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Whey Proteins Cross-linked by Transglutaminase or Glycated with Maltodextrin: Physicochemical Bases of the Improved Heat Stability

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Whey Proteins Cross-linked by Transglutaminase or Glycated with Maltodextrin: Physicochemical Bases of the Improved Heat Stability

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DEDICATION

This dissertation is dedicated to my parents Xitang Wang and Jie Yang, who gave me life and led me to a science world, enabling such a discovery of science to take place today.

And also to my beloved husband, Hua Ouyang, who supported me, encouraged me and comforted me in this separated period of time.

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ABSTRACT

Whey protein, as a byproduct in cheese manufacturing, is an ideal source for producing ready-to-drink protein beverages for different market demands, attributed to its high content of essential amino acids and versatile functionalities, bland flavor and good digestibility. Whey protein is a mixture of proteins, which can be categorized into whey protein concentrate (WPC) with a protein content of 50~80% and whey protein isolate (WPI) with a protein content higher than 90%, depending on different separation techniques. Thermal processing is required to ensure microbiological safety and quality of dairy products, leading to denaturation of whey proteins, especially at pH around its isoelectric point. Denaturation facilitates the aggregation of whey proteins that involves a number of colloidal interactions such as hydrophobic, electrostatic, hydrogen bonding interactions and covalent disulfide bonds, which are significantly influenced by pH, concentration, temperature, ionic strength, and the presence of co-solutes. Based on the understanding of physic forces, the thermal stability of protein can be enhanced via two approaches, which are restricting the denaturation and creating repulsive forces. A combination of preheating and microbial transglutaminase cross-linking can improve the thermal stability of whey proteins at neutral pH after second heating at 80 ℃ or 138 ℃ in the presence of different concentration of NaCl. Whey proteins glycated with saccharides creates a structure with internal whey protein core and an external saccharide shell. Further aggregation of protein molecules is suppressed because the approaching of protein molecules has to overcome the saccharide shell. Glycation with saccharides is markedly affected by reaction conditions such as pH (powder acidity for dry base reaction), temperature, time, protein:saccharide ratio and types of protein and saccharide,

etc. Addition of monosaccharide such as D-glucose and disaccharide such as sucrose in the whey protein aqueous system also improve the thermal stability of protein. When combined with optimized glycation, whey proteins can be stabilized at pH ranging from 4 to 7.

Keywords: whey proteins, thermal stability, preheating, transglutaminase, glycation, co-solutes

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1.1 Introduction

1.1.1 Perspective of shelf-stable ready-to-drink beverages with whey proteins

Shelf-stable beverages and refrigerated beverages belong to ready-to-drink beverages, which offer the advantages of convenience and portability for consumers. The food market of shelf-stable beverages is expanding rapidly as the products offer the ease of distribution and storage. Whey proteins are preferred as a source for manufacturing ready-to-drink protein beverage because of their excellent nutritional quality and versatile functional properties (De Wit, 1998; Ha & Zemel, 2003). Ready-to-drink protein beverages containing whey proteins are developed for different market demands, such as sports beverages, medical and therapeutic nutritional beverages, milk-based infant formulas, and smoothies type beverages (Rittmanic, 2006).

Beverages formulated with whey proteins are generally adjusted to a pH from 2.8 to 3.5, showing a high clarity at a high concentration of protein. Thermally processed acidic beverages are usually filled while hot into containers (hot-fill process) for shelf-stable beverages. The hot liquid product can sterilize the container which can withstand filling temperature, and inactivate molds and yeasts (LaClair & Etzel, 2009; Rittmanic, 2006). Protein beverages at pH between 4.6 and 7.5 must be thermally sterilized via ultra-high-temperature process or retort process for shelf-stable products (Rittmanic, 2006). Beverages containing > 10 g protein and < 18 g sugar per 240 mL can be labeled as “high protein” and “reduced sugar” products (Etzel, 2004).

Furthermore, a large amount of sugar is used to formulate acidic beverages to mask the acid taste and may cause health problems, such as teeth erosion (Mettler, Rusch,

& Colombani, 2006), increased body fat, blood lipid titers, insulin secretion and appetite (Etzel, 2004). Protein beverages with neutral acidity can overcome the drawbacks of acidic beverages. However, whey proteins are not appropriate as a source for producing neutral beverages as they are not heat stable, forming gels or aggregates/precipitates with as little as 2-3% proteins under thermal process conditions unless a stabilizing system is used (Mleko, 1999). Technologies stabilizing whey proteins during heating can expand the application of whey proteins as an ingredient in shelf-stable beverages.

1.1.2 Types and composition of whey proteins ingredients

Whey from cheese manufacturing is commonly used as a source of manufacturing protein ingredients, and the composition in cheese whey depends on cheese making processes (Damodaran & Paraf, 1997). Whey protein ingredients are produced by removing non-protein compounds such as lipids, minerals, and lactose to increase the protein content. The most common whey protein ingredients are whey protein concentrate (WPC), containing 30-80% protein, and whey protein isolate (WPI), containing >90% protein (Foegeding, Davis, Doucet, & McGuffey, 2002). Methods of separation and isolation of whey proteins determine the composition of WPC and WPI, which may affect suitability for a particular application. WPCs, manufactured by membrane separation, such as microfiltration, ultrafiltration and nanofiltration processes, are widely used as ingredients for functions such as gelation, foaming and emulsifying. WPIs produced by ion-exchange and membrane filtration show compositional differences in mineral and glycomacropeptide contents, exhibiting better functional properties than WPCs (Fox & McSweeney, 2003; Fuquay, Fox, & McSweeney, 2011; Rittmanic, 2006).

Proteins in WPC and WPI are a mixture comprised primarily of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) and secondary bovine serum albumin (BSA), immunoglobulin G (IgG), and others (Table 1-1) (Thompson, Boland, & Singh, 2009). β-Lg (~50%) and α-La (~20%) consist approximately 70-80% of total whey proteins and thus are responsible for many physicochemical properties of whey protein ingredients such as gelling, hydration, emulsifying and foaming. Although β-Lg has a number of hydrophobic residues it is soluble in water because most nonpolar amino acid residues are buried in the interior of protein and polar groups are on the surface, contributing a good solubility of whey proteins at pH away from isoelectric point (Cayot & Lorient, 1997).

1.1.3 Structures of whey proteins

β-Lg is the most abundant whey protein in bovine milk, accounting for 10% of total protein or ~50-60% of total whey proteins, depending on the process for isolation of whey proteins (Fuquay, Fox, & McSweeney, 2011). β-Lg has not been detected in the milk of humans, rodents or lagomorphs (Thompson, Boland, & Singh, 2009).

β-Lg has a molecular weight of 18.3 kDa, 162 amino acid residues, one free thiol group and two disulfide bonds (Damodaran & Paraf, 1997). The structure of β-Lg shows a high degree of organization, with 9 anti-parallel strands and 9 β-sheets forming a β-barrel. Each β-sheet has one hydrophobic side and a hydrophilic side, and a hydrophobic cavity is formed by aligning hydrophobic sides of β-sheets facing each other (Allen, 2010). β-Lg contains a large portion of β-sheets (50% of residues) and a small portion of α-helix (10-15%) and β turns (15-20%). There are ten known genetic variants of bovine

β-Lg, with the most abundant variants being β-Lg A and β-Lg B that differ by two amino acid residues, which are Asp64Gly and Val118Ala, respectively (Farrell Jr, Jimenez-Flores, Bleck, Brown, Butler, Creamer, et al., 2004).

The isoelectric point (pI) of β-Lg is around pH 5.2-5.35 (Farrell Jr, et al., 2004; Godovac-Zimmermann & others, 1996). The quaternary structure of protein varies as a result of a delicate balance among hydrophobic, electrostatic and hydrogen-bond interactions, depending on the pH, temperature and ionic strength (Sakurai & Goto, 2002; Sakurai, Oobatake, & Goto, 2001). At pH 5.5-7.5, β-Lg exists as a dimer consisting of two stacked cones that are the prevalent form at physiological conditions in milk (Damodaran & Paraf, 1997; Fox & McSweeney, 2003). At pH 3-5, β-Lg dimers associate to form octomers. β-Lg exists in the monomer form at pH below 3, which resembles that of a cone with a hydrophobic pocket being capable of binding small hydrophobic ligands such as vitamin A and fatty acids (Damodaran & Paraf, 1997; Fox & McSweeney, 2003; Kontopidis, Holt, & Sawyer, 2004). The compact structure of β-Lg, with 9 β-sheets and 2 disulfide bridges, is resistant to complete proteolysis by digestive proteases. At pH >9, the β-Lg molecule is irreversibly denatured (Damodaran & Paraf, 1997).

The denaturation temperature of β-Lg is ~78 ℃, with a loss of solubility at 78-82 ℃ caused by denaturation and aggregation (Allen, 2010). Protein unfolding exposes free thiol groups and hydrophobic residues, leading to formation of a variety of covalent and hydrophobic intermolecular associations and also the homo- and heteropolymeric disulfide-bridges (Thompson, Boland, & Singh, 2009).

α-La is a smaller molecule than β-Lg. α-La has a molecular mass of 14.2 kDa, 123 amino acid residues, and 4 disulfide bridges (Damodaran & Paraf, 1997). Unlike β-

Lg, there is no free thiol groups in α-La. α-La contains a high level of tryptophan and 1.9% of sulfur and is a major protein in human milk. α-La is an elliptical shaped compact protein made up of two lobes: the α-lobe contains 3 α-helices and two short 3_{10}-helices, while a small three-stranded β-sheet and a short 3_{10}-helix make up the β-lobe (Pike, Brew, & Acharya, 1996). α-La exhibits high similarity with lysozyme (Kontopidis, Holt, & Sawyer, 2004). The pI of α-la is around pH 4.5~4.8. Due to the presence of disulfide bonds and its compact structure, α-La is considered to be heat stable with a reversible denaturation temperature of 62 ℃ (Bryant & McClements, 1998; Fox & McSweeney, 2003).

BSA is composed of 583 amino acid residues with an average molecular weight of 66 kDa and in a multi-domain structure with complex ligand-binding specificities. BSA has 17 disulfide bonds and one free sulfhydryl group (Considine, Patel, Anema, Singh, & Creamer, 2007). Serum albumin is found in both blood serum and in the milk of mammals. BSA enter the milk by passive diffusion from blood streams rather than being produced by the mammary gland like other whey proteins (Allen, 2010). BSA is also an elliptically shaped protein and functions as a transporter of hydrophobic molecules such as fatty acids across membranes, but exists at a much lower quantity in milk than in blood (Thompson, Boland, & Singh, 2009). BSA accounts for approximately 5% of the protein in cheese whey and thereby does not have a significant effect on functional properties of whey protein ingredients (Allen, 2010).

Although the three dimensional structure of BSA has not been determined, it shares 75% sequence identity with human serum albumin. The secondary structures of BSA are composed of 76% helix, 10% turn and 23% extended chain, and no β-sheet

(Considine, Patel, Anema, Singh, & Creamer, 2007), with several loops connecting the two sub-domains A and B in the each domain (Fox & McSweeney, 2003). Reversible changes in the conformation of BSA occur between 42 and 50 ℃ (Considine, Patel, Anema, Singh, & Creamer, 2007). When temperature reaches its denaturation temperature of ~64 ℃, both unfolding of BSA and the free thiol group catalyze aggregation (Considine, Patel, Anema, Singh, & Creamer, 2007). Gelation of BSA might occur when heated above 70 ℃ if the concentration of disulfide bonds is sufficiently high (Considine, Patel, Anema, Singh, & Creamer, 2007).

In bovine milk, immunoglobulin account for approximately 2% of total milk protein and 10% of whey proteins with molecular weight varies (Christen & Smith, 2000). IgG, in particular IgG1 with a molecular weight of 161 kDa, is the predominant species of immunoglobulins. IgG contains two heavy chains and two light chains and has predominantly β-sheet structures. The two heavy chains are linked each other by disulfide bonds, and a heavy chain and a light chain are linked via disulfide bonds (Thompson, Boland, & Singh, 2009).

1.2 Physical and chemical interactions relevant to the stability of whey proteins in aqueous solutions

1.2.1 Stability of colloidal particles interpreted by inter-particle interactions

Whey proteins are colloidal particles, and understanding the unique structure and interaction of whey proteins is essential to the development and application of new protein ingredients. A generalized illustration is given in Figure 1-1 to correlate the

stability of two ideal colloidal particles (hard spheres) with the overall interaction energy (V) between them (Walstra, 2002). The V needed to bring two particles into contact can be either positive or negative, resulting from repulsive or attractive inter-particle interactions, respectively. The V is always positive at very close distance (< 0.5 nm) because hard spheres cannot be compressed, but varies at longer separation distance. When the V is always positive (top dashed curve), the dispersion is stable against aggregation. Whereas, the always negative V (bottom dashed curve) indicates the irreversible aggregation of particles being separated at a distance corresponding to the primary minimum. The example (solid curve) between these two extreme cases shows the significance of both attractive and repulsive forces, and the possibility of irreversible (point A) or reversible (point C) aggregation depends on the magnitude of energy barrier (point B) (Walstra, 2002).

The stability and aggregation of colloidal particles in the aspects of thermodynamics and kinetics has been described using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which is the first useful theory that takes into account the combined effect of van der Waals attraction and electrostatic repulsion between molecules in solution (De Young, Fink, & Dill, 1993). For charged particles, an increase in salt concentration results in stronger attraction between particles and faster aggregation since the free energy barrier is reduced. Whey proteins are more complicated than hard spheres because several molecular interactions (Table 1-2) are significant to their stability, discussed below.

1.2.2 Attractive interaction forces

Hydrophobic interactions. Hydrophobic interactions describe the aggregation tendency of nonpolar substances to minimize the contact with water. When a non-polar molecule is introduced in water, hydrogen bonds between water molecules are interrupted to cause the rearrangement of water molecules around the non-polar molecule, and this changes both enthalpy and entropy of the system (Bryant & McClements, 1998). The aggregation of non-polar molecules in aqueous systems is thermodynamically favored because of the significance in reducing enthalpy (Dill, 1990; Evans & Wennerström, 1999). Hydrophobic interactions are influenced by the structure of the non-polar molecules and external factors such as temperature. The hydrophobic interactions between whey proteins are stronger at a higher temperature in the range from 0 to ~60 ℃, reach the maximum at about 60-70 ℃, and are weakened upon further increases in temperature (Bryant & McClements, 1998).

Hydrogen bonds. A hydrogen bond is formed between the electron-rich and electron-depleted portions within a molecular or from two separate polar molecules. Electrostatic, van der Waals and steric interactions can impact hydrogen bond formation (Dill, 1990). Intermolecular hydrogen bonding is responsible for the high boiling point of water, whereas intra-molecular hydrogen bonding partly contributes to the secondary, tertiary and quaternary structures of proteins (Damodaran, 2008). Hydrogen bonds are usually not to be considered as a major force in determining the aggregation of globular protein, however, they are responsible for many unique properties of water and also for stabilizing the structures once formed (Kinsella & Whitehead, 1989). The actual strength

of a particular hydrogen bond depends on the electronegativity and orientation of the donor and the acceptor groups (Baker & Hubbard, 1984). Environmental factors such as pH and ionic strength in the system thus may influence on the strength of hydrogen bonding and heating reduces the number of hydrogen bonds because of the increased molecular mobility at higher temperatures (Tang, Pikal, & Taylor, 2002).

van der Waals forces. The inter-protein van der Waals forces in aqueous systems are always attractive and are contributed by dipole-dipole, dipole-induced dipole, and induced dipole-induced dipole interactions (Walstra, 2002). The strength of van der Waals interactions decays with intermolecular distance. Changes in van der Waals forces during protein denaturation, from folded to unfolded state, is too little to be considered in determining the conformational stability of proteins in solution (Bryant & McClements, 1998; Damodaran, 2008; Walstra, 2002).

Disulfide bonds. Disulfide bonds are formed between amino acids containing thiol group such as cysteine, which is found in whey proteins (Shimada & Cheftel, 1989). Both free thiol groups and disulfide bonds exist in the interior of native whey proteins in the folded structure thereby are not contributing to inter-protein interactions. Unfolding of whey proteins exposes the embedded thiol groups that become available to form inter- and intra-molecular disulfide bonds as long as the thiol groups are brought to close vicinity (Bryant & McClements, 1998; Damodaran, 2008; Walstra, 2002). The strength of disulfide bonds is independent on pH and ionic strength, but can be reduced at extreme high temperatures attributed by the disruption of disulfide bonds and oxidation of cysteine (Betz, 1993; Bryant & McClements, 1998).

1.2.3 Repulsive interaction forces

Electrostatic interactions. Electrostatic interactions can be attractive or repulsive depending on the types of surface charges (Bryant & McClements, 1998). The strength and effective distance of electrostatic interactions vary with the pH and ionic strength (Bryant & McClements, 1998; Walstra, 2002). At the isoelectric point (pI) of whey proteins (Table 1-1), the net charge of protein molecules is zero. At a pH away from pI, protein molecules are either positively (below pI) or negatively (above pI) charged (Bryant & McClements, 1998). The electrostatic interactions are affected by the ionic strength due to electrostatic screening by electrolytes. The strength of electrostatic interactions between charged species increases with the increase of temperature because of the entropic nature of the interactions (Bryant & McClements, 1998; Damodaran, 2008; Walstra, 2002).

Steric repulsion. Steric repulsion is generated due to overlap of electron clouds of molecules. Steric repulsion is very significant in protein conformation as it reflects how close the protein molecules could be packed together. The strength of steric repulsion is indirectly influenced by the pH, ionic strength, and temperature (Bryant & McClements, 1998; Walstra, 2002). The expansion of the chains on the surface of molecules results in an increased steric repulsion at low ionic strength caused by the reduced Debye-Hückel screening of the proteins charges at lower ionic strength (Belfort & Lee, 1991). The intensity of steric interactions showed a decrease at pH around pI, which is caused by the reduced net charge density in the absorbed protein molecules (Belfort & Lee, 1991).

Hydration interactions. Hydration interactions are the result of the energy required to break hydrogen bonds between the molecules and the water molecules in its immediate vicinity (Besseling, 1997). The strength of hydration interactions depends on the degree of protein hydration, which is influenced by pH and ionic strength (Bryant & McClements, 1998).

1.3 Heat stability of whey proteins

Food processing is used to ensure food safety, extend shelf-life, or modify functionality of ingredients. This may involve temperature, pressure, ionic strength, and pH, all of which can result in the denaturation and aggregation of whey proteins (Jelen, Rattray, & Fox, 1995). Effects of heating are discussed in this section.

1.3.1 Heat-induced denaturation and aggregation of whey proteins

Heating strongly affects the protein structures and thereby a variety of protein functionality of commercial importance. The undesirable changes include the (turbid) appearance of protein-containing solutions, precipitation, and loss of flowability (gelation) due to aggregation of denatured whey proteins during heating. Heat-induced denaturation and aggregation of whey proteins can be described by a kinetic model developed by Roefs and de Kruif (1994) at neutral pH and an intermediate temperature (65 ℃) based on a generalized free radical reaction scheme involving steps of initiation, propagation and termination (Roefs & Kruif, 1994). In general, a protein such as β-Lg initially reversibly dissociates from dimers into monomers, which undergo partial unfolding and expose a reactive thiol group. The reactive thiol group then reacts with a

disulfide bond, forming a new thiol to continue the reaction via disulfide interchange. The aggregation is terminated when thiol groups on reactive intermediates are consumed to form bigger structures (Ryan, Zhong, & Foegeding, 2013). However, non-covalent forces are not accounted for in this model. With additional consideration of physical forces such as hydrophobic attraction, the denaturation of β-Lg at neutral pH starts with a dissociation step of dimer into monomers, which occurs at a temperature of 30~50 ℃ (Sawyer, 1969). The conformational changes of monomers occur at a higher temperature around 78 ℃ to expose a reactive thiol group and increase thiol activity (Bryant & McClements, 1998; Zhu & Damodaran, 1994). Reactive monomers then interact via disulfide bonds to form non-native dimers, trimmers and other small aggregates, followed by formation of large aggregates through covalent and non-covalent interactions, corresponding to macroscopic gels or precipitates as end products (Ryan, Zhong, & Foegeding, 2013).

1.3.2 Factors impacting heat-induced denaturation and aggregation of whey proteins

Initial protein concentration. A series of studies on heat-induced denaturation of β-Lg showed that the protein initial concentration had a marked effect on the thermal denaturation and aggregation (Hoffmann, Sala, Olieman, & de Kruif, 1997; Le Bon, Nicolai, & Durand, 1999a). The average molecular mass and the radius of gyration of the heat-induced β-Lg aggregates increased with increasing initial concentration, which may be attributed to the shift of weight fraction of the aggregates towards higher molecular mass (Hoffmann, Sala, Olieman, & de Kruif, 1997; Le Bon, Nicolai, & Durand, 1999a).

The conversion rate of native to aggregated protein is proportional to the initial protein/monomer concentration (Elofsson, Dejmek, & Paulsson, 1996; Le Bon, Nicolai, & Durand, 1999b). A greater intrinsic viscosity was found for the sample with 11% protein than that with 8% protein after treatment for identical heating temperature and duration (Vardhanabhuti & Foegeding, 1999). Protein concentration showed no correlation with the changes in tertiary structure of the protein induced by heating but was correlated to the formation of multimeric species (Iametti, Cairoli, De Gregori, & Bonomi, 1995), with fewer dimers and trimmers when heated at a higher initial protein concentration (Hoffmann, Sala, Olieman, & de Kruif, 1997).

pH. The aggregation of whey proteins, especially β-Lg, is very sensitive to the system pH. The aggregation rate is higher at a pH closer to the isoelectric point of whey proteins (Verheul, Roefs, & de Kruif, 1998). β-Lg bears only positive charges at very acidic pH around 2-3 in contrast to its amphoteric behavior at neutral pH, resulting in a considerable different mechanism of aggregation from that at neutral pH (de la Fuente, Singh, & Hemar, 2002). A lower acidic pH results in an increase in denaturation temperature, with the maximum at pH 3-4 for β-Lg, due to the weakened intra-molecular electrostatic repulsion and enhanced conformational stability, which reduces denaturation/aggregation reaction rate. The properties of whey proteins aggregates formed at acidic pH have been studies for years (Aymard, Nicolai, Durand, & Clark, 1999; Ikeda & Morris, 2002; Schokker, Singh, Pinder, & Creamer, 2000). β-Lg with different initial concentrations heated at pH 2.5 and 80 ℃ for 0-180 min resulted in large aggregates whose dimension increased with increases in heating duration and ionic strength but was independent on protein concentration (Schokker, Singh, Pinder, &

Creamer, 2000). The aggregates formed at acidic pH mostly have a worn-like shape, which may be attributed to association of monomers with a larger aggregates at a fixed location on β-Lg molecules (Schokker, Singh, Pinder, & Creamer, 2000). The end-to-end aggregation is more likely than forming oligomers due to strong electrostatic repulsion at the acidic pH around pH 2-2.5 (Schokker, Singh, Pinder, & Creamer, 2000), which was observed by electron microscopy (Aymard, Nicolai, Durand, & Clark, 1999) and atomic force microscopy (Ikeda & Morris, 2002). The contribution of exchange reaction between the reactive thiol groups and disulfide bonds to the aggregation is negligible for the aggregates formed at pH 2-3 because the thiol groups are very stable at low pH (de la Fuente, Singh, & Hemar, 2002; Schokker, Singh, Pinder, & Creamer, 2000).

Unlike at very acidic pH values, the aggregation of whey proteins at around neutral pH involves the interchange of reactive thiol groups. The reactivity and the accessibility of thiol groups are higher between pH 7 and 8.5 (de la Fuente, Singh, & Hemar, 2002) . Proteins such as β-Lg undergo a conformational change and a refolding process that result in the exposure of responsible amino acid, Glu89 of β-Lg at pH 7.1 (Brownlow, Cabral, Cooper, Flower, Yewdall, Polikarpov, et al., 1997) and 8.2 (Qin, Bewley, Creamer, Baker, Baker, & Jameson, 1998), which is buried at pH 6.2. This conformational change accounts for the physical and chemical pH-dependent properties of β-Lg and has functional implications for the reversible binding and release of ligands (de la Fuente, Singh, & Hemar, 2002). At pH 8, the thiol group of β-Lg is more readily available for reaction since the pK_a of the cysteine is approximately 8. Conversely, proteins have to be heated or unfolded by other mechanisms to expose the thiol group at pH below 7 (de la Fuente, Singh, & Hemar, 2002). A study of β-Lg at 65 ℃ in a pH

range of 6.4 to 8.0 showed that intermolecular disulfide bonds played an important role in the formation of heat-induced β-Lg aggregation, whereas at pH 6.0, both physical forces and thiol/disulfide exchange reaction were involved in aggregation (Hoffmann, Sala, Olieman, & de Kruif, 1997; Hoffmann & van Mil, 1999). At pH 6.0, very large aggregates formed , which can be attributed to secondary, non-covalent interactions between primary, disulfide-linked aggregates (de la Fuente, Singh, & Hemar, 2002). Increase of pH results in an increase in the conversion rate of native β-Lg to aggregates and a decrease in the size of aggregates, however, at pH 6.4-6.8, the aggregates formed with a higher molecular weight and a more compact conformation and less negatively charged when compared with the aggregates formed at higher pH (de la Fuente, Singh, & Hemar, 2002; Hoffmann & van Mil, 1999). The decrease in size of aggregates with an increase of pH was attributed to the fact that a higher pH accelerates the dissociation and initiation reactions to form a large number of reactive intermediates in the early stages of the reaction (Hoffmann & van Mil, 1999). This also increases the probability of termination reaction and thus facilitates the formation of more but smaller disulfide-linked aggregates (de la Futente, Singh, & Hemar, 2002).

Temperature and heating time. Like reactions, the structures of aggregates can be controlled by the rate (temperature) and duration. The aggregation is completed in a shorter time at a higher temperature at neutral pH, because of the slower denaturation and reduced collision frequency of proteins at a lower temperature (Hoffmann, Sala, Olieman, & de Kruif, 1997; McSwiney, Singh, & Campanella, 1994; Zuniga, Tolkach, Kulozik, & Aguilera, 2010). A two-step process, a first-order denaturation reaction followed by a

second-order aggregation reaction, has been proposed to interpret the phenomena during heating β-Lg at various conditions (Verheul, Roefs, & de Kruif, 1998), with the aggregation step activated after reaching a certain extent of denaturation. The reaction rates in both steps are influenced by heating conditions, pH, and ionic strength of the system. The unfolding reaction is rate-limiting at a low temperature, at pH close to pI, and at a high ionic strength, whereas the aggregation is rate limiting at high temperature, a pH further from pI, and a low ionic strength (de la Fuente, Singh, & Hemar, 2002). The contribution of non-covalent interactions to β-Lg aggregation varies with temperature, with an increasing importance at temperature of 90-110 ℃ (Galani, 1999). When temperature exceeds 100 ℃, a reduced aggregate size and viscosity were observed, probably due to the decomposition of protein above 113 ℃ and peptide bond breakage above >140 ℃ (Photchanachai & Kitabatake, 2001).

Ionic strength. Ionic strength impacts the thermal stability of whey proteins by affecting solubility, denaturation, electrostatic interactions, and specific binding. The increased solubility of protein (salting-in) at low ionic strength and reduced solubility (salting-out) are well-known as the Hofmeister effects. The presence of NaCl at high concentrations can stabilize native protein conformation and reduce the denaturation rate, whereas an increased ionic strength promotes aggregation via weakening the electrostatic repulsion due to the screening of surface charge (Ryan, Zhong, & Foegeding, 2013). The critical salt concentration required to induce the formation of aggregates is dependent on types of salt (e.g., lower molar concentration for $CaCl_2$ than NaCl) and increases with increasing pH above pI (Kuhn & Foegeding, 1991; Schmitt & others, 2007). Ions may also act as a ligand binding with protein molecules at a specific position or being a part of

the native structure, which is observed for the better heat stability of α-La when bound with calcium (Hendrix, Griko, & Privalov, 2000).

1.4 Strategies of improving heat stability of whey proteins

1.4.1 Overview

As discussed above, thermal processing conditions are dependent on product acidity, while products are formulated to have quality, safety, nutrition, and sensory characteristics meeting consumer demands. When whey proteins become a part of a product, solution conditions such as pH, ionic strength, and types and concentrations of other solutes can impact the types and magnitudes of protein interactions. Because denaturation precedes aggregation during heating, strategies can be developed to restrict the denaturation of whey proteins. This has been studied by adopting other co-solutes or pretreatments such as preheating and enzymatic cross-linking. The surface properties of proteins can also be fabricate to introduce repulsive forces such as electrostatic and steric repulsions to prevent or reduce protein aggregation. These strategies are briefly discussed below.

1.4.2 Cross-linking by transglutaminase

1.4.2.1 Introduction

Transglutaminase (TGase) is a transferase that forms isopeptide bonds between lysyl and glytaminyl residues. TGase can be produced by both mammals and microorganisms. Compared to mammalian TGase, microbial TGase (mTGase) is smaller, and more stable, and has calcium-independent activity (Ando, Adachi, Umeda, Matsuura,

Nonaka, Uchio, et al., 1989). The mTGase is the most commonly used enzyme in modification of whey proteins, which catalyzes the formation of covalent crosslinking between proteins to modify functional properties such as gelation (Truong, Clare, Catignani, & Swaisgood, 2004), and emulsifying properties (Færgemand, Otte, & Qvist, 1998; Liu & Damodaran, 1999), and thermal stability (Zhang & Zhong, 2010).

1.4.2.2 Reactions catalyzed by transglutaminase

Reactions catalyzed by TGase are summarized in Figure 1-2. TGase catalyzes the acyl transfer reaction between the γ-carboxyamide moiety of protein-bound glutamine residue (acyl donors) and a variety of primary amines (acyl acceptors) (Yokoyama, Nio, & Kikuchi, 2004). At the absence of amino substrates, TGase catalyzes the hydrolysis of the γ-carboxyamide group, resulting in deamination (Yokoyama, Nio, & Kikuchi, 2004). The formation of enzyme catalyzed lysyl-glutamyl bonds attenuates hydrophobic interactions through steric hindrance and formation of compact molecules, which limits the exposure of hydrophobic moieties and thus improves the thermal stability of proteins (Eissa & Khan, 2006).

1.4.2.3 Property changes due to transglutaminase cross-linking

Surface hydrophobicity. The surface hydrophobicity of proteins can either increase or decrease after treatment by mTGase. Babiker et al. (1996) found that surface hydrophobicity of the protease- and acid-pretreated soy proteins decreased greatly after polymerization by mTGase, suggesting that the exposed hydrophobic residues of the pretreated peptides were buried after polymerization by mTGase. A later study of WPI treated by mTGase also reported the decrease of surface hydrophobicity, which however

was concluded to be caused by the occlusion of hydrophobic cavities to the fluorescent probes (Agyare & Damodaran, 2010). Other studies also demonstrated the decrease in surface hydrophobicity after mTGase was applied to treat sodium caseinate (Tang, Yang, Chen, Wu, & Peng, 2005), soy and wheat proteins (Ahn, Kim, & Ng, 2005). The increase in surface hydrophobicity was also reported for whey protein isolate (Hiller & Lorenzen, 2007) and soy protein films (Tang & Jiang, 2007) after mTGase treatment. The increase in surface hydrophobicity may be attributed to the exposure of embedded hydrophobic groups during enzymatic cross-linking at a mild temperature. A few studies also found no significant changes in surface hydrophobicity when β-casein, WPI and 11S soy globulins after being treated with mTGase, suggesting that the cross-linking of lysine and glutamine residues did not alter the exposure of hydrophobic amino acids (Liu & Damodaran, 1999; Yildirim, Hettiarachchy, & Kalapathy, 1996). The difference in changes of surface hydrophobicity may be attributed to the type of proteins based on the premise of same enzymatic cross-linking conditions. Some proteins may not be suitable substrates for enzymatic cross-linking due to lacking of or the embedment of substrate amino acid residues (Ercili-Cura, Partanen, Husband, Ridout, Macierzanka, Lille, et al., 2011; Reddy, Kella, & Kinsella, 1988).

Charges. TGase treatment at neutral pH increases the surface potential as a result of partial deamidation of glutamine and asparagine (Figure 1-2) (Yokoyama, Nio, & Kikuchi, 2004). TGase cross-linked protein results in an increase in overall net charge (more negatively charged) because acyl- reaction can introduce new groups to charged amino acid residues of proteins (DeJong & Koppelman, 2002).

Heat stability. Treatment of proteins with TGase can modify their heat stability through the incorporation of amines, cross-linking and deamidation reaction (O'Sullivan, Kelly, & Fox, 2002). TGase treatment can improved the heat stability of caseins at pH>6.5 (O'Sullivan, Kelly, & Fox, 2002). The solubility and heat stability of sodium caseinate at pH 4.6 were also improved after TGase treatment (Flanagan, Gunning, & FitzGerald, 2003). The mTGase treatment increased the thermal denaturation temperature of glycinin and β-conglycinin component and thus improved the heat stability (Tang, Chen, Li, & Yang, 2006). β-Lg after excessive treatment with mTGase (23 h) also showed the improved heat stability, and the prior treatment by the reducing agent dithiothreitol (DTT) was observed to be not needed (Tang & Ma, 2007). The enhanced heat stability after TGase treatment may be attributed to the partial unfolding of protein molecules and the subsequent re-arrangement of protein conformation (Tang & Ma, 2007).

Rheology. Applying mTGase in dairy products provides an alternative for improving texture and functional properties. A number of studies showed that proteins cross-linked with mTGase generally increased gel strength and improved viscosity (Dickinson & Yamamoto, 1996; Hernàndez-Balada, Taylor, Phillips, Marmer, & Brown, 2009). The viscosity of whey protein isolate at pH 6-7 was not significantly influenced after mTGase treatment, but a considerable increase was observed when the solution pH was 8, implying the pH-dependence of polymerization reaction (Eissa, Bisram, & Khan, 2004). Increasing incubation time from 0 to 8 h at pH 7.5 and 40 ℃ also increased the apparent viscosity of WPI cross-linked by mTGase (Truong, Clare, Catignani, & Swaisgood, 2004). The firmness of mTGase-treated protein gels is much higher than

those formed from untreated protein solutions (Jaros, Partschefeld, Henle, & Rohm, 2006) with a markedly increase in gelation temperature of WPI. However, a high level of mTGase resulted in the loss of gelation properties after heating (Truong, Clare, Catignani, & Swaisgood, 2004).

1.4.3 Co-solutes

1.4.3.1 Types of co-solutes studied for heat stability of whey proteins

Co-solutes can be categorized into four classes depending on whether their effects on protein stability is neutral, favorable, unfavorable or combined (McClements, 2002). The stabilizing co-solutes are those that oppose protein denaturation. This can be described by the transfer free energy, the energy change when protein molecules are transferred from a pure solvent to a co-solute solution (McClements, 2002). When the transfer free energy is positive, the transfer of proteins is thermodynamically unfavorable, that is, protein molecules prefer to be surrounded by solvent molecules, and co-solute molecules are excluded from protein molecules, forming a region that protein molecules cannot get through and thus cannot aggregate. For stabilizing co-solutes, the transfer free energy is greater for the native state than for the denatured state; these co-solutes therefore restrict proteins from denaturation.

Sugars or sugar alcohols, used as sweeteners, are a category of stabilizing co-solutes. Generally, an increase of sugar/sugar alcohol concentration leads to an increased denaturation temperature of proteins and therefore the enhanced thermal stability (Baier & McClement, 2001; Kulmyrzaev, Bryant, & McClements, 2000). Disaccharides such as sucrose and trehalose reduce the thermal aggregation rate of β-Lg, whereas

polysaccharide dextran did not prevent the aggregation during heating (Perez-Moral, Adnet, Noel, & Parker, 2011). Sugar alcohols with molecular weight less than 200 g/mol such as xylitol, sorbital, erythitol and mannitol also showed better effects in improving heat stability of whey proteins than higher molecular weight sugar alcohols such as maltitol, lactitol and isomalt (LaClair & Etzel, 2009).

Other co-solutes have also shown the ability to improve the stability of whey proteins after heating. Glycerol at a concentration of 20% v/v can improve the heat stability whey protein solutions at 2% protein, pH 4, attributed to the increase of denaturation temperature (Gekko, 1982). 4 M of Urea, 10% v/v of propylene glycol and 10% v/v of ethanol also improved the thermal stability of whey proteins at pH 4 after heating at 88 ℃ for 2 min, resulting in reduced whey protein solution turbidity (LaClair & Etzel, 2009). β-casein with >90% purity is a feasible component to stabilize higher concentrations of whey proteins in beverages (Yong & Foegeding, 2008). Hydrophilic amino acids such as proline (Samuel, Ganesh, Yang, Chang, Wang, Hwang, et al., 2000), asparagine and glutamine (LaClair & Etzel, 2009) when used at a sufficiently high concentration also are effective in decreasing the turbidity of protein solution after heating. Surfactants, such as sodium alkyl sulfates and alkyl maltopyranosieds reduce aggregation, suggesting that surfactants may modulate whey protein properties (Giroux & Britten, 2004; Hansted, Wejse, Bertelsen, & Otzen, 2011; Tran Le, Sabatino, Heyman, Kasinos, Dinh, Dewettinck, et al., 2011).

1.4.3.2 Mechanisms of heat stability improvements of whey proteins by co-solutes

One of the most accepted mechanisms of simple sugars stabilizing whey proteins is that sugar molecules are preferentially excluded from the region immediately surrounding the proteins, due to a combination of excluded volume effect, caused by steric exclusion of larger sugar molecules, and differential interaction effects (McClements, 2001). Sugars increase the thermal denaturation temperature of proteins because their interactions with native proteins are thermodynamically more favorable than with denatured proteins (Baier & McClements, 2006). The excluded volume effect leads to molecular arrangements around proteins to reduce the contact between proteins and surrounding co-solute molecules (McClements, 2001). The inclusion of co-solutes also decreases the collision frequency of native and denatured globular protein molecules due to the increased viscosity of the continuous phase (LaClair & Etzel, 2009; McClements, 2002; Semenova, Antipova, & Belyakova, 2002). In the presence of glycerol, the interaction between glycerol molecules hydrophobic groups on protein surfaces is not preferred, resulting in less exposure of nonpolar groups involved in protein aggregation (McClements, 2002). A study on sucrose and glycerol used at different levels revealed that sucrose was more effective than glycerol in increasing the thermal stability of globular BSA because of the stronger steric exclusion effect (Baier & McClements, 2006).

At 70 ℃, the dimers of β-Lg solution (6% w/v) were partially unfolded and formed opaque particulate aggregates (Yong & Foegeding, 2008).In the presence of β-casein, the formation of particulate aggregates of β-Lg is blocked but formed clear fine-

stranded aggregates (Yong & Foegeding, 2008). Amino acids stabilize proteins based on different mechanisms. Proline can suppress protein aggregation by interacting with hydrophobic amino acids exposed during heating (Samuel, et al., 2000), while arginine can prevent the heat-induced aggregation of lysozyme at neutral pH via the hindrance of intermolecular interaction between unfolded molecules (Matsuoka, Tomita, Hamada, & Shiraki, 2007).

Urea stabilizes proteins through the rupture of hydrogen bonds and interference with hydrophobic interactions, resulting in completely denatured proteins with high solubility (Damodaran, 2008). Propylene glycol and ethanol also stabilize protein due to the favorable interaction between glycols/ethanol and exposed hydrophobic amino acid residues of denatured proteins (LaClair & Etzel, 2009).

Surfactants interact with proteins and modulate the thermal aggregation depending on charge properties (Hansted, Wejse, Bertelsen, & Otzen, 2011). Cationic surfactants promote protein aggregation at pH 6.5 after heating at 85 ℃ by a combination of destabilization and charge neutralization. Anionic and non-ionic surfactants stabilize proteins through solubilizing unfolded proteins and inhibiting protein aggregation. Anionic surfactants suppress the protein aggregation by not only stabilizing potential aggregation sites of proteins but also altering charge properties of proteins (Hansted, Wejse, Bertelsen, & Otzen, 2011), with the latter strengthening electrostatic repulsion that is not possible for non-ionic surfactants.

1.4.3.3 Property changes due to co-solutes

Solubility. Water solubility of proteins during heating usually decreases due to the increased surface hydrophobicity and weakened electrostatic repulsion due to ionic co-solutes causing the charge screening. However, co-solutes influence the water solubility of proteins in different ways, either by altering the balance of the soluble-insoluble protein equilibrium or by altering the protein conformation (McClements, 2002). In addition to types of proteins and co-solutes, the water solubility of proteins is dependent on external conditions such as pH and temperature. The water solubility of different globular proteins was found to increase by adding sugar (sorbitol, saccharose, sorbose and sucrose) and glycols (glycerol, propylene glycol and ethylene glycol) near their pI (Antipova & Semenova, 1997; McClements, 2002) but sucrose decreased the solubility of ovalbumin at pH away from the pI (Antipova, Semenova, & Belyakova, 1999). Charged amino acids such as L-Arg and L-Glu at 50 mM dramatically increased the concentration of soluble protein, and long-term stability of protein (Golovanov, Hautbergue, Wilson, & Lian, 2004). Surfactants also can improve the solubility of proteins. Sodium dodecyl sulfate (SDS) binds with hydrophobic sites of soy proteins and increases negative charges on proteins and thereby increases the protein solubility (Malhotra & Coupland, 2004; Nakai, Ho, Tung, & Quinn, 1980). Lecithin can largely minimize the heat-induced protein intermolecular interactions thus prevent protein aggregation during heating at 80 ℃ for 15 min at pH 6.55, thus increases the solubility of proteins after heating (Tran Le, et al., 2011)

Rheology. In addition to increasing the viscosity of protein solutions to improve heat stability, co-solutes can alter the gelation of proteins through increasing the temperature of protein unfolding and the magnitude of attractive and repulsive forces between protein molecules (McClements, 2002). This can increase the rigidity of gels after heating (McClements, 2002). Sucrose increased the temperature of BSA gelation when 2% w/w BSA solution was heated at pH 7 from 30-90 ℃ and held at 90 ℃ for various durations (Baier & McClements, 2001). For the protein-SDS system, the viscosity of protein solution was not significantly changed when SDS was used at a low concentration; the denaturation of proteins at higher SDS concentration, also possibly by binding with SDS micelles, significantly increase the viscosity (Malhotra & Coupland, 2004).

