 and hydrophobic residues become solvent accessible (Sava et al., 2005). Denatured β-lg molecules then aggregate, leading to formation of β-lg oligomers and polymers. Thiol-disulfide interchange reactions and non-covalent interactions both play an important role in aggregation; however, their relative roles are unknown (Mulvihill and Donovan, 1987; Hoffmann et al., 1997; Galani et al., 1999). At neutral pH, thiol-disulfide interchange interactions are believed to play an important role in β-lg aggregation, as this reaction is favored at neutral to alkaline pH (Creighton, 1988; Schokker et al., 2000). β-Lg forms particulated networks in the pH range of 4-6 and fine stranded networks are formed above and below this region (Langton and Hermansson, 1992). Kinetics of β-lg thermal denaturation and aggregation has been mostly studied near neutral pH between pH 6.5 and 7.5 (Roefs and De Kruif, 1994; Oldfield et al., 1998; Oldfield et al., 2005; Anema et al, 1996; Verheul et al, 1998; Galani et al., 1999; Dannenberg et al., 1988; Hoffmann et al., 1997) and more recently at pH 2 (Arnaudov et al., 2003, 2007; Bolder et al., 2007). Limited studies of β-lg aggregation exist between pH 3 and 4 (Mudgal et al., 2009). Most commonly, a

simplified single differential equation kinetic model is used to describe the denaturation and aggregation of β-lg relating the disappearance of native β-lg with time (Verheul et al, 1998; Galani et al., 1999; Arnaudov et al., 2007).

$$\frac{dc}{dt} = -k_n c^n \quad \text{Eq. 1}$$

Where c is the protein monomer concentration (g/L), k_n is the rate constant, and n is the order of the reaction. Whey protein denaturation, especially β-lg denaturation, has been studied during milk pasteurization and is well documented (Oldfield et al., 2005; Anema et al, 1996; Oldfield et al., 1998). Denaturation kinetics follows a 1st order reaction at lower temperatures (70-90 °C) and a higher order in the temperature range of (90-130 °C) with a break around 90 °C (Dannenberg et al., 1988; Oldfield et al, 1998).

Aggregation follows denaturation and results in disappearance of β-lg monomers during heating, and thus may also be modeled with a simple kinetic model (Eq. 1). The order of the rate of reaction for aggregation has been found between 1 and 2, based on the fits to the experimental data (Verheul et al, 1998; Galani et al., 1999). It is also influenced by order of different reactions during different steps of denaturation and aggregation (Verheul et al., 1998).

Other types of models used to describe β-lg aggregation include radical polymerization (Roefs and De Kruif, 1994), polycondensation, and nucleation and growth (Clark et al., 2001; Arnaudov et al., 2003, 2007, Bromley et al., 2005, 2006). Roefs and De Kruif, (1994) reported a thiol-catalyzed polymerization model for β-lg at neutral pH, leading to formation

of disulfide linked aggregates. A nucleation and growth model by Arnaudov et al., 2003 described gelation of β-lactoglobulin at acidic pH (2) in three main stages: an initial unfolding or dimerization step, a step of linear fibrillar aggregation via nucleation and growth, and lastly a step of random association of the fibrils.

Quantitative models describing the nucleation and growth models for β-lg fibril formation at low pH (2-2.5) have also been reported (Bromley et al., 2005, Clark et al., 2001). In a simple model, fibril formation at pH 2.5 was modeled using a set of three ordinary differential equations (ODE's) following some assumptions (Bromley et al., 2005). These equations described the nucleation and growth process in three steps: (1) formation of active species (2) nucleation by active species (3) fibril growth. Based on the analytical solution of their model, Bromley et al., 1995 reported a critical nucleus size of about 4 needed at pH 2.5 when β-lg was heated at 70-75 °C at low ionic strength conditions and reported that nucleation was a rate limiting process under the conditions studied. Kinetics of β-lg aggregation has been studied using size exclusion chromatography at pH 2.5 (Schokker et al, 2000) and pH 7 (Hoffmann et al., 1997). The rate of β-lg aggregation increased with increasing protein concentration at pH 2.5 (Schokker, 2000), and the size of aggregates formed after heating also increased with an increase in protein concentration at pH 2.0 (Aymard, 1999) and at neutral pH (Hoffmann et al., 1997).

The objective of this study was to investigate kinetics of β-lg aggregation during a whey modification process (pH 3.35) as a function of heating time and protein concentration at low

ionic strength conditions. β-Lg network development with time was studied using high performance liquid chromatography- Multi-angle laser light scattering (HPLC-MALS), and rheology at pH 3.35 to further understand the cold-thickening mechanism associated with the product from the modification. Finally, a nucleation and growth kinetic model for β-lg thermal aggregation at pH 3.35 explaining concentration dependent effects was evaluated.

6.3 MATERIAL AND METHODS

6.3.1 Protein Material

β-lactoglobulin (BioPure^{R}, ~ 94 % pure, total protein 98 % dry basis) was donated by Davisco Foods, Inc. The wet basis sample protein content was determined by micro-Kjeldahl (AOAC, 1984) to be 92.81 %.

6.3.2 Solution preparation

β-Lg solutions of different concentrations (2, 4 and 8 % w/w solids) were prepared by dissolving β-lg in de-ionized (DI) water by continuous stirring at room temperature for 1-2 hours. Sodium azide (0.02 %) was added to all samples to prevent microbial growth. Thereafter, solutions were adjusted to pH 3.35 using 6 N HCl. Following pH adjustment,

solutions were heated at 85 °C for desired times in a water bath. Then, these modified β-lg solutions were cooled and stored in a 4 °C refrigerator for 24 h prior to any analysis.

6.3.3 High performance liquid chromatography-Multi-angle laser light scattering (HPLC-MALS)

To characterize distribution of molar mass of aggregates formed after heating at different times and varying concentrations, light scattering studies were performed. β-Lg solutions at varying concentrations (2, 4, and 8 % w/w solids) were prepared in 40 mM sodium citrate/citric acid buffer at pH 3.35. Then these samples were heated for varying times (30 min – 6 h). Following heating, samples were cooled and stored at 4° C for 24 h. Next day, samples were diluted to 5 mg/ml final concentration and filtered through a 0.45 micron filter. A Bicinchoninic acid (BCA) assay (Methodology developed by Thermo Scientific Inc., Rockford, IL) was performed prior and after filtration to determine protein loss during filtration. Two independent replicates were performed.

The HPLC–MALS was calibrated with 200 µl of buffer and 100 µl of BSA (Bovine serum albumin) standard (5 mg/ml), and data were normalized based on BSA. A gel filtration column (Shodex, KW-804) was linked to a photodiode detector (Waters 2996) coupled with refractive index detector (Waters 2414) and MALS detector (Waters Technology, Dawn EOS Enhanced Optical Systems) to determine molar mass and concentration distribution of the samples. Twentyfive µl of samples at 5 mg/ml concentrations were injected into the column.

6.3.4 Shear rate sweeps

Shear rate sweeps were performed on all solutions at 25 °C using a stress controlled rheometer (ATS Rheosystems, Bordentown, NJ) to characterize flow behavior and viscosities of heated β-lg solutions. A smooth, 25 mm concentric cylindrical geometry was used, and shear rates were varied from 1 to 100 s^{-1} using a constant rate program to minimize inertial effects. A pre-shear condition (15 s^{-1} for 30 s) was applied to all solutions to obtain uniform solutions prior to measurements with a consistent baseline shear history. A thin film of mineral oil was applied to the sample surface to minimize sample dehydration.

