

**Preparation and Characterization of Acid-induced Gels at pH
1.0-4.0 and Thermal-stable Dispersions at pH 5.5-6.0 using
Preheated Whey Protein**

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

KangKang Li

May 2015

Acknowledgements

I highly appreciate my advisor, Dr. Qixin Zhong, for his patience, understanding and tolerance during my M.S. study. My sincere thanks go to my committee members: Dr. Svetlana Zivanovic and Dr. Guoxun Chen. I also acknowledge my labmates, faculty members and staff at UT, and friends and family.

I do appreciate Food Science and Technology Department, University of Tennessee, and Knoxville for providing a home for two years.

ABSTRACT

Whey protein (WP) is a good source for producing protein-rich products, including satiety foods and beverages. Thermal aggregation of WP during sterilization or pasteurization impacts food quality important to shelf-stable beverages. Preheating WP improves the thermal stability at neutral acidity, and forming complexes with other molecules to provide charge and/or steric hindrance can be used to improve thermal stability of WP at acidic conditions. Conversely, aggregation properties of preheated WP upon acidification can be used to create unique functional properties. Therefore, studying properties of preheated WP can broaden its application in the food industry.

Gelation of preheated WP and pectin mixtures at gastric acidity (pH 1.0-4.0) was studied in the first part of this thesis because of the potential to control satiety. The fast-acidified gels had less homogenous microstructures and lower strengths than gels slowly-acidified by glucono-delta-lactone. Preheated WP gelled at pH 3.5-4.0, while the WP-pectin mixture at mass ratios of 20:1, 10:1 and 5:1 formed gels at pH 2.0-3.5, 1.5-3.0 and 1.5-2.5, respectively. WP-pectin gels were weakened by NaCl but strengthened by CaCl_2 , indicating the significance of electrostatic attraction on gelation. Without hydrophobic attraction and hydrogen bonds, gelation was not observed for preheated WP but was evident for the WP-pectin mixtures at pH 2.5 and 3.0. The findings suggest the potential application of preheated WP-pectin mixtures to increase the viscosity in the stomach.

The second part of this thesis research was focused on combining sodium stearyl lactylate (SSL) and preheating treatment to improve thermal stability of WP at pH 5.5-6.0. The binding between SSL and WP was promoted by heating based on particle size and zeta-potential results. Circular dichroism spectroscopy indicated the formation of a more ordered secondary structure of WP after binding with SSL. SSL increased the zeta-potential magnitude, reduced the extent of denaturation, the exposure of sulfhydryl groups, and surface hydrophobicity of WP. These properties in turn improved the thermal

stability of WP to make it possible to produce transparent fluids containing 4.5% WP and 0.2% SSL at pH 5.5-6.0.

Keywords: whey protein, denaturation, gelation, thermal stability, preheating, sodium stearyl lactylate.

TABLE OF CONTENTS

Chapter 1. Introduction and Literature Review.....	1
1.1 . Introduction.....	2
1.1.1. Perspective of whey protein (WP).....	2
1.1.2. Production, types and compositions of WP ingredients	2
1.2. Physical and chemical interactions during thermal processing	3
1.2.1. Stability of WP interpreted by inter-particle interactions	3
1.2.2. Attractive forces	4
1.2.3. Repulsive forces	6
1.3. Thermal aggregation of WP above pI.....	8
1.3.1. Thermal aggregation of β -Lg above pI	8
1.3.2. Kinetics of thermal aggregation of β -Lg	9
1.3.3. Influence of other components of WP	11
1.4. Acid-induced gelation of WP.....	11
1.5. Strategies of improving thermal stability of WP	13
1.5.1. Preheating treatments	13
1.5.2. Modification of amino groups of WP	14
1.5.3. Binding WP with anionic surfactants	15
1.6. Conclusions.....	16
1.7. Research scope.....	16
References	18
Appendix.....	26
Chapter 2. Aggregation and Gelation Properties of Preheated Whey Protein and Pectin	
Mixtures at Gastric Acidity	28
2.1. Abstract	29
2.2. Introduction.....	30
2.3. Materials and methods	31
2.3.1. Materials	31
2.3.2. Preparation of preheated WP	31
2.3.3. Aggregation properties	32
2.3.4. Gelation properties.....	32
2.3.5. Confocal laser scanning microscopy (CLSM)	33
2.3.6. Stability of gels upon dilution and mixing.....	33
2.3.7. Statistical analysis.....	33
2.4. Results and discussion	34
2.4.1. Aggregation properties of WP and pectin mixtures.....	34
2.4.2. Formation of self-standing gels	36
2.4.3. Microstructures of gels	38
2.4.4. Rheological properties of gels.....	39
2.4.5. Dilution stability of gels	41
2.5. Conclusions	41

References	43
Appendix	48
Chapter 3. Sodium Stearoyl Lactylate Improves Thermal Stability of Whey Protein at pH	
5.5-6.0	65
3.1. Abstract	66
3.2. Introduction	67
3.3. Materials and methods	69
3.3.1. Materials	69
3.3.2. Sample preparation	69
3.3.3. Analysis of particle size and zeta-potential	69
3.3.4. Circular dichroism (CD) spectroscopy	70
3.3.5. Intrinsic fluorescence intensity	70
3.3.6. Determination of sulfhydryl group (-SH) contents	70
3.3.7. Surface hydrophobicity (H_0)	71
3.3.8. Statistical analysis	71
3.4. Results and discussion	71
3.4.1. Influence of SSL on thermal stability of native WP (nWP)	71
3.4.2. Binding between SSL and nWP	72
3.4.3. Influence of SSL on properties of preheated WP (dWP)	73
3.4.4. Thermal stability of preheated WP-SSL	75
3.5. Conclusions	76
References	77
Chapter 4. Concluding Remarks and Future Work	94
4.1. Conclusions	95
4.2. Future work	96
Vita	97

LIST OF TABLES

Table 1-1. Typical protein compositions of whey protein and their properties (Bryant et al., 1998; Fuquay et al., 2011)	26
--	----

LIST OF FIGURES

Figure 1-1. Model of β -Lg denaturation and aggregation at pH>5.7. Adapted from (Nicolai et al., 2011).	27
Figure 2-1. Absorbance at 600 nm (Abs_{600}) of (A) dispersions with 0.45% w/v pectin, 0.45% w/v native or preheated WP, and their mixtures at a WP:pectin mass ratio of 10:1; (B) dispersions with 0.45% preheated WP and pectin at WP:pectin mass ratios of 1:0, 20:1, 10:1, and 5:1. Error bars are standard deviations (n=4).	48
Figure 2-2. Abs_{600} of dispersions of (A) native WP and pectin, (B) preheated WP, and (C) preheated WP and pectin with various concentrations of NaCl or $CaCl_2$. WP content was 0.45% w/v in all treatments, and the WP:pectin mass ratio was 10:1. Error bars are standard deviations (n=2).	51
Figure 2-3. Photographs of samples with pectin (0.63% w/v), native or preheated WP, and their mixtures at different WP to pectin mass ratios. WP content was 6.3% w/v in all treatments.	55
Figure 2-4. Photographs of mixtures with 6.3% w/v preheated WP and 0.63% w/v pectin supplemented with various concentrations of NaCl or $CaCl_2$. .	56
Figure 2-5. CLSM images of (A) preheated WP at pH 3.5, (B) preheated WP at pH 4.0, (C) preheated WP and pectin at pH 2.5, and (D) preheated WP and pectin at pH 3.0. WP (stained by red Rhodamine B) and pectin concentrations were 6.3% and 0.63% w/v, respectively. Bar = 10 μ m.	57
Figure 2-6. Storage modulus of samples with 6.3% w/v preheated WP adjusted to pH 3.5 (A and C) or 4.0 (B and D) during time sweep (A and B) at 37 °C or temperature ramp (C and D) from 37-80 °C at 5 °C/min. Samples were also added with 10% w/v urea or 5 mM NEM. Three parallel tests were performed and representative curves are shown.	58
Figure 2-7. Storage modulus of mixtures with 6.3% w/v preheated WP and 0.63% w/v pectin adjusted to pH 3.5 (A and C) or 4.0 (B and D) during time sweep at 37 °C (A and B) or temperature ramp from 37-80 °C at 5 °C/min (C and D). Samples were added with 10% w/v urea or 5 mM NEM. Three	

parallel tests were performed and representative curves are shown.	61
Figure 2-8. Photographs of 5 mL 6.3% w/v preheated WP (top) and 6.3% w/v preheated WP and 0.63% w/v pectin mixtures (bottom) with 10mM CaCl ₂ , after mixing with 15 mL deionized water.	64
Figure 3-1. Photographs of native WP (4.5%) and SSL (0-1.0%) mixtures heated at pH 5.25-6.0 and 95 °C for 1 min.....	82
Figure 3-2. Diameters of mixtures with SSL and native or preheated WP at various mass ratios, before and after heating at 90 °C and pH 7.0 for 20 min. Preheated WP was prepared by heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n = 2).....	83
Figure 3-3. Zeta-potential of native and preheated WP with SSL before and after heating at 90 °C and pH 7.0 for 20 min. Preheated WP was prepared by heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n=2).	84
Figure 3-4. CD signals of mixtures with various mass ratios of native WP and SSL (A) before or (B) after heating at 90 °C and pH 7.0 for 20 min.....	85
Figure 3-5. CD signals of mixtures with various mass ratios of native WP and SSL at 217 nm before and after heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n=2). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).....	86
Figure 3-6. Intrinsic fluorescence intensities of WP before and after heating at 80-95 °C for 20 min, in comparison to a mixture with WP and SSL after heating together at identical conditions (WP+SSL). Error bars are standard deviations (n = 2). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).....	87
Figure 3-7. Surface and total –SH contents of WP before and after heating at 80-95 °C for 20 min, in comparison to a mixture with WP and SSL after heating together at identical conditions (WP+SSL). Error bars are standard deviations (n = 2).	88
Figure 3-8. (A) Particle size distributions of SSL and preheated WP (at 90 °C	

and pH 7.0 for 20 min) without and with SSL (dWP-SSL), in comparison to a mixture with WP and SSL after heating together at identical conditions (dWP+SSL). Plot B shows diameters of the highlighted peak in A of these three samples preheated at 80-95 °C and pH 7.0 for 20 min (B). Error bars are standard deviations (n = 2). The data point labeled with an asterisk represents significant differences ($P < 0.05$) from the other two samples at the same preheating temperature.89

Figure 3-9. Surface hydrophobicity (H_0) of WP after heating at 80-95 °C and pH 7.0 for 20 min without and with 0.2% SSL (dWP+SSL), in comparison to treatments after adding SSL in WP preheated at same conditions (dWP-SSL). Error bars are standard deviations (n = 2). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$). ...92

Figure 3-10. Photographs of WP after heating at 80-95 °C and pH 7.0 for 20 min without and with 0.2% SSL (dWP+SSL), in comparison to treatments after adding SSL in WP preheated at same conditions (dWP-SSL), after adjusting to pH 5.25-6.0 and heating at 95 °C for 1 min. Concentrations of WP and SSL were 4.5 % and 0.2 % w/v, respectively.93

Chapter 1. Introduction and Literature Review

1.1. Introduction

1.1.1. Perspective of whey protein (WP)

The market of protein-enriched food is growing that meal-replacement and sports beverages with a high protein claim has posted a staggering 37% growth in 2007-2012 (Frank, 2013). Food proteins promote bone growth, wound healing, muscle building and body shaping, and stave off the effect of aging (Krissansen, 2007). WP, as a byproduct of cheese industry, is a preferred protein source because of abundant supply of essential amino acids, bland flavor, digestibility and health benefit (Kinsella & Morr, 1984).