1.4.4 Forming soluble aggregates by preheating

1.4.4.1 Concept of soluble aggregates

Whey protein soluble aggregates (WPSAs) are defined as thermally-formed intermediates between monomeric proteins and an insoluble gel network or precipitate (McSwiney, Singh, & Campanella, 1994). The heating step used to form WPSAs changes protein structures and confers the improved structural and thermal stability upon further processing. The formation of WPSAs and the resulting properties are related to both internal (whey protein type and concentration) and external factors (solution chemistry, co-solutes, heating conditions). WPSAs are formed below the protein concentration corresponding to gelation at a specific combination of heating temperature and duration (McSwiney, Singh, & Campanella, 1994). Besides the protein concentration and heating

temperature/time, physicochemical properties of WPSAs including surface hydrophobicity, zeta-potential, size and shapes can be fabricated by controlling pH, ionic strength, ion type and co-solutes during heating (Ryan, Zhong, & Foegeding, 2013).

1.4.4.2 Mechanisms of improving heat stability by forming soluble aggregates

WPSAs can be formed by heating whey protein aqueous solutions at a pH approximately from 6.0 to 7.5 (Ryan, Zhong, & Foegeding, 2013). A proposed mechanism of improved salt and thermal stability of whey protein after forming WPSAs is outlined in Figure 1-3 (Ryan, Zhong, & Foegeding, 2013). The hydrophobic amino acid residues initially embedded at the native state become exposed after thermal denaturation. The denatured proteins, behaving like anionic polymers with abundant charges, aggregate only when hydrophobic patches on separate molecules approach each other, and the aggregation of unfolded whey proteins now allows the formation of inter-protein disulfide bonds at the exposed thiol groups (Vardhanabhuti & Foegeding, 1999). The WPSAs formed at neutral pH and low ionic strength have the increased zeta-potential, which, together with the formed covalent disulfide bonds, increases the heat stability of whey proteins at an increased salt concentration (Ryan & others, 2011; Schmitt & others., 2007).

1.4.4.3 Changes in some properties

Surface hydrophobicity. The surface hydrophobicity of WPSAs is typically determined indirectly through the binding with hydrophobic probes such as 8-anilino-1-naphthalenesulfonic acid (ANS) (Ryan, Zhong, & Foegeding, 2013). The surface hydrophobicity of WPSAs is markedly impacted by pH, temperature and ionic strength

during preparation (Ryan, Zhong, & Foegeding, 2013). Heating of whey proteins at pH 6.0-7.0 corresponded to a reduction in surface hydrophobicity at pH 6.0, forming spherical and dense WPSAs possibly due to limited exposure of hydrophobic patches (Schmitt & others., 2007). At pH 7.0, heating facilitates the exposure of hydrophobic patches and therefore increases surface hydrophobicity; extended heating however may result in the decrease in surface hydrophobicity because aggregation via hydrophobic amino acid residues on surfaces reduces the sites for binding with hydrophobic probes (Ryan, Zhong, & Foegeding, 2013).

Zeta-potential. The zeta-potential is calculated by determining the electrophoretic mobility, which is obtained by performing an electrophoresis experiment on the sample and measuring the velocity of the particles using laser Doppler velocimetry (LDV) (Ryan, Zhong, & Foegeding, 2013). The magnitude of zeta-potential, either positive or negative, indicates the strength of electrostatic repulsion. Zeta-potential of whey protein solutions can increase or decrease after thermal treatments, depending on solution and heating conditions. The zeta-potential of native β-Lg, ~-25 mV at pH 7.0, was observed to have decreased after heating at 70 ℃ for 24 h but have increased after heating at 85 ℃ for 8 h (Donato, Schmitt, Bovetto, & Rouvet, 2009). Zeta-potential is affected by the presence of salt, because charge screening by the added salt lowers the electrophoretic mobility of proteins (Schmitt & others., 2007).

Morphology. The morphology of WPSAs depends strongly on a number of factors, mostly pH, initial concentration, temperature, and ionic strength during preheating. At pH 2-3, large, rigid and rod shaped aggregates are formed after preheating; the aggregates become more spherical when pH gets closer to pI. At pH above pI, the

shape of aggregates varies depend on protein concentration during preheating. Small and elongated aggregates are observed at lower protein concentrations, while larger and randomly branched aggregates with self-similar structures are formed at higher protein concentration (Phan-Xuan & others., 2011). Generally, a higher temperature during preheating results in large aggregates, but heating at temperatures above ~140 ℃ can results in the cleavage of peptide bonds and thus a reduction in aggregate size (Photchanachai & Kitabatake, 2001). A longer heating time may have little influence on the aggregates size but facilitate the yield of aggregates, indicating aggregate growth phase reached a steady state (Le Bon, Nicolai, & Durand, 1999a, 1999b). The size of aggregates increases with NaCl concentration, but different shapes can be observed during heating at different pH conditions due to the effects on electrostatic interactions (Schmitt & others., 2007). For WPSAs formed by heating at pH 7.0 with NaCl, the WPSAs were denser, more curved and thinner than those formed with the absence of NaCl, suggesting that the oriented aggregation at the absence of salt is no longer the case due to charge screening (Schmitt & others., 2007).

1.4.5 Glycation with reducing sugars

1.4.5.1 A brief overview of the Maillard reaction

Glycation, also known as the Maillard reaction, occurs between the ε-amino groups of the lysine residues in food proteins and the reducing-end carbonyl group of a sugar (Liu, Ru, & Ding, 2012). The Maillard reaction starts with a condensation reaction between a reducing sugar and an amino group to yield a Schiff base (formation of N-glycoside). After formation of N-glycoside, Amodori rearrangement takes place for

isomerization, leading to formation of Amodori rearrangement products (ARPs), 1-amino-1-deoxy-2-ketose (Ames, 1992). Subsequently, ARPs undergo a series of reactions, depending on the pH of the system, forming complex products for Strecker degradation. In the advanced stage, some of the Maillard reaction products with dark color are formed in various reactions and are responsible for the brown pigments and aromas which can be desirable or undesirable (Liu, Ru, & Ding, 2012).

Glycation can occur either in the aqueous solution (wet base) or spray-dried/freeze-dried powder (dry base). Temperature, duration, pH, water activity, intrinsic properties of protein and sugar, and the protein:sugar ratio are factors that influence physicochemical properties of glycated proteins. Glycation by heating powder mixtures of protein and reducing sugar is faster than in aqueous solutions because of the lower concentration of reactants in the latter (Liu, Ru, & Ding, 2012). Additionally, the rate of initial ARPs formation can be reduced due to the presence of water that further slows later steps in the Maillard reaction (Van Boekel, 2001).

Attachment of oligosaccharides to proteins through the Maillard reaction is more limited when compared to monosaccharides because of the steric hindrance of the initially attached carbohydrate chains (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). However, side reaction products and color developments may be suppressed in the subsequent reactions when bigger reducing saccharides are used. Glycation of protein with reducing saccharides greatly enhances the solubility, thermal stability, and emulsifying properties of proteins (Kato & Kobayashi, 1991; Liu, Ru, & Ding, 2012). The rate of reaction increases with increases in temperature due to an increase of the reactivity (Damodaran, 2008). In aqueous solution, more brown color compounds are

formed during reaction at a high temperature for a short time when compared to a mild temperature for a long heating time. The rate of Maillard reaction is influenced by pH (Damodaran, 2008). At a lower pH, the amino group is more protonated and becomes less nucleophilic. In contrast, the amino group is deprotonated at alkaline pH and more nucleophilic and is reactive with the carbonyl group of reducing saccharides (Nemet, Šošo, & Lazić, 2010). A number of reaction pathways can result in the formation of brown pigments. The rate of color formation is reduced by decreasing pH because the pathway of forming melanoidins is favored under alkaline conditions (Dattatreya & Rankin, 2006) . The fate of the ARPs is also determined by pH (Martins, Jongen, & Van Boekel, 2000). The effect of water on Maillard reaction is complicated as water is involved in various reaction mechanisms. The rate of Maillard reaction increases from the dry state with an initial water activity of 0.2-0.3, is optimum at a water activity of 0.6-0.7, and decreases at a higher water activity due to the dilution of reactants and hindrance of further stages of reactions (Damodaran, 2008; Labuza & Saltmarch, 1981; Van Boekel, 2001). The ratio of protein to saccharide is considered as an important factor determining the rate of Maillard reaction. At low protein:saccharide ratios, saccharide molecules are sufficient for reaction with available amino groups (Jones & McClements, 2010), requiring a shorter reaction time (Carabasa-Giribet & Ibarz-Ribas, 2000).

1.4.5.2 Principles of improving thermal stability of proteins by glycation

The improved thermal stability of whey proteins has been observed after glycation with reducing saccharides (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Liu & Zhong, 2012, 2013). This can be attributed to steric effects based on the

well-established theory that polymers grafted on a colloidal particle provide steric hindrance against aggregation with the premise that the solvent condition allows the extension of polymer chains to the continuous phase (Israelachvili, 2011). The reducing saccharide molecules attached on protein molecules act as a hairy layer (Israelachvili, 2011). Because the strength of steric hindrance is a function of polymer chain density on colloidal particle surface (Israelachvili, 2011), a higher degree of glycation facilitates the improvement of functional properties of proteins.

1.4.5.3 Changes in some properties due to glycation

Surface hydrophobicity. With the attachment of hydrophilic reducing saccharides during glycation, the surface hydrophobicity of proteins changes, affecting greatly their functional properties (Liu, Ru, & Ding, 2012). The measured surface hydrophobicity was reported to either increase or decrease after glycation due to factors such as types of protein and reducing sugar and the degree of glycation (Liu, Ru, & Ding, 2012). The increase in surface hydrophobicity was found to be related to the increased availability for binding fluorescence probes due to the exposure of hydrophobic patches resulting from protein denaturation. The extent of surface hydrophobicity increase depends on the protein:sugar ratio and glycation duration (Tang, Sun, & Foegeding, 2011). Many studies on different proteins reported the maximum surface hydrophobicity at an intermediate reaction duration, followed by a gradual decrease at longer glycation duration (Sava & others., 2005; Sun, Hayakawa, & Izumori, 2004; Tang, Sun, & Foegeding, 2011). The reduction of surface hydrophobicity after glycation has been attributed to the blocking of fluorescent probes to bind lysine and/or arginine residues by the attached saccharides

(Gasymov & Glasgow, 2007) and the aggregation of proteins resulting from attraction between hydrophobic patches exposed during glycation (Sava & others., 2005).

Isoelectric point (pI). Glycation of proteins results in the blockage of basic lysine by reducing saccharides and a shift of protein pI to a lower pH (Liu, Ru, & Ding, 2012). The extent of pI shift depends on the chemistry of protein and reducing sugars and glycation conditions. The pI of WPI shifted from pH 4.86 to pH 4.28 after partial glycation (Wang & Ismail, 2012). The pI of phaseolin progressively decreased within reaction duration, from 4.85 to 4.27 and 4.45 at a protein/glucose molar ratio of 1:100 and 1:50, respectively, after incubation of 10 h, and a greater reduction of pI was due to a higher degree of glycation (Tang, Sun, & Foegeding, 2011).

Heat stability. Heat-induced aggregation of protein can be controlled by formation of protein-polysaccharide conjugate. The thermal aggregation can be inhibited by the interactions of globular proteins and charged polysaccharides by lowering the accessibility of protein reactive sites, which has been demonstrated in the case of β-Lg and Sodium polypectate system (Ndi, Swanson, Dunker, & Luedecke, 1996). Glycation of β-Lg (2 mg/mL) exhibited a better thermal stability when heated at pH 5, 25-90 ℃ as compared to native protein (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001). β-Lg glycated with dextrans at 60 ℃ and water activity of 0.44 showed an improved thermal stability at pH 5 above 85 ℃ (Jiménez-Castaño, Lopez-Fandino, Olano, & Villamiel, 2005). In our group, whey proteins glycated with different sugars (D-glucose, sucrose, lactose) under various reaction conditions have been studied, suggesting that a steric hindrance was created during glycation thus enhanced the heat stability of whey proteins (Liu & Zhong, 2012, 2013).

Rheology. Several studies reported the increased viscosity and viscoelastic properties after glycation. The increase of viscosity of glycated proteins may be attributed to the increased dimension of proteins after glycation and possibly cross-linked proteins (Liu, Ru, & Ding, 2012). A longer period of incubation and a longer chain length of reducing saccharides result in a greater increase of viscosity, suggesting the viscosity is related to the number of reducing sugars attached to the protein (Corzo-Mart ńez, Moreno, Villamiel, & Harte, 2010; O'Regan & Mulvihill, 2009). It was also observed for the increased viscosity and gelation after reconstituting sodium caseinate powder glycated with maltodextrin for a longer duration (O'Regan & Mulvihill, 2009). For egg white, the increase in gel strength formed by heating glycated proteins was observed to be a function of saccharide structure, following the order of psicose>fructose>glucose (Sun, Hayakawa, & Izumori, 2004).

1.5 Conclusions

Thermal stability of whey proteins is critically needed to produce shelf-stable protein beverages. Thermal stability of whey proteins is a function of pH, ionic strength, thermal treatment conditions, and the presence of other co-solutes. Thermal stability of whey proteins at neutral pH can be improved by adopting conditions restricting protein denaturation during heating such as dissolving co-solutes, enzymatic crosslinking, or preheating to form soluble aggregates. Glycation of whey proteins with appropriate saccharides can improve thermal stability at various acidic and ionic conditions. These strategies may also be used in combination. Much work however is needed to improve

the properties of these systems and understand physicochemical mechanisms associated with the property improvements.

1.6 Scope of dissertation research

Heat-induced aggregation of whey proteins can be suppressed through a variety of approaches based on the understanding of colloidal interactions in the system. Although these strategies result in small particles and lower turbidity after heating, most of them were employed at low whey protein concentration and at pH far away from pI. The current work focused on the physicochemical bases of improved heat stability of whey proteins, at high concentration (5% w/v), different pH conditions (pH 4-7), after heating at temperatures above the denaturation temperature of native whey proteins (80 ℃, 15 min), simulated hot-filling process (88 ℃, 2 min), and simulated UHT process (138 ℃, 1 min).

The heat stability of whey proteins was first studied at neutral pH through sequential preheating and mTGase pretreatments (Chapter 2 and 3). Preheating whey proteins at neutral pH in the absence of salt leads to partial unfolding of proteins, which alters the overall charge on the protein surface, as a result, affecting the electrostatic interactions between protein molecules. Additionally, substrate amino acids embedded in the interior can be exposed during preheating, which could increase the accessibility of reactive amino acids (lysine and glutamine) for mTGase cross-linking. TGase cross-linking limit the further exposure of hydrophobic moieties of whey proteins, thus improved the heat stability of whey proteins. Chapter 2 focused on the physicochemical

properties of whey proteins after heating at 80 ℃, with determination of changes in turbidity, particle size, charges, surface hydrophobicity, denaturation behavior, rheology, and particle morphology, etc. A more severe heating process (138 ℃) was applied on sequentially pretreated whey proteins, revealing the physicochemical changes in a high temperature environment, presented in Chapter 3.

Heat-induced aggregation of whey proteins can be inhibited at pH 4-7 via glycation with reducing saccharides, that is, Maillard reaction (Chapter 4 and 5). Whey proteins glycated with saccharides creates a structure with internal whey protein core and an external saccharide shell. The aggregation of proteins therefore can be prevented since protein molecules have to overcome the saccharide shell to aggregate. Reaction conditions significantly affect the glycation of whey protein and reducing saccharides, including temperature, relative humidity and pH, resulting in final products with a variety of functional properties. The pathway of Maillard reaction varies under different pH conditions that determine the reaction rate and color formation. Combined with addition of co-solutes in the system, whey proteins can be stabilized at pH 4-7. In Chapter 4, the impact of powder acidity on the glycation of whey protein and maltodextrin was studied. In Chapter 5, the effects of four types of saccharides: D-glucose, sucrose, lactose and D-cellobiose on the improvement of heat stability of whey proteins were studied at pH 5.0.

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Appendix

Table 1-1. Typical protein composition of whey (Bryant & McClements, 1998; Thompson, Boland, & Singh, 2009).

Protein	Proportion by mass (%)	No. amino acids	Molecular mass (kDa)	Isoelectric point	Disulfide bonds/thiols	T_d (℃)	Comments
β-Lg	60	162	18.2[a]	5.2 - 5.4	2/1	78	Two common variants, A and B
α-La	20	123	14.2	4.8 - 5.1	4	62	About 10% of molecules are glycosylated
BSA	3	583	66.4	4.8 - 5.1	17/1	64	Also present in blood serum
IgG	10	>500	161 (G1)[b]	Many isoforms 5.5 - 6.8	-	72	Passive transfer of immunities

[a] Molar mas for the A variant.
[b] G1 is the major immunoglobulin; two other classes, IgM and IgA, are present in much lower abundance.

Table 1-2. Characteristics of molecular interactions between two similar protein molecules in aqueous solutions and the impacts of environmental conditions on force strength (Bryant & McClements, 1998).

Type	Sign	Strength	Range	pH	Ionic strength	Temperature
Hydrophobic	Attractive	Strong	Long	No	No	Increases
Electrostatic	Repulsive	Weak→ Strong[a]	Short→ Long[a]	Yes	decreases	Increases
Hydrogen Bonding	Attractive	Weak	Short	No	No	Decreases
Hydration	Repulsive	Strong	Short	Yes	Yes	Decreases
Van der Waals	Attractive	Weak	Short	No	No	_
Steric repulsion	Repulsive	Strong	Short	No	No	_
Disulfide bonds	Attractive	Very	Short	Yes	Yes	No

[a] Dependent on pH and ionic strength.

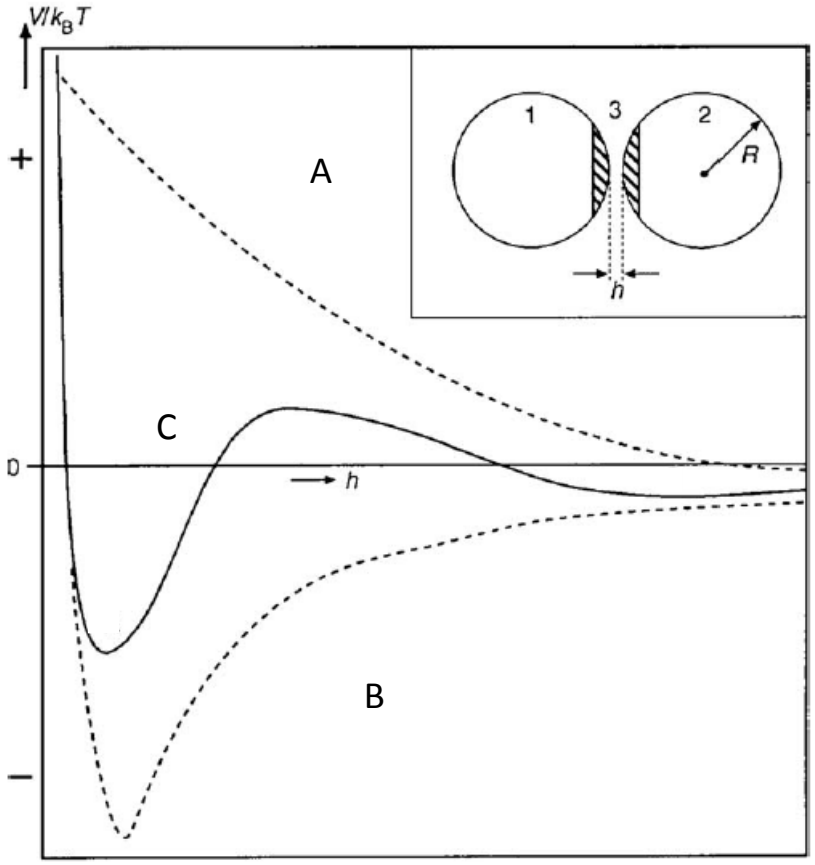

Figure 1-1. The colloidal interaction free energy *V* between hard spheres with a radius of R separation distance *h* (Walstra, 2002) .

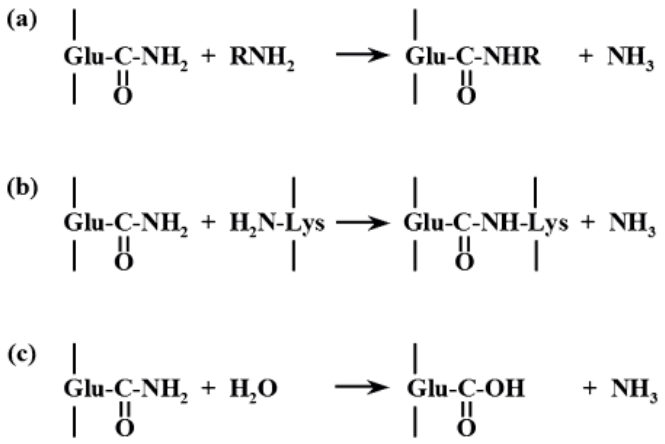

Figure 1-2. Reactions catalyzed by transglutaminase (TGase): (a) Acyl transfer; (b) Crosslinking of Gln and Lys residues in proteins or peptide and (c) Deamidation (Yokoyama, Nio, & Kikuchi, 2004).

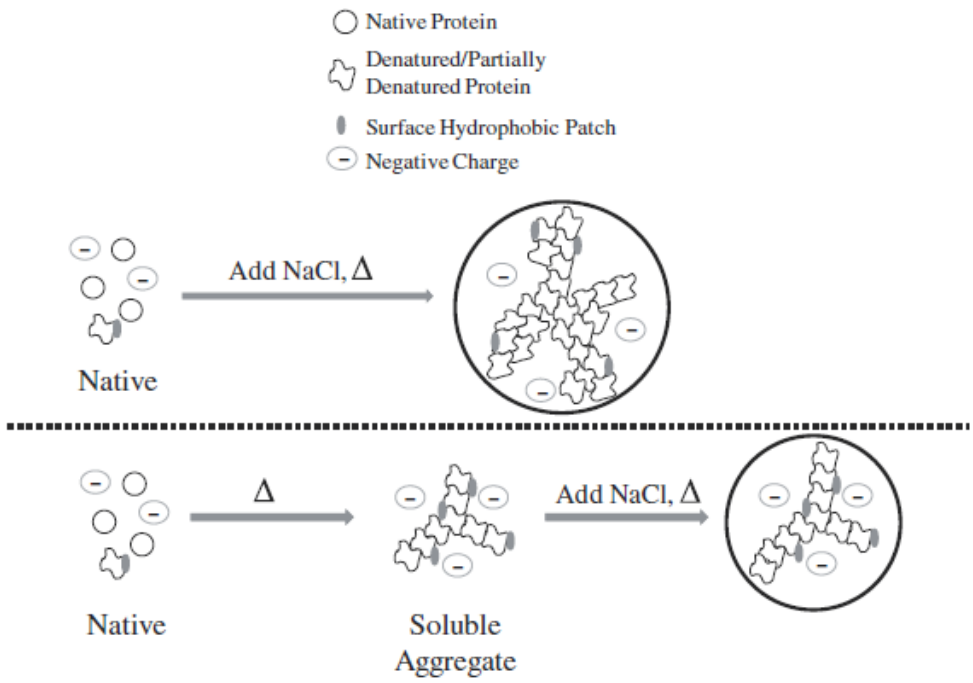

Figure 1-3. Proposed mechanisms of whey protein aggregation during heating at neutral pH in the absence or presence of NaCl as impacted by preheating. Δ indicates heat treatment and negative charges indicate the overall protein charge (Ryan & others, 2011).

Chapter 2. Nanoscale Understanding of Thermal Aggregation of Whey Proteins Pretreated by Transglutaminase

2.1 Abstract

Nanoscale structures of whey protein isolate (WPI) pretreated by microbial transglutaminase (mTGase) and subsequent heating were studied in this work and were correlated to zeta-potential, surface hydrophobicity, thermal denaturation properties, and macroscopic turbidity and viscosity. Dispersions of 5% w/v WPI were pretreated by individual or sequential steps of preheating at 80 °C for 15 min and mTGase, used at 22-110 U/g-WPI for 1-15 h, before adjusting to pH 7.0 and 0-100 mM NaCl for heating at 80 °C for 15 and 90 min. Zeta-potential and surface hydrophobicity of WPI increased after all pretreatment steps. Preheating increased cross-linking reactivity of WPI by mTGase, corresponding to significantly increased denaturation temperature. Particle size analysis and atomic force microscopy revealed that structures of sequentially-pretreated WPI remained stable after heating at 100 mM NaCl, corresponding to transparent dispersions. Conversely, WPI pretreated by one step only aggregated at 100 mM NaCl and resulted in turbid dispersions. The present study provides both physical chemical and structural information, which are essential for better understanding the mechanisms of WPI aggregation at neutral pH and offers a feasible way for food industrial applications of WPI as the major composition.

Keywords: whey protein, thermal aggregation, enzymatic cross-linking, rheology, nanostructure, surface properties

2.2 Introduction

Whey proteins are among the most studied biopolymers because of their properties to form emulsions and nanoemulsions (Lee, Choi, Li, Decker, & McClements, 2011), nanofibers (Loveday, Su, Rao, Anema, & Singh, 2011), hydrogels (Betz, Hörmansperger, Fuchs, & Ulrich Kulozik, 2012), and nanostructures for delivering nutraceuticals and pharmaceuticals (Chen & Subirade, 2005, 2006). Cheese whey is the major source for commercial production of whey protein ingredients, and whey protein isolates (WPI) refer to products purified to a protein content higher than 90%. WPI are composed of a mixture with β-lactoglobulin, α-lactalbumin, and bovine serum albumin being the major components (Foegeding, Davis, Doucet, & McGuffey, 2002). WPI have been used to fabricate a variety of structures by engineering their aggregation induced by acidity, electrolytes and temperatures (Bryant & McClements, 1998; Foegeding, Davis, Doucet, & McGuffey, 2002). Particularly, heating is a common approach to form fibrillar or particulate aggregates of whey proteins by controlling repulsive and attractive intra/intermolecular physical forces such as electrostatic interactions, hydrophobic interactions, and hydrogen bonding and intra- and intermolecular disulfide bonds via sulfhydryl-disulfide interchange (Bryant & McClements, 1998). The property of concern in the present study is the dispersibility after heating whey proteins. In a concentrated dispersion, thermal aggregation of whey protein into a three-dimension network is important to the formation of hydrogels but is problematic for systems requiring the maintained fluidity and low viscosity. Furthermore, because micrometer-sized structures deflect light to cause turbidity and precipitate during storage, fabricating nanostructures

of whey proteins after thermal processing is needed to maintain the visual transparency of dispersions, commonly referred as heat stability.

Thermal aggregation of native whey protein at neutral pH has been well-studied (Bryant & McClements, 1998). At low ionic strength, WPI dispersions typically remain transparent after heating because the strong repulsive electrostatic interactions correspond to reaction-limited aggregation and the formed fibril structures are too small to reflect visible light. At high ionic strength, the shortened Debye length favors protein aggregation that is diffusion-limited, forming particulates that results in turbid dispersions or gels. Reducing thermal aggregation of whey proteins has been studied after incorporation of small molecule co-solutes such as sucrose (Baier & McClements, 2001; Kulmyrzaev, Bryant, & McClements, 2000), glycerol (Chanasattru, Decker, & McClements, 2007; Chantrapornchai & McClements, 2002), and sorbitol (Chanasattru, Decker, & McClements, 2007), or chaperones (Yong & Foegeding, 2008). These compounds increase denaturation temperatures of whey proteins. However, it remains a challenge to maintain dispersion transparency after heating at neutral pH and increased ionic strength. We recently applied microemulsions as templates to produce whey protein nanoparticles by preheating, with and without prior pretreatment by microbial transglutaminase (mTGase), and observed that nanoparticles had much improved heat stability at 100 mM NaCl (Zhang & Zhong, 2009; Zhang & Zhong, 2010). However, the capacity of producing nanoparticles in microemulsions is limited and is an obstacle for practical applications.

TGase (EC 2.3.2.13, protein-glutamine γ-glutamyltransferase) catalyzes the crosslinking reaction between protein molecules through acyl transfer reactions to form

inter- or intramolecular ε-(γ-glutamyl)lysine isopeptidic bonds (Jaros, Partschefeld, Henle, & Rohm, 2006). Although the enzyme is commonly used to cross-link proteins to strengthen gel networks, mTGase has been reported to improve the heat stability of whey protein (Jaros, Partschefeld, Henle, & Rohm, 2006). The study on the specificity of mTGase inducing protein cross-linking varied with the type of proteins. β-lactoglobulin, the major type of proteins in WPI, can be significantly cross-linked by mTGase after preheating, whereas no prerequisite of heating for α-lactalbumin which is another type of proteins in WPI (Rodriguez-Nogales, 2006). The direct treatment of WPI by mTGase was observed to increase thermal denaturation temperature of whey proteins (Agyare & Damodaran, 2010). The reasonable explanation of the increase of denaturation temperature was attributed to covalent cross-linking without any major structural change, however, the cross-linked protein exhibited maximum turbidity at pH 4.0-4.5, indicating that the pI was shifted (Agyare & Damodaran, 2010). The 5% β-lactoglobulin (Tanimoto & Kinsella, 1988) and WPI solutions reduced by 10 mM dithiothreitol (DTT) (Truong, Clare, Catignani, & Swaisgood, 2004) no longer formed gels at pH 7.5 after extensive cross-linking by mTGase. Intra- and intermolecular bonds formed by mTGase were proposed to impact the unfolding of whey proteins during heating (Truong, Clare, Catignani, & Swaisgood, 2004). Tanimoto and Kinsella (Tanimoto & Kinsella, 1988) separated β-lactoglobulin, with prior reduction by DTT and addition of $CaCl_2$ and cross-linking by guinea pig liver TGase, into two fractions based on molecular weight (MW) and evaluated the stability of fractions constituted to 5% protein by heating at 90 °C for 30 min. The fraction bigger than 100 kDa was stable, but a soft gel was observed for the other smaller MW fraction. Moderate mTGase pretreatment conditions (10 U/g-protein,

6-9 h at 37°C and pH 7.4) were observed to reduce the heat stability of β-lactoglobulin (Tang & Ma, 2007), but mTGase cross-linking for 23 h or longer improved the heat stability, at which conditions the prior denaturation by DTT was not required (Tang & Ma, 2007). Despite these related studies, much work is needed to technologically improve the heat stability of WPI solutions with 5% or more protein, at neutral acidity and increased NaCl concentration, and without using toxic DTT. Scientifically, the formation of supramolecular structures of whey proteins as impacted by mTGase pretreatment is to be studied and interpreted by integration with information of protein denaturation properties and colloidal chemistry.

The objective of this work was to study thermal aggregation of WPI after being pretreated by mTGase at different enzyme levels and durations, with and without prior preheating. Preheating was studied because of the improvement in reactivity of whey protein as a substrate for mTGase (Rodriguez-Nogales, 2006a, 2006b). Physicochemical changes were interpreted using several complimentary techniques illustrating rheological, surface-charge, and thermal denaturation properties of whey proteins, as well as supramolecular structures studied using particle size measurements by dynamic light scattering (DLS) and atomic force microscopy (AFM).

2.3 Materials and Methods

2.3.1 Materials

The WPI was a product from Hilmar Ingredients (Hilmar, CA). The powdered mTGase sample (product Activa TG-TI) was a gift from Ajinomoto Food Ingredients

LLC (Chicago, IL). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

2.3.2 Determination of mTGase activity

The freshly-prepared mTGase solution was quantified for activity using the literature method (Folk & Cole, 1966), with modification of pH and temperature. One unit (U) of activity was defined as the grams of powdered enzyme able to produce 1 μmol of hydroxamate per min at pH 7.0 and 50 ℃ when substrates of N-carbobenzoxy-glutaminyl-glycine and hydroxylamine were catalyzed by the enzyme.

2.3.3 Pretreatment protocol

The WPI powder was hydrated at 5% w/v in 20 mL of a 50 mM sodium phosphate buffer overnight at room temperature (21 °C). For sequential pretreatments of preheating and enzymatic cross-linking, samples were adjusted to pH 7.5 using 1 N NaOH and preheated for 15 min in a water bath maintained at 80 °C, followed by cooling immediately in a room temperature water bath. The mTGase powder was dissolved at 2, 5, and 10% mass of WPI. If necessary, the pH was readjusted to 7.5 (Truong, Clare, Catignani, & Swaisgood, 2004) before incubation in a 50 °C shaking water bath for 1-15 h. After immersion in a room-temperature water bath, samples were adjusted to pH 7.0 and 2.0 mL of solution was transferred into 4.0 mL vials. The vials were supplemented with 0, 20, or 40 μL of a 5.0 M NaCl solution (in distilled water), corresponding to a final NaCl concentration of 0, 50, and 100 mM.

To compare with sequentially-pretreated samples, a set of samples were prepared by heating the pH 7.5 solutions at 80 °C for 15 min only, while another set of samples were directly pretreated by dissolving the enzyme powder in the pH 7.5 solutions, followed by incubation at 50 °C for cross-linking. These samples were adjusted to pH 7.0 and NaCl concentrations as above. The control without pretreatment was the 5% w/v WPI solution directly adjusted to pH 7.0 and 0, 50, and 100 mM NaCl.

2.3.4 Evaluation of heat stability

The vials prepared as above were heated for 15 min in a water bath pre-equilibrated to 80 °C. After cooling to room temperature in a water bath, the vials were compared for visual appearance by photographing or for absorbance acquired at 400 and 600 nm using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

2.3.5 Differential scanning calorimetry (DSC)

Thermal denaturatation properties of WPI after different pretreatments were studied using a differential scanning calorimeter (VP-DSC, MicroCal, Northamption, MA). The WPI dispersions after different pretreatments were diluted to 2% w/v using the pH 7.0 phosphate buffer. Samples were degassed at 20 ℃ for 10 min before injection into the sample cell, with deionized water used as a reference. The scanning was conducted from 25 to 105 ℃ at a rate of 1.0 ℃/min. The total calorimetric apparent enthalpy change (ΔH) and the denaturation temperature (T_d) were determined via analysis of the thermographs, which was performed by using two-state model (supplied by MicroCal).

2.3.6 Surface hydrophobicity

Surface hydrophobicity of WPI was determined using fluorescence probe 1-anilino-8-naphtalene-sulphonate (ANS) according to a literature method (Alizadeh-Pasdar & Li-Chan, 2000), with modification. Each sample was diluted to 5 concentrations from 0.005 to 0.2% w/v using a 10 mM phosphate buffer at pH 7.0. The ANS solution was prepared at 8 mM in the same buffer. Ten μL ANS solution was added to 2 mL of each protein solution. The fluorescence intensity was measured at excitation wavelength of 365 nm and emission wavelength of 484 nm using a RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The background fluorescence was calibrated using distilled water. Surface hydrophobicity was calculated by linear regression analysis, using the initial slope of the fluorescence intensity-protein concentration plots as an index of surface hydrophobicity (S_0).

2.3.7 Particle size analysis (DLS)

The native WPI dispersion with 50 mM sodium phosphate and 100 mM NaCl formed a gel after heating at 80 ℃ for 15 min. To overcome this difficulty, this 5% w/v WPI dispersion was prepared without sodium phosphate but with 100 mM NaCl to produce a flowable sample after heating. Other samples were prepared as in the pretreatment protocol and adjusted to 100 mM NaCl. Protein dispersions before and after heating at 80 ℃ for 15 min were measured using a Delsa^{TM} Nano particle size/zeta potential analyzer from Beckman Coulter, Inc. (Brea, CA). The volume-length mean particle diameter was calculated using eq 1.

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

where n_i is the number of particles with a diameter d_i.

2.3.8 Zeta-potential

WPI dispersions were diluted to 0.5% w/v using a 50 mM sodium phosphate buffer adjusted to pH 7.0 before zeta-potential measurements using the Delsa^{TM} Nano instrument (Beckman Coulter Inc., Brea. CA). Samples containing 100 mM NaCl also were desalted by 40-h dialysis in deionized water using a membrane with a molecular weight cut-off of 3,500 Da (Fisher Scientific, Fair Lawn, NJ, USA). The bulk deionized water was replaced every 8 h. After dialysis, WPI dispersions were diluted to 0.5% w/v using the phosphate buffer and adjusted to pH 7.0 for zeta-potential analysis. Two replicates were tested for three times each, and the averages from 6 measurements were reported.

2.3.9 Atomic force microscopy (AFM)

The WPI dispersions were diluted in deionized water to an overall protein concentration of 10 ppm. For a gelled sample, the gel was added with deionized water and vortexed overnight. Four μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA). The samples were scanned using a rectangular cantilever probe (FESPA, Bruker Corp., Santa Barbara, CA) with aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m. The imaging was conducted using a Multimode microscope

(Veeco Instruments, Inc., Plainview, NY) operated using the tapping mode. Both height and phase images with a preset scan area of 5×5 or 2×2 μm were generated. Height images were used to measure heights of individual particles using the instrument software and the averages of particle heights were reported.

2.3.10 Rheology

Fifteen mL samples were contained in 20 mL vials and pretreated using the above protocols. After heating at 80 °C for 15 min, samples were transferred into the sample cup of a Searle setup (bob outer diameter = 28 mm and cup inner diameter = 30 mm) of an AR2000 rheometer (TA Instruments, New Castle, DE). Shear rate ramps were performed from 0.1 to 1000 s^{-1} at 20 °C.

2.3.11 Statistical analysis

Results were presented as averages and standard deviations from replicates. All statistical analyses were performed using SAS software (version 9.2, SAS Institute, Cary, NC). A two-way analysis of variance (ANOVA) was carried out to test significance between treatments. Statistical differences among comparable data points for various treatments were calculated by post-hoc comparison of means according to the least-significant-difference (LSD) mean separation method at a *p* level of 0.05.

2.4 Results and Discussion

The mTGase activity was measured to be 102.1 U/g powder. The levels of mTGase were thus 2.0, 5.1, and 10.2 U/g-WPI when the mTGase powder was used at 2,

5, and 10% mass of WPI. Absorbance values at 400 and 600 nm for heated samples followed similar trends after pretreatment at various conditions. The absorbance at 600 nm was smaller than that at 400 nm, and only the results at 400 nm are presented here. The results are first presented for heat stability in two groups for samples with and without preheating, followed by properties probed by DLS, AFM, zeta potential, surface hydrophobicity, DSC, and rheology.

2.4.1 Heat stability of samples pretreated by mTGase without preheating

Heat stability was compared for absorbance because bigger structures formed during thermal treatment cause higher absorbance/turbidity. Samples pretreated by three levels of mTGase without preheating were heated at 80 °C for 15 min, and the absorbance values are shown in Figure 2-1A, B, and C for pH 7.0 samples at 0, 50, and 100 mM NaCl, respectively. A higher level of mTGase and a longer treatment time generally resulted in a lower absorbance value. Samples at 0 mM NaCl were all clear after heating, corresponding to an absorbance value lower than 0.45 at 400 nm (Figure 2-1A) and below 0.1 at 600 nm (not shown). The heat stability of WPI at a low ionic strength and neutral pH is well-known, due to strong electrostatic repulsion (Bryant & McClements, 1998). At 50 mM NaCl, samples pretreated by mTGase to a greater extent had lower absorbance (Figure 2-1B), consistent with improved visual clarity of dispersions. At 100 mM NaCl, the control sample and the sample treated with the least amount of mTGase for the shortest duration (at 2.0 U/g-WPI for 1 h, Figure 2-1C) formed gels after heating, while other samples were fluidic, with improved clarity after being pretreated by a higher level of mTGase for a longer time. The statistical analysis

showed the significances ($p < 0.05$) of mTGase level and incubation time during pretreatments and significant impacts ($p < 0.05$) of NaCl concentration on sample turbidity.

2.4.2 Heat stability of samples sequentially pretreated by preheating and mTGase

After preheating at 80 °C for 15 min at pH 7.5 and 0 mM NaCl, samples were pretreated by mTGase before adjusting to pH 7.0 and 0-100 mM NaCl for a second time heating at 80 °C for 15 min. The control with preheating only was transparent at 0 mM NaCl, slightly turbid at 50 mM NaCl and turbid but flowable at 100 mM NaCl. Preheating alone thus improved heat stability, when comparing to the gel formation at 100 mM NaCl for native WPI (Figure 2-1C). All sequentially pretreated samples were visually clear, with absorbance values presented in Figure 2-2. At 0 mM NaCl, the absorbance values of samples (Figure 2-2A) were similar to those without preheating (Figure 2-1A). Opposite to observations from samples directly pretreated by mTGase (Figure 2-1), samples preheated and pretreated by a higher mTGase level overall had a higher absorbance value (Figure 2-2), with a reduction in absorbance from the control (with preheating only) much less significant than the corresponding treatment without preheating.