6.3.5 Kinetic data analysis

Molar mass distributions were obtained from HPLC-MALS data. Conversion of native-like β-lg to β-lg polymers was calculated from the relative concentration ratio of higher molecular weight β-lg to low molecular weight non-aggregated β-lg (< 36 kDa) (Mudgal et al., 2009). Fractions of varying size β-lg aggregates (< 10^5 Da, 10^5-10^6 Da, and > 10^6 Da) were determined based on the weight fraction vs. molar mass data. Initial rate of reaction order was calculated by plotting the rate of change of non-aggregated fraction (inversely correlated with increase in conversion) with respect to the initial protein concentration of heated β-lg solutions on a double log plot similar to methodology used by Verheul et al., 1998.

6.4 RESULTS

6.4.1 Molar mass distribution

HPLC coupled with MALS was used to identify molar mass distribution of aggregates formed after heating of β-lg solutions at varying concentrations for different heating times. At all concentrations studied (2, 4, and 8 % w/w solids), the amount of aggregated β-lg increased continuously with increasing heating times at 85 °C and pH 3.35 (Figure 6.1). The degree of aggregation (ratio of aggregated to non-aggregated β-lg fraction) also increased with initial protein concentration. From the results, the molar mass of the aggregates formed after heating was found to be dependent on the initial protein concentration. From the cumulative weight fraction (cwf) vs. molar-mass curves in Figures 6.1A and 6.1B, aggregate molar-mass reached a maximum (~ 10^7 Da) at lower protein concentrations (2 and 4 % w/w). While at 8 % w/w, aggregate molar-mass increased continuously with heating times, eventually leading to the formation of a gel for heating times greater than 4 hour (Figure 6.1C).

6.4.2 Conversion

To quantify the degree of aggregation in heated β-lg solutions, ratios of aggregated to non-aggregated β-lg (conversion) were determined at varying heating times and initial concentrations (Figure 6.2A). Conversion increased with increasing heating time at all concentrations. However, the rate of increase in conversion was also enhanced with an

increase in the initial protein concentration. At 2 % w/w, conversion increased from approximately 9 % after 30 minutes of heating to approximately 25 % after 6 hours of heating. Similarly for 4 % w/w solutions, conversion increased from 12 % at 30 minutes to 37 % after 6 h of heating. At 8 % w/w, increase in conversion was from ~ 21 % at 30 minutes to ~ 49 % after 4 h of heating. The conversion values for all treatments along with their respective standard of deviations are listed in Table 6.1.

6.4.3 Order of the rate of aggregation reaction

To determine the order of the rate of aggregation, relative loss of non-aggregated β-lg was calculated with heating times at different concentrations and initial rate of reaction was calculated using eq. 1, according to Verheul et al., 1998 (Figure 6.2B). The order of the rate of β-lg aggregation was determined to be 1.01, and a R^2 value of ~ 1 was obtained for the linear fit (Figure 6.2B).

6.4.4 β-Lg oligomers and polymers

Based on the aggregate molar mass, three categories for β-lg aggregates were defined: monomers/oligomers (< 10^5 Da), intermediates (10^5 -10^6 Da) and large aggregates (> 10^6 Da). For all treatments studied, a majority of the protein (> 50 %) was observed in the low molecular weight form (< 10^5 Da) indicating that aggregation under the conditions studied was relatively a slow process (Figure 6.3). At 2 % w/w, significant fraction (> 10 %) of the large aggregates (> 10^6 Da) was observed only after 6 h of heating, otherwise β-lg existed

primarily in the low molecular weight form (Figure 6.3A). A fraction of the intermediates (10^5-10^6 Da) remained relatively unchanged and low (< 5 %) for all heating times studied. At 4 % w/w, the fraction of low molar-mass aggregates decreased with heating time, while concentration of large aggregates increased with time (Figure 6.3B). The fraction of intermediates (10^5-10^6 Da) remained relatively unchanged and low (< 5 %) for all heating times studied, similar to the 2 % w/w results. A significant fraction (> 10 %) of the large aggregates was seen only after 4 h of heating.

At 8 % w/w, above the identified critical concentration (6.9 % w/w) for this system (Mudgal et al., 2009), a significant fraction (> 10 %) of large aggregates (> 10^6 Da) was observed as early as 30 minutes of heating (Figure 6.3C). A fraction of the large aggregates increased with increasing heating time continuously from 12 % at 0.5 h to 43 % after 4 h, while the low molecular weight monomer-oligomer fraction decreased with heating time (Figure 6.3C). Similar to the other concentrations studied, a fraction of the intermediates remained low (< 5 %) and relatively unchanged with heating time, confirming the presence of a bimodal distribution (Figure 6.3C). To quantify the bimodal distribution, a correlation between the loss of low molar-mass fraction and the appearance of the large aggregates was determined at 4 and 8 % w/w (Figure 6.4). The rate of disappearance of low-molar mass aggregates indicated by the negative slope value was similar to the rate of formation of the large aggregates (positive slope value) at both 4 and 8 % w/w concentrations. The coefficient of determination (R-square) values greater than 0.96 were obtained for all fits (Figure 6.4). These results supported that following heating, β-lg either remained in the

monomeric/oligomeric form or was incorporated into large aggregates of molar mass > 10^6 Da.

6.4.5 Root mean square (RMS) radii of aggregates

From the HPLC-MALS data, RMS radii of β-lg aggregates formed during heating were determined. In Figure 6.5A, cumulative weight fraction vs. RMS radii plot is shown for heated 8 % w/w β-lg solutions. The size of the aggregates increased continuously with the time of heating consistent with the molar-mass distribution data. A cumulative weight fraction (cwf) of 0.95 or a 95 percentile value means that the 95 % of the total fraction is smaller than that value. The RMS radii corresponding to cwf of 0.95 increased from 44 nm +/- 6 after 30 minutes of heating to 66.5 nm +/- 2.5 after 4 h of heating (Figure 6.5B). The rms radii corresponding to cwf of 0.8, 0.9 and 0.95 were plotted with varying times of heating for 8 % w/w solutions (Figure 6.5B). Parallel trend lines in Figure 6.5B suggested continual formation of the large aggregates with heating time, validating the molar-mass data.

6.4.6 Heating time effects on apparent viscosity of β-lg solutions

Apparent viscosity of heated β-lg solutions at varying concentrations and heating times were measured and results were correlated to the light scattering data. At lower protein concentrations, viscosities of heated solutions remained similar and no significant difference was discernable between the lowest and highest heating times at 2 % w/w. A small increment

was observed when a 4 % sample was heated for 6 h (Figure 6.6A). While for 8 % w/w β-lg samples, the viscosity increased continuously with heating time, eventually leading to formation of a gel after 6 hours of heating (Figure 6.6B).

6.5 DISCUSSION

Since conversion of β-lg to large aggregates increased with increasing heating times at all concentrations, it was concluded that aggregation continued to occur at all concentrations for the conditions studied. The increase in conversion values with heating times were previously reported at pH 2.5 (Schokker et al., 2000). However, after 6 h of heating, a majority of the protein remained non-aggregated especially at lower concentrations, suggesting a relatively slower kinetic process of aggregation at pH 3.35. At pH 2, using atomic force microscopy data, Arnaudov et al. (2003) reported that even after prolonged heating (24 h) a considerable fraction of β-lg remained non-aggregated when heated at concentrations up to 3 % w/w. Arnaudov et al. (2003) attributed this observation to formation of β-lg 'dead-end' species which can not participate in aggregation, and these 'dead-end' species could be either denatured monomers or oligomers.

In this study, the size and molar mass of aggregates formed was found to be dependent on the initial protein concentration. Aymard et al. (1999) reported increased size of aggregates with increasing initial protein concentration at pH 2. As the initial reaction order was of first order

with respect to the initial concentration, at higher concentrations, the rate of reaction was higher leading to the formation of larger aggregates (Figure 6.2C). The fraction of intermediate sized aggregates (10^5-10^6 Da) remained very low (< 5 %) and relatively unchanged at all treatments studied, indicating a bimodal distribution. Similar observations were reported previously at pH 2 (Arnaudov et al., 2003) and at pH 2.5 and low ionic strength conditions (Schokker et al, 2000).