1.1.2. Production, types and compositions of WP ingredients

Whey from cheese manufacturing is commonly used as a source of manufacturing WP ingredients. When casein is formed into a cheese network or removed from milk, the remaining whey fluid contains approximately 0.6% protein and 93% water (Fuquay, Fox, & McSweeney, 2011). Whey can be processed into a food ingredient by simple drying, or the protein content can be further increased by removing lipid, minerals and lactose. The most common WP ingredients are whey protein concentrate (WPC), containing 30-80% protein, and whey protein isolate (WPI), containing >90% protein (Foegeding, Davis, Doucet, & McGuffey, 2002).

WP is a mixture of globular proteins, including β -lactoglobulin (β -Lg), α -lactalbumin (α -La), and lesser amounts of bovine serum albumin (BSA), immunoglobulin, and others (De Wit, 1981). B-Lg is the most abundant WP in bovine milk, accounting for 50-60% of total WP, depending on the process for isolation of WP (Fuquay et al., 2011). It has a molecular weight of 18.3 kDa, 162 amino acid residues, an isoelectric point (pI) at about pH 5.2, one free sulfhydryl group (-SH) and two disulfide bonds (Damodaran, 1997). The -SH is important since it facilitates sulfhydryl interchange reactions which allow the formation of new structures (Kinsella & Morr, 1984). B-Lg exists as a noncovalent dimer at the pH between 5.5 and 7.5 (McKenzie & Sawyer, 1967). Native β -Lg (Figure 1) has nine β strands that are folded into two β sheets.

Each β -sheet has a hydrophobic side and a hydrophilic side, creating a very hydrophobic cavity (Considine, Patel, Singh, & Creamer, 2005). Although β -Lg has a number of hydrophobic residues, it is soluble in a broad pH range 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 WP at pH away from pI (Damodaran, 1997). A-La has a molecular weight of 14.2 kDa, 123 amino acid residues, a pI at pH 4.8-5.1 and 4 disulfide bridges (Bryant, McClements, & Julian McClements, 1998; Damodaran, 1997). It is an elliptical shaped compact protein made up of two domains by a deep cleft (Pike, Brew, & Acharya, 1996). It is stable against thermal aggregation since it renatures easily when cooled (Brown, 1988).

1.2. Physical and chemical interactions during thermal processing

1.2.1. Stability of WP interpreted by inter-particle interactions

WP molecules dispersed in a liquid are considered as colloidal particles. The aggregation of WP depends on the relative magnitude of attractive and repulsive forces. In general, aggregation occurs when attractive forces are stronger than the repulsive forces.

The stability and aggregation of colloidal particles in the aspects of thermodynamics and kinetics have been described using the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. The basic idea of the DLVO theory is that the stability of hydrophobic colloids in aqueous systems is determined by the combination of van der Waals attraction and electrostatic repulsion (De Young, Fink, & Dill, 1993). The DLVO theory has been very successful in predicting stability against aggregation for many, especially inorganic systems (Walstra, 2002). However, it cannot be applied in WP since the major attractive force in thermal aggregation of WP is not van der Waals interaction (Bryant et al., 1998).

1.2.2. Attractive forces

Hydrophobic attraction and disulfide bonds are major attractive forces during thermal aggregation between WP during heating, while van der Waals interactions and hydrogen bond are minor attractive forces (Bryant et al., 1998).

Hydrophobic attraction. Hydrophobic attraction causes the aggregation tendency of nonpolar substances to minimize the contact with water (Woodbury, 2011). Inter- or intra-molecular hydrophobic attraction acts between aliphatic chains or between aromatic groups of amino acid residues of WP. Many of the nonpolar groups are located in the interior of WP, so it is usually necessary to promote some degree of protein unfolding before aggregation occurs (De Wit, 1981). Hydrophobic attraction is influenced by external factors such as temperature. The hydrophobic attraction between WP is stronger at a higher temperature in the range from 0 to ~60 °C (Baldwin, 1986), so heating enhances WP aggregation.

Hydrophobic attraction also exists between WP and other material with hydrophobic groups. Surfactants such as sodium dodecyl sulfate (SDS), lauric acid, diacetyl tartaric acid ester of monoglyceride and sodium stearyl lactylate (SSL) have a hydrophilic head group and a hydrophobic alkyl chain. Hydrophobic attraction can be present between the aliphatic chains of the surfactants and the non-polar protein surface regions that are adjacent to cationic sites (Lundahl et al., 1986).

Disulfide bond (S-S). A S-S is formed between two -SH in cysteine through -SH oxidation into S-S bonds and/or -SH/S-S interchange reactions. B-Lg contains one free cysteine (cys121) which contributes 90–95% of the -SH groups in milk and milk-derived ingredients (Larsen, Jenness, & Geddes, 1949). The –SH is buried inside, about 9 Å from the dimer interface, so it is not reactive at native status. During heating at neutral pH, the native β -Lg partially unfolds, leading to exposure of the –SH (Livney, Verespej, & Dalgleish, 2003). The –SH is important in aggregation of WP, and a replacement of the cys121

for a serine residue completely blocks the heat-induced irreversible aggregation of β -Lg (Jayat et al., 2004). Since α -La has no $-\text{SH}$, its solutions (0.7%; 0.1 M phosphate buffer, pH 7.0) remain clear and do not gel after heating for 10 min at 100 °C (Chaplin & Lyster, 1986). Comparing to formation of S-S from two $-\text{SH}$ groups, SH/S-S interchange reactions is predominant during aggregation (Schokker, Singh, Pinder, Norris, & Creamer, 1999) and gelation of WP (Shimada & Cheftel, 1989).

pH is a key factor for the reactivity of $-\text{SH}$ groups. The pKa of a fully water exposed $-\text{SH}$ is around 8.3, which implies that the reactivity is greatly reduced under mild acidic conditions (Visschers & de Jongh, 2005). At pH 9-11, $-\text{SH}$ induced polymerization occurs at room temperature (22 °C), while at pH 3, 5, and 7, $-\text{SH}$ induced polymerization is only evident after heating to 85, 75, and 70 °C. At pH 9-11, significant $-\text{SH}$ oxidation occurs even at room temperature, while $-\text{SH}/\text{S-S}$ interchange predominates at pH 3-5 (Monahan, German, & Kinsella, 1995).

It is generally considered that formation of S-S is mostly responsible for large aggregates at neutral pH, while noncovalent interactions (hydrophobic attraction, ionic, and Van der Waals) become more important at lower pH (Hoffmann & van Mil, 1997). However, contributions of hydrophobic attraction and S-S bond to aggregation of WP are not fully understood (de la Fuente, Singh, & Hemar, 2002). Monahan (1996) claimed non-covalent interactions such as hydrophobic attraction and van der Waals forces are responsible of the initial formation of aggregates and later S-S bond strengthens the already formed aggregates. While Bryant (1998) claimed that hydrophobic attraction is responsible for initiation of aggregation, and S-S bond plays a major role in strengthening these aggregates. Livney (2003) claimed that hydrophobic attraction may be important within the aggregates, but the role of S-S bond in both initial aggregation stages and in attaching large aggregates in later stages appears to be dominant. Upon treatment with urea (dissociating agent for non-covalent aggregates linked via hydrogen bonds), a small decrease in the

molecular weight, indicating that some (primary) S-S linked aggregates had formed larger aggregates via non-covalent interactions (Hoffmann, Sala, Olieman, & de Kruif, 1997). The contributions of noncovalent interactions to aggregation increased with an increase of temperature from below 75 °C to 90–110 °C (Galani, 1999) or with pH values closer to the pI and/or with higher ionic strengths (Verheul, Roefs, & de Kruif, 1998).

van der Waals forces. The inter-protein van der Waals interactions act between all molecules, are always attractive, and consist of dipole-dipole, dipole-induced dipole, and induced dipole-induced dipole interactions (Walstra, 2002) At the molecular level, van der Waals interactions tend to have fairly similar magnitudes for all types of molecules (Israelachvili, 2011). Consequently, they only play a minor role in determining the conformation of WP in solution, since they have little change in the folded and unfolded states of WP (Bryant et al., 1998).

Hydrogen bonds. A hydrogen bond is formed between the electron-rich and electron-depleted portions within a molecular or between two separate polar molecules. Extensive hydrogen bonds occur in protein solution. hydrogen bonds play an important role in stabilizing the structures once formed, but they are not usually the major driving force determining the conformation and aggregation of globular proteins (Kinsella & Whitehead, 1989)

1.2.3. Repulsive forces

Electrostatic interactions. Electrostatic interactions act between species with a permanent electrical charge, for example, dipoles or ions. The interaction is repulsive when the charges have the same sign; and the interaction is attractive when they have different signs (Bryant et al., 1998). WP has both acidic and basic amino acid residues which are generally positively and negatively charged at neutral pH, respectively. There is an attraction between groups of WP with opposite charges. Additives with positive charges (Ca (II) and chitosan) or negative charges (pectin, alginate, phosphate, lauric

acid and SSL) may also have an attraction with groups of WP with different charges. The number of charges depends of the degree of ionization which is a function of pH. WP has a pI of about 5.2 where WP has a zero net charge. At a pH away from pI, WP carries either net positive (below pI) or negative (above pI) charges which provide electrostatic repulsion between molecules. Electrostatic interactions are the sum of both attraction and repulsion. Electrostatic interactions are particularly sensitive to the ionic strength because the magnitude and range of the interactions can be reduced appreciably by electrostatic screening, but they are strengthened with increasing temperature (Evans & Wennerström, 1999).

Ion bridging is another type of molecular interaction which involves electrostatic interactions (Jeyarajah & Allen, 1994). It occurs when a polyvalent ion simultaneously binds to the surface of two molecules/particles by electrostatic attraction. These polyvalent ions may be low molecular weight species, such as Ca^{2+} or high molecular weight biopolymers, such as pectin (Bryant et al., 1998). The ability of polyvalent ions to form ion bridges is superimposed on their ability to reduce the magnitude of the electrostatic interactions through electrostatic screening (Jeyarajah & Allen, 1994).

Steric interactions. Steric interactions are extremely strong repulsive interactions between atoms or molecules at close separations because of the overlap of their electron clouds (Israelachvili, 2011). In dispersion of colloidal particles, if the proximity of a second particle restricts the volume in which the protruding polymer chains can be, this means that the number of conformations that a chain can assume is restricted, hence the entropy of these chains is lowered and a repulsive force will act (Walstra, 2002). In addition, if the osmotic pressure increases in the gap between the particles, solvent will be drawn into the gap to equilibrate osmotic pressure around particles, which guarantee a higher dynamic force separating particles (Walstra, 2002). Steric interactions also govern -SH/S-S interchange reaction of β -Lg and α -La during thermal treatment (Livney et al., 2003). The steric

interactions decrease with a higher ionic strength or pH around pI, because the reduced net charge density of protein (Belfort & Lee, 1991).