Overall, the sequentially-pretreated WPI had much improved heat stability than those with preheating or mTGase pretreatment only, demonstrated in Figure 2-3 corresponding to an enzyme level of 5.1 U/g-WPI and a cross-linking duration of 4 h. It has been shown that native whey proteins are not very susceptible to cross-linking by mTGase (Han & Damodaran, 1996), particularly the most abundant β-lactoglobulin

(Ercili-Cura, Partanen, Husband, Ridout, Macierzanka, Lille, et al., 2012; Reddy, Kellam & Kinsella, 1988), possibly because glutamine and lysine groups involved in the cross-linking reaction are buried within the protein core and inaccessible by the enzyme (Cozzolino, Di Pierro, Mariniello, Sorrentino, Masi, & Porta, 2003). The mTGase cross-linking is much facilitated after preheating to a temperature above the denaturation temperatures of whey proteins (Eissa, Puhl, Kadla, & Khan, 2006; Han & Damodaran, 1996; Rodriguez-Nogales, 2006b; Sharma, Zakora, & Qvist, 2002). This was verified in our preliminary experiments using sodium-dodecyl-sulfite polyacrylamide gel electrophoresis that demonstrated no, limited, and extensive cross-linking for samples pretreated by preheating only, mTGase only, and sequential steps, respectively. To further understand structural formation as impacted by pretreatments, the following sections present findings from treatments using mTGase at 5.1 U/g-WPI for 4 h.

2.4.3 Particle size changes measured by DLS

Table 2-1 shows $d_{4,3}$ of WPI dispersions after various pretreatments. The $d_{4,3}$ increased from ~10 nm of the untreated WPI dispersion to ~32 nm after preheating at 80 ℃ for 15 min. Results in Table 2-1 are slightly higher than the hydrodynamic radius of β-lactoglobulin, α-lactalbumin, and bovine serum albumin that is 2.6-4.9 (Parker, Noel, Brownsey, Laos, & Ring, 2005), 2.0 (Molek & Zydney, 2007), and 3.7 nm (Brownsey, Noel, Parker, & Ring, 2003), respectively, possibly because the dispersion contained 50 mM sodium phosphate that causes some flocculation of proteins. The increase in particle size after preheating is expected because whey proteins are known to form filament-type aggregates after heating at neutral or slightly alkaline pH and low ionic strength (Bryant

& McClements, 1998; Foegeding, Davis, Doucet, & McGuffey, 2002). When pretreated by mTGase directly, the $d_{4,3}$ increased slightly to ~12 nm, indicating a small degree of cross-linking that resulted in a structure smaller than that after preheating. The sequentially-pretreated WPI dispersion had the largest $d_{4,3}$ of ~37 nm, indicating the further formation of structures from preheated whey protein by mTGase cross-linking. When comparing the sequentially-pretreated WPI and that directly pretreated by mTGase, the particle size and the net increase in particle size (from preheated sample or control) were much bigger for the sequential pretreatment, further verifying the increased reactivity of whey protein after preheating. After adjusting to 100 mM NaCl and heating at 80 ℃ for 15 min, the sequentially-pretreated dispersion had an insignificant change in $d_{4,3}$, while the increase was significant for other samples, following the order of control without pretreatment > mTGase pretreatment > preheating. Because bigger structures scatter light to a greater extent, DLS results are in agreement with visual appearance (Figure 2-3) and absorbance (Figures 2-1 and 2-2).

2.4.4 Structures of whey proteins studied by AFM

Nanoscale structures of whey proteins after various pretreatments were studied using AFM for samples before and after heating at 100 mM NaCl. Images scanned at 2×2 μm are presented in Figure 2-4, while those at 5×5 μm are shown in supplementary Figure 2-S1. Images scanned at 2×2 μm were also used to measure average heights of particles that are listed in Table 2-2. Native whey proteins were mostly monomeric, with some dimer-like structures. The average height of whey proteins was 3.37 nm, which is similar to the reported dimension of individual whey protein molecules, particularly that

of β-lactoglobulin (Aymard, Nicolai, Durand, & Clark, 1999; Elofsson, Dejmek, Paulsson, & Burling, 1997). After heating at pH 7.0 and 100 mM NaCl, WPI formed a turbid gel. The vortexed gel revealed coarse particulate aggregates with a height of ~33 nm, consistent with the literature studies (Ikeda & Foegeding, 1999; Ikeda & Morris, 2002).

Figure 2-4B shows preheated WPI before and after heating at 80 ℃ for 15 min. Preheating WPI at pH 7.0 in the absence of NaCl corresponded to clusters of granular aggregates with a height of ~7 nm. Aggregates with an increased height of 16 nm were observed after heating for a second time in the presence of 100 mM NaCl, corresponding to translucent appearance (Figure 2-3B). Ikeda and Morris (Ikeda & Morris, 2002) previously revealed similar heat-induced structures of WPI and β-lactoglobulin, presumably through steps of the initial formation of granular aggregates with varying sizes and the subsequent aggregation between these granular aggregates. This results in the co-existence of small particles with varied sizes depending on ionic strength and duration of heat treatment and much larger aggregates of small particles.

After being pretreated with mTGase without preheating, the structure of whey proteins (Figure 2-4C, left) was similar to that of native WPI (Figure 2-4A, left), but the particle height increased to 6.45 nm, indicating limited cross-linking. After heating at 100 mM NaCl, aggregates were observed (Figure 2-4C, right), with an average height of 17 nm (Table 2-2), which was smaller than the structure formed from native WPI.

After sequential pretreatments, granular aggregates with an average height of ~10.73 nm were observed (Figure 2-4D, left; Table 2-2; supplementary Figure 2-S1). Both morphology (supplementary Figure 2-S2) and height (Table 2-2) suggest a greater

extent of aggregation than a single pretreatment step. This verifies the improved enzymatic reactivity of WPI after preheating (Eissa, Puhl, Kadla, & Khan, 2006; Han & Damodaran, 1996; Rodriguez-Nogales, 2006b; Sharma, Zakora, & Qvist, 2002) and crosslinking of structures formed after preheating. After the second heating in the presence of 100 mM NaCl, the morphology of aggregates was similar to that before heating (image D in Figure 2-4 and supplementary Figure 2-S1). The average height increased to 12 nm, but the increase was not significant (Table 2-2).

To further study the aggregation ability of WPI at 100 mM NaCl, pretreated samples were heated at 80 °C for 90 min. The samples (supplementary Figure 2-S2) had similar appearance as in Figure 2-3, except that samples with one pretreatment step only became more turbid than those in Figure 2-3. Changes in sample appearance were in agreement with $d_{4,3}$ data that showed a constant dimension for the sequentially-pretreated sample but increased dimensions at a longer heating time for those with only one pretreatment step (Table 2-1). The morphology based on AFM generally agreed with the DLS (Figure 2-5, supplementary Figures 2-S3 and 2-S1). For the sample pretreated by mTGase only, the morphology (Figures 2-4 and 2-5) and particle height (Table 2-2) after heating for 90 min was similar to the native WPI heated for 15 min. This indicates that WPI cross-linked by mTGase to a limited extent aggregates similarly to native WPI, except at a slower rate. In contrast, AFM particle heights (Table 2-2) of the sequentially-pretreated samples were very similar at heating durations of 15 and 90 min, which, together with constant $d_{4,3}$, suggests that cross-linked structures are stable during heating at 100 mM NaCl. For the preheated WPI, the $d_{4,3}$ was bigger at a longer heating duration but AFM particle heights remained unchanged at the two heating durations. Therefore,

denatured whey proteins after preheating at studied conditions can still aggregate at 100 mM NaCl, with possible interpretation given in the later section.

Overall, the measured particle heights from AFM (Table 2-2) are much smaller than the dimensions measured in DLS (Table 2-1). In a dispersion, the measured dimension (hydrodynamic diameter) in DLS indicates the overall space occupied by the aggregated particles and is expected to be bigger than the height of granular aggregates (Table 2-2) that is smaller than the dimension suggested by aggregate morphology (Figures 2-4 and 2-5). Furthermore, the 5% w/v dispersion used in particle size measurements was diluted to 10 ppm in AFM, and weakly flocculated aggregates may be measured in DLS but not AFM. The drying process also can reduce the dimension of aggregates, resulting in smaller particle heights measured in AFM.

2.4.5 Zeta potential and surface hydrophobicity

The long-range electrostatic repulsion and shorter range hydrophobic attraction are critical for the stability of whey protein (Bryant & McClements, 1998; Rabiey & Britten, 2009). Table 2-3 shows the zeta-potential of WPI samples at pH 7.0 after various pretreatments and subsequent heating at pH 7.0 and 0 or 100 mM NaCl for 15 min at 80 °C. The zeta-potential of native WPI dispersion was -27 mV at pH 7.0 and became more negative after pretreatments, with the magnitude increased further after heating at 0 mM NaCl. The results in Table 2-3 are higher than the -24.6 mV of native WPI and -26.6 mV of WPI heated at 90 °C for 10 min at pH 6.8 (Ryan, Vardhanabhuti, Jaramillo, van Zanten, Coupland, & Foegeding, 2012), and the difference can be due to different supplies of WPI, pH, and heating conditions. For the crosslinking catalyzed by mTGase,

the reaction between an acyl donor (glutamine) and an acyl acceptor (lysine) results in the loss of an amino group (Jaros, Partschefeld, Henle, & Rohm, 2006), which reduces the number of positive charges. Cysteine, with pKa of 8.5, is an amino acid with free thiol groups available for formation of intra- and intermolecular disulfide bonds after heating (Bryant & McClements, 1998), which may reduce the surface positive charges. Furthermore, denaturation causes the redistribution of surface amino acids and the number of negatively-charged surface amino acids can increase after heating and/or mTGase cross-linking. Because zeta potential increased more significantly after heating than after mTGase treatments (Table 2-3), whey protein structure changes due to thermal denaturation are the major cause of increased zeta-potential. The increased zeta-potential, i.e. electrostatic repulsion of pretreated WPI can reduce the aggregation during heating but is not the major cause of the improvement in heat stability, because the preheated WPI (with zeta potential of -33.50 mV) formed bigger structures (Tables 2-1 and 2-2) and more turbid dispersion (Figure 2-3) than the sequentially-pretreated WPI (with zeta potential of -32.72 mV).

For the samples heated at 100 mM NaCl, the zeta-potential was significantly lower than that before heating when samples were measured directly. After dialysis, the measured zeta-potential was significantly higher than that before dialysis and that before heating, and was similar to that heated at 0 mM NaCl. The increased zeta-potential measured after dialysis is expected because colloidal particles have an increased mobility at decreased ionic strength due to the shortened Debye length, which was observed in the electrophoresis study (Israelachvili, 1992).

As for surface hydrophobicity, the S_0 increased after all pretreatment steps (Table 2-4). Heating caused a greater increase in S_0 than mTGase, and the increase in S_0 was greater when heated at 0 mM NaCl (the preheated sample) than at 100 mM NaCl (the control sample). Thermal denaturation causes the exposure of initially embedded hydrophobic amino acid residues and therefore the increase in S_0 (Zhu & Damodaran, 1994). The protection of NaCl against thermal denaturation has been reported for a higher extent of whey protein denaturation at 0 mM NaCl (~100%) than at 100 mM NaCl (~10%) (Nicorescu, Loisel, Vial, Riaublanc, Djelveh, Cuvelier, et al., 2008). The overall trend in Table 2-4 is consistent with the results of Ryan *et al*. (Ryan, Vardhanabhuti, Jaramillo, van Zanten, Coupland, & Foegeding, 2012). Because an increase in S_0 favors protein aggregation, changes in S_0 are not responsible for the observed heat stability of WPI after pretreatment.

2.4.6 Thermal denaturation properties of whey proteins after pretreatments

All DSC thermograms of samples at pH 7.0 and 100 mM NaCl showed an endothermic peak (Figure 2-6), with the estimated ΔH and T_d summarized in Table 2-5. Thermal denaturation of whey proteins involves both endothermic and exothermic processes, with the former attributed by protein unfolding due to mechanisms such as disruption of intramolecular hydrogen bonds and the latter resulting from hydrophobic attraction and formation of covalent (disulfide) bonds during the aggregation of denatured protein (Fitzsimons, Mulvihill, & Morris, 2007; Nicorescu, et al., 2008; Unterhaslberger, Schmitt, Sanchez, Appolonia-Nouzille, & Raemy, 2006). The overall thermogram depends on dispersion pH, ionic strength, protein concentration, and heating

rate (Unterhaslberger, Schmitt, Sanchez, Appolonia-Nouzille, & Raemy, 2006). The T_d of native WPI at 100 NaCl was at 77 ℃, which is in a good agreement with the literature (Haug, Skar, Vegarud, Langsrud, & Draget, 2009; Nicorescu, et al., 2008; Unterhaslberger, Schmitt, Sanchez, Appolonia-Nouzille, & Raemy, 2006). The center of endothermic peak of preheated WPI shifted to 80 ℃, with a much reduced intensity when compared to native WPI, corresponding to increased T_d and decreased ΔH that indicate improved thermal stability of WPI after preheating. Preheated WPI is highly charged due to increased electrostatic repulsion and formation of covalent disulfide bonds during preheating, thus exhibited greater resistant to salt-induced aggregation. The T_d and ΔH of WPI cross-linked by mTGase directly were not statistically different from those of native WPI, suggesting limited improvement of heat stability. This agrees with studies showing that native α-lactalbumin and β-lactoglobulin are not very reactive substrates during mTGase cross-linking (Han & Damodaran, 1996). Preheating partially unfolds whey protein and increase the susceptibility to cross-linking by mTGase (Rodriguez-Nogales, 2006b; Sharma, Zakora, & Qvist, 2002; Zhang & Zhong, 2010), corresponding to significantly increased T_d and decreased ΔH of sequentially-pretreated WPI. Because zeta-potential alone is insufficient to interpret the most improved heat stability after sequent pretreatments, the denaturation properties of whey protein are responsible for thermal stability observed in Figure 2-3.

2.4.7 Viscosity of samples heated at 80 ℃ for 15 min after various pretreatments

Shear rate ramps of WPI samples after heating at 80 °C for 15 min showed typical shear-thinning properties, i.e., a reduced viscosity at a higher shear rate, shown in

supplementary Figure 2-S4 for those with 0 mM NaCl. Whey proteins are globular proteins, and the aggregation of whey proteins during heating has been described in the theoretical framework of colloidal interactions (Bryant & McClements, 1998). For colloidal dispersions, the dependence of apparent viscosity on shear rate measured using a mechanical rheometer typically shows a plateau regime at the low shear rate regime, called zero-shear viscosity (η_o) and another plateau at the high shear rate regime, called infinite-shear viscosity (η_∞), illustrated in supplementary Figure 2-S4. There was no identifiable η_o for all samples tested, possibly because the lowest shear rate used in tests, 0.1 s^{-1} (10 times of the instrument limit of 0.01 s^{-1}), was not sufficiently low.

The impact of various pretreatment conditions on whey protein structures is best indicated by η_o that is not interfered by external shear force. The apparent viscosity of samples at 0.1 s^{-1} did not show any trend as impacted by the studied pretreatment conditions and is thus not reported here. This may have been caused by two factors. First, the apparent viscosity at 0.1 s^{-1} was not η_o, as discussed above. Second, samples were heated in vials before being loaded in the sample cup of rheometer because of the need to adjust solvent conditions during sample preparation. Transferring samples during the process may interrupt some structures formed during pretreatments and the final heating step. On the other hand, η_∞ is another indicator of sample characteristics because weaker interactions are disrupted by shear, which also can overcome the errors resulting from sample preparation.

Apparent viscosities at 1000 s^{-1} are treated as η_∞ for samples heated at 80 °C for 15 min after pretreatment at various conditions. For samples without preheating (Figure

2-7), the general trend was similar to absorbance values as impacted by pretreatments (Figure 2-1): lower η_∞ at a greater extent of enzymatic treatment. For preheated samples (Figure 2-8), samples treated by a higher enzyme level showed higher η_∞. At each NaCl concentration, the maximum η_∞ was observed for WPI pretreated by mTGase for 4 h at the highest level of enzyme and 8 h at two other enzyme levels, and the overall trend is similar to the impact of pretreatment on sample absorbance (Figure 2-2).

The agreement between viscosity and absorbance data and the different trend observed for the treatments with and without preheating are to be interpreted carefully. A higher extent of aggregated structures is correlated to a higher viscosity because of increased inertia against fluid flow (McClements, 2005). Because WPI without preheating is not an active substrate of mTGase, the extent of cross-linking is expected to follow reaction kinetics that is a function of reaction time and mTGase concentration. Since cross-linked whey proteins are stable against aggregation during heating at 80 °C for 15 min, thermal aggregation is less extensive for samples subjected to a higher degree of enzyme treatment, corresponding to a lower absorbance value (Figure 2-1) and viscosity after heating (Figure 2-7). In contrast, the preheated WPI is cross-linked by mTGase easily and becomes resistant against aggregation during the final heating step at 80 °C for 15 min. The extent of mTGase cross-linking then determines absorbance and viscosity, which shows a higher absorbance value and viscosity at a higher mTGase level at the same ionic strength. At the same level of mTGase, the highest absorbance and viscosity were observed at an intermediate cross-linking duration. It is possible that individual aggregates formed during preheating are cross-linked initially, corresponding

to an increase in viscosity, followed by cross-linking between initially cross-linked structures, which reduces viscosity if cross-linking is not end-to-end. This corresponds to the maximum space occupied by aggregates at an intermediate cross-linking duration, which is shorter when mTGase is used at a higher level, as indicated by absorbance and viscosity data (Figures 2-2 and 2-8).

2.4.8 Physical interpretation of thermal aggregation of whey protein at 100 mM NaCl and neutral pH as impacted by mTGase pretreatment

Based on the nanoscale structural information reported in this work, the impacts of pretreatments on thermal aggregation of whey protein at 100 mM NaCl can be summarized in Figure 2-9. Aggregation between colloidal particles is either reaction- or diffusion-limited (Russel, Saville, & Schowalter, 1989). For native WPI, proteins are denatured during heating and become more hydrophobic, and the weakened electrostatic repulsion at an increased ionic strength (100 mM NaCl) favors protein aggregation. This corresponds to diffusion-limited aggregation, resulting in turbid gels formed of particular aggregates with much increased $d_{4,3}$ and AFM particle height (Tables 2-1 and 2-2). Heating whey proteins at low ionic strength is known to be diffusion-limited, forming filament-type aggregates (Bryant & McClements, 1998). In this work, preheating was conducted at pH 7.5 with 50 mM sodium phosphate for limited duration, forming flake-like aggregates (Figure 2-4 and supplementary Figure 2-S1) with mostly two-three layers of denatured proteins (Table 2-2). The remaining protein molecules can still be denatured and join the aggregates during the second heating with 100 mM NaCl, corresponding to both increased $d_{4,3}$ and AFM particle height (Tables 2-1 and 2-2) when heated for 15 min.

At an elongated heating duration, the initially formed aggregates form bigger structures laterally due to excluded volume, corresponding to increased $d_{4,3}$ and similar AFM particle height (Tables 2-1 and 2-2). Compared to native WPI, the increased zeta-potential and T_d after preheating reduce the extent of aggregation, corresponding to fluidic translucent dispersion.

For samples directly pretreated by mTGase, the aggregation characteristics are similar to native WPI, except to a less extent due to a portion of cross-linked structures that are stable against thermal aggregation. For the sequentially-pretreated sample, it can be deduced that mTGase crosslinking occurs mostly between proteins within flakes formed during preheating and those between flakes and individual proteins, corresponding to bigger AFM particle height and $d_{4,3}$ than the preheated sample. The cross-linked whey proteins have much higher T_d, corresponding to practically constant particle heights and $d_{4,3}$ during heating with 100 mM NaCl (Tables 2-1 and 2-2) and transparent dispersions (Figure 2-3 and supplementary Figure 2-S2).

2.5 Conclusions

Individual or combined pretreatment methods of preheating and mTGase improved heat stability of WPI to different degrees. The sequential pretreatment showed the best improvement as transparent dispersions were observed for the three NaCl concentrations tested. When comparing to increased zeta-potential and surface hydrophobicity after different pretreatments, the reduced protein aggregation resulted from the increased T_d. The excellent agreement between absorbance and viscosity results revealed the structure formation kinetics as catalyzed by mTGase. Nanostructures

assessed by DLS and AFM revealed nanoscale phenomena underlying macroscopic properties of whey protein aggregation as impacted by prior pretreatments. Native WPI and that pretreated by mTGase directly to a limited extent aggregated similarly, forming particulate structures. Preheating increased the resistance of whey protein against thermal aggregation, and the subsequent heating at increased ionic strength involved the addition of free proteins and lateral aggregation of initially formed structures. Sequential pretreatments induced the formation of aggregates resistant against aggregation, enabling transparent dispersions regardless of heating duration and ionic strength. Findings of this work may be used to produce transparent beverages with added salts such as sports drinks.

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Appendix

Table 2-1. The $d_{4,3}$ of whey protein dispersions after pretreatments and after further heating at pH 7.0 and 100 mM NaCl for 15 and 90 min at 80 °C.

Pretreatment conditions [a]	$d_{4,3}$ (nm) [b]		
	After pretreatment	After heating for 15 min + 100 mM NaCl	After heating for 90 min + 100 mM NaCl
None (control)	9.8 ± 2.6^{G}	93.0 ± 3.9^{A}	-
Preheating only	31.7 ± 1.3^{F}	52.1 ± 3.8^{D}	68.7 ± 2.6^{C}
mTGase only	11.9 ± 2.3^{G}	65.2 ± 3.4^{C}	82.0 ± 5.0^{B}
Sequential preheating and mTGase	$37.0 \pm 3.3^{E,F}$	36.7 ± 1.0^{E}	36.5 ± 4.4^{E}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are mean ± standard deviation from two replicates, each measured for three times. Different superscripts indicate statistical difference in mean ($p < 0.05$).

Table 2-2. Particle heights estimated from AFM for WPI after pretreatments and after further heating at pH 7.0 and 100 mM NaCl for 15 and 90 min at 80 °C.

Pretreatment conditions [a]	Particle height (nm) [b]		
	After pretreatment	After heating for 15 min	After heating for 90 min
None (control)	3.37 ± 0.26^{F}	32.59 ± 1.10^{A}	-
Preheating only	$7.44 \pm 0.69^{D,E}$	16.46 ± 0.40^{B}	16.32 ± 1.58^{B}
mTGase only	$6.45 \pm 0.37^{E,F}$	17.23 ± 3.34^{B}	30.16 ± 1.27^{A}
Sequential preheating and mTGase	$10.73 \pm 1.99^{C,D}$	12.37 ± 1.82^{C}	12.50 ± 2.02^{C}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are mean ± standard deviation from duplicate measurements. Different superscript letters represent significant difference in mean ($p < 0.05$).

Table 2-3. Zeta-potential of WPI dispersions at pH 7.0 after various pretreatments and subsequent heating at pH 7.0 and 0 or 100 mM NaCl for 15 min at 80 °C.

Pretreatment conditions [a]	Zeta-potential (mV) [b]			
	After pretreatment	After heating with 0 mM NaCl	After heating with 100 mM NaCl	After heating with 100 mM NaCl, dialyzed [c]
None (control)	-26.98 ± 0.38^{G}	-35.10 ± 4.04^{C,D}	-26.86 ± 0.39^{G}	-38.58 ± 0.37^{A,B}
Preheating only	-33.50 ± 0.79^{D,E}	-43.58 ± 2.32^{A}	-30.48 ± 1.61^{F,G}	-40.98 ± 1.71^{A,B}
mTGase only	-30.35 ± 0.80^{E,F,G}	-40.18 ± 0.46^{A,B}	-29.03 ± 1.45^{G}	-40.31 ± 2.05^{A,B}
Sequential preheating and mTGase	-32.75 ± 1.51^{D,E,F}	-40.03 ± 0.34^{A,B}	-32.27 ± 0.94^{D,E}	-37.82 ± 1.03^{B,C}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are mean ± standard deviation from two replicates, each measured for three times. Different superscripts indicate statistical difference in mean ($p < 0.05$).
[c] Samples heated at 100 mM NaCl were dialyzed using a membrane with a molecular weight cut-off of 3.5 kDa.

Table 2-4. Surface hydrophobicity (S_0) of WPI measured at pH 7.0 after pretreatments and subsequent heating at 80 ℃ for 15 min in the presence of 100 mM NaCl.

Pretreatment conditions [a]	S_0 (slope $\times 10^6$) [b]	
	After pretreatment	After heating with 100 mM NaCl
None (control)	1.85 ± 0.02^{D}	2.10 ± 0.01^{C}
Preheating only	3.71 ± 0.01^{A}	-
mTGase only	2.29 ± 0.01^{B}	-
Sequential preheating and mTGase	3.66 ± 0.02^{A}	3.76 ± 0.32^{A}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are mean ± standard deviation from duplicate measurements. Different superscript letters represent significant difference in mean ($p < 0.05$).

Table 2-5. Denaturation temperature (T_d) and enthalpy change (ΔH) at pH 7.0 and 100 mM NaCl for WPI dispersions pretreated at different conditions.

Pretreatment conditions [a]	T_d (℃) [b]	ΔH (kcal/g) [b]
None	77.45 ± 0.53^{C}	12.40 ± 0.77^{A}
Preheating only	79.98 ± 0.40^{B}	3.58 ± 0.08^{B}
mTGase only	77.76 ± 0.11^{C}	11.10 ± 0.38^{A}
Sequential preheating and mTGase	82.76 ± 0.06^{A}	2.71 ± 0.10^{B}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are mean ± standard deviation from duplicate measurements. Different superscript letters in each column represent significant difference in mean ($p < 0.05$).

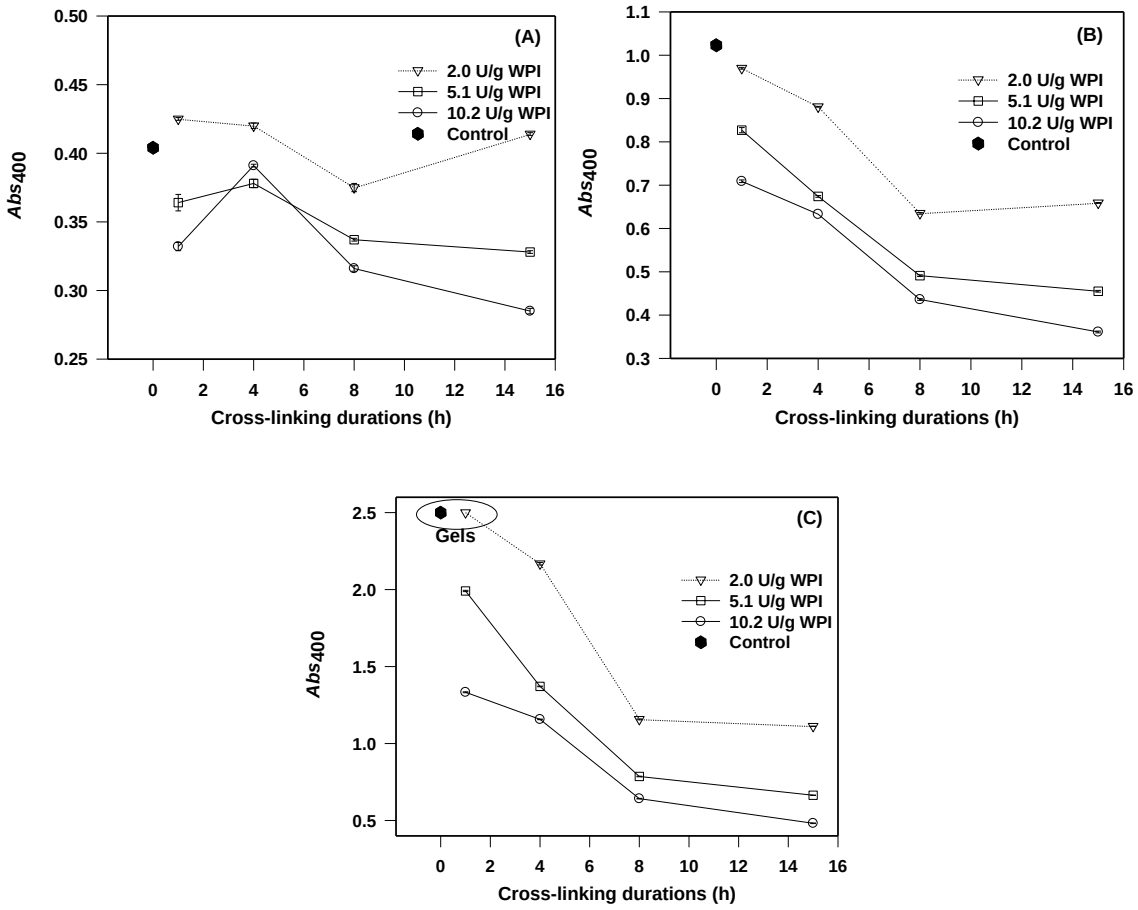


Figure 2-1. Absorbance of 5% w/v WPI dispersions at 400 nm (Abs_{400}) after heating at 80 °C for 15 min. Before heating, samples were adjusted to pH 7.5 and cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g-WPI for 1-15 h at 50 °C before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl. Control samples were heated directly after adjusting pH and NaCl concentration.

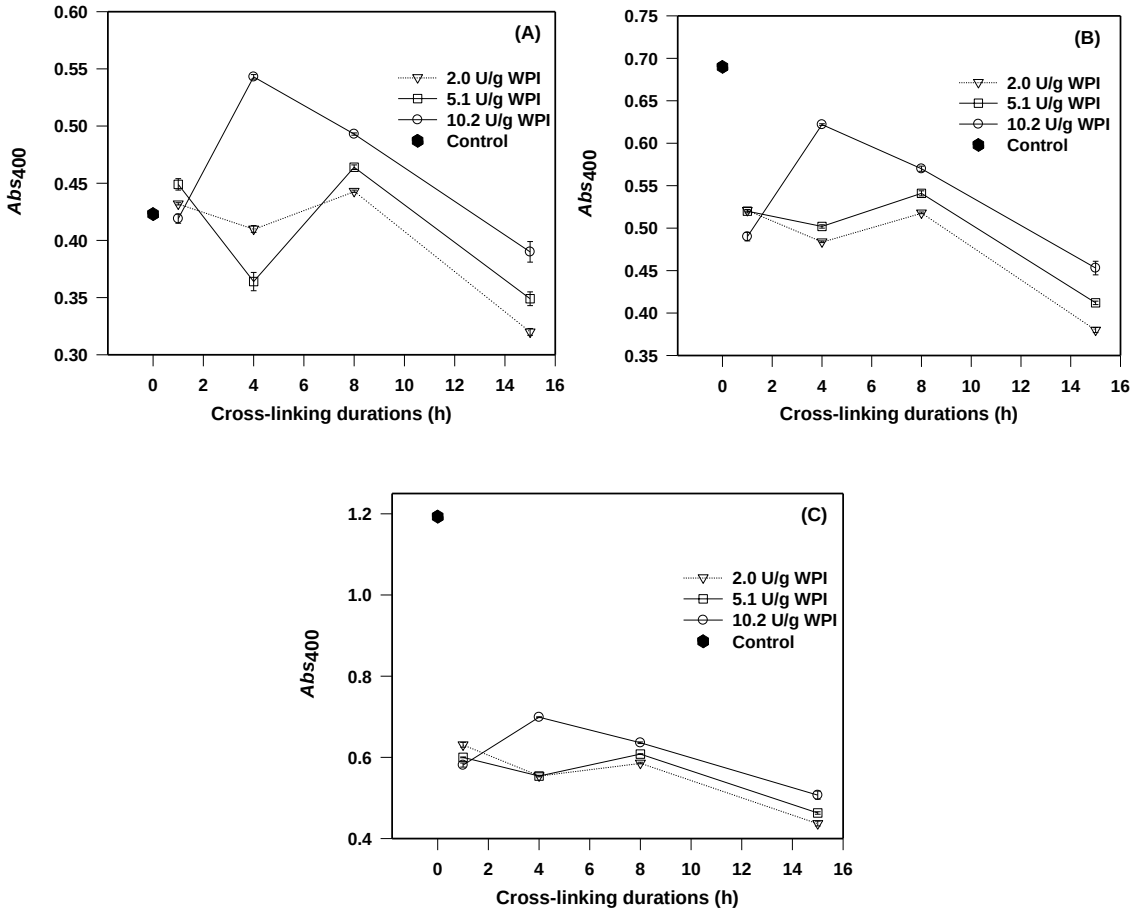


Figure 2-2. Absorbance of 5% w/v WPI dispersions at 400 nm (Abs_{400}) after heating at 80 °C for 15 min. Before heating, samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, and then cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g-WPI for 1-15 h at 50 °C before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl. Control samples were preheated at the same conditions before adjusting pH and NaCl concentration.

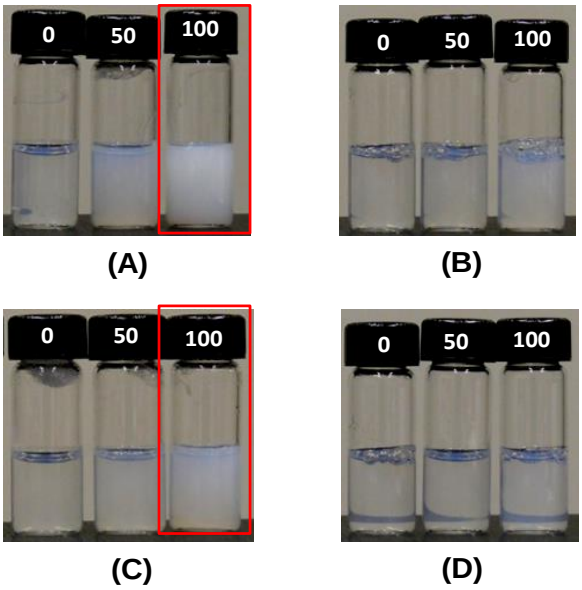


Figure 2-3. Photographs showing appearance of 5% w/v WPI dispersions, adjusted to pH 7.0 and 0, 50, and 100 mM NaCl (as labeled on vials), after being heated at 80 °C for 15 min. Image (A) shows samples without pretreatment. Samples in (B-D) were pretreated differently before heating. Samples in (B) were adjusted to pH 7.5 and preheated for 15 min at 80 °C before pH and NaCl adjustment for second-time heating at 80 °C for 15 min. Samples in (C) were pretreated by mTGase using a level of 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h before adjusting pH and NaCl concentration as in (B). Samples in (D) were preheated as in (B) and then pretreated by mTGase as in (C) before adjusting pH and NaCl concentration as in (B). Samples formed gels are indicated with red rectangles.

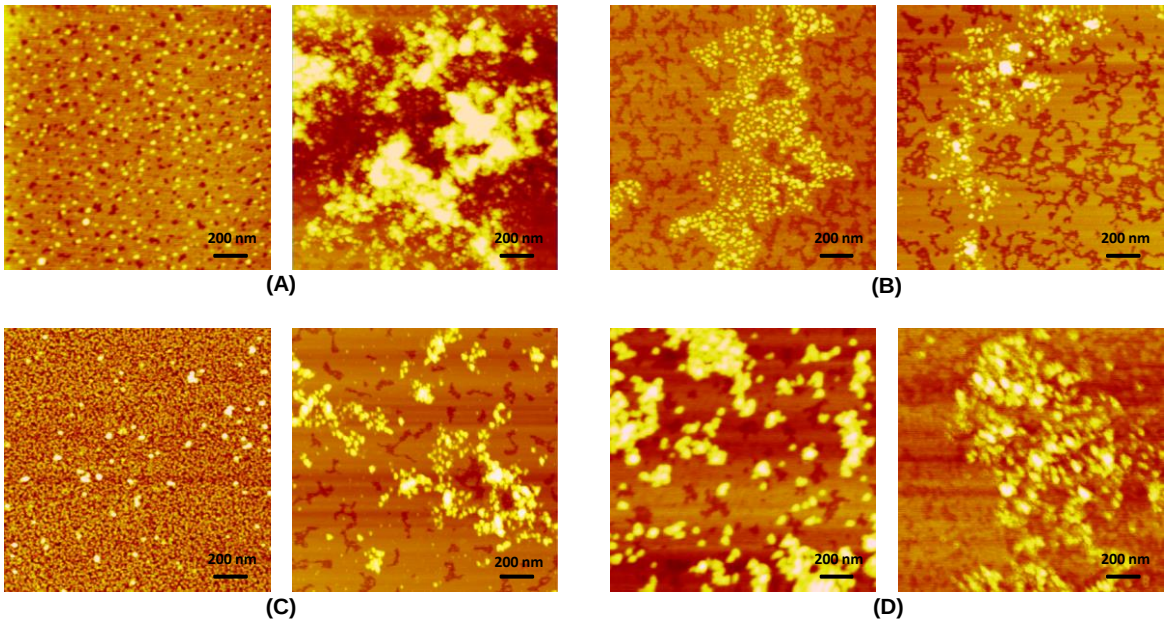


Figure 2-4. AFM height images of whey protein samples before (left) and after (right) heating at 80 °C for 15 min at pH 7.0 and 100 mM NaCl. Before pH and NaCl adjustments, the 5% w/v WPI dispersions were subjected to (A) no pretreatment, (B) preheating at pH 7.5 for 15 min at 80 °C, (C) pretreatment by mTGase at 5.1 U/g-WPI for 4 h, or (D) sequential pretreatments as in (B) and (C). The scanned dimension is 2 ×2 μm in all images

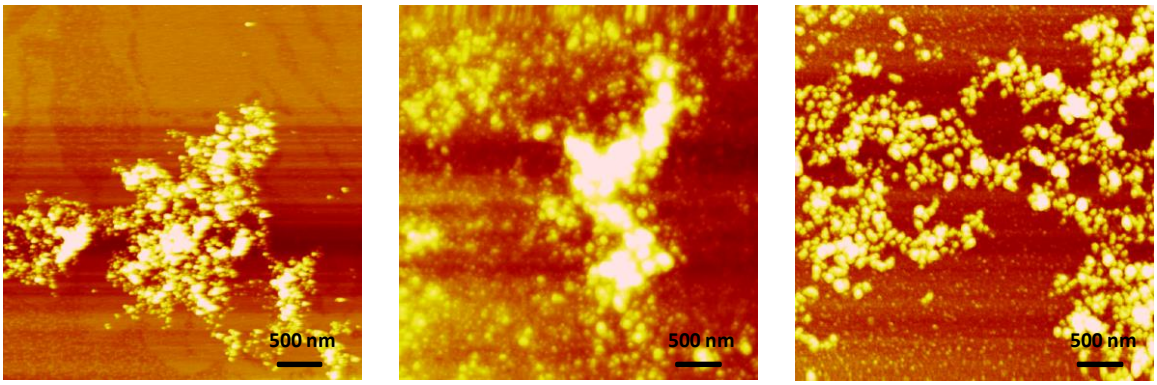


Figure 2-5. Samples in Figure 2-4, at pH 7.0 and 100 mM NaCl, after heating at 80°C for 90 min: (A) preheating only, (B) mTGase pretreatment only, and (C) sequential pretreatments. The scanned dimensions are 5×5 μm in (A) and 2×2 μm in (B) and (C).

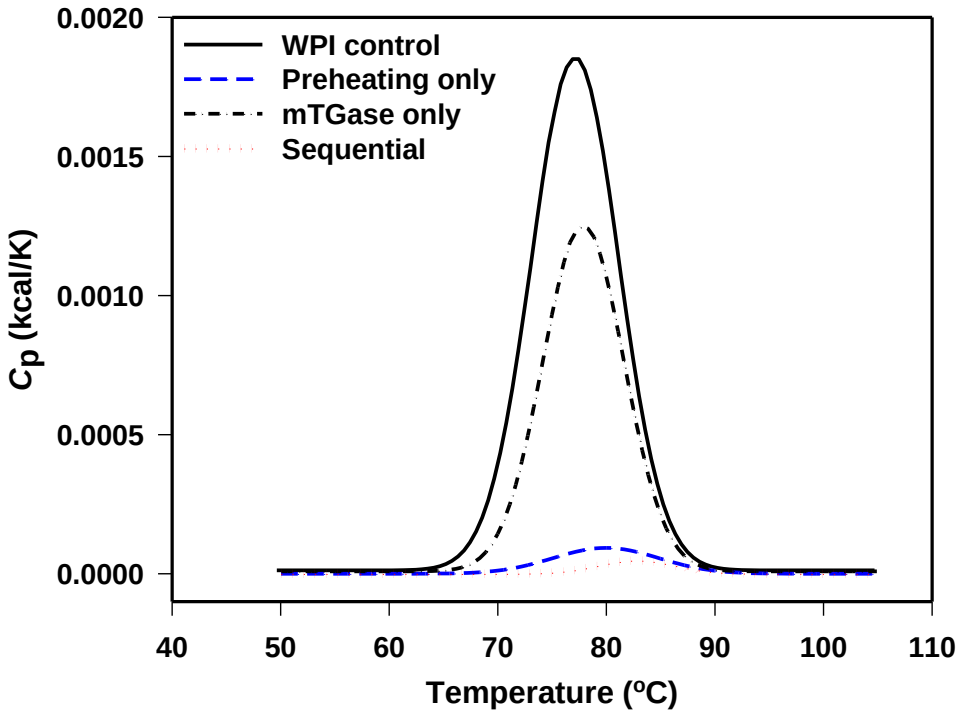


Figure 2-6. Comparison of DSC thermograms of WPI at pH 7.0 and 100 mM NaCl before and after pretreatments as in Figure 2-4.

