THERMALLY INDUCED AGGREGATION OF WHEY PROTEINS: CHARACTERIZATION OF PROTEIN ISOLATES AND BETA-LACTOGLOBULIN/ PECTIN INTERACTIONS

by

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(Under the direction of Milena Corredig)

ABSTRACT

Despite its widespread utilization, information relating to the characterization of commercially available whey protein isolate (WPI) is limited. Further insight into its behavior in mixed systems can enhance its use as a "value-added" ingredient. The thermal aggregation behavior of WPI was examined, as well as interactions between β-lactoglobulin, the main functional protein in whey, with a high methoxyl and a pectinesterase-modified pectin at low pH, and stability of orange juice with added whey protein.

Molecular weight averages and distribution of WPI aggregates varied significantly with temperature and concentration. Upon heating 10% WPI solutions, native protein decreased with increasing temperature and hydrodynamic diameters increased with temperature. Protein composition of the aggregates did not change upon heating. Pectin charge distribution affected the formation of soluble complexes with β-lactoglobulin. Fortification was most stable upon addition of β-lactoglobulin and WPI at pH 3.0 and a heat treatment of 85°C.

INDEX WORDS: Whey protein isolate, Heat-induced aggregation, Pectin-protein interactions, Juice stability.

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Dedicated To:

My parents Kristin and Dale Curtsinger, sister Steffani, brother Sean, Aunt Kelly and other family without whose love and support this would not have been possible as well as my friends for their inspiration and support.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

For many years, whey proteins were treated as by-products of cheese processing and either discarded or used as animal feed. Today, whey is an important value-added ingredient due to its nutritional benefits and excellent functional properties.

Whey proteins are high quality and considered "complete" proteins in that they contain all of the essential amino acids (Werner, 1981). They are used as functional ingredients in foods because they possess water-binding, gelation, foaming/whipping, emulsification and other texture-enhancing properties. Pure whey protein fractions can be prepared using industrial-scale processes for their separation.

When separating whey proteins, different isolates can be produced. Whey protein concentrate (WPC) can be formulated to specific protein levels ranging from 34-90%, with the rest of its composition being attributed to fat and ash. Whey protein isolate (WPI) contains protein concentrations greater than 90%, with limited fat and ash.

Whey proteins are soluble over a wide pH range, making them ideal for use in acidified beverages. Pectin has long been known to stabilize these beverages (Kravtchenko *et al*., 1995). Studies with model systems may help elucidate the mechanisms by which pectin and the main functional whey protein, β- lactoglobulin, interact.

The hypotheses of this research are:

i) Denaturing and subdenaturing temperatures have different effects on the aggregation mechanism of WPI.

ii) The pH of the system has an effect on the interactions produced in β-lactoglobulin/pectin mixtures.

iii) Charge interactions have an effect on the colloidal stability of whey fortified orange juice.

The goal of these studies were to determine protein behavior upon processing conditions both in the presence of and in the absence of pectin- for this reason we studied model systems. The objectives of this research were:

i) To study the heat-induced interactions and aggregation behavior of WPI.

ii) To understand the interaction mechanisms of β-lactoglobulin with pectins of different charge densities.

iii) To investigate the role of different whey protein isolates in Valencia orange juice and their effect on stability.

Whey protein aggregates were studied using size exclusion chromatography in combination with multi-angle laser light scattering, dynamic light scattering and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. β-lactoglobulin/pectin interactions were analyzed by size exclusion chromatography, dynamic light scattering, and uronic acid assay. The stability of whey fortified orange juice was determined by measuring uronic acid content, titratable acidity, % transmission and capillary electrophoretic analysis.

Results from this research will show how heating temperature affects the aggregation behavior of WPI, as well as provide information on the types of interactions

that β-lg and pectin display under various environmental conditions. These data will aid in the application for the novel utilization of whey proteins.

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CHAPTER 2

LITERATURE REVIEW

2.1 Milk Proteins

Milk protein contribution in the human diet can be much appreciated when one considers that almost 20% of American's daily protein comes from dairy products (Smit *et al*., 1999). Its composition is well understood and it is known that behavioral and physico-chemical differences exist within the two classes of proteins that are found in milk. These classes of protein include the caseins, which constitute 80% of the total protein content and the whey proteins, the serum portion that remains in solution after removal of caseins, comprising 20% of the milk proteins (Swaisgood, 1996).

2.2 Nutritional and Functional Implications of Whey Proteins

There is an increasing demand for the production of food made with low-cost, high-quality protein as an ingredient. One reason for this is the nutritional importance of protein in the diet. According to Werner (1981), the biological value of whey protein is superior to other common dietary proteins such as egg, beef, soy, casein and wheat. This contributes to key nutrition enhancing benefits found in whey protein. For instance, α-lactalbumin a whey protein typically found in infant formula, has increased amounts of tryptophan, a building block of the vitamin niacin. Niacin, an essential co-enzyme in fat synthesis, tissue respiration and utilization of carbohydrates, is important in maintaining vital roles in the human body (Heine *et al*., 1995; Heine, 1999). Lactoferrin, an

antioxidant and iron-building protein in whey, has been found to exhibit bacteriostatic and bacteriocidal activity against a variety of pathogenic bacteria, including those responsible for gastroenteric infections, food poisoning, listeriosis and mastitis (Dionysius *et al*., 1993). Whey protein has also exhibited anticarcinogenic effects, for example on the incidence of colon tumorogenesis. One study tested the chemical induction of tumors in the colon of rats by injecting them subcutaneously with the carcinogen-dimethylhydrazine (McIntosh *et al*., 1995). It was found that only 30% of rats fed a diet rich in whey protein developed colon cancer compared to 55-60% of rats consuming diets rich in meat or soy protein. Furthermore, those rats consuming a diet rich in whey protein experienced a significant reduction in the total number of tumors compared with those fed meat or soy protein (McIntosh *et al*., 1995).

In addition to their nutritional implications, food technologists are interested in the utilization of whey protein in products because of the functional properties that they impart in foods. For instance, β-lactoglobulin and α-lactalbumin, the main proteins found in whey, are primarily responsible for the foaming/whipping, water-binding, gelation, emulsification and other structural and textural properties (Kinsella, 1976; Voutsinas *et al*., 1983; Cayot and Lorient, 1997). Furthermore, they are light in color, bland, do not contribute to strong flavors in products and are relatively soluble over a wide range of pH (Regester *et al*., 1996; Cayot and Lorient, 1997). Several intrinsic factors that affect their functional properties include amino acid sequence and composition, structure (secondary and tertiary), hydrophilic/hydrophobic character of protein surface, net charge and distribution and molecular rigidity/flexibility (Phillips *et al*., 1994). These characteristics make whey protein ideal for use in many foods that require stabilization. For these

reasons, whey has shifted from being treated as a disposal problem in the industry to that of an ideal functional protein with unique properties and low economic cost (Regester *et al*., 1996).

2.3 Whey Protein Composition

Knowledge of structure and conformation is necessary in understanding the functionality of whey proteins. The major whey proteins are β-lactoglobulin and α-lactalbumin, composing 54% and 21%, respectively, of the total whey proteins (Kinsella, 1984). The minor whey protein components include serum albumin, lactoferrin, immunoglobulins and phospholipoproteins (Kinsella, 1984).

2.3.1 β-lactoglobulin (β-lg)

β-lactoglobulin is the most abundant whey protein in milk and many of its structural and functional properties have been elucidated. It is a globular protein with a monomeric molecular weight of 18,300 Da and primarily exists as a dimer at room temperature and a pH between ~5.5 and ~7.5 (McKenzie, 1971; Kinsella, 1984; Hambling *et al*., 1992). Native β-lg is made up of 162 amino acid residues and possesses two disulfide bonds (residues 66-120 and 121-106) and one free thiol group (residue 119), buried within the protein molecule (Swaisgood, 1982). These groups are important for heat-induced thiol disulfide interchange reactions, allowing intermolecular and intramolecular disulfide-bond interactions as well as heat-induced interaction of the free thiol group with κ-casein (Kinsella, 1984). Thiol-disulfide bond exchange has also been shown to convert native monomer β-lactoglobulin to non-native monomers (Iametti *et al*., 1996; Schokker *et al*., 1999; Havea *et al*., 2000). Raising the temperature above ~60°C causes a dissociation of the dimer to non-covalent monomers (Verheul *et al.*,

1998), a necessary step for polymerization of the protein (Iametti *et al*., 1996). Upon further heating, the molecule unfolds and exposes the free thiol group and interior hydrophobic groups (McKenzie, 1971; Iametti *et al*., 1996). The free thiol group is necessary for intramolecular disulphide interactions to occur.

A kinetic model for its denaturation/aggregation behavior has been proposed by Roefs and de Kruif (1994), involving initiation, propagation and termination steps, with the free thiol group of β-lactoglobulin being responsible for further polymerization of the protein (aggregate formation). The first step involves reversible reactions, in which the β-lg dimer is split into two separate monomers. Next, initiation takes place, an irreversible first-order reaction in which the conformation of native β-lg is transformed so that the free thiol group is exposed and highly reactive. In the propagation step, the reactive thiol group interacts via a thiol/disulfide exchange reaction with one of the two intramolecular disulfide bonds of a nonreactive β-lg molecule. This forms the intermolecular disulfide bond and, upon a new thiol group becoming exposed, the process continues leading to the formation of linearly linked aggregates. Upon increasing temperatures, unfolding occurs concomitant with increased activity and oxidation of the thiol group (Hambling *et al*., 1992).

Thermal induced aggregation may also be caused by non-covalent interactions (such as ionic, van der Waals and hydrophobic) caused by physico-chemical conditions of the environment. Factors such as concentration of protein, pH, heating temperature and ionic strength have an effect on the types of interactions that can occur, and many researchers have examined the aggregation behavior of β-lg upon changes of

environmental conditions (Hambling *et al*., 1992; Verheul *et al*., 1998; Verheul *et al*., 1999; Hoffmann and van Mil, 1999; Schokker *et al*., 2000a; Uhrinova *et al*., 2000).

β-lg is mainly dimeric between the pH range of 5 to about 8 (McKenzie, 1971; Morr & Ha, 1993), however, its structure is a function of the pH of the system. Well below its isoelectric point (pH 5.2), at pH 3.5 and above pH 7.5, the protein dissociates into monomers (Hambling *et al*., 1992; Aymard *et al*., 1996). Between pH 3.7 and 5.2, β-lg is present in octameric form with a molecular weight of 144 kDa (Hambling *et al*., 1992; Morr and Ha, 1993).

The secondary structure of β-lg has been studied by a variety of techniques. Crystallographic studies of have elucidated the orientation of the dimers and the location of specific residues. It has been shown to possess 10% α-helix, 43% anti-parallel β-sheet, 15-20% reverse turn and 15-20% aperiodic structure (Papiz *et al*., 1986). The anti-parallel β-sheet, formed by 9 strands (A-I), wraps around to form a flattened cone or calyx, which includes the hydrophobic residues of the protein (Papiz *et al*., 1986; Phillips *et al*., 1994). Dimeric protein is formed when anti-parallel interactions are made by strand I of each monomer. The interior hydrophobic calyx of the protein is an eight-stranded, twisted anti-parallel β-barrel (Papiz *et al*., 1986). At basic pH, the open end of the calyx provides an access route to a cavity, known as the "open" conformation in which small, hydrophobic molecules are thought to bind. At acidic pH, the protein exists in its "closed" conformation, caused by strands E-F occluding the open end of the calyx (Papiz *et al*., 1986). β-lg is in the family of lipocains and a possible binding site for retinol has been identified by model-building. However, its function in vivo is still not understood (Papiz *et al*., 1986).

Bovine β-lg possesses genetic polymorphism. This leads to different variants of the protein, of which at least 7 are known, with A and B considered the most ubiquitous (Eigel *et al*., 1984). The differences between these two variants lie in the amino acid sequence of the protein. Aspartic acid and valine, located in positions 64 and 118, respectively, of β-lg A, are replaced by glycine and alanine in variant B, giving rise to differences in molecular weight and affecting thermal stability (Eigel *et al*., 1984; Huang *et al*. 1994; Manderson *et al*., 1998).

2.3.2 α-lactalbumin (α-lac)

α-lactalbumin is a small, compact globular protein of 14,200 Da molecular weight, with an isoelectric point around a pH of 4.2 (Brew and Grobler, 1992; Bramaud *et al*., 1995). It contains 123 amino acids, four intramolecular disulphide bonds and no free thiol group (Brew and Grobler, 1992). Three aspartic acid residues surround an atom of calcium, which help stabilize the tertiary structure of the protein and protects against thermal denaturation (Morr and Ha, 1993, Wong *et al*., 1996). The protein is also capable of binding to other divalent and monovalent ions. Upon binding to these ions, conformational transitions take place, which are dependent upon the concentration of ions (Wong *et al*., 1996).

α-lac has an apo-like conformation near its isoelectric point and its hydrophobic properties increase strongly below pH 4.5. Previous studies show that hydrophobicity is an important characteristic of α-lac when incorporating into aggregated whey proteins (Havea *et al*., 1998). The binding of calcium to apo-α-lactalbumin leads to a more compact and less hydrophobic conformation, known as halo-α-lac (Shanbhag *et al*., 1991; Eynard *et al*., 1992). Both apo and halo forms of the protein increase in surface

hydrophobicity upon heat treatment due to "swelling" or relaxation of the protein structure, however halo-α-lac displays reversible heat-induced modifications upon cooling, while apo-α-lac does not return to its original conformation upon cooling after heat treatment (Eynard *et al*., 1992).

2.4 Processing of Whey Protein Isolate and Concentrates

Upon manufacturing of cheese around the world, whey solids result in over 500,000 T of whey protein and many processes have been created to recover this protein for use as a value-added food ingredient (Muller, 1982). Manufacturing of whey proteins produces two different types of dry product: whey protein concentrate (WPC) and whey protein isolate (WPI), each defined by the concentration and type of protein contained in the system. WPC can be formulated to specific protein levels ranging from 34-90%. WPI contain a higher concentration of protein (ie. greater than 90%). These concentrations may go up to 95% protein, with limited fat and ash, when WPI are required to perform specific function in food products.

Protein and fat can be separated from the whey using ion exchange chromatography or filtration techniques such as ultrafiltration, diafiltration, and microfiltration. The main process for separation is ultrafiltration (UF), in which low molecular weight compounds such as lactose, non-protein nitrogen, vitamins and minerals, permeate from the milk, while the remaining proteins concentrate in a retentate (Maubois *et al*., 1987). This is generally carried out using semipermeable membranes with molecular cut-off limits of 10-50 kDa, separating the whey protein from the low molecular weight compounds (Morr and Ha, 1993). The process is performed between 10 and 50°C and at a pH of 4.6-6.5 (Maubois *et al*., 1987). After separation, retentate is

diafiltered with deionized water to further remove lactose and other low molecular weight species. Finally, the whey protein is spray-dried at low temperatures, so as to prevent protein denaturation, giving a powder with a final protein content of $\geq$ 75% (Morr, 1989).

Another technique used to process whey proteins in industry is ion exchange chromatography. The pH of the system is adjusted so as to obtain the necessary charge of the ampholytic protein. At pH values lower than 5.2, the positively charged proteins absorb onto cation exchangers, while at pH values above 5.2, the negatively charged proteins bind to anion exchangers (Maubois *et al*., 1987; Rossomando, 1990). Proteins adsorb to the column and are subsequently displaced by readjusting the pH of the solution. The desorbed proteins elute from the ion exchanger, are concentrated by UF and are spray dried (Morr and Ha, 1993). This results in a product with protein concentrations greater than 90%, often characterized by a more controlled protein composition.

2.5 Studies Involving Heat Treatment of Whey Protein Isolates

The effect of heat treatment on the functionality of whey proteins has been the subject of investigation to determine the extent of denaturation, the mechanisms involved in the aggregation steps and factors which lead to gelling of whey proteins, especially under conditions used in food systems.

Denaturation is the first step in the aggregation process for milk proteins (Dalgleish *et al*., 1982; Ma and Harwalkar, 1991). Following denaturation, the mechanisms and kinetic of interactions depend on the environmental conditions. For instance, the type of gel network formed is determined by pH, solutes and heating conditions, ultimately creating fine-stranded, opaque or translucent gels (Foegeding *et al*., 1998; Ikeda and Morris, 2002).

It is known that increased protein-protein interactions can affect the rate of aggregation, as well as the microscopic network structure of globular protein gels (Langton and Hermansson, 1992; Ikeda *et al*., 1999; Ikeda and Morris, 2002). As for other functional properties, it is known that heating has a negative impact on solubility, yet moderate heat treatment may enhance emulsification, foaming capacity and can produce soluble aggregates which can enhance viscosity in food products (de Wit and Klarenbeek, 1984; Vardhanabhuti & Foegeding, 1999).

The protein concentration has an effect on the polymerization process. β-lg, at a concentration of 10 g/L, heated at 65°C at pH 6.5 forms small oligomers (di-, tri, and tetramers) (Hoffmann *et al*., 1997). Upon increasing concentration (30-100 g/L), mainly larger aggregates with a molecular mass of 3 x 10^5 Da form primarily by intermolecular disulfide bridging (Hoffmann *et al*., 1997). It has been shown that at low ionic strength, the size of the protein particles grows rapidly and after a short time, reaches a relatively constant value, increasing in number rather than size (Verheul *et al*., 1998; Schokker *et al*., 1999). β-lg aggregates heated at neutral pH in the temperature range of 60-70°C tend to be less than 100 nm in size (Roefs and de Kruif, 1994; Hoffmann *et al*., 1996). Vardhanabhuti & Foegeding (1999) have also demonstrated that increasing concentration of β-lg also leads to an increase in viscosity upon heating.

The network structure of a heat-denatured globular protein gel depends greatly on the balance of attractive and repulsive forces among the protein molecules, as determined by pH and ionic strength (Bryant & McClements, 2000; Ikeda and Morris, 2002). When heating the proteins near their isoelectric point or increasing the ionic strength, there is a decreased intermolecular repulsion between the protein molecules (Kella & Kinsella,

1988). This has a pronounced effect on the β-lg aggregation process. The hydrodynamic diameter of protein aggregates, as determined by dynamic light scattering, shows an increase with increasing NaCl concentration, due to shielding and salting-out effects. This leads to decreased intermolecular repulsion and promotes chemical and physical aggregation (Phillips *et al*., 1994; Verheul *et al*., 1998). Aggregates can become so large that determination of the molecular mass distribution by chromatography coupled with multi-angle laser light scattering becomes problematic; this is primarily due to aggregates eluting in the excluded void volume of the columns, making quantitative analysis of the aggregates difficult (Hoffmann *et al*., 1997).

Larger aggregates are also formed as a result of increasing pH. This is due to the degree of dissociation of the thiol group increasing and leading to an increased rate of propagation and termination steps (Hoffmann and Van Mil, 1999). Many studies have been carried out on β-lg aggregation at neutral pH values (de Wit and Klarenbeek, 1984; McSwiney *et al*., 1994; Roefs and de Kruif, 1994; Iametti *et al*., 1995; Nielsen *et al*., 1996; Hoffmann *et al*., 1997; Manderson *et al*., 1998; Verheul *et al*., 1998; Hoffmann & van Mil, 1999; Schokker *et al*., 1999). Few researchers have studied the aggregation behavior of the protein at acidic pH. At pH values lower than the pI, the thermal stability of the protein increases and it is suggested that extra hydrogen bonding is responsible (Kella and Kinsella, 1988). β-lg maintains monomeric conformation and much of the hydrophobic core appears intact, contributing to little sulfhydryl-mediated disulfide exchange reactions at low pH (Harwalkar and Kalab, 1985; Uhrinova *et al*., 2000). Heating β-lg at pH 2.5 and 80°C, forms aggregates with molecular masses of 10^6-10^7, with non-covalent interactions, mainly of hydrophobic nature (Schokker *et al*., 2000a).

2.6 Pectin Structure

Pectins are plant-derived, heterogenous polysaccharides with functional properties related to their chemical and physical structure. They contain a chain structure of α-(1,4) linked D-galacturonic acid units, interrupted with (1,2)-linked L-rhamnopyranosyl (rhamnose) residues (Neukom *et al*., 1980; Rolin, 1993). Pectins include various types of sugars attached as side chains that can be linked to the rhamnose groups or to the galacturonic acid groups. They are concentrated in "hairy regions" and the most common ones are D-galactose, L-arabinose and D-xylose (Voragen *et al*., 1995). Unsubstituted "smooth" regions, containing mainly galacturonic acid units (homogalacturonan), separate the "hairy" regions on the molecule (Voragen *et al*., 1995).

Knowledge of the methoxyl ester content in the "smooth" regions, expressed as degree of methylation (DM), plays a major role in the gelling properties of pectin. Pectins with a DM greater than 50% are classified as high methoxyl pectin (HMP) while low methoxyl pectin (LMP) contains a methoxyl concentration less than 50%. HMP forms gels based on hydrogen bonds and hydrophobic interactions in the presence of sugars or other co-solutes and low pH, which are necessary for chain-chain interactions to occur (Oakenfull, 1991, Rolin, 1993). LMPs have a high charge density and gels are formed mainly electrostatically in the presence of cations, acting as a bridge between pairs of carboxyl groups along the homogalacturonan chain, as described by the "egg box model" (Grant *et al*., 1973; Rees, 1982; Morris *et al*., 2000). It has recently been demonstrated that LMP pectins can bind without the presence of Ca^{++}, however a low pH environment and high concentration of co-solute is needed to observe chain-chain interactions (Gilsenan *et al*., 2000).

Not only do the number of charges affect the functional properties of pectins, but differences in the distribution of the methoxyl esters also have an influence on the chemical and physical structure of the molecules. Depending on the type of process used to produce the pectins, different esterification patterns can be found. Enzymatic deesterification using plant pectinesterases cause a blockwise or sequential distribution of the methoxyl ester groups, whereas fungal pectinesterases and chemical deesterification (using alkali) results in a random distribution (Taylor, 1982; Ralet *et al*., 2001).

The distribution of methoxyl ester groups can be intermolecular or intramolecular. Distribution over a variety of pectin molecules in a mixture gives an intermolecular distrubution, while intramolecular distribution relates to the distribution within one molecule (de Vries *et al*., 1986; Voragen *et al*., 1995). This can cause differences in populations of pectins impacting molar mass or charge density, and thus functional properties. For instance, intermolecular distribution of sugars and methoxyl groups in "homogeneous" pectins was studied in relation to calcium binding properties and it was found that plant pectinesterase caused higher calcium binding properties than fungal pectinesterase, even for HMP, due to plant enzymes contributing to a wide range of charge density on the pectin molecule (Ralet *et al*., 2001). These factors can all significantly affect the functional behavior of pectin when used as a stabilizer or thickener in food products.

2.6.1 Applications of pectin in acidified systems

The earliest application of pectin in foods was in fruit jams with high soluble solids and a pH between 3.0-3.3. HMP is required in this system because it forms a gel in the presence of sugar and acid (May, 2000). LMP is used in low sugar jams and jellies.

They are less sensitive to sugar concentration, yet require the presence of Ca^{++} to form a gel (May, 2000).

Pectin is also often added to dairy products because it interacts with the proteins and causes stability of the dispersions. HMP is added as a stabilizer, and it prevents precipitation of protein in low pH milk beverages such as fruit-flavored milk, sour milk and yogurt drinks (Glahn, 1982, Pereyra *et al*., 1997). LMP can provide a gel in milk or acid products by interaction with Ca^{++} (May, 2000). A better knowledge of the interaction occurring between these biopolymers is important to determine their behavior as functional ingredients in food systems.

2.7 Protein-Polysaccharide Interactions

The interaction of protein-polysaccharide complexes in solution can be described by fundamental principles of colloid science. The mixing behavior is primarily controlled by enthalpic effects given by the relative strength of interactions of polymers with themselves and with the solvent (Syrbe *et al*., 1998). The result of these effects contributes to either segregative behavior in which the biopolymers repel each other and are non-compatible, or associative behavior wherein the biopolymers attract one another (de Kruif & Tuinier, 2001).

Three situations that may arise from the mixing of two biopolymers (see Figure 2.1).

2.7.1 Incompatibility

When biopolymers are incompatible because of molecular repulsion, the mixing causes instability and eventually segregation of two immiscible aqueous phases, one containing mainly the protein and the other containing mostly the polysaccharide (Syrbe

et al., 1998; Tolstoguzov, 1986; Dickinson & McClements, 1995). Factors that may contribute to inhibition of interactions include a high bulk concentration of biopolymers, high ionic strength to screen charges or protein-protein induced interactions (Grinberg & Tolstoguzov, 1997; de Kruif & Tuinier, 2001).

2.7.2 Complex coacervation

If attractive forces between the biopolymer molecules exist, complexation can occur. The interactions of such complexes lead to the formation of soluble or insoluble coacervates. The coacervate may also phase separate, with one phase containing both polymers, and the other devoid of any (Syrbe *et al*., 1998). Generally this effect is shown in mixed systems of oppositely charged protein-anionic polysaccharide complexes and is typically driven by electrostatic interactions (Tolstogusov, 1986; Schmitt, et al., 2000).

2.7.3 Co-solubility

Homogenous stable solutions of proteins and polysaccharides can also be formed (Tolstoguzov, 1986). These systems are stable even at high polymer concentrations, but molecular weight plays a major role in stability. In general, the higher the molecular weight, the less likely the two biopolymers are to balance between not too repulsive and not too attractive interactions, thus leading to either complex coacervation or incompatibility (Syrbe *et al*., 1998).

2.7.4 β-lactoglobulin/pectin interaction studies

Studies carried out on proteins and anionic polysaccharide mixed systems, have led researchers to agree that interactions are mainly electrostatic. These interactions depend on enviromental conditions, such as pH and ionic strength (Tolstoguzov, 1986). In pectin, not only the molecular weight, but also the charge on the backbone (HMP vs.

LMP) has an effect on the interactions with β-lg, due to differences in their charge densities. Ndi *et al*. (1996) showed that interactions between sodium polypectate (SPP), a polygalacturonic acid molecule with a high charge density, with β-lg at pH 6.5 were limited, due to both biopolymers possessing net negative charges. Similarly, Wang and Qvist (2000) studied the interactions of β-lg with both LMP and HMP at the same pH, but used various ionic strengths to manipulate the interactions taking place. They found that no phase separation occurred at low ionic strength with the HMP, while stability with LMP at this pH required the presence of 0.3 M NaCl. The increase in ionic strength results in a screening of the negative charges of LMP, allowing for co-solubility of the two biopolymers.

Upon lowering the pH, coulombic interactions change between β-lg and pectins with varying degrees of methanol substitutions. At pH 3.5, β-lg is positively charged while pectin possesses a net negative charge. When combining SPP with β-lg at this pH, interactions between the two biopolymers resulted in the formation of a cloudy white precipitate (Ndi *et al*., 1996). On the other hand, Wang and Qvist (2000) found, when mixing β-lactoglobulin with HMP, a white homogenous dispersion formed with no phase separation, despite the opposite net charges. Its apparent stability was attributed to the formation of soluble complexes further associating to large aggregates.

The charge densities of the polymers are important in maintaining stability of protein-pectin mixtures, as it has been illustrated by these and other studies involving the mixing of β-lg with other anionic polysaccharides (Schmitt *et al*., 2000; Croguennoc *et al*., 2001). Studies on the interactions of β-lg with HMP and LMP are scarce, and the mechanism of mixing is not fully understood. No studies have currently been conducted

on the effect of thermally induced interactions of β-lactoglobulin with HMP and LMP at low pH.

2.8 Analytical Techniques Used for the Study of Whey Protein and Pectin Systems

2.8.1 Chromatography

Chromatography is a type of separation used for macromolecules, which separate based on solute adsorption. High-performance liquid chromatographic (HPLC) elution is separation process in which solution is forced through chromatography media, at a controlled velocity, through a column. The type of media used is dependent upon structural characteristics of the macromolecules, such as size or charge, which determine its relative adsorption.

Macromolecules can be separated depending on there shape and size by using size exclusion chromatography (SEC). In this technique, molecular species diffuse into matrices of the media, which have controlled porosity (Chicz and Regnier, 1990). The smaller molecules permeate within the pores, while macromolecules, which are too large to penetrate the pores, are excluded and travel between the matrices (Chicz and Regnier, 1990). The movement of the sample is dependent on the flow rate of the mobile phase and the degree of permeation into and out of the pores of the stationary phase, thus larger molecules are eluted first, followed by smaller molecules. This is because larger molecules travel faster through the column than smaller molecules (Li-Chan, 1996). Mobile phases used for SEC should minimize the potential for solute/column interactions (Stoll and Blanchard, 1990). In order to prevent solute/column interactions, mobile-phases should have an ionic strength sufficiently high (>0.1 M NaCl) to inhibit hydrophobic and electrostatic interactions with the gel matrix (Chicz and Regnier, 1990).

SEC has been employed in many studies for the separation of whey proteins (Nielsen *et al*., 1996; Hoffmann *et al*., 1997; Hoffmann and van Mil, 1999; Schokker *et al*., 1999; Schokker *et al*., 2000a; Schokker *et al*., 2000b).

Ion-exchange chromatography (IEC) separates macromolecules on the basis of their surface charge and corresponding electrostatic interactions with a charged matrix. Separation takes place by the binding of the solute to the fixed charges of the resin and elution of the solute from the fixed charges by displacement with salt or change in solvent (Rossomando, 1990). The pI of the protein, as well as the pH of the mobile phase, determine the type of resin that is used. In anion IEC, the fixed charges are positive and in cation IEC the fixed charges are negative. Thus, macromolecules that bind to the fixed resin in anion IEC will have a net negative charge (where pH > pI), while those that bind in cation IEC will carry a net positive charge (pH < pI) (Rossomando, 1990). This technique has not only been used for milk proteins, but also for pectins (Andrews *et al*., 1985; Schols *et al*., 1989; Hollar *et al*., 1991; Kravtchenko *et al*., 1992; Ralet *et al*., 2001).