6.5.1 Nucleation and growth model

A nucleation and growth mechanism for the β-lg aggregation was proposed at the pH of the modification process, 3.35 and low ionic strength conditions. From the results of this study, it was observed that β-lg either remained non-aggregated in low molecular weight form or was incorporated into large aggregates (> 10^6 Da). A high correlation between disappearance of low molar-mass β-lg fraction and increase in the fraction of large aggregates (> 10^6 Da) (Figure 6.4) quantified the bimodal distribution. From these results, it appeared that at lower protein concentrations, nucleation is the rate limiting step. According to the nucleation and growth model, once aggregates of certain sizes are formed, β-lg is incorporated into the nuclei. Because, nucleation is the rate limiting step, at low protein concentrations nucleation can occur only after long periods of heating. From results offered in this study, it seemed that at 2 % w/w, nucleation could only occur after 6 h of heating (Figure 6.3A), while at 4 % w/w it may have occurred after 3-4 hours of heating (Figure 6.3B). At 8 % w/w, nucleation occurred as early as 30 minutes of heating, and therefore was not a rate limiting step. In a previous study, a critical concentration of 6.9 % w/w solids (6.4 % protein) was suggested for

this system, above which the viscosity of heated solutions increased quickly. Above the critical concentration, nucleation is not a rate limiting step. This proposition seems plausible, as with an increase in initial concentration, rate of aggregation increases with a first order rate of reaction (Figure 6.2C). Above the critical concentration, nucleation is not the rate limiting step, aggregation can occur rapidly leading to higher conversion values and formation of large aggregates. Also, above the critical concentration, inter-particle distances are smaller and stronger colloidal interactions exist (Mudgal et al., 2009), leading to further association of these aggregates and eventually a higher viscosity. Mudgal et al. (2009) reported that after heating of 4 % w/w β-lg at pH 3.35, isolated flexible fibrils were formed, while at 8 % w/w 'microgel' aggregates were observed. The formation of 'microgels' can be explained by further association of large aggregates/isolated flexible fibrils as explained above. The nucleation and growth mechanism explained above addresses the β-lg aggregation at pH 3.35 and low ionic strength conditions. Nucleation and growth mechanism for the formation of fibrils at pH 2 has been previously reported (Aymard et al., 1999; Clark et al., 2001; and Bolder et al., 2007). It was also proposed that nucleation was a rate limiting step at pH 2 and low ionic strength conditions (Aymard et al., 1999; Bolder et al., 2007). Since, long linear fibrils are formed at pH 2 and a low ionic strength condition while flexible fibrils are formed at pH 3.35, the network characteristics are different. However, results from this study further supported the nucleation and growth mechanism of β-lg aggregation at acidic pH and low ionic strength conditions suggesting there are some similarities in aggregation kinetics at pH 2 and 3.35.

6.5.2 Nucleation and growth mathematical model for β-lg aggregation at pH 3.35

As discussed in previous sections, the majority of the β-lg remained non-aggregated under the conditions studied. After heating for various times, β-lg either existed in low molecular weight form or formed large aggregates. A simple nucleation and growth model describing β-lg aggregation at pH 3.35 was formulated. Since the temperature of heating is 85 °C, it is reasonable to assume that protein denaturation/unfolding is a relatively fast process and of a time scale much less than that of the aggregation. Arnaudov et al., 2007 also reported that protein denaturation occurred very quickly when β-lg was heated at 70 °C, pH 2 and made a similar assumption in their model. Therefore, the first assumption for this model is that all β-lg is quickly denatured and is available in active state upon heating. Second assumption is that the concentration of intermediates is much smaller as compared to the sum total concentration of the non-aggregated fraction and the large aggregates as also evident from the light scattering data.

According to the nucleation and growth theory, large aggregates are formed after nuclei are formed and thus have greater molar mass than the nuclei. The rate of aggregation was modeled to be of first order with respect to the non-aggregated fraction and fraction of nuclei similar to the methodology used by Bromley et al., 2005. The nucleation was modeled as an n^{th} order reaction with respect to the active monomeric/dimeric β-lg (< 36 kDa), where n is the critical number of active species needed to form a nucleus. The fractional concentration of active β-lg (non-aggregated monomeric/dimeric) is denoted by m, where, m can be measured from the cumulative weight fraction vs. molar mass plots (< 36 kDa). N represents

the fraction of nuclei at any point of time, while '*A*' represents aggregates of molar mass greater than M_c, where, M_c is the critical size of nucleus. '*I*' denotes the fractional concentration of intermediates between *m* and *A*, including *N*. The fractional concentration of *I* is assumed small in comparison to the total concentration of *m* and *A* as also evident from the molar mass distributions. The model is described as follows:

$$\frac{dN}{dt} = k_1 m^n \qquad \text{Eq. 2}$$

Integrating Eq. 2 gives:

$$N = k_1 m^n t \qquad \text{Eq. 3}$$

$$\frac{dA}{dt} = k_2 (1 - A)(N) \qquad \text{Eq. 4}$$

$$\frac{dA}{dt} = k_2 (1 - A) k_1 m^n t \qquad \text{Eq. 5}$$

$$\frac{dA}{dt} = k_2 (1 - A) k_1 (1 - A)^n t \qquad \text{Eq. 6}$$

As $m + A + I = 1$ and assuming $I << m + A$, $m = 1 - A$

$$\frac{dA}{dt} = k_2 k_1 (1 - A)^{n+1} t \qquad \text{Eq. 7}$$

Integrating Eq. 7 gives:

$$\frac{1}{n} \frac{1}{(1 - A)^n} = k_1 k_2 \frac{t^2}{2} \qquad \text{Eq. 8}$$

Rearranging and applying natural logarithm *Ln* gives:

$$Ln\, n + nLn(1-A) + 2Ln\, t = Ln\frac{2}{k_1 k_2}$$

$$Ln(1-A) = -\frac{2}{n}Ln t + \frac{1}{n}Ln\frac{2}{k_1 k_2 n} \qquad \text{Eq. 9}$$

Assuming *n* is constant for a specific concentration, pH and temperature, *n* can be estimated from the gradient of the plot of *Ln* (1-*A*) with *Ln t* (Figure 6.7). From the gradients of the plots in Figure 6.7, *n* was estimated to be 12.6 at 8 % w/w, 25.7 at 4 % w/w, and 76.6 at 2 % w/w respectively. These results indicated that the critical nucleus size was dependent on the protein concentration with a larger critical nucleus size needed at lower protein concentrations. The nuclei size were determined to be between (2.3-4.6 X 10^5 Da) at 8 % w/w, (4.7-9.4 X 10^5 Da) at 4 % w/w, and (1.4-2.8 X 10^6 Da) at 2 % w/w respectively. At lower protein concentrations, the rate of aggregation is low and also the size of nucleus needed is large. Therefore at low protein concentrations nucleation is the rate limiting step. While at higher protein concentrations, rate of aggregation is high and the critical size of nucleus is small resulting in fast nucleation. Thus nucleation is not a rate limiting step at higher protein concentrations and large aggregates are formed quickly. The order of the nucleation reaction at pH 2-2.5 was determined to be 4 (Bromley et al., 2005, Clark et al., 2001), which is much smaller than the orders obtained at pH 3.35 and thus explains extremely slow kinetics low protein concentrations during heating at pH 3.35, low conversion and formation of shorter fibrils. As observed in figure 6.7, the high R^2 values of linear fit suggest that this model explains aggregation at pH 3.35 suitably.