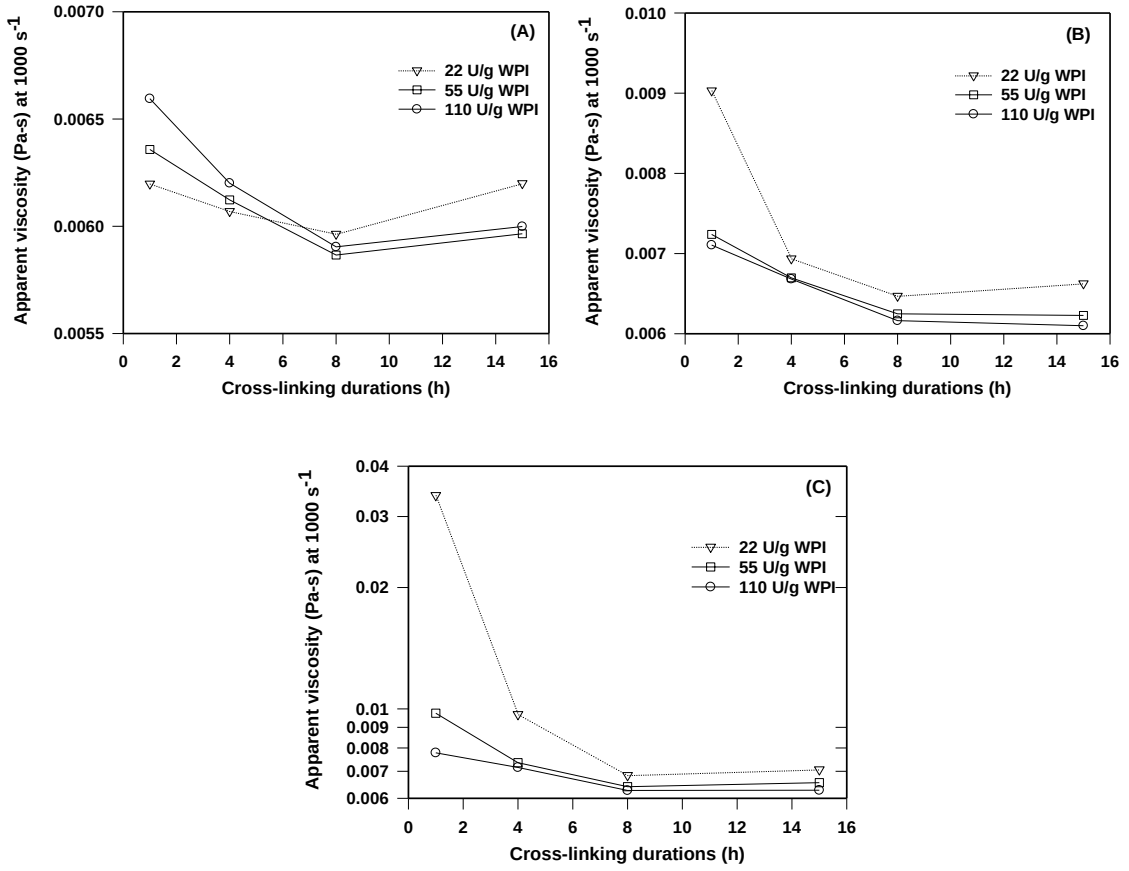


Figure 2-7. Apparent viscosities at 20 °C and 1000 s^{-1} for 5% w/v WPI dispersions heated at 80 °C for 15 min. Before heating, WPI samples were adjusted to pH 7.5 and cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1-15 h at 50 °C before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl.

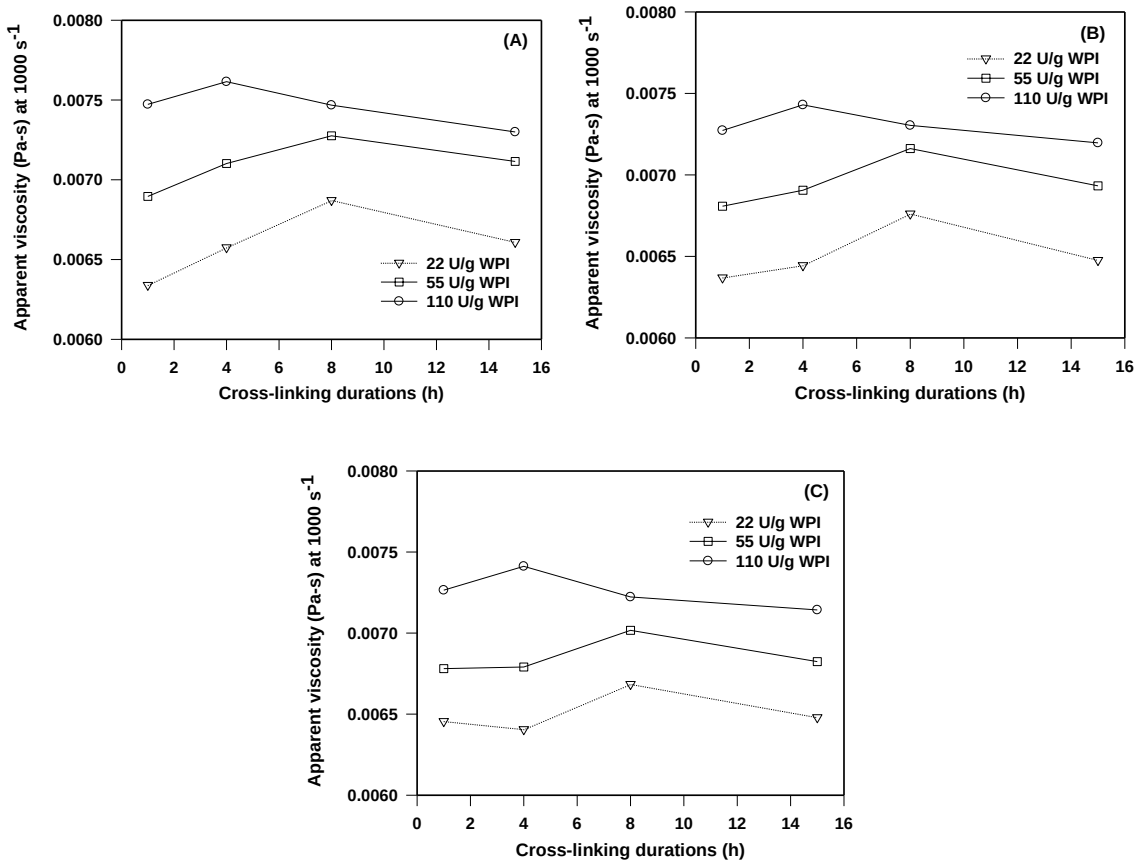


Figure 2-8. Apparent viscosities at 20 °C and 1000 s^{-1} for 5% w/v WPI dispersions heated at 80 °C for 15 min. Before heating, WPI samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1-15 h at 50 °C, and adjusted to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl.

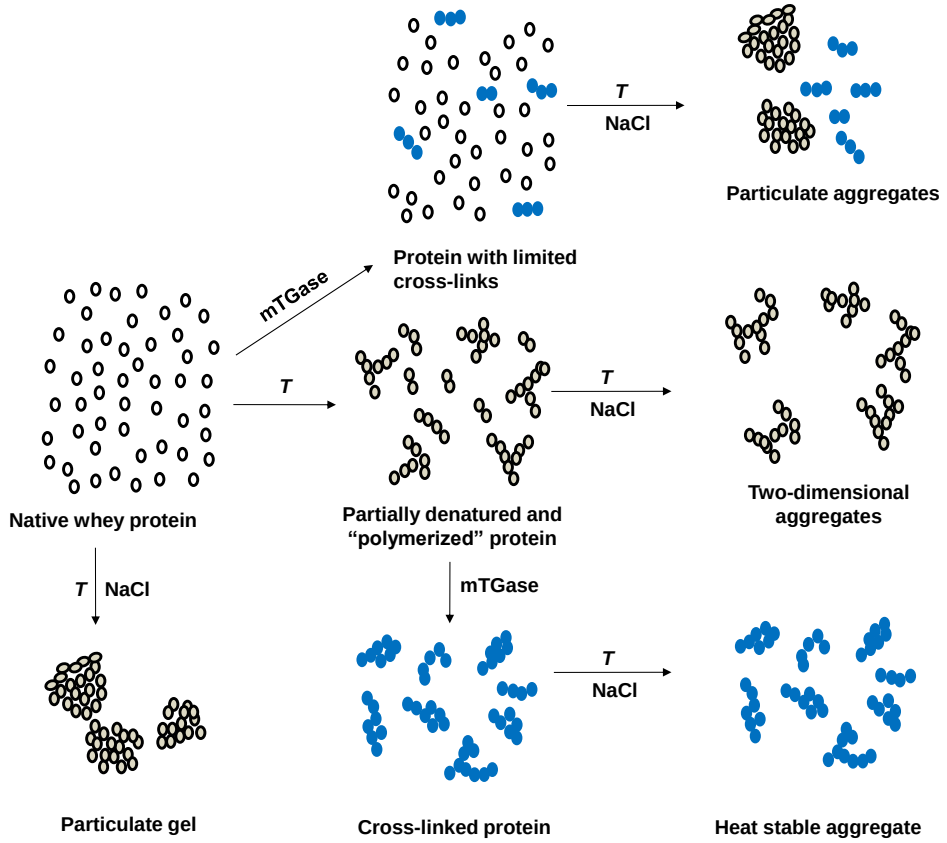


Figure 2-9. Proposed mechanisms of whey protein aggregation during heating at 80 ℃, pH 7.0, and 100 mM NaCl as impacted by individual or sequential preheating and mTGase pretreatments.

Supplementary information

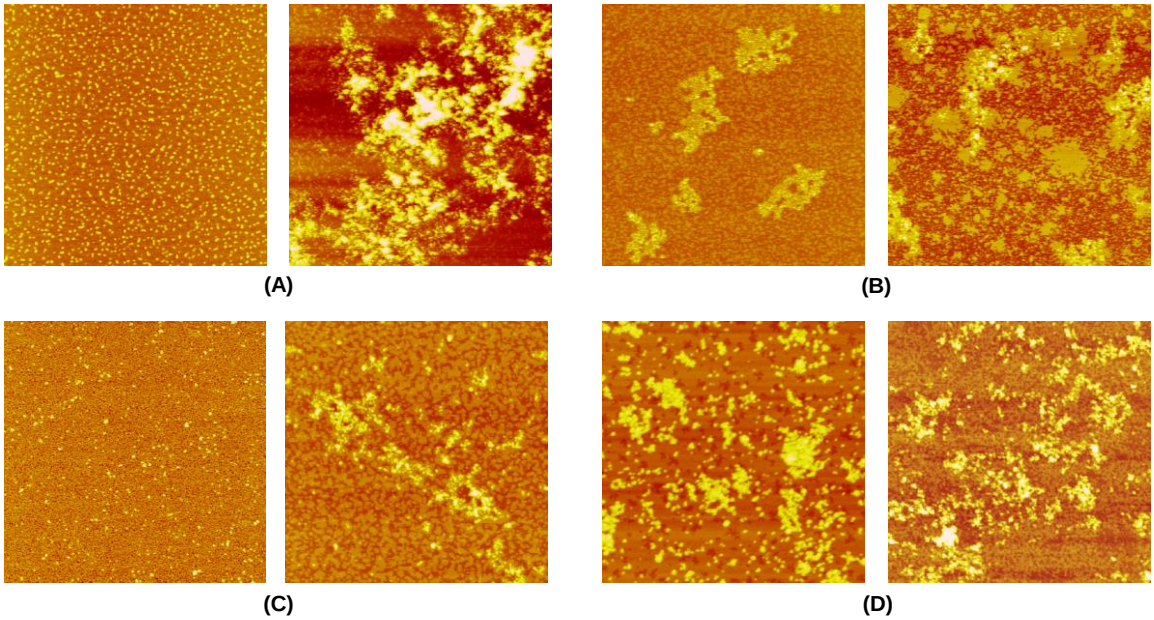


Figure 2-S1. AFM height images of whey protein samples before (left) and after (right) heating at 80 °C for 15 min at pH 7.0 and 100 mM NaCl. Before pH and NaCl adjustments, the 5% w/v WPI dispersions were subjected to (A) no pretreatment, (B) preheating at pH 7.5 for 15 min at 80 °C, (C) pretreatment by mTGase at 5.1 U/g-WPI for 4 h, or (D) sequential pretreatments as in (B) and (C). The scanned dimension is 5×5 μm in all images.

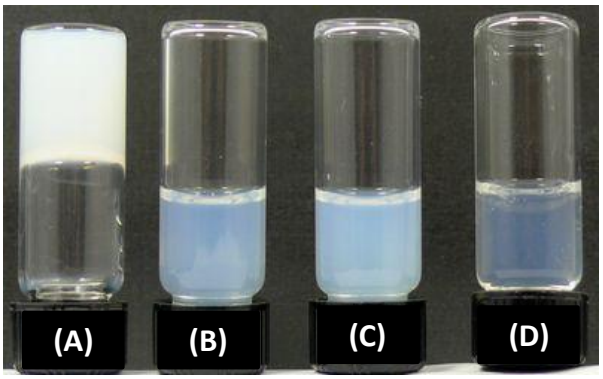
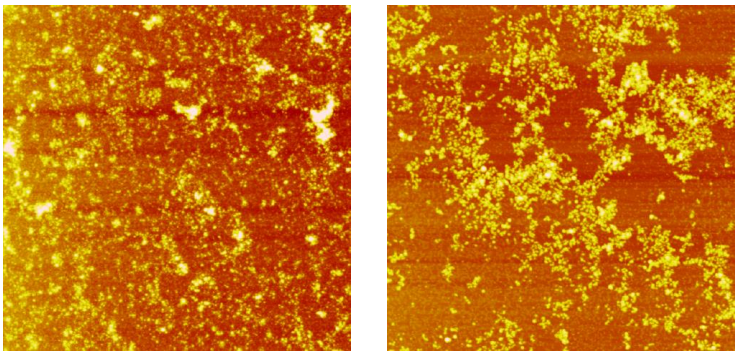


Figure 2-S2. Photographs showing appearance of 5% w/v WPI dispersions, adjusted to pH 7.0 and 100 mM NaCl, after being heated at 80 °C for 90 min. Image (A) shows the sample without pretreatment. Sample (B) was adjusted to pH 7.5 and preheated for 15 min at 80 °C before pH and NaCl adjustment for second-time heating at 80 °C for 90 min. Sample (C) was pretreated by mTGase using a level of 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h before adjusting pH and NaCl concentration. Sample (D) was preheated as in (B) and then pretreated by mTGase as in (C) before adjusting pH and NaCl concentration.



(A) (B)

Figure 2-S3. AFM height images of whey protein samples after heating at 80 °C for 90 min at pH 7.0 and 100 mM NaCl. Before pH and NaCl adjustments, the 5% w/v WPI dispersions were subjected to (A) pretreatment by mTGase at 5.1 U/g-WPI for 4 h, or (B) sequential pretreatments of preheating at pH 7.5 for 15 min at 80 °C and mTGase as in (A). The scanned dimension is 5×5 μm in both images.

Figure 2-S4. Shear rate ramps at 20 °C for 5%w/v WPI dispersions at pH 7.0 and 0 mM NaCl after heating at 80 °C for 15 min. Before heating, samples were pretreated by mTGase at 5.1 U/g-WPI for 1-15 h (A) without or (B) with preheating at 80 °C for 15 min. Figure C is a schematic illustration of shear-rate dependence of apparent viscosity for a typical colloidal dispersion, showing zero-shear (η_o) and infinite-shear (η_∞) viscosities at low and high shear rate regimes.

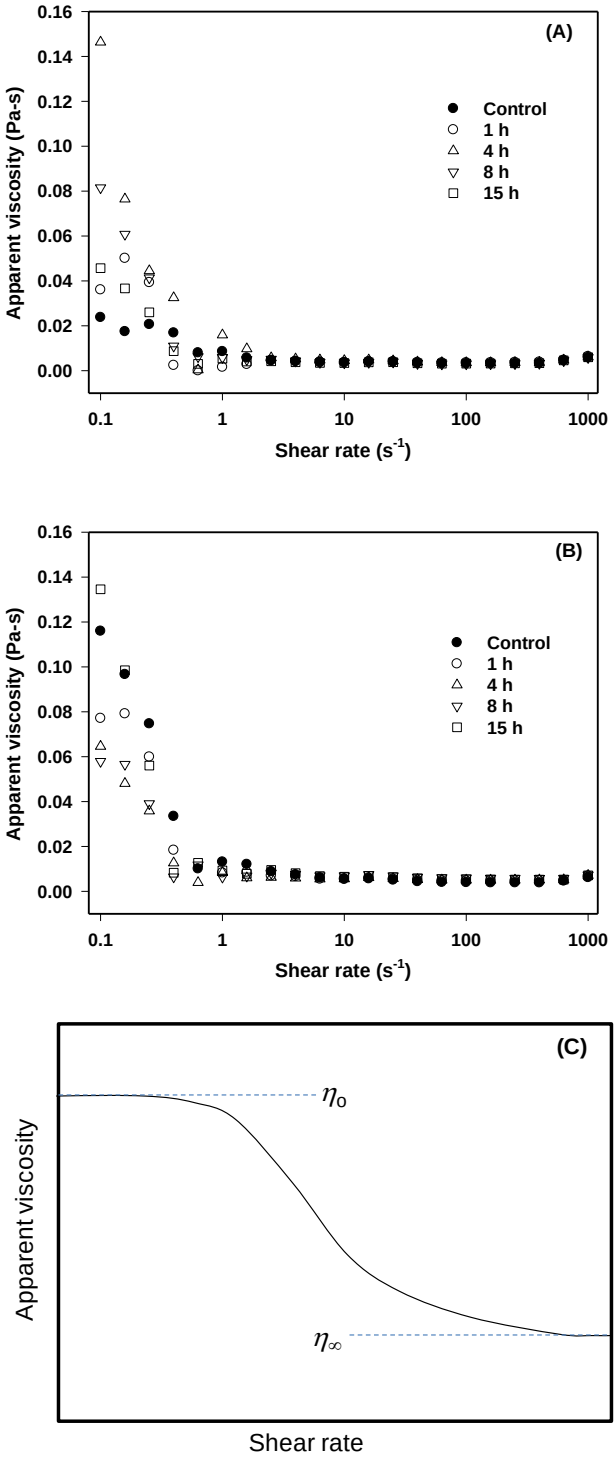
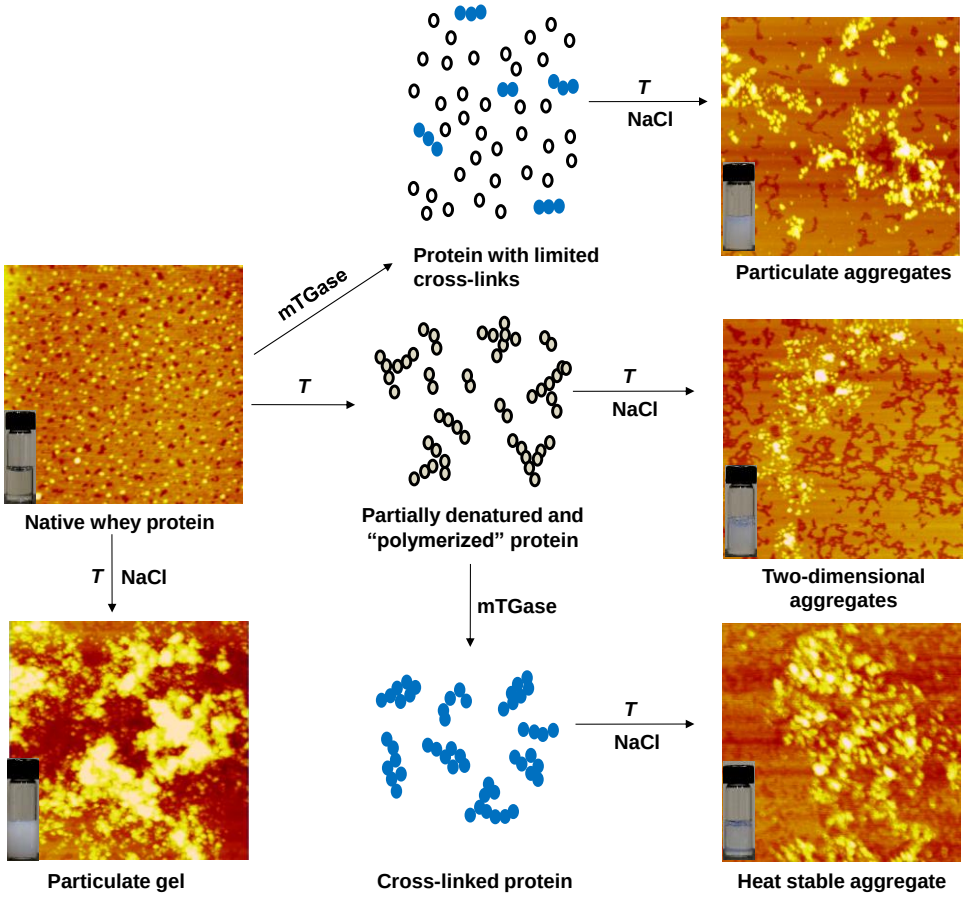


Figure 2-S4. Continued.

TOC graphic


T
NaCl
Protein with limited cross-links
Particulate aggregates
mTGase
T
Native whey protein
T
NaCl
Partially denatured and "polymerized" protein
T
NaCl
Two-dimensional aggregates
mTGase
T
NaCl
Particulate gel
Cross-linked protein
Heat stable aggregate

Chapter 3. Sequential Preheating and Transglutaminase Pretreatments Improve Stability of Whey Protein Isolate at pH 7.0 During Thermal Sterilization

3.1 Abstract

Whey protein isolate (WPI) is a potential ingredient to manufacture shelf-stable transparent beverages if proteins are heat stable, i.e., without causing turbidity, precipitation and gelation after the required thermal processing to obtain commercial sterility (138 °C for 8 s or longer). However, the information is lacking about stability of WPI during heating at 138 °C. Furthermore, novel technology and mechanistic understanding on how to produce clear products after heating systems with >5% WPI, particularly with salt, is needed. In this work, 5%w/v WPI was pretreated by microbial transglutaminase (mTGase) at three levels for 1-15 h at 50 °C, with and without prior preheating at 80 °C for 15 min. Heat stability of pretreated samples at pH 7.0 and 0, 50, and 100 mM NaCl was evaluated at 138 °C. Samples directly pretreated by mTGase for a greater extent demonstrated improved heat stability. For samples after sequential preheating and mTGase pretreatments, clear dispersions were observed at 0 and 50 mM NaCl even after heating at 138 °C for 30 min. All pretreatments increased the magnitude of zeta-potential and resistance against thermal denaturation. The sequentially-pretreated WPI was the most heat-resistant, with decreasing hydrodynamic diameter (< 36 nm) during extended heating at 138 °C and 50 mM NaCl and the initially increased particle height (< 12 nm) assessed in atomic force microscopy. The nanoscale particles enabled transparent dispersions after heating. In contrast, other samples showed different extents of aggregation at 50 mM NaCl, forming turbid dispersions or gels.

Keywords: whey protein, transglutaminase, thermal pretreatment, sterilization, transparency

3.2 Introduction

Shelf-stable protein beverages as replacement diets supply required nutrients and consumption convenience. To be shelf-stable, beverages are preferably clear after the required sterilization using processes such as ultra-high temperature (UHT) processing at 138 °C (280 °F) for 8 s (McGarrahan, 1982). Therefore, heat stable protein ingredients are needed to prevent turbidity, precipitation and gelation after thermal sterilization. Based on our experience with several commercial whey protein isolate (WPI) supplies, most WPI products maintain clarity after thermal treatment if pH is 3.5 and below, which makes WPI a common choice to manufacture protein-based or -fortified clear beverages. Furthermore, protein-containing beverages with acidity closer to neutral conditions may reduce the consumption of carbohydrate and increase that of proteins (Etzel, 2004), and those with additional mineral salts such as those found in sports drinks may present an opportunity for product development.

At neutral pH, whey proteins, mainly β-lactoglobulin, α-lactalbumin, and bovine serum albumin, exist at the molten globular state when heated at a temperature above about 65 °C (Bryant & McClements, 1998). The thermally denatured whey protein has the increased zeta-potential and surface hydrophobicity than the native protein (Ryan, Vardhanabhuti, Jaramillo, van Zanten, Coupland, & Foegeding, 2012). The extent of whey protein aggregation depends on heating temperature and duration and is determined by the overall impact of three types of molecular forces: electrostatic repulsion, hydrophobic attraction, and intra- and inter-molecular disulfide bonds formed via sulfhydryl-disulfide interchange (Baier & McClements, 2005; Bryant & McClements, 1998; de la Fuente, Singh, & Hemar, 2002; Foegeding, Davis, Doucet, & McGuffey,

2002). An increase in ionic strength shortens the Debye length and thus the effective distance of electrostatic repulsion, favoring whey protein aggregation and forming bigger aggregates after heating (Bryant & McClements, 1998; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). Coarse protein aggregates cause turbid appearance and precipitate during storage, which are infeasible for clear beverage products. Additionally, thermal gelation of whey protein samples is commonly observed at neutral acidity, moderate ionic strength and protein concentration (Bryant & McClements, 1998; Foegeding, Davis, Doucet, & McGuffey, 2002). Therefore, maintaining dispersibility of whey proteins and visual transparency of fluidic dispersions after thermal treatment, referred as heat stability in this work, is important for producing relevant beverage products.

Improving heat stability of whey proteins has been an active research topic in the past two decades. Heat stability of single whey protein components and WPI at neutral pH was improved after incorporating an appropriate amount of co-solutes such as sucrose, glycerol, sorbitol (Baier & McClements, 2001; Chanasattru, Decker, & McClements, 2007; Chantrapornchai & McClements, 2002; Kulmyrzaev, Bryant, & McClements, 2000), and β-casein with >90% purity (Yong & Foegeding, 2008). Preheating WPI solutions dispersed in microemulsions to form nanometer-sized whey protein particles was recently observed to improve the heat stability, further improved by prior treatment using microbial transglutaminase (mTGase) (Zhang & Zhong, 2009; Zhang & Zhong, 2010).

The mTGase is commonly used to cross-link proteins for strengthening gel networks (Jaros, Partschefeld, Henle, & Rohm, 2006) but has also been observed to

improve heat stability of whey proteins (Tanimoto & Kinsella, 1998; Truong, Clare, Catignani, & Swaisgood, 2004). Some studies applied denaturing agents such as dithiothreitol (DTT) prior to mTGase pretreatment (Tanimoto & Kinsella, 1998; Truong, Clare, Catignani, & Swaisgood, 2004), while others observed heat stability improvement after extensive mTGase pretreatment, without the use of DTT (Agyare & Damodaran, 2010; Lorenzen, 2007; Tang & Ma, 2007). At present, it has not been reported for WPI dispersions that maintain visual clarity after UHT processing at neutral acidity, moderate NaCl concentration, and 5% or more protein. Supramolecular structure formation as impacted by mTGase pretreatment also is to be studied.

The first objective of this work was to study heat stability of WPI at UHT conditions after individual or sequential pretreatments of preheating and mTGase. The second objective was to understand physicochemical changes due to pretreatments and supramolecular structural changes during UHT processing. Heat stability of samples was evaluated for 5% w/v WPI dispersions at pH 7.0 and with 0-100 mM NaCl after heating at 138 °C for 1-30 min. Physicochemical properties were characterized using a variety of techniques interpreting changes of primary structures, surface charge properties, thermal denaturation properties, and nanoscale structures. Surface charge properties determine the magnitude of repulsive electrostatic interactions between whey proteins and are commonly measured for zeta-potential that is assessed based on electrophoretic properties of colloidal particles (Ryan, Vardhanabhuti, Jaramillo, van Zanten, Coupland, & Foegeding, 2012; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007). Dynamic light scattering (DLS) and atomic force microscopy (AFM) were used to characterize nanoscale structures. DLS is commonly used to evaluate hydrodynamic

radius of whey protein structures after heating in dispersions (Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007; Unterhaslberger, Schmitt, Sanchez, Appolonia-Nouzille, & Raemy, 2006). AFM has been used to characterize detailed topographical information of heat-induced structural formation of whey proteins without complicated sample preparations, e.g., fine-stranded and particulate structures in our early study after heating at neutral acidity with low and high ionic strengths, respectively (Ikeda & Morris, 2002).

3.3 Materials and methods

3.3.1 Materials

WPI was obtained from Hilmar Ingredients, Inc (Hilmar, CA). The product had 95% protein (dry-basis) according to the manufacturer. The Activa TG-TI mTGase in the powdered form was a product from Ajinomoto Food Ingredients, LLC (Chicago, IL). The brochure from the manufacturer indicated that the enzyme activity is optimum at 50 °C and pH 7.0. Double-distilled water and other chemicals were procured from Fisher Scientific (Pittsburgh, PA).

3.3.2 Determination of mTGase activity

The freshly-prepared mTGase solution was quantified for activity using the method of Sigma-Aldrich for TGase (EC 2.3.2.13). One unit (U) of activity was defined as the ability of an enzyme sample to catalyze the formation of 1 μmol hydroxamate per min when substrates of N-carbobenzoxy-glutaminyl-glycine and hydroxylamine were incubated with the enzyme at pH 7.0 and 50 °C.

3.3.3 Pretreatment protocol

WPI powders were hydrated at 5% w/v in 50 mM sodium phosphate overnight at room temperature. For preheating whey proteins, samples were adjusted to pH 7.5 using 1 N NaOH, heated in an 80 °C water bath for 15 min, and immediately cooled in a room temperature water bath. To cross-link whey proteins by mTGase, WPI samples, with or without preheating, were adjusted to pH 7.5 using 4 N NaOH, dissolved with mTGase powder to 2, 5, and 10% mass of WPI, incubated in a 50 °C water bath for different durations, and cooled in a room temperature water bath. pH 7.5 was chosen from the literature mTGase cross-linking conditions (Truong, Clare, Catignani, & Swaisgood, 2004). The pretreated samples were adjusted to pH 7.0 using 1 N HCl, and 1.0 mL aliquots of samples were transferred into 4.0 mL vials. To adjust NaCl concentration, a 5.0 M NaCl solution was pre-prepared in distilled water. Each WPI sample vial was supplemented with 10 or 20 μL of the 5.0 M NaCl solution, yielding a final NaCl concentration of 50 or 100 mM, respectively. The untreated control was the 5% w/v WPI solution directly adjusted to the same pH and NaCl concentrations using similar procedures.

3.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed under the reducing conditions using a Mini Protean Tetra Cell (Bio-Rad Laboratories, Inc., Hercules, CA) at a constant voltage of 200 V. The 18% Tris-HCl gel (Ready Gel Precast Gel) was from Bio-Rad Laboratories, Inc. (Hercules, CA). The gel was stained with Coomassie Blue.

3.3.5 Evaluation of heat stability

Although the continuous UHT process requires 138 °C for 8 s (McGarrahan, 1982), samples were heated for 1, 5, or 30 min in a glycerol bath maintained at 138 °C. The shortest time of 1 min was longer than 8 s because of the need to address the come-up time required to reach the target temperature in entire sample vials. The two additional heating durations were used to better understand the structural changes of whey protein during sterilization. After heating, the vials were immediately cooled in a room-temperature water bath. Samples were then photographed and measured for absorbance at 400 and 600 nm using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

3.3.6 Differential scanning calorimetry (DSC)

The influence of pretreatment on thermal denaturation properties of whey protein was studied using a differential scanning calorimeter (model Q2000, TA Instrument, New Castle, DE). The samples containing 5% w/v protein were pretreated as above and adjusted to pH 7.0 with 100 mM NaCl. For samples pretreated by mTGase alone or sequential preheating and mTGase, the enzyme powder was used at 5% of WPI mass and the cross-linking duration was 4 h. The scanning was conducted from 25 to 105 ℃ at a rate of 1.5 ℃/min, and an empty cell was used as a reference. To facilitate the examination of samples after various pretreatments, baseline adjustment was applied (Bruylants, Wouters, & Michaux, 2005; Gauche, Barreto, & Bordignon-Luiz, 2010; Giancola, De Sena, Fessas, Graziano, & Barone, 1997). The thermographs were processed by using the instrument analysis software (TA Instrument, New Castle, DE).

3.3.7 Dynamic light scattering (DLS)

The particle size distribution of WPI dispersions was measured using a model Delsa™ Nano particle size/zeta-potential analyzer (Beckman Coulter, Inc., Brea, CA). To prevent gelation, the WPI dispersions were prepared at 5% w/v protein in deionized water instead of 50 mM phosphate buffer. After pretreatments and supplementing with 0 and 50 mM NaCl, samples were heated at 138 ℃ for 1, 5 and 30 min and diluted using 0.01 M phosphate buffer (pH 7.0) to an appropriate concentration for DLS. The measurements were carried out on two replicates and the volume-length mean particle diameter ($d_{4,3}$) was calculated according to equation (1).

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

where n_i is the number of particles of a diameter d_i.

3.3.8 Zeta-potential

Impacts of pretreatments on zeta-potential of WPI were analyzed using the Delsa™ Nano particle size/zeta-potential analyzer (Beckman Coulter Inc., Brea. CA). The dispersions were adjusted to pH 7.0 and diluted to 0.5% w/v using a 50 mM sodium phosphate buffer adjusted to pH 7.0. Two replicates were tested for three times each.

3.3.9 Atomic force microscopy (AFM)

The WPI dispersions were diluted in deionized water to an overall protein concentration of 10 ppm. Four μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA) for AFM. A rectangular cantilever having an aluminum reflective coating on the

backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp., Santa Barbara, CA) and a Multimode microscope (Veeco Instruments, Inc. Plainview, NY) operated in the tapping mode were used to scan the sample. Both topographical and phase images were generated with a preset scan area of 2×2 μm. Topographical images were used to measure heights of individual particles.

3.3.10 Statistical analysis

Statistical differences were analyzed using post-hoc comparison of means according to the least-significant-difference (LSD) mean separation method at a *p* level of 0.05. The SAS software (version 9.2, SAS Institute, Cary, NC) was used in analyses.

3.4 Results and discussion

3.4.1 mTGase levels used in pretreatment

The mTGase activity was estimated to be 102.1 U/g powder. When the powdered mTGase sample was used at 2, 5, and 10% mass of WPI, this corresponded to an activity/substrate level of ca. 2.0, 5.1, and 10.2 U/g-WPI.

3.4.2 Structures of WPI analyzed by SDS-PAGE

SDS-PAGE patterns of selected samples are presented in Figure 3-1. Compared to native WPI, preheating alone did not apparently change the pattern of protein bands because the SDS-PAGE buffer disrupts any possible physical and chemical (disulfide) bonds formed during heating, as reported elsewhere (Zhang & Zhong, 2009). For the WPI sample directly treated by mTGase at a level of 5.1 U/g-WPI for 4 h without preheating, the lower molecular weight band corresponding to α-lactalbumin (MW = 14.1 kDa (Swaisgood, 1996) became very vague, and the density of band corresponding

to β-lactoglobulin (MW = 18.4 kDa (Swaisgood, 1996) decreased slightly. The upper section of the lane showed smearing areas, indicating the weakened bands of α-lactalbumin and β-lactoglobulin resulted from cross-linking by mTGase. For samples sequentially treated by preheating and mTGase at a level of 5.1 U/g-WPI, the band patterns were similar for the 4 treatment durations from 1 to 15 h that showed significant reduction of bands corresponding to α-lactalbumin and β-lactoglobulin, significant smearing in the upper molecular weight regimes of lanes, and a significant portion of cross-linked whey proteins that remained in the sample loading wells. The mTGase has a molecular weight of ~40 kDa (Jaros, Partschefeld, Henle, & Rohm, 2006) and appeared in the gel for samples applying the enzyme.

Native whey proteins are not very susceptible to cross-linking by mTGase (Han & Damodaran, 1996) possibly because glutamine and lysine groups involved in the cross-linking reaction are buried within the protein core and inaccessible for the enzyme (Cozzolino, Di Pierro, Mariniello, Sorrentino, Masi, & Porta, 2003). It was also recognized that native and preheated α-lactalbumin is cross-linked by mTGase more easily than β-lactoglobulin (Rodriguez-Nogales, 2006a). Therefore, the band corresponding to α-lactalbumin diminished to a greater extent than β-lactoglobulin after mTGase treatment (Figure 3-1). Preheating (Han & Damodaran, 1996; Rodriguez-Nogales, 2006b; Sharma, Zakora, & Qvist, 2002) or denaturation using a reducing agent such as DTT (Aboumahmoud & Savello, 1990; Gauche, Vieira, Ogliari, & Bordignon-Luiz, 2008; Sharma, Zakora, & Qvist, 2002; Truong, Clare, Catignani, & Swaisgood, 2004) facilitates the cross-linking of whey proteins greatly. Although the SDS-PAGE

showed similar bands of sequentially pretreated whey protein samples (Figure 3-1), it should be noted that the portion of proteins that did not enter the gel may have different molecular weights after different treatment durations. Unfortunately, we were not capable of identifying these structures using advanced instruments such as capillary electrophoresis (Rodriguez-Nogales, 2006a) or size-exclusion chromatography (SEC) combined with on-line multi-angle laser light scattering (Tang & Ma, 2007).

3.4.3 Heat stability of samples pretreated by mTGase directly

Impacts of direct pretreatment with mTGase on the absorbance after sterilization at 138 °C were examined at 400 (Abs_{400}) and 600 nm and found to follow similar trends, with absorbance values at 600 nm being much smaller. The results hereafter are presented for Abs_{400} only. Figure 3-2 shows Abs_{400} of samples that were directly pretreated with mTGase before heat stability test at 138 °C for 1 min. At 0 mM NaCl (Figure 3-2A), all samples were clear, corresponding to Abs_{400} values lower than 0.55. A higher level of mTGase applied for a longer time resulted in a lower Abs_{400} in many cases. The control WPI sample without mTGase pretreatment had Abs_{400} comparable to those pretreated with mTGase. It has been reported in the literature that whey proteins form filamentous aggregates with diameters of about 11 nm upon heating at neutral pH and a low ionic strength (Ikeda & Morris, 2002). Since such fine structures would not effectively scatter light, dispersions of whey protein normally remain transparent after heating around neutral pH and a low ionic strength. At 50 mM NaCl (Figure 3-2B), the control sample and the sample treated with the lowest level of mTGase (2.0 U/g-WPI) for the shortest duration (1 h) formed gels, while other samples remained flowable. The samples pretreated with 5.1 U/g-WPI of mTGase for 1 h and those pretreated with 10.2 U/g-WPI

for 1, 8, or 15 h showed mostly transparent appearance with Abs_{400} values around 0.5. At 100 mM NaCl (Figure 3-2C), the control, samples treated with the lowest level of mTGase (2.0 U/g-WPI) for all examined durations, and those treated with increased levels of mTGase (5.1 and 10.2 U/g-WPI) for less than 4 h formed gels. Samples treated with 5.1 or 10.2 U/g-WPI for more than 8 hours remained flowable, with reduced turbidity at a higher level of enzyme treatment. The results indicate improved heat stability of WPI after direct pretreatment by mTGase. As discussed previously, mTGase has limited activity in cross-linking native whey proteins. However, commercial WPI products are usually spray-dried to form powdered products, and exposure of whey proteins to the drying hot air can cause a certain extent of denaturation that was estimated to be approximately 5% (Lorenzen, 2007). Therefore, a limited extent of cross-linking is expected when WPI is directly pretreated by mTGase, as shown in SDS-PAGE (Figure 3-1).

Heating at 138 °C for 5 min enhanced gelation, particularly in the presence of added NaCl (Figure 3-3). At 0 mM NaCl (Figure 3-3A), all samples remained clear, with Abs_{400} values smaller than approximately 0.3. The Abs_{400} values shown in Figure 3-3A are consistently smaller than those shown in Figure 3-2A at an identical level and duration of mTGase pretreatment, suggesting that the extended heating at 138 °C caused degradation of protein aggregates. At 50 mM NaCl (Figure 3-3B), the control and the samples treated with 2.0 and 5.1 U/g-WPI of mTGase formed gels at all examined durations of the mTGase pretreatment, while those pretreated with 10.2 U/g-WPI of mTGase transformed from a gel to a clear solution with increasing duration of the pretreatment. At 100 mM NaCl, all examined samples formed gels (Figure 3-3C).