2.8.2 Light scattering

Light scattering techniques have been widely used in the determination of molecular weights and radii of gyration of polymers. The degree to which a macromolecule scatters the light depends on particle diameter, shape and volume. This can be described by the theory of light scattering, which states that light is scattered as a result of changes of polarizability of solution due to fluctuations caused by concentrations of solute in small elements (Burchard, 1992).

One example of light scattering techniques is multi-angle laser light scattering (MALLS), which measures the intensity of scattered volume from 18 different light scattering detectors. It can determine absolute polymer averages and distribution of molecular weight (MW). When combined with SEC-HPLC, it is possible to obtain differential and cumulative distributions of the MW and mean square radii (Wyatt, 1993). Several studies have used MALLS to characterize pure β-lactoglobulin aggregates under various environmental conditions (Hoffmann and van Mil, 1999; Schokker *et al*., 2000a; Hoffmann *et al*., 1997; Schokker *et al*., 2000b).

Another light scattering technique widely used to characterize particle size is dynamic light scattering (DLS). DLS measures the diffusion of particles moving under Brownian motion, so that the larger the particle is, the slower it moves (Claes *et al*., 1992). The hydrodynamic diameter of the particles is calculated using the translational diffusion coefficient, temperature and viscosity of the solution. Previous studies have used DLS to obtain information for pure β-lg (Verheul *et al*., 1998; Schokker, 2000a) and β-lg/pectin solutions (Wang and Qvist, 2000).

2.8.3 Capillary electrophoresis

Capillary electrophoresis is a relatively new separation technique, which combines gel electrophoresis and chromatography principles. Like gel electrophoresis, separations are dependent on differential migration in an electric field, however, separations are carried out in free solution without the need for casting gel. Similar to HPLC, detection is accomplished as the separation is carried out, with resolved zones producing an electronic signal as they migrate past the detector. The electrophoretic migration velocity depends on the magnitude of the electric field and electrophoretic

mobility (Wher *et al*., 1999). When separating whey proteins, a high pH and coated capillaries are necessary to suppress protein adsorption to the inner capillary (Dong, 1999). Several researchers have used capillary electrophoresis for the separation and quantification of milk proteins (de Jong *et al*., Fairise and Cayot, 1998) and whey proteins (Recio, *et al*., 2000).

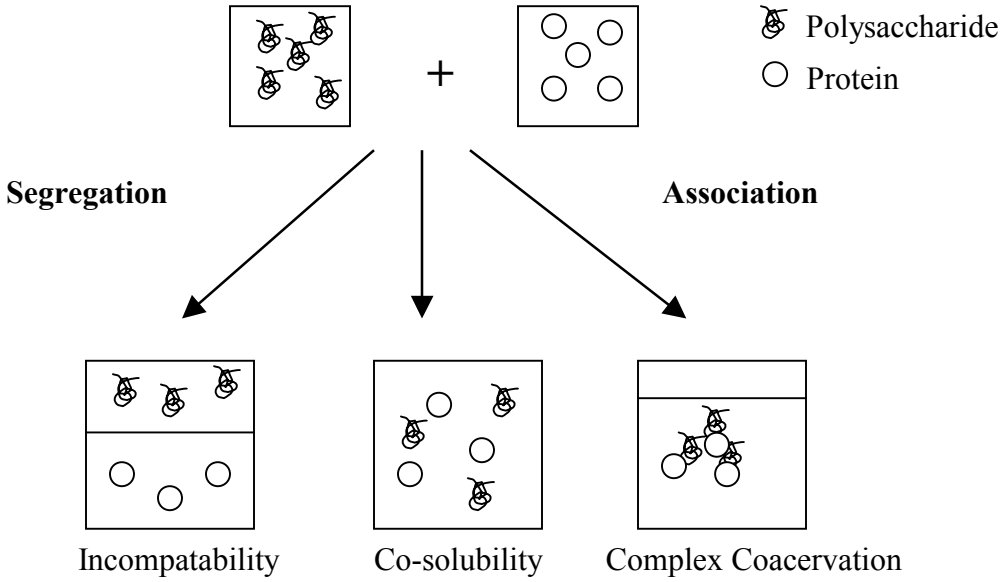

Figure 2.1: Behavior of protein/polysaccharide mixtures upon mixing (adapted from De Kruif and Tuinier, 2001)

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CHAPTER 3

MOLECULAR WEIGHT DISTRIBUTION OF HEAT-INDUCED AGGREGATES OF WHEY PROTEIN ISOLATE[1]

[1]Kazmierski, M. and M. Corredig. Submitted to the *Journal of Dairy Research*.

Abstract 3.1

Heat-induced interactions of a whey protein isolate (WPI) were studied at neutral pH using size exclusion chromatography in combination with multi-angle laser light scattering. Temperature, time and concentration of protein were the conditions tested. While temperature significantly affected the molecular sizes of the aggregates, within the same temperature and concentration, heating time was not significant. No intermediate size aggregates were found, and WPI aggregates with a molar mass of $> 10^7$ g/mol were separated from the residual native protein using a Biosep S3000 size exclusion column. Concentration had a significant effect on the size and number of the aggregates formed, with larger aggregates forming at higher concentrations.

3.2 Introduction

When heated in solution, whey proteins partially unfold, aggregate and, if the concentration is higher than a critical value, they form a gel. The structure of the network that whey proteins form depends on the balance between attractive and repulsive forces of the proteins present in the system (Foegeding *et al*. 1998). For example, opaque or clear gels form depending on pH and ionic strength (Langton & Hermansson, 1992; Foegeding *et al.* 1995, Lefevre & Subirade, 2000; Ikeda & Morris, 2002).

The heat-induced gelation of whey proteins is of great interest because of their widespread utilization as food ingredients. Although whey protein isolates (WPI) and whey protein concentrates (WPC) are the whey protein forms commercially available, fundamental studies have been limited, for the most part, to the heat-induced aggregation and gelation of β-lactoglobulin (β-lg), the primary functional protein in whey (Iametti *et al.*, 1995; Hoffmann *et al.*, 1995). At neutral pH and room temperature, β-lg is primarily in the dimer form, each monomer has two disulphide bonds and one free thiol group. Its mechanism of aggregation has been described: aggregates form via covalent and non-covalent interactions, and at neutral pH the thiol catalized reaction seems to be the driving force of the aggregation (McSwiney *et al*. 1994; Cairoli *et al*. 1994).

Being the most abundant protein in whey, β-lg dominates the overall gelation behavior of WPI. For this reason, the heat-induced aggregation of WPI has many features common to pure β-lg. However, the presence of other proteins modifies the aggregation of WPI (Hines & Foegeding, 1993). In a previous study on protein preparations containing β-lg and α-lactalbumin (α-lac) mixtures (Dalgleish *et al*. 1997), it was shown that α-lac does not form aggregates on its own, and that the ratio of α-lac/β-lg in the

original mix has an effect on the aggregation. The presence of α-lac enhances the formation of heat-induced aggregates of β-lg, held together both by hydrophobic interactions and disulfide bridges. Recently, it was reported that while α-lac seems to react with native β-lg, it does not react with non-native β-lg (Hong & Creamer, 2002). Studies on WPC heated at 75°C have also demonstrated that the aggregates contain β-lg, α-lac and other minor components such as BSA (Havea *et al*. 1998).

A better characterization of these soluble aggregates upon heating will relate to the mechanical and microstructural properties of the various gels which can be prepared with WPI. Size exclusion chromatography combined with multi-angle laser light scattering (MALLS) has been proven to be a powerful technique for characterizing the aggregation of proteins. By using MALLS, β-lg aggregation was studied at neutral and acidic pH (Schokker *et al*. 2000 a, b). It allows the determination of the molecular weight of the aggregates independently from their hydrodynamic radius and also from the separation power of the column (Hoffmann, *et al*. 1997; Hoffmann & Van Mil, 1999; Schokker *et al.* 1999, 2000 a,b; Wyatt,1993).

The objective of the present study was to characterize the molecular weight distribution of the soluble WPI polymers formed at neutral pH using size exclusion chromatography in combination with a MALLS detector. The present work reports on the influence of time, temperature and concentration on the molecular weight distribution of the soluble aggregates formed during heating of WPI. Understanding how these factors affect the aggregation process is of practical interest since thermal treatments are known to modify the functional properties of WPI.

3.3 Materials and Methods

Appropriate quantities of commercial whey protein isolate (Bipro, Davisco Foods International, Inc. Eden Prairie, MN 55344) were dissolved in deionized water, extensively dialyzed and freeze-dried. The freeze-dried powder contained 92% protein (by N analysis) and residual calcium and sodium content were 70.2 μg/g and 380.5 μg/g, respectively (as determined by atomic absorption spectroscopy at the Chemical Analysis Laboratories at The University of Georgia).

Freeze-dried powder was dissolved at concentrations of 25, 50 and 100 mg/ml in HPLC-grade water, and the pH was adjusted to 7.0 using 0.1 M NaOH. Aliquots (2 ml) of sample were filtered through a 0.2 μm filter (PVDF, Millipore Corporation, Bedford, MA 01730) and heated in a water bath at 65, 70, 80 and 90°C for times ranging from 5 to 60 min (time taken to reach the desired temperature was 40 s). The heated samples were immediately cooled in an ice bath and stored at 4°C until further analysis.

Heated solutions were diluted with HPLC-grade water to a final concentration of 5 mg/ml and filtered through a syringe filter (0.45 μm, PVDF, Millipore Corporation) before injection. Aliquots (50 μL) were injected onto a size exclusion column (Biosep SEC-S3000, Phenomenex, Torrance, CA 90501) with a Spectrasystem autosampler (AS3000, Thermo Separation Products, San Jose, CA 95134). 50 mM Na phosphate buffer, pH 7.0 containing 0.15 M NaCl was delivered at a flow rate of 0.5 ml/min with a Waters P500 HPLC pump attached to an in-line degasser (Waters, Milford, MA 01757) and two in-line filters (0.22 and 0.1 μm, Millipore Corporation). The detector was a multi-angle, digital signal processing laser light scattering detector (DAWN DSP-F, Wyatt Technologies, Santa Barbara, CA 93117), which contained a F2 flow cell and a

He-Ne laser light source (Wyatt, 1993). The refractive index (RI) detector used was the Optilab DSP interferometric refractometer (Wyatt Technologies), which operated at 633 nm and a constant temperature of 40ºC. The specific refractive index increment (d*n*/d*c*) was determined to be 0.166 cm^3/g by injecting known concentrations of WPI in the Optilab and using d*n*/d*c* for Windows (version 5.2) software (Wyatt Technologies). This value, required for molecular weight distribution calculations, is comparable to that used by Hoffmann *et al*. (1997) for pure β-lg. Given the low concentration of the protein eluting in the chromatography, the value of A2 (second virial coefficient) was considered null. Molecular weight distribution calculations were carried out with the mass Astra/EASI (version 4.1) software (Wyatt Technologies). Weight averaged molecular masses (M_w) were calculated using the equation: $M_w = \sum c_i M_i / \sum c_i$ and analysis of variance was performed on the values using the general linear model procedure of SAS (version 8.0, Cary, NC 27513). The effects of temperature, time and concentration were tested in the model.

3.4 Results and Discussion

Representative profiles of WPI eluted by size exclusion chromatography are shown in Figure 3.1. The samples (100 mg/ml) depicted were heated at 70ºC for different times. The RI (Figure 3.1 A) relates to the amount of protein eluting, while the light scattering signal (Figure 3.1 B) determines the intensity of the light scattered. The latter is proportional to the average size of the scattering molecules and the amount of molecules present. Our work using RI as the concentration detector resulted in values of molecular weight similar, in terms of size, to those determined for pure β-lg by using UV as the detection method (Schokker *et al*. 1999, 2000). The RI signal (Figure 3.1A) showed two

protein populations eluting at 5.0 ml and 8.0 ml corresponding to aggregated protein and residual native protein, respectively. The concentration of native protein decreased with time of heating, while the amount eluting in the aggregate peak seemed to increase with time.

The intensity of the MALLS signal for the WPI aggregate peak increased with heating time (Figure 3.1B). However, time did not affect the molecular weight averages (M_w), because of the simultaneous increase in the number of aggregates as determined by the concentration detector (RI, Figure 3.1A). In addition, no "intermediate" size aggregates could be separated. This differed from the results reported for purified β-lg: after heating β-lg under similar conditions, large amounts of oligomers and aggregates of intermediate size formed (Cairoli *et al*. 1994; Schokker *et al*. 1999). The absence of aggregates of intermediate size in our study suggested that the aggregation occurred rapidly and passed through the intermediate stage very efficiently, when compared to β-lg heated in isolation (Schokker *et al*. 1999). This absence of intermediates confirmed what was reported previously using other chromatography columns (Kazmierski & Corredig, 2003).

Temperature effects are depicted in Figure 3.2 for 50 mg/ml WPI solutions heated at 65, 70 and 80ºC. The molecular weight is shown in combination with the elution pattern determined with the RI detector and is presented to illustrate the relative concentrations of the WPI aggregates. A decrease in the concentration of residual native molecules is shown over heating time, as already reported for β-lg (Roefs & de Kruif, 1994). This loss of native protein caused a shift to larger peaks where the aggregates eluted not only with increasing heating time, but also with increasing temperature,

confirming that, like pure β-lg, WPI becomes more reactive over temperature of heating (Paulsson *et al*. 1986; Cairoli, 1994).

Table 3.1 lists the average molecular weights, indicated as M_w, for the aggregate peaks of 100 mg/ml (the highest concentration tested) solutions of WPI at the four temperatures tested and at various times. Values varied no more than 10% from one injection to the next. It has been shown in previous studies that 100 mg/ml preparation gives a good signal to noise ratio, and therefore is an optimal concentration to obtain accurate molecular mass data (Hoffmann *et al*. 1997)

Figure 3.3 shows the molecular weight distribution calculated for the native WPI peak with the data collected by MALLS/RI. Two populations with different molecular weight distributions were shown, with the first being attributed to the presence of native α-lac, while the second peak of an average molecular weight (M_w) of $3*10^4$g/mol indicated that β-lg was present mainly in the dimeric form. The molecular weight distribution of WPI aggregates after heating at four different temperatures is presented in Figure 3.4, for WPI solutions heated at a concentration of 100 mg/ml. At 65°C, most protein was still present in the native form, and a small population of larger aggregates was observed. The WPI soluble polymers formed upon heating seemed to be more widely distributed at lower temperature of heating than at 90°C (Figure 3.4). Temperature had a significant effect ($p < 0.05$) on the formation of large aggregates, and the M_w of the WPI aggregates increased to $1*10^6$ - $1*10^7$ g/mol, perhaps indicating a change in the mechanism of intermolecular aggregation at higher temperatures (Figure 3.4). This is in agreement with previous research on pure β-lg indicating that temperature affects its aggregation mechanism characterized by thiol-disulfide exchange reactions and non-

covalent interactions (McSwiney *et al*. 1994; Hoffman *et al*. 1995; 1997; Verheul *et al*. 1998). However, in comparison with pure β-lg samples heated under similar conditions, in which molecular masses are reported to be in the range of 10^5 to $2*10^6$ g/mol (Hoffmann *et al.* 1997; Schokker *et al.* 1999), WPI aggregates were larger, most likely because of the presence of α-lac. The molecular weight distribution of heated WPI was less polydispersed than that of pure β-lg, since intermediate size aggregates are present in the latter system (Schokker *et al.* 1999). Heated WPI solutions contained much larger "intermediates" forming the string of beads, as the WPI aggregates are often referred to. Larger soluble aggregates than those separated by size exclusion chromatography may have existed, but were filtered out prior to injection with the 0.45 μm filter.

Ikeda and Morris (2002) have suggested that the aggregation mechanism of WPI at neutral pH also follows a two-step aggregation model, but larger aggregates ("beads") formed the string of beads. These large beads are present together with some elementary particles not incorporated into the aggregate. By heating at various temperatures our work demonstrates that temperature affects the size and the number of the beads formed during the gelation of WPI, perhaps affecting the microstructure of the gels.

The effect of concentration on the aggregation of whey proteins was also studied and Figure 3.5 illustrates the elution of 25, 50 and 100 mg/ml WPI solutions heated at 80ºC for 15 min. After heating, samples were equally diluted to a final concentration of 5 mg/ml before injection, therefore a higher intensity in the RI peaks correspond to the "true" presence of a larger number of aggregates in solution. Aggregation was accelerated by increasing the concentration of initial protein during heating (Figure 3.5). The chromatograms illustrated that a higher concentration of WPI led to an increase in

the amount of the aggregates, as shown by the increased RI signal. The LS signal also increased with concentration. Concentration had a significant effect ($p < 0.05$) on the average M_w of the aggregates formed, further indicating that the increase in M_w of WPI aggregates is dependent not only on temperature, but also on the number of particles colliding during heating. Vardhanabuti and Foegeding (1999) have shown that increasing protein concentrations affects the viscosity of soluble WPI polymers formed at low ionic strength, 80°C for 60 min. It was hypothesized that WPI polymerization is a two-step process, in which whey monomers first interact through disulfide and non-covalent interactions to form primary polymers. Our study supportes their hypothesis and confirms that concentration affects the size of the primary WPI polymers, as the LS signal increased with increasing concentration of protein (Figure 3.5). Iametti *et al*. (1995) showed, by a series of different techniques, that the temperature required for initiating the formation of associated forms of β-lg increases with protein concentration. Our results on WPI indicate that heating a WPI solution at neutral pH results in large aggregates with heating time having no effect on the size of the soluble polymers. However, very short heating times result in a number of aggregates too small to obtain accurate molecular weight data.

3.5 Conclusions

The mechanism of formation of WPI soluble aggregates differs from that of pure β-lg in that aggregation seems to occur without the formation of an intermediate aggregate. In addition, in contrast with what was previously shown for pure β-lg, no significant differences in the molecular weight of the soluble aggregates were seen with time of heating, indicating that the process of aggregation leading to large soluble

polymers is more rapid than that of β-lg in isolation. It is possible that at longer heating times the aggregates formed, larger than those determined by MALLS, were excluded from the measurements with a filtration step. Temperature had a significant effect on the molecular weight of WPI aggregates, with larger values than those previously shown for β-lg, because of the inclusion of α-lac in the aggregate. Increasing the concentration of WPI in solution caused an increase in the number of aggregates produced, as well as the size of the aggregates. Temperature and concentration have been also shown to increase the size of β-lg, because of the increased reactivity of the free thiol group. A similar mechanism is responsible for the heat-induced aggregation of WPI. The changes in the molecular characteristics of the soluble polymers formed upon heating ultimately influence their functional properties in terms of viscosity and gelling behavior. However, a correlation between the molecular weight averages and molecular weight distributions of the soluble aggregates and the microstructure and rheological properties of the gels formed is still to be determined.

Table 3.1: Average values of weight averaged molecular mass [M_w] for 100 mg/ml WPI heated at various temperatures and times.†

	M_w values [10^6] [g/mol]			
Time [min]	**65°C**	**70°C**	**80°C**	**90°C**
10	1.071	1.435	1.836	3.638
15	1.068	1.265	1.69	3.52
20	0.7694	1.326	1.57	3.352
30	0.8071	1.477	2.011	
40	0.8543	2.139	2.73	
60	0.965	1.737		

†Coefficient of variance between replicates was 10%.

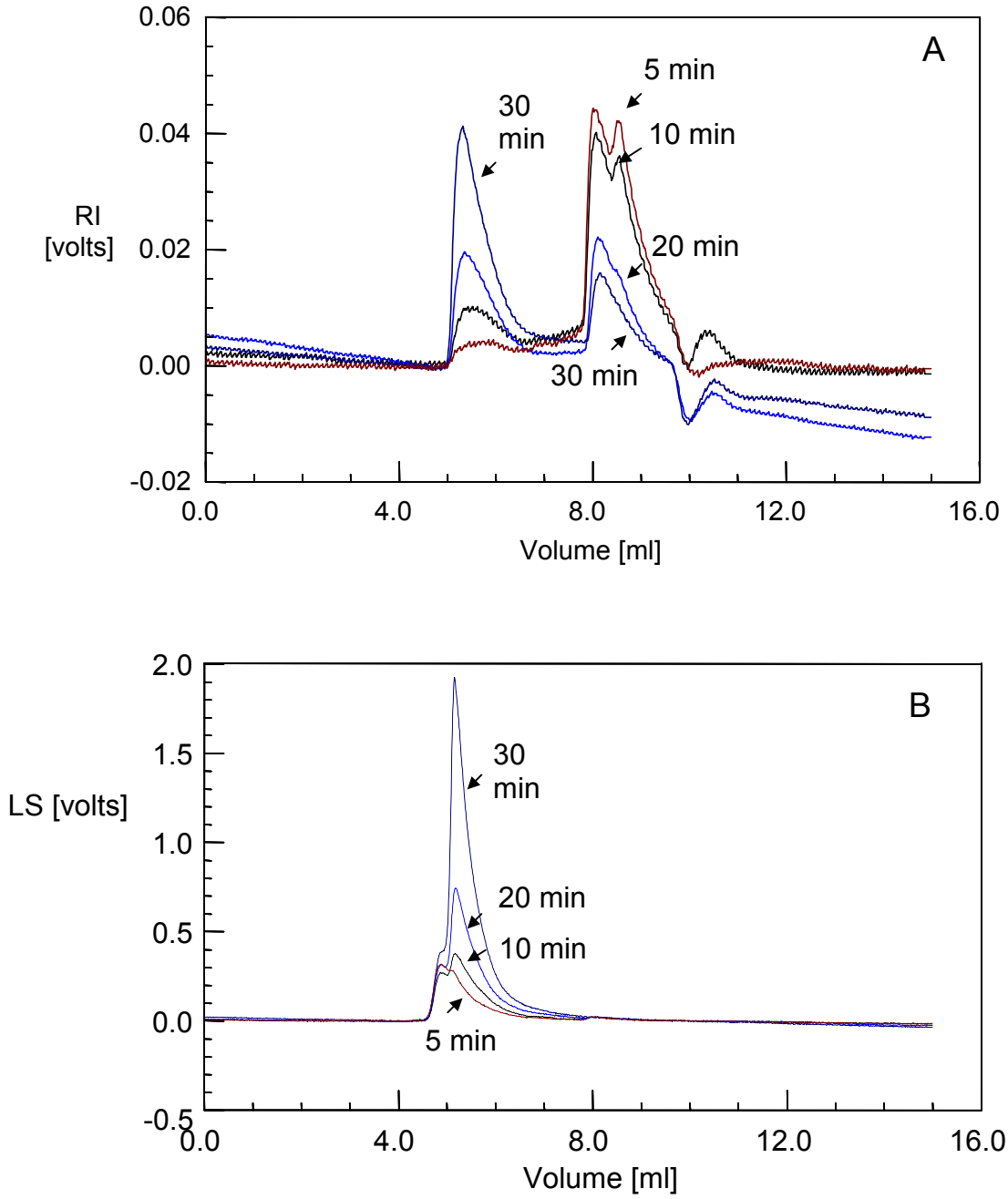

Figure 3.1: Size exclusion chromatography profiles of WPI (100 mg/ml) heated at 70°C for 0, 10, 20 and 30 min. RI (A) and light scattering (90° angle) (B) signals.

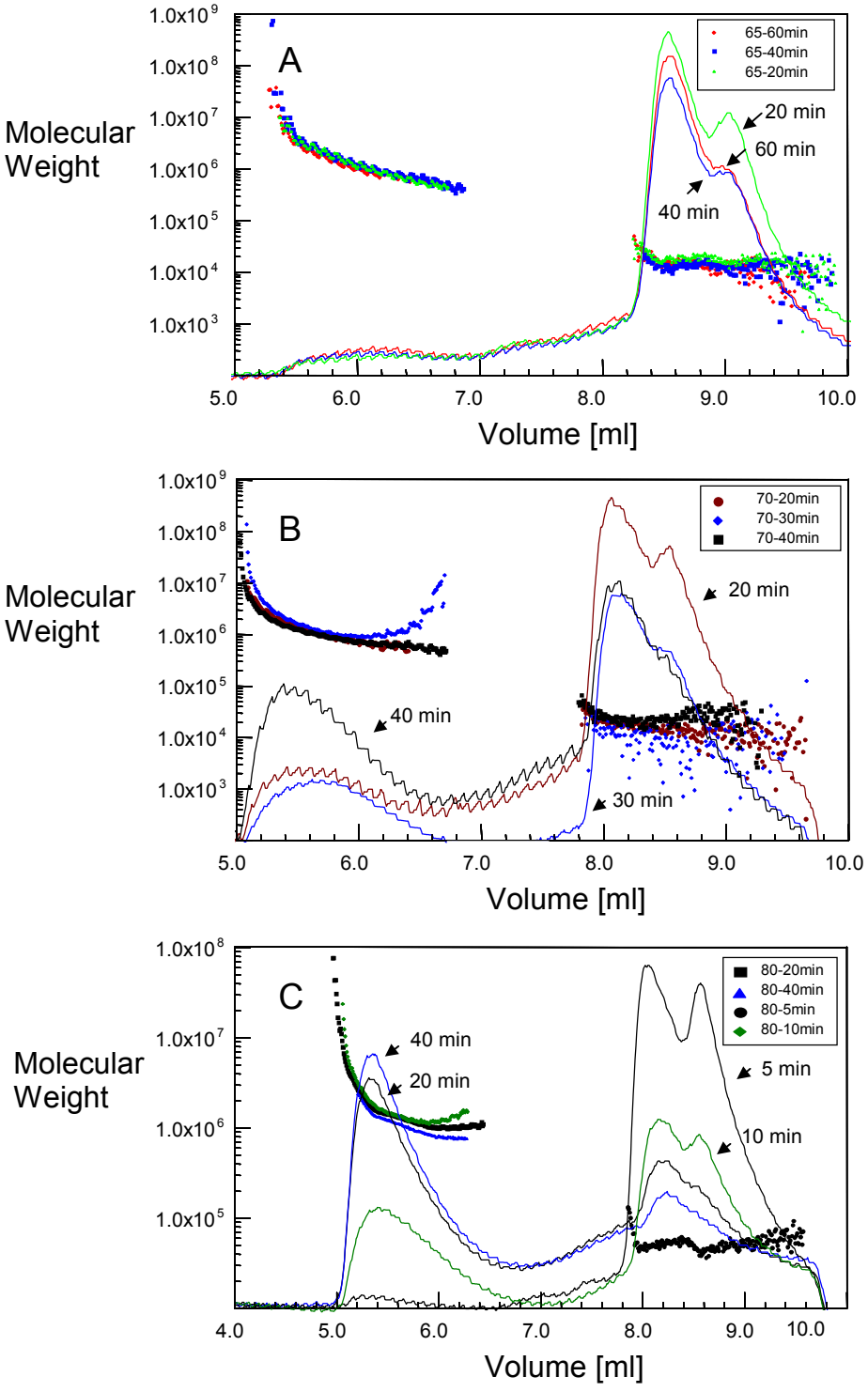

Figure 3.2: SEC-RI elution profiles of the peaks obtained for 50 mg/ml WPI concentrations heated at 65 (A), 70 (B) and 80°C (C) for various times (as indicated in the legend). Signal is overlaid with the calculated molecular mass.

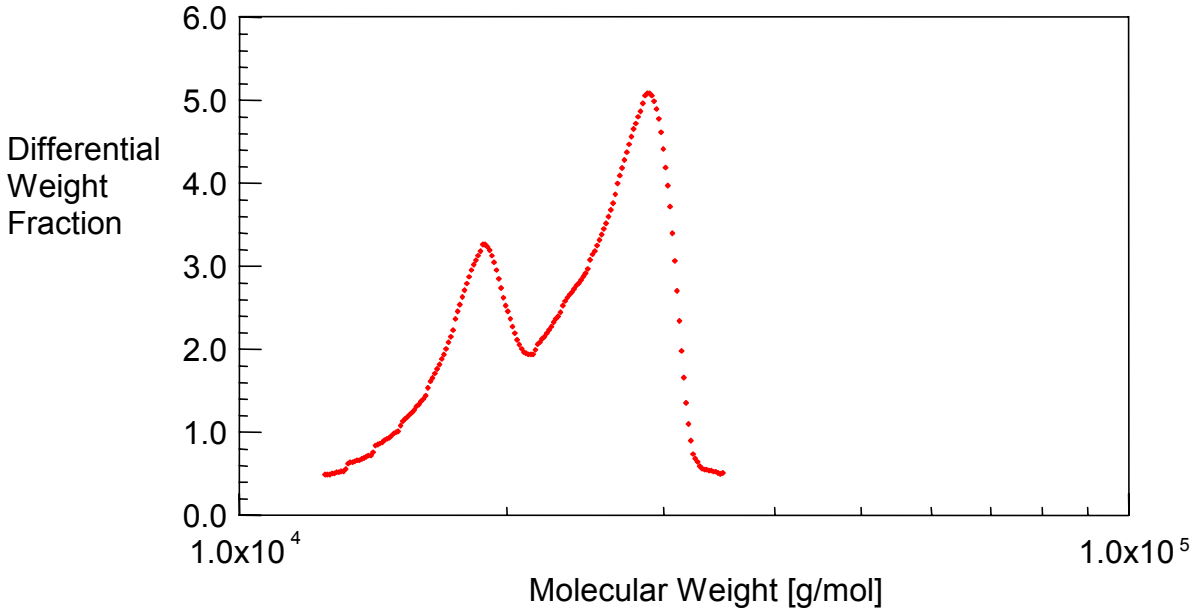

Figure 3.3: Molecular weight distribution of the native WPI.