1.3. Thermal aggregation of WP above pI

1.3.1. Thermal aggregation of β -Lg above pI

As the major content and –SH source of WP, β -Lg dominates the overall gelling behavior of WP (Mulvihill & Kinsella, 1987). Observations from β -Lg are therefore a good first approximation for WP (Ryan, Zhong, & Foegeding, 2013). Thermal aggregation of β -Lg is interpreted in 2 steps: denaturation and aggregation (Verheul et al., 1998).

Denaturation. Denaturation indicates a process that the tightly folded conformation of native proteins changes into a more or less unfolded conformation (Walstra, 2002). In native status, the attractive forces between β -Lg molecules are not sufficiently strong to overcome the repulsive forces, so the molecules exist either as individual entities or as small aggregates (Kinsella & Whitehead, 1989). The aggregation of native β -Lg is completely reversed when the pH is increased or decreased away from pI (Majhi et al., 2006). Reactive amino-acids (non-polar and –SH) are located in the interior of the globular proteins, and it is usually necessary to promote some degree of protein unfolding before aggregation will occur (De Wit, 1981).

At 30 to 50 °C and neutral pH, dimers of β -Lg are known to dissociate to monomers (Sawyer, 1969). Upon heating to a temperature above 60 °C, β -Lg partially unfolds to a molten globule and exposes its hydrophobic groups and –SH (Iametti et al., 1996). Molten globule is characterized by a partially folded conformation with retention of the secondary structural elements whereas the tertiary structure becomes much more fluid with consequent slight swelling (Hirose, 1993). This step is also called unfolding or activation or formation of reactive monomers.

Aggregation. Aggregation of WP is summarized in Figure 1-1 (Ryan et al., 2013). Firstly denatured monomers form non-native dimers, trimers, oligomers

and other small molecular weight aggregates via S-S. Proteins forming these oligomers still have a large amount of secondary structures, but the structure is thought to be more mobile (Bauer, Hansen, & Øgendal, 1998). Secondly oligomers associate into larger aggregates through covalent and noncovalent interactions when the concentration of oligomers exceeds a critical amount (Nicolai, Britten, & Schmitt, 2011). The aggregates are called primary aggregates or soluble aggregates, containing about 100 monomers and with a hydrodynamic radius of about 15 nm. The soluble aggregates are curved strands at pH 7.0 with a length of about 50 nm and a diameter of about 10 nm, and approximately spherical with radii up to 150 nm at pH 5.8 (Durand, Christophe Gimel, & Nicolai, 2002). Finally soluble aggregates may associate further into larger polydisperse self-similar aggregates by covalent and noncovalent interactions (Nicolai et al., 2011). Above a critical concentration, a gel is formed when a system-spanning network is formed and the sample doesn't flow upon tilting, while below the concentration large aggregates can precipitate or form heterogeneous structures under gravity (Nicolai et al., 2011; Ryan et al., 2013).

1.3.2. Kinetics of thermal aggregation of β -Lg

The kinetics of aggregation depends on heating conditions. When heating β -Lg at 65 °C and neutral pH without addition of salt, noncovalent interactions are neglectable, and aggregation is relatively slow (Nicolai et al., 2011). The kinetics of the aggregation fits a model with a reaction order of 1.5. The model proposes, by analogy with polymer radical chemistry, an initiation step of exposure of the free -SH of native β -Lg, a propagation step of -SH/S-S interchange reactions, and a termination step of formation of S-S by two -SHs (Roefs & Kruif, 1994). This model gives a correct description of the decrease in concentration of native β -Lg and the increase in scattered intensity, as measured by in situ light scattering during heating (Hoffmann et al., 1996). This model however has some limitations. Firstly, the initial aggregation is different at higher temperatures. At 65 °C, reactive monomers are dominant,

while aggregation occurs via many intermediates at 78.5 °C (Schokker et al., 1999). The reason would be that the formation of reactive monomers (initiation reaction) would be very fast at higher temperature. The rate of sulfhydryl oxidation (termination reaction) would also be faster (i.e. very short propagation), and only relatively small aggregates would be formed via this pathway (de la Fuente et al., 2002). Secondly, non-covalent interactions are involved in the aggregation at high temperatures. Though non-covalent interactions contribute little to aggregation at temperatures below 75 °C, they become important at temperatures above 90 °C (Galani, 1999). Finally, the reaction scheme accounts for the formation of aggregates in which monomers are linearly linked, but aggregates are not stiff rods and may even have a spherical shape (de la Fuente et al., 2002).

Based on these limitations, an expanded model was established to describe β -Lg denaturation/aggregation under a wide range of conditions (pH, temperature and ionic strength) (Verheul et al., 1998). There are two limiting cases concerning the overall reaction kinetics. With low heating temperatures, pH closer to the pI of the protein, and at high NaCl concentrations, denaturation step is rate limiting, leading to an overall reaction order of 1.0. With high heating temperatures, pH further from the pI, and a low ionic strength, the aggregation reactions are rate limiting, with an overall reaction order of 2.0. In addition, non-covalent bonding becomes increasingly important at high NaCl concentrations. Two phases are observed in the aggregation step, including primary aggregates with a diameter under 100nm, and secondary aggregates with a large size.

In kinetics models, pH and ionic strength are the most important parameters. In some sense, adding salt at a fixed pH is equivalent to lowering or raising the pH towards pI at a fixed ionic strength at pH 7.0 (Baussay et al., 2004; Pouzot, Nicolai, Visschers, & Weijers, 2005). In both cases electrostatic interactions are reduced either by screening or decreasing the charge density.

1.3.3. Influence of other components of WP

B-Lg dominates the behavior of WP aggregation, but other proteins influence the aggregation. The structure of large aggregates formed at steady state in WPI at pH 7.0 was found to be almost identical to those formed in β -Lg solutions (Mahmoudi et al, 2007). The dependence of a diameter of aggregates on protein concentration was the same in WPI as in pure β -Lg solutions if no salt was added though the dependence becomes weaker with 0.1 M NaCl (Mahmoudi et al., 2007).

A-La and β -Lg form mixed oligomers and large aggregates in their mixture or WPI by both covalent (Hong and Creamer 2002, Livney et al 2003) and non-covalent bonds (Livney et al., 2003) at neutral pH. Effects of adding α -La in β -Lg solutions at pH 5.7 were studied at a fixed protein concentration of 40 g/L after heating at 80 °C for 15 min and no salt (Schmitt et al., 2011). With an increase in the amount of α -La, the fraction of protein that formed particles decreased and the fraction that formed small aggregates increased. Particle size was insensitive to the fraction of α -La up to 50%, but the shape was less spherical and they had a tendency to cluster.

Schmitt (2011) compared aggregation of WPI at pH 5.7 with their results obtained from mixtures of α -La and β -Lg with the same ratio. More and somewhat larger particles were formed in WPI, which was attributed to the presence of BSA and minerals in WPI. Subtle differences were found for different commercial WPI samples, due to differences in compositions and mineral contents.

1.4. Acid-induced gelation of WP

Aggregation of WP can be enhanced to form WP gels by acidification. The acid-induced process belongs to cold gelation, in two steps. In the first step, a solution of native proteins is preheated and soluble aggregates are formed by heating at a pH distant from the pI and at a low ionic strength. Upon cooling, the aggregates remain soluble and no gelation occurs. In the second step,

gelation is induced at an ambient temperature by reduction of electrostatic repulsion, by changing the pH toward the pI of the proteins. Gelation caused by lowering the pH is called acid-induced gelation (Alting et al., 2002).

Acid-induced gels prepared by WP or its major component β -Lg have the same aggregation characteristics (Alting et al., 2002; Alting et al., 2003).

Glucono delta-lactone (GDL), which slowly hydrolyses to gluconic acid, causes a gradual reduction of pH and therefore acidification by GDL results in a regular gel (de Jong, Klok, & van de Velde, 2009). The GDL induced acidification is a static process generally in 12-24 h.

In the acid-induced cold gelation process, firstly a protein network is formed by physical interactions, which is subsequently stabilized by the formation of disulfide bonds. The initial morphology of the network was established by non-covalent interactions, as confirmed by confocal laser scanning microscopy observation of gel after blocking $-SH$ (Alting, Hamer, de Kruif, & Visschers, 2000). Sulfhydryl oxidation or $-SH/S-S$ interchange reactions normally occur under alkaline conditions, but the proximity of the aggregates enables the subsequent formation of covalent $S-S$, even at acidic pH values. $-SH/S-S$ interchange reactions occur at pH higher than 3.9 (Alting et al., 2004). Adding sulfhydryl blocking agents to WP aggregate dispersions resulted in a $\sim 90\%$ decrease in gel hardness (Alting et al., 2003).

Salts shield the electrostatic repulsion between the charged protein aggregates and cause them to aggregate. Comparing to monovalent ions, divalent ions are much more effective at screening electrostatic interactions and because of their ability to form salt bridges (Bryant et al., 1998). Divalent cations, such as Ca^{2+} may also induce aggregation because of their ability to act as bridges between negatively charged carboxylic groups on neighboring WP molecules (Jeyarajah & Allen, 1994).

Electrostatic attraction between acidic and basic amino acid residues at the pI contributes to increasing gel strengths, and the maximum strength of acid-induced WP gel is observed at $\sim pH$ 5, close to the pI of β -Lg (Ju and

Kilara 1998; Alting et al 2004; Bryant et al 1998).

Addition of polysaccharides alters the properties of acid-induced WP gels. Electrostatic attraction occurs between negatively charged groups of polysaccharides and positively charged protein groups, especially at pH around or below pI. In a study on mixed gel at pH 4.8 (de Jong et al., 2009), interactions between protein aggregates and polysaccharides mainly depend on the charge density of polysaccharides because interactions were strengthened with increasing charge density of polysaccharides, while no interaction is observed for neutral polysaccharides. The charge density of the polysaccharides dominates the microstructure of the mixed WP gels that in turn determines the large deformation properties and texture characteristics (de Jong et al., 2009).

1.5. Strategies of improving thermal stability of WP

Thermal stability is the ability of proteins to survive heat processing without detrimental changes such as excessive turbidity, increased viscosity, phase separation, precipitation or gelation (Burrington, 2012). From the perspective of interactions, thermally stable proteins in solutions are proteins or protein aggregates with strong repulsive colloidal interactions (Ryan et al., 2013). Non-modified WP will not be stable as the sole protein in gradient at levels above 3% protein at neutral pH using a retort or UHT process (Rittmanic, 2006). To improve thermal stability of WP, the aggregation of WP can be inhibited via following strategies.

1.5.1. Preheating treatments

Thermally formed intermediates between monomeric proteins and an insoluble gel network or precipitate are defined as WP soluble aggregates (McSwiney, Singh, & Campanella, 1994). They are also called WP polymers or polymerized WP, which implies covalent links among protein molecules; preheated WP, describing the process used; and nanoparticles, indicating the average size (Ryan et al., 2013). In this thesis, “preheated WP” is used to

contrast with native WP. Preheated WP can be formed by heating WP solutions at a pH approximately from 6.0 to 7.5 (Ryan et al., 2013).