6.6 CONCLUSIONS

The rate of β-lg aggregation at pH 3.35 was found to be of the first order with respect to the initial concentration. The conversion of β-lg into aggregates increased with increasing heating times at 85 °C, pH 3.35 at all concentrations. However, the size of the aggregates formed was dependent on the initial protein concentration. A very small fraction (< 5 %) of intermediates was present at all concentrations and heating times studied indicating a bimodal distribution. A nucleation and growth model was proposed for the β-lg aggregation at the pH of the modification procedure (3.35). It was found that above the critical concentration, nucleation was not a rate limiting step and aggregation occurred rapidly leading to the formation of larger aggregates. The critical size of nuclei was found dependent on the protein concentration with larger nucleus size needed at lower protein concentrations. Above the critical concentration, smaller inter-particle distances, higher conversion, the formation of large aggregates, and stronger colloidal interactions lead to further association among these aggregates, forming microgels and eventually leading to gelation.

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Table 6.1.

Conversion values with heating time at varying concentrations. Values in brackets indicate standard deviations.

Heating Time h	Concentration (% w/w solids)		
	2	4	8
0.5	9 (0.4)	12 (2.0)	21 (2.8)
1	10 (0.7)	14 (0.7)	28 (2.1)
2	11 (0.0)	20 (3.5)	35 (1.4)
3	-	22 (0.7)	41 (3.5)
4	14 (2.1)	25 (1.1)	49 (2.1)
6	25 (3.9)	37 (6.4)	-

Figures:

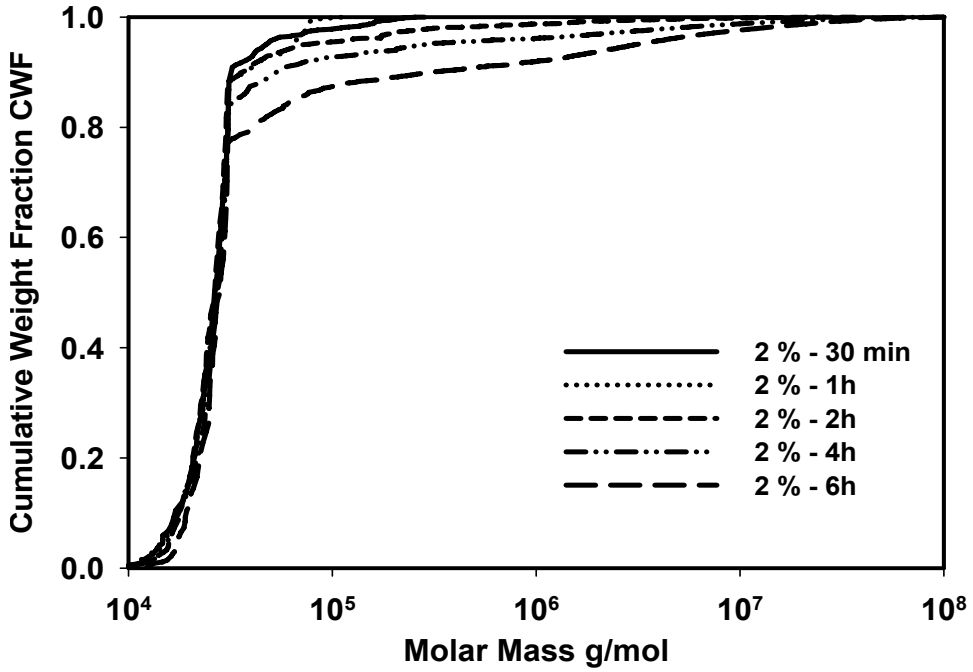

Figure 6.1A. Molar mass distribution of aggregates formed after heating of β-lg solutions at 2 % w/w for varying times at pH 3.35

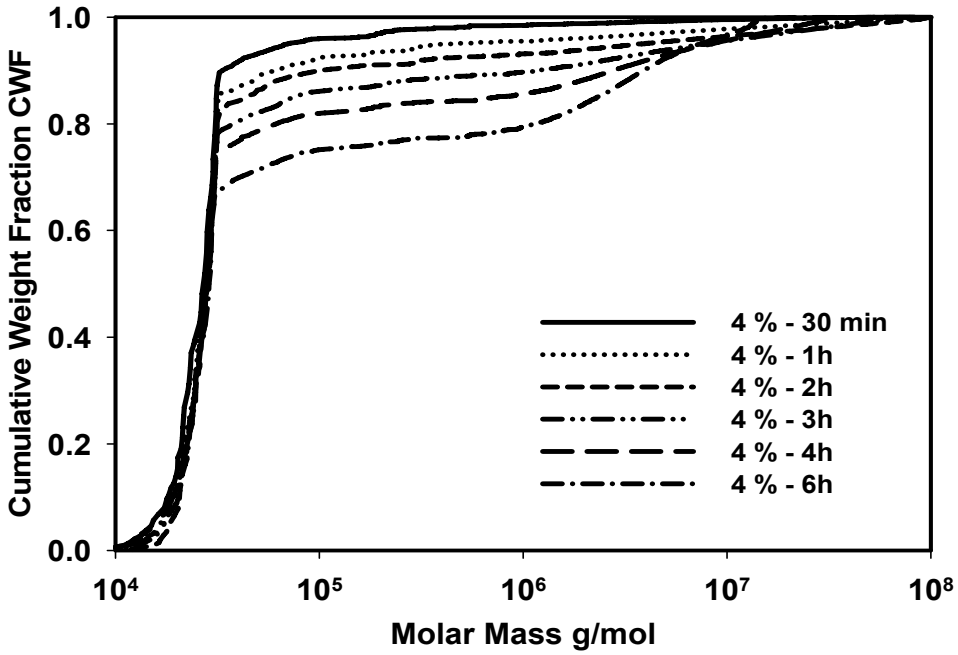

Figure 6.1B. Molar mass distribution of aggregates formed after heating of β-lg solutions at 4 % w/w for varying times at pH 3.35

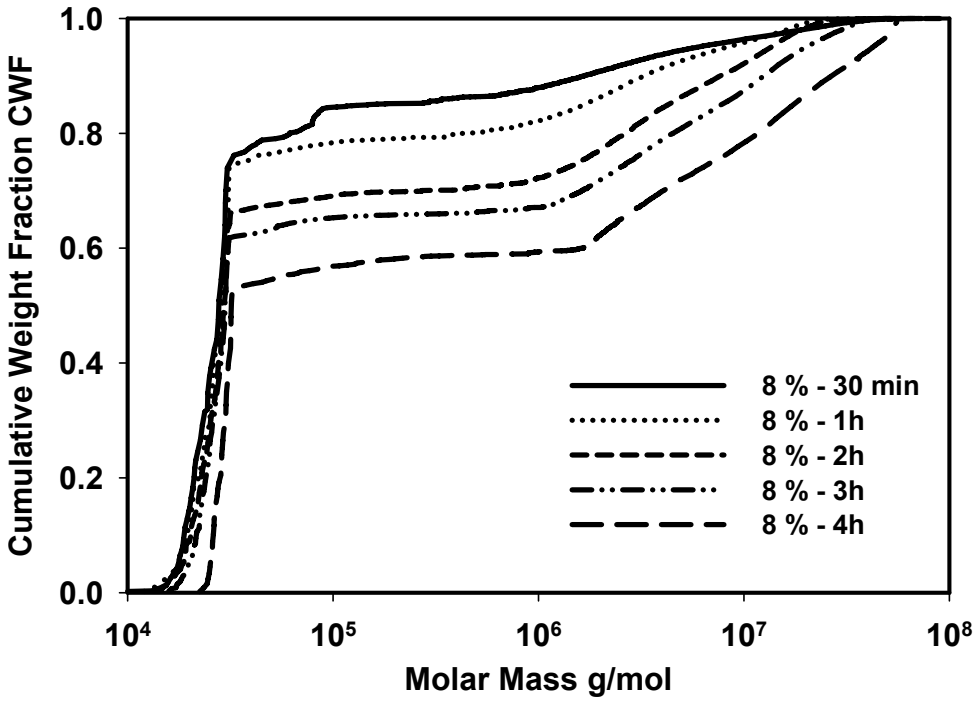

Figure 6.1C. Molar mass distribution of aggregates formed after heating of β-lg solutions at 8 % w/w for varying times at pH 3.35