3.4.4 Heat stability of samples pretreated by sequential preheating and mTGase

Whey proteins are denatured during thermal pretreatment, exposing internal amino acids available for reactions catalyzed by mTGase (Han & Damodaran, 1996; Rodriguez-Nogales, 2006b; Sharma, Zakora, & Qvist, 2002). In this set of pretreatments, whey proteins were preheated at 80 °C for 15 min before applying mTGase for cross-linking at 50 °C. The pretreated samples were then adjusted to pH 7 and 0-100 mM NaCl, and heated at 138 °C for evaluating heat stability. Figure 3-4 shows Abs_{400} of the samples heated at 138 °C for 1 min. At 0 mM NaCl (Figure 3-4A), all samples were clear, corresponding to Abs_{400} values lower than 0.55. A higher level of mTGase and a longer cross-linking duration resulted in a larger Abs_{400}, which is opposite to the trend observed in Figure 3-2A. Additionally, almost all Abs_{400} values shown in Figure 3-4A are larger than those shown in Figure 3-2A at an identical mTGase level and duration of the pretreatment, confirming that preheating prior to enzyme treatments enhanced cross-linking by mTGase. The control without mTGase pretreatment shown in Figure 3-4A also shows an Abs_{400} larger than that of the control shown in Figure 3-2A, suggesting that the preheating prior to mTGase pretreatment caused some extent of aggregation between protein molecules. At 50 mM NaCl, the control shown in Figure 3-2B formed a gel, while the preheated control shown in Figure 3-4B turned to be slightly turbid but remained flowable. The samples preheated and then pretreated with mTGase for 4 h showed remarkably lower values of Abs_{400} (Figure 3-4B) compared to those pretreated directly with mTGase (Figure 3-2B). Preheating also led to prevention of gelation at 100 mM NaCl even without mTGase pretreatment (Figure 3-4C). All the mTGase-pretreated

samples did not gel in the presence of 100 mM NaCl after heating at 138 °C for 1 min (Figure 3-4C). While most samples became turbid, the sample pretreated with 10.2 U/g-WPI of mTGase for 15 h remained clear. These results suggest that preheating itself has a positive impact on the heat stability of WPI, that preheating facilitates cross-linking catalyzed by mTGase, and that cross-linked whey proteins are more heat stable than native ones.

All samples remained clear after heating at 138 °C for 5 min in the absence of NaCl, with Abs_{400} values around 0.2 (Figure 3-5A). These Abs_{400} values were generally smaller than those after heating at 138 °C for 1 min (Figure 3-5A), suggesting once again degradation of protein aggregates during extended heating. At 50 mM NaCl (Figure 3-5B), the control and the sample treated with the lowest level of mTGase for the shortest duration formed gels, while most other samples remained clear after heating at 138 °C for 5 min, with Abs_{400} values less than 0.5. At the NaCl level of 100 mM, all examined samples formed gels after heating at 138 °C for 5 min (Figure 3-5C). The results indicate that the sequentially-pretreated WPI is still able to aggregate at 100 mM NaCl, which coincides with an earlier work showing that WPI aggregates to the highest extent at 100 mM NaCl when examined at pH 7.0 and 0-1000 mM NaCl (Ikeda, Foegeding, & Hagiwara, 1999).

3.4.5 Thermal denaturation studied by using DSC

When three major whey proteins are studied individually, the denaturation temperature of β-lactoglobulin, α-lactalbumin, and bovine serum albumin is 78, 62, and 64 °C, respectively (Bryant & McClements, 1998). When WPI is measured, the

denaturation temperature represents the overall mixture, which was measured to be 74.6 °C in a study at pH 7.0 and 0 mM NaCl (Ibanoglu, 2005). In the present study, DSC profiles of WPI after different pretreatments were characterized at 100 mM NaCl. A sharp endothermic peak centered on ca. 77 °C was observed for untreated WPI (Figure 3-6A), slightly higher than the reported 74.6 °C at 0 mM NaCl (Ibanoglu, 2005). The WPI pretreated by mTGase only at 5.1 U/g-WPI for 4 h also showed an endothermic peak at around 75 °C (Figure 3-6C), likely because of limited cross-linking at these conditions (Figure 3-1). This peak became broader for the preheated WPI (Figure 3-6B) and almost unidentifiable for the sequentially-pretreated WPI (Figure 3-6D). The DSC results indicate that treatment using mTGase only for 4 h did not significantly change denaturation properties of whey protein. Preheating caused irreversible denaturation of whey protein, but the preheating conditions in this work (80 °C for 15 min) did not induce complete denaturation. The sequentially-pretreated whey protein demonstrated very limited further thermal denaturation. The DSC profiles generally agree with observations of heat stability evaluated at 138 °C for 1 min for samples with 100 mM NaCl, in the order of sequentially-pretreated > preheated > mTGase-pretreated > untreated (Figures 3-2 and 3-4).

3.4.6 Particle size and zeta-potential after pretreatments

The $d_{4,3}$ of WPI dispersions before and after pretreatments at pH 7.0 is listed in Table 3-1. Direct pretreatment of WPI using 5.1 U/g-WPI of mTGase for 4 h increased $d_{4,3}$ slightly but the increase was insignificant. Preheating significantly increased $d_{4,3}$ that further increased significantly after mTGase pretreatment. The $d_{4,3}$ changes agree with

SDS-PAGE in Figure 3-1 and the literature showing native WPI is not an active substrate of mTGase (Han & Damodaran, 1996) but is reactive after preheating (Han & Damodaran, 1996; Rodriguez-Nogales, 2006a; Sharma, Zakora, & Qvist, 2002).

Table 3-2 presents zeta-potential of WPI dispersions at pH 7.0 before and after pretreatments. The -24.45 mV of native WPI dispersion at pH 7.0 is in agreement with the -24.6 mV at pH 6.8 in a study (Ryan, Vardhanabhuti, Jaramillo, van Zanten, Coupland, & Foegeding, 2012). The increase in zeta-potential was insignificant after direct pretreatment of WPI using 5.1 U/g-WPI of mTGase for 4 h. Preheating significantly increased zeta-potential of WPI that remained unchanged after further mTGase cross-linking. Thermal denaturation is thus the major factor in changing the distribution of charged surface amino acids of whey proteins. The sequentially-pretreated WPI was more resistant to thermal aggregation than the preheated WPI at increased ionic strength (Figures 3-4, 3-5, and 3-7; Table 3-1), but both samples had similar zeta-potential (Table 3-2). This suggests that electrostatic repulsion is not the mechanism responsible for the improved heat stability of the sequentially-pretreated WPI. Conversely, because denaturation precedes aggregation during heating at neutral pH, the increased resistance against thermal denaturation (Figure 3-6) is the major contributor to the improved heat stability of WPI after sequential pretreatments. However, because denaturation properties were evaluated below 100 °C, structural changes during heating at 138 °C were further studied, presented below.

3.4.7 Structure changes of pretreated whey proteins after heating at 138 ℃

Figure 3-7 shows photographs of WPI dispersions after heating at 138 °C for up to 30 min in the absence or presence of 50 mM NaCl. All the dispersions without NaCl remained clear after heating at 138 °C for 30 min. In the presence of 50 mM NaCl, dispersions without any pretreatment and those treated with either preheating at 80 °C for 15 min or mTGase turned into turbid dispersions or even gelled after heating at 138 °C for 5 min. The samples sequentially pretreated first with heating at 80 °C for 15 min and then with 5.1 U/g-WPI of mTGase remained clear after heating at 138 °C for 30 min even in the presence of 50 mM NaCl. Samples heated for 30 min had darkened color, likely due to thermal decomposition of organic matter.

The $d_{4,3}$ changes after heating at 138 °C are presented in Table 3-1. Native WPI and that directly pretreated by mTGase showed initial aggregation after heating for 1 min, followed by the decrease in particle size upon further heating. In contrast, preheated WPI, with and without subsequent mTGase pretreatment showed a monotonic decrease in $d_{4,3}$ when heated at 0 mM NaCl. At 50 mM NaCl, the $d_{4,3}$ of preheated WPI increased after 1-min heating, likely because there is a fraction of WPI that can still be denatured after preheating at the studied conditions (Figure 3-6) and aggregate similarly to native WPI during heating at 138 °C. Conversely, the sequentially-pretreated WPI showed no increase in $d_{4,3}$ after heating. The reduction in particle size at 0 mM NaCl is responsible for the lowered absorbance of samples when heating was increased from 1 min to 5 min (Figures 2-5). Furthermore, the significantly bigger particles ($P < 0.05$) after heating at 50 mM NaCl than at 0 mM NaCl for samples with preheating or mTGase pretreatment only

agree with the expectation that a higher ionic strength weakens electrostatic repulsion and facilitates protein aggregation (Bryant & McClements, 1998; Schmitt, Bovay, Rouvet, Shojaei-Rami, & Kolodziejczyk, 2007).

When β-lactoglobulin A was heated at pH 6.4 up to 180 °C, complimentary data from native and reducing SDS-PAGE, with and without 2-mecaptoethanol, DSC, viscosity, amino acid analysis, and particle size analysis suggested that protein aggregation occurred up to 113 °C, followed by collapsing of initially formed aggregates at a temperature higher than about 113 °C and decomposition of β-lactoglobulin A above around 140 °C due to hydrolysis of peptide bonds (Photchanachai & Kitabatake, 2001). By reviewing studies of heating β-lactoglobulin at pH above 6.8, it was suggested that, in addition to primary unfolding events at around 65-75 °C, decomposition of disulfide bonds formed at lower temperatures is possible above 125 °C, which is accompanied by complete unfolding of all amino acid residues (de Wit, 2009). At the early stage of heating at 138 °C, native WPI and those pretreated by preheating or mTGase only are denatured to different extents, and the denatured proteins aggregate. As aggregation and decomposition are both controlled by reaction kinetics, it is likely that aggregation dominates initially, followed by the domination of structural reorganization and thermal decomposition of aggregated proteins at longer heating times. This is evident for macroscopic observations (Figure 3-7) of samples heated with 50 mM NaCl at different durations, especially for the native WPI sample that formed a gel after heating for 5 min, followed by macroscopic structure collapse at a longer heating time. This also agrees with $d_{4,3}$ data in Table 3-1. Conversely, the sequentially pretreated WPI is resistant

against thermal denaturation and therefore subsequent aggregation, showing transparent samples (Figure 3-7) and a monotonic decrease in $d_{4,3}$ at a longer heating duration (Table 3-1).

To further understand nanoscale structure changes of whey proteins, AFM studies were carried out for samples sequentially pretreated with heating and mTGase. The AFM image shows that native whey proteins were mostly spherical particles, with a portion present as dimers or trimers (Figure 3-8A), corresponding to particle heights smaller than 3.5 nm. The estimated particle heights are similar to the literature AFM study of β-lactoglobulin (height = 2.2 nm) (Ikeda & Morris, 2002) and light scattering studies that reported the hydrodynamic radius of β-lactoglobulin, α-lactalbumin, and bovine serum albumin to be 2.6-4.9 (Parker, Noel, Brownsey, Laos, & Ring, 2005), 2.0 (Molek & Zydney, 2007), and 3.7 nm (Brownsey, Noel, Parker, & Ring, 2003), respectively. The presence of dimeric whey protein structures also agrees with the well-known fact that β-lactoglobulin forms dimers at pH 7 (Adams, Anderson, Norris, Creamer, & Jameson, 2006; Haug, Skar, Vegarud, Langsrud, & Draget, 2009). After sequential pretreatment, aggregates (Figure 3-8B) with particle height of 6.34 nm (Table 3-3) were observed. The AFM and DLS results suggest that cross-linking of preheated whey proteins by mTGase occurs among a few to several whey protein molecules. Furthermore, the particle heights measured in AFM were consistently smaller than $d_{4,3}$ from DLS. In DLS, $d_{4,3}$ is an indication of hydrodynamic diameter occupied by the entire structure, while the particle height indicates the dimension at only one direction. Sample drying in AFM can also reduce the overall dimension. Additionally, samples were diluted to 10 ppm in AFM, and the dilution may separate weakly flocculated structures that are measured in DLS.

After heating at 138 °C for 1 and 30 min, particular structures were observed (Figure 3-8). At 0 mM NaCl, particle heights did not increase significantly after heating (Table 3-3). At 50 mM NaCl, particle heights almost doubled after heating for 1 min, followed by no significant increase after heating for 30 min. The AFM images suggest that the cross-linked whey proteins are resistant against aggregation and are disassociated during heating at 138 °C. The increased height of mostly spherical particles likely results from the melting and reorganization of aggregated whey proteins during heating at 138 °C. The complimentary results from AFM and DLS thus unveiled nanoscale structure changes of WPI after pretreatments and during UHT processing.

3.5 Conclusion

Preheating WPI at 80 °C for 15 min or direct pretreatment with varied levels of mTGase was observed to have improved the stability of WPI dispersions during sterilization at neutral pH to certain extents, while the sequential pretreatments first with heat and then with mTGase significantly enhanced the heat stability of WPI. The sequentially-pretreated WPI dispersions remained transparent after heating at 138 °C for 30 min in the presence of 0 and 50 mM NaCl at pH 7. Analysis of nanostructure changes revealed that cross-linking by mTGase occurred between a few to several whey protein molecules. The increased resistance against thermal denaturation was responsible for the improved heat stability of sequentially-pretreated WPI at 50 mM NaCl. When heated for a longer time at 138 °C, the cross-linked whey proteins were dissociated and reformed to bigger particles, and the resultant nanoscale particles (< 40 nm based on DLS, < 13 nm

based on AFM) enabled transparent dispersions. The present study demonstrates the feasibility of using sequential pre-heating and mTGase pretreatments to develop sterilized beverage products with a high content (5%w/v) of whey protein and yet of transparent appearance at ambient temperatures.

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Appendix

Table 3-1. The $d_{4,3}$ of WPI dispersions at pH 7.0 after heating at 138 ℃ for 0, 1, 5 and 30 min.

Pretreatment conditions [a]	NaCl (mM)	$d_{4,3}$ (nm) after heating for different durations (min) [b]			
		0	1	5	30
Untreated	0	9.8 ± 2.6^{K}	47.8 ± 3.6^{C}	$22.4 \pm 0.8^{H,I}$	27.7 ± 2.5^{G}
	50	-	-	-	-
Preheating only	0	31.7 ± 1.3^{F}	25.9 ± 0.5^{G}	11.6 ± 0.9^{I}	$12.9 \pm 1.5^{J,K}$
	50	-	52.46 ± 2.36^{B}	-	-
mTGase only	0	$11.9 \pm 2.3^{J,K}$	21.0 ± 3.4^{I}	19.3 ± 1.1^{I}	$14.0 \pm 0.6^{J,K}$
	50	-	95.6 ± 5.2^{A}	-	-
Sequential preheating and mTGase	0	40.6 ± 1.7^{D}	32.6 ± 0.8^{F}	$13.0 \pm 0.5^{J,K}$	$12.2 \pm 4.2^{J,K}$
	50	-	35.9 ± 1.5^{E}	32.1 ± 2.3^{F}	$25.4 \pm 2.4^{G,H}$

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.

[b] Numbers are averages ± standard deviations from two replicates, each measured for three times. Different superscript letters indicate statistical difference ($p < 0.05$).

Table 3-2. Zeta-potential of WPI dispersions at pH 7.0 after various pretreatments.

Pretreatment conditions [a]	Zeta-potential (mV) [b]
Untreated	-24.45 ± 1.41^{B}
Preheating only	-36.91 ± 0.82^{A}
mTGase only	-27.24 ± 2.82^{B}
Sequential preheating and mTGase	-35.88 ± 3.20^{A}

[a] Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are averages ± standard deviations from two replicates, each measured for three times. Different superscript letters indicate statistical difference ($p < 0.05$).

Table 3-3. Average particle heights from AFM for the sequentially-pretreated[*] WPI at pH 7.0 with 0 or 50 mM NaCl after heating at 138 ℃ for 0, 1, and 30 min.

NaCl (mM)	Heating duration (min) [b]		
	0	1	30
0	6.34 ± 0.41^{B}	7.35 ± 1.48^{B}	7.58 ± 0.18^{B}
50	-	11.61 ± 0.69^{A}	12.29 ± 0.79^{A}

[*]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, while the mTGase pretreatment was conducted using 5.1 U/g-WPI at pH 7.5 and 50 °C for 4 h.
[b] Numbers are averages ± standard deviations of particle heights measured from AFM topographical images. Different superscript letters indicate statistical difference ($p < 0.05$).

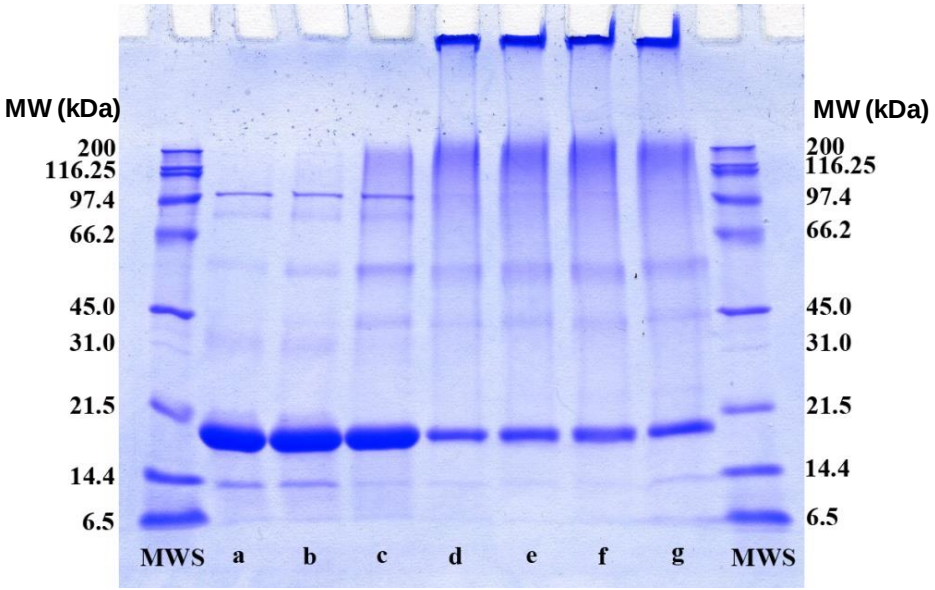

Figure 3-1. SDS-PAGE patterns of WPI after various treatments. Lane codes: MWS = molecular weight standard; a = native WPI; b = WPI after 15-min preheating at 80 °C; c = WPI after 4 h cross-linking by mTGase at 5.1 U/g WPI; d,e,f,g = WPI preheated at 80 °C for 15 min then cross-linked by mTGase at 5.1 U/g WPI for 1, 4, 8, and 15 h, respectively.

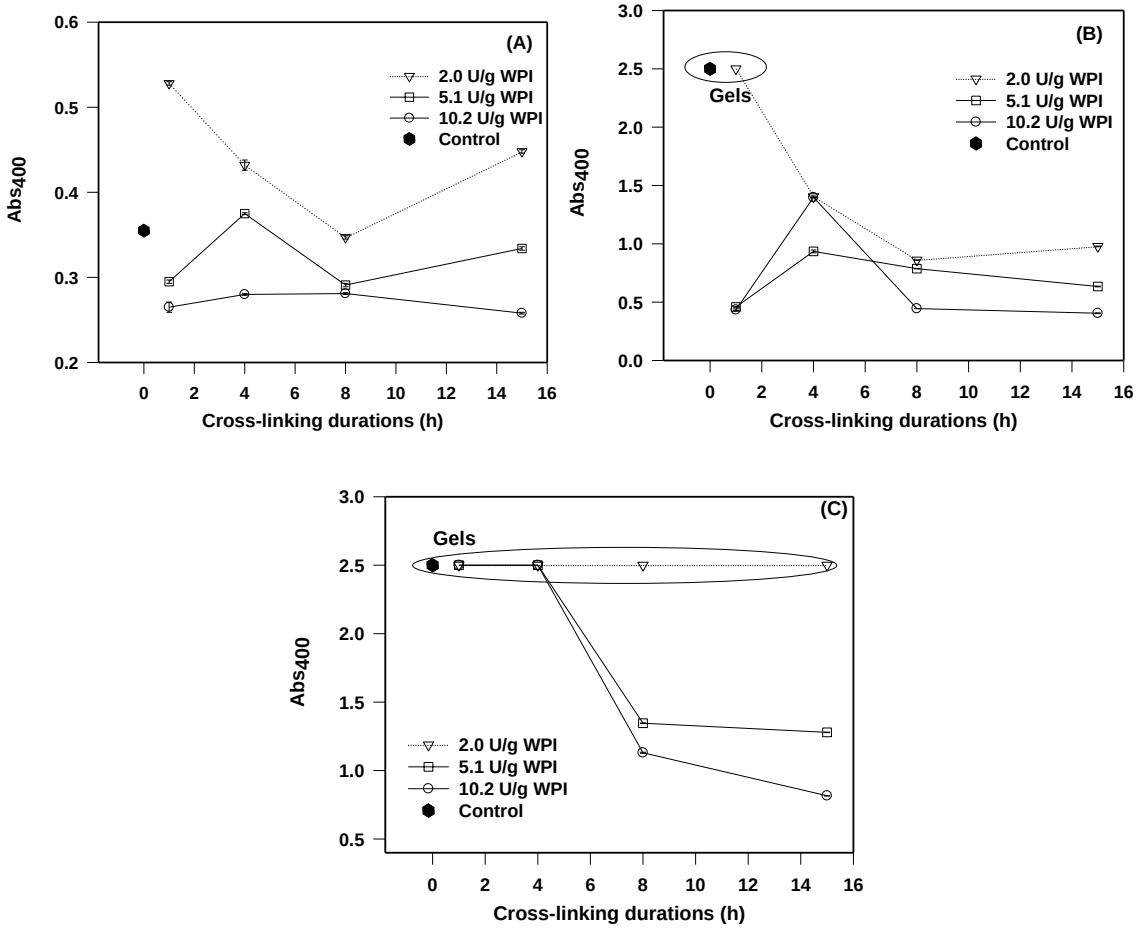

Figure 3-2. Absorbance of samples at 400 nm (Abs_{400}) after heating at 138 °C for 1 min. The 5% w/v WPI samples were adjusted to pH 7.5 and cross-linked by varied concentrations of mTGase for 1-15 h before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl for heat stability tests. Error bars are standard deviations from three replicates.

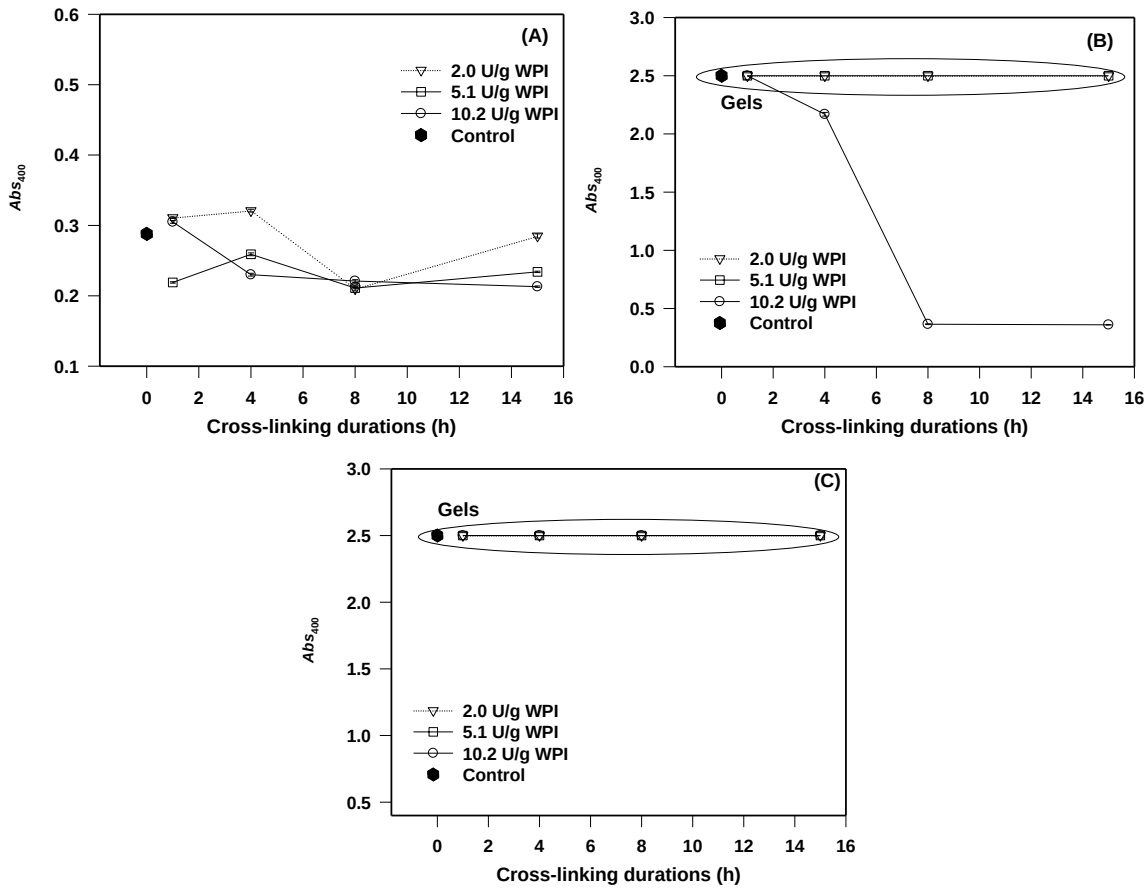

Figure 3-3. Absorbance of samples at 400 nm (Abs_{400}) after heating at 138 °C for 5 min. The 5% w/v WPI samples were adjusted to pH 7.5 and cross-linked by varied concentrations of mTGase for 1-15 h before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl for heat stability tests. Error bars are standard deviations from three replicates.

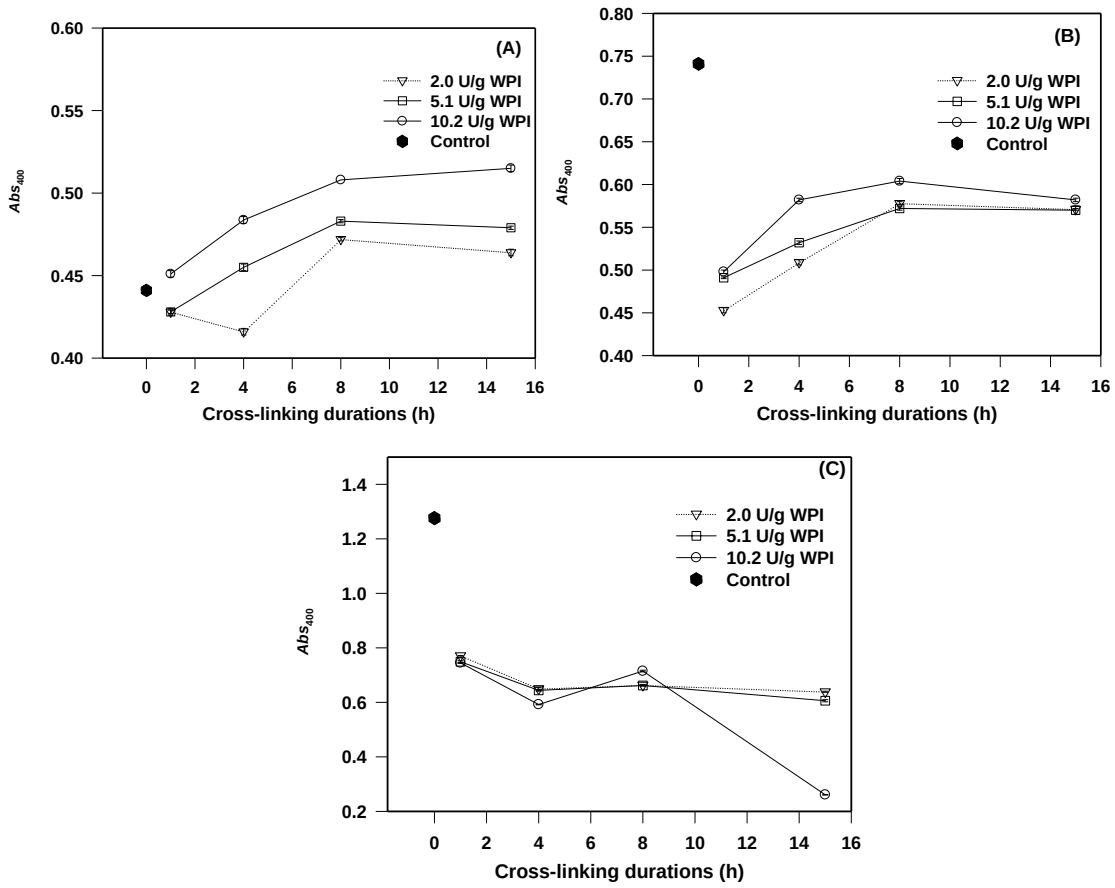

Figure 3-4. Absorbance of samples at 400 nm (Abs_{400}) after heating at 138 °C for 1 min. The 5% w/v WPI samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, and then cross-linked by varied concentrations of mTGase for 1-15 h before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl for heat stability tests. Error bars are standard deviations from three replicates.

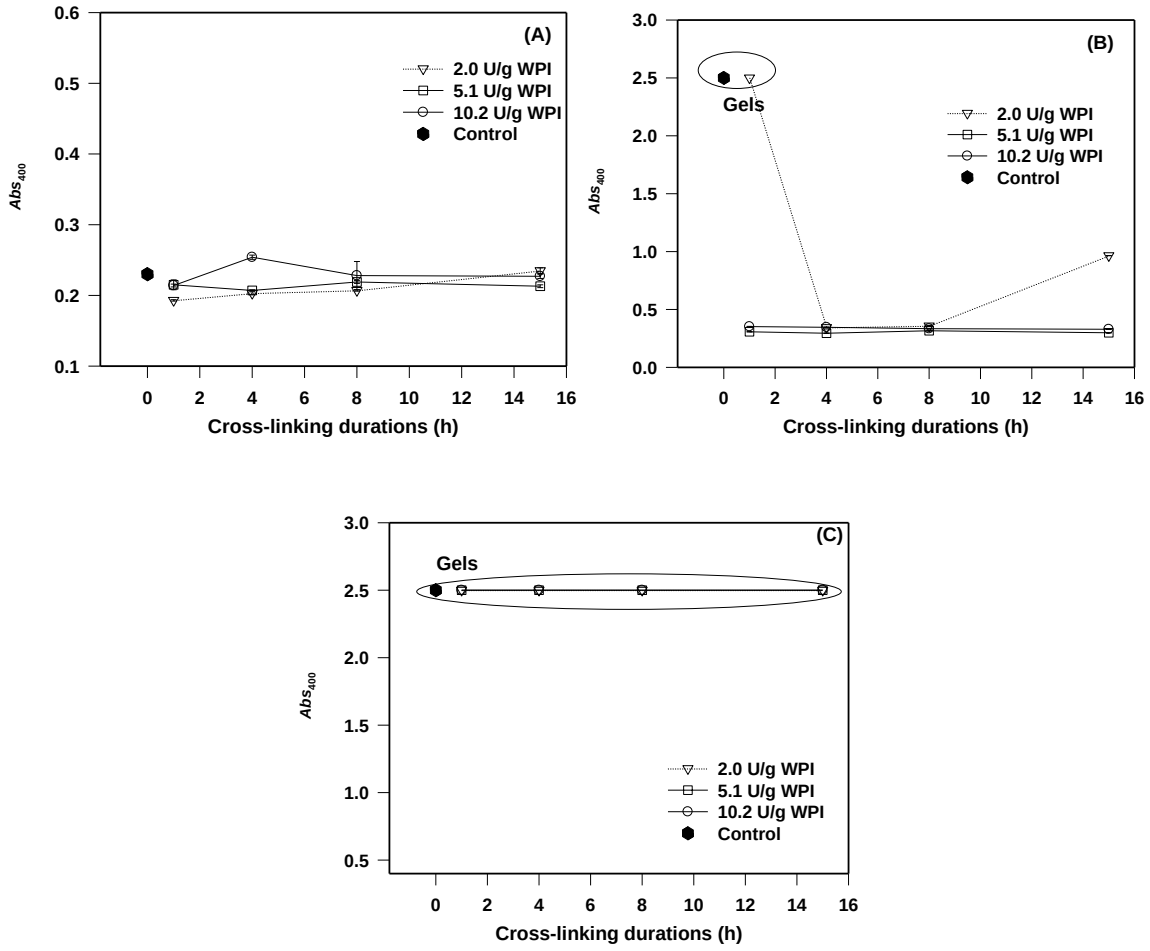

Figure 3-5. Absorbance of samples at 400 nm (Abs_{400}) after heating at 138 °C for 5 min. The 5% w/v WPI samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, and then cross-linked by varied concentrations of mTGase for 1-15 h before adjusting to pH 7.0 and (A) 0, (B) 50, and (C) 100 mM NaCl for heat stability tests. Error bars are standard deviations from three replicates.

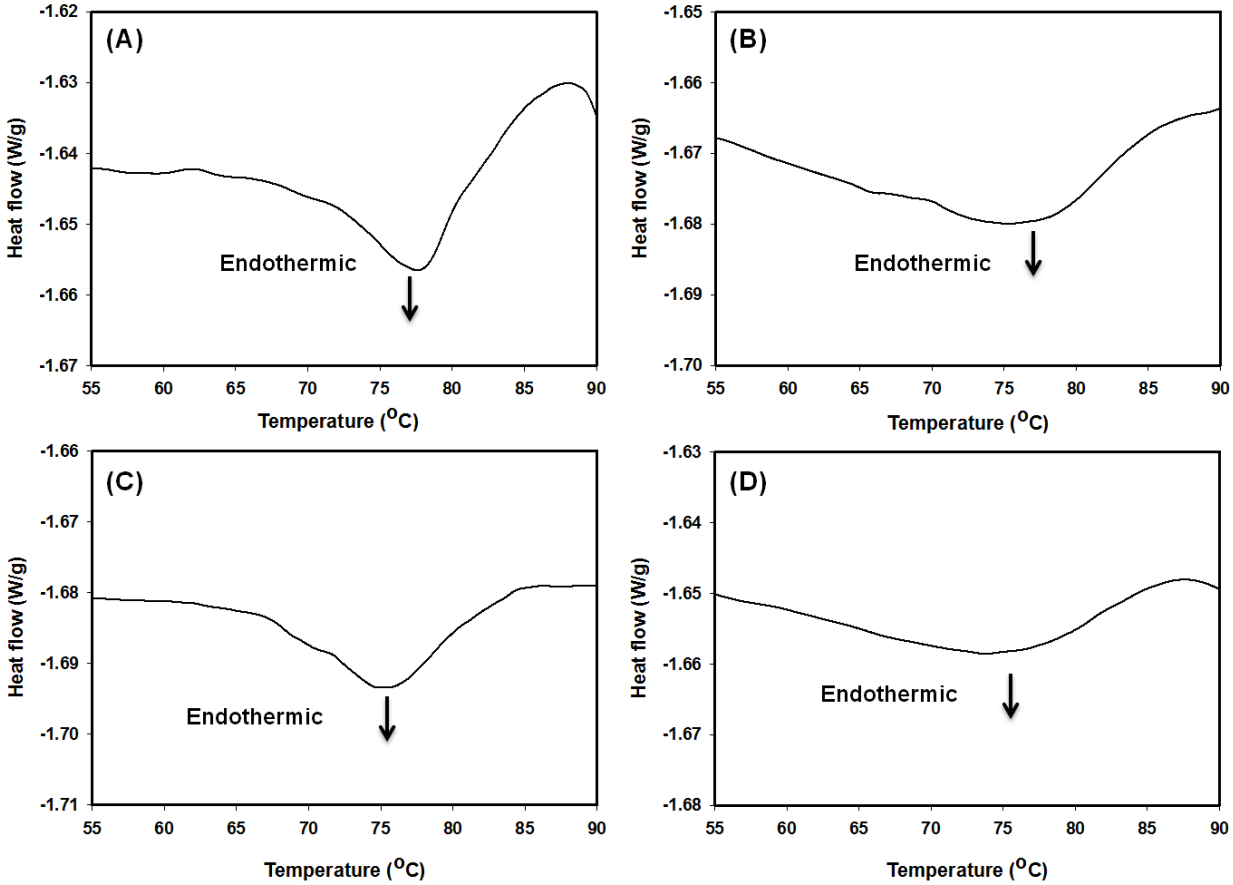

Figure 3-6. DSC profiles of 5% w/v (A) native WPI, (B) preheated WPI, (C) mTGase-pretreated WPI, and (D) sequentially-pretreated WPI dispersions at pH 7.0 and 100 mM NaCl. The mTGase pretreatments were conducted at 5.1 U/g-WPI for 4 h. The DSC scans were conducted at a heating rate of 1.5 ℃/min.

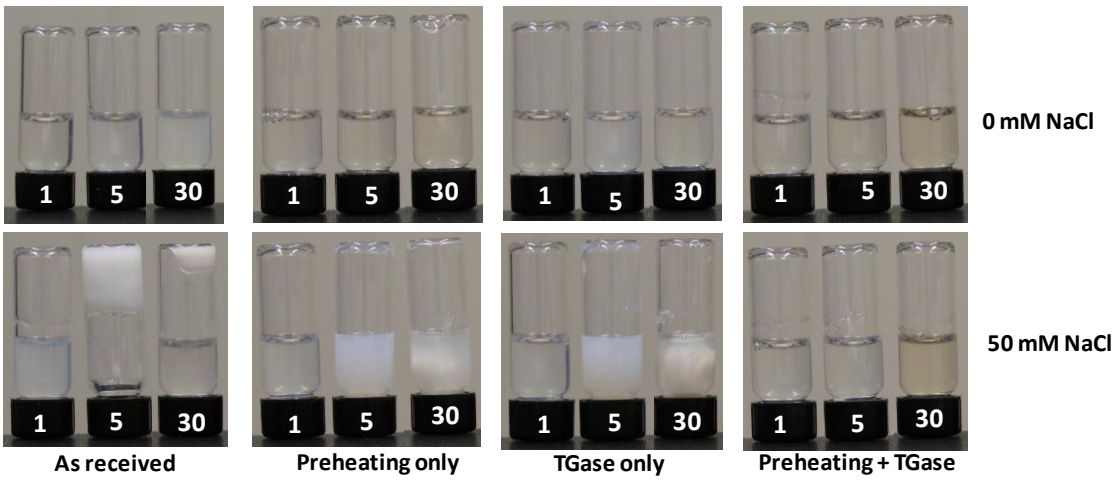

Figure 3-7. The 5% w/v WPI at pH 7.0 after heating at 138 °C for 1, 5, or 30 min, as labeled on vials. Samples were pretreated by individual or sequential steps of preheating and mTGase cross-linking. Preheating was performed at 80 °C for 15 min. The mTGase pretreatment was conducted at 50 °C for 4 h with an mTGase level of 5.1 U/g-WPI. Salt concentrations were adjusted before heat stability tests at 138 °C.

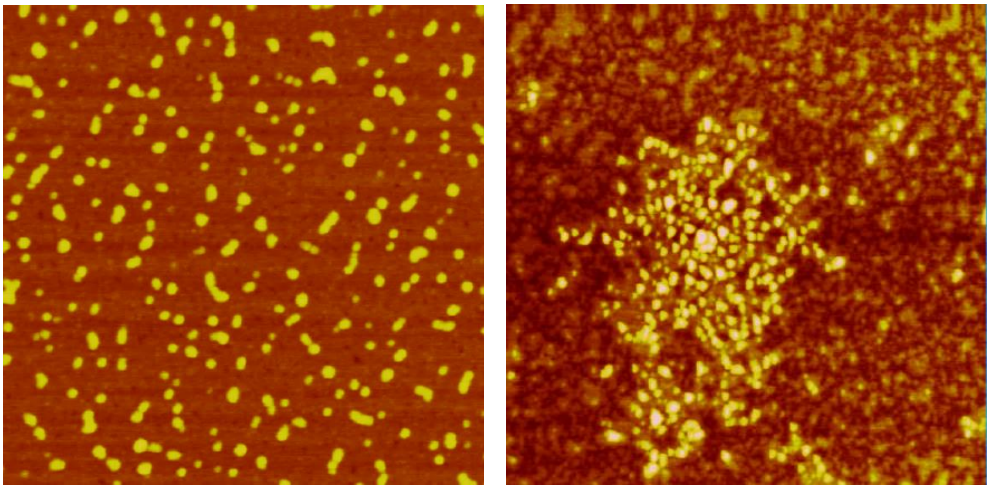

Figure 3-8. Topographical AFM images of WPI before (A) and after (B) sequential pretreatments using conditions as in Figure 3-7. The scanned area is 2 μm × 2 μm in each image.