Dispersions with preheated WP have smaller particle sizes and lower viscosity than that of native WP after heating at pH 6.8 with 100mM NaCl (Ryan et al., 2012). However, the mechanism of improved thermal stability by preheating are not fully understood. The exact surface topology of soluble aggregates including the identity and location of charged and hydrophobic functional groups is unknown (Ryan et al., 2012). Electrostatic repulsion between soluble aggregates may be strengthened due to the increased magnitude of negative zeta potential from -24.6 to -26.6 mv after preheating, but hydrophobic attraction can be enhanced as evidenced by a significantly increase in surface hydrophobicity from 1.72 to 7.17 slope $\times 10^6$ (Ryan et al., 2012). Other factors including alteration in kinetic metastability of aggregation (Ryan et al., 2012) and the quantity and location of disulfide bonds may contribute to thermal stability of WP.

1.5.2. Modification of amino groups of WP

Crosslinking by Transglutaminase (TGase). TGase is a transferase that forms isopeptide bonds between a glutamine residue and a free amine group (e.g., the amine group of lysine) (Jaros et al., 2006). It can be potentially applied in the food industry to modify food proteins (Yokoyama, Nio, & Kikuchi, 2004). The formation of TGase-catalyzed bonds attenuates hydrophobic attraction through steric hindrance and formation of compact molecules, which limits the exposure of hydrophobic groups and thus improves the thermal stability of proteins (Eissa & Khan, 2006). TGase also improves thermal stability of preheated WP. The Absorbance at 400nm of 5% w/v preheated WP with 100mM of NaCl after heating at 80 °C and pH 7.0 decreased from about 1.2 to 0.5 with crosslinking by 10.2 U/g TGase for 15 h (Wang, Zhong, & Hu, 2012).

Glycation. Glycation, also known as the Maillard reaction, occurs between the amine groups of the lysine residues in food proteins and the reducing-end

carbonyl groups of a sugar (Liu, Ru, & Ding, 2012). The improved thermal stability of WP has been observed after glycation with reducing saccharides (Chevalier et al., 2001; Liu & Zhong, 2012). This can be attributed to steric effects based on a 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. Because the strength of steric hindrance is a function of polymer chain density on colloidal particle surface, a higher degree of glycation facilitates the improvement of functional properties of proteins (Israelachvili, 2011). The restriction of chemical modification of amine groups is the loss of lysine which generally is the limiting amino acid.

1.5.3. Binding WP with anionic surfactants

The binding of anionic surfactants with proteins were extensively studied between SDS and proteins including β -Lg (Jones, 1975), bovine serum fetuin (Zaidi et al., 2014), ovalbumin, bacterial α -amylase, and papain (Su & Jirgensons, 1977). SDS bind with protein through hydrophobic attraction between the alkyl chain and hydrophobic groups of proteins and electrostatic attraction between anionic head and positively charged groups of proteins (Jones, 1975; Zaidi, Nusrat, Zaidi, & Khan, 2014). The relative importance of hydrophobic and electrostatic contributions to binding, and the mechanism of induced conformational change are incompletely understood despite extensive investigation (Goddard & Ananthapadmanabhan, 1993; Jones, 1992). In general, the binding of surfactant to proteins can be divided into three steps: specific binding at a low surfactant concentration, non-cooperative binding at a higher surfactant concentration and cooperative binding at an even higher concentration (Jones, 1975). For a native β -Lg dimer, two anionic surfactants are bound initially, following by a second step with approximately 22 anionic surfactants, and a third micellar-like binding step (Hill & Briggs, 1956).

Thermal stability of WP could be improved by binding anionic surfactants.

SDS and SSL inhibited denaturation of 9% w/v WP, increased denaturation temperatures, and improved solubilities above pI (Giroux & Britten, 2004).

1.6. Conclusions

Thermal aggregation properties of WP are crucial for properties of WP-based foods requiring sterilization and pasteurization. Both enhancing aggregation for a semi-solid product and inhibiting aggregation for shelf-stable beverages have practical significance. This will require the understanding and controlling of the interactions forces of WP including electrostatic interactions, disulfide bonds, hydrophobic attraction, hydrogen bonds and van der Waals interactions. These interactions are functions of protein concentration, pH, ionic strength, and thermal treatment conditions. Therefore these factors can be used to develop different strategies for enhanced or inhibited aggregation.

1.7. Research scope

WP enhances satiety over a short-term period when compared to carbohydrates but there was no consistent effect of WP alone (Lam et al., 2009). Since viscosity or gelation may prolong satiety (Solah et al., 2010), the second chapter in the thesis aims at inducing gelation of WP at gastric conditions. Because WP does not form gels at gastric pH due to strong positive charges, an anionic polysaccharide (pectin) was used studied to neutralize positive charges of WP, screen electrostatic repulsion and induce gelation.

On the other hand, ready-to-drink beverages offer a fast, convenient and portable way to intake protein. However, WP dispersions become turbid or even form gel at pH 4.0-6.0 after thermal sterilization and pasteurization. Improvements in thermal stability of WP at pH 5.5-6.0 will broaden its application in ready-to-drink beverages. Improvements of thermal stability of WP by preheating or anionic surfactants have been studied individually. In the

third chapter, preheating treatments and an anionic surfactant were used to further improve thermal stability of WP at pH 5.5-6.0.

References

- Alting, A. C., de Jongh, H. H. J., Visschers, R. W., & Simons, J.-W. F. A. (2002). Physical and chemical interactions in cold gelation of food proteins. *Journal of Agricultural and Food Chemistry*, 50(16), 4682–4689.
- Alting, A. C., Hamer, R. J., de Kruif, C. G., Paques, M., & Visschers, R. W. (2003). Number of sulfhydryl groups rather than the size of the aggregates determines the hardness of cold set whey protein gels. *Food Hydrocolloids*, 17(4), 469–479.
- Alting, A. C., Hamer, R. J., de Kruif, C. G., & Visschers, R. W. (2000). Formation of disulfide bonds in acid-induced gels of preheated whey protein isolate. *Journal of Agricultural and Food Chemistry*, 48(10), 5001–5007.
- Alting, A. C., Van Der Meulena, E. T., Hugenholtz, J., & Visschers, R. W. (2004). Control of texture of cold-set gels through programmed bacterial acidification. *International Dairy Journal*, 14(4), 323–329.
- Baldwin, R. L. (1986). Temperature dependence of the hydrophobic interaction in protein folding. *Proceedings of the National Academy of Sciences*, 83(21), 8069–8072.
- Bauer, R., Hansen, S., & Øgenda, L. (1998). Detection of intermediate oligomers, important for the formation of heat aggregates of β -lactoglobulin. *International Dairy Journal*, 8(2), 105–112.
- Baussay, K., Le Bon, C., Nicolai, T., Durand, D., & Busnel, J.-P. (2004). Influence of the ionic strength on the heat-induced aggregation of the globular protein β -lactoglobulin at pH 7.0. *International Journal of Biological Macromolecules*, 34(1), 21–28.
- Belfort, G., & Lee, C.-S. (1991). Attractive and repulsive interactions between and within adsorbed ribonuclease A layers. *Proceedings of the National Academy of Sciences*, 88(20), 9146–9150.
- Bryant, C. M., McClements, D. J., & Julian McClements, D. (1998). Molecular basis of protein functionality with special consideration of cold-set gels

- derived from heat-denatured whey. *Trends in Food Science & Technology*, 9(4), 143–151.
- Burkhard, R. K., & Stolzenberg, G. E. (1972). Interaction between sodium dodecyl sulfate and ferricytochrome. *Biochemistry*, 11(9), 1672–1677.
- Burrington, K. (2012). Technical Report: Whey Protein Heat Stability. Retrieved from <http://www.usdairy.com/~media/usd/public/technicalreportwheyproteinheatstability.pdf.pdf>
- Chaplin, L. C., & Lyster, R. L. J. (1986). Irreversible heat denaturation of bovine α -lactalbumin. *Journal of Dairy Research*, 53(02), 249–258.
- Chevalier, F., Chobert, J.-M., Popineau, Y., Nicolas, M. G., & Haertlé, T. (2001). Improvement of functional properties of β -lactoglobulin glycosylated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11(3), 145–152.
- Chung Chun Lam, S., Moughan, P. J., Awati, A., & Morton, H. R. (2009). The influence of whey protein and glycomacropeptide on satiety in adult humans. *Physiology & Behavior*, 96(1), 162–168.
- Considine, T., Patel, H. A., Singh, H., & Creamer, L. K. (2005). Influence of binding of sodium dodecyl sulfate, all-trans-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of β -lactoglobulin B. *Journal of Agricultural and Food Chemistry*, 53(8), 3197–3205.
- Damodaran, S. (1997). *Food proteins and their applications* (Vol. 80). CRC Press.
- De Jong, S., Klok, H. J., & van de Velde, F. (2009). The mechanism behind microstructure formation in mixed whey protein–polysaccharide cold-set gels. *Food Hydrocolloids*, 23(3), 755–764.
- De la Fuente, M. A., Singh, H., & Hemar, Y. (2002). Recent advances in the characterisation of heat-induced aggregates and intermediates of whey protein. *Trends in Food Science & Technology*, 13(8), 262–274.

- De Wit, J. N. (1981). Structure and functional behaviour of whey protein. Netherlands Milk and Dairy Journal (Netherlands).
- Durand, D., Christophe Gimel, J., & Nicolai, T. (2002). Aggregation, gelation and phase separation of heat denatured globular proteins. *Physical A: Statistical Mechanics and Its Applications*, 304(1), 253–265.
- Wennerstrom, H.; Evans, D. The colloidal domain: where physics, chemistry, biology, and technology meet, 2nd ed.; Wiley-VCH, New York, 1999.
- Foegeding, E. A., Davis, J. P., Doucet, D., & McGuffey, M. K. (2002). Advances in modifying and understanding whey protein functionality. *Trends in Food Science & Technology*, 13(5), 151–159.
- Frank, J. N. (2013). US Consumers Have a Healthy Appetite for High Protein Food. The US Leads the Way in Global Launches of High Protein Products. . Retrieved from <http://www.mintel.com/press-centre/food-and-drink/us-consumers-have-a-healthy-appetite-for-high-protein-food-the-us-leads-the-way-in-global-launches-of-high-protein-products>
- Fuquay, J. W., Fox, P. F., & McSweeney, P. L. H. (2011). *Encyclopedia of Dairy Sciences* 2nd Edition, Four-Volume set. Academic Press.
- Galani, D. (1999). Heat-induced denaturation and aggregation of β -Lactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregates. *International Journal of Food Science & Technology*, 34(5–6), 467–476.
- Giroux, H. J., & Britten, M. (2004). Heat treatment of whey protein in the presence of anionic surfactants. *Food Hydrocolloids*, 18(4), 685–692.
- Hill, R. M., & Briggs, D. R. (1956). A study of the interaction of n-Octylbenzene-p-sulfonate with β -Lactoglobulin¹, 2. *Journal of the American Chemical Society*, 78(8), 1590–1597.
- Hirose, M. (1993). Molten globule state of food proteins. *Trends in Food Science & Technology*, 4(2), 48–51.