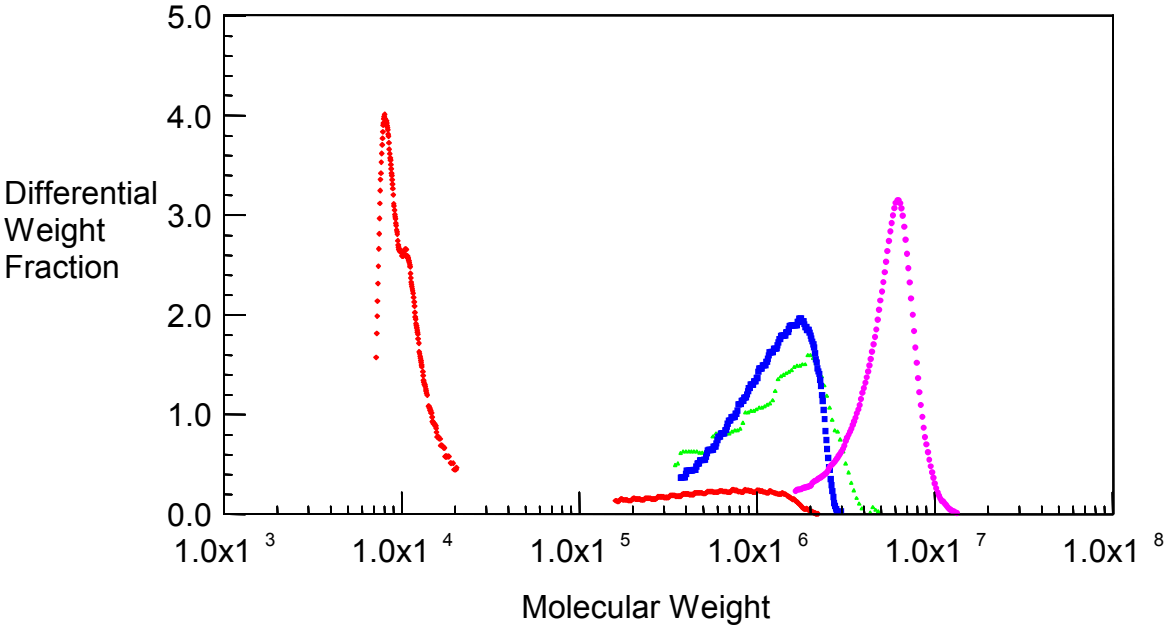

Figure 3.4: Molecular weight distribution of aggregates of a 100 mg/ml solution of WPI heated at different temperatures (65, 70, 80 and 90°C) for 20 min.

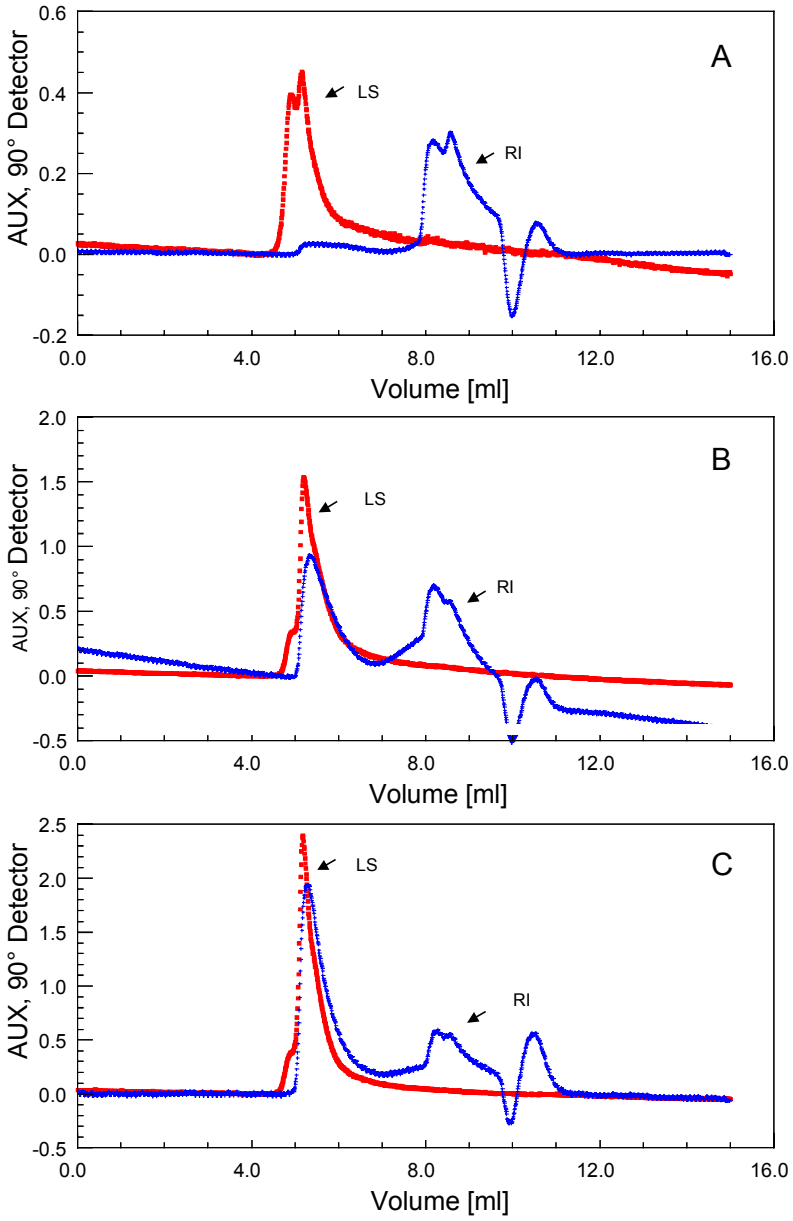

Figure 3.5: Elution profiles (RI and 90°C angle LS) of WPI heated at 80°C for 15 min at 3 different concentrations: 25 (A), 50 (B), and 100 mg/ml (C).

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CHAPTER 4

CHARACTERIZATION OF SOLUBLE AGGREGATES FROM WHEY PROTEIN ISOLATE[1]

[1]Kazmierski, M., and M. Corredig. Submitted to *Food Hydrocolloids*.

4.1 Abstract

The aggregation behavior of a commercially available whey protein isolate upon heat treatment at neutral pH and low ionic strength was determined using a combination of size exclusion chromatography, multi-angle laser light scattering, dynamic light scattering and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The objective of the work was to examine the effect of heating on the denaturation and subsequent aggregation of 10% (w/v) WPI solutions at neutral pH and low ionic strength. Previous work on heat-induced whey protein aggregation has focused in particular on β-lactoglobulin (β-lg), which is the main protein responsible for heat-induced aggregation; however, because of the presence of α-lactalbumin (α-lac) in WPI, differences in the aggregation behavior occur. Time and temperature affected the amount of residual native α-lac and β-lg, showing a decrease in native protein as aggregates formed. The aggregates differed in molecular weight and hydrodynamic diameter depending on temperature of heating. Electrophoresis of soluble aggregates, collected by preparative size exclusion chromatography, showed a ratio of about 2.0 β-lg/α-lac, regardless of temperature or time of heating. On the other hand, the composition of the residual native protein suggested higher α-lac reactivity at 65°C than at 75 and 85°C compared to β-lg, with generally faster rates of denaturation for both proteins with increasing temperature of heating.

4.2 Introduction

Whey proteins are among the most employed functional food proteins in food formulations. The major proteins in whey are β-lactoglobulin (β-lg), α-lactalbumin (α-lac) and bovine serum albumin (BSA). It has long been known that when elevating temperatures beyond room temperature, β-lg dissociates from a dimer to a monomer, exposing its thiol group and interior hydrophobic residues, enabling thiol/disulfide exchange reactions (Gough & Jenness 1962; McKenzie, 1971). The hydrophobic, thiol-disulfide and electrostatic interactions induced by thermal aggregation have since been examined under various conditions for β-lg (McSwiney, Singh & Campanella, 1994; Iametti, Cairoli, De Gregori & Bonomi, 1995; Qi, Brownlow, Holt & Sellers, 1995; Hoffmann & van Mil 1997; Verheul, Roefs & de Kruif, 1998; Schokker, Singh, Pinder, Norris & Creamer, 1999; Fessas, Iametti, Schiraldi & Bonomi, 2001) and α-lac (Gezimati, Singh & Creamer, 1996; Anema, 2001) to further understand their changes in heat-induced interactions. Notwithstanding this, the complete mechanism of heat denaturation and aggregation of the isolated proteins is still far from being understood. Calvo, Leaver and Banks (1993) determined that the aggregation of α-lac is dependent upon the presence of BSA and/or β-lg, due to lack of a free thiol group and the aggregation behavior of mixtures of these whey proteins somewhat differs from that of β-lg taken in isolation (Matsudomi, Oshita, Kabayashi & Kinsella, 1993; Dalgleish, Senaratne & Francois, 1997; Boye & Alli, 2000). Recently it has been reported that when heating β-lg in the presence of α-lac, the proteins form heterogeneous aggregates (Gezimati, Creamer & Singh, 1997; Dalgleish et al., 1997; Kavanagh, Clark, Gosal &

Ross-Murphy, 2000). It is also known that changes in β-lg and α-lac ratios in WPI result in aggregates with different protein composition (Dalgleish et al., 1997).

Changes in molecular weight distribution of heat-induced aggregates of isolated β-lg have been determined by multi angle laser-light scattering coupled with size exclusion chromatography, and it has been reported that the molecular masses of aggregates are dependent on concentration, pH, ionic strength and temperature of heating (Hoffmann, Sala, Olieman & de Kruif, 1997). Intermediates at the early stages of β-lg aggregation have also been observed by many authors (Iametti et al., 1995; Schokker et al., 1999). Schokker et al. (1999) determined the size of these intermediate aggregates of β-lg by size exclusion chromatography and multi-angle laser light scattering, and reported that the aggregates formed were mainly pentamers of β-lg. One limitation of using size exclusion chromatography is the volume exclusion limits of the columns. Large soluble aggregates, such as those produced by WPI, may be separated only using columns operating with a separation range extending to $5\text{-}10^6$ kDa.

It has been reported that heat-induced aggregation of WPI results in the formation of gels, and that the structure and properties of the gel depend on the medium composition, heating conditions and mechanism of aggregation (Mleko & Foegeding, 2000; Vardhanabhuti, Foegeding, McGuffey, Daubert & Swaisgood, 2001; Puyol, Perez & Horne, 2001). At neutral pH and low ionic strength, WPI is characterized by strong electrostatic repulsion forces and transparent gels, with a fine stranded structure form (Ikeda & Morris, 2002). In spite of the interest in the rheological and microstructural characteristics of WPI gels, there is still limited knowledge of the mechanism of association of β-lg and α-lac upon heating. A better understanding of WPI aggregation

behavior upon heating would be of great significance to the food industry because WPI is used more often than isolated proteins due to cost and availability. It is important to note that whey protein concentrate is the most widespread ingredient, however, the study of heat-induced aggregation of whey protein concentrate would be hindered by the presence of fat and other components, which may modify the aggregation behavior and the results may not adequately reflect the role of the primary functional proteins.

The objective of the present work was to examine the effect of heating on denaturation and subsequent aggregation of WPI solutions at neutral pH and low ionic strength. We attempted to study the behavior leading up to gelation by examining the molecular weight (MW) changes of the aggregates formed and the residual native protein in WPI solutions heated at 65, 75 and 85°C. In addition, to further characterize the soluble aggregates, preparative size exclusion chromatography was carried out and the fractions collected and analyzed.

4.3 Materials and Methods

Commercial WPI (BIPRO^{TM}) was provided by Davisco Foods International, Inc. (Le Sueur, MN). WPI was solubilized to approximately 15% (w/v) in deionized water, extensively dialyzed against deionized water and freeze dried. The freeze dried sample contained 92% protein (as determined by N analysis) and residual calcium and sodium were 70.2 μg/g and 380.5 μg/g, respectively (determined by atomic absorption spectroscopy at the Chemical Analysis Laboratories of the University of Georgia).

All chemicals were of reagent grade and purchased from J.T. Baker (Phillipsburg, NJ). Unless otherwise indicated, the mobile phase for chromatographic analysis consisted of 50 mM $NaPO_4$ buffer from dibasic and monobasic salts, pH 7.0 containing 0.15 M

NaCl. It is likely that aggregates analyzed using this concentration of salt buffer may undergo some level of polymerization within the column (Barbut & Drake, 1997), however protein elution was obtained only preventing interactions of the proteins with the gel matrix.

4.3.1 Sample preparation

WPI solutions (10% w/v) were prepared at room temperature by dissolving freeze-dried WPI in deionized water and adjusting to pH 7.0 using 0.1 M HCl. For each experiment, duplicate samples (3 mL each) of the mixture were filtered with a syringe filter (0.22μm, Millipore Corporation, Bedford, MA) into clean, dry 10 mL test tubes, covered with aluminum foil and heated at 65, 75 and 85ºC for times ranging from 5 to 120 min in a water bath (come-up time of approximately 45 s). Heated samples were cooled on ice and stored at 20ºC until further analysis. Unheated, filtered 10% native WPI suspensions were used as control samples.

4.3.2 Determination of residual native protein

WPI solutions after heat treatment were diluted to 2.5 mg/mL and then filtered with 0.45 μm (PVDF, Millipore Corporation, Bedford, MA). Separations were carried out by size exclusion chromatography (Superdex 75, AP Biotech, Piscataway, NJ), at a flow rate of 0.5 mL/min. Filtered samples were injected (100 μL) and eluted with sodium phosphate buffer using an Akta purifier system consisting of a P-900 HPLC pump, a UV-900 detector and integrating software (Unicorn, version 3.1, AP Biotech). The peak area was determined at a wavelength of 280 nm and residual native protein was calculated as % area relative to unheated protein area (mAU*mL).

4.3.3 Molecular weight determination with SEC-MALLS

Heated solutions were diluted with HPLC-grade water to a final concentration of 5 mg/mL and filtered through a syringe filter (0.45 μm, PVDF, Millipore Corporation, Bedford, MA) before injection. Aliquots (50 μL) were injected onto a Superdex 200 size exclusion column (AP Biotech, Piscataway, NJ), with a Spectrasystem autosampler (AS3000, Thermoseparation Products, San Jose, CA). The Superdex 200 column was selected because of its ability to separate within a molecular weight fractionation range of 100 to 1500 kDa. Buffer (phosphate buffer as described above) was delivered at a flow rate of 0.5 mL/min with a Waters P500 HPLC pump attached to an in-line degasser (Waters, Milford, MA) and two in-line filters (0.22 and 0.1 μm, Millipore, Bedford, MA). The detector was a multi-angle, digital signal processing light scattering detector (DAWN DSP-F, Wyatt Technologies, Santa Barbara, CA), which contained a F2 flow cell and a He-Ne laser-light source (Wyatt, 1993). The refractive index detector used was the Optilab DSP interferometric refractometer (Wyatt Technologies), which operated at 633 nm and a constant temperature of 40ºC. The specific refractive index increment (d*n*/d*c*) was determined to be 0.166 cm^3/g by injecting known concentrations of WPI in the Optilab and using d*n*/d*c* for Windows (version 5.2) software (Wyatt Technologies). This value, required for molecular weight distribution calculations, is comparable to that used by Hoffmann et al. (1997) for pure β-lg. Molecular weight distribution calculations were carried out with the Astra/EASI (version 4.1) software (Wyatt Technologies)

4.3.4 Preparative chromatography of soluble aggregates

In the attempt to collect large aggregates of WPI, a preparative size exclusion column was employed. Undiluted 10% WPI samples, heated or unheated, were filtered

(0.8 μm PVDF filters, Millipore) and injected (1 mL injection loop) onto a preparative column (XK16/70, AP Biotech) packed with Sephacryl S-500 chromatography media (AP Biotech). Samples were injected at a high concentration, so that the eluted fractions after collection would contain protein aggregates in amounts high enough for further analysis to be carried out. This preparative size exclusion media was selected because of its fractionation range between 2 x 10^4 and 10^8 Da. Samples were eluted at 1 mL/min with an Akta purifier system consisting of a P-900 HPLC pump, and monitored with a UV-900 detector (AP Biotech). Elution was monitored at 280 nm with Unicorn software (AP Biotech). Fractions (10 mL) were collected over the entire elution volume in a Frac-900 fraction collector (AP Biotech). Fractions were immediately analyzed by dynamic light scattering (DLS), then desalted using econo-pac 10 DG disposable chromatography columns (Bio-Rad, Hercules, CA) and subsequently freeze-dried. Samples were stored at -20ºC for SDS-PAGE analysis. Molecular weight standards (5 mg/mL) were also injected to monitor the performance of the column.

4.3.5 Dynamic light scattering

Fractions containing WPI soluble aggregates, collected from the elution by preparative chromatography, were analyzed using a Particle Size Analyzer (90-Plus, Brookhaven Inst., Holtsville, NY) with a 50 mW diode laser (90º angle) and a BI-9000AT correlator (Brookhaven Instruments). Experiments were carried out at 25ºC with the laser beam operating at 659.0 nm and 1.330 as the refractive index. After SEC separation, samples were filtered through 5 μm filters (Pall Corporation, Ann Arbor, MI) in 10 mm polystyrene cuvettes for analysis. WPI solutions were measured with no further dilution. Photon counts were monitored to ensure that the sample signal was optimal, so

that the photons of light would be scattered only once by the sample (Dalgleish & Hallett, 1995). Measurements were carried out in triplicate with 10 runs of 2 min each and 1 min between each run. The effective diameter of the particles in solution was calculated from a cumulant fit of the intensity autocorrelation function obtained by the intensity fluctuations of the scattered light (Dalgleish & Hallett, 1995) with 90-Plus particle sizing software (version 3.37, Brookhaven Instruments).

4.3.6 Electrophoresis of soluble aggregate fractions

Electrophoresis (SDS-PAGE) experiments were performed in the presence of SDS and under reducing conditions to determine the ratio of α-lac and β-lg present in the various fractions collected during preparative chromatography. 1.5 mg of desalted, freeze-dried samples were dispersed in 100 μL electrophoresis buffer (12.5% Tris-HCl pH 6.8, 20% SDS, 1.25% bromophenol blue, 5% β-mercaptoethanol, 25% Glycerol) and denatured in a boiling water bath for 5 min. Samples were then loaded onto a 10-40% gradient Tris-HCl acrylamide gel (Biorad, Hercules CA). Gels were run at 200 V, stained with Coomassie blue and destained according to manufacturer's instructions (Biorad). After staining, gels were scanned with an imaging densitometer (Model GS-700, Biorad) and intensity of the bands was quantified using molecular imaging software (Molecular Analyst®, version 1.5, Biorad).

4.3.7 Statistical Analysis

All data were analyzed by the general linear model procedure to determine the significant differences between samples. Significance was defined at $p < 0.05$. The SAS program (version 8.0, Cary, NC, USA) was used for all the calculations.

4.4 Results and Discussion

4.4.1 Determination of residual soluble protein

Heating temperature significantly affected the elution profiles of WPI (Figure 4.1). The area of the peaks of residual native β-lg (eluting as 11 mL) and α-lac (eluting at 12.5 mL) varied depending on heating temperature. In addition, an increase in the area of the soluble aggregates eluting as a single peak at about 8 mL was shown. Time of heating was also significant, as was previously shown for pure and mixed systems of α-lac and β-lg (Dalgleish et al. 1997; Hoffmann et al. 1997; Anema, 2001). The small peaks in between the native and the large aggregates eluting at the void volume of the column indicated in very few cases the presence of intermediate aggregates. These intermediates, as well as a time/temperature dependence of the heat-induced aggregation, were shown in pure, heated, β-lg samples and were attributed to exposed nucleophilic groups of β-lg, linking the monomeric subunits of β-lg together (Schokker et al., 1999).

As shown in Figure 4.1, WPI seemed to quickly react at higher temperatures forming α-lac/β-lg complexes, with very little intermediates. Intermediates did not appear at high temperatures (for example 85°C), nor were different WPI aggregate peaks as apparent as those previously seen in studies of pure β-lg (Schokker et al., 1999). On the other hand, Dalgleish et al. (1997) showed lack of intermediates in mixtures heated at 75°C containing α-lac and β-lg, when β-lg was the main component. Statistical analysis of residual native protein data indicated that time and temperature were significant effects in the denaturation behavior of WPI ($p<0.05$). Results shown in Figure 4.1 seemed to indicate the formation of small aggregate peaks for samples treated at 65°C for 15 min and much larger peaks were shown for WPI heated at 75 and 85°C for the same time.

These differences in the heat-induced aggregation behavior of WPI at various temperatures are the result of unfolding and aggregation of β-lg through disulphide exchange reactions. The aggregation of β-lg is known to involve initiation, propagation and termination steps (Roefs & de Kruif, 1994; Hoffmann, Roefs, Verheul, van Mil & de Kruif, 1996). Because the reactivity of the thiol group is greater at 85°C than 65°C, there is an increased probability of collisions at higher temperatures, leading to increased formation of WPI aggregates.

Figure 4.2 illustrates the decrease in the amount of whey protein eluting in the "native" peak after heating. The initial amount of total native β-lg and α-lac in solution decreased upon heating as aggregates began to form. At 65ºC (Figure 4.2 A), the decline in % area of β-lg was considerably slower than that of α-lac. At 60 min, close to 60% β-lg protein was still eluting in the native peak while very little native α-lac was present. Similarly, Hoffmann et al. (1996) reported that over 60% β-lg in solution did not aggregate after heating at 68.5ºC for over 1 h, and the denaturation of β-lg reached a plateau only after longer heating times. At 65°C, β-lg seemed less affected by temperature than α-lac in WPI solutions. This is in agreement with studies showing that dimeric β-lg possesses a high stabilization free energy at neutral pH in phosphate buffer (Apenten, Khokhar & Galani, 2002). These results are also in agreement with the differences in the temperature of denaturation of α-lac and β-lg (De Wit & Klarenbeek, 1981).

These differences in the kinetics of denaturation of β-lg from those of α-lac at 65°C strongly affected the denaturation behavior of WPI. While β-lg was still present in large amounts in its undenatured form, α-lac no longer eluted in the native peak after 60

min of heating. This suggested that α-lac incorporated into the aggregate more quickly and in higher proportions than β-lg at 65°C compared to 75 and 85°C (Figure 4.2B and C).

Due to lack of sulfhydryl-containing proteins, purified α-lac does not form aggregates when heated alone (Calvo et al., 1993), but it has been shown that the presence of α-lac can make β-lg even less thermally stable (Boye & Alli, 2000). In addition, the denaturation of α-lac may be encouraged by the free sulphydryl groups of a denatured β-lg (Paulsson, Hegg & Castberg, 1986; Matsudomi et al., 1993). These factors may cause the formation of much larger aggregates in WPI than those observed in isolated β-lg.

At 75 and 85ºC, β-lg denaturation occured at a faster rate than at 65°C as it has already been reported in the literature (Hambling, Mc Alpine, & Sawyer, 1992; Phillips, Whitehead & Kinsella, 1994; Qi et al., 1995). The increase in thermal reactivity of β-lg caused a parallel % area decrease for α-lac and β-lg (Figure 4.2). At higher temperatures, a synergistic interaction seems to occur between α-lac and β-lg when heated in the presence of one another, as it was discussed for heating at 75ºC by Dalgleish et al. (1997). At 85ºC, the residual native protein seemed to decrease in a parallel fashion, and both proteins denatured at a fast rate, with little residual protein left after 10-15 min. At these temperatures, the denaturation behavior of WPI was similar to that reported for a solution of β-lg (Hambling et al., 1994; Qi et al., 1995).

4.4.2 Molecular weight distribution of soluble aggregates

Heat denaturation of WPI led to the formation of large aggregates, which were separated from the native protein using size exclusion chromatography. Figure 4.3

depicts a representative chromatogram of WPI showing the signal from the multi angle light scattering (90° angle) and the refractive index detectors. The soluble aggregates prepared at low temperature of heating, eluted at the excluded volume of the column but displayed two separate peaks, perhaps as a result of separation of a smaller aggregate from dust particles. These larger particles eluting before the protein aggregate were present in some of the low temperature treatment samples did not show any concentration signal therefore were not included in the molecular weight calculations. All peak calculations, for all the samples at different time and temperature of heating, were determined starting with the concentration signal peak. The protein aggregates were analyzed and the MW distributions calculated. In spite of the poor chromatographic separation of the aggregates (eluting in the void volume of the column) MALLS detection allows an absolute determination of molecular weight distributions. This overcomes the problems of conventional MW calibration with standards, considering that the WPI aggregates are larger than the highest MW standard eluted in the column (Thyroglobulin 669 kDa).

Heating temperature affected the MW distribution of the soluble aggregates eluted in the excluded volume, as shown in Figure 4.4. The aggregates formed at 65°C displayed an average MW of 1.6 x 10^6 g/mol, while those formed at 85ºC showed larger aggregates with an average MW of 4.5 x 10^6 g/mol. These results suggested that higher MW aggregates formed as a function of temperature and was in agreement with previous studies on isolated β-lg (Hoffmann et al., 1997; Verheul et al., 1998). In particular, Hoffmann et al. (1997) found that β-lg heated at 65°C for 24 h formed aggregates of 4 x 10^6 Da. Our data indicated a lower MW average for WPI heated at 65°C for 1 h. Perhaps

the smaller size distribution in WPI aggregates was caused by the presence of α-lac. The MW distributions of WPI aggregates showed a significant ($p<0.05$) increase with heating temperature (Figure 4.4). Our results did not show significant differences in the MW distribution over time. In addition, no intermediate aggregates could be measured in contrast with what was reported by Schokker *et al*. (1999). It is known that β-lg plays a major role in WPI aggregation; it may be possible that in WPI solutions, large aggregates may form faster than in solutions containing β-lg in isolation. It is important to note that the limitation of using size exclusion chromatography remains, in that the filtration through a 0.45 μm filter allows only relatively small aggregates to be measured.

4.4.3 Fractionation of the soluble aggregates and their characterization

To overcome some of the limitations of HPLC size exclusion chromatography, preparative chromatography was carried out with Sephacryl S-500. The estimated MW separation range of this gel is between 2×10^4 and 10^8 Da, therefore the excluded volume and the separation range is much larger than those of the columns previously used in this work or by other authors. Undiluted samples of heated WPI were injected to obtain maximum yield of protein following elution. The high amount of protein injected sacrificed the resolution of the chromatography, however various fractions were collected. The elution patterns obtained by fractionating these large soluble aggregates are shown in Figure 4.5 for samples treated at 65, 75 and 85°C for 15 min. Results for the injected samples at these temperatures for 30 min were similar to those collected after 15 min of heating. The UV elution profiles of the preparative scale separation displayed wide peaks, with a shift to lower elution volumes at higher heating temperatures. WPI treated at 65°C eluted at about 55 mL, while samples treated at 75 and 85°C eluted at

about 40 mL. WPI heated at 85°C exhibited a broad elution volume from 40-100 ml, indicating a wider distribution of aggregates at that temperature than at the lower temperatures.

Fractions (10 mL) collected at 50, 60, 70 and 80 ml, corresponding to fractions indicated as 5, 6, 7 and 8 in Figure 4.5, were analyzed by DLS to determine possible differences in hydrodynamic diameter. Figure 4.6 summarizes the average diameter measured for each fraction collected during chromatography. Fraction 5 was not measured for samples heated at 65°C, because of the low protein concentration eluting at 50 mL. A significant decrease in the hydrodynamic diameter of the aggregates with increasing fraction number was shown at each temperature. This confirmed that, even though no clear peak separation was shown under the conditions used for the elution, the WPI aggregates were separated during chromatography.

The hydrodynamic diameters for fraction 5 of WPI samples heated for 15 min at 75 and 85°C were 61 nm and 120 nm, respectively (Figure 4.6A). Smaller aggregates formed at 65°C, while large aggregates formed at 85°C. Within each fraction, significant differences ($p<0.05$) were shown in the diameter size with temperature. At longer heating time (30 min, Figure 4.6B), fraction 5 showed a diameter of 67 nm at 85°C and 58 nm at 75°C. When compared to fraction 5 heated at 85°C for 15 min, the values of hydrodynamic diameter were smaller. On the other hand, fractions 6, 7 and 8 were not significantly different with heating time (Figure 4.6). The difference in fraction 5 may be caused by differences in the elution of the largest aggregates of WPI heated at 85°C, perhaps shape differences. In fact, not only MW differences, but also shape, surface

hydrophobicity and charge of the aggregates may have played a role in the separation of WPI aggregates by chromatography.

A two-step model has been proposed for β-lg where, at neutral pH, the native protein, with a stokes radius of 2.7 nm (Hambling et al., 1992), forms primary globular particles which subsequently aggregate (Roefs & de Kruif, 1994). The presence of primary globular particles forming aggregates has also been recently shown by atomic force microscopy (Ikeda & Morris, 2002). The authors showed that a 11% solution of WPI, heated at neutral pH and 80°C forms aggregates with diversity of size and shape, though their elemental units primarily globular in shape. A few individual globular aggregates not incorporated into the aggregate were also present (Ikeda & Morris, 2002). Formation of large particles in WPI samples may also occur in a similar fashion, as a result of temperature, via aggregation of primary particles eluting at the later stages of the chromatographic separation (fractions 7 and 8).