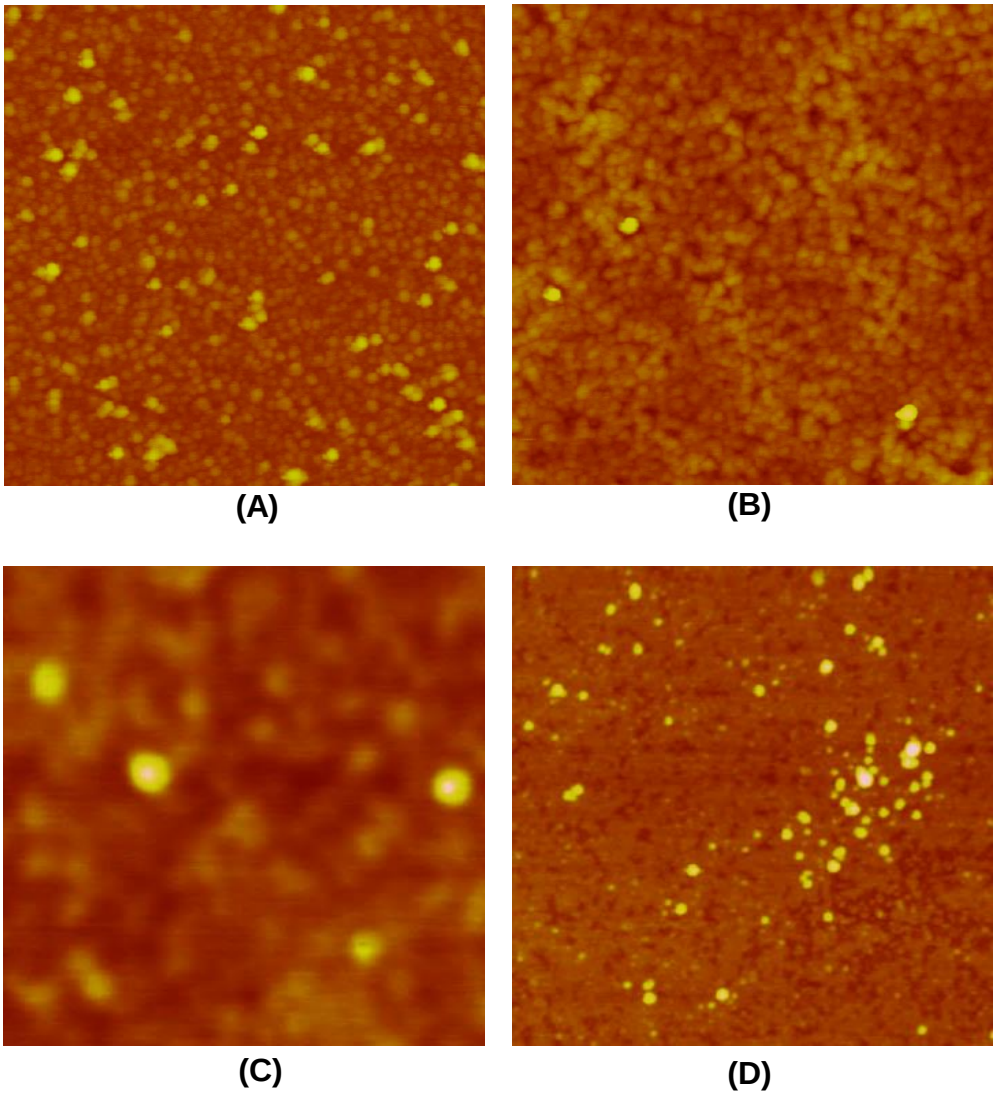

Figure 3-9. Topographical AFM images of sequentially-pretreated WPI shown in Figure 3-7 after heating at 138 °C for 1 (A and B) and 30 (C and D) min with 0 (A and C) and 50 (B and D) mM NaCl. The scanned area is 2 μm × 2 μm in each image.

Chapter 4. Properties of Whey Protein-Maltodextrin Conjugates as Impacted by Powder Acidity during the Maillard Reaction

4.1 Abstract

Heating the powder of whey protein isolate (WPI)-maltodextrin (MD) mixture, the Maillard reaction, improves thermal stability of WPI, but the effects of powder acidity have not been studied. In this work, solutions with WPI and MD were adjusted to pH 4-7 (m-pH) to obtain spray-dried powder that was glycated at 80 ℃ and 65% relative humidity for 1-4 h. The conjugates were evaluated for physicochemical properties. A higher m-pH and a longer glycation resulted in a darker color. The m-pH 6 treatment had the highest degree of glycation, lowest surface hydrophobicity, lowest isoelectric point, and highest denaturation temperature, which contributed to the best heat stability evaluated at 5% protein, pH 4-7 and 0-150 mM NaCl by heating at 138 ℃ for 1 min. The results indicate that adjusting WPI-MD mixture solution to pH 6.0 to prepare powder for glycation can reduce the color of protein ingredients while providing heat stability for transparent beverage applications.

Keywords: whey protein, glycation, powder acidity, thermal stability, color, degree of conjugation

4.2 Introduction

Whey protein isolate (WPI), containing more than 90% protein, is a common protein ingredient used to manufacture protein beverages. Beverages formulated with WPI are generally highly acidic to receive high clarity and heat stability, and a large amount of sugar is used to mask acid taste, which may cause a number of health problems such as teeth erosion and diabetes (Etzel, 2004; Mettler, Rusch, & Colombani, 2006). Low-acid beverages, with pH above 4.6, may reduce concerns about sugars. Compared to no thermal requirements of the US Food and Drug Administration for acid foods (pH < 4.6), the production of shelf-stable low-acid foods requires thermal processing to ensure microbiological safety, such as ultra-high temperature (UHT) processing of dairy products at 280 ℉ (138 ℃) for at least 8 s (McGarrahan, 1982). This presents a challenge for whey proteins that undergo denaturation and aggregation during heating to cause turbidity and storage instability, and possibly gelation, especially for high protein beverages with more than 4.2%w/v protein (Etzel, 2004).

The folded (native) state of proteins is only slightly more thermodynamically favorable than at the unfolded (denatured) state, and protein denaturation during thermal treatments is thus common (Chi, Krishnan, Randolph, & Carpenter, 2003). The thermally-induced denaturation and aggregation of whey proteins are generally attributed to several molecular forces such as van der Waals, hydrophobic, and electrostatic interactions and intra- and inter-molecular disulfide bonds via sulfhydryl-disulfide interchange, which can be altered by solution pH, protein concentration, ionic strength and temperature (Baier & McClements, 2005; Bryant & McClements, 1998). At neutral

pH, the clarity of whey protein solution after heating can be retained by supplementing co-solutes such as sucrose, glycerol, sorbitol, and polysaccharides, as a result of the increased denaturation temperature (T_d) of whey protein (Baier & McClements, 2001; Chanasattru, Decker, & McClements, 2007; Chantrapornchai & McClements, 2002; Kulmyrzaev, Bryant, & McClements, 2000). Preheating of whey proteins improves the heat stability and sequential mild preheating and transglutaminase pretreatments can stabilize WPI dispersions after heating at 138 °C and neutral pH for 1- 30 min (Wang, Zhong, & Hu, 2013; Zhang & Zhong, 2010; Zhong, Wang, Hu, & Ikeda, 2012). When solution pH is around the isoelectric point (pI) of whey proteins (β-lactoglobulin (β-Lg): 5.2; α-lactalbumin: 4.5-4.8; bovine serum albumin: 4.7-5.1) (Bryant & McClements, 1998), protein aggregation can be extensive because of the weakened electrostatic repulsion at the overall net charge being close to zero.

Glycation of proteins with reducing carbohydrates via the Maillard reaction is an effective method to modify protein functionality and has received much attention in recent years. The ε-amino group of the lysine residues is the primary glycation site (Ames, 1992). Other groups such as the imidazole group of histidine, the indole group of tryptophan, and the guanidine group of arginine residues also participate the reaction, but with a lesser extent (Ames, 1992). The Maillard reaction can take place in solutions (Li, Zhong, Ji, Yokoyama, Shoemaker, Zhu, et al., 2012; Zhu, Damodaran, & Lucey, 2008) and powder (Akhtar & Dickinson, 2003). In aqueous solutions, the glycation rate is low because water inhibits the initial Amadori reaction and subsequent reactions (Liu, Ru, & Ding, 2012; Van Boekel, 2001). In solutions, the Maillard reaction is favored at a higher pH from 4.0 to 12.0, generating undesired darker brown color (Ajandouz, Tchiakpe, Ore,

Benajiba, & Puigserver, 2001; Ajandouz & Puigserver, 1999; Damodaran, 2008; BeMiller & Huber, 2007). Conversely, conjugates produced from powder with neutral acidity improved the thermal stability of whey proteins (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001), showing transparent dispersions after heating samples at pH 3.0-7.0 and 0-150 mM NaCl and $CaCl_2$ (Liu & Zhong, 2012, 2013; Wang & Ismail, 2012). Besides the origin and ratio of substrates (proteins and reducing carbohydrates), reaction temperature and duration, water activity and reaction pH affect the glycation kinetics and thus functionality of reaction products significantly (Sanmartín, Arboleya, Villamiel, & Moreno, 2009). A higher temperature and a longer reaction duration increase the extent of Maillard reactions, which not only increases the degree of glycation (Schiff base) but also generates darkened colors associated with advanced reactions that have caused worldwide concerns about the probable carcinogenicity in humans (BeMiller & Whistler, 1996; Mottram, Wedzicha, & Dodson, 2002). The pathway of forming melanoidins, major pigments responsible for the brown color of Maillard-reaction products, is favored under more alkaline conditions, as being reported for glycation in solutions (Ajandouz, Tchiakpe, Ore, Benajiba, & Puigserver, 2001). However, impacts of powder acidity on color formation and thermal stability of whey proteins glycated with reducing saccharides at dry conditions have not been studied.

The major objective of this work was to characterize and understand thermal stability and color formation of WPI-maltodextrin (MD) conjugates produced by heating spray-dried powder with different acidity at 80 ℃ and 65% relative humidity for 1-4 h. To study powder acidity, WPI and MD were dissolved in deionized water and adjusted to pH 4-7 before spray drying. Physicochemical bases of powder acidity impacts were

studied for the degree of glycation, surface hydrophobicity, isoelectric point, and denaturation temperature of conjugates. Because Maillard reactions impact nutritional quality of proteins such as the loss of lysine and other essential amino acids and the reduced bioavailability (Hurrell, 1990), the secondary objective was to study amino acid compositions and *in vitro* digestibility of conjugates, with the digestibility evaluated for conjugates before and after the simulated UHT processing.

4.3 Materials and methods

4.3.1 Materials

The WPI was obtained from Hilmar Ingredients (Hilmar, CA). MD with a dextrose equivalent (DE) of 18 was acquired from Grain Processing Corporation (Muscatine, IA). Pepsin, 1-anilino-8-naphtalene-sulphonate (ANS), and pancreatin were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

4.3.2 Production of WPI-MD conjugates

WPI and MD18 were hydrated overnight at room temperature (~21 ℃) at 2.5%w/v each in deionized water. The mixture solution was adjusted to pH 4, 5, 6, and 7 using 10 N and 4 N NaOH and was spray-dried at an inlet temperature of 160 ℃, an outlet temperature of ca. 90 ℃, an air flow rate of 20 m^3/h, and a feeding rate of 250 mL/h (model B-290 Mini-Spray Dryer, Büchi Laboratoriums-Technik, Flawil, Switzerland). The spray-dried powder was incubated at 80 ℃ and 65% relative humidity for 1, 2 and 4 h in a humidity-controlled incubator (model IG420U Environmental

chamber, Yamato Scientific America Inc., Santa Clara, CA) for glycation. The powder was then collected and stored at -20 ℃ in a freezer.

4.3.3 Preparation of conjugate solution and heat stability test

The conjugates were prepared at 5%w/v protein in deionized water and hydrated overnight at ~21 °C. Solutions were measured for pH before adjusting to pH 7 using 4 N and 1N NaOH to prepare transparent solutions and filtration through a #1 filter paper (particle retention: > 11 μm, Whatman, Clifton, NJ) to remove visible particulates such as brush debris resulting from powder collection in sample preparation. Subsequently, samples were adjusted to pH 4-7 using 1 N and 0.25 N HCl or NaOH, 0-100 mM NaCl, and 0 or 5%w/w sucrose. The 1 mL solutions were contained in 4 mL glass vials and were heated in a glycerol bath at 138 ± 1 ℃ for 1 min to simulate UHT processing that is equivalent to 138 ℃ for at least 8 s for dairy products (McGarrahan, 1982). The 1 min duration was used to ensure sufficient thermal treatment throughout the vials. The visual appearance of samples was compared by photographing.

4.3.4 Color measurements

The extent of advanced Maillard reactions (brown color) was measured using the UV/Vis spectrophotometer (ThermoScientific, Waltham, MA) at 420 nm (BeMiller & Huber, 2007; Damodaran, 2008; Martins, Jongen, & Van Boekel, 2000). The color of protein solutions at pH 7.0 without sucrose and NaCl was measured before and after heating at 138 °C for 1 min using a MiniScan XE Plus Hunter Colorimeter (Hunter Associates Laboratory, Inc., Reston, VA). The parameters *L*, *a*, *b* were determined twice

each for two independent conjugate replicates and the color intensity (C^*) was calculated as follows (Medrano, Abirached, Panizzolo, Moyna, & Añón, 2009).

$$C^* = (a^2 + b^2)^{1/2}$$

4.3.5 Attenuated Total Fourier Transform Infrared (FTIR) Spectroscopy

The WPI-MD conjugate structure was studied using a Nicolet Nexus 670 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). WPI and its conjugates were prepared at 2%w/v protein in deuteroxide to reduce the strength of intra-molecular hydrogen bonds (Yost, Tejedor-Tejedor, & Anderson, 1990). A drop of each protein sample solution was placed on the ATR accessory, and over 64 scans at a 4 cm^{-1} resolution were collected and averaged to obtain the spectrum. The original spectrum was smoothed using the OMNIC software. The FTIR spectra at the amide I and II region (1400-1800 cm^{-1}) were analyzed to reflect changes in secondary structures of WPI before and after glycation.

4.3.6 Degree of glycation

The degree of glycation was measured for unreacted amines using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method (Tainturier, Roullier, Martenot, & Lorient, 1992), with some modifications. A fresh working solution of TNBS was prepared prior to assays by diluting the 1% TNBS solution (Geno Technology, Inc., St Louis, MO) in 0.1 M sodium bicarbonate aqueous solution to an overall TNBS concentration of 0.01% w/v. Each conjugate solution was prepared at 200 μg/mL in the 0.1 M sodium bicarbonate solution at pH 8.5, followed by mixing with the TNBS working solution at a volume ratio of 2:1. Subsequently, the mixture was incubated at 37

℃ in a water bath for 2 h, and the reaction was terminated by adding 10%w/v sodium dodecyl sulfate (SDS) solution and 1 N HCl to a volume ratio of mixture : SDS : HCl = 6:2:1. The final mixture solution was measured for absorbance at 335 nm using the above UV/Vis spectrophotometer.

4.3.7 Amino acid composition

Amino acid compositions of proteins were analyzed by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT) using the following procedures. "A 1-2 mg potion of protein powder was mixed with 0.3 mL of hydrolysis acid (6 N HCL, 0.2%v/v phenol, 1 nmol norvaline as internal standard) in a rimless 10 × 75 mm Pyrex ™ tube. After flame-sealing of the tube by vacuuming, the sample was hydrolyzed at 115 ℃ for 16 h. After cooling, the sample was opened and the hydrolyzate was evaporated to dryness in a heated vacuum centrifuge. The dried sample was dissolved in 0.5 mL of 0.02 M HCl (pH 1.5) and serially diluted so that a 20 μL injection would be 0.1% of the hydrolyzate. 0.5% of each sample aliquot was placed in the 0.3 mL limited volume insert of an 11 mm Snap-Cap glass vial after filtration through a 0.22 μm centrifugal-filter for loading onto the Hitachi L-8900 PH amino acid analyzer (Tokyo, Japan) equipped with a Hitachi 2622SPH ion-exchange column (4.6 mm ID × 60 mm) for amino acid composition analysis. Data was collected using EZChrom Elite for Hitachi software. The tryptophan and cysteine were partially destroyed due to the HCl hydrolysis, thus are not presented."

4.3.8 Differential scanning Calorimetry (DSC)

The thermal denaturation properties of WPI and its conjugates were studied using an ultrasensitive VP-DSC (MicroCal, LLC, Northampton, MA). Samples prepared at 1%w/v protein, pH 5.0 or 7.0, and 100 mM NaCl were degased at 20 ℃ for 10 min before injection into the sample cell. Deionized water was used as a reference. The temperature ramp from 25 to 105 ℃ was scanned at a rate of 1.0 ℃/min. The denaturation temperature (T_d) and total calorimetric apparent enthalpy change (ΔH) were determined using a two-state model supplied by MicroCal. All determinations were performed in two replications.

4.3.9 Zeta-potential measurement

The zeta potential was measured using a Delsa^{TM} Nano instrument (Beckman Coulter Inc., Brea. CA). WPI and its conjugates were prepared at 2%w/v protein and diluted to 1%w/v protein dispersions, with a pH adjustment to 3-7, followed by filtration through a 0.45 μm PVDF syringe membrane (EMD Millipore Corp., Billerica, MA) before measurements. For each sample, the zeta-potential – pH plot was used to extrapolate the pH corresponding to a zeta-potential value of zero and was treated as the pI. Two replicates were tested for three times each, and the averages from 6 measurements were reported.

4.3.10 Surface hydrophobicity

Surface hydrophobicity of WPI and its conjugates was evaluated using ANS as a fluorescence probe according to a literature method (Alizadeh-Pasdar & Li-Chan, 2000), with modification. The 2%w/v protein solutions at pH 5 and 7 were diluted to five

concentrations from 0.01 to 0.2% w/v using either a 10 mM phosphate-citrate buffer solution (pH 5.0) or a 10 mM phosphate buffer solution (pH 7.0). The ANS solution was prepared at 8 mM in the same buffer solutions. Fifteen μL ANS solution was added to 3 mL of each protein solution. The fluorescence intensity of samples was recorded at an excitation wavelength of 363 nm using a RF-150 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The emission spectra were recorded between 400 and 600 nm. The corresponding buffer solution was used as a blank. The initial slope of the fluorescence intensity-protein concentration plots after linear regression analysis was used as an index of surface hydrophobicity (S_0).

4.3.11 Digestibility of WPI and its conjugates

The stimulated in vitro protein digestion was performed according to a literature method (Garc á-Rico, Tejeda-Valenzuela, V élez, & Montoro, 2012), with some modifications. One gram of pepsin was suspended in 10 mL of 0.1 N HCl as the simulated gastric fluid, while the simulated intestinal fluid was prepared by dissolving 0.04 g of pancreatin and 0.25 g of bile extract in 10 mL of 0.1 M $NaHCO_3$. WPI or conjugate sample was suspended in 9 mL deionized water to an overall protein concentration of 5%w/v and adjusted to pH 2.0 using 6 N and 1 N HCl. The pH value was readjusted to pH 2.0 after 15 min of stirring if necessary. The protein solution was mixed with 80 μL of freshly prepared pepsin solution to a level of 0.008 g pepsin per 5 g protein. The sample was made up to 10 g with deionized water, followed by incubation in a shaking water bath operating at 37 ℃ and 120 rpm. After 2-h simulated gastric digestion, the reaction was stopped by adjusting pH to 5.0. The subsequent simulated

intestinal digestion was proceeded by adding 500 μL of the simulated intestinal fluid to the sample. After incubating for 2 h in the same shaking water bath, the sample was adjusted to pH 7.2 to stop the reaction by drop-wise addition of 0.5 M NaOH. The digested samples were centrifuged at 14129*g* for 30 min (Centrifuge MiniSpin, Eppendorf, Hamburg, Germany), and the supernatants were collected for SDS-PAGE analysis.

To perform SDS-PAGE, a protein sample was mixed with the loading buffer to obtain a protein concentration of 1 mg/mL, followed by heating at 95 ℃ for 5 min. Tris-HCl gels (15% precast linear gradient polyacrylamide gel) from Bio-Rad Laboratories (Hercules, CA) were used in electrophoresis at 120 V for about 80 min. Coomassie Brilliant Blue staining was carried out according to the gel manual. The stained gels were scanned (CanoScan 8600F, Canon U.S.A., Inc., New York, NY) and bands were quantified using Image J software (National Institute of Health, Bethesda, MD).

4.3.12 Statistical analysis

Statistical differences were analyzed using post-hoc comparison of means according to the least-significant-difference (LSD) mean separation method at a *p* level of 0.05. The SAS software (version 9.2, SAS Institute, Cary, NC) was used in analyses.

4.4 Results and discussion

4.4.1 Changes in powder acidity after glycation

Typically, WPI solutions are adjusted to neutral pH to prepare powder for glycation (Corzo-Martínez, Moreno, Villamiel, & Harte, 2010; Jiménez-Castaño,

Villamiel, & López-Fandiño, 2007; Morales & Jiménez-Pérez, 2001). In this study, the protein solution before spray-drying was adjusted to pH 4.0-7.0, referred as m-pH hereafter, to prepare powder for glycation. After hydrating conjugates in deionized water overnight, the solution pH was measured, presented in Figure 4-1. Glycation increased powder acidity, more significant at a longer glycation duration and higher m-pH. The decrease in pH during glycation of porcine plasma protein-monosaccharides (Benjakul, Lertittikul, & Bauer, 2005), bovine serum albumin-xylose (Easa, Armstrong, Mitchell, Hill, Harding, & Taylor, 1996) and fructose-glycine (Matmaroh, Benjakul, & Tanaka, 2006) solutions has been reported previously. The decrease in pH has been discussed due to the formation of acids, the conversion of basic amines to other compounds, and the condensation reaction between free amines of protein and carbonyl groups of reducing carbohydrates (Beck, Ledl, Sengl, & Severin, 1990; Matmaroh, Benjakul, & Tanaka, 2006). The greater decrease in the solution pH for the treatment of higher m-pH indicates a greater extent of the Maillard reaction.

4.4.2 Thermal stability and color formation

Photographs of selected samples after heating at 138 ℃ for 1 min are shown in Figure 4-2. The m-pH 6.0 treatment showed the best heat stability, while the m-pH 4.0 treatment was the least stable. For the m-pH 6 treatments, the sample glycated for a longer duration had a darker color and better thermal stability, with the 4-h treatment being stable after heating at all solution pH (s-pH) and 0-100 mM NaCl and the 2-h treatment, with a much lighter color, being unstable only at pH 5.0 and 0 mM NaCl.

Samples were compared for turbidity at 600 and 420 nm, shown in Figure 4-3 for those glycated for 2 h and heated at s-pH 4.0-7.0 and 50 mM NaCl. The Abs_{600} was much lower (not displayed) than Abs_{420}, with similar trends. Abs_{420} is also commonly used as an indicator of brown pigments formed in the final stage of Maillard reaction (Lerici, Barbanti, Manzano, & Cherubin, 1990). Generally, Abs_{420} increased with an increase in glycation time and m-pH. The m-pH 6 treatment overall had lower Abs_{420} than other treatments. The addition of 5% sucrose significantly reduced the turbidity of samples after heating. For the m-pH 6 treatment at s-pH 5.0 and 50 mM NaCl, the addition of 5% sucrose enabled a transparent dispersion after heating, contrasting with the sample without sucrose (Figure 4-2). Co-solutes such as sucrose, typically at higher than 10%, have been reported to improve the thermal stability of whey proteins at neutral acidity (Baier & McClements, 2001; Kulmyrzaev, Bryant, & McClements, 2000; McClements, 2002).

The color measurements (Table 4-1) generally agreed with visual observations, showing greater C^* at a higher m-pH and a longer glycation duration. Results in Table 4-1 also showed no significant changes in C^* before and after heating at 138 °C for 1 min. This group of studies shows that m-pH can be controlled to reduce the extent of glycation and color formation. Co-solutes can be used to reduce the extent of glycation while obtaining sufficient UHT heat stability, which are being studied in greater details and will be reported elsewhere. The rest of this paper is focused on the impacts of m-pH on the structures of glycated WPI.

4.4.3 Structural changes of glycated WPI analyzed by FTIR

Glycation of WPI results in an increase or decrease in contents of secondary structures, in terms of α-helix, β-sheet, β-turn, and random coil, modifying functional properties of WPI (Liu, Ru, & Ding, 2012). In this study, FTIR spectra in the amide I and II region (1400-1800 cm^{-1}, Figure 4-4) were compared for changes in secondary structures of WPI after glycation (Liu, Ru, & Ding, 2012). The peaks located around 1644 and 1529 cm^{-1} are characteristics of proteins (Liu & Zhong, 2013). The peaks at 1687 and 1529 cm^{-1} represent β-sheet and α-helix structures (Liu, Ru, & Ding, 2012; Surewicz, Mantsch, & Havel, 1996). After glycation, the intensity of peaks at 1687 cm^{-1} and 1549 cm^{-1} decreased, and shifts of peaks at 1644 cm^{-1}, to 1630 cm^{-1}, and 1529 cm^{-1}, to 1531 cm^{-1}, were observed. The changes in FTIR spectra indicate secondary structure changes of WPI after glycation, with a decrease in the peak at 1530 cm^{-1} (Liu, Ru, & Ding, 2012). Previously, we showed glycation led to the decreased content of ordered structures (β-sheet and α-helix) and the increased content of aperiodic structures (Liu & Zhong, 2013), which can result from the reduced hydrophobicity of whey protein after glycation (Monahan, German, & Kinsella, 1995).

4.4.4 Degree of glycation

In the present study, the degree of glycation was measured based on the reaction of free amines with TNBS, with a higher absorbance value at 335 nm (Abs_{335}) indicating a greater amount of unreacted amines and thus a lower degree of glycation. Abs_{335} decreased with an increase of m-pH until m-pH 6, before increasing at m-pH 7 (Figure 4-5). The degree of glycation generally agrees with heat stability results in Figure 4-2, since

a greater number of MD molecules attached to protein molecules provides stronger steric hindrance against protein aggregation during heating (Liu & Zhong, 2013).

4.4.5 Amino acid composition changes after glycation

Amino acid compositions of WPI before and after glycation are listed in Table 4-2. Lysine content decreased significantly after glycation, indicating it is the primary amino acid in the Maillard reaction (Ames, 1992). The lysine content was lower at a higher m-pH but was not significantly different ($p > 0.05$) for conjugates prepared at m-pH 5, 6 and 7.

4.4.6 Thermal denaturation properties of glycated WPI

The DSC thermograms of samples at pH 5.0 and 7.0 with 100 mM NaCl are shown in Figure 4-6, with the obtained T_d and ΔH summarized in Table 4-3. Generally, glycation led to an increase of T_d and a decrease of ΔH, indicating the improved thermal stability or tertiary conformational stability (Liu, Ru, & Ding, 2012). The T_d at pH 5.0 was higher than at pH 7.0, as observed for β-Lg that is the most abundant whey protein (Haug, Skar, Vegarud, Langsrud, & Draget, 2009). The impacts of m-pH on T_d (Table 4-3) show the same trend as the degree of glycation (Figure 4-5). The T_d is all below the 138 °C used in heat stability test (Figure 4-2), and therefore the increase of T_d after glycation only contributes partially to the improved heat stability after glycation at various m-pH.

4.4.7 Surface properties of glycated WPI

Glycation of WPI alters the distribution of protein surface charges, leading to decreases of pI and surface hydrophobicity (Achouri, Boye, Yaylayan, & Yeboah, 2005; Pedrosa, Trisciuzzi, & Ferreira, 1997). Zeta potential profiles of conjugates are presented in Figure 4-7, and the estimated pI (pH corresponding to zero zeta potential) is summarized in Table 4-4. The lowest pI, around 4.07, was observed for the m-pH 6.0 treatment, indicating the most significant changes in protein structure that is in agreement with the highest degree of glycation and best heat stability, as discussed previously. The decrease of pI is attributed to the decreased content of basic lysine on protein surface after glycation with MD, which is supported by the lowered magnitude of zeta-potential of glycated WPI than WPI at pH below pI (Figure 4-7). Because the m-pH 6 treatment was stable at pH 5.0 (with close–to-zero zeta-potential, i.e., weak electrostatic repulsion), the improved thermal stability of glycated WPI is mainly attributed to the steric repulsion generated by MD covalently bonded on whey proteins (Liu & Zhong, 2013).

Protein samples were further measured for surface hydrophobicity, with the obtained S_0 at pH 5 and pH 7 shown in Table 4-5. The decrease of S_0 after glycation is expected (Broersen, Voragen, Hamer, & de Jongh, 2004; Hiller & Lorenzen, 2010), and the higher S_0 at pH 5 than at pH 7 is due to reduced surface charge at acidity near pI (Corzo-Martínez, Moreno, Olano, & Villamiel, 2008). The m-pH 6 treatment showed the relatively lower S_0 than other treatments, agreeing with the highest degree of glycation (Figure 4-5).

4.4.8 In vitro digestibility

The lowered reactivity of trypsin on lysine and arginine residues (Liu & Zhong, 2013; Luz Sanz, Corzo-Mart ńez, Rastall, Olano, & Moreno, 2007) and conformational changes of proteins can decrease and increase, respectively, the digestibility of WPI after glycation (Corzo-Mart ńez, Soria, Belloque, Villamiel, & Moreno, 2010). In the present study, the in vitro digestibility of WPI glycated at m-pH 4.0-7.0 was studied before and after the simulated UHT processing (heating at 138 °C for 1 min) of protein solutions at pH 7.0, partially characterized using SDS-PAGE (Figure 4-8). The protein bands were analyzed for distributions (Table 4-6). The increase and broadening of protein MW of glycated WPI confirmed the glycation. After simulated gastric digestion of native WPI, the band corresponding to β-Lg remained mostly intact, the α-lactalbumin band disappeared, while high MW bands showed the decreased intensity. After the second step of simulated intestinal digestion for 2 h, residual β-Lg was visible, while other proteins became small molecular mass. For glycated WPI, the major smearing zone became narrower only after the simulated intestinal digestion but was still wider than the WPI (Figure 4-8), with molecular mass bigger than that of WPI (Table 4-6). This indicates that glycated WPI can be partially digested but is less digestible than WPI, suggesting that glycation provides a protection against proteolysis (Corzo-Mart ńez, Soria, Belloque, Villamiel, & Moreno, 2010). For samples heated at 138 °C for 1 min, β-Lg became susceptible to digestion by pepsin, with only vague bands remaining. The digestibility of proteins is significantly influenced by both the accessibility of enzymes to the reactive sites and protein structures. The native β-Lg is resistant to pepsin hydrolysis in the gastric

environment due to its stable globular structure that can be thermally denatured to expose susceptible peptide bonds for proteolysis (O'Loughlin, Murray, Kelly, FitzGerald, & Brodkorb, 2012). After the second step digestion of heated samples, there was a vague β-Lg band observed for WPI but no visible bands were detected for all conjugate treatments (Figure 4-8, Table 4-6). Since UHT processing is needed to produce shelf-stable beverages with pH greater than 4.6, the excellent digestibility and heat stability of glycated WPI suggest the potential application in production of transparent beverages. Sensory analysis of the eventual model beverage products however is needed for consumer acceptance.

4.5 Conclusions

Thermal stability and color intensity of glycated WPI were observed to be significantly affected by the acidity of spray-dried powder used in the Maillard reaction. WPI glycated at m-pH 6 exhibited the best thermal stability, particularly at pH 5.0, due to the highest degree of glycation, the highest increase of T_d, and the lowest $\triangle H$. The m-pH 6 treatment also had a relatively lighter color than that at m-pH 7. With addition of co-solutes like 5% sucrose, the powder acidity and glycation conditions can be carefully chosen to produce whey protein ingredients with excellent digestibility and light colors to manufacture transparent, shelf-stable beverages after UHT processing.

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Appendix

Table 4-1. Color measurement of WPI and WPI-MD conjugate solutions with 5% protein, without sucrose and adjusted to pH 7.0, before and after heating at 138 ℃ for 1 min.[ζ]

Samples	Glycation duration (h)	Before heating				After heating			
		L (Lightness)	*a* (Redness)	*b* (Yellowness)	*C** (Color intensity)	*L* (Lightness)	*a* (Redness)	*b* (Yellowness)	*C** (Color intensity)
WPI	0	70.44 ±0.66	-1.23 ±0.06	3.97 ±0.05	4.15 ±0.06^{L}	70.38 ±0.71	-1.24 ±0.05	4.43 ±0.57	4.61 ±0.54^{K,L}
Conjugate,	1	69.97 ±0.08	-1.67 ±0.00	7.98 ±0.76	8.15 ±0.75^{I,J,K}	70.00 ±0.22	-1.74 ±0.03	7.88 ±0.64	7.63 ±0.00^{J,K,L}
m-pH 4	2	68.48 ±0.85	-1.50 ±0.23	11.80 ±0.77	11.89 ±0.74^{H,I}	68.77 ±0.80	-1.53 ±0.29	11.62 ±0.72	11.31 ±0.09^{H,I,J}
	4	67.64 ±0.82	-1.25 ±0.30	17.39 ±3.49	17.44 ±3.46^{F,G}	68.13 ±1.05	0.30 ±0.24	16.63 ±3.63	19.55 ±0.47^{F}
Conjugate,	1	69.95 ±0.78	-1.72 ±0.10	9.99 ±0.39	10.13 ±0.37^{H,I,J}	69.46 ±0.40	-1.66 ±0.08	9.54 ±0.56	9.59 ±0.41^{I,J}
m-pH 5	2	67.54 ±0.52	-1.45 ±0.21	16.00 ±0.30	16.06 ±0.28^{F,G}	67.51 ±0.33	-1.33 ±0.12	15.87 ±0.39	15.76 ±0.15^{F,G}
	4	64.80 ±1.12	0.39 ±0.26	31.00 ±2.85	31.00 ±2.85^{D,E}	64.77 ±0.54	0.21 ±0.28	30.37 ±2.08	28.94 ±0.06^{E}
Conjugate,	1	69.49 ±0.71	-1.95 ±0.06	11.41 ±0.98	11.58 ±0.95^{H,I}	69.45 ±0.56	-1.78 ±0.05	10.84 ±0.76	10.68 ±0.31^{H,I,J}
m-pH 6	2	67.57 ±0.40	-1.75 ±0.10	18.83 ±0.33	18.91 ±0.34^{F}	67.74 ±0.30	-1.59 ±0.11	17.98 ±0.17	18.66 ±0.69^{F}
	4	63.99 ±1.46	0.33 ±1.60	38.24 ±5.11	38.26 ±5.12^{C}	63.96 ±1.17	0.75 ±0.92	37.23 ±4.78	34.24 ±0.56^{D}
Conjugate,	1	68.55 ±1.36	-1.89 ±0.22	15.71 ±3.25	15.82 ±3.20^{F,G}	68.39 ±0.68	-1.73 ±0.16	15.22 ±2.11	13.71 ±0.20^{G,H}
m-pH 7	2	65.89 ±0.25	-0.99 ±0.04	27.35 ±1.00	27.36 ±0.99^{E}	66.16 ±0.18	-0.11 ±1.32	25.66 ±1.09	27.25 ±1.15^{E}
	4	61.49 ±1.56	3.24 ±1.49	48.02 ±4.97	48.13 ±5.06^{A}	61.47 ±1.19	3.20 ±1.22	46.47 ±4.45	43.97 ±0.83^{B}
Deionized water	-	71.93 ±0.08	-0.78 ±0.01	0.50 ±0.01	0.92 ±0.01	-	-	-	-

[ζ] Numbers are mean ±standard deviation from two replicates. Different subscript letters represent significant difference in mean ($p < 0.05$).

Table 4-2. Amino acid compositions of WPI and WPI-MD conjugates prepared at different m-pH.

Amino acid	Composition (mole%)[ζ]					Normalized by mole% of WPI			
	WPI	Conjugate, m-pH 4	Conjugate, m-pH 5	Conjugate, m-pH 6	Conjugate, m-pH 7	Conjugate, m-pH 4	Conjugate, m-pH 5	Conjugate, m-pH 6	Conjugate, m-pH 7
Asx	9.7 ±0.7^{A}	9.9 ±0.3^{A}	10.0 ±0.3^{A}	10.0 ±0.3^{A}	10.0 ±0.3^{A}	1.02	1.03	1.03	1.03
Thr	6.3 ±0.4^{A}	6.5 ±0.2^{A}	6.6 ±0.2^{A}	6.5 ±0.2^{A}	6.6 ±0.2^{A}	1.03	1.05	1.03	1.05
Ser	4.6 ±0.2^{A}	4.7 ±0.1^{A}	4.7 ±0.1^{A}	4.8 ±0.1^{A}	4.7 ±0.1^{A}	1.02	1.02	1.04	1.02
Glx	16.1 ±1.3^{A}	16.7 ±0.6^{A}	16.6 ±0.5^{A}	16.8 ±0.6^{A}	16.8 ±0.6^{A}	1.04	1.03	1.04	1.04
Gly	2.5 ±0.1^{B}	2.5 ±0.0^{B}	2.5 ±0.0^{B}	2.5 ±0.0^{B}	2.5 ±0.0^{B}	1.00	1.00	1.00	1.00
Ala	9.4 ±0.4^{A}	9.3 ±0.2^{A}	9.4 ±0.2^{A}	9.4 ±0.2^{A}	9.4 ±0.2^{A}	0.99	1.00	1.00	1.00
Val	6.6 ±0.4^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	1.03	1.03	1.03	1.03
Met	2.4 ±0.2^{A}	2.4 ±0.1^{A}	2.5 ±0.1^{A}	2.5 ±0.1^{A}	2.5 ±0.1^{A}	1.00	1.04	1.04	1.04
Ileu	6.3 ±0.5^{A}	6.4 ±0.2^{A}	6.5 ±0.2^{A}	6.6 ±0.2^{A}	6.5 ±0.2^{A}	1.02	1.03	1.05	1.03
Ieu	11.8 ±0.9^{A}	12.0 ±0.4^{A}	12.1 ±0.3^{A}	12.1 ±0.4^{A}	12.1 ±0.4^{A}	1.02	1.03	1.03	1.03
Tyr	2.2 ±0.2^{A}	2.1 ±0.1^{A}	2.1 ±0.1^{A}	2.1 ±0.1^{A}	2.1 ±0.1^{A}	0.95	0.95	0.95	0.95
Phe	2.4 ±0.2^{A}	2.4 ±0.1^{A}	2.5 ±0.1^{A}	2.5 ±0.1^{A}	2.5 ±0.1^{A}	1.00	1.04	1.04	1.04
Lys	9.3 ±0.8^{A}	8.1 ±0.3^{A,B}	7.6 ±0.2^{B}	7.4 ±0.3^{B}	7.1 ±0.3^{B}	0.87	0.82	0.80	0.76
His	1.3 ±0.1^{A}	1.4 ±0.1^{A}	1.4 ±0.0^{A}	1.4 ±0.1^{A}	1.4 ±0.1^{A}	1.08	1.08	1.08	1.08
Trp	-	-	-	-	-	-	-	-	-
Arg	1.9 ±0.2^{A}	2.0 ±0.1^{A}	2.0 ±0.1^{A}	2.0 ±0.1^{A}	2.0 ±0.1^{A}	1.05	1.05	1.05	1.05
Pro + Cys	7.2 ±0.4^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	6.8 ±0.2^{A}	0.94	0.94	0.94	0.94

[ζ] Numbers are mean ±standard deviations from two replicates. Different superscript letters in the same row indicate significant difference (p <0.05).

Table 4-3. Denaturation temperature (T_d) and enthalpy change (ΔH) of WPI and conjugate dispersions at pH 5.0 and 7.0, with 100 mM NaCl. [ζ]

Samples	pH 5		pH 7	
	T_d (℃)	ΔH (kcal/g)	T_d (℃)	ΔH (kcal/g)
WPI	80.61 ± 0.66^{D}	20.52 ± 1.80^{A}	77.45 ± 0.53^{C}	12.40 ± 0.77^{A}
Conjugate, m-pH 4	88.20 ± 0.06^{C}	14.25 ± 1.17^{B}	82.66 ± 0.11^{B}	11.55 ± 0.60^{A}
Conjugate, m-pH 5	89.22 ± 0.17^{B,C}	11.55 ± 0.81^{B,C}	83.83 ± 0.18^{A,B}	8.33 ± 0.14^{B}
Conjugate, m-pH 6	90.77 ± 0.19^{A}	8.70 ± 0.60^{C}	84.62 ± 0.50^{A}	5.52 ± 0.38^{C}
Conjugate, m-pH 7	89.69 ± 0.12^{A,B}	9.33 ± 0.63^{C}	82.57 ± 0.93^{B}	4.73 ± 0.14^{C}

[ζ] Numbers are mean ± standard deviation from two replicates. Different superscript letters in each column represent significant difference in mean ($p < 0.05$).

Table 4-4. Isoelectric point of WPI and WPI-MD conjugates estimated from zeta potential profiles.

Sample	Isoelectric point
WPI	4.63 ± 0.14^{A}
Conjugate, m-pH 4	4.43 ± 0.03^{B}
Conjugate, m-pH 5	$4.29 \pm 0.05^{B,C}$
Conjugate, m-pH 6	4.07 ± 0.07^{D}
Conjugate, m-pH 7	$4.17 \pm 0.02^{C,D}$

Table 4-5. Surface hydrophobicity (S_0) of WPI and WPI-MD conjugates measured at pH 5.0 and 7.0.

Sample [ζ]	S_0 (slope $\times 10^6$) [#]	
	pH 5.0	pH 7.0
WPI	2.08 ± 0.08^{A}	1.85 ± 0.02^{B}
Conjugate, m-pH 4	2.10 ± 0.06^{A}	1.56 ± 0.01^{E}
Conjugate, m-pH 5	1.99 ± 0.03^{A}	1.54 ± 0.01^{E}
Conjugate, m-pH 6	$1.78 \pm 0.04^{B,C}$	$1.57 \pm 0.01^{D, E}$
Conjugate, m-pH 7	1.81 ± 0.20^{B}	$1.68 \pm 0.00^{C,D}$

[ζ] Conjugates were prepared at m-pH from 4 to 7, 80 °C, and 65% relative humidity for 2 h.
[#] Numbers are mean ± standard deviation from duplicate measurements. Different superscript letters represent significant difference in mean ($p < 0.05$).

Table 4-6. Comparison of SDS-PAGE band patterns of WPI and WPI-MD conjugates, with and without heat treatment at 138 °C for 1 min, before and after simulated gastric and intestinal digestions.