- Hoffmann, M. A. M., Roefs, S., Verheul, M., Van Mil, P., & De Kruif, K. G. (1996). Aggregation of beta-lactoglobulin studied by in situ light scattering. *Journal of Dairy Research*, 63, 423–440.
- Hoffmann, M. A. M., Sala, G., Olieman, C., & de Kruif, K. G. (1997). Molecular mass distributions of heat-induced β -lactoglobulin aggregates. *Journal of Agricultural and Food Chemistry*, 45(8), 2949–2957.
- Hoffmann, M. A. M., & van Mil, P. J. J. M. (1997). Heat-induced aggregation of β -lactoglobulin: role of the free sulfhydryl group and disulfide bonds. *Journal of Agricultural and Food Chemistry*, 45(8), 2942–2948.
- Hong, Y.-H., & Creamer, L. K. (2002). Changed protein structures of bovine β -lactoglobulin B and α -lactalbumin as a consequence of heat treatment. *International Dairy Journal*, 12(4), 345–359.
- Iametti, S., Gregori, B., Vecchio, G., & Bonomi, F. (1996). Modifications occur at different structural levels during the heat denaturation of β -Lactoglobulin. *European Journal of Biochemistry*, 237(1), 106–112.
- Israelachvili, J. N. (2011). *Intermolecular and surface forces: revised third edition*. Academic press.
- Jaros, D., Partschfeld, C., Henle, T., & Rohm, H. (2006). Transglutaminase in dairy products: chemistry, physics, applications. *Journal of Texture Studies*, 37(2), 113–155.
- Jayat, D., Gaudin, J.-C., Chobert, J.-M., Burova, T. V, Holt, C., McNae, I., Haertlé, T. (2004). A recombinant C121S mutant of bovine β -lactoglobulin is more susceptible to peptic digestion and to denaturation by reducing agents and heating. *Biochemistry*, 43(20), 6312–6321.
- Jeyarajah, S., & Allen, J. C. (1994). Calcium binding and salt-induced structural changes of native and preheated. beta.-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 42(1), 80–85.
- Jones, M. N. (1975). A theoretical approach to the binding of amphipathic molecules to globular proteins. *Journal of Biochemistry*, 151, 109–114.

- Ju, Z. Y., & Kilara, A. (1998). Textural properties of cold-set gels induced from heat-denatured whey protein isolates. *Journal of Food Science*, 63(2), 288–292.
- Kinsella, J. E., & Morr, C. V. (1984). Milk proteins: physicochemical and functional properties. *Critical Reviews in Food Science & Nutrition*, 21(3), 197–262.
- Kinsella, J. E., & Whitehead, D. M. (1989). Proteins in whey: chemical, physical, and functional properties. *Advances in food and nutrition research*, 33, 343-438.
- Krissansen, G. W. (2007). Emerging health properties of whey protein and their clinical implications. *Journal of the American College of Nutrition*, 26(6), 713S–723S.
- Larsen, R. A., Jenness, R., & Geddes, W. F. (1949). Effect of heat treatment on the sulfhydryl groups of milk serum proteins. *Cereal Chemistry*, 26(4), 287–297.
- Liu, G., & Zhong, Q. (2012). Glycation of whey protein to provide steric hindrance against thermal aggregation. *Journal of Agricultural and Food Chemistry*, 60(38), 9754–97762.
- Liu, J., Ru, Q., & Ding, Y. (2012). Glycation a promising method for food protein modification: physicochemical properties and structure, a review. *Food Research International*, 49(1), 170–183.
- Livney, Y. D., Verespej, E., & Dalgleish, D. G. (2003). Steric effects governing disulfide bond interchange during thermal aggregation in solutions of β -lactoglobulin B and α -lactalbumin. *Journal of Agricultural and Food Chemistry*, 51(27), 8098–8106.
- Lundahl, P., Greijer, E., Sandberg, M., Cardell, S., & Eriksson, K.-O. (1986). A model for ionic and hydrophobic interactions and hydrogen-bonding in sodium dodecyl sulfate-protein complexes. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, 873(1), 20–26.

- Mahmoudi, N., Mehalebi, S., Nicolai, T., Durand, D., & Riaublanc, A. (2007). Light-scattering study of the structure of aggregates and gels formed by heat-denatured whey protein isolate and β -lactoglobulin at neutral pH. *Journal of Agricultural and Food Chemistry*, 55(8), 3104–3111.
- Majhi, P. R., Ganta, R. R., Vanam, R. P., Seyrek, E., Giger, K., & Dubin, P. L. (2006). Electrostatically driven protein aggregation: β -lactoglobulin at low ionic strength. *Langmuir*, 22(22), 9150–9159.
- McKenzie, H. A., & Sawyer, W. H. (1967). Effect of pH on beta-lactoglobulins. *Nature*, 214, 1101–1104.
- McSwiney, M., Singh, H., & Campanella, O. H. (1994). Thermal aggregation and gelation of bovine β -lactoglobulin. *Food Hydrocolloids*, 8(5), 441–453.
- Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on protein unfolding and sulfhydryl/disulfide interchange reactions during heat-induced gelation of whey protein. *Journal of Agricultural and Food Chemistry*, 43(1), 46–52.
- Monahan, F. J., McClements, D. J., & German, J. B. (1996). Disulfide-mediated polymerization reactions and physical properties of heated WPI-stabilized emulsions. *Journal of Food Science*, 61(3), 504–509.
- Mulvihill, D. M., & Kinsella, J. E. (1987). Gelation characteristics of whey protein and beta-lactoglobulin. *Food Technology (USA)*.
- Nicolai, T., Britten, M., & Schmitt, C. (2011). β -Lactoglobulin and WPI aggregates: formation, structure and applications. *Food Hydrocolloids*, 25(8), 1945–1962.
- Oakes, J. (1974). Protein-surfactant interactions. Nuclear magnetic resonance and binding isotherm studies of interactions between bovine serum albumin and sodium dodecyl sulphate. *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases*, 70, 2200–2209.

- Pike, A. C. W., Brew, K., & Acharya, K. R. (1996). Crystal structures of guinea-pig, goat and bovine α -lactalbumin highlight the enhanced conformational flexibility of regions that are significant for its action in lactose synthase. *Structure*, 4(6), 691–703.
- Pouzot, M., Nicolai, T., Visschers, R. W., & Weijers, M. (2005). X-ray and light scattering study of the structure of large protein aggregates at neutral pH. *Food Hydrocolloids*, 19(2), 231–238.
- Rittmanic, S. (2006). U.S. whey protein in ready-to-drink beverages (pp. 1-8). Retrieved from <http://www.usdec.org/files/Publications/BEVERAGESwebversion8-16-06.pdf>
- Roefs, S. P. F. M., & Kruif, K. G. (1994). A model for the denaturation and aggregation of β -Lactoglobulin. *European Journal of Biochemistry*, 226(3), 883–889.
- Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein soluble aggregates thermally treated with salts. *Food Hydrocolloids*, 27(2), 411–420.
- Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of whey protein soluble aggregates for thermal stability—a hypothesis paper. *Journal of Food Science*, 78(8), R1105–R1115.
- Sawyer, W. H. (1969). Complex between β -lactoglobulin and κ -casein. A review. *Journal of Dairy Science*, 52(9), 1347–1355.
- Schmitt, C., Bovay, C., Vuillomenet, A.-M., Rouvet, M., & Bovetto, L. (2011). Influence of protein and mineral composition on the formation of whey protein heat-induced microgels. *Food Hydrocolloids*, 25(4), 558–567.
- Schokker, E. P., Singh, H., Pinder, D. N., Norris, G. E., & Creamer, L. K. (1999). Characterization of intermediates formed during heat-induced aggregation of β -lactoglobulin AB at neutral pH. *International Dairy Journal*, 9(11), 791–800.

- Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *Journal of Agricultural and Food Chemistry*, 37(1), 161–168.
- Solah, V. A., Kerr, D. A., Adikara, C. D., Meng, X., Binns, C. W., Zhu, K., Prince, R. L. (2010). Differences in satiety effects of alginate-and whey protein-based foods. *Appetite*, 54(3), 485–491.
- Verheul, M., Roefs, S. P. F. M., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of β -lactoglobulin. *Journal of Agricultural and Food Chemistry*, 46(3), 896–903.
- Visschers, R. W., & de Jongh, H. H. J. (2005). Disulphide bond formation in food protein aggregation and gelation. *Biotechnology Advances*, 23(1), 75–80.
- Walstra, P. (2002). *Physical chemistry of foods* (Vol. 121). CRC Press.
- Wang, W., Zhong, Q., & Hu, Z. (2012). Nanoscale understanding of thermal aggregation of whey protein pretreated by transglutaminase. *Journal of Agricultural and Food Chemistry*, 61(2), 435–446.
- Yokoyama, K., Nio, N., & Kikuchi, Y. (2004). Properties and applications of microbial transglutaminase. *Applied Microbiology and Biotechnology*, 64(4), 447–454.

Appendix

Table 1-1. Compositions of whey protein and their properties (Bryant et al., 1998; Fuquay et al., 2011)

Protein	WPC %	WPI %	M.W (kDa)	-SH/S-S	pI
β -Lg	50-60	44-69	18.3	1/2	~5.2
α -La	12-16	14-15	14.2	0/4	4.8-5.1
BSA	3-5	1-3	66	17/1	4.8-5.1
Immunoglobulins	5-8	2-3	150-960	-	5.5-6.8

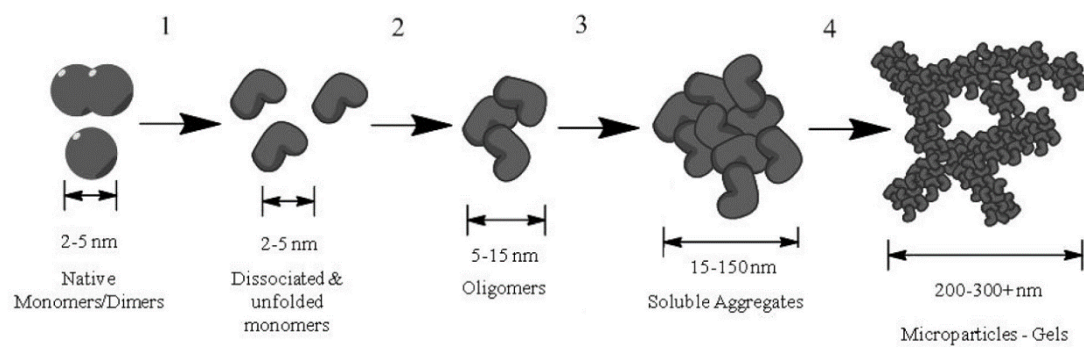


Figure 1-1. Model of β -Lg denaturation and aggregation at pH > 5.7. Adapted from (Nicolai et al., 2011).