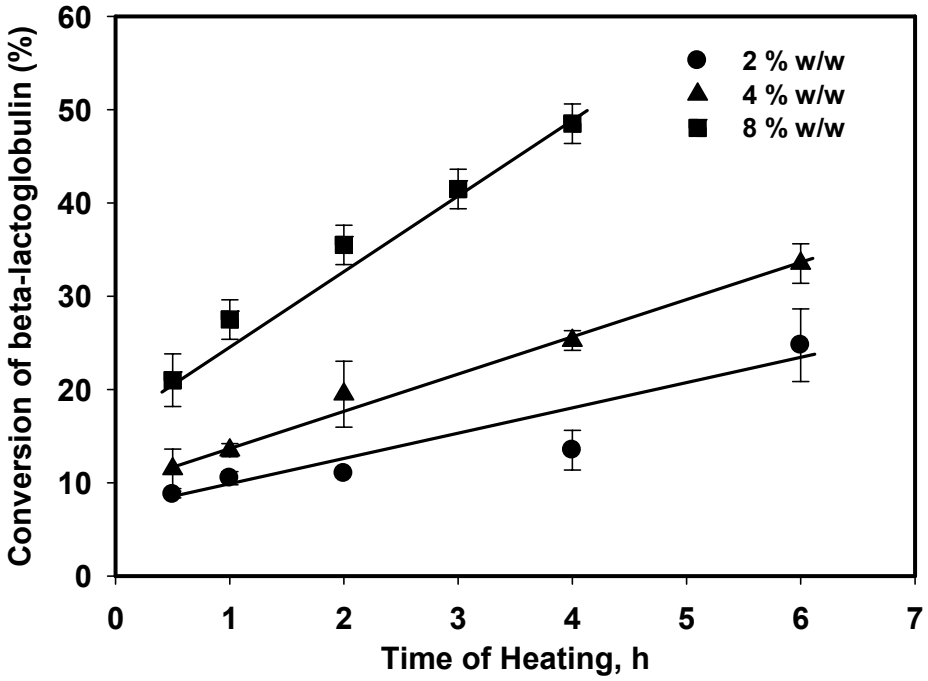

Figure 6.2A. Conversion of β-lg to aggregates with heating time at varying β-lg concentrations. Error bards denote one standard deviation.

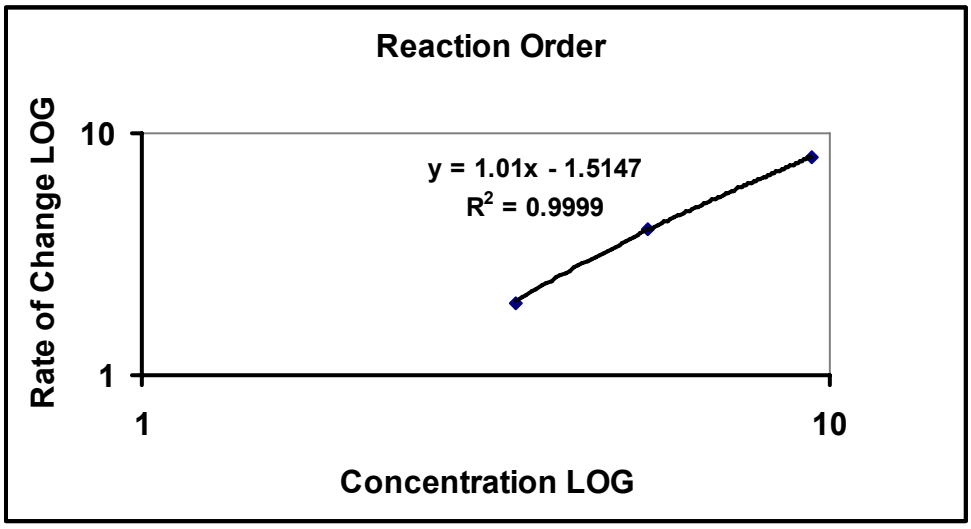

Figure 6.2B. Rate of reaction order for aggregation of β-lg at pH 3.35

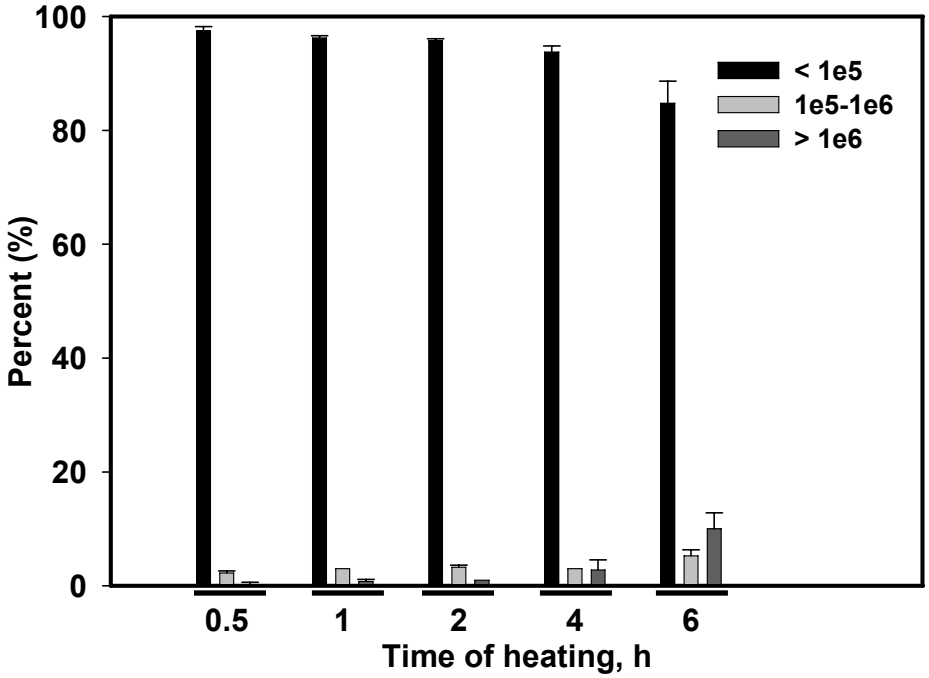

Figure 6.3A. Aggregate size as a function of heating times at 2 % w/w concentrations. Error bars represents one standard deviation.