	Before digestion		After gastric digestion		After intestinal digestion	
	MW of protein bands (kDa)	% of band intensity	MW of protein bands (kDa)	% of band intensity	MW of protein bands (kDa)	% of band intensity
Samples without heating						
WPI	75	4.0	-	-	-	-
	>50	2.6	-	-	-	-
	37	5.4	37	7.0	-	-
	25	7.7	-	-	-	-
	16	73.7	16	93.0	16	38.6
	13	6.7	-	-	13	7.8
	-	-	-	-	12	53.6
Conjugate, m-pH 4	75	5.3	-	-	-	-
	50	4.5	50	5.8	-	-
	18-28	90.2	18-28	94.2	19-23	100.0
Conjugate, m-pH 5	75	5.7	-	-	-	-
	50	6.5	50	5.6	-	-
	18-28	87.8	18-28	94.4	19-23	100.0
Conjugate, m-pH 6	75	2.2	-	-	-	-
	50	5.4	50	4.5	-	-
	18-28	92.4	18-28	95.5	19-23	100.0
Conjugate, m-pH 7	75	2.5	-	-	-	-
	50	5.5	50	5.5	-	-
	18-28	92.0	18-28	94.5	19-23	100.0

Table 4-6. Continued.

	Before digestion		After gastric digestion		After intestinal digestion	
	MW of protein bands (kDa)	% of band intensity	MW of protein bands (kDa)	% of band intensity	MW of protein bands (kDa)	% of band intensity
Samples heated at 138 °C for 1 min						
WPI	75	5.3	-	-	-	-
	>50	1.6	-	-	-	-
	37	8.0	-	-	-	-
	25	3.5	-	-	-	-
	16	75.2	16	69.4	-	-
	13	6.4	-	-	-	-
	-	-	<10	30.6	-	-
Conjugate, m-pH 4	75	0.8	-	-	-	-
	37	2.7	37	5.9	-	-
	13-20	96.5	13-20	94.1	-	-
Conjugate, m-pH 5	75	1.2	-	-	-	-
	37	4.6	-	-	-	-
	13-20	94.2	13-20	100.0	-	-
Conjugate, m-pH 6	-	-	-	-	-	-
	13-20	100.0	13-20	100.0	-	-
Conjugate, m-pH7	37	4.0	-	-	-	-
	13-20	96.0	13-20	100.0	-	-

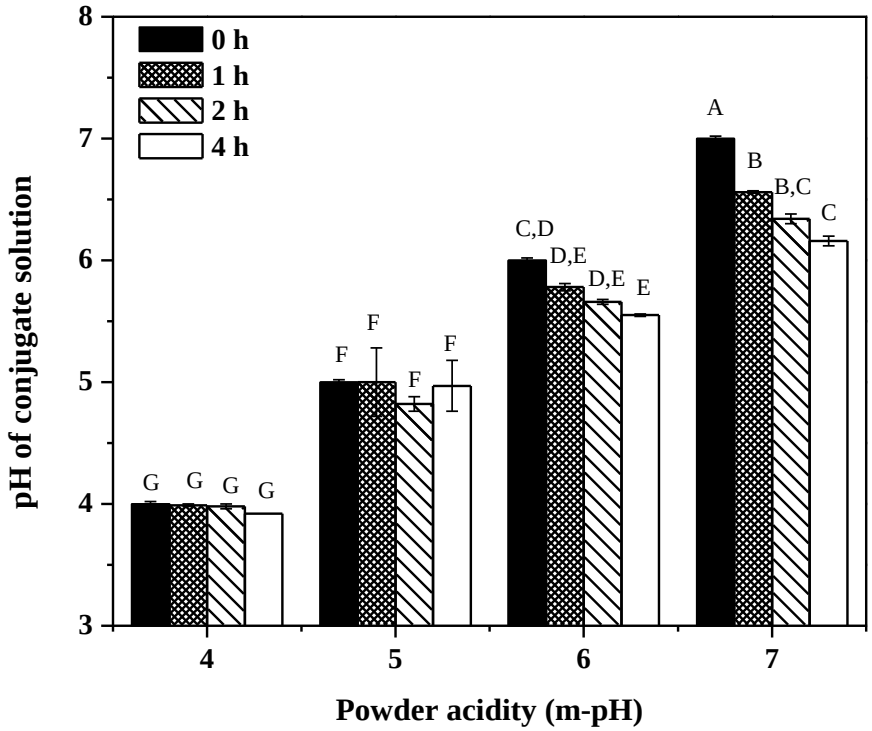

Figure 4-1. Comparison of solution pH after hydrating conjugate powder at 5%w/v protein in deionized water. Conjugates were prepared by incubating powder spray-dried from WPI and MD mixture solutions adjusted to pH 4-7 (m-pH, the 0-h treatments) at 80 ℃ and 65% relative humidity for 1-4 h. Error bars are standard deviations from two replicates. Different letters above bars indicate significant difference in mean ($p < 0.05$).

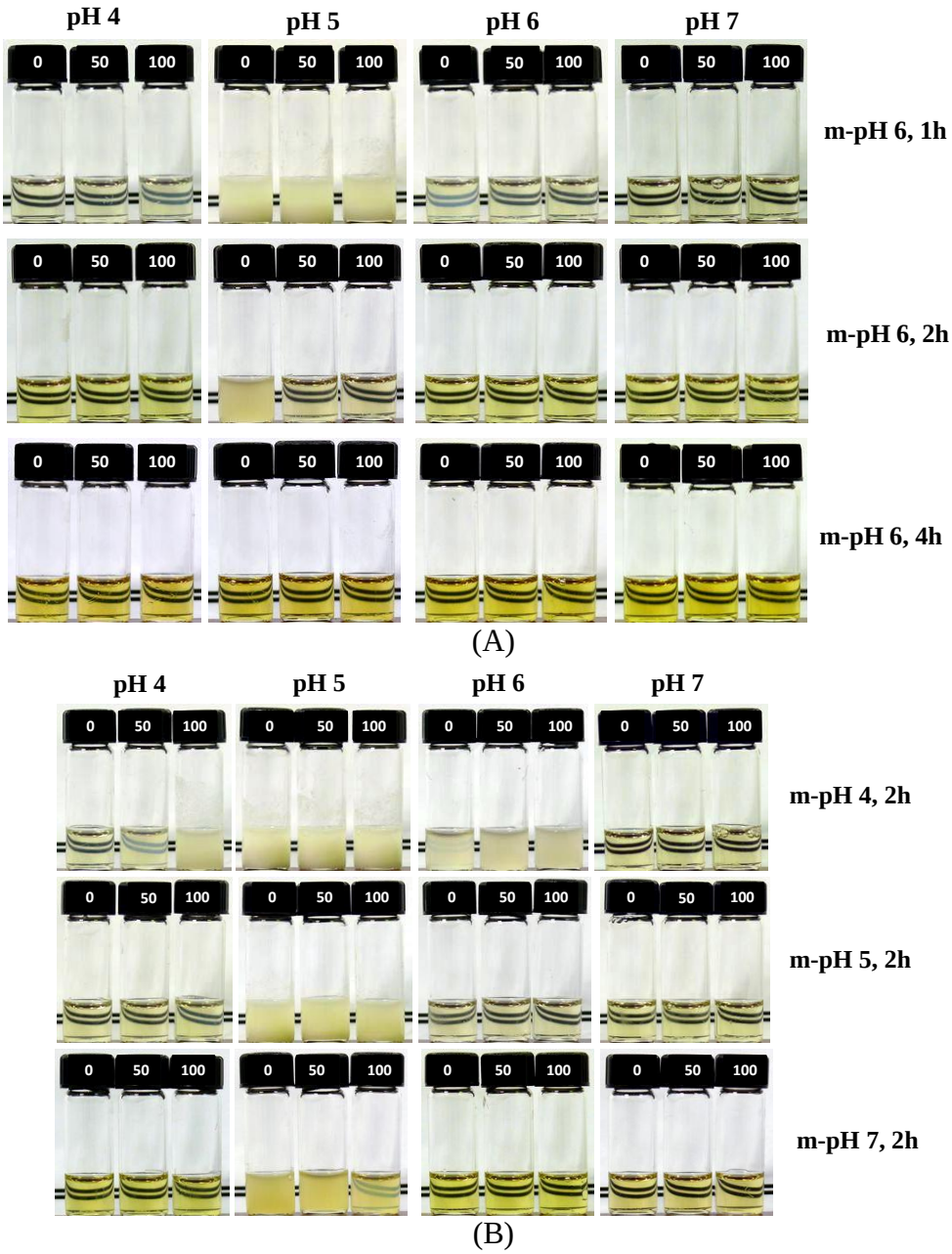

Figure 4-2. Appearance of WPI-MD conjugate solutions after heating at 138 ℃ for 1 min. Conjugate solutions were prepared with 5%w/v protein, adjusted to pH 4-7 (labeled above the vials), 0-100 mM NaCl (labeled on vial caps) and 5%w/w sucrose prior to heating. The conjugates were prepared at 80 ℃ and 65% relative humidity for 1, 2, and 4 h for the m-pH 6.0 treatment (A) or 2 h for the m-pH 4.0, 5.0, and 7.0 treatments (B).

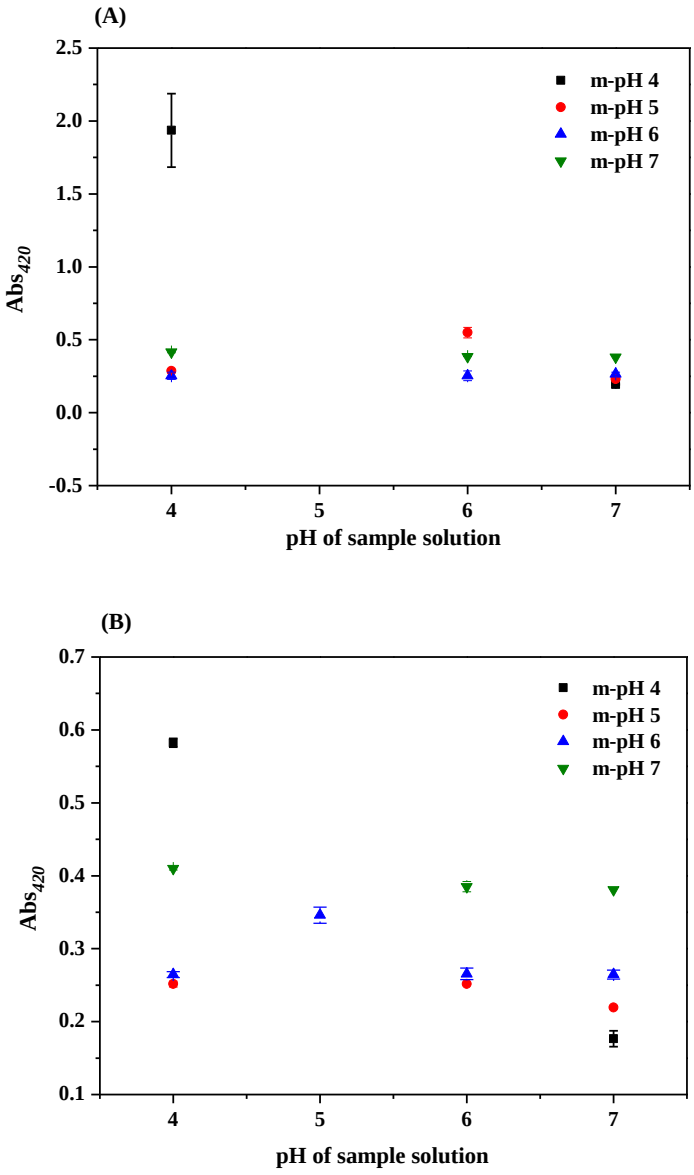

Figure 4-3. Absorbance at 420 nm (Abs_{420}) of conjugate solutions, prepared at 5% w/v protein, pH 4-7 (X-axis), 50 mM NaCl, and 0 (A) or 5% (B) w/w sucrose, after heating at 138 °C for 1 min. Conjugates were prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH, as in the legend) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h. Samples with Abs_{420} higher than 2.0 or forming gels are not plotted.

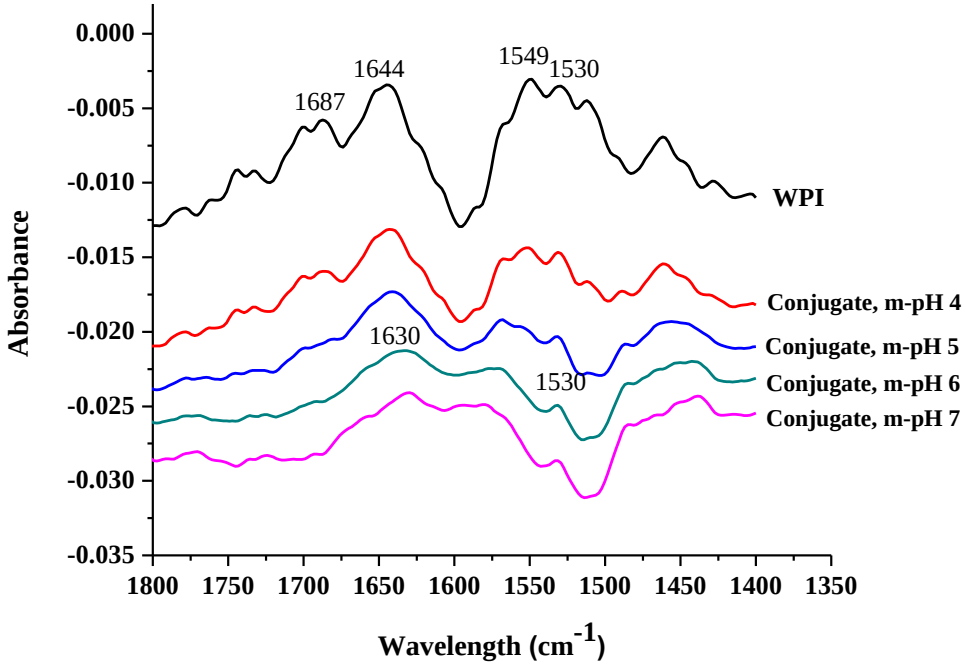

Figure 4-4. Comparison of FTIR spectra of WPI and the WPI-MD conjugate prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH, as in the legend) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h.

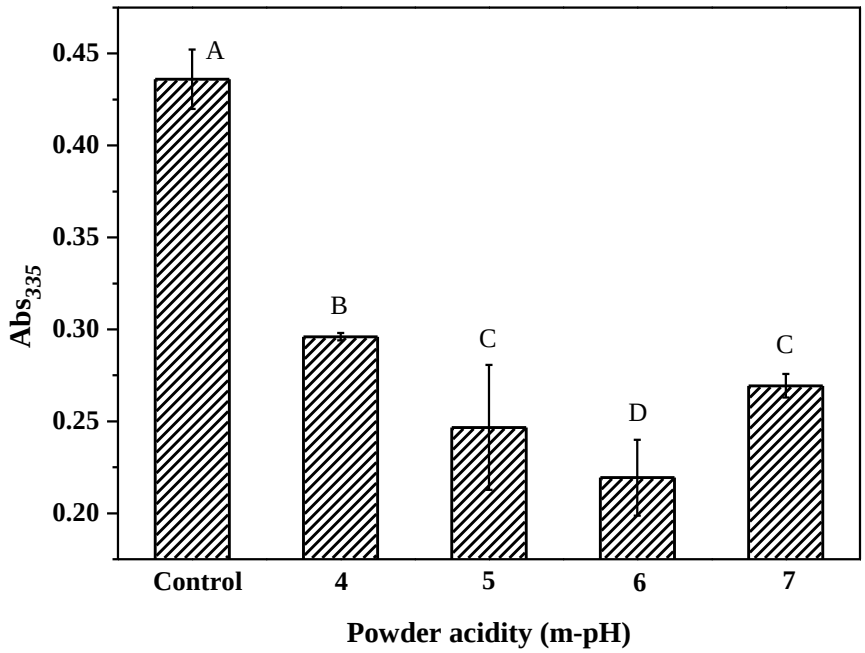

Figure 4-5. Absorbance at 335 nm (Abs_{335}) of WPI (control) and WPI-MD conjugates reacted with TNBS for measuring the degree of glycation. Conjugates were prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH, as in the X-axis) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h

Figure 4-6. Comparison of DSC thermograms of WPI solutions at pH 5 and 7 (A) and WPI-MD conjugate solutions at pH 5 (B) and 7 (C). Conjugates were prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH, as in the legend) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h. All samples were prepared at 1%w/v protein with 100 mM NaCl for DSC.

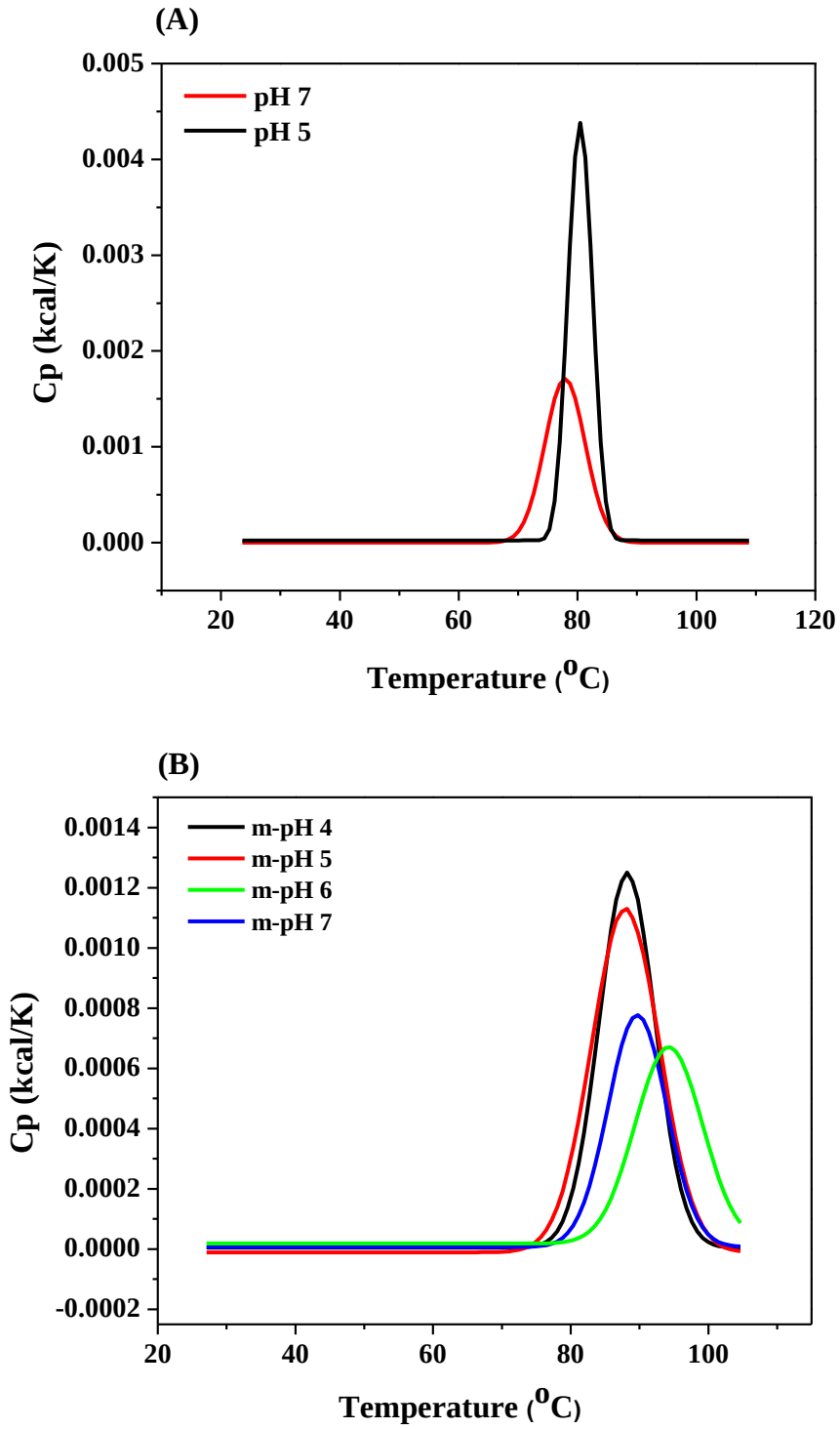

Figure 4-6. Continued.

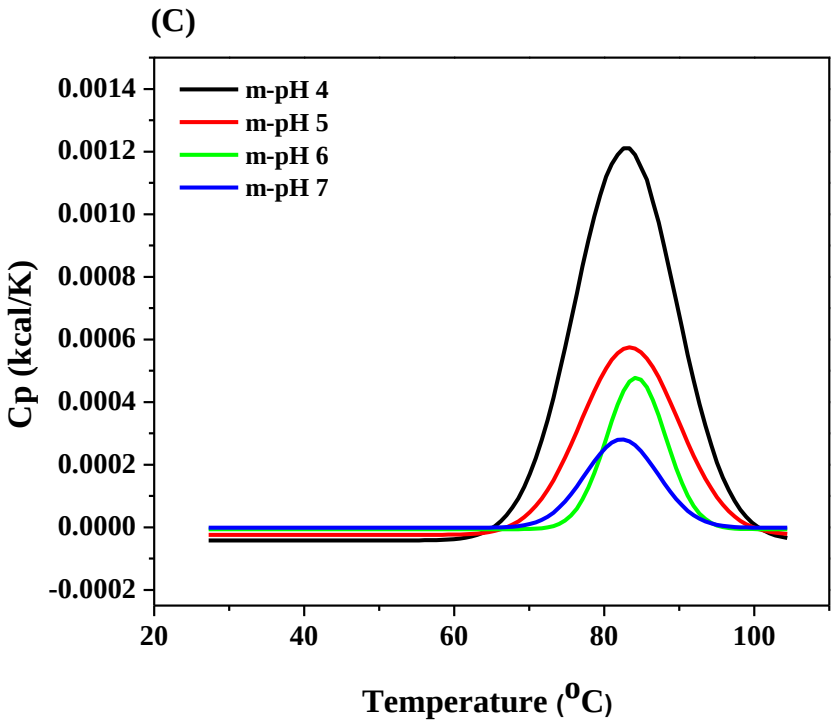

Figure 4-6. Continued.

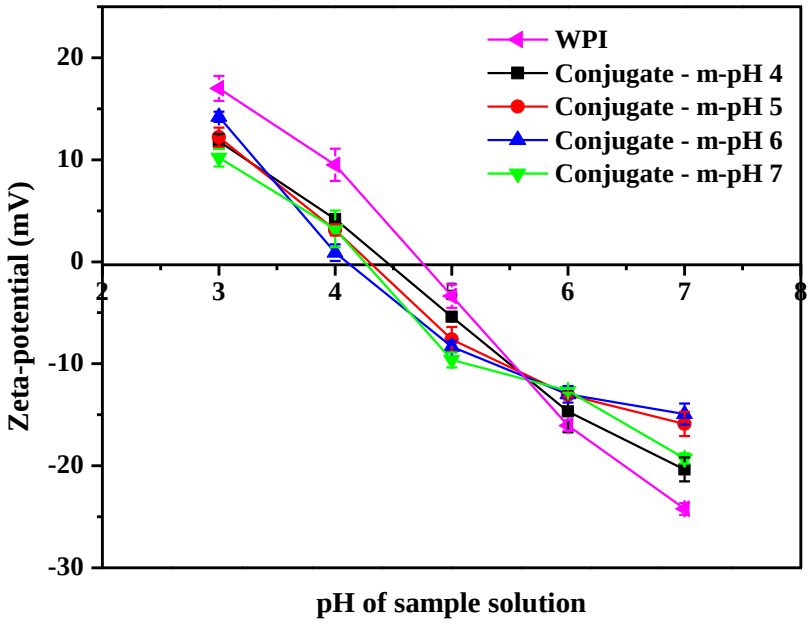

Figure 4-7. Zeta-potential profiles of WPI-MD conjugates prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH, as in the legend) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h, with comparison to WPI.

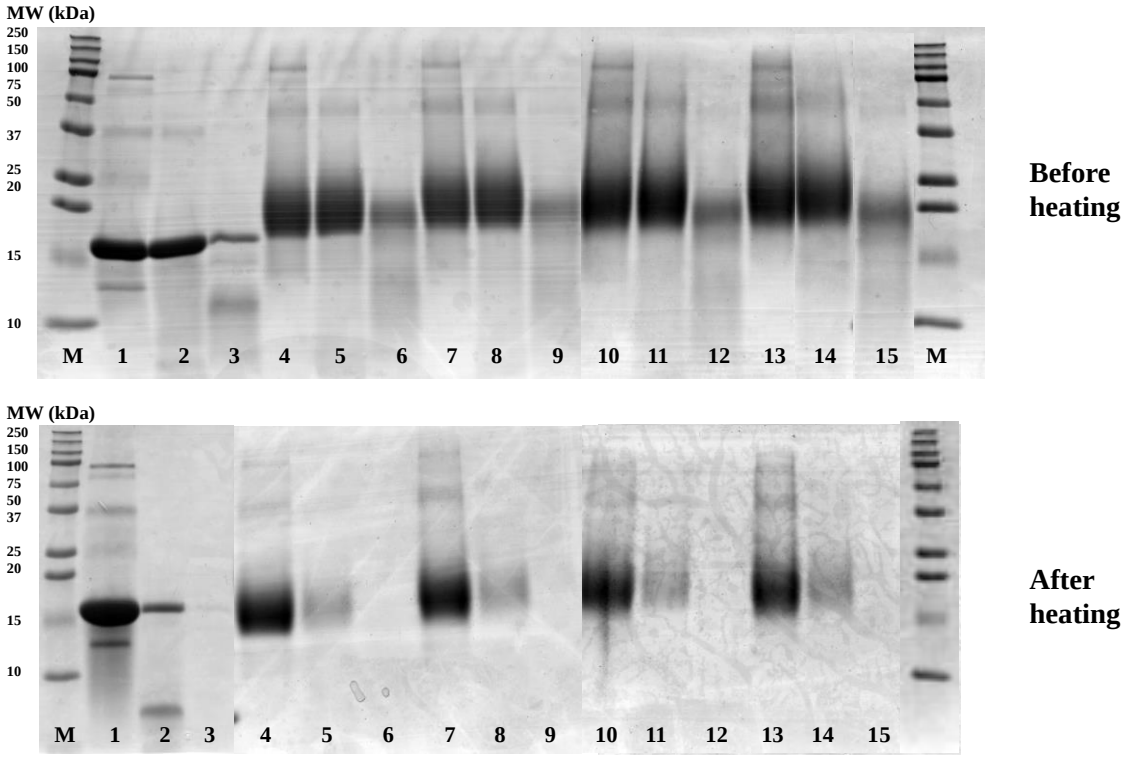

Figure 4-8. SDS-PAGE analysis of WPI and WPI-MD conjugates before and after simulated digestions. Conjugates were prepared by spray-drying WPI-MD solutions adjusted to pH 4-7 (m-pH) to obtain powder for glycation at 80 °C and 65% relative humidity for 2 h. Protein solutions at pH 7.0 before (top panel) and after (bottom panel) heating at 138 °C for 1 min were compared for digestibility. Lanes 1-3 are WPI samples, while lanes 4-6, 7-9, 10-12, and 13-15 represent conjugates prepared at m-pH 4, 5, 6, and 7, respectively. For each sample group, lanes from left to right represent protein samples before digestion, after the simulated gastric digestion, and after sequential steps of simulated gastric and intestinal digestions. Lanes labeled with "M" represent protein markers.

Chapter 5. Improved Thermal Stability of Whey Protein-Maltodextrin Conjugates at pH 5.0 by D-Glucose, Sucrose, D-cellobiose, and Lactose

5.1 Abstract

Thermal aggregation of whey proteins is a challenge for applications in clear food products with acidity around their isoelectric points (~pH 5.0). Glycating whey proteins with sufficient maltodextrins by the Maillard reaction effectively prevents thermal aggregation but produces undesirable color and byproducts. Co-solutes improve heat stability of whey proteins at neutral pH but have not been studied at pH 5.0. In this work, mono- and di-saccharides were studied for heat stability improvements of whey protein-maltodextrin conjugates at pH 5.0. The saccharides improved heat stability of conjugates by increasing the viscosity and denaturation properties (increased denaturation temperature and reduced enthalpy change), corresponding to smaller aggregates. Sucrose was more effective in improving thermal stability than D-cellobiose, lactose and D-glucose and was interpreted by the stronger preferential interactions with conjugates. Therefore, the saccharides can be used to stabilize conjugates during heating so that milder conjugation conditions can be adopted to reduce the color and byproduct formation during the Maillard reaction.

Keywords: WPI-MD conjugates, co-solute, heat stability at pH 5.0, preferential interactions, thermal denaturation properties, atomic force microscopy

5.2 Introduction

Whey proteins, with β-lactoglobulin and α-lactalbumin being the most abundant, are utilized widely in food and health-care products due to their nutritional and functional properties. Heat treatment is used to ensure product safety and shelf life and improve functional properties (Jelen, Rattray, & Fox, 1995). However, whey proteins can be readily denatured and aggregate during heating, which limits their application in food products, especially those with pH around the isoelectric point (pI). β-Lactogloblin, which is 5.2, and 4.8-5.1 for α-Lactalbumin, and bovine serum albumin, respectively (Bryant & McClements, 1998). The thermal stability of whey proteins is determined by the overall impacts of attractive and repulsive forces (Bryant & McClements, 1998; Semenova, Antipova, & Belyakova, 2002). Hydrophobic attraction is strengthened after thermal denaturation of whey proteins because of the exposure of initially embedded hydrophobic amino acids (Bryant & McClements, 1998). At pH around pI, electrostatic repulsion is weakened due to the reduced net charge, and whey proteins aggregate easily during heating (Bryant & McClements, 1998).

Glycation is an effective approach to prevent whey protein aggregation during thermal treatment because of the introduction of steric repulsion on protein surface (Liu & Zhong, 2012, 2013). Glycation of proteins with reducing saccharides is based on the Maillard reaction between an available amino group and a carbonyl-containing saccharide, with the reaction scheme and rate being dependent on temperature, duration, acidity, water activity, protein:saccharide ratio, and intrinsic properties of proteins and saccharides (BeMiller, 1996). In general, a higher temperature, a longer glycation duration, and a higher pH favor the production of Maillard reaction products that have

been linked to undesired brown color and negative health concerns such as carcinogenicity (Mottram, Wedzicha, & Dodson, 2002). Therefore, glycation conditions shall be chosen based on the balance of enabling functional properties and minimizing negative impacts due to the Maillard reaction.

Co-solutes such as sucrose, glycerol, sorbitol, and polysaccharides can increase the denaturation temperature and improve heat stability of whey proteins at neutral pH (Baier & McClements, 2001; Chanasattru, Decker, & McClements, 2007; Chantrapornchai & McClements, 2002; Kulmyrzaev, Bryant, & McClements, 2000). Co-solutes influence the conformation of proteins and thus protein interactions and functionalities by directly binding to surface groups of proteins or indirectly altering the structure of water around proteins (Timasheff, 1998). The improved thermal stability of whey proteins by sucrose was proposed to be attributed by the increased viscosity of continuous phase, causing a decrease in the frequency of protein-protein collisions, the increased protein unfolding temperature, and the reduced extent of protein unfolding (Kulmyrzaev, Bryant, & McClements, 2000). The latter two can be caused by the preferential exclusion of sucrose from the vicinity immediately surrounding proteins (Arakawa & Timasheff, 1982; Baier & McClements, 2001; Lee & Timasheff, 1981), causing protein molecules to fold more tightly or aggregate to reduce the surface area (Baier & McClements, 2001; Parsegian, Rand, & Rau, 1995; Timasheff, 1998). The chemical potential (μ) difference of protein molecules in a pure solvent and those in a co-solute solution is the transfer free energy (ΔG_{tr} – eq. 1) (McClements, 2002; Timasheff, 1993, 1998). When ΔG_{tr} is negative, protein molecules prefer to be surrounded by co-solute molecules (McClements, 2002), and it is therefore possible to change the

conformation, aggregation, and thermal stability of proteins (Timasheff, 1998). ΔG_{tr} is affected by the type and concentration of co-solutes.

$$\Delta G_{\mathrm{tr}} = \mu_2\,(\mathrm{co-solute}) - \mu_2(\mathrm{solvent}) \quad (1)$$

Impacts of co-solutes on protein heat stability and interactions at acidity near pI have not been studied. In this study, the first objective was to study the improvement of thermal stability of whey protein isolate-maltodextrin (WPI-MD) conjugates during the simulated ultra-high temperature (UHT) processing at pH 5.0 by D-glucose, sucrose, lactose and D-cellobiose. This acidity, near the whey protein pI, has been observed to require the most extensive glycation to obtain heat stability (Damodaran, 2008). UHT is required by the US Food and Drug Administration for low acid foods (pH > 4.6) and is equivalent to 138 °C for at least 8 s for dairy products (McGarrahan, 1982). To study conditions expected in applications, unconjugated WPI and MD were not removed, and the added mono- and di-saccharides are referred to co-solutes hereafter. The second objective was to understand the mechanism of thermal stability improvement of conjugates at pH 5.0 by studying rheological and thermal denaturation properties of conjugates based on the preferential interaction theory (Baier & McClements, 2001). The structures after heating were characterized using atomic force microscopy (AFM) to better understand impacts of co-solutes on protein aggregation during heating. Findings from this work can be used to reduce the extent of Maillard reaction to achieve thermal stability of conjugates while reducing the negative impacts due to glycation.

5.3 Material and Methods

5.3.1 Materials

The WPI was provided by Hilmar Ingredients (Hilmar, CA). MD with a dextrose equivalent (DE) of 18 was obtained from Grain Processing Corp (Muscatine, IA). D-cellobiose was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Deionized water was used throughout all experiments.

5.3.2 Preparation of glycated WPI

WPI and MD were dissolved at 2.5% w/v each in deionized water and hydrated overnight at room temperature (21 °C). The mixture solution was adjusted to pH 6.0 and spray dried at an inlet temperature of 160 ℃, an outlet temperature of ca. 90 ℃, an air flow rate of 20 m^3/h, and a feed rate of 250 mL/h (model B-290 Mini-Spray Dryer, Büchi Laboratoriums-Technik, Flawil, Switzerland). The spray-dried powder was incubated in a humidity-controlled incubator (model IG420U Environmental chamber, Yamato Scientific America Inc., CA) at 80 ℃ and 65% relative humidity for 2 h to produce the glycated WPI. The powder was then collected and stored at -20 ℃ in a freezer.

5.3.3 Preparation of protein solution for thermal stability test

Glycated WPI powder was dissolved at 5%w/v protein either in deionized water (solution A) or an aqueous solution with 0.8 M co-solutes - D-glucose, sucrose, lactose, or D-cellobiose (Solution B). Solutions A and B were mixed at different volume ratios to obtain a protein concentration of 5% w/v and a co-solute concentration of 0, 0.2, 0.4, 0.6

and 0.8 M. Protein solutions were adjusted to pH 5.0 using 1 N and 0.25 N HCl and 0-100 mM NaCl. The final mixtures were heated at 138 ± 1 ℃ for 1 min in a glycerol bath. The simulated heating duration was longer than the 8 s required for dairy products (McGarrahan, 1982) to provide sufficient thermal treatment throughout the vials. The visual appearance of samples after heating was photographed for comparison.

5.3.4 Turbidity measurement

Samples after heating were diluted with 10 mM phosphate citrate buffer (pH 5.0) to a protein concentration of 1.0%w/v. Absorbance of the dispersions was determined at 600 nm using a UV/Vis spectrophotometer (ThermoScientific, Waltham, MA) and used as an indicator of turbidity. All samples were measured in duplicate.

5.3.5 Viscosity measurement

The viscosity of protein solutions before and after heating at 138 ℃ for 1 min was measured using an AR2000 rheometer (TA Instrument, New Castle, DE) and a cone and plate geometry. The cone diameter was 40 mm and the angle was 1 °. Approximately 500 μL of a sample was transferred on the Peltier plate and a shear rate ramp from 0.1 to 100 s^{-1} was performed at 20 ℃. Each sample was measured in duplicate and the averages were reported.

5.3.6 Differential scanning calorimetry (DSC)

A VP-DSC calorimeter (MicroCal, Northamption, MA) was used to study thermal denaturation properties of glycated WPI as affected by co-solutes. Solutions were prepared at 1%w/v protein, pH 5.0, and 0-0.8 M co-solutes. Approximately 1 mL of a

solution was injected into the sample cell and degassed for 10 min at 25 ℃. Deionized water was used as a reference. The scanning was conducted from 25 to 115 ℃ at a rate of 1.5 ℃/min, with temperature equilibrium for 5 min both before and after scanning. The denaturation temperature (T_m) was determined from each thermogram, and the enthalpy change (ΔH) was calculated from the area of the endothermic peak using a two-state model (supplied by MicroCal).

5.3.7 Water activity measurement of co-solute solutions

Water activity (a_w) of co-solute solutions at room temperature (~25 ℃) was measured using an AquaLab water activity meter (Series 3TE, Decagon Devices, Inc., Pullman, WA). The instrument was calibrated using a 6 M NaCl solution with a_w of 0.760 before measurement.

5.3.8 Morphology of aggregated particles

The aggregate structure after heat treatment was studied using a Multimode VIII AFM (Bruker Inc., Billerica, MA). A rectangular cantilever probe (FESPA, Bruker Corp, Santa Barbara, CA) with a length of 200-250 μm and a quoted force constant of 2.80 N/m was used for scanning at the tapping mode. Two μL of a sample solution, which was diluted to a protein concentration of 10 ppm with deionized water previously, was spread evenly onto a freshly cleaved mica sheet. Heights of individual particles in the topography images were determined using section analysis provided by the NanoScope Analysis software of AFM instrument (version 1.40, Bruker, Inc., Billerica, MA).

5.3.9 Statistical analysis

All results were presented as averages and standard deviations from replicates. The analysis of the variance (ANOVA) of the data was conducted using the SAS software (version 9.2, SAS institute, Cary, NC). Significant differences were analyzed using the least significant difference (LSD) test with a confidence interval of 95%.

5.4 Results and discussion

5.4.1 Heat stability of conjugates as impacted by co-solutes

The appearance and absorbance at 600 nm (Abs_{600}) of samples with 0-0.8 M co-solutes after heating at 138 ℃ for 1 min and dilution to 1% w/v protein are presented in Figures 5-1 and 5-2, respectively. In general, a decrease in turbidity after heating was observed for samples with a higher co-solute and/or NaCl concentrations. At 0 mM NaCl, samples with sucrose showed the sharpest decrease in Abs_{600} after heating, followed by those with D-cellobiose, lactose and D-glucose (Figure 5-2A). At 50 and 100 mM NaCl, the effects of co-solute types became less significant, especially for those between sucrose and cellobiose, and heat stable samples were observed for treatments with 0.4 M or higher co-solutes (Figure 5-1), showing no significant difference in Abs_{600} ($P > 0.05$). The ability of cellobiose in stabilizing conjugates is attractive for food applications because it is not digestible by humans (Nakamura, Oku, & Ichinose, 2004; Sheriff., 2004), does not increase the calorie intake (Stoker, 2011), and is bland in taste (Sheriff., 2004).

At pH 5.0, around the pI of whey proteins, electrostatic repulsion is expected to be weak, and the heat-induced aggregation of WPI-MD conjugates is the balance of hydrophobic attraction and repulsive steric interactions provided by the MD moiety on proteins (Liu and Zhong, 2012). Before heating, hydrophobic interactions are not stronger than steric repulsion for the WPI-MD conjugates produced at the studied glycation conditions, as indicated by transparent samples in Figure 5-1. Thermal denaturation of conjugates strengthens hydrophobic interactions that are a function of environmental conditions such as pH, ionic strength, co-solutes, and temperature (Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013; Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2012). The extent of aggregation also is a function of protein and co-solute concentrations. These properties are presented below.

5.4.2 Viscosity of WPI-MD conjugate solutions with co-solutes

The rheograms of samples with 0 mM NaCl at a shear rate ramp of 0.1 – 100 s^{-1}were determined before heating at 138 ℃ for 1 min, presented in Figure 5-3. Generally, a higher shear stress therefore apparent viscosity at a same shear rate was observed at a higher co-solute concentration (Figure 5-3), as expected. WPI solution showed the Newtonian behavior, while the gradual increase in co-solute concentration resulted in shear-thinning behavior. The samples with glucose had the lowest viscosity, and those with disaccharides did not show significant differences. This is expected because glucose samples had lower mass concentrations than other treatments with same molar concentrations of co-solutes. A higher viscosity at a higher co-solute concentration generally agreed with heat stability improvements in Figures 5-1 and 5-2, except the

better heat stability of glucose treatments than lactose treatments at 0 mM NaCl (Figure 5-2A). Therefore, viscosity alone is insufficient to interpret the improved heat stability of WPI-MD conjugates by co-solutes.

5.4.3 Thermal denaturation properties of WPI-MD conjugates as impacted by co-solutes

The influences of co-solutes and their concentrations on the denaturation properties of WPI-MD conjugates at pH 5.0 and 0 mM NaCl are summarized in Table 5-1. The T_m shifted from ~ 81 ℃ of WPI to ~89 ℃ of WPI-MD conjugates, and ΔH decreased from ~21 kcal/g to ~15 kcal/g after glycation. Therefore, glycation of WPI with MD reduces the degree of protein denaturation during heating. However, glycation at the studied conditions alone is insufficient to prevent protein aggregation during the simulated UHT processing (Figures 5-1 and 5-2), likely because the number of MD glycated on whey proteins did not provide sufficiently strong steric repulsion (Akhtar & Dickinson, 2003; Liu & Zhong, 2012; Mulsow, Jacob, & Henle, 2009; Zhu, Damodaran, & Lucey, 2010). Extensive glycation, e.g., with MD at 60 °C for 72 h, was observed to stabilize WPI after heating at 138 °C for 1 min, but the sample color was dark (Liu & Zhong, 2012).