Chapter 2. Aggregation and Gelation Properties of Preheated Whey Protein and Pectin Mixtures at Gastric Acidity

2.1. Abstract

Viscous foods in the stomach enhance satiety and can be an intervention strategy to control the diet intake. The objective of this work was to characterize gelation properties of mixtures with preheated whey protein (WP) and pectin at 0:1-1:5 mass ratios quickly acidified to pH 1.0-4.0 by HCl under intensive agitation. The microstructure of gels was studied using confocal laser scanning microscopy, and gelation properties were characterized using small strain oscillation tests and formation of self-standing gels in vials. Urea (10%) or N-ethylmaleimide (5 mM) was supplemented in WP-pectin mixtures to study the roles of hydrogen bonds, hydrophobic attraction, and formation of disulfide bonds on gelation. The fast-acidified gels had less homogenous microstructures and lower strength than those using slow acidification enabled with glucono-delta-lactone. Preheated WP gelled at pH 3.5-4.0, while the WP-pectin mixture at mass ratios of 20:1, 10:1, and 5:1 formed gels at pH 2.0-3.5, 1.5-3.0, and 1.5-2.5, respectively. WP-pectin gels were weakened by NaCl but strengthened by CaCl_2 based on the observation of gels sustaining gravity, indicating the significances of electrostatic attraction and ionic bridges on gelation properties. Without hydrophobic attraction, gelation was not observed for preheated WP but was evident for the WP-pectin mixtures at pH 2.5 and 3.0. Additionally, gels with preheated WP only were dissolved after dilution in water, while those of preheated WP-pectin formed with 10 mM CaCl_2 at pH 2.0 and 2.5 remained partially undissolved. The findings suggest the potential application of preheated WP-pectin mixtures to increase the viscosity in the stomach.

Keywords: preheated whey protein, pectin, gelation, gastric conditions, satiety

2.2. Introduction

More than one-third of adults in United States are obese (Ogden, Carroll, Kit, & Flegal, 2014) which causes significant health, social, and economic consequences (Finkelstein, Trogdon, Cohen, & Dietz, 2009). The consumption of foods with high contents of digestible carbohydrates results in a rapid increase of glucose in the blood shortly after a meal and subsequent storage in the body, which also leads to a rapid onset of hunger and a desire to eat again (Norton, Moore, & Fryer, 2007). Enhancing satiety of diets can be an effective intervention strategy to fight against obesity, and viscous beverages or semi-solid foods can provide sustained satiety (Solah et al., 2010). Conversely, highly viscous beverages and large particulates can cause negative sensory qualities. An alternative approach is to develop ingredients that provide quality in foods but can increase viscosity markedly or form digestible gels in the stomach.

Acid-induced gelation of preheated whey proteins (WP) is a well-studied topic (Alting et al., 2002) and is a potential strategy to provide low viscosity in neutral food systems but high viscosity at gastric acidity (Zhang & Vardhanabhuti, 2014b). To facilitate acid-induced gelation, anionic polyelectrolytes can be added in preheated WP, because the two groups of biopolymers become oppositely charged once pH is below the protein pI (Norton & Frith, 2001). Pectin is such a non-digestible polyelectrolyte that can form gels with preheated WP after acidification (de Jong et al., 2009; Zhang et al., 2014; Zhang & Vardhanabhuti, 2014a). The delayed digestion of WP after gelation with pectin (Zhang & Vardhanabhuti, 2014b), the lowered energy density, and the increased viscosity (Fizman & Varela, 2013) are additional features making the mixture of pectin and preheated WP a good combination to prolong satiety.

Acid-induced gelation of preheated WP has been studied at pH 6.0-8.0 (Mleko & Foegeding, 2000), and 5.0 (Alting, Hamer, de Kruif, & Visschers, 2003; Alting et al., 2004). Gelation properties of preheated WP and

polysaccharide mixtures were reported at pH 4.0-5.6 (de Jong et al., 2009) and 4.7 (Zhang et al., 2014; Zhang & Vardhanabhuti, 2014a). However, gastric pH ranges from 1.0 to 4.0 during meal time (Mattioli et al., 1990), and gelation properties of WP with and without polysaccharides at this pH range are unknown. In addition, gels in the above studies were prepared under slow (12-24 h) and static acidification enabled by glucono-delta-lactone (GDL). These laboratory conditions are important to understand fundamental properties but are different from gastric conditions involving agitation and fast acidification.

The objective of the present work was to study aggregation and gelation characteristics of mixtures of pectin and preheated WP after fast acidification to pH 1.0-4.0. Fast acidification with hydrochloric acid under agitation was used to simulate gastric conditions. Aggregation and gelation properties of WP and pectin mixtures were studied at pH 1.0-4.0 with various concentrations of sodium chloride or calcium chloride, and molecular interactions were probed after supplementing urea and N-ethylmaleimide (NEM).

2.3. Materials and methods

2.3.1. Materials

Whey protein isolate (WPI) powder was obtained from Hilmar Ingredients, Inc. (Hilmar, CA). The product contains 91.0% w/w protein, 0.2% w/w lactose, 0.2% w/w fat, 4.0% w/w moisture, and 1.0% w/w ash according to the manufacturer. Low-methoxyl pectin from citrus peel with an esterification degree of 32% (Lot# SLBF4758V) was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Urea (99% purity) and NEM (99% purity) were purchased from Acros Organic (Morris Plains, NJ). Unless otherwise stated, all chemicals were of analytical grade.

2.3.2. Preparation of preheated WP

Fifty grams of WPI powder was dissolved in 500 mL deionized water and adjusted to a total volume of 550 mL and a protein concentration of about

9% w/v. Protein solutions were adjusted to pH 7.0 using 5 M NaOH and hydrated overnight at 4 °C, heated for 30 min in a water bath after reaching the set temperature of 80 °C, immediately cooled in an ice/water bath, and stored overnight at 4 °C before following studies.

2.3.3. Aggregation properties

Mixtures with 0.045% w/v pectin, 0.45% w/v WP (native or preheated), and their mixtures were prepared from stock solutions. The mixture solutions were adjusted to pH 1.0-4.0 using 1.0 M HCl, and measured for absorbance at 600 nm (Abs_{600}) within 1 h after pH adjustment using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA). Averages of four sample replicates were reported. Additional samples were prepared for 0.45% w/v preheated WP and 0.0225, 0.045 and 0.09% w/v pectin, corresponding to respective WP:pectin mass ratios of 20:1, 10:1, and 5:1. To study the influence of ionic strength, NaCl (25, 50 or 100 mM) or $CaCl_2$ (10 or 20 mM) was dissolved in solutions with preheated WP. Averages of two sample replicates were reported.

2.3.4. Gelation properties

Samples were prepared from stock solutions to 6.3% w/v WP and 0.315, 0.63, or 1.26% w/v pectin, corresponding to WP:pectin mass ratios of 20:1, 10:1, and 5:1, respectively. The adjustment of pH to 1.0-4.0 was conducted using 6.0 and 1.0 M HCl within 1 min under agitation at 700-1100 rpm on a stir plate. To study the influence of ionic strength, NaCl was added at 25, 50 or 100 mM, and $CaCl_2$ at 5 or 10 mM in the mixture with preheated WP and pectin at a mass ratio of 10:1. Two milliliters of these samples were pipetted into vials, incubated at room temperature (20 °C) for one hour, and photographed after inverting vials. Slow-acidified gels were prepared with 6.3% preheated WP and 0.2 g/protein GDL, and its final pH was 4.0 (Eissa & Khan, 2005).

Small strain oscillatory rheology was also applied to study gelation properties. Tests were performed using an AR2000 rheometer (TA Instruments, New Castle, DE) with a concentric cylinder geometry (30 mm in

the stator diameter, 28 mm in the rotor diameter, 42 mm in the height of cylinder). Samples were also added with 10% w/v urea or 5 mM NEM and adjusted to pH 1.0-4.0. Oscillatory tests included a time sweep over 60 min at 37 °C and a temperature ramp from 37 to 80 °C with a heating rate of 5 °C/min. An oscillation frequency of 1 Hz and a strain amplitude of 0.1% were used in tests. Two sample replicates were tested and representative samples were presented in the data.

2.3.5. Confocal laser scanning microscopy (CLSM)

Microstructures of gels were studied on a Leica TCS SP2 microscope and a 63x objective lens (Leica Microsystems Inc., Heidelberg, Germany). The excitation wavelength was 543 nm for Rhodamine B (Salcedo et al., 1999). The emission from 562 to 650nm was collected using a triple dichroic mirror at 488/543/633 nm. Digital images were acquired in 1024 × 1024 pixel resolution. Specimens of 20 × 20 mm square strips were prepared for CLSM observation, and images of 7.8 × 7.8 μm were acquired. Fourteen milliliters of 6.3% w/v preheated WP were stained with 20 μL 0.2% w/v Rhodamine B in distilled water. The stained WP was stirred in dark for 1 h, mixed with deionized water or pectin solution, adjusted for pH, and allowed to form gels. The gels were placed on slides and examined for CLSM.

2.3.6. Stability of gels upon dilution and mixing

Gels at pH 1.0-4.0 were prepared with 6.3% w/v preheated WP, with and without 0.63% pectin and 10 mM CaCl₂. Then 5 mL of each sample was pipetted into 20 mL glass vials, mixed with 15 mL of deionized water, and vigorously hand-shaken for 5 s.

2.3.7. Statistical analysis

Statistical analyses were performed using the SAS Enterprise Guide program (version 6.1, SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using Tukey's test. The significance level was set at 0.05.

2.4. Results and discussion

2.4.1. Aggregation properties of WP and pectin mixtures

2.4.1.1. Aggregation at low ionic strength

The aggregation between pectin and WP at pH 3.5-8.0 is a result of electrostatic attraction between negative charged pectin and positive charged amino acid residues of WP and has been studied using parameters of zeta-potential, turbidity and particle size (Jones, Decker, & McClements, 2009; Jones & McClements, 2011). In the present work, the aggregation properties below pH 3.5 were studied using samples with 0.45% w/v protein and measured for Abs₆₀₀. An increase in sample turbidity indicates the increase in quantity or dimension of aggregates which are large enough to scatter light (Jones, Lesmes, Dubin, & McClements, 2010). The aggregation properties were then used to guide later gelation studies.

Native WP has a hydrodynamic diameter of 10 nm at neutral acidity, corresponding to transparent samples with low turbidity (Wang et al. 2012). The clear native WP samples with Abs₆₀₀ below 0.05 at pH 4.0 to 1.0 (Fig. 2-1A) indicated the absence of protein aggregation at pH below 4.0, which is expected (Ju & Kilara, 1998). Solutions with 0.45% w/v pectin were also clear at pH 1.0-4.0. The high Abs₆₀₀ (2.15) of a solution with 0.45% w/v WP and 0.045% pectin at pH 4.0 indicates that WP and pectin, with positive and negative net charges, respectively, formed large complexes. The mixture was less turbid when pH decreased from 4.0 to 1.0, indicating the reduced extent of aggregation. The overall charge of the WP-pectin mixture at a mass ratio of 10:1 is positive at pH 4.0 (Jones et al., 2010), and WP has more positive charges at a lower pH between 4.0 and 1.0. The lower Abs₆₀₀ at lower pH therefore resulted from the weakened electrostatic attraction due to protonation of carboxylate groups of pectin that has galacturonic acid with pKa of 3.47 at 20 °C (Holvik & Høiland, 1977).