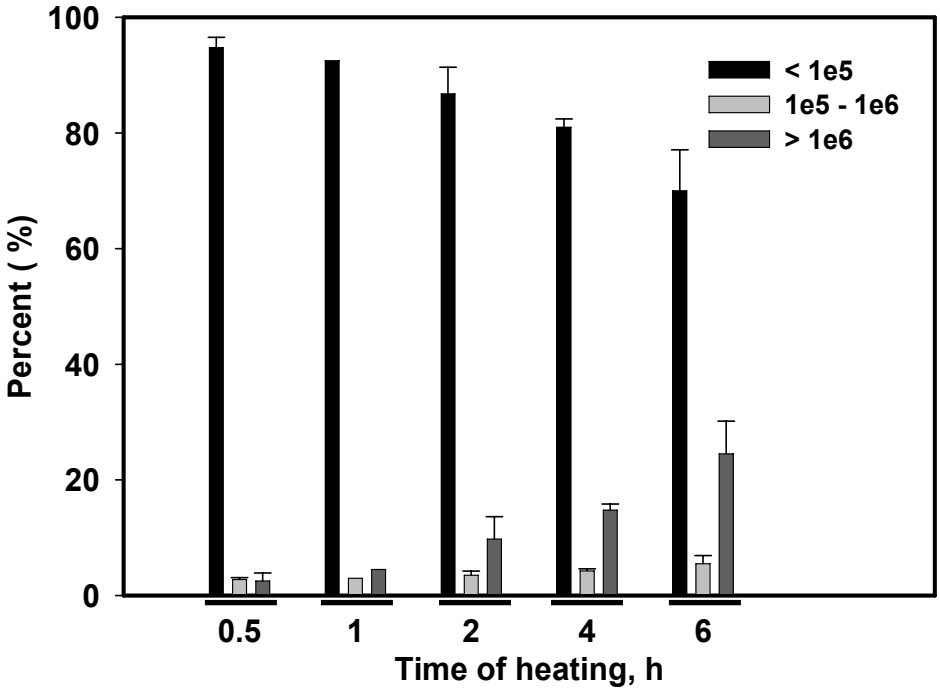

Figure 6.3B. Aggregate size as a function of heating times at 4 % w/w concentrations. Error bars represents one standard deviation.

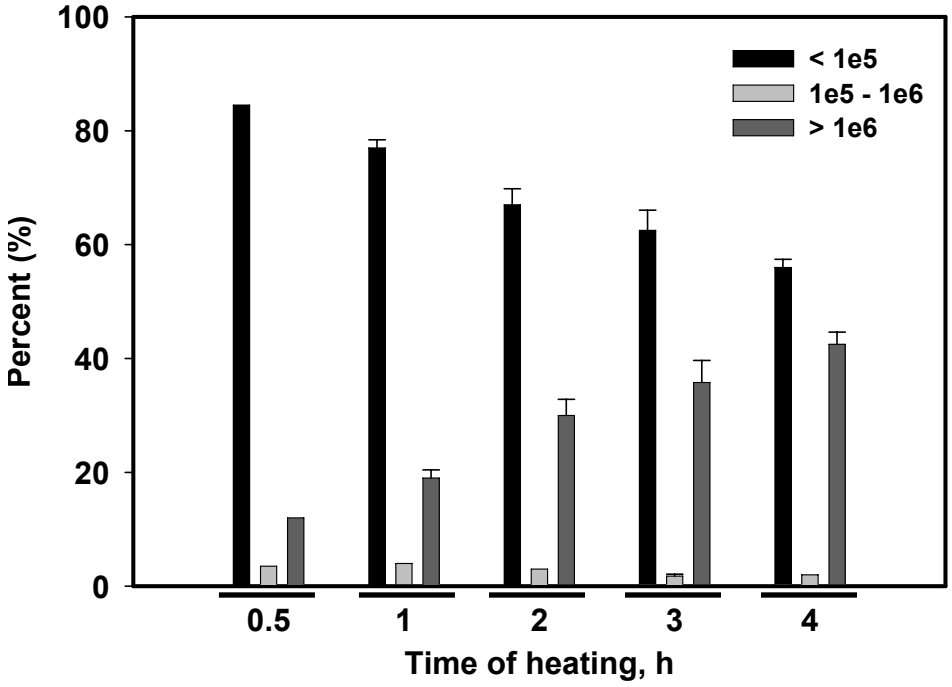

Figure 6.3C. Aggregate size as a function of heating times at 8 % w/w concentrations. Error bars represents one standard deviation.

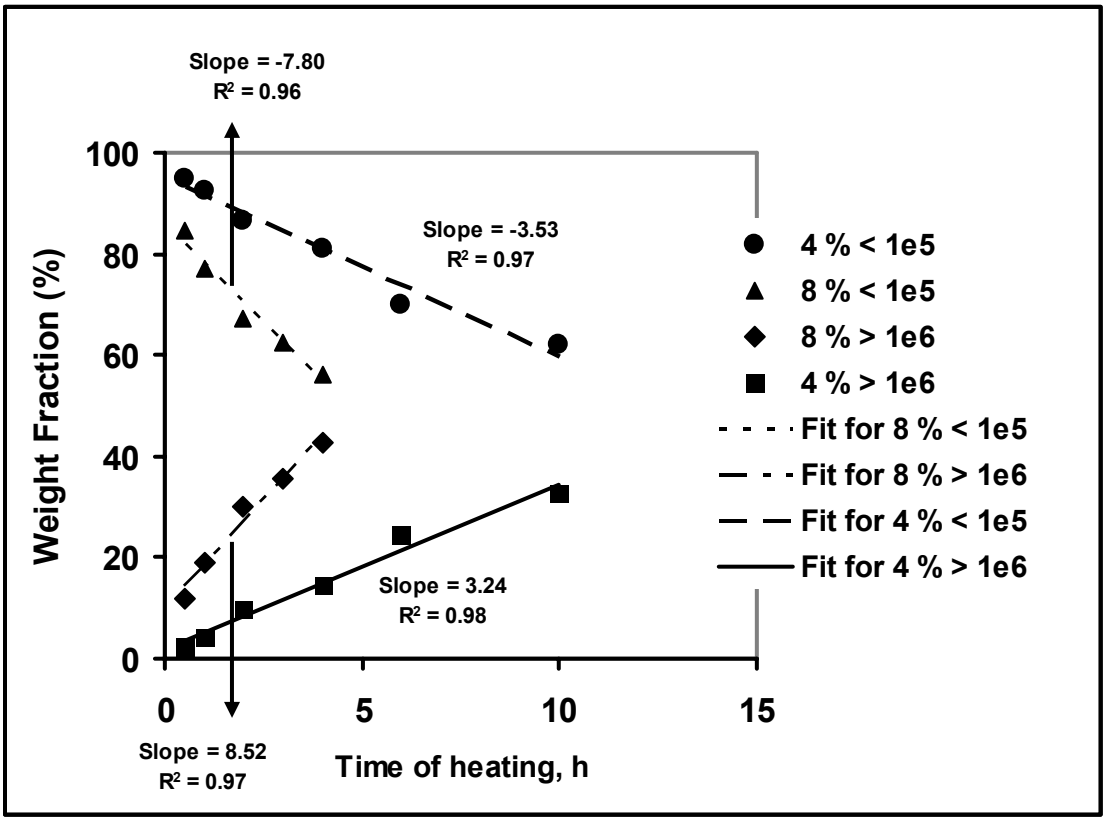

Figure 6.4. Correlation between aggregate sizes with heating time quantifying a bimodal distribution and supporting the nucleation and growth mechanism