The fractions collected by preparative chromatography were freeze-dried and aliquots of protein aggregate were analyzed by SDS-PAGE electrophoresis. The ratios of protein present in the fractions from WPI heated at 65, 75 and 85°C were then calculated by quantifying the protein migration by scanning densitometry and image analysis. The composition of the aggregate was determined as ratio of β-lg/α-lac for each fraction collected, as a function of temperature, and values for fraction 6 and 8 are shown in Table 4.1. Fraction 6 contained the largest aggregates separated by chromatography, as determined by DLS. Statistical analysis of values of β-lg/α-lac determined in the various fractions showed no significant differences between temperatures or time of heating. Peak 6 contained a ratio of β-lg/α-lac comparable to that present in the original WPI

solution. Similar results were also determined for fraction 7. These results are somewhat in disagreement with those reported by Dalgleish et al. (1997) where after heating at 75°C whey proteins solution with different β-lg to α-lac ratios, earlier aggregation steps seemed to produce aggregates richer in β-lg, and α-lac seemed to react more as the reaction proceeded. However, shorter heating times were examined in their case, and after 4 min of heating the ratio was approximately 2 β-lg/α-lac.

Table 4.1 also shows the whey protein composition of fraction 8 (mostly unaggregated protein) after 15 and 30 min heating time. After 30 min, a higher β-lg/α-lac ratio was present at lower temperature of heating (65°C) (Figure 4.8). This confirmed that, at high temperatures, β-lg rapidly unfolds and α-lac is incorporated in the aggregate as the reaction proceeds, as shown previously. In fraction 8 of WPI heated for 30 min at 65°C, the ratio of β-lg/α-lac was significantly higher than those fractions heated at 75 and 85°C, and this is in agreement with results shown in Figure 4.2. Results for fraction 8 at 15 min showed no significant differences in the ratio of β-lg/α-lac, however according to results shown in Figure 4.2, at that time the reaction had not proceeded enough to show differences in the β-lg/α-lac ratios.

4.5 Conclusions

Upon heating 10% WPI (w/v) solutions, many similarities were found in the denaturation behavior of WPI when compared to that of isolated β-lg. The presence of α-lac however, strongly influences the denaturation behavior of β-lg. At 65°C, β-lg denaturation occurred at a slower rate than that of α-lac. The MW distribution of the soluble aggregates at 65°C displayed an average of 1.6×10^6 g/mol, while those formed at 85ºC showed larger aggregates with an average MW of 4.5×10^6 g/mol. The data

supported the two step model of a complex formed by covalent interactions upon heating, and WPI showed larger aggregates when compared with aggregates formed by isolated β-lg. Preparative SEC proved to be a valuable approach in collecting the WPI aggregates allowing for further analysis by DLS and SDS-PAGE. An increase in the hydrodynamic diameter was found with temperature of heating, and perhaps shape differences among the large aggregates. Electrophoresis showed no differences in the β-lg/α-lac ratio in the collected aggregates, indicating that there may be a preferred stochiometry of interaction between β-lg and α-lac in the formation of the intermediate particles. The incorporation of α-lac in WPI aggregates depended upon the amount of denatured β-lg. Perhaps the reaction involved the formation of a β-lg aggregate before α-lac interaction, as recently hypothesized for aggregates of BSA-α-lac by Havea et al. (2000).

4.6 Acknowledgements

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Table 4.1: Ratio of β-lg/α-lac in the aggregates determined by SDS-PAGE for fractions 6 and 8 collected by size exclusion chromatography of WPI heated at 65, 75 and 85°C for 15 and 30 min.

Ratio β-lg/α-lac	Fraction 6		Fraction 8	
	15 min	30 min	15 min	30 min
65°C	2.5 ± 0.70	2.11± 0.25	1.90 ±0.18	2.37 ±0.06
75°C	2.15±0.37	1.69±0.00	1.41±0.22	1.73 ±0.29
85°C	2.13±0.19	2.32 ±0.42	1.36±0.60	1.10±0.10

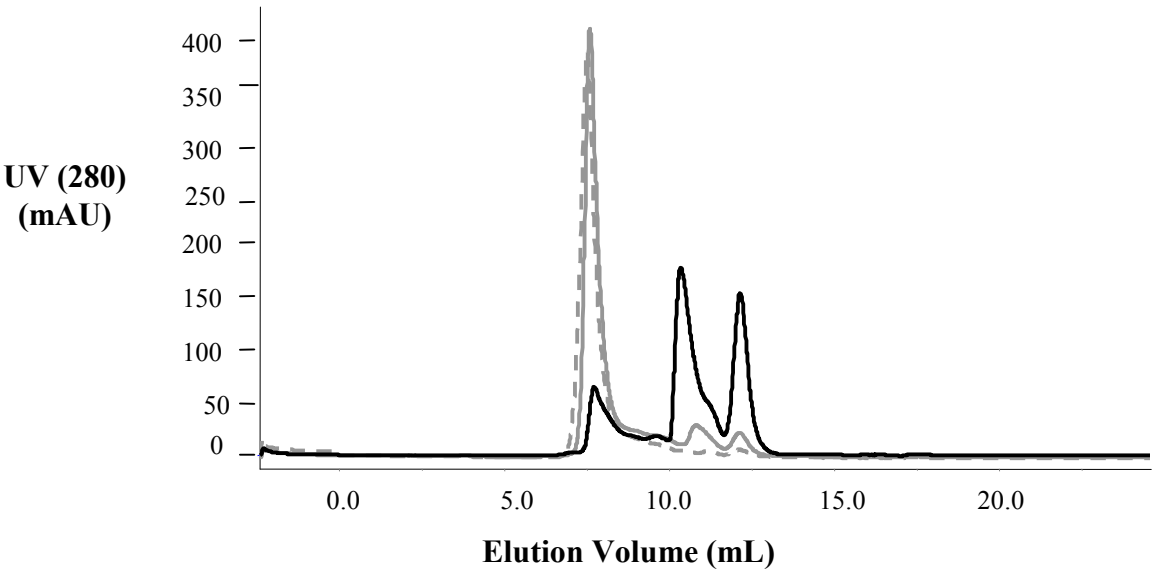


Figure 4.1: Size exclusion chromatography profiles (Superdex 75 column) of WPI heated for 15 min at various temperatures: 65ºC (black line); 75ºC (gray line); 85ºC (dashed gray line).

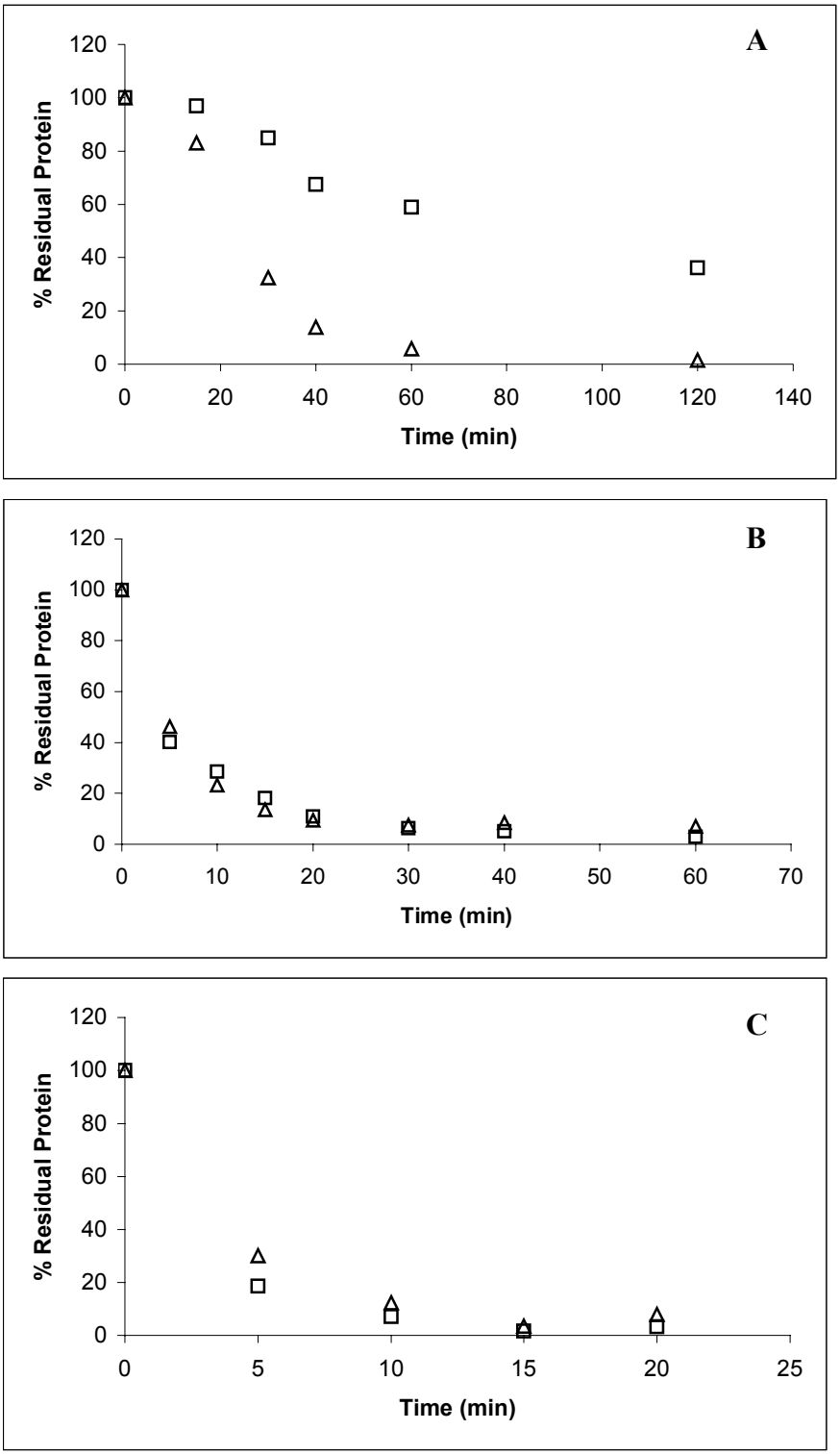


Figure 4.2: Residual protein peak (% peak area /peak area of unheated protein) as a function of heating time for (A) 65°C; (B) 75°C; (C) 85°C. β-lg (□);α-lac (Δ). Results are the average of two chromatographic separations.

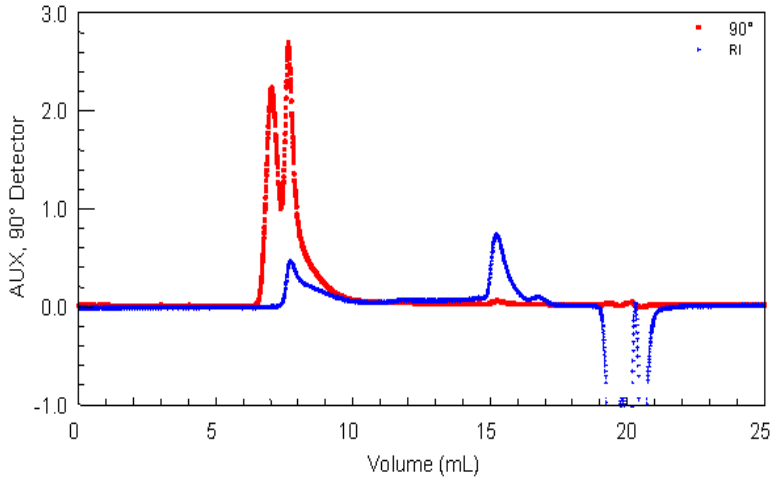


Figure 4.3: Size exclusion chromatography profile of a WPI sample heated at 65°C for 40 min detected by RI (black line) and MALLS (90° angle) (gray dotted line).

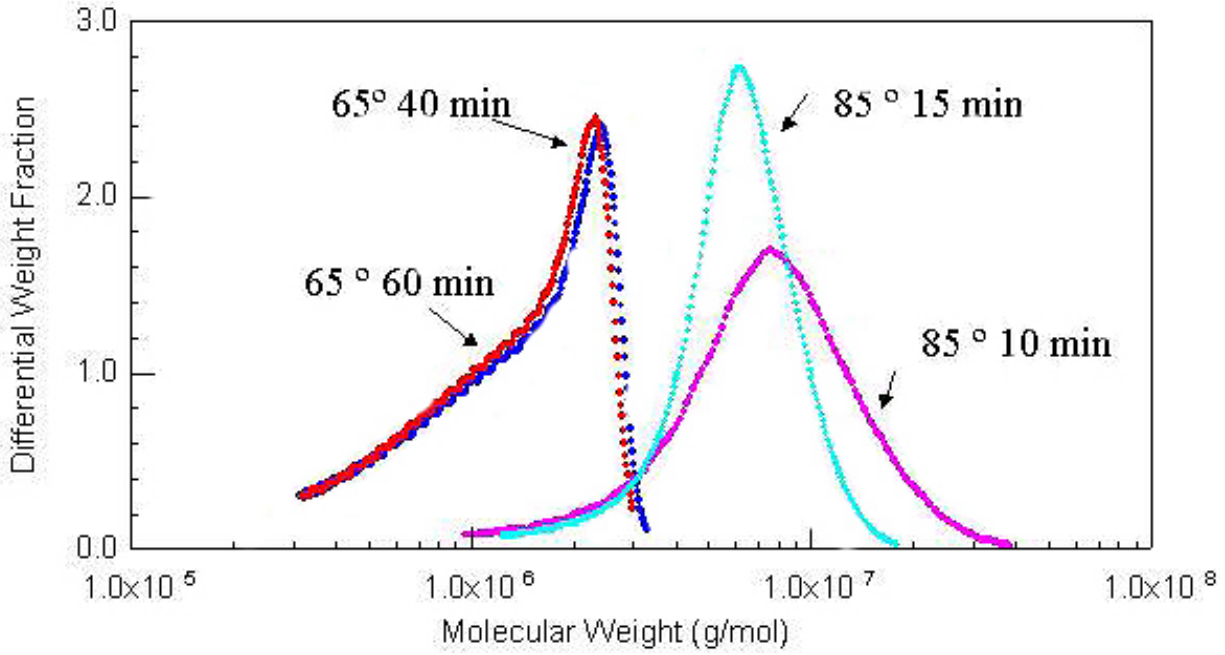


Figure 4.4: Molecular weight distributions of aggregates of WPI heated at 65 and 85°C for various times, calculated from the aggregate peaks eluted by the Superdex 200 column.

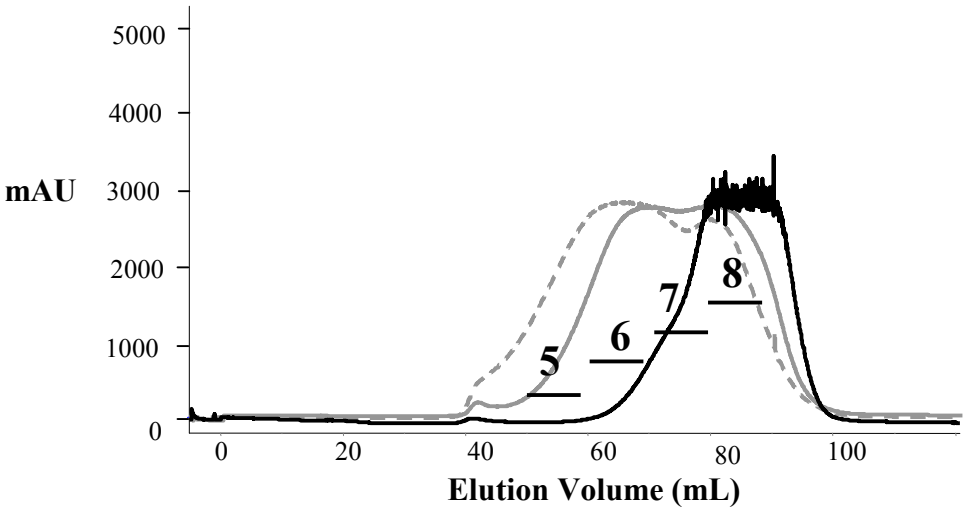
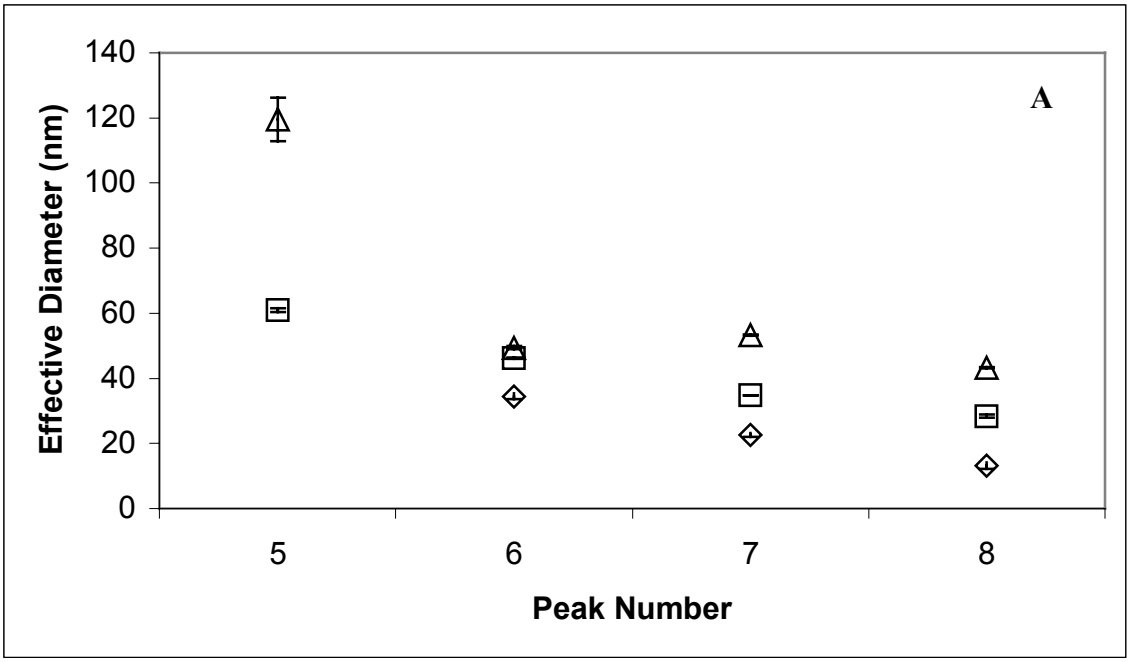


Figure 4.5: Elution profiles of undiluted WPI (10% w/v) aggregates using preparative size exclusion chromatography (Sephacryl 500). Samples treated for 15 min at 65°C (solid black line); 75ºC (solid gray line); 85ºC (dashed gray line). Fractions 5, 6, 7 and 8 collected at 50, 60, 70 and 80 ml elution volumes, respectively.



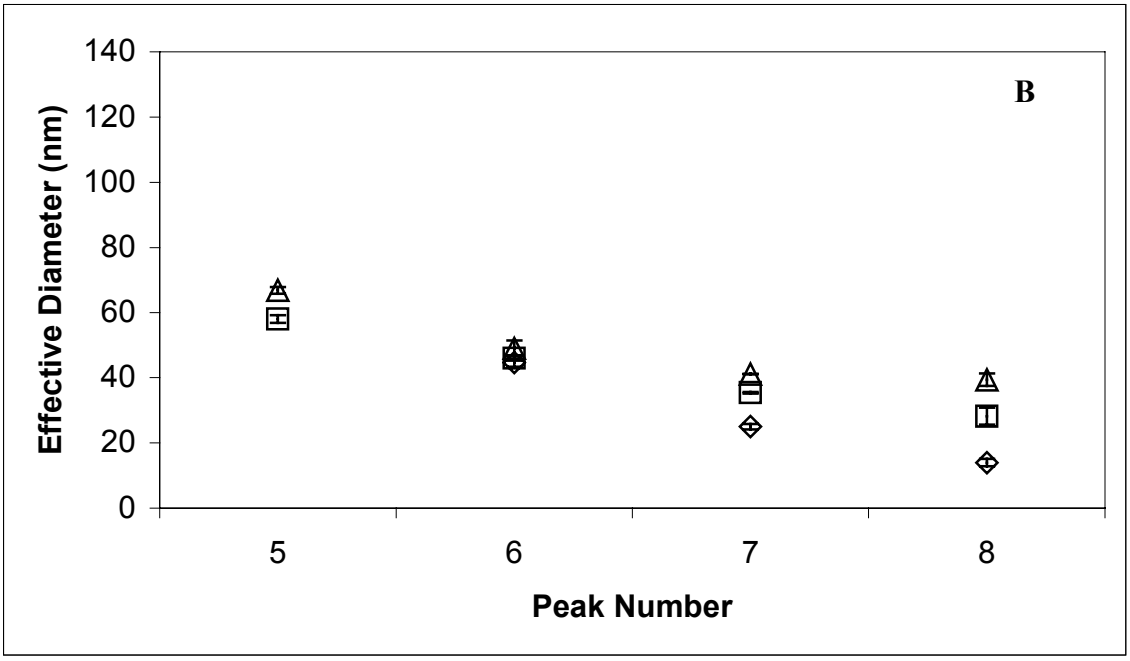


Figure 4.6: Average diameter measured by DLS of fractions eluted of WPI heated at ◊, 65ºC; □, 75ºC; Δ, 85ºC; for 15 min (A) and 30 min (B). Error bars show standard deviation.

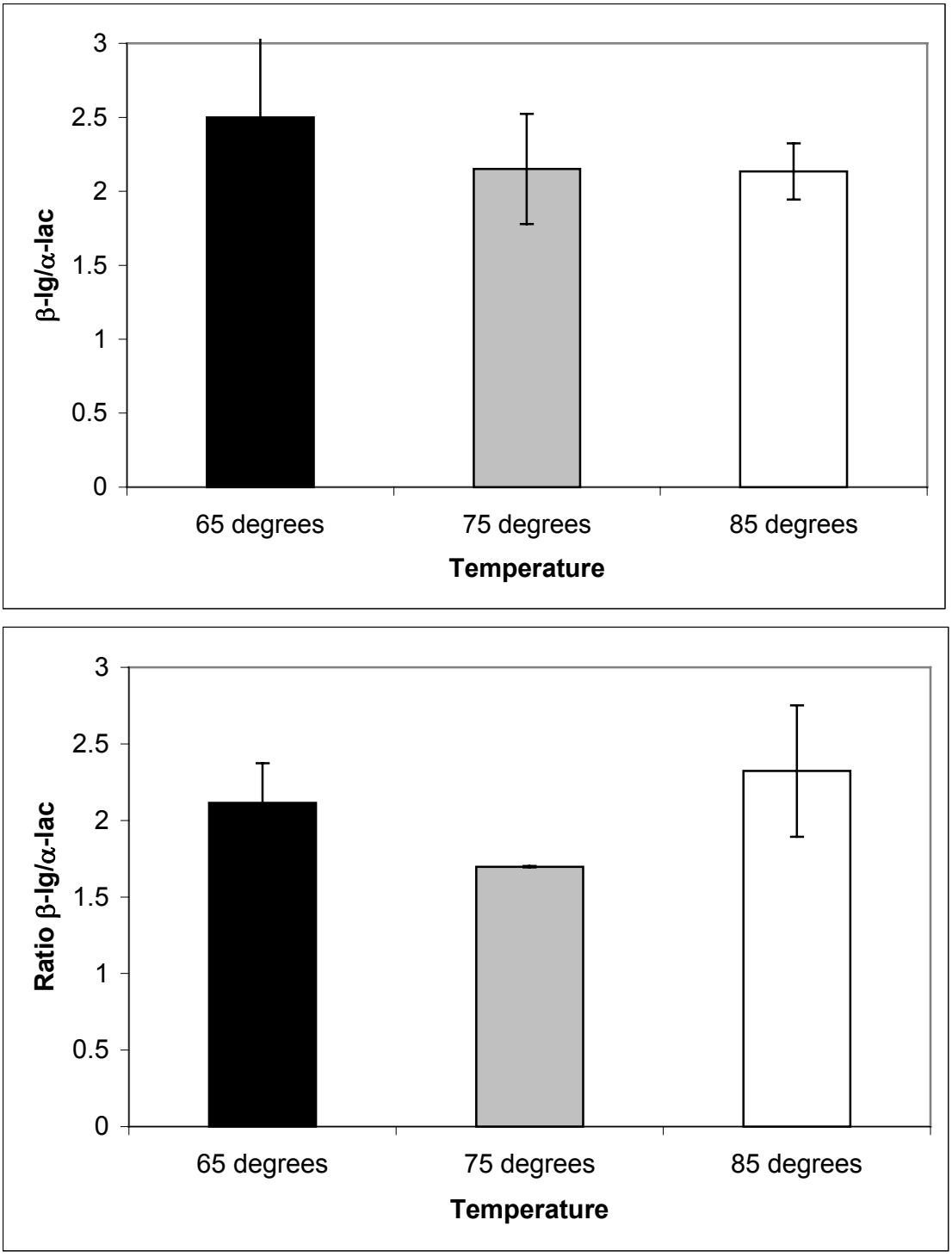


Figure 4.7: Composition of the aggregates determined by SDS-PAGE. β-lg/α-lac for fraction 6 of WPI heated at 65°C (black); 75°C (gray); 85°C (white); for 15 min (A) and 30 min (B).

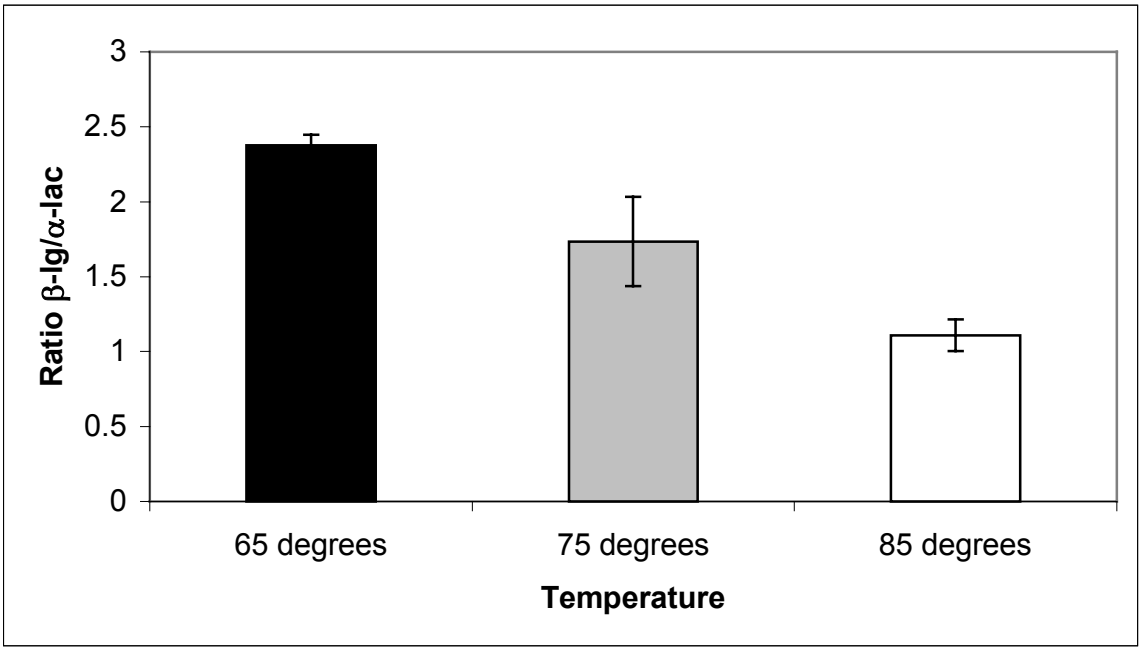


Figure 4.8: Composition of the aggregates determined by SDS-PAGE. β-lg/α-lac for fraction 8 of WPI heated at 65°C (black); 75°C (gray); 85°C (white) for 30 min.

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CHAPTER 5

PURIFICATION OF CASEIN AND WHEY FRACTIONS

5.1 Introduction

During the course of study, it was necessary to purify proteins using optimum methods of isolation. This chapter provides a summary of the method development for separation of the casein and whey fractions of milk proteins, as well as techniques used to test the purity of the samples, such as electrophoresis and column chromatography. The isolated whey proteins were to be used for future studies requiring this level of purity.

5.2 Isolation of Milk Proteins

Bovine caseins were isolated using the method described by Hollar and others (1991). Low heat, non-fat dry milk (Dietrich's milk products Inc., Reading, PA) was dissolved as 15% solids in 2 L of deionized H_2O and heated to 30°C. Milk pH was adjusted to 4.6 with 1 N HCl, the isoelectric point of casein. Whey was drained twice with cheesecloth and the casein precipitate was washed with deionized H_2O. Precipitate was then resuspended with distilled water to 2 L and neutralized to pH 6.7 with 1 N NaOH. Casein was then freeze-dried and stored at –20°C.

Two batches of whey protein were separated by anion exchange chromatography, using the method described by Andrews and others (1985). A 15 mg/mL solution of commercial whey protein isolate (Land O' Lakes, St. Paul, MN) was prepared in 275 mL of 20 mM Tris-HCl, pH 7.0. The protein solution was stirred for one hour, filtered with a 0.8 μm filter (Millipore, Bedford, MA), freeze-dried and stored at –20°C until further analysis.

5.3 Separation of Casein Fractions

Casein fractions were separated according to the method of Hollar and others (1991), with slight modifications. A preliminary separation was carried out with a small

pre-packed cation exchange column (HiTrap™ SP HP, 1 mL, AP Biotech, Piscataway, NJ) to optimize the separation of the four different casein fractions. The conditions tested were concentration of casein and the gradient used for chromatographic elution. Separations were conducted using an Akta purifier system consisting of a P-900 HPLC pump, a UV-900 detector measuring at 280 nm. Data was collected with Unicorn software (version 3.1, AP Biotech, Piscataway, NJ).