Co-solutes further improved denaturation properties of conjugates, shown for thermograms in Figure 5-4 and the determined T_m and ΔH in Table 5-1. Overall, T_m increased while ΔH decreased with an increase in co-solute concentration, with the extent varying with the co-solute type. The interactions between protein molecules and surrounding water molecules can be altered in the presence of co-solutes, resulting in

significant changes in the magnitude and slope of specific heat (Figure 5-4) (Freire, 1995). The decrease of ΔH indicates a more compact conformation of protein molecules and the contact between protein surface and water molecules becomes less favorable at a higher co-solute concentration. When heat stability and denaturation properties are correlated, it appears that a sufficient increase of T_m (to ~ 92 ℃ at 0 mM NaCl) and a sufficient decrease of ΔH (to 7.8 kcal/g at 0 mM NaCl) by co-solutes are needed to produce transparent samples after the simulated UHT processing. This was enabled by supplementing no lower than 0.4 M sucrose, 0.6 M cellosbiose, or 0.8 M of other three co-solutes (Table 5-1). It was also observed that 0.2 M cellobiose was the least effective in increasing T_m but most effective in reducing ΔH. Proteins start unfolding when heated to its T_m and ΔH is correlated with the content of ordered secondary structures (Koshiyama, Hamano, & Fukushima, 1981). The ΔH is a net result of endothermic reactions, such as disruption of hydrogen bonds (Privalov & Khechinashvili, 1974), and exothermic reactions, such as breakup of hydrophobic interactions (Arntfield & Murray, 1981; Jackson & Brandts, 1970). D-cellobiose may be more effective than other co-solutes in suppressing the breakage of hydrogen bonds or the breakup of hydrophobic interactions, thus reducing conformational changes of WPI during heating.

5.4.4 Estimation of changes in preferential interactions between conjugates and co-solutes

The increase of T_m by co-solutes has been proposed to be caused by the preferential exclusion of co-solutes from the region surrounding proteins (Baier & McClement, 2001; Semenova, Antipova, & Belyakova, 2002). This can be caused by

steric exclusion because sugar molecules are considerable bigger than water molecules. The preferential interactions between proteins and neighboring co-solute or water molecules may cause differences in electrostatic, van der Waals, hydrogen bonding and hydrophobic interactions to either favor or oppose protein unfolding (Timasheff, 1993, 1998). For weakly interacting co-solutes, the preferential interaction can be studied by Γ_{23}, the preferential interaction coefficient (Kovrigin & Potekhin, 1997).

$$\Gamma_{23} = (\partial v_3/\partial v_2)_{T,\mu_3} \tag{2}$$

where v_2, v_3 represent the number of protein and co-solute molecules, respectively. *T* is the absolute temperature, and μ_3 is the chemical potential of co-solutes.

The Γ_{23} describes the amount of co-solutes that must be added or removed from the bulk phase in order to maintain the chemical potential when the protein concentration is slightly increased (McClements, 2002). The Γ_{23} provides quantitative information about preferential exclusion (negative Γ_{23}) or accumulation (positive Γ_{23}) of co-solute molecules at protein surface (Record, Zhang, & Anderson, 1998; Baier & McClement, 2001; McClement, 2002). Denaturation of protein molecules increases the surface hydrophobicity, resulting in changes in Γ_{23} (Kovrigin & Potekhin, 1997) as follows:

$$\Delta\Gamma_{23} = -\frac{\Delta H \left(\frac{\partial T_m}{\partial x_3}\right)_{pH}}{RT_m^2(\partial \ln a_3/\partial x_3)_{T_m}} \tag{3}$$

where R is the gas constant, x_3 is the co-solute mole fraction, a_3 is the activity of co-solute. ΔH and T_m can be determined directly using the DSC, while a_3 can be calculated from the Gibbs-Duhem equation (eq. 4) as long as the water activity of aqueous sugar solution at T_m is obtained:

$$x_1 \frac{\partial \ln \gamma_1}{\partial x_1} = -x_3 \frac{\partial \ln \gamma_3}{\partial x_3} \quad (4)$$

where γ is the activity coefficient ($a_i = \gamma_i x_i$), and subscript 1 represents water.

Although a higher activity coefficient is expected at a higher temperature (Gharsallaoui, Rogé, Génotelle, & Mathlouthi, 2008), the effect of temperature on water activity was found almost negligible for dilute solutions but significant for very concentrated solutions, e.g., >80% w/w sucrose (Starzak & Mathlouthi, 2006). It was also observed that the activity coefficient of sucrose in aqueous solutions with <50% w/w sucrose is similar between 0 and 80 °C (Gharsallaoui, Rogé, Génotelle, & Mathlouthi, 2008). Because the maximum co-solute concentration is 0.8 M (higher mass concentrations for disaccharides, equivalent to 21.5% w/w), the temperature effects on water activity are treated to be negligible in the present study.

The $\Delta\Gamma_{23}$ values at various molar concentrations of co-solutes are shown in Figure 5-5. Generally, $\Delta\Gamma_{23}$ became more negative as the co-solute concentration increased, suggesting the preference of the native state over the denatured state of proteins. A series of studies revealed that co-solutes are preferentially excluded from the vicinity of a globular protein at relatively low temperatures but accumulate at higher temperatures (Xie & Timasheff, 1997). The developed thermodynamic model suggests the accumulation of co-solutes on protein surface is not thermodynamically favored because this creates a gradient of co-solute concentration between the region next to the protein surface and the bulk phase. The accumulation of co-solutes on protein surface also reduces the contact between protein and water. The $\Delta\Gamma_{23}$ is a result of combined effects due to changes in the exposure of ionic, polar and nonpolar groups of proteins to nearby

molecules (Baier & McClement, 2001). The magnitudes of $\Delta\Gamma_{23}$ (Figure 5-5) followed the order of sucrose > D-cellobiose > lactose > D-glucose. Therefore, sucrose is the most effective in preventing whey proteins from unfolding, which agrees with the observed heat stability (Figures 5-1 and 5-2).

5.4.5 Structure of whey proteins with sugars studied by AFM

The morphology of WPI-MD conjugates after heating with co-solutes was studied using AFM, with images presented in Figure 5-6 and the analyzed average particle heights listed in Tables 5-2 and 5-3. The WPI-MD conjugate without thermal treatment had individual particles (Figure 5-6a) with an average height of 3.5 nm, which is slightly bigger than native whey proteins with an average height of ~3.4 nm reported in our previous study (Wang, Zhong, & Hu, 2013). After heating at pH 5.0 without co-solutes, irregularly-shaped aggregates (Figure 5-6b) with an average height of ~10 nm were observed. The addition of a higher concentration of D-glucose gradually reduced the irregularity of aggregate morphology (Figure 5-6c-f) and particle height (Table 5-2), eventually resulting in uniform particles (Figure 5-6f) with an average height of ~5 nm at 0.8 M D-glucose. The trend is in agreement with the turbidity data in Figure 5-2a.

The structures of WPI-MD conjugates after being heated with 0.6 M of different co-solutes are shown in Figure 5-6g-I and the average particle heights are presented in Table 5-3. Particle heights are the most reliable measurements in AFM (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). The much smaller particle heights of cellobiose and sucrose treatments than those of lactose and D-glucose treatments (Table 5-3) agreed with the transparent vs. turbid appearance of these samples (Figure 5-1). The

AFM analysis showed that the thermal aggregation of WPI-MD conjugates occurred in all treatments, was less significant at a higher co-solute concentration, and was a function of co-solute structure. The AFM results indirectly suggest differences in the interactions between co-solutes and conjugates, as revealed by above analyses.

5.5 Conclusions

The current study showed that heat stability of WPI-MD conjugates at pH 5.0 can be improved by supplementing a sufficient quantity of mono- or disaccharides. Sucrose was the most effective stabilizer, followed by D-cellobiose, lactose and D-glucose. In addition to the reduced collision frequency between conjugate molecules due to the increased viscosity, co-solutes impacted the denaturation of conjugates differently. The differences in heat stability improvements by co-solutes were correlated with preferential interaction coefficients analyzed based on thermal denaturation properties of conjugates and activity of co-solutes. Findings from this study can be used to prepare clear beverages at around pH 5.0 that would otherwise require extensively-reacted Maillard-type products.

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Appendix

Table 5-1. Heat denaturation properties of WPI and WPI-MD conjugate at pH 5.0 as impacted by co-solutes. *

Sample	Co-solute concentration (M)	T_m (℃)	ΔH (kcal/g)
Native WPI	0	80.6 ± 0.7^{J}	20.5 ± 1.8^{A}
Glycated WPI	0	89.3 ± 0.1^{I}	14.6 ± 0.2^{B}
Glycated WPI + D-glucose	0.2	90.3 ± 0.2^{H}	$11.3 \pm 0.1^{D,E}$
	0.4	$91.0 \pm 0.1^{F,G}$	$10.3 \pm 0.6^{D,E,F}$
	0.6	92.6 ± 0.1^{E}	$9.3 \pm 0.6^{F,G}$
	0.8	94.2 ± 0.1^{C}	$7.9 \pm 1.0^{G,H}$
Glycated WPI + Sucrose	0.2	91.4 ± 0.1^{F}	$11.8 \pm 0.2^{C,D}$
	0.4	92.3 ± 0.3^{E}	$7.8 \pm 0.2^{G,H}$
	0.6	95.6 ± 0.3^{B}	$4.5 \pm 0.1^{K,L}$
	0.8	96.4 ± 0.2^{A}	3.7 ± 0.0^{L}
Glycated WPI + Lactose	0.2	$90.7 \pm 0.1^{G,H}$	$13.1 \pm 0.1^{B,C}$
	0.4	92.1 ± 0.1^{E}	$10.1 \pm 0.9^{E,F}$
	0.6	93.3 ± 0.1^{D}	$9.0 \pm 0.4^{F,G}$
	0.8	96.6 ± 0.1^{A}	$6.2 \pm 0.5^{I,J}$
Glycated WPI + D-cellobiose	0.2	89.5 ± 0.1^{I}	$7.0 \pm 0.2^{H,I}$
	0.4	$90.8 \pm 0.3^{F,G,H}$	$6.6 \pm 0.1^{H,I}$
	0.6	92.0 ± 0.3^{E}	$5.8 \pm 0.2^{I,J,K}$
	0.8	95.5 ± 0.2^{B}	$4.8 \pm 0.4^{J,K,L}$

* Numbers are mean ± standard deviation from duplicate measurements. Different superscript letters represent significant difference in the mean ($p < 0.05$).

Table 5-2. AFM particle heights of WPI-MD conjugates in solutions with 0-0.8 M D-glucose before and after heating at 138 ℃ for 1 min at pH 5.0.

	NaCl (mM)	D-glucose (M)	Average particle height (nm)*
Before heating	0	0	3.47 ± 0.43^{D}
After heating	50	0	10.12 ± 1.10^{A}
		0.2	9.79 ± 1.15^{A}
		0.4	8.66 ± 0.71^{B}
		0.6	8.13 ± 1.15^{B}
		0.8	5.12 ± 0.73^{C}

* Numbers are mean ± standard deviation from >80 particles on four images, two images each from duplicate protein samples. Different superscript letters represent significant difference in the mean ($p < 0.05$).

Table 5-3. AFM particle heights of WPI-MD conjugates in solutions with 0.6 M co-solutes and 50 mM NaCl after heating at 138 ℃ for 1 min at pH 5.0.

Co-solute type	Average particle height (nm)*
D-glucose	8.13 ± 1.15^{A}
Sucrose	5.91 ± 0.95^{B}
Lactose	$6.40 \pm 0.74^{A,B}$
D-cellobiose	5.48 ± 1.02^{B}

* Numbers are mean ± standard deviation from >80 particles on four images, two images each from duplicate protein samples . Different superscript letters represent significant difference in the mean ($p < 0.05$).

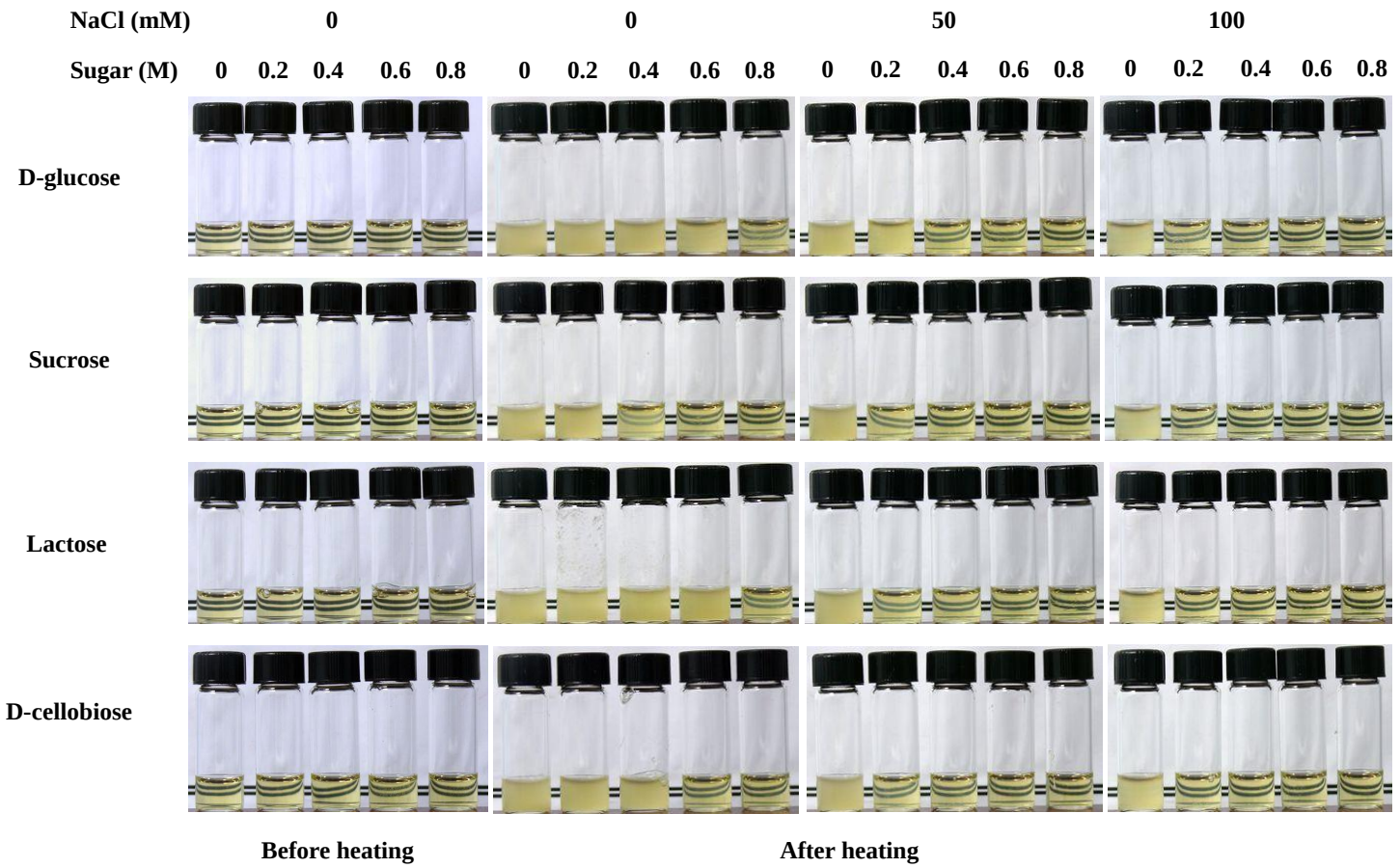
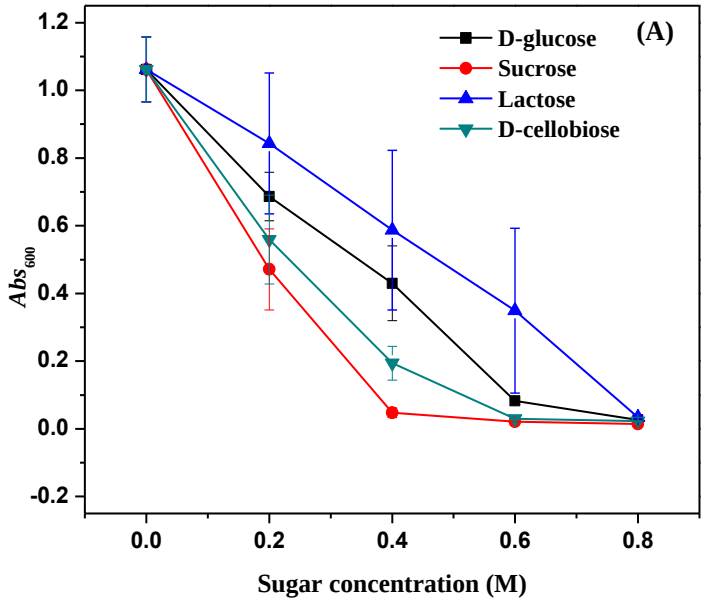


Figure 5-1. Visual appearance of WPI-MD conjugate solutions before and after heating at 138 ℃ for 1 min. Solutions were prepared with 5% w/v protein, 0, 50, and 100 mM NaCl, and 0, 0.2, 0.4, 0.6 and-0.8 M co-solutes (from left to right in each image), and adjusted to pH 5.0 prior to heating.

Figure 5-2. Absorbance at 600 nm (Abs_{600}) of WPI-MD conjugate solutions after heating at 138 °C for 1 min. Solutions were prepared at 5% w/v protein, pH 5.0, 0-0.8 M co-solutes and (A) 0, (B) 50, and (C) 100 mM NaCl after heating and diluted 5 times using 10 mM phosphate citrate buffer (pH 5.0). Error bars are standard deviations from two replicates.



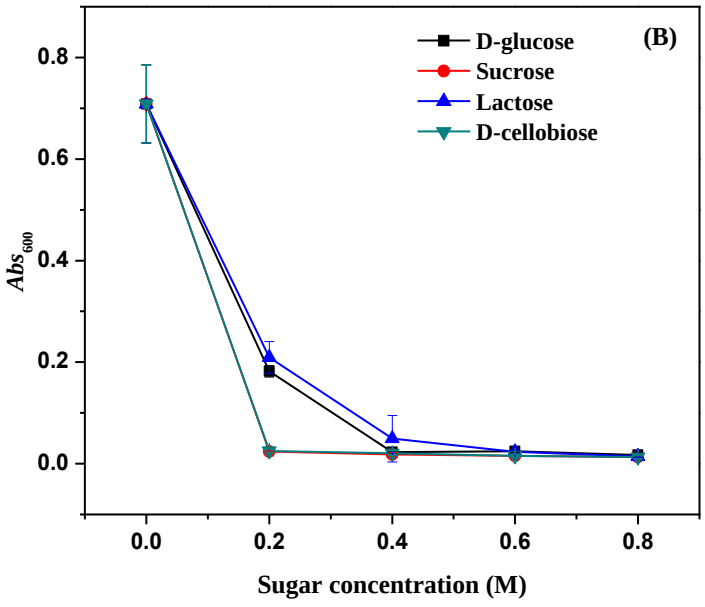


Figure 5-2. Continued.

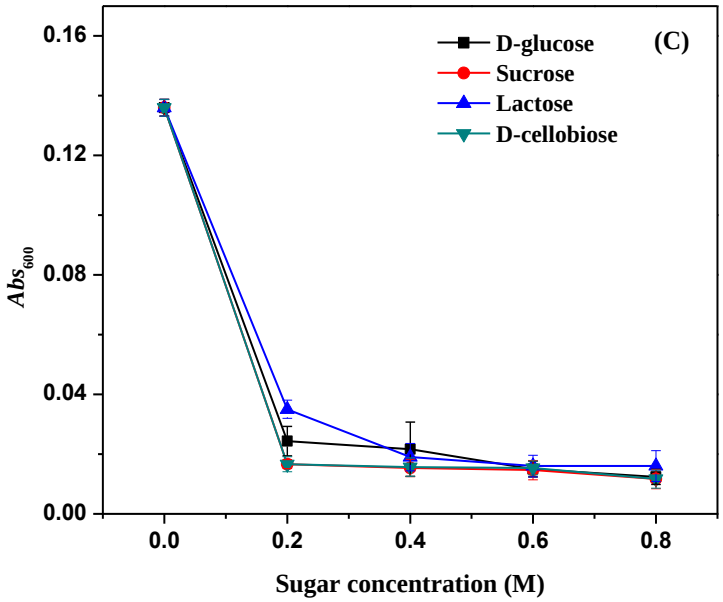


Figure 5-2. Continued.

Figure 5-3. Rheograms of WPI-MD conjugate solutions measured at a sheer rate ramp of 0.1-100 s^{-1}. Sample were prepared with 5% w/v protein, pH 5.0, 0 mM NaCl, and 0-0.8 M of (A) D-glucose, (B) sucrose, (C) lactose, and (D) D-cellobiose. The control sample was 5% w/v WPI solution at pH 5.0 and 0 M NaCl.

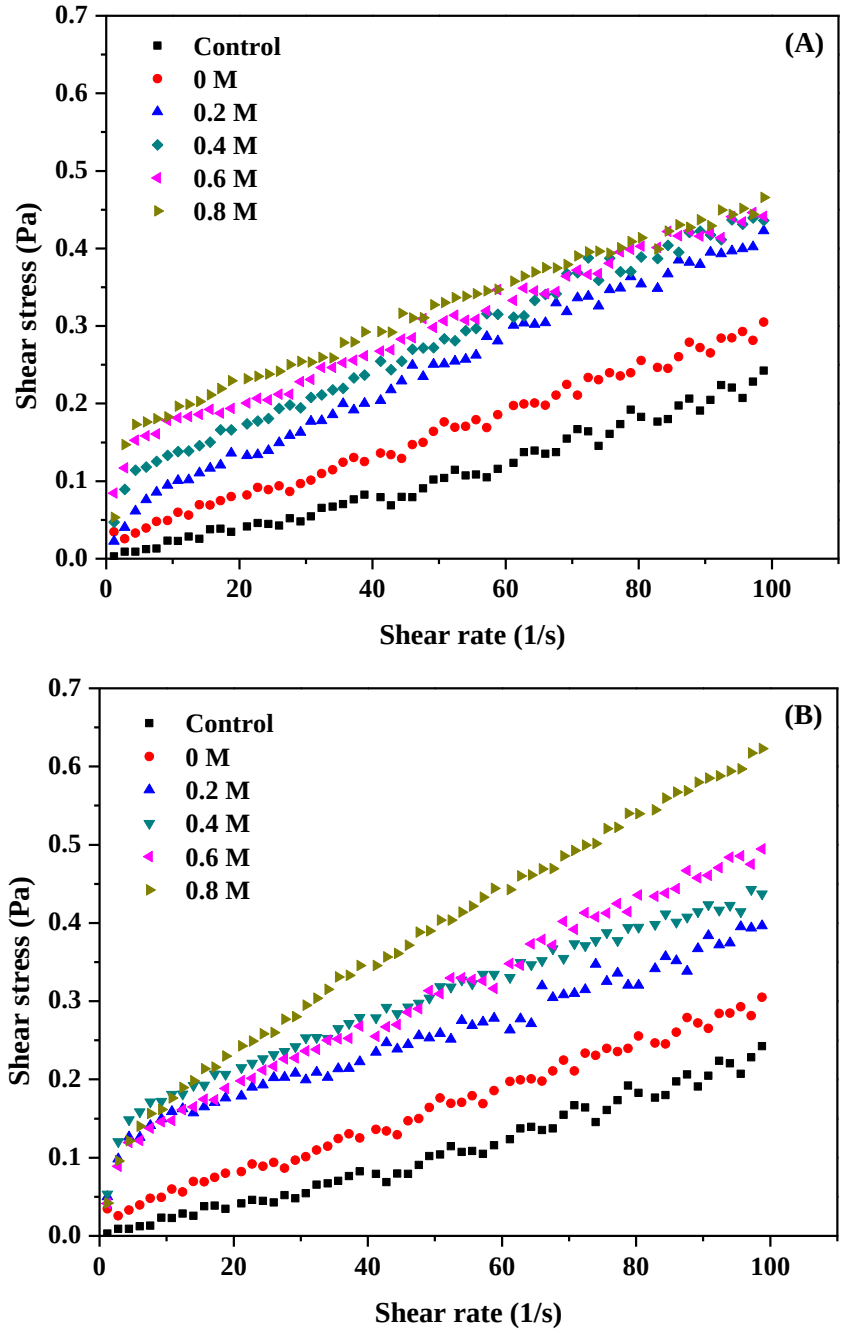
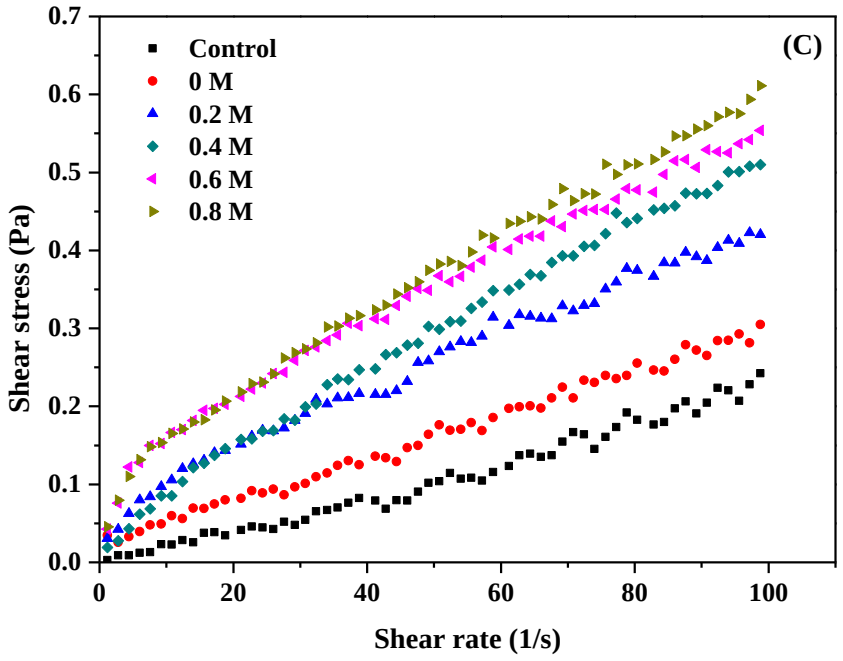


Figure 5-3. Continued.



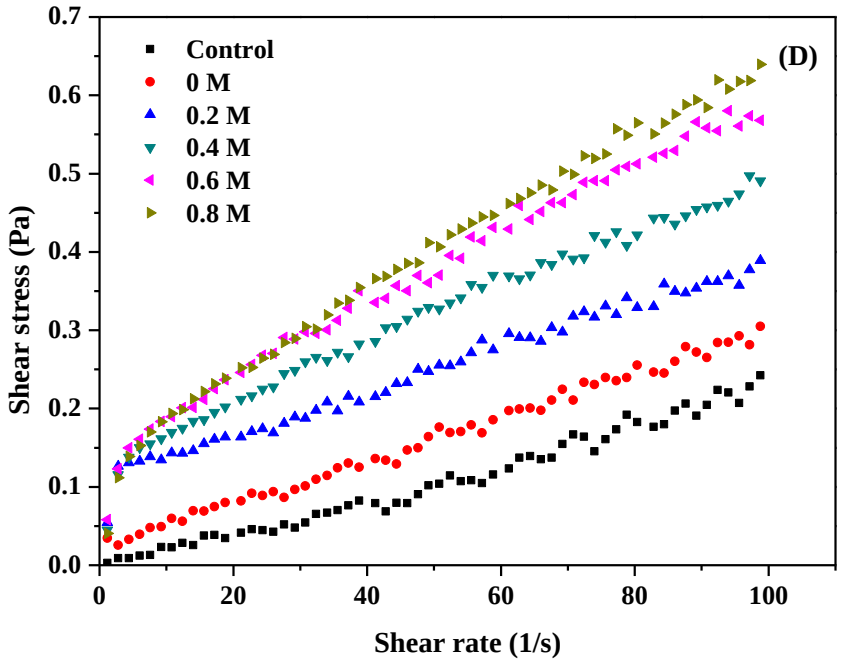


Figure 5-3. Continued.

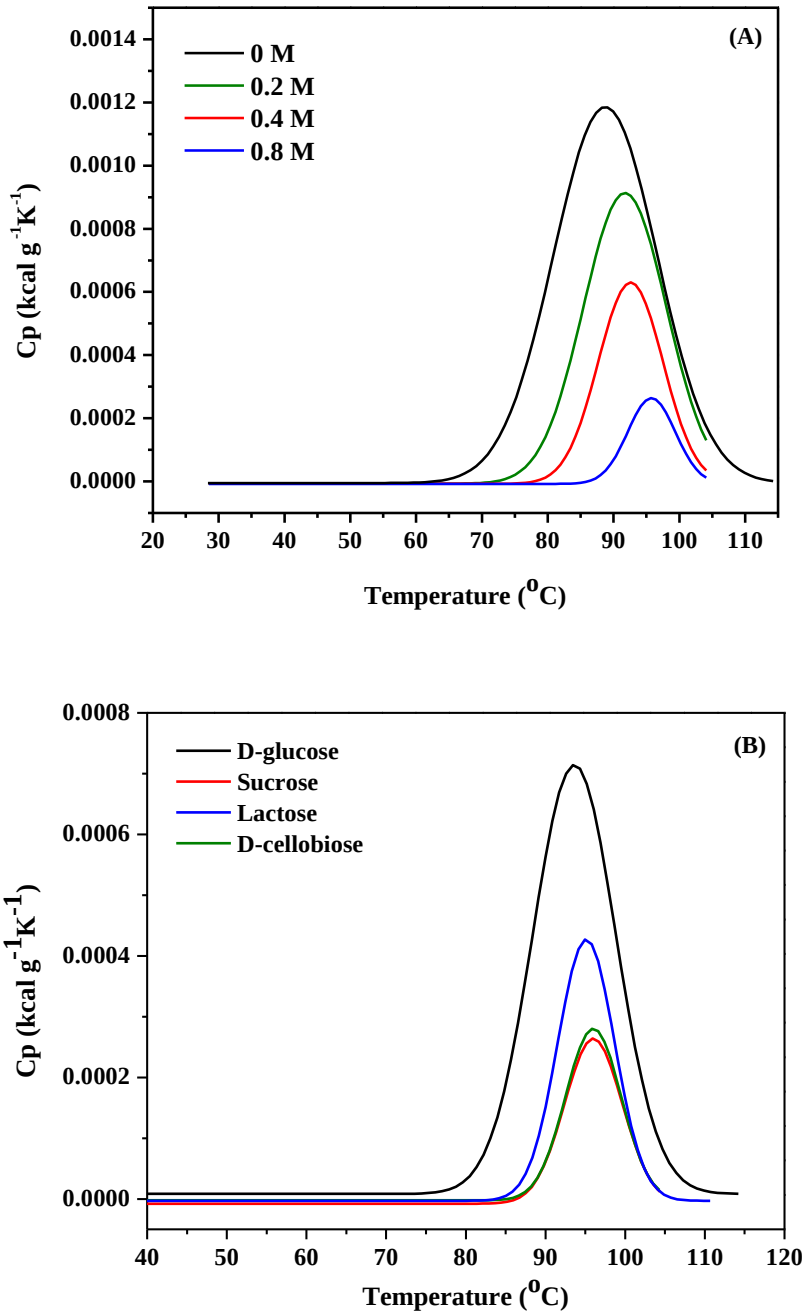


Figure 5-4. DSC thermograms of WPI-MD conjugate solutions with (A) 0-0.8 M sucrose and (B) 0.6 M of various co-solutes at pH 5.0.

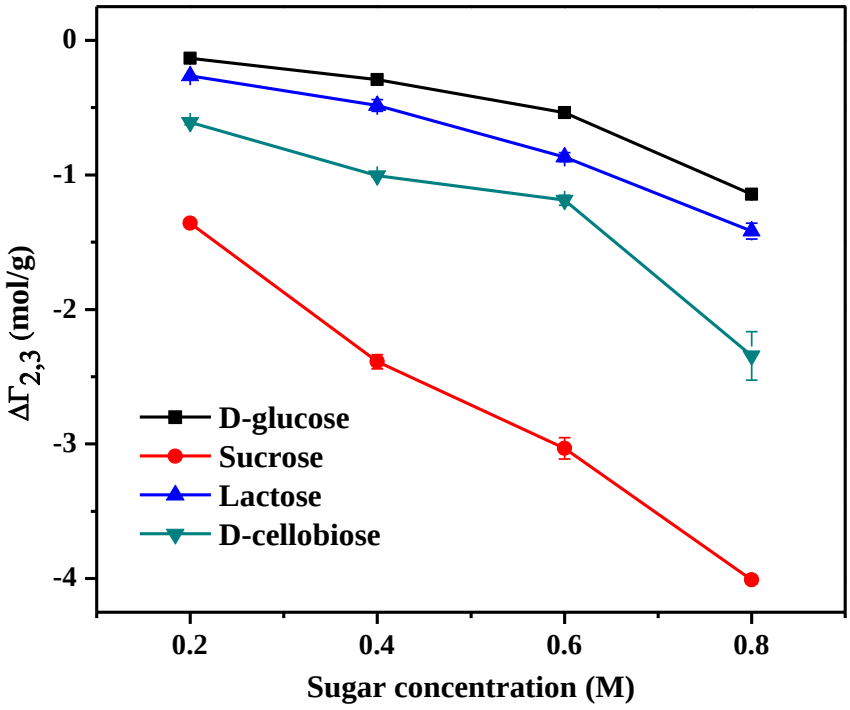


Figure 5-5. Changes in preferential interaction coefficient ($\Delta\Gamma_{2,3}$) of WPI-MD conjugates in pH 5.0 solutions with 0.2-0.8 M co-solutes. Error bars are standard deviations from two replicates.

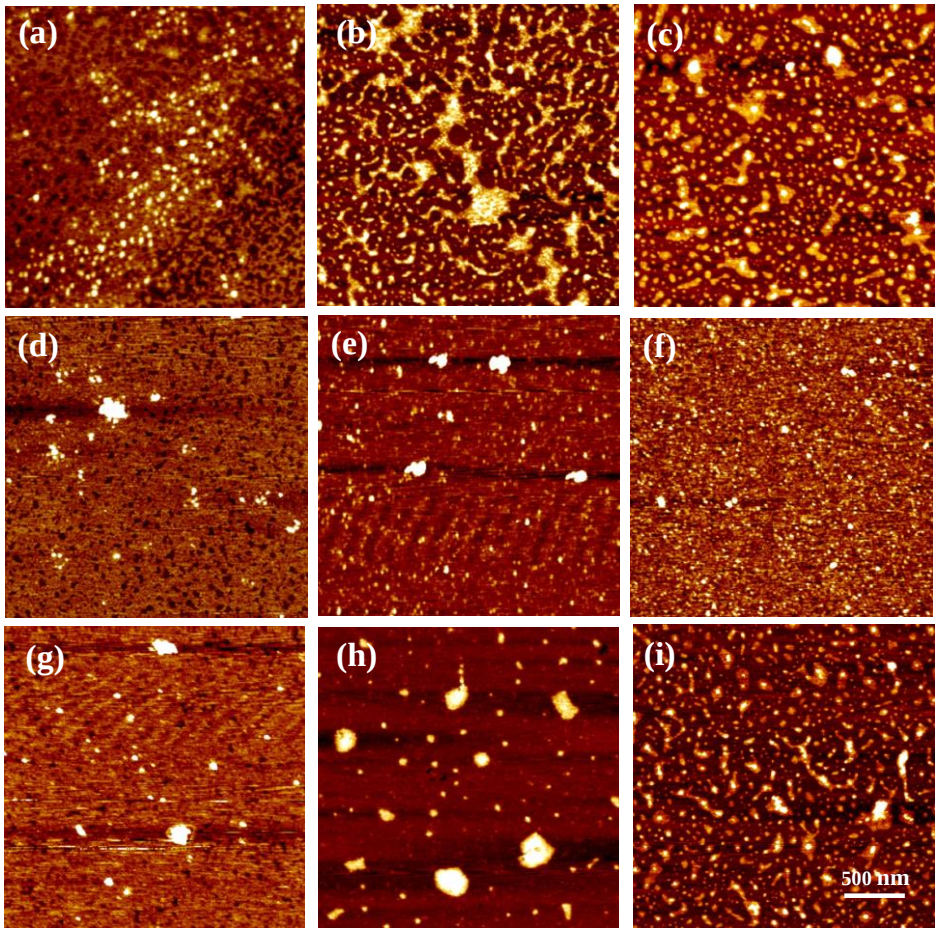


Figure 5-6. AFM topography images of WPI-MD conjugates at pH 5.0 and 50 mM NaCl, before (a) and after heating at 138 °C for 1 min. Samples after heating are shown for treatments with (b) 0, (c) 0.2, (d) 0.4, (e) 0.6, and (f) 0.8 M D-glucose, or 0.6 M (g) sucrose, (h) lactose, or (i) D-cellobiose.

Chapter 6. Concluding Remarks and Future Work

Conclusions

Heat stability of whey proteins can be improved by a number of ways, depending on the desired pH conditions. The combination of preheating and enzymatic cross-linking (sequential pretreatment) can stabilize whey proteins at neutral pH by suppressing both heat- and salt-induced aggregations. Preheating treatment at neutral pH and temperature above denaturation temperate of whey proteins resulted in partial unfolding of the structure. Embedded reactive sites of whey proteins for enzymatic cross-linking were exposed after preheating and became more accessible. This sequential pretreatment increased the degree of enzymatic cross-linking and facilitated the whey protein stabilization during heating at 80 ℃ in the presence of 0-100 mM NaCl. Heating whey proteins at 138 ℃ caused turbidity, precipitation and gelation at a shorter time than at 80 °C. The sequentially-pretreated whey proteins exhibited good resistance against aggregation during heating at 138 °C for up to 30 min, showing smaller particle dimension at a longer heating time which can be caused by the dissociation of cross-linked whey proteins and reformation of nanoscale particles.

Glycating whey proteins with reducing saccharides such as MD can improve heat stability at pH ranging from 4 to 7. Glycation can be conducted using conditions based on the solution chemistry of target beverages. Heat stability of conjugates can be further controlled by studying the acidity of spray-dried mixture powder of WPI and MD. WPI glycated with MD at m-pH 6 showed an improvement in heat stability after heating at 138 ℃ and had a lighter color when compared to that glycated at m-pH 7. This was attributed to a higher degree of glycation at m-pH 6 than at m-pH 7 and the color

formation can be suppressed at more acidic conditions. Supplementing co-solutes with and without calories further improved the heat stability of WPI-MD conjugates at pH 4-7, which enables the glycation at milder conditions to reduce the undesired brown color and possibly other glycation byproducts while achieving heat stability. Co-solutes stabilized WPI-MD conjugates by excluded volume and preferential interactions, in addition to the increased viscosity reducing the frequency of collision and the increased denaturation temperature at a higher co-solute concentration. The strengthened preferential interactions by co-solutes prevented the transfer of whey protein molecules from the solvent to co-solute, forming a region that whey protein molecules cannot get close to aggregate.

Physicochemical properties of whey proteins presented in this dissertation provide scientific bases heat stability improvement by TGase cross-linking and glycation with reducing saccharides. The information will be useful to future studies illustrating molecular and sub-molecular phenomena related to whey protein heat stability improvement. These conditions can be further optimized to prepare ingredients for the production of high quality, nutritious, and shelf-stable whey protein-based Ready-to-drink beverages during thermal processes.

Future work

Extending form the present work, the impacts of nanoparticle size and shape formed s at different treatments on heat stability of whey proteins can be further studied. Other microscopy techniques, such as TEM, can be applied to study additional structure information of particles after different treatments. Whey protein nanoparticles with improved heat stability can be fabricated via controlling the environmental factors such as pH, ionic strength, heating conditions and the presence of co-solutes for other applications such as emulsification. Furthermore, interactions between whey proteins and surfactants such as cationic lauric arginate can be studied for whey proteins preheated at various pH conditions, temperatures for different durations, to study the possibility of surfactants improving heat stability. In addition, emulsifying properties antioxidant properties, and byproducts of whey proteins after various pretreatments and glycations. can be characterized. In order to test the pratical application, in vitro digestibility of TGase cross-linked or glycated whey proteins and shelf stability of model beverages also require future work.

VITA

Wan Wang entered College of Food Science and Technology, Huazhong Agricultural University in 2003 to pursue a Bachelor degree of Food Science and Engineering. After graduating from the university, she continued her study for a Master of Science degree, focusing on the Food Science and Nutrition at the University of Leeds, UK. Her thesis project of Master's study is a combination of food chemistry and sensory evaluation. In January 2011, she began her doctoral program in the Department of Food Science and Technology at the University of Tennessee, Knoxville, working with Dr. Qixin Zhong in the Food Physics and Nanotechnology Lab. She mainly focused on the physicochemical properties of whey proteins, conformational changes and interactions of whey proteins with other biopolymers, in order to enhance the thermal stability of whey proteins, therefore broaden the applications of whey proteins as a widely used source in protein beverages.