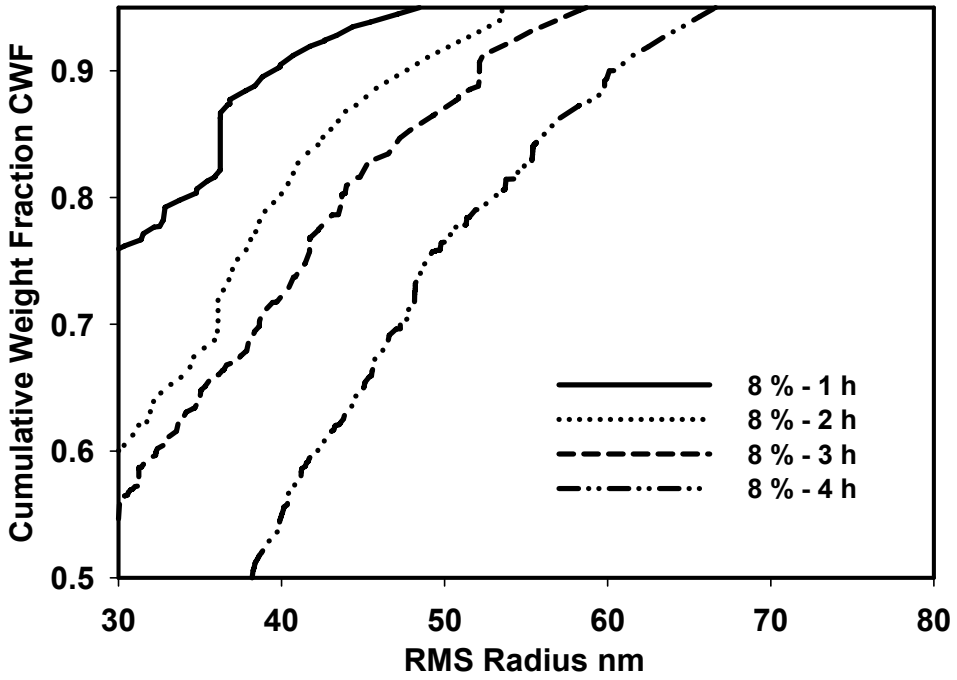

Figure 6.5A. RMS radii of aggregates formed during heating of 8 % w/w (solids) β-lg solutions at pH 3.35

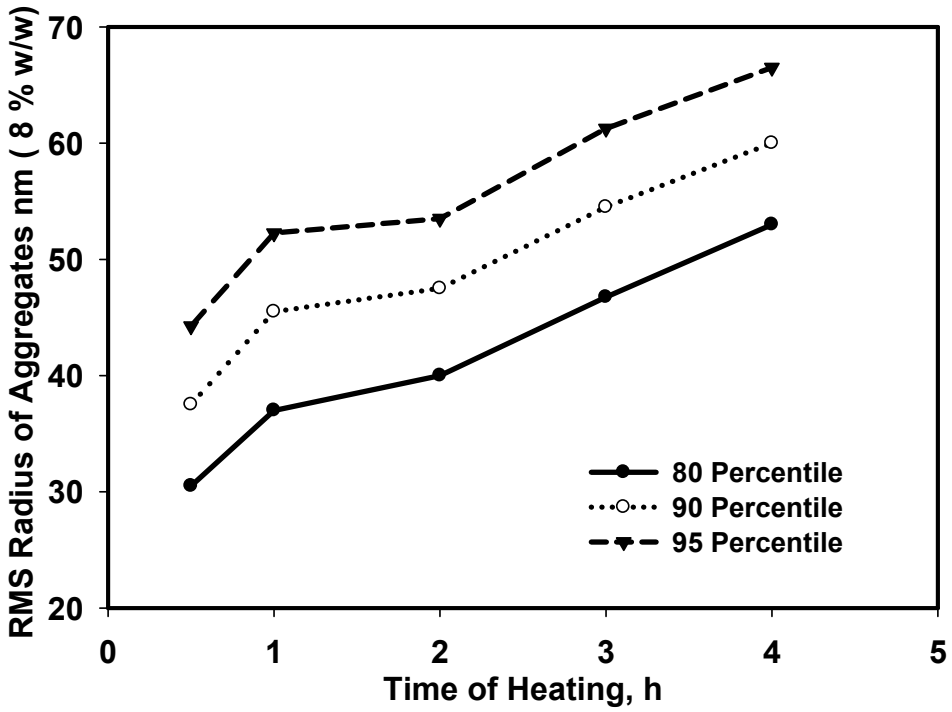

Figure 6.5B. RMS radii of aggregates formed during heating of 8 % w/w (solids) β-lg solutions at pH 3.35. Parallel trends indicating continual growth of aggregates with heating time

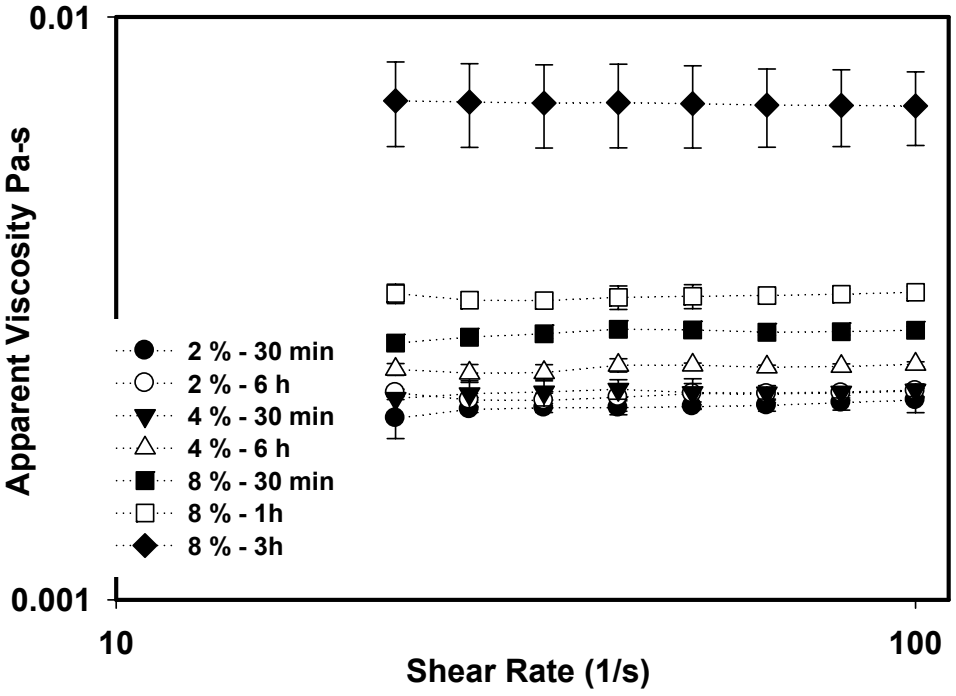

Figure 6.6A. Apparent viscosity of heated β-lg solutions at different concentrations (2, 4 and 8 % w/w) and heating times at pH 3.35. Error bars denote one standard deviation.

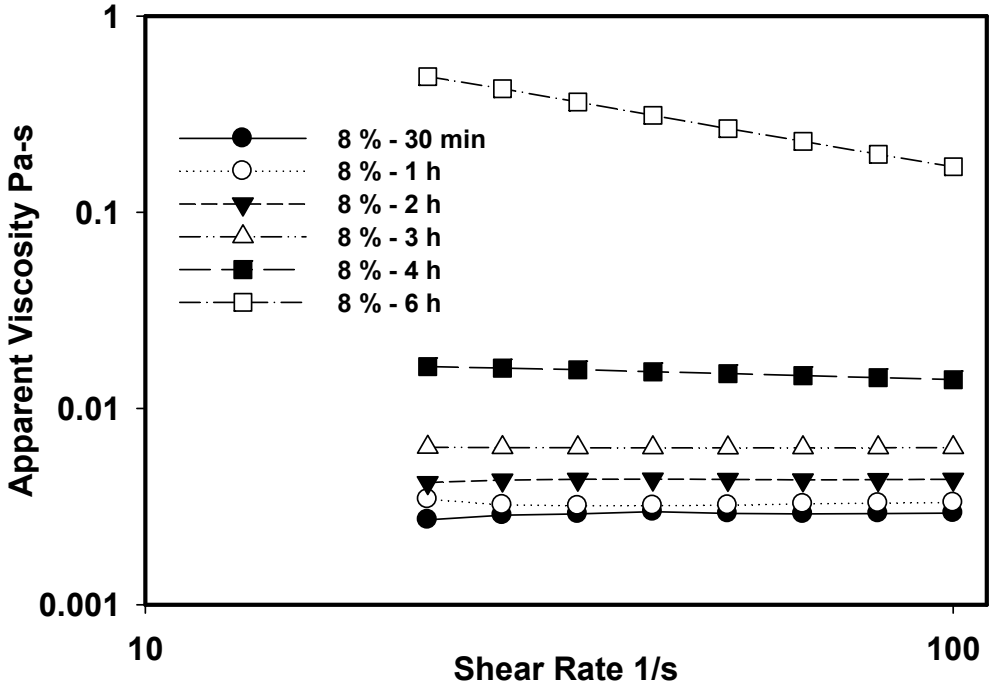

Figure 6.6B. Apparent viscosity of heated 8 % w/w (solids) β-lg solutions at pH 3.35