Native WPs are denatured and form soluble aggregates during heating at neutral pH (Marangoni et al., 2000). WPs aggregate as strands after heating

at pH 7.0 with a low ionic strength (Ikeda & Morris, 2002). The width of the strands is so small that they do not scatter light strongly (Bryant & McClements, 1998). The turbid appearance and high Abs_{600} of preheated WP (Fig. 2-1A) at pH 3.5 and 4.0 indicated the aggregation of WP strands. Below pH 3.5, samples were clear and had Abs_{600} of about 0.15, suggesting the redispersion of flocculated strands due to strengthened electrostatic repulsion. The overall trend was similar when pectin was added at one-tenth mass of preheated WP, except for the increased turbidity at pH 2.0-3.0 (Fig. 2-1A) which indicates complexation between pectin and WP strands. The complexation was further verified in Fig. 2-1B that showed higher turbidity at pH 2.0-4.0 with a higher content of pectin. Results in Fig. 2-1 suggest the feasibility to form gels from preheated WP and pectin at gastric acidity.

2.4.1.2. Aggregation at increased ionic strengths

Impacts of ionic strength on WP aggregation and gelation were studied for 25-100 mM NaCl or 10-20 mM $CaCl_2$. For solutions with 0.45% w/v native WP or 0.45% w/v pectin, no apparent change in Abs_{600} was observed (data not shown), indicating these ionic conditions are insufficient to cause aggregation of each biopolymer. Fig. 2-2A shows the Abs_{600} of dispersions with 0.45% w/v native WP and 0.045% w/v pectin after addition of NaCl and $CaCl_2$. At pH 3.0-4.0, an increase in ionic strength generally resulted in a decrease in Abs_{600} , which is expected because the charge screening effects weaken the electrostatic attraction between WP and pectin and cause the decoupling of complexes (Jones & McClements, 2011). At pH 1.0-2.5, no significant impacts of ionic strength on Abs_{600} were observed, resulting from the insignificance of complex formation, as discussed previously.

Fig. 2-2B shows a significant increase in Abs_{600} of preheated WP alone at pH 3.0 and 3.5 with an increase in ionic strength, indicating that weakened electrostatic repulsion between preheated WP induced further aggregation (Bryant & McClements, 1998). The Abs_{600} of the mixtures of preheated WP and pectin (10:1) increased at pH 2.5-3.5, but decreased at pH 2.0 with the

increase of ionic strength (Fig. 2-2C). The phenomenon can be explained by the relative significance of aggregation and decoupling of WP-pectin complexes. At pH 2.5-3.5, the aggregation dominated in the system and led to an increase in Abs₆₀₀, while decoupling dominated at pH 2.0 due to weakened electrostatic attraction between WP and pectin.

2.4.2. Formation of self-standing gels

2.4.2.1. Gel formation at a low ionic strength

Gels were first evaluated for visual inspection of self-standing gels from samples with 6.3% w/v WP (Fig. 2-3). All samples were well-dispersed at pH 7.0 but behaved differently after acidification. Pectin or native WP did not aggregate by itself at pH 1.0-4.0. The mixture with native WP and pectin did not form gels at pH 1.0-4.0 but became more turbid when compared to samples with only one biopolymer. This observation agrees with the turbidity at a low protein concentration (Fig. 2-1A) and suggests these complexes cannot form a gel network.

Preheated WP formed gels at pH 3.5 and 4.0 (Fig. 2-3) due to sufficient aggregation (Fig. 2-1A) (Bryant & McClements, 1998). Aggregation of preheated WP was limited below pH 3.5 due to strong electrostatic repulsion (Fig. 2-1A), corresponding to fluid samples (Fig. 2-3). When pectin was mixed with preheated WP at a mass ratio of 20:1, gels were observed at pH 2.0-3.5 and pH 1.0 (Fig. 2-3). When the WP to pectin mass ratio decreased to 10:1 and 5:1, the gelation pH range shifted to pH 1.5-3.0 and 1.5-2.5, respectively (Fig. 2-3). Below pH 4.0, pectin neutralizes positive charges of WP and facilitates aggregation (Fig. 2-1A), and a larger amount of pectin facilitates aggregation at pH 2.0-4.0 (Fig. 2-1B), which explains the shifting in gelation pH of preheated WP-pectin with an increase of the pectin content. Preheated WP formed gels at pH 1.0 regardless of pectin contents (Fig. 2-3). The conformation of WP at this extreme pH is a possible reason of gelation, which is not discussed in this study. The gelation pH theoretically has strong impacts on satiety. The gastric pH is around 1.0 before a meal and can gradually

increase to pH 4.0 during a meal (Mattioli et al., 1990). The observations in Fig. 2-3 indicate mixtures of preheated WP and pectin, with a lower gelation pH, can aggregate more easily than preheated WP alone when encountering gastric juice, which will impact satiety differently. The ability of WP to aggregate quickly once entering the stomach is important because of the non-enzymatic and enzymatic hydrolysis of protein at gastric conditions.

No gelation but precipitation occurred at pH 4.0, pH 3.5-4.0 and pH 3.0-4.0 when WP to pectin mass ratios were 1:20, 1:10, and 1:5, respectively. The WP-pectin mixture has zero net charge at around pH 4.0 (Jones et al., 2009). Intense aggregation to form large and dense structures is expected at an acidity corresponding to zero net charge of protein systems (Weinbreck, 2004), which results in precipitation rather than gelation (Kallala, Jullien, & Cabane, 1992). At a pH below 4.0, complexes have net positive charges that result in reduced and less compact structures than at pH 4.0 (Weinbreck, 2004), due to moderate intra- and intermolecular electrostatic repulsion, which favors gelation.

It is well established that transparent gels can be formed by heating WP above pH 6 and below pH 4 due to formation of fine strands, while turbid or opaque gels are formed between pH 4 and 6 due to formation of particulates (Langton & Hermansson, 1992). In the present study, strands are pre-formed during preheating, and gel opacity is a result of higher-ordered structures formed from preheated WP strands. Preheated WP formed translucent and opaque gels at pH 3.5 and 4.0, respectively (Fig. 2-3), which can be explained by differences in structures aggregated from fine strands at these two pHs. A lower magnitude of net charge at pH 4.0 likely results in easier aggregation to bigger aggregates corresponding to an opaque gel. The appearance of WP-pectin gels changed from translucent to opaque when pH increased from 2.0 to 3.5 (Fig. 2-3). In addition, gels at pH 1.5-3.0 became opaque at a WP:pectin mass ratio of 5:1 (Fig. 2-3), indicating formation of bigger structures with an increase of pectin content. In a previous study, the transition from

transparent to opaque appearance of gels was also observed after heating mixtures of β -lactoglobulin and dextran sulfate with a constant protein concentration and an increase of concentrations of dextran sulfate at neutral pH (Zhang & Foegeding, 2003). It was interpreted as a transition of gel structure from 'fine stranded' to 'particulate', which may also be the case of our WP-pectin system.

2.4.2.2. Gel formation at increased ionic strengths

Figs. 2-4 show the influence of ionic strength on gelation of mixtures with 6.3% w/v preheated WP and 0.63% w/v pectin. Addition of NaCl appeared to weaken the structure at pH 3.0 and 1.5, while gels were still observed at pH 1.0-3.0 after addition of 5 and 10 mM CaCl_2 . As presented previously, preheated WP-pectin mixtures can precipitate, form gels or remain fluidic depending on the extent of aggregation. NaCl decoupled complexes or facilitated the aggregation of WP and complexes by screening electrostatic attraction and inter-particle repulsion, respectively (Fig. 2-2). At pH 3.0, WP-pectin mixtures are less-charged than at lower pH. When electrostatic repulsion is screened by NaCl, intense aggregation occurs, corresponding to precipitation instead of gelation. Since pectin is less-charged at pH 1.5, the effects of NaCl on electrostatic attraction become visually observable. CaCl_2 also increases ionic strength, but divalent calcium ions can serve as cross-linkers for anionic low-methoxyl pectin to strengthen the network (Kohn, 1987), which may strengthen the gels at pH 1.0-3.0.

2.4.3. Microstructures of gels

Preheated WP-pectin (10:1) gels at pH 3.0 and 2.5 and preheated WP gels at pH 4.0 and 3.5 were studied in CLSM, with images shown in Fig. 2-5. Red areas represent protein matrices stained by Rhodamine B, and black areas are devoid of protein. Irregular structures with void areas were observed for preheated WP at pH 3.5 and 4.0 (Fig. 2-5A and 2-5B). In previous CLSM studies, preheated WP acidified using GDL generally had a uniform structure (Alting et al., 2003; Zhang et al., 2014), which is a result from homogeneous

distribution of GDL and slow acidification without descriptive forces. In our study, gels were prepared by fast acidification with agitation, which results in an instantaneous regional decrease of the pH (Alting et al., 2004) and heterogeneous structures that can be disrupted and reformed.

Irregular structures were also observed in preheated WP-pectin gel at pH 2.5 (Fig. 2-5C) and 3.0 (Fig. 2-5D). Compared to preheated WP only samples (Fig. 2-5A and 2-5B), the preheated WP-pectin mixture at pH 3.0 appeared to have formed denser and smaller aggregates (Fig. 2-5C). At pH 2.5 (Fig. 2-5D), both islands and small aggregates were observed, with void space throughout the sample. In a previous study, phase separation was observed in CLSM images of WP/polysaccharide gels with a protein continuous network and discontinuous serum phase, and sharp phase boundaries were observed at pH 4.8 and a charge density of below 0.7 for polysaccharides of guar gum, gellan gum, xanthan gum, high methoxyl pectin, and k-carrageenan (de Jong & van de Velde, 2007). In contrast, phase separation was not observed for low methoxyl pectin with an esterification degree of 37% (de Jong & van de Velde, 2007). Pectin used in the present study had an esterification degree of 32%, and no macroscopic phase separation was observed.

2.4.4. Rheological properties of gels

Rheological properties were used to investigate gel structures and formation mechanisms. Variations of the storage modulus (G') as a function of time and temperature are shown in Fig. 2-6 and 2-7. The initial G' of preheated WP at pH 3.5 (Fig. 2-6A) was much lower than that of the gel at pH 4.0 (Fig. 2-6B), which agrees with the higher hardness of WP gels at a pH closer to 5 (Cavallieri et al., 2007). The G' of preheated WP at pH 4.0 after incubating for 60 min was about 450 Pa (Fig. 2-6B), while the G' of GDL-induced preheated WP gels with a final pH of 4.0 after incubating for 60 min was about 4700 Pa. The difference can also be explained by differences in acidification conditions. The slow acidification using GDL favors formation of disulfide bonds because

sulfhydryl groups are more reactive at a higher pH (Monahan et al., 1995) which allows a longer time during GDL-acidification. Apart from disulfide bonds, fast acidification with agitation in the present study can break-up physical bonds to lower G' .

To investigate the interactions in gels, NEM or urea was added to inhibit formation of disulfide bonds (Alting et al., 2003) or hydrophobic attraction and hydrogen bonds (Lapanje, 1978), respectively. Preheated WP gels at both pH 4.0 and 3.5 became fluid after addition of urea, showing low G' during time or temperature sweep tests (Fig. 2-6), indicating hydrogen bonds and hydrophobic attraction are indispensable for gel network formation. On the other hand, the addition of NEM lowered G' of gels at pH 3.5 and 4.0 during time sweep at 37 °C and heating from 37 to 80 °C (Fig. 2-6). The phenomenon indicates disulfide bonds were formed at pH 3.5 and 4.0 and contributed to the formation of gel networks.