Two dilutions of 20 and 40 mg/mL of freeze-dried casein were prepared with urea-acetate (UA) buffer containing 6 M urea and 0.02 M sodium acetate at pH 5.0. The pH was adjusted to 7.0 using 1 N NaOH and 0.02 to 0.04 mL of β-mercaptoethanol was added. The samples were stirred for 30 min and then filtered with a 0.8μm filter (Syringe PVDF, Millipore, Bedford, MA). The pH was adjusted back to pH 5.0 with 1 N HCl and applied to the column equilibrated with 1 CV UA buffer. Elution was carried out with a gradient of UA containing 1 M NaCl (buffer B). A 1 mL injection was performed with a flow rate of 1 mL/min. Gradient tested included a) a gradient of 0-100% buffer B in 8 column volumes (CV); b) 0-40% in 4 CV with an increase to 100% in 6 CV; c) 0-40% in 10 CV with an increase to 100% for 2 CV. Figure 5.1 depicts representative elution profiles from 3 chromatographic separations. A 40 mg injection (Figure 5.1A) overloaded the column, while a fast gradient (Figure 5.1B) did not give adequate separation of casein fractions. Optimum separation was achieved as shown in Figure 5.1C with a 20 mg/mL dilution and a gradient of 0-40% buffer B for 10 CV and an increase to 100% for 2 CV (See Figure 5.1C).

After optimization on a small scale, caseins were isolated on a larger scale using a preparative column (XK 50/30, AP Biotech, Piscataway, NJ) with 300 mL of SP

Sepharose resin (AP Biotech, Piscataway, NJ). Freeze-dried casein (10 g) was dissolved in 500 mL of UA buffer (pH 5.0), the pH was adjusted to pH 7.0 with 1 N NaOH and 2 ml of β-mercaptoethanol was added. Sample was stirred for 1 hour and then filtered with a 0.8μm filter (47 mm White Gridded AAWG, Millipore, Bedford, MA). The pH was adjusted back to pH 5.0 with 1 N HCl and applied to the column equilibrated with 1 CV UA buffer.

Elution was carried out at a flow rate of 6 mL/min, starting with 3% of buffer B and increasing to 40% in 10 CV and terminating the elution with 2 CV of a gradient concentration of 40-100% buffer B. The resin was re-equilibrated with 1 CV of UA buffer. The fractions collected were dialyzed extensively with milli-Q water to remove urea and mercaptoethanol, freeze dried and stored at –20°C.

Figure 5.2 shows the elution profile of the casein separation over elution volume. The first two peaks were well resolved, while peaks eluting at 2500-3500 mL were not resolved. The first peak corresponds to β-casein, followed by k-casein, α_{s1} and α_{s2}-casein, respectively. The elution profile corresponds to what shown previously by Hollar and others (1991).

5.4 Separation of Whey Proteins

As already described for the casein separation, whey proteins were also first separated on a small pre-packed column and later isolated by preparative chromatography. An anion exchange HiTrap™ Q HP column (5 mL, AP Biotech, Piscataway, NJ) was employed following the method of Andrews and others (1985). Several conditions of protein loading and buffer gradient were used to optimize the

elution in 20 mM tris buffer at pH 7.0. Whey protein isolate concentrations of 12.5 and 25 mg/mL were injected (1 mL loop) and eluted using a gradient built with 20 mM tris buffer, pH 7 and 20 mM tris buffer containing 1 M NaCl (buffer B), delivered at 0.5 mL/min. Various gradient combinations were tested, as for example a) From 0-20% buffer B in 8 CV with an increase to 100% in 2 CV; b) From 0-30% in 8 CV with an increase to 100% in 2 CV; c) From 0-40% in 8 CV with an increase to 100% in 2 CV. Figure 5.3 illustrates some examples of these whey protein separations. The optimum conditions selected were with a sample dilution of 12.5 mg/mL, with a gradient elution of 0-20% buffer B in 8 CV and a subsequent increase to 100% buffer B in 2 CV (elution shown in Figure 5.3C).

After establishing the optimal gradient and loading conditions, a large-scale separation of the whey proteins was conducted using a preparative anion exhange column (XK 50/30, AP Biotech, Piscataway, NJ) with 300 ml of Q Sepharose^{TM} XL resin (AP Biotech, Piscataway, NJ). Protein was separated using the Akta purifier (AP Biotech, Piscataway, NJ) as described above, monitoring the elution with a UV detector at 280 nm. The column was equilibrated with 20 mM Tris buffer, pH 7.0. Elution was carried out with a flow rate of 6.5 mL/min in a total of 10 CV, with a start buffer composed of 10% Tris buffer with 1 M NaCl (buffer B) and an increase to 20% B in 8 CV, followed by a 2 CV gradient of 20-100% buffer B. The column was cleaned with 1 CV buffer B and re-equilibrated with 2 CV of 20 mM Tris buffer. 10 mL fractions were collected and dialyzed extensively against distilled H_2O, freeze dried and stored at -20°C. Figure 5.4 illustrates the elution profile of the protein isolation which corresponds to that seen

previously by Andrews and others (1992), including the middle peak, thought to be immunoglobulins.

5.5 Analysis of Isolated Fractions

For analysis of casein purity, the PhastSystem^{R} (AP Biotech, Piscataway, NJ) was used to carry out a urea-PAGE electrophoresis, as described by Van Hekken and Thompson (1992). In brief, 8-25% gradient gels were modified by soaking for 15 min in 6.6 M urea, 0.112 M Tris-HCl, 0.112 M acetate at pH 6.4 and subsequently air dried for 3 to 5 min. Native buffer strips were then used for electrophoretic separation (AP Biotech, Piscataway, NJ). Samples (1.5mg/75µl) were dissolved in urea-Tris-acetate buffer with 10% 2-mercaptoethanol and 0.25% bromophenol blue, boiled for 5 min and 1 µl was loaded into each lane. Electrophoretic conditions were programmed at 300 volts, 7.5 mA, 2.5 W at 15C for 10 volt-hours. Gels were run for 110 accumulated volt-hours. Bands were stained and destained according to manufacturer's instruction (AP Biotech, Piscataway, NJ). Figure 5.5 depicts the electrophoretic migration of the fractions isolated by ion exchange chromatography. The various proteins were identified according to Van Hekken and Thompson (1992).

Casein fractions collected from cation exchange chromatography were also tested for purity using a second ion exchange chromatography on a Hitrap Q HR (1 mL anion-exchange column, AP Biotech, Piscataway, NJ), as described by Andrews and others (1985). 5 mg/mL each of the four freeze-dried fractions isolated by preparative chromatography were dissolved in 20 mM Tris-HCl urea buffer pH 7.0, and 100 µl was injected into the column, after equilibration with 3 ml of running buffer.

Separation was carried out at 1.0 mL/min with a gradient of 0-35% buffer B for 15 CV and a target concentration of 100% buffer B reached after 5 CV. Elution profiles of purified caseins are shown in Figure 5.6.

The quality of the whey proteins isolated by ion exchange chromatography was tested using a size exclusion chromatography column (Superdex 200, AP Biotech, Piscataway, NJ). Samples (3 mg/mL of β-lactoglobulin or α-lactalbumin) were injected (100 μl loop) onto the column after equilibration with 50 mM $NaPO_4$ buffer containing 0.15 M NaCl, pH 7.0. Elution was carried out 0.5 mL/min. As shown by the elution profiles of the whey proteins fractions (Figure 5.7), both α-lac and β-lg were present in a purified form.

5.6 Conclusions

The optimization procedures were effective for isolation of the whey and casein fractions of milk protein. The casein and WPI proteins were successfully separated into fractions of β-casein, κ-casein, α_{s1}-casein, α_{s2}-casein, β-lactoglobulin and α-lactalbumin. In addition, chromatography and electrophoresis were employed for the identification of isolated fractions and determination of their purity. The isolated whey proteins were further used for protein-protein and protein-polysaccharide interaction experiments.

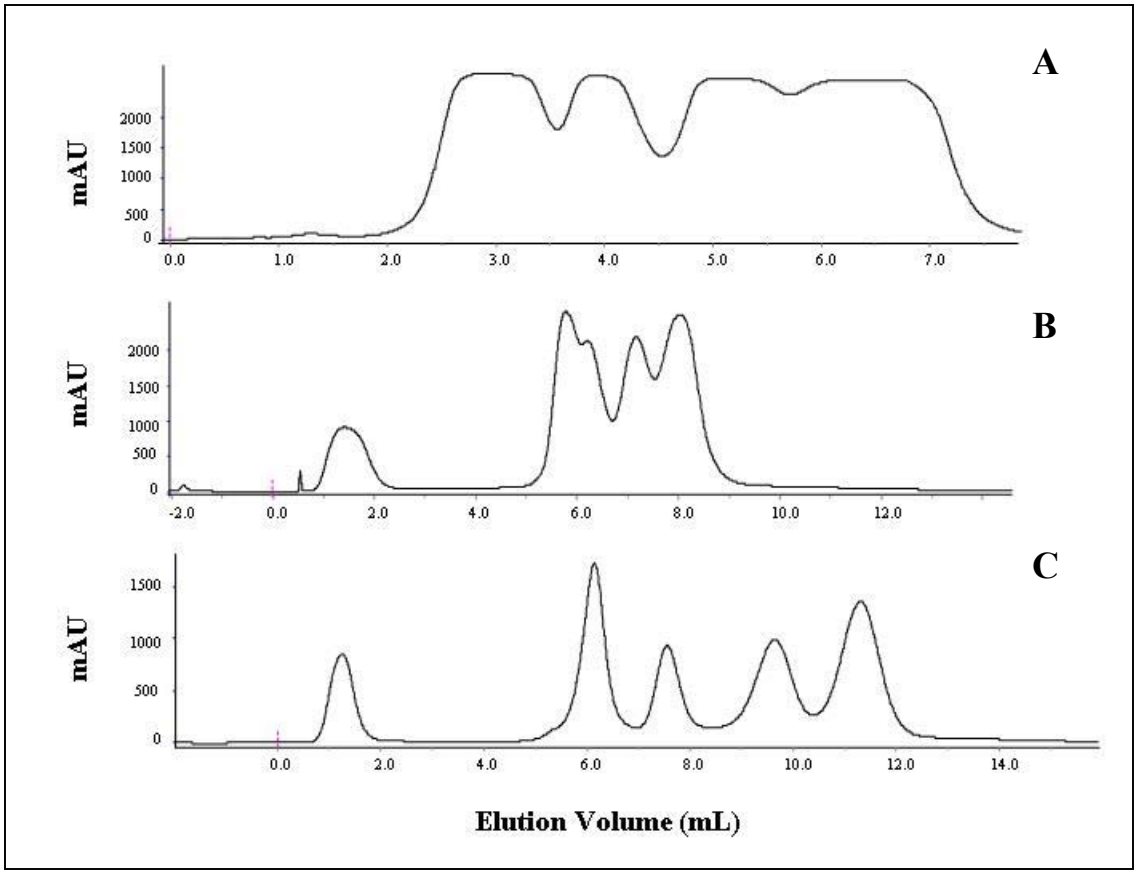

Figure 5.1: Optimization of different dilutions of casein separated using various gradients for elution. A) 40 mg loaded, 0-100% 8 CV; B) 20 mg loaded, 0-40% 4 CV and 40-100% 6 CV; C) 20 mg loaded, 0-40% 10 CV and 40-100% 2 CV. 1 CV=5 mL.

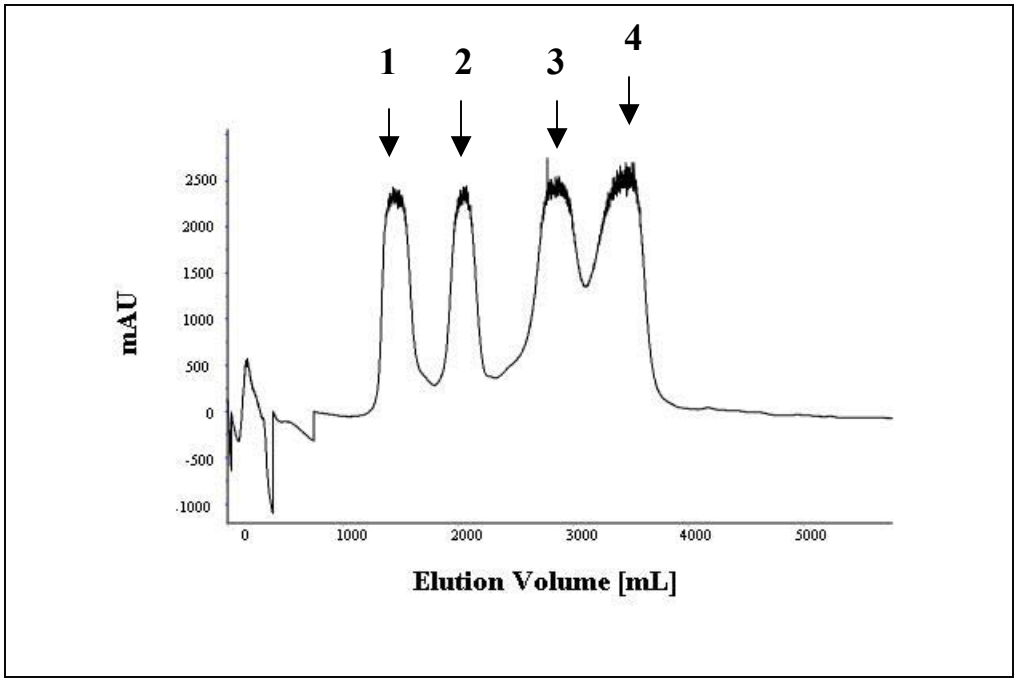

Figure 5.2: Elution profile of casein separation using preparative size exclusion chromatography. 1) β-casein; 2) κ-casein; 3) α_{s1}-casein; 4) α_{s2}-casein.

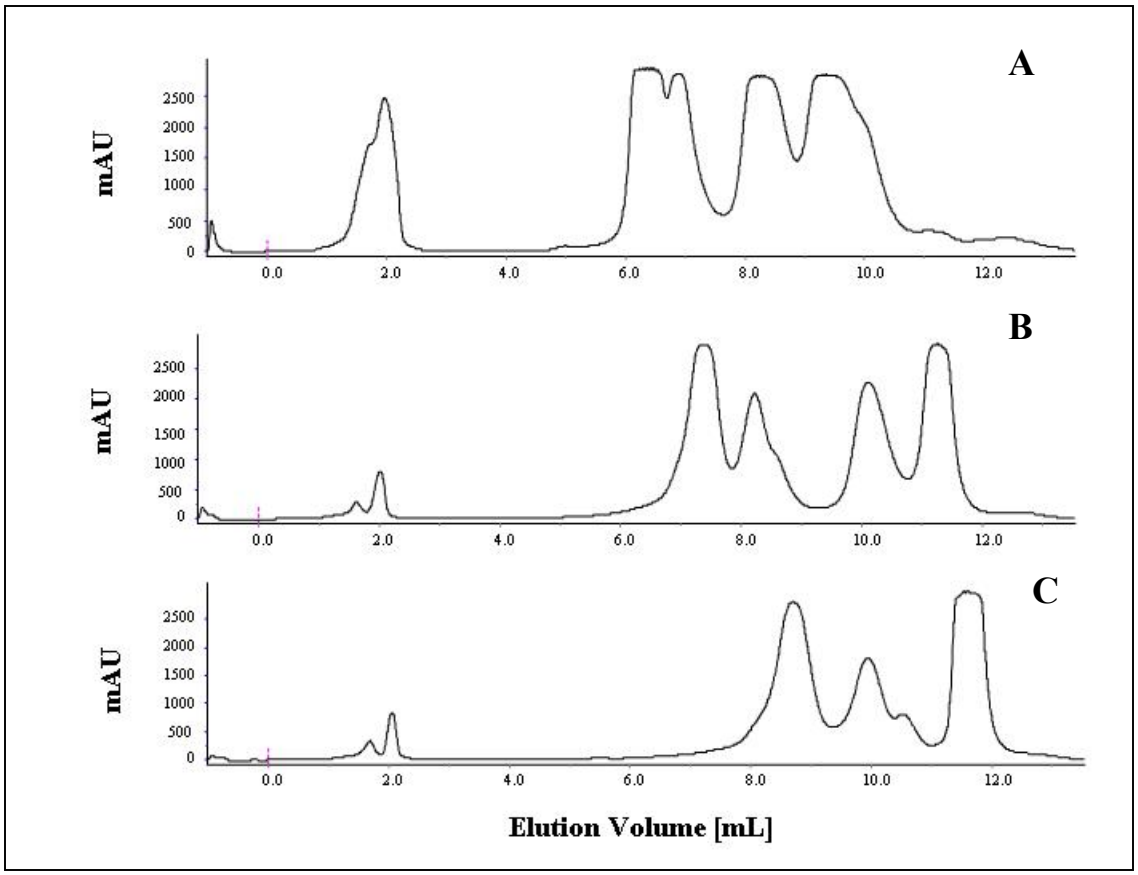

Figure 5.3: Example of whey protein elution profiles obtained using protein loads and gradient elutions. A) 25 mg WPI, 40% 8 CV and 100% 2 CV; B) 12.5 mg WPI, 30% 8 CV and 100% 2 CV; C) 12.5 mg WPI, 20% 8 CV and 100% 2 CV.

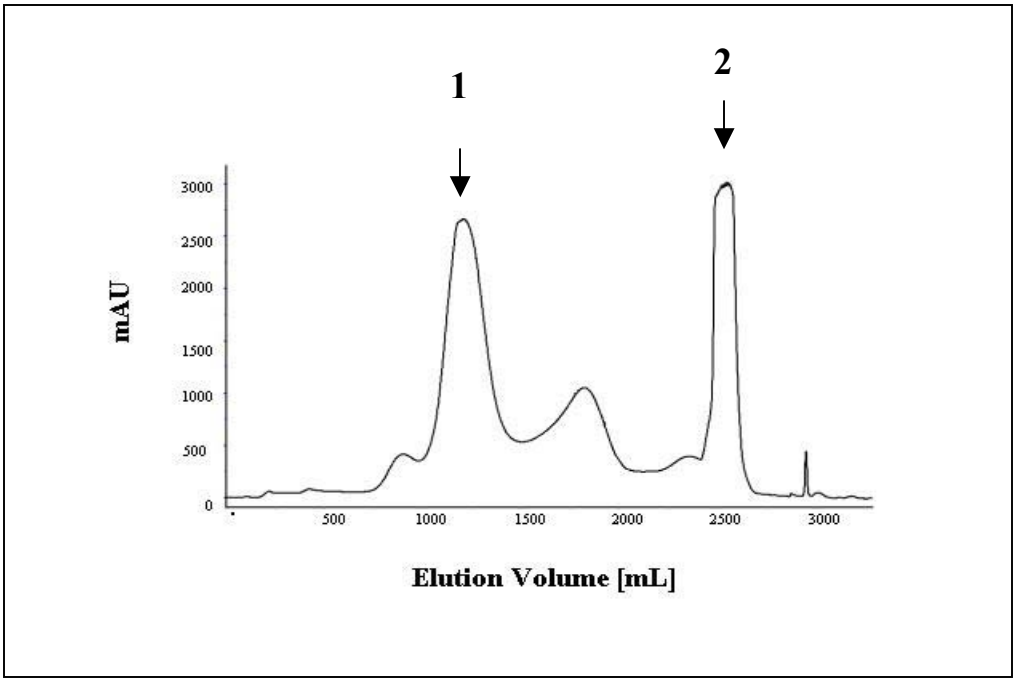

Figure 5.4: Elution profile of whey separation using anion exchange chromatography. 1) α-lactalbumin; 2) β-lactoglobulin.

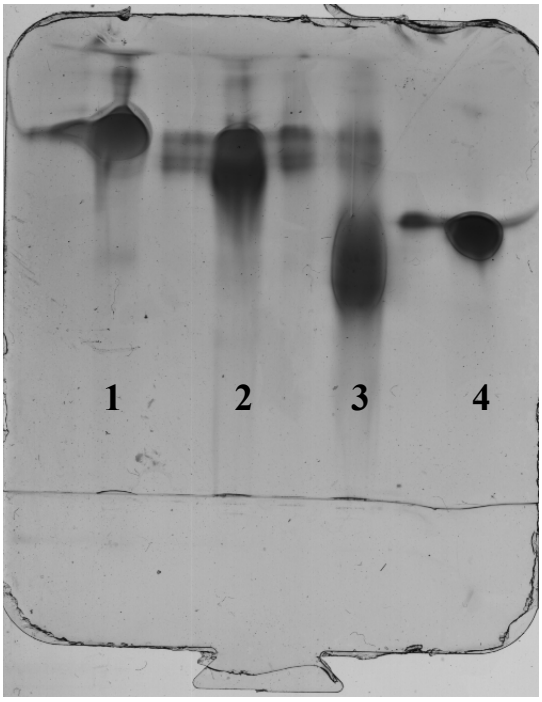

Figure 5.5: Urea-PAGE profile of the isolated fractions of bovine casein using 8-25% gradient gel and the PhastSystem^{R}. Lane 1) α_{s1}-casein; 2) α_{s2}-casein; 3) κ-casein; 4) β-casein.

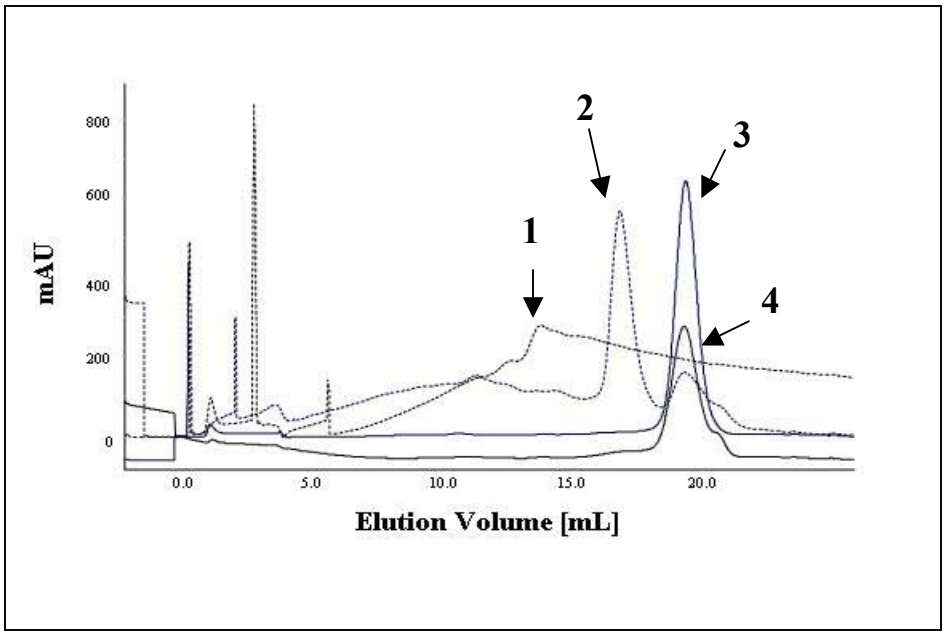

Figure 5.6: Elution profile of purified caseins on the HiTrap Q column. 1) β-caseins; 2) κ-caseins; 3) α_{s1}; 4) α_{s2}.

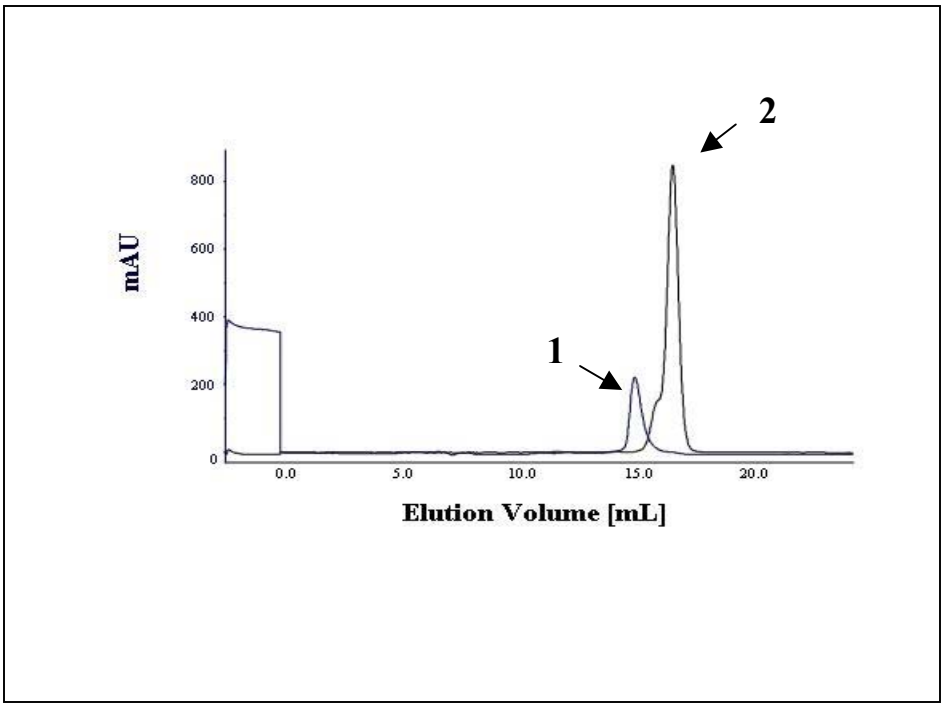

Figure 5.7: Size exclusion profile of the fractions isolated by ion-exchange chromatography. 1) α-lactalbumin; 2) β-lactoglobulin.

5.7 References

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CHAPTER 6

INTERACTIONS OF BETA-LACTOGLOBULIN AND PECTINS IN ACIDIFIED SYSTEMS[1]

[1]Kazmierski, M., L. Wicker and M. Corredig. To be submitted to the *Journal of Food Science*.

6.1 Abstract

Interactions of β-lactoglobulin (β-lg) with a high methoxyl pectin (HMP) and modified pectin (mHMP) were examined at pH 3.8 upon heat treatment using size exclusion chromatography and dynamic light scattering. β-lg-HMP mixtures formed soluble aggregates with larger hydrodynamic radius at a heating temperature of 65°C than 90°C. β-lg aggregation was enhanced in the presence of HMP, while β-lg-mHMP mixtures phase separated, and did not form soluble aggregates. Adjustment of the β-lg-HMP mixtures to pH 6.0 suggested that the heat-induced polymer complexes were more stable (did not dissociate) at 90°C than 65°C. The insoluble pellets of the heated β-lg-mHMP mixtures, resuspended at pH 6.0 indicated extensive interactions. Differences in behavior between the two pectins were attributable not only to the lower degree of methylation of the mHMP, but to the charge distribution of the pectin, which was modified by a plant pectinesterase.

Key Words: Pectin-protein interactions, beta-lactoglobulin, modified pectin, acidified system.

6.2 Introduction

There has been considerable progress in understanding the interaction of biopolymers and their role in aqueous systems. In particular, substantial advances have been made in understanding the behavior of polysaccharides and globular proteins. In mixed systems, their interactions affect the macroscopic properties of food, such as stability, texture, viscosity and mouthfeel (Tolstoguzov 1986; Harding and others 1992; Syrbe and others 1995; Syrbe and others 1998; de Kruif and Tuiner 2001). Three effects may occur upon mixing of protein with a polysaccharide: phase separation (leading to a protein-enriched and a polysaccharide-enriched phase), co-solubility (the biopolymers will be present in a single phase), and complexation. The latter is typically driven by electrostatic interactions and is dependent on the pH and ionic strength (Syrbe and others 1998; Burgess 1990; Galazka and others 1999; Schmitt and others 2000). Complex formation has been described upon mixing of anionic polysaccharides and proteins (Imeson and others 1977).

Pectins, anionic polysaccharides derived from plants, are often used as gelling agents and stabilizers in foods. The pectic backbone is composed of α-(1,4) D-galacturonosyl residues interrupted with (1,2)-linked L-rhamnopyranosyl residues. “Hairy regions” are characterized by sugars such as D-galactose, L-arabinose and D-xylose attached to the pectin backbone (Voragen and others 1995).

Pectins are employed as a means of stability in low pH food products such as acidified milk drinks and yogurt (Glahn 1982; Cai and Arntfield 1997; Pereyra and others 1997; Mishra and others 2001). The study of the interaction of pectins with milk proteins is important for understanding the behavior of dairy systems. It has been recently

demonstrated that at low pH, pectin interacts with caseins via electrostatic forces, leading to casein stabilization (Maroziene and de Kruif 2000; Tuinier and others 2002). The absorption of pectin around the casein occurs at or below pH 5.0. (Tuinier and others 2002).

β-lactoglobulin (β-lg) interactions with pectins have also been studied at low and high pH. The methoxyl ester content (DM) of pectin is an important factor in affecting the phase behavior of mixed systems at pH less than the isoelectric point of β-lg. While at pH 6.0 the two polymers coexist, upon mixing low methoxyl pectin (LMP) with β-lg at pH 3.5, complexation occurs, leading to a white precipitate. On the other hand, homogenous dispersions are obtained if high methoxyl pectin (HMP) is used at the same pH (Wang and Qvist 2000).