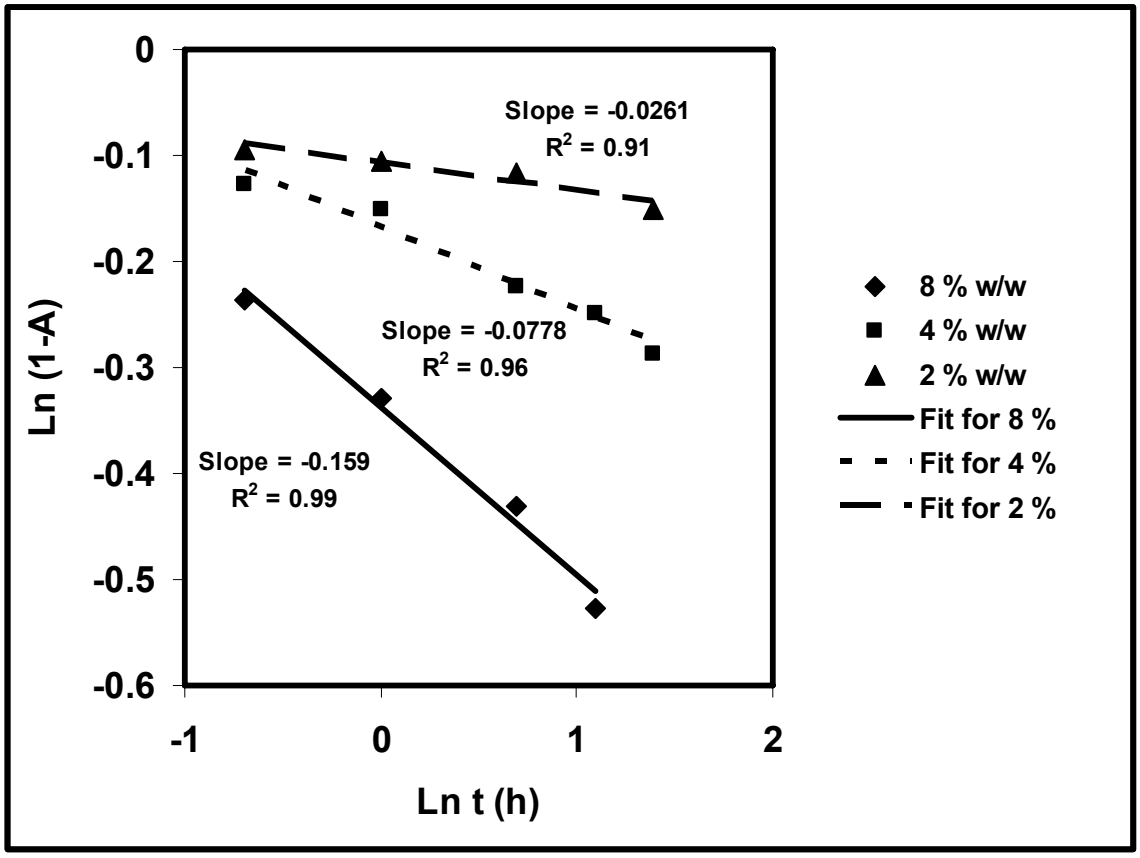

Figure 6.7. Plot of Ln (1-A) with Ln t to determine the critical size of nucleus at varying (2, 4, and 8 % w/w) protein concentrations

CHAPTER 7.

CONCLUSIONS

Concentration effect studies on the modification process identified a critical concentration (C_c ~ 6.4 % protein) below which no significant thickening function could be achieved from modified β-lg powders. While at pH 2 and low ionic strength conditions, β-lg forms rigid rods or linear fibrils, more flexible fibrils were formed at pH 3.35, the pH of the modification procedure. The diameter of these fibrils was approximately 5 nm, similar to what is obtained at pH 2, while the persistence length of about 35 nm much smaller than lengths at pH 2. The length of these flexible fibrillar aggregates formed at pH 3.35 was concentration dependent. Below the critical concentration, isolated fibrils of molar mass up to 10^7 Da were observed, while above the critical concentration, more connected aggregates ('microgels') composed of these fibrils were created. It appeared that the formation of these microgels was necessary to achieve the desired thickening function.

The addition of $CaCl_2$ during the modification process resulted in an increase in ionic strength. This increase resulted in screening of electrostatic repulsions at pH 3.35 and thus higher aggregation of β-lg. Adding small amount of $CaCl_2$ up to 6.67 mM (20 mM *I*) above the critical concentration led to an improved thickening function from modified β-lg powders. The network characteristics after the addition of this small amount of $CaCl_2$ were still fine stranded with formation of flexible fibrillar aggregates. Increasing ionic strength up to 0.08 M at lower concentrations did not increase the viscosity of heated solutions. The presence of large fibrillar aggregates (microgels) prior to the drying step was essential to obtain any thickening from subsequently dried powders. During the freezing step, freeze concentration effects led to further aggregation of microgels, as was observed from the

transmission electron microscopy of reconstituted modified powders. This effect led to a further increase in thickening capacity of modified β-lg powders. Similar network characteristics were observed in rehydrated modified powders prior to and following drying.

Upon heating during modification, disulfide linked aggregates were formed as observed from the electrophoresis data. However, comparing the protein band profiles of rehydrated modified β-lg powders prepared from different concentration, it was confirmed that disulfide linked aggregates alone did not contribute to the thickening function of modified powders but non-covalent interactions also seemed to play an important role. Acid hydrolysis was observed at both pH 2 and 3.35 with a more prominent effect at pH 2, substantiated by electrophoresis data. Partial hydrolysis of β-lg with pepsin, resulting in formation of more peptides, seemingly promoted aggregation and increased viscosity by about two logs above the critical concentration.

Finally, from kinetic studies it was found that the nucleation and growth mechanism described the thermal aggregation of β-lg at pH 3.35. The size of the flexible fibrillar aggregates formed was dependent on the initial protein concentration, and the nucleation was determined to a rate limiting step below the critical concentration. Above the critical concentration, nucleation occurred rapidly, leading to a higher degree of aggregation and thus formation of large aggregates. Nuclei sizes were found to be dependent on the protein concentration and a bimodal distribution of aggregates was formed after heating at all

concentrations. By manipulating concentration and heating it is possible to control the degree of aggregation and the size of aggregates between 10^6 to 10^8 Da.

This dissertation contributes to the existing literature on β-lg aggregation mechanisms between pH 2 and 7 and also explains the mechanisms behind the cold-thickening function of the modified whey powders. To modify the functional properties of whey proteins, formation of soluble aggregates is critical. In conventional, cold-gelation studies, these soluble aggregates were formed at pH 7, and then cold-gelation was induced. At low pH, one way to modify functional properties of whey proteins is to form long rigid fibrils, which increase viscosity on a weight efficient basis. This dissertation provides another way to form soluble aggregates which are composed of flexible fibrillar strands. As described herein, the properties and size of these soluble aggregates can be altered as desired by manipulating ionic strength, enzymatic hydrolysis, concentration, and heating time to manipulate functionality of modified whey ingredients.