The effect of heat on β-lg mixed with sodium polypectate (SPP) was evaluated by Ndi and others (1996). Charged interactions between the polymers were studied at pH 3.5 and 6.5 using gel permeation chromatography and it was shown that heat-induced aggregation of β-lg is "enhanced" by the presence of SPP, depending on concentration (Ndi and others 1996). Similarly, in the presence of κ-carrageenan, an anionic polysaccharide, it was shown that the thermal aggregation behavior (at high pH) of β-lg is accelerated, and the heating rate affects the size of the microdomains that form (Croguennoc and others 2001a, b).

The objective of this study was to investigate the interactions occurring in mixed systems containing β-lg, HMP and a modified HMP (mHMP) at pH 3.8 and after heat treatment. Knowledge of protein-pectin interactions under different conditions has important implications for the application of whey proteins as functional ingredients in

low pH foods. By determining optimal interactions between whey protein and pectin, foods can be formulated so as to exhibit a certain texture and appearance.

6.3 Materials and Methods

6.3.1 Pectin and protein sample preparation

β-lactoglobulin was separated by preparative anion exhange chromatography from WPI (Land O' Lakes, St. Paul, MN). 300 ml of Q Sepharose^{TM} XL resin (AP Biotech, Piscataway, NJ) were employed and the separation followed the procedure described by Andrews and others (1985). Protein was separated using an Akta purifier system consisting of a P-900 HPLC pump, a UV-900 detector measuring at 280 nm and Unicorn (version 3.1) software (AP Biotech, Piscataway, NJ). The column (XK 50/30, AP Biotech, Piscataway, NJ) was equilibrated with 20 mM Tris buffer, pH 7.0. 400 mL of a 1% dilution of commercial whey protein isolate was prepared in 20 mM Tris-HCl, pH 7.0 and loaded onto the system. Elution was carried out with a flow rate of 6.5 mL/min in a total of 10 column volumes (CV), with a gradient elution to 0.2 M NaCl in 8 CV and a subsequent increase to 1 M NaCl in 2 CV. Protein fractions were collected and, after extensive dialysis against deionized H_2O, were freeze dried and stored at -20°C.

High methoxyl pectin (HMP) was prepared from a 4% (w/v) pectin (Citrus Colloids, 72% DE, Hereford, United Kingdom) dispersion made in 0.01 M EDTA. After hydration, the dispersion was precipitated and washed on sintered glass funnels with 95% ethanol. Acetone was used to further dry the pectin. Washed pectin was dried within 24 hours, ground with a mortar and pestle and screened through a USA standard sieve (No. 50 Newark Wire Cloth Company, Newark, NJ).

Modified high methoxyl pectin (mHMP) was made by treating HMP with pectin methylesterase (PME) purified from Valencia orange pulp (Citrus World, Lake Wales, FL) as described by Wicker and others (2002).

A 1.0% (w/v) of the HMP dispersion was prepared in 0.1 M NaCl and equilibrated to 30^{o}C with a water bath. PME (57.6 Units/ specific activity, 142.2 U/mg) was added and the pH was maintained at 7.5 using an automatic titrator (718 STAT Titrino, Metrohm, Herisau, Switzerland) with 0.1 N NaOH. The reaction was run for 2h 36min to give an estimated release of 8910 μmol ester in 19 g of pectin. To stop the reaction, the pH was adjusted to 4.0 with 4 M HCl and ethanol (3x initial volume) was added. The modified pectin was boiled for 10 min, washed with ethanol and then dried with acetone using sintered glass funnels. Both pectins (HMP, 72% DE and mHMP, 67% DE, as determined by titration and Fourier transform infrared spectroscopy) were stored at –20^{o}C until use (Hunter and others 2003).

6.3.2 Pectin/protein interactions

A 100 mg/mL stock solution of β-lg and a 5 mg/mL stock solution of high methoxyl or modified high methoxyl pectin were prepared in 50 mM sodium acetate buffer (pH 3.8) and filtered (0.45 μm PVDF, Millipore, Bedford, MA). Preliminary experiments were carried out on β-lg heated at pH 3.8 and 4.0 in both water and 50 mM sodium acetate. Buffer was chosen to better maintain the pH of the samples during experiments. Mixes of protein-pectin containing 10 mg/mL protein with a 1:10 ratio pectin:protein were prepared. Solutions were placed into clean, dry test tubes and solutions containing only protein (10 mg/mL) or pectin (1 mg/mL) were also prepared in buffer and used in control experiments. 2 mL samples were vortexed, covered and heated

in a water bath at 65°C and 90°C for times ranging from 5 to 30 min and then immediately cooled in an ice bath. Experiments were carried out in duplicates and unheated mixtures were also analyzed. All the analysis was carried out within 24 hours from heating.

Samples were transferred in eppendorf tubes and centrifuged at 10,000 rpm for 15 min at 25°C (Beckman Coulter Inc., Fullerton, CA). After centrifugation, supernatants were filtered (0.45 μm PVDF, Millipore, Bedford, MA) and injected (100 μL) onto a size exclusion column (Superdex 75, AP Biotech). Elution was carried out at a flow rate of 0.5 mL/min with 50 mM sodium acetate buffer containing 0.15 M NaCl at either pH 3.8 or 6.0 (see below), using the Akta purifier system described above (AP Biotech, Piscataway, NJ). The column was cleaned after each use with 0.5 M NaOH. Area of the peaks was determined integrating the elution chromatograms (mAU*mL) and the amount of residual native protein was calculated as % of the peak of an unheated pure β-lg sample. Fractions (1 mL) were collected for further analysis, stored at 4°C and analyzed within 48 hours after elution.

Samples containing high methoxyl pectin (HMP) had a turbid appearance and their aggregate size was measured by dynamic light scattering (DLS) (Particle Size Analyzer, 90 Plus, Brookhaven Inst., Holtsville, NY) with a 50m Watt diode laser (90º angle) and a BI-9000AT correlator (Brookhaven Instruments). Experiments were carried out at 25°C with the laser beam operating at 659.0 nm and 1.330 as the refractive index. Samples were filtered through 5 μm filters (Pall Corporation, Ann Arbor, MI) and added (400 μL) into 10 mm polystyrene cuvettes containing 1 ml of 50 mM sodium acetate buffer, pH 3.8, filtered with a 0.1 μm filter (Whatman, Clifton, NJ).

The β-lg-HMP supernatants at pH 3.8 were then adjusted to pH 6.0 and centrifuged at 10,000 rpm for 15 min at 25°C. No visible pellet was present, and supernatants were filtered (0.45 μm PVDF, Millipore, Bedford, MA) and injected (100 μL) onto the Superdex 75 column, as described previously. Elutions were carried out using 50 mM sodium acetate buffer, pH 6.0 and 1 mL fractions were collected and stored at 4°C for further analysis.

After centrifugation, modified high methoxyl pectin (mHMP) samples showed a pellet and a clear serum, and DLS measurements could not be performed on these supernatants. The pellets were washed with 1.5 ml of 50 mM sodium acetate buffer (pH 3.8), centrifuged using the conditions described above and resuspended in sodium acetate buffer at pH 6.0. Samples were vortexed until no visible pellet was present and then recentrifuged at 10,000 rpm for 15 min at 25°C. After centrifugation no visible pellet was present. Chromatography was carried out by injecting (100 μL) filtered (0.45 μm, Millipore, Bedford, MA) supernatants onto the Superdex 75 column. After elution with sodium acetate buffer, pH 6.0, 1 mL fractions were collected and stored at 4°C.

The amount of pectin present in supernatants was determined by quantifying the amount of uronic acid present (Blumenkrantz and Asboe-Hansen 1973). Assays were also carried out on the fractions collected from size exclusion chromatography, to determine the elution behavior of the pectin. Appropriate dilutions of supernatants (200 μL aliquots) were added to 2.2 g of a cold 0.0125 M sodium tetraborate in concentrated sulphuric acid. During sample addition, the tubes were refrigerated on crushed ice to prevent charring. The mixture was vortexed, covered with glass marbles and heated in a water bath at 100°C for 5 min. After cooling in an ice-water bath to room temperature, meta-

hydroxydiphenyl (MHDP) (0.15 % solution in 0.5 % NaOH) (20 μL) was added to the sample. Absorbance was measured at 520 nm after 3 min of incubation at room temperature. A blank sample was obtained for each measurement by addition of 20 μL of 0.5% NaOH instead of the MHDP reagent. Two calibration curves were obtained by preparing a series of dilution of galacturonic acid in a concentration range from 0-18 μg in either deionized H_2O or a 0.1 mg/mL solution of β-lg to determine if the presence of protein affected the pectin determination. The protein standard curve showed an average loss of 11%. The amount of pectin in the samples was quantified using the calibration curves obtained with deionized H_2O.

All data were analyzed by the general linear model procedure to determine if pectin type, temperature or time of heating were significant effects. Significance was defined at $p < 0.05$. The SAS program (version 8.0, Cary, NC, USA) was used for statistical analysis.

6.4 Results and Discussion

After centrifugation of the samples containing β-lg and HMP no visible precipitate formed, except for a small amount in the samples heated at 90°C. The homogeneous white supernatants were measured by dynamic light scattering (DLS) and results shown in Figure 6.1.

In contrast with the stable suspensions formed in the β-lg-HMP at pH 3.8, the mixtures containing mHMP formed a solid pellet after centrifugation with a clear supernatant. These mixtures could not be analyzed due to the low photon counts of scattered light, which are necessary for the determination of particle size (Dalgleish and Hallett 1995).

6.4.1 Dynamic light scattering measurements

The average values of diameter of the aggregates of heated and unheated β-lg-HMP samples are shown in Figure 6.1. Upon mixing of HMP with β-lg, an aggregate of 204 nm formed and after heating, the size increased from an average diameter of 204 nm to > 300 nm. Statistical analysis demonstrated that while temperature significantly affected the size of the aggregate, a longer heating time did not further affect the size. The average size of the aggregates at 65°C was 350 nm and was significantly different (p-value < 0.05) from the 300 nm diameter of the aggregates formed at 90°C.

Larger diameter aggregates are shown in solutions of pure β-lg heated at high temperature and low pH (Schokker and others, 2000) than the aggregates heated at low temperatures, however this does not seem to be the case when HMP pectin is present with β-lg. Higher heating temperatures produced aggregates with a smaller effective diameter as shown in Figure 6.1. This could be partly explained by the presence of a small pellet in the samples heated at 90°C, which could be composed of the largest aggregates precipitating upon centrifugation, leaving smaller soluble aggregates in the supernatant. On the other hand, the samples heated at 65°C may have only soluble aggregates. It could also be hypothesized that stronger interactions occurred between the two polymers upon heating at 90°C, while the complexes formed at 65°C were not as strong, forming larger hydrodynamic diameters. Unfortunately these results do not allow pectin-protein and/or protein-protein aggregates to be differentiated. Size exclusion chromatography was then employed on both β-lg-HMP and β-lg-mHMP supernatants to further elucidate differences between the samples, in terms of residual native protein and the amount of aggregate present in the soluble phase (shown in figures 6.3 and 6.4).

6.4.2 Uronic acid measurements of soluble supernatants

Table 6.1 summarizes the effect of heating, pH and type of pectin on the uronic acid content in the supernatants after centrifugation. Upon mixing of β-lg with mHMP at pH 3.8, most of the pectin was present in the precipitate, and by increasing the pH from 3.8 to 6.0, pectin resuspended into the soluble phase, as shown by the increase in pectin content of the supernatants from pH 3.8 to 6.0 (Table 6.1). This suggested that the interactions, which led to complex formation and phase separation of the β-lg-mHMP mixtures, were coulombic in nature, and were reversible upon changing the pH to 6.0. Temperature of heating affected the resolubilization of the pectin in the supernatant upon pH change. Heating at 90°C showed less reversibility of the β-lg-mHMP complexes at pH 6.0 compared to the amount of pectin recovered in the samples heated at 65°C. Perhaps the interactions occurring at the higher temperature caused a stronger complex to form, causing decreased reversibility of the ionic interactions.

Table 6.1 also summarizes results on the amount of pectin present in the soluble phase of the β-lg-HMP mixtures. The supernatants showed no variation in the amount of pectin present after centrifugation. Uronic acid contents in the β-lg-HMP mixtures corresponded to a high recovery of pectin as the complexes were soluble (Table 6.1).

6.4.3 Size exclusion of β-lg mixtures with HMP and mHMP at pH 3.8

Figure 6.2 represents the elution of supernatants of β-lg solutions with added HMP and mHMP at pH 3.8 after mixing, with no heat applied. The native β-lg peak was shown at 12 mL and when HMP was mixed with β-lg, most of the β-lg maintained its solubility in the supernatant and eluted as a native peak with a peak area comparable to that of the unheated β-lg in isolation. The large aggregates described in Figure 6.1

indicated soluble protein-pectin interactions in these supernatants as already described by other authors (Syrbe and others 1998), and the elution chromatograms depicted in Figure 6.2 demonstrated that the addition of pectin did not induce protein-protein interactions in the unheated samples.

In contrast to solubility β-lg-HMP mixtures, β-lg-mHMP samples showed a solid-white pellet upon centrifugation. Thus, low amounts of native protein were recovered in the soluble supernatant as shown by the small native peak size compared to the β-lg-HMP and the control samples (Figure 6.2). The complex formation between β-lg and mHMP resembled the mixing behavior described upon mixing β-lg with low methoxyl pectin (LMP) where, at low pH and low ionic strength, LMP forms insoluble complexes with β-lg causing phase separation (Wang and Qvist 2000). The different behaviors observed between the protein with mHMP and unmodified HMP at pH 3.8 were caused not only by the lower number of methoxyl groups present in the mHMP compared to HMP, but perhaps, by the difference in charge density of the modified pectin. The pectin, still high in the number of methoxyl groups present, was modified with a plant methylesterase, which creates regions of demethoxylated carboxylic groups in a blockwise pattern (Ralet and others 2001). This caused the formation of complex coacervates of β-lg and mHMP by electrostatic interactions (Syrbe and others 1998) similar to those with LMP, as the two polymers carry opposite charges at low pH.

Figure 6.3 depicts the elution profile of soluble phases of β-lg in isolation, and mixed with HMP and mHMP after heating at 65° and 90° C for 20 min. In the samples heated at 65°C (Figure 6.3A) a small aggregate peak eluting at 7 mL was shown for β-lg-HMP samples, however most of the protein was still present in native form in the mix.

When compared to the elution of a pure β-lg solution heated at the same temperature and time, it appeared that HMP facilitated the formation of soluble aggregates. A similar behavior was observed by Croguennoc and others (2001) in the presence of another negatively charged polysaccharide, κ-carrageenan, during heating of β-lg at neutral pH. The presence of κ-carrageenan seemed to accelerate the growth of the aggregates formed during heat treatment. When observing the amount of pectin eluted in the various fractions it was shown that in the β-lg-HMP sample heated at 65°C there was a pectin peak at 7 mL of about 0.04 mg/mL (Figure 6.3B). The pectin peak eluted at the same elution volume as the protein aggregate peak. This may be an indication of soluble protein-pectin complexes not dissociated by the elution buffer (which contained 0.15 M NaCl). The complexes eluted in the excluded volume of the column and the two polymers may simply co-exist in the soluble phase. However, control HMP samples (1 mg/mL) injected in the column showed no indication of a large peak eluting at this elution volume (7 mL), leading to the conclusion that an interaction may exist. HMP also demonstrated a protective effect on the denaturation of β-lg when heated to 65°C, as shown by the large amount of native protein retained in the supernatant (Figure 6.4).

When compared to the unheated samples depicted in Figure 6.2, samples of β-lg-mHMP heated at 65°C for 20 min did not show differences in the amount of protein left in the soluble phase (Figure 6.3A). A small amount of eluted at 7 mL (Figure 6.3B) indicating that most pectin precipitated with β-lg upon mixing of mHMP with β-lg. Only a small amount of residual native β-lg was still present in the mix heated at 65°C.

Compared to the protein aggregate peak at 65°C, the elution peak at 7 mL greatly increased after heating to 90°C in the β-lg-HMP mixtures (Figures 6.3A and C). On the

other hand while a larger number of protein-containing aggregates were present at 90°C, the pectin peak was comparable to that shown in the β-lg-HMP samples heated at 65°C (Figures 6.3B and D). It appeared that increased denaturation of native protein at 90°C led to the formation of soluble protein aggregates. No pellet was collected from these samples, and no residual native protein was shown upon chromatographic separation of β-lg-HMP heated at 90°C for 20 min. If interactions were simply electrostatic in nature and were characterized by pectin/unaggregated protein complexes, the elution with the acetate buffer containing 0.15 M NaCl would still separate a fraction of native β-lg.

Heat treatment at 90°C enhanced aggregation and phase separation of the β-lg-mHMP samples: the supernatants, after heating at 90°C for 20 min did not elute either a residual protein or pectin peak.

Statistical analysis of the residual native protein area demonstrated that temperature had a significant effect ($p < 0.05$) on the aggregation of β-lg. In addition, there were behavioral differences in the aggregation and the protein-pectin interactions upon heating, but time did not seem to be a factor in the amount of residual unaggregated protein. Figure 6.4 illustrates the amount of residual native β-lg (calculated as area of residual native peak relative to the unheated β-lg control) as a function of heating temperature and time. Protein denaturation in the β-lg-HMP mixtures was greater at 90°C compared to 65°C (Figure 6.4B), as suggested by the low concentrations of native protein after heating at the higher temperature. Reactivity of the sulphydryl group of the β-lg molecule is limited at low pH (Schokker and others 2000), and the type of interactions occurring during heating of β-lg in the presence of HMP and mHMP may be attributed to a balance of associative and non-associative forces between protein-protein and protein

and pectin. HMP did not seem to affect the denaturation behavior of β-lg, as the amount of residual native protein in the β-lg-HMP mixtures was similar to the native protein content in the heated β-lg control (Figure 6.4A).

While heating the samples at 65°C in the presence of HMP did not seem to affect the amount of residual native protein present in the soluble phase, heating at the same temperature in the presence of mHMP showed a 30% recovery of native β-lg in the supernatant, as most of the protein precipitated with mHMP upon its addition (Figure 6.4C). After heating at 90°C no residual native protein was shown in the β-lg control and in either HMP or mHMP mixtures.

6.4.4 Size exclusion of β-lg mixtures with HMP and mHMP after adjustment to pH 6.0

To evaluate the electrostatic nature of the interactions occurring between β-lg and the HMP and mHMP pectin, the pH was adjusted from 3.8 to 6.0 after heating. In β-lg-HMP, the soluble phases containing the protein-pectin complexes were simply adjusted to pH 6.0, while in the β-lg-mHMP samples, the pellet was resuspended to pH 6.0. After centrifugation, the supernatants were then analyzed by size exclusion chromatography, to determine the presence/absence of pectin and native or aggregated proteins, and elution profiles are shown in Figures 6.5 and 6.6.

Figure 6.5 illustrates the elution profile of β-lg-HMP supernatants after adjustment to pH 6.0. After heating at 65°C for 20 min, the native protein content in the heated β-lg-HMP was comparable to the unheated mixture and pectin eluted in the excluded volume (7 mL) with a peak concentration of uronic acids of 0.04 mg/mL (Figure 6.5B). When comparing to the elution of the same β-lg-HMP mixture at pH 3.8 (Figure 6.3A) the protein aggregate peak at 7 mL, present at pH 3.8, was absent in the

elution of the same sample adjusted to pH 6.0 (Figure 6.5A). This confirmed the non-covalent nature of the protein aggregates and that upon heating at 65°C ionic interactions occurring between protein and HMP could be disrupted with a pH change. After adjustment from 3.8 to 6.0, the overall net charge of the two polymers changes, causing the electrostatic complexes to dissociate. In addition, the native protein at pH 6.0 eluted earlier (around 11 mL) than the native protein eluting at 12 mL at pH 3.8. This suggested conformational changes occurring to β-lg at pH 6.0 and is in agreement with non-native monomers observed previously (Iametti and others 1995).

In contrast to what was observed after heating at 65°C, the β-lg-HMP mixtures heated at 90°C for 20 min showed the presence of large aggregates eluting at 7 mL (Figure 6.5C) and the uronic acid assays on the various fractions showed that pectin elution was not affected by heat treatment (Figure 6.4 B). The elution profile of β-lg-HMP samples adjusted to pH 6.0 was comparable to that at pH 3.8 (Figure 6.3C) indicating that the heat-induced aggregates formed at 90°C did not dissociate after pH adjustment. These heat-induced aggregates were stable in the soluble phase at both pH.

The pellets collected after centrifugation of β-lg-mHMP mixture at pH 3.8 were resuspended at pH 6.0. After resuspension of the pellet of the samples heated at 65°C, some native protein was recovered in the supernatant (Figure 6.6A), indicating that the interactions between protein and mHMP were electrostatic in nature, and did not cause further aggregation of β-lg. The aggregates were held by electrostatic forces, and the protein dissociated upon pH change with a possible conformational change, as shown by the earlier elution of native protein occurring at 11 mL (Figure 6.6A and C). A small amount of large aggregates, eluting at 7 mL, suggested that the resuspended coacervates

were rich in pectin and protein, as indicated by the pectin eluting in the same aggregate peak (Figure 6.7B). This elution behavior suggested that some β-lg was still interacting with pectin or with other β-lg molecules, by electrostatic forces (Schokker and others 2000).

After heating of the β-lg-mHMP at 90°C, the resuspended β-lg-mHMP pellet showed a different elution profile from that of the same samples heated at 65°C. In addition, differences were shown also from the elution of the soluble phase at pH 3.8. The supernatant was practically devoid of native protein (Figure 6.6C) and only soluble aggregates eluting with large molecular weight were present, and in much larger amounts than in the samples heated at 65°C (Figures 6.6A and C). On the other hand, the amount of pectin eluted in the aggregate peak was lower than that resuspended at pH 6.0 from samples heated at 65°C (Figure 6.6D). These results suggested that more protein-protein interactions occurred during heating at 90°C, as demonstrated by the low amount of residual native protein. Even though the presence of mHMP caused precipitation upon mixing, heat treatment still affected the β-lg aggregation behavior, since no native protein was recovered by resuspending the pellet, insoluble at pH 3.8, to pH 6.0. No changes occurred in the amount of residual native protein resuspended after pH adjustment from the insoluble pellet with time of heating. As shown in Figure 6.7, upon heating at 65°C, more than 50% of the β-lg could be resolubilized in its native form. On the other hand, at 90°C, all the protein was aggregated. This behavior did not show an effect of time, but temperature caused a change in the first 5 min of heating.

6.5 Conclusions

Our results indicated that soluble complexes are formed between β-lg and HMP while insoluble complexes are formed with β-lg and mHMP, similarly to the behavior described upon mixing β-lg with a low methoxyl pectin by Wang and Qvist (2000). The size of soluble aggregates produced in β-lg-HMP mixtures at pH 3.8 was shown to decrease with increasing temperature as measured by DLS. The presence of HMP seemed to enhance the aggregation of β-lg, while mHMP prevented formation of soluble aggregates at pH 3.8. By adjusting the pH to 6.0 it was possible to determine the role played by electrostatic interactions in the formation of the pectin/protein complexes. Modification of pH of the soluble β-lg-HMP mixtures showed that the native protein denaturation is protected by HMP and the heat-induced complexes are reversible when heated at 65°C with conformational changes occurring to the protein, while the heat-induced polymer complexes of β-lg and HMP are not disrupted by pH change after heating at 90°C. Resuspending the insoluble pellets obtained from heated β-lg-μHMP mixtures indicated more extensive interactions. In spite of the high amount of methoxyl groups still present in the mHMP (65%DE) this pectin showed strong electrostatic interactions with β-lg, probably due to the difference in charge density induced by the enzyme (a plant pectinesterase) used to modify the polymer. Pectin-protein interactions seemed to be strongly affected by temperature, and while at 65°C native protein was recovered upon disruption of the electrostatic interactions, at 90°C only large protein aggregates were recovered. Conformational changes also occurred to the protein upon resuspension to pH 6.0. These results have important implications in the formulation of

thermally processed foods. By utilizing whey protein and different types of pectins it is possible to provide a wide variety of textural characteristics and appearances.

6.6 Acknowledgements

We would like to thank Ms. Janice Hunter for preparing the pectin samples, and for perfoming the titration and infrared spectroscopy analyses for measurement of the degree of esterification of the pectins.

Table 6.1: Uronic acid content of supernatants of β-lg-HMP mix at pH 3.8; β-lg-mHMP at pH 3.8 and after resuspension of the pellet at pH 6.0. Unheated mix, heated at 65°C and 90°C for 20 min.

Uronic Acid [mg/mL]			
	β-lg-mHMP mix		
	No Heat	**65°C**	**90°C**
pH 3.8	0.11 ± 0.00	0.16 ± 0.06	0.07 ± 0.03
pH 6.0	0.68 ± 0.19	0.46 ± 0.21	0.27 ± 0.02
	β-lg-HMP mix		
pH 3.8	0.62 ± 0.09	0.58 ± 0.01	0.65 ± 0.20

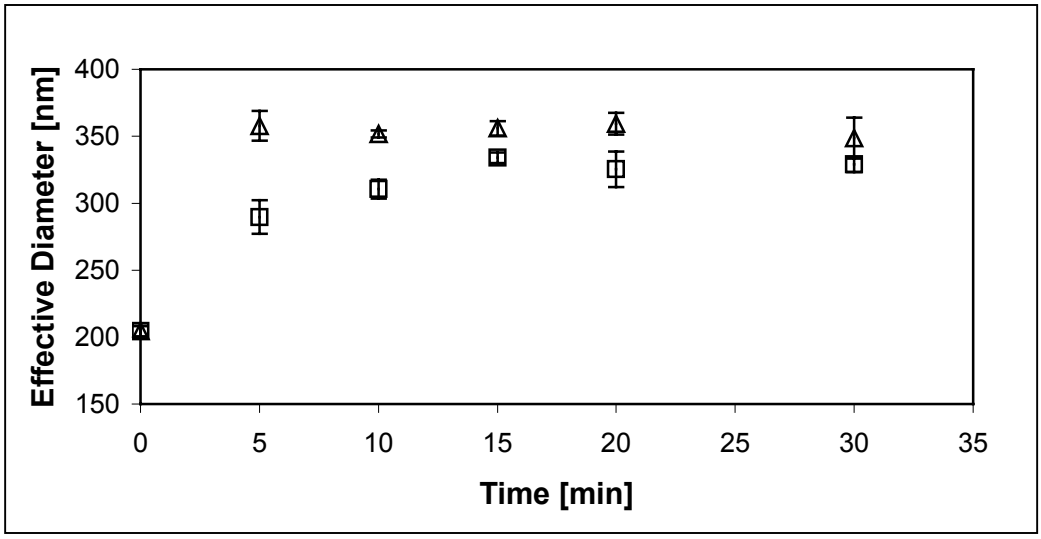


Figure 6.1: Effect of heating temperature and time on the diameter of β-lg-HMP aggregates; 65°C (Δ); 90°C (□). Bars represent standard deviations.

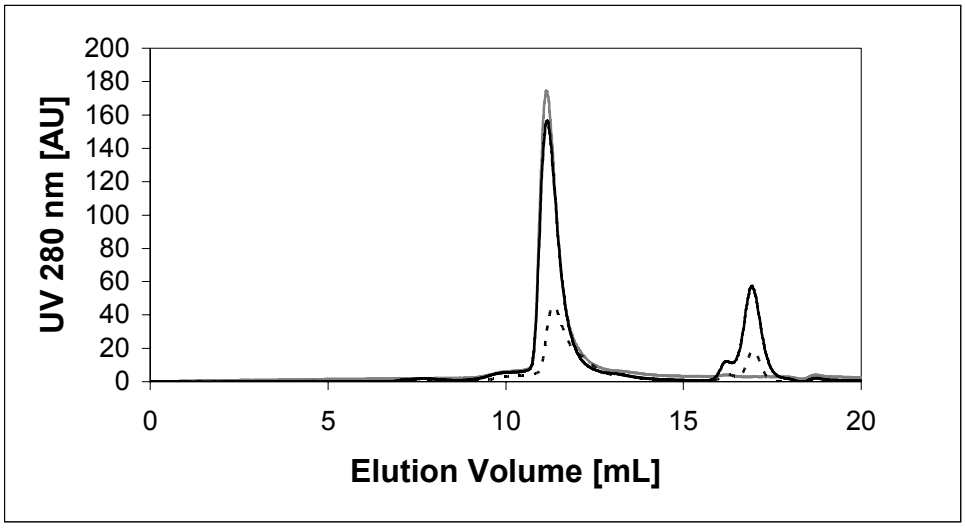
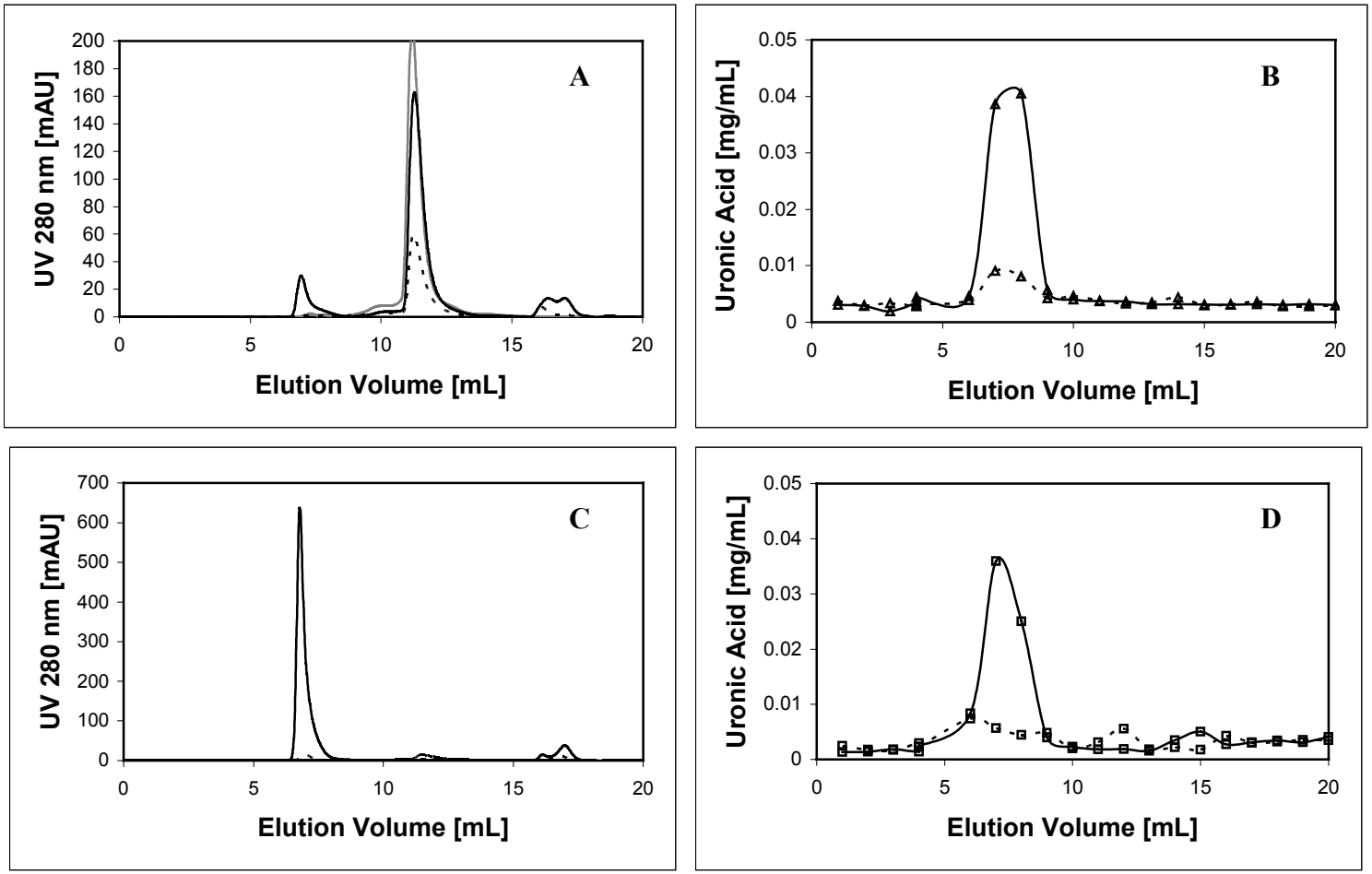


Figure 6.2: Elution profile of unheated protein-pectin samples at pH 3.8 using the Superdex 75 column. β-lg (solid gray line), β-lg-HMP (solid black line) and β-lg-mHMP (dotted black line).

Figure 6.3: Elution profiles of heated protein/pectin mixtures at pH 3.8 using the Superdex 75 column. β-lg (solid gray line), β-lg-HMP (solid black line) and β-lg-mHMP (dotted black line). Left (A,C) UV 280 signal; right (B,D) uronic acid in fractions. (A,B) 65°C for 20 min; (C,D) 90°C for 20 min.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UV 280 nm [mAU]
200
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160
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120
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80
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0
5
10
15
20
Elution Volume [mL]
B
Uronic Acid [mg/mL]
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Elution Volume [mL]
C
UV 280 nm [mAU]
700
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500
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100
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Elution Volume [mL]
D
Uronic Acid [mg/mL]
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Elution Volume [mL]

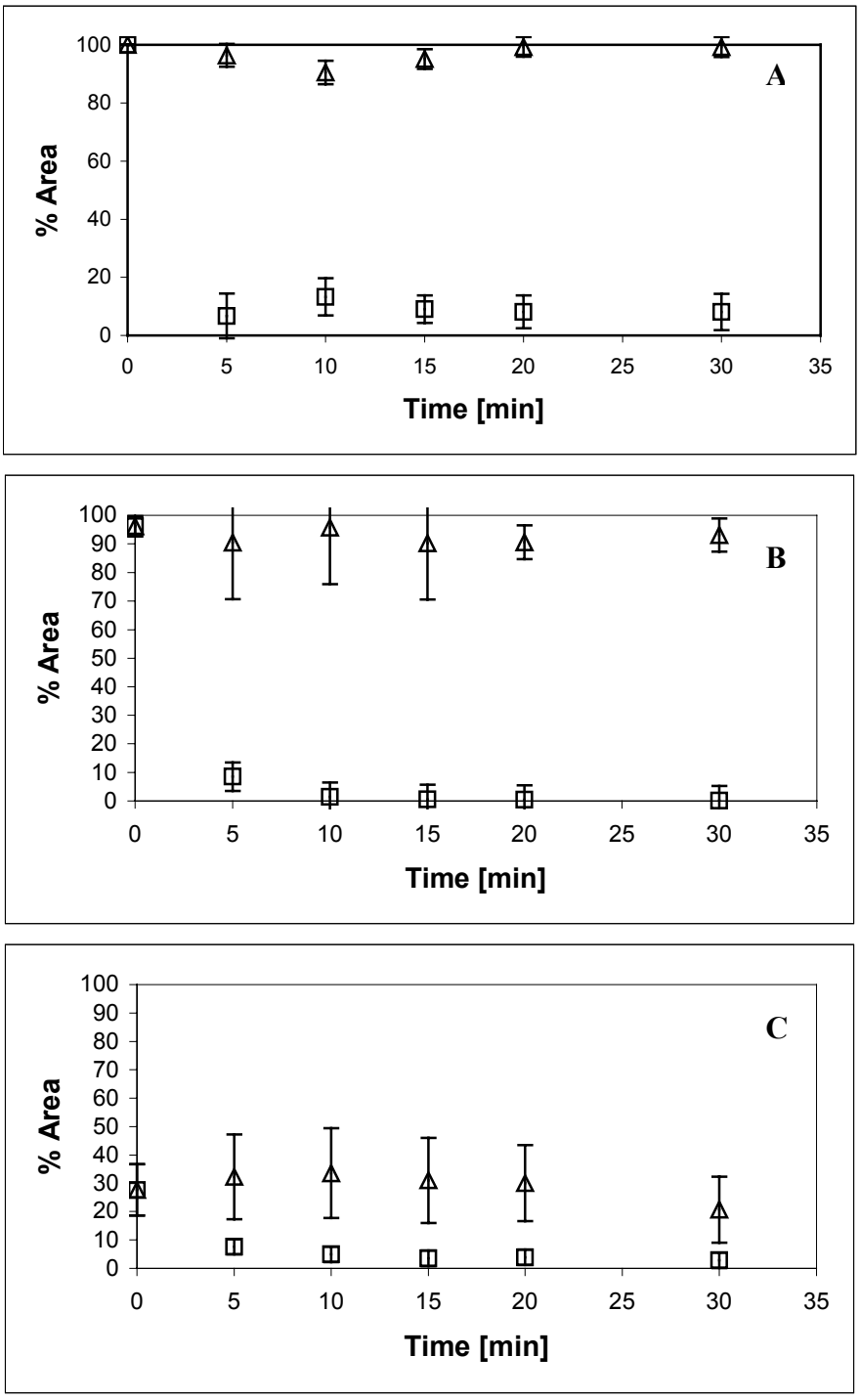
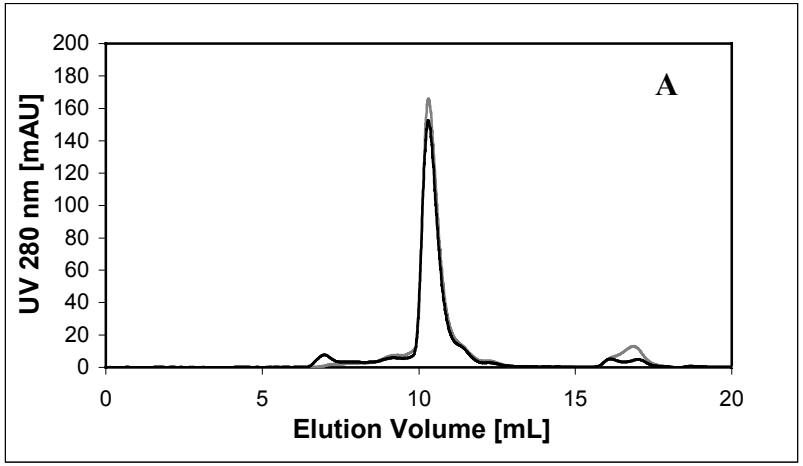


Figure 6.4: Residual native protein (calculated as % area of corresponding pure β-lg control peak) of β-lg (A) β-lg-HMP (B) and β-lg-mHMP (C) at pH 3.8 after heating at (Δ) 65°C; (□) 90°C for various times.

Figure 6.5: Elution profiles of unheated β-lg-HMP mixtures (solid gray line) and heated β-lg-HMP mixtures (solid black line) after adjustment of supernatants to pH 6.0 using the Superdex 75 column. Left (A,C) UV 280 signal, right (B,D) uronic acid content in fractions. (A,B) 65°C for 20 min; (B,D) 90°C for 20 min.


A
UV 280 nm [mAU]
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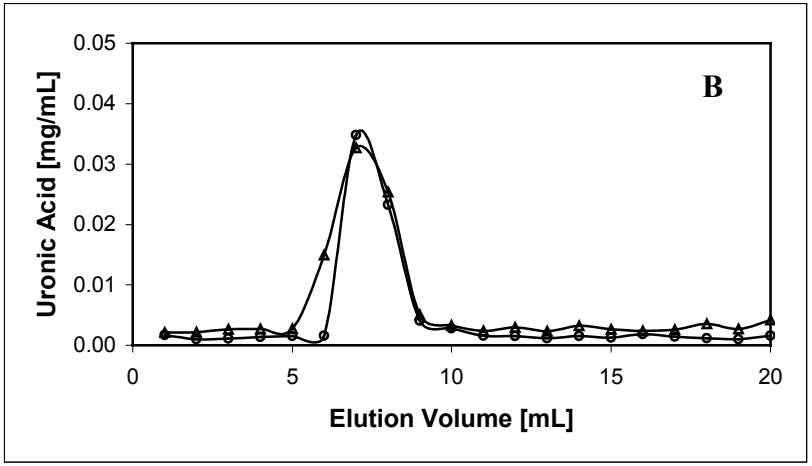
20
Elution Volume [mL]


B
Uronic Acid [mg/mL]
0.05
0.04
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0.02
0.01
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5
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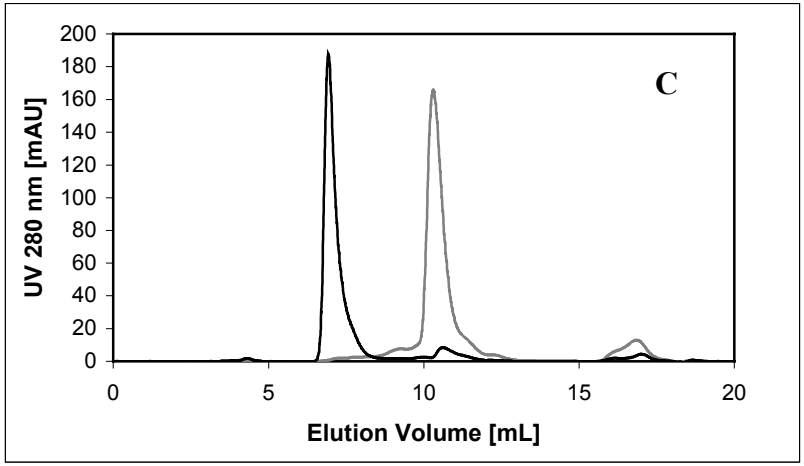
15
20
Elution Volume [mL]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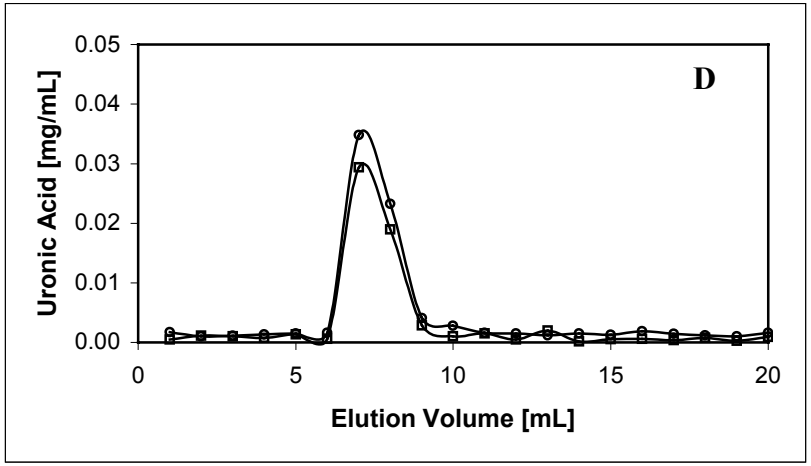
Elution Volume [mL]


D
Uronic Acid [mg/mL]
0.05
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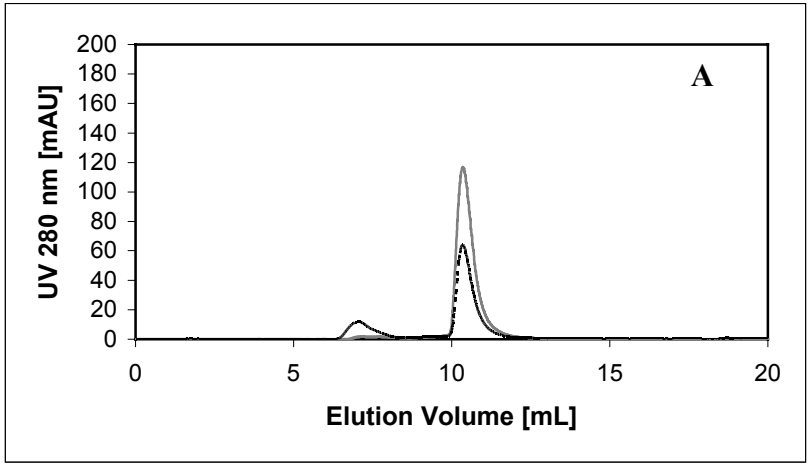
Elution Volume [mL]

Figure 6.6: Elution profiles of unheated β-lg-mHMP mixtures (solid gray line) and heated β-lg-mHMP mixtures (dotted black line) after resuspension of the pellet in acetate buffer at pH 6.0 using the Superdex 75 column. Left (A,C) UV 280 nm signal, right (B,D) uronic acid in fractions. (A,B) 65°C for 20 min; (C,D) 90°C for 20 min.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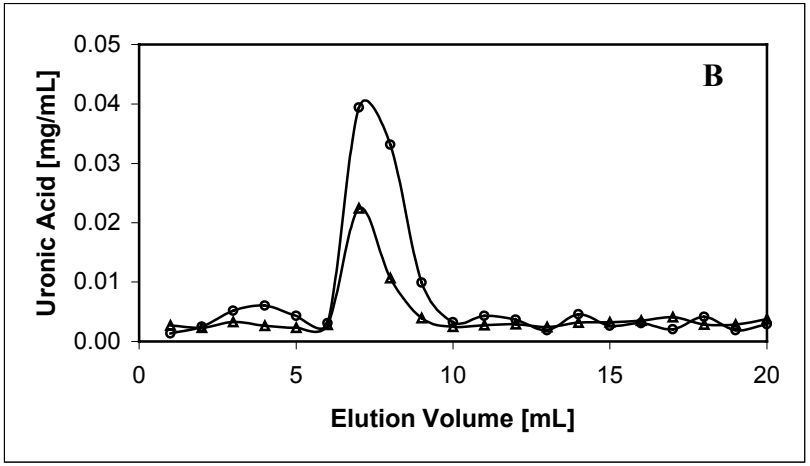
Elution Volume [mL]


B
Uronic Acid [mg/mL]
0.05
0.04
0.03
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0.01
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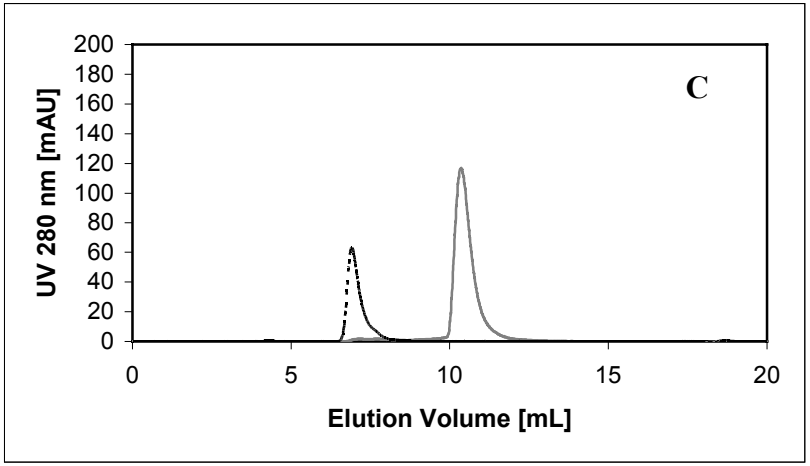
Elution Volume [mL]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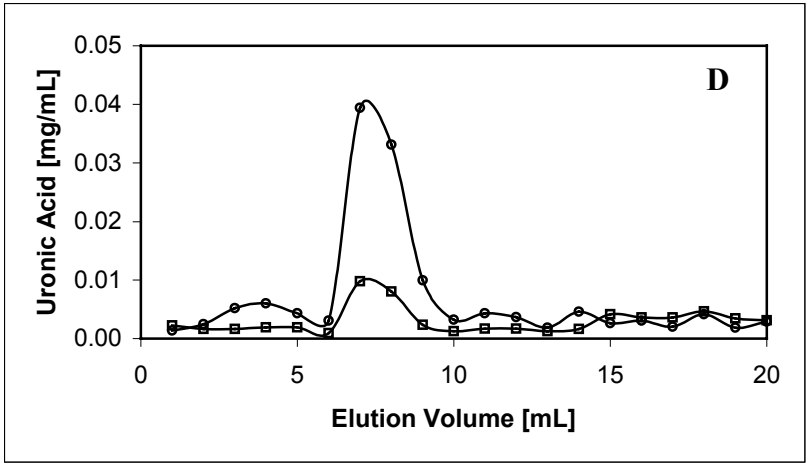
Elution Volume [mL]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Elution Volume [mL]

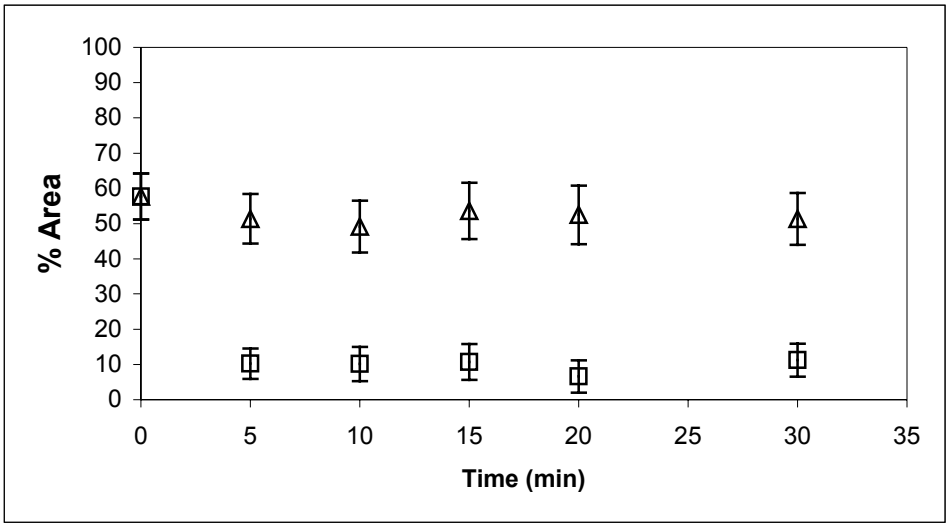

Figure 6.7: Residual native protein (calculated as % area of corresponding to pure β-lg control peak) of β-lg-mHMP pellet, resuspended at pH 6.0. Samples heated at (Δ) 65°C and (□) 90°C for various times.

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CHAPTER 7

OPTIMIZING STABILITY OF ORANGE JUICE FORTIFIED WITH WHEY PROTEIN AT LOW pH VALUES[1]

[1]Kazmierski, M., S. Agboola, and M. Corredig. Submitted to the *Journal of Food Quality*.

7.1 Abstract

The influence of temperature, heating time and pH on the stability of whey protein-fortified Valencia orange juice was determined by uronic acid content, degree of esterification (DE), % transmission measurements (%T) and capillary electrophoretic analysis of the juice-protein supernatants. Uronic acid content and charge of pectins showed no significant change in heat-treated samples with added proteins. The %T decreased with decreasing pH and increasing temperature and heating time for α-lactalbumin (α-lac), β-lactoglobulin (β-lg) and whey protein isolate (WPI). The lowest transmission values were shown at pH 3.0 and 85C. Capillary electropherograms confirmed more extensive juice-protein interactions in WPI and β-lg added juices than in those containing α-lac, especially at low pH, resulting in more stable juice-protein mixtures.

7.2 Introduction

The use of "value-added ingredients" is designed to meet specific nutritional demands and increasing consumer markets. Whey proteins possess a significant source of nutritional and functional characteristics and are often employed as protein ingredients in many food products. Their bland flavor and solubility over a wide pH range make their utilization ideal in juice systems. Whey proteins may be added to most types of juice and sensory studies indicate that the addition of whey to orange juice results in satisfactory evaluations in terms of clarity, odor and flavor (Vojnovic *et al*. 1993).

One challenge, however, in preparing juice products, is to maintain the juice cloud stability, as cloud particles need to remain suspended in the serum phase. Juice cloud is composed of 52% protein, 4.5% pectin, 25% lipid, 1.5% hemicellulose, 5.7 nitrogen and 2% ash (Baker and Cameron 1999). When cloud destabilizes, juice clarification occurs, and this results in low consumer acceptability (Baker and Cameron 1999). The most important cause of cloud de-stabilization and clarification of fresh orange juice is the activity of the enzyme pectinmethylesterase (PME) on pectin molecules (Joslyn and Sedky, 1940; Rouse *et al.* 1952). PME cleaves methylated esters from the galacturonic acid backbone of pectin and causes the acid groups to bridge with cations, e.g., with Ca^{2+} they form calcium pectate gels, which will precipitate (Baker and Cameron 1999). It is therefore important to heat inactivate the PME enzyme to minimize its activity on the juice pectins (Wenzel *et al.* 1951; Rouse *et al.* 1952; Baker and Cameron 1999; Schmelter *et al.* 2001). However, even in enzyme-inactivated, cloud-stable juice, the introduction of whey proteins could influence the stability due to possible

interactions between whey protein and juice components, eg., pectins (Wang and Qvist 2000).

Structural modifications of whey proteins cause many changes in their physical and functional properties. For example, at processing temperatures greater than 65C, β-lactoglobulin (β-lg) undergoes conversion from a dimer to a monomer conformation, resulting in the formation of large molecular weight aggregates (Hambling *et al*. 1992). The free thiol group of β-lg is fundamental in the formation of disulfide bonds with other cysteine containing proteins during heating (Phillips *et al*. 1994). The rate of denaturation and coagulation of whey proteins is also dependent on pH, because of changes on the net charge of the proteins (Kinsella 1984; Verheul *et al*. 1999).

Studies on mixtures of β-lg and pectin in aqueous solutions have shown that pH and ionic strength greatly influence the presence of single-phase solutions and insoluble complexes (Wang and Qvist 2000). In a protein fortified orange drink product, this would result in precipitation of the protein and cause a decrease in sensory quality and consumer appeal (Jelen 1992). Coexisting macromolecules may be thermodynamically incompatible due to different physico-chemical properties possessed by α-lac and β-lg (isoelectric point, charge distribution, particle size, etc.). This can be expected to impact the stability of whey-juice blends differently, causing phase separation primarily due to specific proteins exhibiting distinct intermolecular and intramolecular changes during heating at the low pH environment of the juice. It is therefore important to understand phase behavior at the molecular level of these biopolymers in order to prevent separation from the serum phase and limit loss of turbidity, cloud destabilization and protein flocculation.

The objective of this work was to investigate the effect of the addition of three different commercial protein isolates: α-lac, β-lg and a whey protein isolate to Valencia orange juice as a function of pH, temperature and time of heating in order to determine protein behavior in this environment and to understand how the association of these macromolecules may affect juice stability.

7.3 Materials and Methods

7.3.1 Materials

Valencia oranges were obtained from Woolworth's grocery store, Wagga Wagga, Australia. Juice was extracted in batches using a Santos juicer (Santos 11p citrus juicer, Lyon, France) and was filtered 2 times through cheesecloth to remove pulp. The unstable pulp was further removed by centrifugation at 20C for 5 minutes at 1000*g* using a Beckman Avanti J-25 centrifuge (Beckman Coulter Inc., Gladesville, NSW, Australia). Inactivation of PME was carried out as described by Rouseff and Ting (1986) with slight modifications. Optimal PME inactivation was determined by heating the juice at various times/temperature and testing for its residual activity using a 0.1 M NaOH solution containing methyl red as indicator. A treatment of 90C for 5 minutes was carried out to ensure complete PME inactivation. After this pasteurization treatment, the juice was rapidly cooled in an ice bath to 20C. Whey Protein Isolate (WPI), (Land O' Lakes Dairy Proteins Group, St. Paul, MN) and β-lg and α-lac protein fractions (Davisco Foods International, Inc. Eden Prairie, MN), were used to fortify orange juice samples. All reagents and chemicals were purchased from Sigma-Aldrich Ltd. (Sydney, Australia).

7.3.2 Preparation of juice samples

Single strength orange juice was diluted 1:1 with 4% (w/v) protein solution dissolved in H_2O to obtain a fortified orange drink with 2% protein. Control samples of 2% protein in deionized H_2O were also prepared. The original pH of the juice was 4.1 and, after adding protein, the samples were adjusted to a pH of 3, 4 or 5 using 2.5 M citric acid or 1 M NaOH. Samples were heated at 65, 75, or 85C for 10, 20, or 30 min and immediately cooled in an ice bath to 20C. The mixtures were then centrifuged at 20C for 15 min at 3000 x *g* and the soluble phase (supernatant) was further analyzed.

7.3.3 Pectin

Pectin was measured by determining the concentration of uronic acid (Blumenkrantz and Asboe-Hansen 1973). The amount of uronic acid in supernatant was based on the meta-hydroxydiphenyl (MHDP) assay with slight modifications. To 0.2 ml of the sample, aliquots (1.2 ml) of a cold 0.0125 M solution of sodium tetraborate in concentrated sulphuric acid were added. The tubes were immediately refrigerated in crushed ice to prevent charring. The mixture was vortexed and heated in a water bath at 100C for 5 min. After cooling in an ice-water bath, 20 µl of the MHDP reagent (0.15 % solution in 0.5 % NaOH) was added to the sample and absorbance was measured at 520 nm after 2 min. The calibration curve was obtained by preparing a series of dilutions of 0.1 mg/ml galacturonic acid standard in a concentration range from 150 to 600 µg/ml with deionized H_2O. Aliquots of standard solution (200 µl) were treated and measured as described above for the samples. A blank sample was obtained for each measurement by addition of 20 µl of 0.5 % NaOH, instead of the MHDP reagent. All determinations were

carried out on four sub-samples. The uronic acid content in the supernatants was quantified before and after the addition of protein.

7.3.4 Titration of soluble phases

After centrifugation, samples were titrated following the procedure for determination of the degree of esterification described by Tuerena *et al*. (1984), to test if there were any differences in methoxyl ester distribution in pectin between juice samples. An aliquot (20 ml) of sample (centrifuged juice supernatant) was titrated with 0.1 N NaOH (x-value) using phenolphtalein as indicator. Next, 50 ml of 0.1 N NaOH were added and the solution was incubated for one hour at room temperature in a stoppered vessel. The excess base will create a favorable environment for alkaline demethoxylation of the pectin esters. Following this incubation, 50 ml of 0.1 M HCl were added to the mixture, which was then titrated with 0.1 N NaOH (y-value) to the phenolphtalein end point. The value of titratable acid groups present in the juice was calculated by dividing the y value by a sum of x and y and is presented as a % ratio.

7.3.5 Cloud stability

Transmission values of juice supernatants are an indicator of cloud stability (Baker and Bruemmer, 1969; Rouseff and Ting, 1986; Baker and Cameron, 1999). As an estimation of cloud loss and stability in the protein added juices, % T was determined in the supernatants. A UV-VIS Scanning Spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) was used to measure the transmission of the supernatant immediately after centrifugation of the juice-protein mixture. Control measurements were also taken for the single strength orange juice supernatant and the unheated protein supernatants. % T measurements were carried out at 650 nm using deionized H_2O as a

reference (Huggart et al. 1975). An increase in the value of transmission of the supernatant was considered an indication of loss of cloud stability and represented clarification of juice.

7.3.6 Analysis by capillary electrophoresis

Analysis of juice supernatants by capillary electrophoresis (CE) was performed to determine changes in the elution of protein present in the soluble phase of juices upon heating at various pH, temperature and time. Supernatants were diluted in a 1:1 ratio with sample buffer composed of 8 M urea, 10 mM dithiothreitol and 100 mM phosphate buffer at pH 8.0 and incubated for one hour at room temperature. Running buffer was prepared by dissolving 294 mg of trisodium citrate dihydrate and 25 mg of hydroxypropylmethylcellulose (HPMC) in 37.5 ml of 8 M urea. HPMC was added to prevent binding of the charged proteins to the walls of the fused silica capillaries. The sample's pH was adjusted to 3.00 with 2.5 M citric acid and brought to 50 ml volume (De Jong *et al*. 1993). Electrophoresis was conducted on a P/ACE System 5510 Beckman CE Unit (Beckman Coulter Inc, Gladesville, NSW, Australia). Protein separation was performed in a fused silica uncoated (50 μm internal diameter) capillary at 45C with an applied voltage of 25 kV and absorbances were recorded at a wavelength of 214 nm. Samples were injected with constant nitrogen pressure for 20 seconds and separated in 25 minutes. After each separation, the capillary was flushed with 1 M NaOH for one minute, rinsed with deionized H_2O for 3 minutes and reconditioned for 2 minutes with running buffer. Prior to storage the capillary was flushed for 5 minutes with 1 M HCl and rinsed for 5 minutes with deionized H_2O. Data were collected and processed with the P/ACE System 5000 series Gold Software.

7.3.7 Statistical analysis

All data were analyzed by the general linear model to determine the significance of pH, temperature and time effects. Significance was defined at $p < 0.05$. Linear and quadratic regressions were also tested for values of %T and uronic acid content. The SAS program (version 8.0, Cary, NC, USA) was used for all the calculations.

7.4 Results and Discussion

7.4.1 Changes in uronic acid content

The amount of pectin residual, determined as uronic acid content, in the non-heat treated orange juice supernatant was 1.38 mg/ml, after centrifugation. This value decreased upon addition of protein. Uronic acid content decreased by an average of 25% for the α-lac/juice samples, 21% for the β-lg/juice samples and 24% for the WPI/juice samples. However, these values showed no significant differences in the amount of residual pectin shown in the soluble phase as a function of protein type, pH, temperature or heating time. Statistical analysis did not determine any significant effect for pH and temperature, temperature and time or pH and time interactions, either.

Although there were no differences in the quantity of uronic acid left in the protein-added soluble phases, based on the given variables, these data suggested that the addition of protein, irrespective of protein type, caused a dramatic decrease in the amount of uronic acid when compared to the natural orange juice supernatant.

7.4.2 Titration of soluble phases

Titration was performed to evaluate changes in the charge of juice pectin during heating after protein-pectin interaction. Variations in charge, due to the deesterification of

the methylated carboxylic groups, would result in a change of titratable acid groups over time of heating. In the original juice pectin may be present in a wide distribution of charges and sizes (Baker, 1979; Kravtchenko *et al*., 1992). Not only the amount of pectin residual in the supernatant, but also the type of charges still present in the pectin backbone may have an effect on the polymer interactions with the proteins. For example, the interaction of protein with a pectin molecule containing a high level of methoxyl groups, may be enhanced due to lower charge density of the pectin.

Values of acidity for each supernatant pH, temperature and heating time are depicted in Figure 7.1 for juice containing α-lac, β-lg or WPI. Within the same pH, temperature and time did not show an effect on the titration of acid groups. These results indicated that there were no significant changes in the charge density of the soluble pectin molecules, when in the presence of protein, over heating time and temperature.

Although no significant changes in the amount of uronic acid and titratable acidity were seen in the soluble fractions over time and temperature of heating in all cases considered in our study, the data suggested that changes in protein type may have affected titratable acid groups in the protein/juice samples at each pH. Figure 7.1 shows the contribution of protein inducing changes in titration. WPI required lower levels of alkali at pH 3.0 than α-lac or β-lg (Figure 7.1 C); however, WPI and β-lg exhibited similar values at pH 4.0 and 5.0 (Figure 7.1 B and C). These effects may be in part attributed to the buffering capacity of each protein, indicating that protein type, rather than pectin charge, had an effect on the titratable acid groups of the juice supernatants over heating time or temperature.

7.4.3 Cloud stability

%T of the soluble phases was used to determine differences in stability of juice as a function of the type of protein added. Table 7.1 summarizes results of statistical analysis of %T values. For all protein treatments, heating time had no significant effect on stability.

For each protein-juice mix, pH had significant effect in varying %T in the supernatants (Table 7.1). Figure 7.2 shows the effect of pH on the %T of juice supernatant with added α-lac, β-lg and WPI. For β-lg and WPI, as the pH decreases, so does the %T, with the lowest value of %T shown at pH 3.0. %T values for α-lac at pH 3.0 were significantly lower than pH 4.0 and 5.0. Lower %T values in the soluble phases at low pH, suggested the presence of more soluble aggregates at this pH. The isoelectric point (pI) of pectin is around 3.2 and the whey proteins have a pI around 5.0 (β-lg~5.2, α-lac~4.2-4.5). At pH 3.0, pectin possesses a near neutral or positive charge, while the whey proteins have a positive charge, giving rise to electrostatic interactions that may provide the driving force to stabilize particles in solution. As the net charge of the proteins tend towards zero (as the pH increases towards the pI), the hydration capacity of the proteins decreases. A decrease in repulsive forces and in hydration shell would favor protein-protein interactions (Mangino 1984; Robin et al. 1993).

Figure 7.3 illustrates the effect of temperature on changes in %T of the juice-protein supernatant after heating for 30 min at pH 3.0. Temperature effects were shown to be significant for every protein treatment (Table 7.1), with %T decreasing with increasing temperature. The formation of soluble aggregates in juice samples was more extensive at higher heating temperatures. The presence of pectin and other orange juice constituents

such as organic/phenolic compounds and salts, may provide a protective effect, leading to increased interactions at elevated temperatures. Thus, the %T values would be lower in the soluble phase upon increasing heating temperature.

Upon graphing the statistical data, an orderly representation was shown for the pH * temperature interaction, therefore only the main effects of Table 7.1 will be discussed.

7.4.4 Stability of orange juice with α-lactalbumin addition

Figure 7.4 illustrates the capillary electropherograms of supernatants of heated protein at pH 3.0 and heated juice-protein at pH 3.0 and 4.0. Analysis of supernatants at pH 5.0 showed no significant peaks, again leading evidence to formation of insoluble complexes at this pH. Minimum transmission of the sample containing α-lac was reached at pH 3.0, 85C and heating for 30 min.

When the heated solution of α-lac was analyzed, the pure fraction eluted as a single peak (Figure 7.4 A, electropherogram 1). This elution pattern of the heated protein suggested that aggregation of α-lac did not occur and is in agreement with Gezimati *et al*. (1997). When analyzing the supernatant of heated juice with added proteins, capillary electropherograms displayed a wide peak and a larger distribution of charge/mass ratios (Figure 7.4 A, electropherogram 2). CE profiles for the mixtures of juice with added α-lac at pH 3.0 and 4.0 showed greater separation of protein fractions, with smaller peaks at pH 4.0 and a lower amount of soluble complexes present in supernatants than at pH 3.0 (Figure 7.4 A, electropherogram 3). These differences can be attributed to the composition of the supernatants, especially the presence of pectin and the difference in ionic strength. The broader, larger peaks at pH 3.0 and 85C suggested better retention of

soluble complexes and greater colloidal stability of α-lac than at the other pH or temperature, and was in agreement with the %T data presented earlier.

7.4.5 Stability of orange juice with β–lactoglobulin addition

Similar to the addition of α-lac, the lowest values of %T were shown for β-lg added juice at pH 3.0 and 85C. However, the electropherogram at pH 3.0, 85C and 30 min heating for the mixture containing β-lg fraction showed a larger number of peaks and a longer elution profile (Figure 7.4 B, electropherogram 2) than the supernatant of juice containing α-lac (Figure 7.4 A, electropherogram 2). This indicated that the aggregates of β-lg have a wide range of charges, unlike that of the heated α-lac elution profile.

The intensity of the peaks in the control and mixed soluble phases of the β-lg fractions indicated that protein amounts present remained relatively consistent in both environments at pH 3.0 (Figure 7.4 B, electropherograms 1 and 2). It has been shown that β-lg, when heated alone, is more thermally stable at high temperatures and low pH, being attributed to an increase of hydrogen bonding (Kella and Kinsella 1988).

The capillary electropherogram of protein-juice supernatant at pH 4.0 showed fewer peaks and less protein than the supernatant at pH 3.0 (Figure 7.4 B, electropherograms 3 and 4). These results indicated that an increase in pH caused more instability in the juice, as also shown with α-lac. However, β-lg displayed higher retention of protein-juice complexes at pH 3.0 than α-lac (Figure 7.4 A and B, electropherogram 2). This may be related to the pH-dependent structural changes of whey proteins where β-lg dissociates into monomers at pH 3.0 and interactions occur by a different mechanism than for α-lac (Kilara and Harwalker 1996; Verheul *et al*. 1999; Schokker *et al*. 2000).

7.4.6 Stability of orange juice with WPI addition

% T values of WPI also followed that seen for α-lac and β-lg samples, with a minimum transmission at pH 3.0 and 85C. The electropherogram for this protein in the absence of orange juice depicted several peaks at this temperature and pH (Figure 7.4 C, electropherogram 1). The protein-juice supernatant at pH 3.0 showed a longer elution profile eluting with a wide charge/mass distribution of protein present in the soluble phases (Figure 7.4 C, electropherogram 2). Similar to the β-lg system, the electropherogram at pH 3.0 showed higher amounts of protein in the supernatant of the heated WPI-juice mixture than pH 4.0 (Figure 7.4 C, electropherogram 3 and 4).

The behavior of WPI added juices was similar to that of β-lg added juices because of the larger amounts of protein retention seen in the electropherograms of protein-juice mixture at pH 3.0 (Figure 7.4 C, electropherogram 2) than retention in the protein-juice mixture for α-lac (Figure 7.4 A, electropherogram 2). This indicated that interactions occurred to a larger extent in soluble β-lg and WPI mixtures than in the soluble α-lac mixtures, probably due to increased interactions of β-lg. This is in agreement with previous studies suggesting thiol-disulfide interchange reactions, hydrophobic interactions and hydrogen bonding of β-lg are primarily responsible for the aggregation behavior of β-lg, in which structure can be changed extensively due to the pH and ionic strength of the environment (Phillips *et al*. 1994; Monahan *et al*. 1995; Verheul *et al*. 1999; Schokker *et al*. 2000).

7.5 Conclusions

Results show that changes in uronic acid content and DE values in the supernatant, upon addition of α-lactalbumin, β-lactoglobulin and WPI, did not change

significantly over time or temperature. Statistical analysis demonstrated that the interactions of protein with pectin in the juice system, as well as other juice components, have lead to differences in % transmission and electropherogram patterns, which have been used to characterize the destabilization of the soluble phases. These differences were caused by pH and temperature, which may have caused chemical changes of the pectin/protein aggregates. Capillary electrophoresis and % transmittance showed the optimum stability of the system to be at pH 3.0 and 85C, indicating more stable protein-protein and protein-pectin complexes under these conditions. Capillary electrophoresis of the supernatants containing β-lg and WPI exhibited higher retention than α-lac supernatants, probably due to specific structural changes occurring in the β-lg molecule at low pH values.

7.6 Acknowledgements

LiHua Wang (University of Georgia, Department of Statistics) is kindly acknowledged for her support during statistical analysis.

Table 7.1: Statistical Analysis (general linear models) of main effects (pH, temperature and time) and interactions for % Transmission of supernatants of juice with added α-lac, β-lg and WPI.

Variable	p value* α-lac	Order of % T α-lac	p value* β-lg	Order of % T β-lg	p value* WPI	Order of % T WPI
pH	0.0015	pH 3<4=5	0.0078	pH 3<4<5	0.0022	pH 3<4<5
Temperature	0.0400	65=75>85	0.0023	65>75>85	0.0001	65>75>85
Time	ns	Ns	ns	ns	ns	ns
pH x Temperature	0.017	**	0.0020	**	0.0001	**
pH x Time	ns	Ns	ns	ns	ns	ns
Temperature x Time	ns	Ns	ns	ns	ns	ns

* Significance is shown when $p<0.05$, no significance is shown as ns. DF of model = 18.

** Interaction of pH x Temperature expressed an orderly representation.

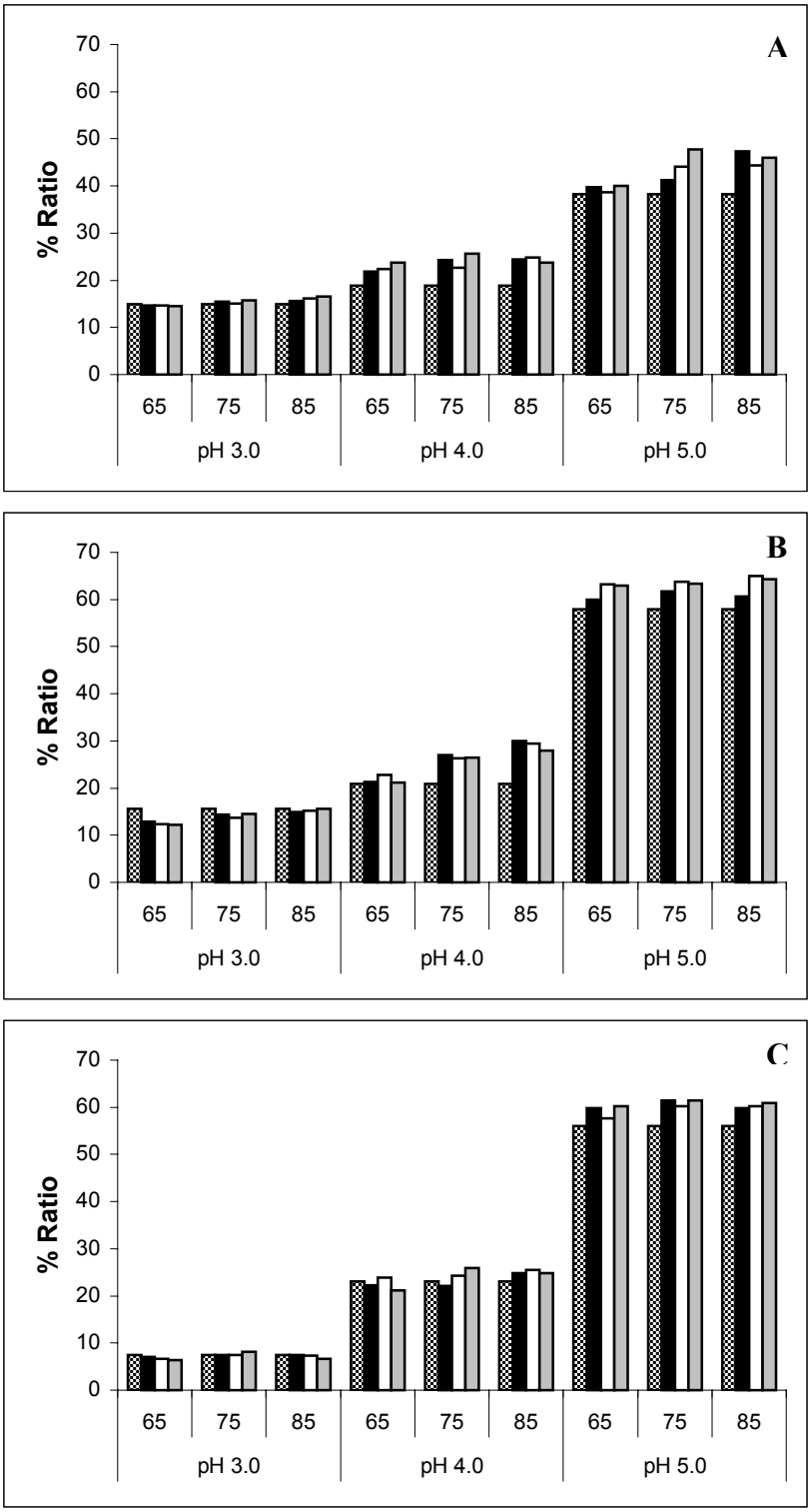

Figure 7.1: Effect of pH, temperature and heating time on acidity (calculated as % ratio, see methods) of supernatants of orange juice fortified with (A) α-lactalbumin; (B) β-lactoglobulin; (C) WPI; with different heating times. Legend: control, no heat applied (checkered bars); 10 min (black); 20 min (white); 30 min (gray).

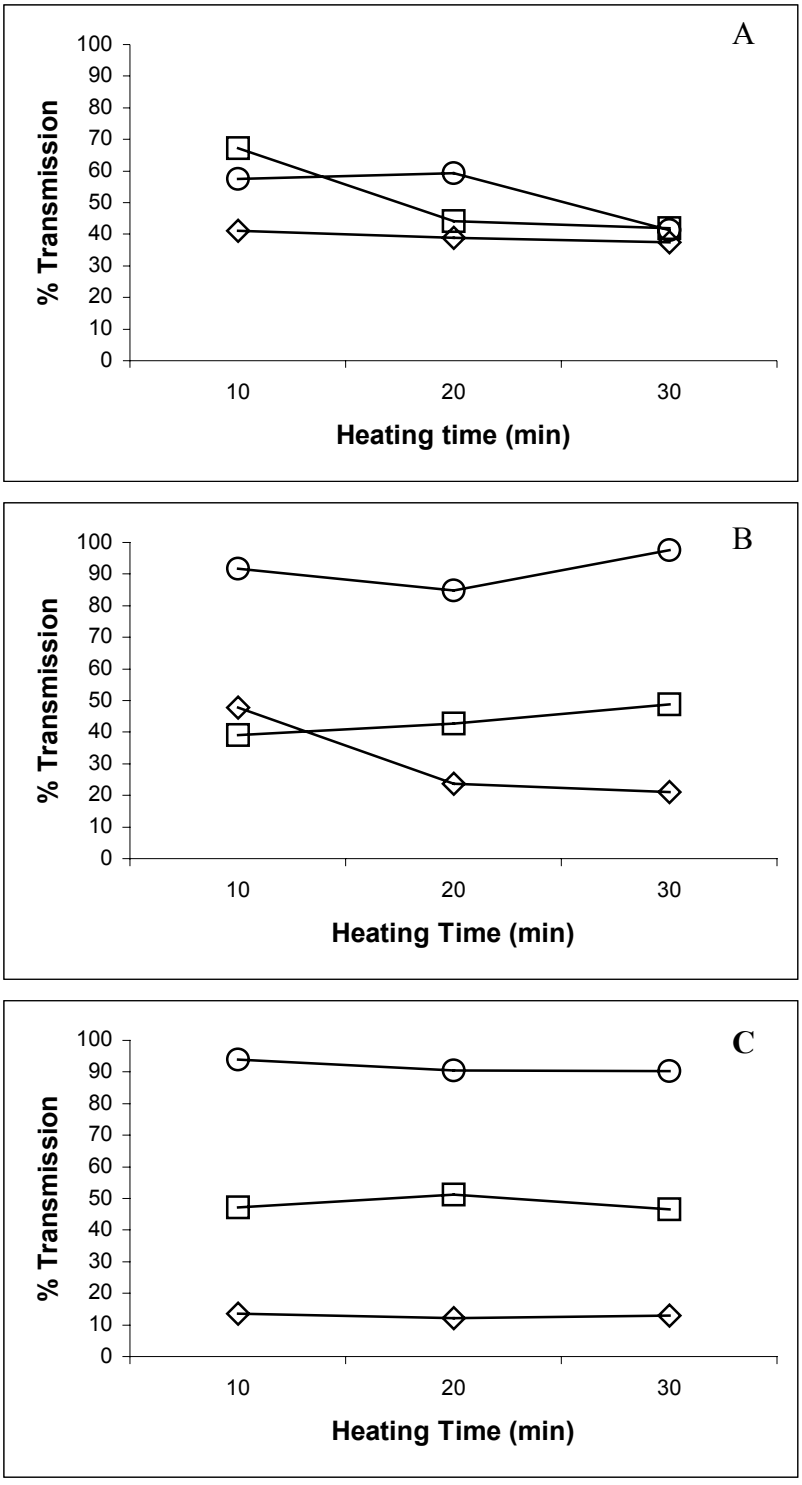

Figure 7.2: Effect of pH and heating time on % Transmission of supernatants of orange juice fortified with (A) α-lactalbumin; (B) β-lactoglobulin; (C) WPI at 85C. (◊) pH 3.0; (□) pH 4.0; (○) pH 5.0.

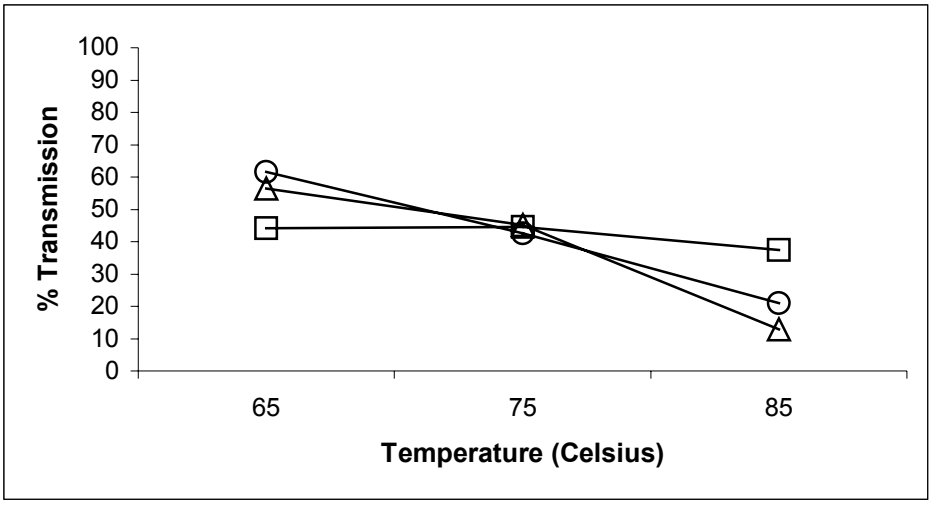

Figure 7.3: Effect of temperature on % transmission of supernatants obtained from protein-juice mixtures at pH 3.0 after heating for 30 min. (□) α-lactalbumin; (○) β-lactoglobulin; (Δ) WPI.

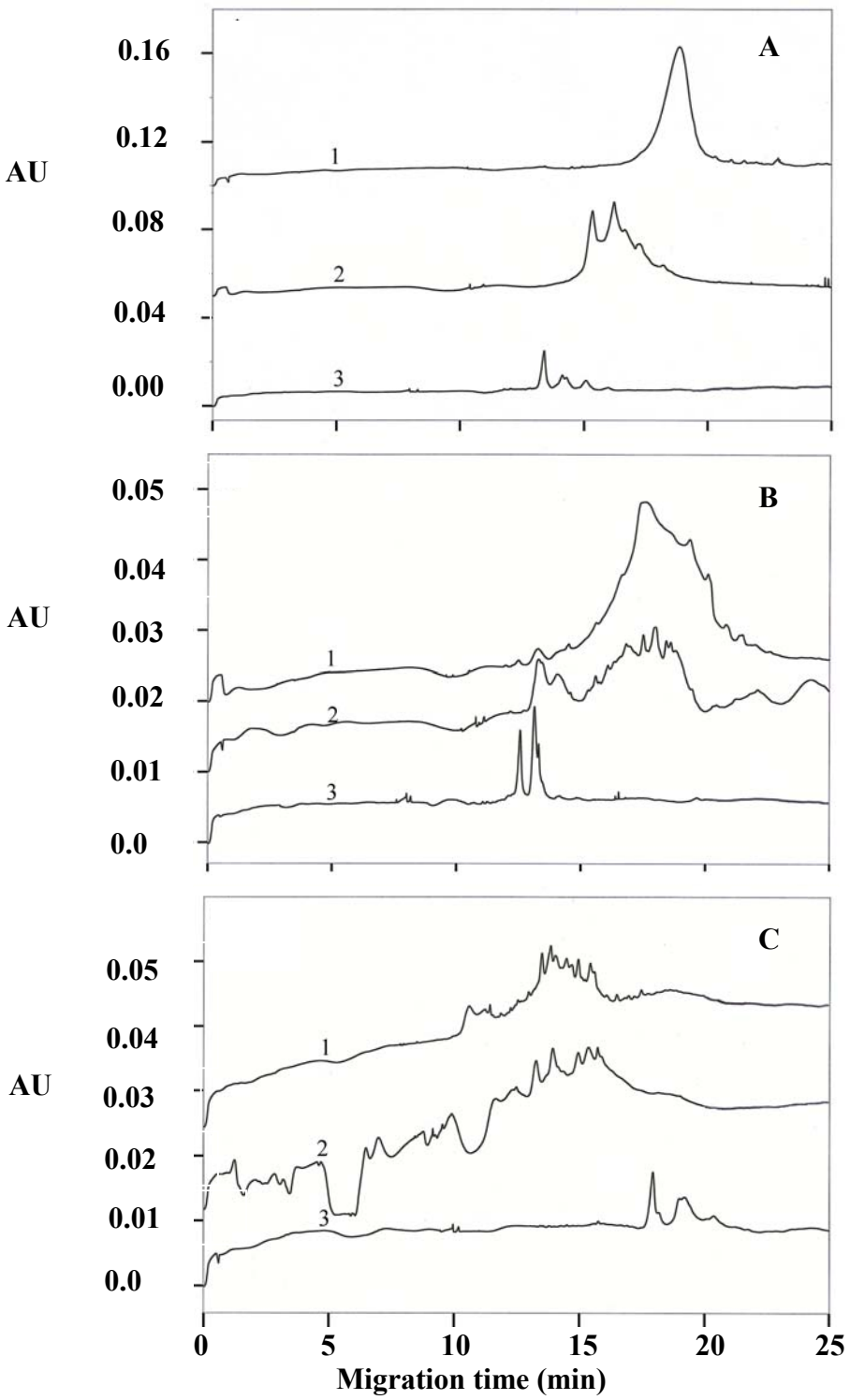

Figure 7.4: Capillary electropherograms of supernatants of orange juice fortified with (A) α-lactalbumin; (B) β-lactoglobulin; (C) WPI, after heating for 30 min at 85C. Electropherogram 1) supernatant of heated protein (no juice added) at pH 3.0; 2) Supernatant of juice with added protein at pH 3.0; 3) Supernatant of juice with added protein at pH 4.0. Absorbance measured at 214 nm.

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CHAPTER 8

CONCLUSIONS

This research focused on protein interactions during heat treatment. We studied the effects of various temperatures on the heat-induced aggregation of whey protein isolate, looked at the interactions between β-lactoglobulin and pectins and we investigated the effect of different types of whey protein isolates on stability of Valencia orange juice.

The effect of heating temperature and time on the aggregation behavior of whey proteins was examined. Temperature had an effect on the formation of aggregates which were as large as $1*10^6$ – $1*10^7$ g/mol after 5-60 min heating at 65-90°C. Thermal aggregation of whey protein isolate (WPI) heated at neutral pH occurred without the formation of intermediate sized aggregates and heating time did not seem to have a significant effect on the molecular weight of the soluble aggregate. Increasing concentration of protein caused an increase in the number of aggregates produced, as well an increase in the size of the aggregates. When heating 10% WPI solutions, residual native proteins decreased while soluble aggregates increased, and the hydrodynamic diameters of the aggregates varied with heating temperature. Electrophoretic analysis of the soluble aggregate fractions, collected by preparative size exclusion chromatography, showed a ratio of about 2.0 β-lactoglobulin/α-lactalbumin, regardless of temperature or time of heating.

Pure β-lactoglobulin was isolated from WPI and used in combination with pectin (both high methoxyl pectin (HMP) and a pectinesterase-modified pectin (mHMP)) to understand the interaction mechanisms of β-lactoglobulin with pectins at low pH. Dynamic light scattering of the soluble aggregates produced in mixtures of β-lactoglobulin with HMP showed decreasing hydrodynamic diameters with increasing temperature, probably due to separation of the large, more unstable aggregates at high temperature. Upon heating, HMP increased the aggregation of β-lactoglobulin. On the other hand, the addition of a plant pectinesterase modified pectin to β-lactoglobulin caused the formation of a precipitate and a clear supernatant. Resuspension of this pellet at pH 6.0 indicated the reversibility to this complex in the presence of mHMP. This behavior was comparable to that of pectins containing a very low number of methoxyl groups. The differences in the aggregation behavior shown in β-lactoglobulin systems with added HMP and mHMP can be attributed to the different charge densities possessed by the two pectins employed in the study.

In the final chapter we summarize our investigations on the role of different whey protein isolates upon addition to Valencia orange juice. It was hypothesized that the presence of a fraction rich in α-lactalbumin, rich in β-lactoglobulin or whey protein isolate in the juice would affect the stability of the mixture. In fact, the orange juice turbidity was dependent upon pH, temperature of heating and the type of protein added. Uronic acid and the charge of the pectins did not show a significant change with pH or by increasing heating temperature. Stability, determined by the lowest values of transmission of the soluble mix, was found for juices at pH 3.0, heated at 85°C. Capillary electrophoresis results suggested that more extensive protein-pectin interactions occurred

with β-lactoglobulin and WPI than with α-lactalbumin at this pH and temperature, because of opposite net charges of the polymers.

These studies demonstrated the importance of studying not only the biopolymer/biopolymer interactions, but also the effects of thermal processing on aggregation behavior of whey proteins and the complex formation with pectin Studies need to be carried out not only on pure model systems, but also on systems containing commercially available fractions (such as protein isolates) and on real systems. By examining its thermal aggregation behavior, further insight on the interaction of β-lactoglobulin and pectins in heated systems at low pH were elucidated. Results from such studies will lead to a better understanding of how to control the stability of more complex food systems. For example, our novel investigation on the stability of orange juice with added whey protein will lead to the determination of the optimal whey protein fraction to be added to orange juice creating a value-added ingredient for orange juice.