Unlike preheated WP gels, the opaque preheated WP-pectin gel at pH 3.0 (Fig. 2-7A) had a lower G' than the gel at pH 2.5 (Fig. 2-7B). This phenomenon confirms that moderate aggregation favors the gelation of preheated WP-pectin mixtures. For both gels at pH 2.5 (Fig. 2-7A and 2-7C) and 3.0 (Fig. 2-7B and 2-7D), NEM had a smaller influence on G' during time sweeps and temperature ramps than the treatments without pectin (Fig. 2-6), indicating disulfide bonds are insignificant for preheated WP-pectin gels. The sulfhydryl groups are less active at a lower pH (Frank J Monahan et al., 1995), and the coverage of pectin on the surface of WP may hinder formation of disulfide bonds. Preheated WP-pectin gels at pH 2.5 and 3.0 were not destroyed by urea (Fig. 2-7), suggesting other interactions are critical in formation of gel networks of preheated WP-pectin mixture. Because hydrophobic attraction and intermolecular disulfide bonds are not significant to gel formation, and electrostatic attraction between preheated WP and pectin is significant to gelation, which is supported by the loss of gelation properties at pH 3.0 after addition of a sufficient amount (100 mM) of NaCl (Fig. 2-4).

2.4.5. Dilution stability of gels

After initial gelation under gastric conditions, gels could be diluted by drinks such as water. Therefore, it is necessary to study the ability of structures after dilution. All preheated WP and preheated WP-pectin (10:1) samples with a protein concentration of 6.3% w/v at pH 1.0-4.0 were dissolved in water (data not shown). Disulfide bonds play a major role in formation of insoluble WP gels (Xin, Chen, & Özkan, 2002). Disulfide bonds in WP gels are formed during heating native WP (Xin et al., 2002) or GDL-acidification of preheated WP (Alting et al., 2003). In our study, samples were prepared from preheated WP by fast acidification, and the formation of disulfide bonds was limited, leading to the quick dissociation after dilution in water.

When preheated WP and preheated WP-pectin (10:1) with 10 mM CaCl_2 were mixed with water (Fig. 9), samples without pectin were also dissolved after mixing with water, while those with pectin at pH 2.0 and 2.5 had undissolved pieces. Therefore, calcium bridges between negatively charged carboxylate groups of pectin strengthened the structures to enable some stability against dilution in water.

2.5. Conclusions

Gelation properties of preheated WP were affected by pectin at gastric pH after fast acidification with agitation. Fast-acidified WP gels had different structures and properties from the GDL-induced gels reported in the literature. Intense agitation reduced the strength and homogeneity of WP gels, and fast acidification limited formation of disulfide bonds. Pectin neutralized positive charges of WP, induced gelation at pH 1.5-3.0, and changed properties of preheated WP gels. At a pH around 3.5 and 4.0, preheated WP formed a gel while preheated WP-pectin did not. Hydrophobic attraction and disulfide bonds were important to the gelation of preheated WP at pH 3.5-4.0, while electrostatic attraction was important to the gelation of preheated WP-pectin mixture. Bridging pectin by divalent calcium ions further changed the gel

properties, and enabled the partial stabilization of network formed at pH 2.0 and 2.5 after dilution in water. This research proves that preheated WP and pectin mixtures have promising properties for satiety application.

References

- Alting, A. C., de Jongh, H. H. J., Visschers, R. W., & Simons, J.-W. F. A. (2002). Physical and chemical interactions in cold gelation of food proteins. *Journal of Agricultural and Food Chemistry*, 50(16), 4682–4689.
- Alting, A. C., Hamer, R. J., de Kruif, C. G., & Visschers, R. W. (2003). Cold-set globular protein gels: interactions, structure and rheology as a function of protein concentration. *Journal of Agricultural and Food Chemistry*, 51, 3150–3156.
- Alting, A. C., Van Der Meulena, E. T., Hugenholtz, J., & Visschers, R. W. (2004). Control of texture of cold-set gels through programmed bacterial acidification. *International Dairy Journal*, 14(4), 323–329.
- Alting, A. C., Weijers, M., de Hoog, E. H. A., van de Pijpekamp, A. M., Cohen Stuart, M. A., Hamer, R. J., Visschers, R. W. (2004). Acid-induced cold gelation of globular proteins: effects of protein aggregate characteristics and disulfide bonding on rheological properties. *Journal of Agricultural and Food Chemistry*, 52(3), 623–631.
- Bryant, C. M., & McClements, D. J. (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. *Trends in Food Science & Technology*, 9(4), 143–151.
- Cavallieri, A. L. F., Costa-Netto, A. P., Menossi, M., & Da Cunha, R. L. (2007). Whey protein interactions in acidic cold-set gels at different pH values. *Le Lait*, 87(6), 535–554.
- De Jong, S., Klok, H. J., & van de Velde, F. (2009). The mechanism behind microstructure formation in mixed whey protein–polysaccharide cold-set gels. *Food Hydrocolloids*, 23(3), 755–764.
- De Jong, S., & van de Velde, F. (2007). Charge density of polysaccharide controls microstructure and large deformation properties of mixed gels. *Food Hydrocolloids*, 21(7), 1172–1187.

- Eissa, A. S., & Khan, S. A. (2005). Acid-induced gelation of enzymatically modified, preheated whey protein. *Journal of Agricultural and Food Chemistry*, 53(12), 5010–5017.
- Finkelstein, E. A., Trogon, J. G., Cohen, J. W., & Dietz, W. (2009). Annual medical spending attributable to obesity: payer-and service-specific estimates. *Health Affairs*, 28(5), w822–w831.
- Fizman, S., & Varela, P. (2013). The role of gums in satiety/satiation. A review. *Food Hydrocolloids*, 32(1), 147–154.
- Holvik, H., & Høiland, H. (1977). Acidity constants of galacturonic and glucuronic acids in water at 298.15 K from conductance and emf measurements. *The Journal of Chemical Thermodynamics*, 9(4), 345–348.
- Ikeda, S., & Morris, V. J. (2002). Fine-stranded and particulate aggregates of heat-denatured whey protein visualized by atomic force microscopy. *Biomacromolecules*, 3(2), 382–389.
- Jones, O. G., Decker, E. a., & McClements, D. J. (2009). Formation of biopolymer particles by thermal treatment of β -lactoglobulin–pectin complexes. *Food Hydrocolloids*, 23(5), 1312–1321.
- Jones, O. G., Lesmes, U., Dubin, P., & McClements, D. J. (2010). Effect of polysaccharide charge on formation and properties of biopolymer nanoparticles created by heat treatment of β -lactoglobulin-pectin complexes. *Food Hydrocolloids*, 24(4), 374–383.
- Jones, O. G., & McClements, D. J. (2011). Recent progress in biopolymer nanoparticle and microparticle formation by heat-treating electrostatic protein--polysaccharide complexes. *Advances in Colloid and Interface Science*, 167(1), 49–62.
- Ju, Z. Y., & Kilara, A. (1998). Aggregation induced by calcium chloride and subsequent thermal gelation of whey protein isolate. *Journal of Dairy Science*, 81(4), 925–931.

- Ju, Z. Y., & Kilara, A. (1998). Gelation of pH-aggregated whey protein isolate solution induced by heat, protease, calcium salt, and acidulant. *Journal of Agricultural and Food Chemistry*, 46(5), 1830–1835.
- Kallala, M., Jullien, R., & Cabane, B. (1992). Crossover from gelation to precipitation. *Journal de Physique II*, 2(1), 7–25.
- Kohn, R. (1987). Binding of divalent cations to oligomeric fragments of pectin. *Carbohydrate Research*, 160, 343–353.
- Langton, M., & Hermansson, A.-M. (1992). Fine-stranded and particulate gels of β -lactoglobulin and whey protein at varying pH. *Food Hydrocolloids*, 5(6), 523–539.
- Lapanje, S. (1978). Physicochemical aspects of protein denaturation.
- Marangoni, A. G., Barbut, S., McGauley, S. E., Marcone, M., & Narine, S. S. (2000). On the structure of particulate gels—the case of salt-induced cold gelation of heat-denatured whey protein isolate. *Food Hydrocolloids*, 14(1), 61–74.
- Mattioli, S., Pilotti, V., Felice, V., Lazzari, A., Loria, P., Tripodi, A., & Gozzetti, G. (1990). Ambulatory 24-hr pH monitoring of esophagus, fundus, and antrum. *Digestive Diseases and Sciences*, 35(8), 929–938.
- Mleko, S., & Foegeding, E. A. (2000). pH induced aggregation and weak gel formation of whey protein polymers. *Journal of Food Science*, 65(1), 139–143.
- Monahan, F. J., German, J. B., & Kinsella, J. E. (1995). Effect of pH and temperature on protein unfolding and sulfhydryl/disulfide interchange reactions during heat-induced gelation of whey protein. *Journal of Agricultural and Food Chemistry*, 43(1), 46–52.
- Norton, I., Moore, S., & Fryer, P. (2007). Understanding food structuring and breakdown: engineering approaches to obesity. *Obesity Reviews*, 8(s1), 83–88.
- Norton, I. T., & Frith, W. J. (2001). Microstructure design in mixed biopolymer composites. *Food Hydrocolloids*, 15(4), 543–553.

- Ogden, C. L., Carroll, M. D., Kit, B. K., & Flegal, K. M. (2014). Prevalence of childhood and adult obesity in the United States, 2011-2012. *JAMA*, 311(8), 806–814.
- Salcedo, R., Wasserman, K., Young, H. A., Grimm, M. C., Howard, O. Z., Anver, M. R., & Oppenheim, J. J. (1999). Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1 α . *The American journal of pathology*, 154(4), 1125-1135.
- Solah, V. A., Kerr, D. A., Adikara, C. D., Meng, X., Binns, C. W., Zhu, K., Prince, R. L. (2010). Differences in satiety effects of alginate-and whey protein-based foods. *Appetite*, 54(3), 485–491.
- Stading, M., Langton, M., & Hermansson, A.-M. (1993). Microstructure and rheological behaviour of particulate β -lactoglobulin gels. *Food Hydrocolloids*, 7(3), 195–212.
- Weinbreck, F. C. J. (2004). Whey-protein/polysaccharide coacervates: structure and dynamics.
- Xin, H., Chen, X. D., & Özkan, N. (2002). Whey protein - based gel as a model material for studying initial cleaning mechanisms of milk fouling. *Journal of Food Science*, 67(7), 2702–2711.
- Zhang, G., & Foegeding, E. A. (2003). Heat-induced phase behavior of β -lactoglobulin/polysaccharide mixtures. *Food Hydrocolloids*, 17(6), 785–792.
- Zhang, S., Hsieh, F. H., & Vardhanabhuti, B. (2014). Acid-induced gelation properties of heated whey protein-pectin soluble complex (Part I): Effect of initial pH. *Food Hydrocolloids*, 36, 76–84.
- Zhang, S., & Vardhanabhuti, B. (2014a). Acid-induced gelation properties of heated whey protein– pectin soluble complex (Part II): Effect of charge density of pectin. *Food Hydrocolloids*, 39, 95–103.

Zhang, S., & Vardhanabhuti, B. (2014b). Intragastric gelation of whey protein–pectin alters the digestibility of whey protein during in vitro pepsin digestion. *Food & Function*, 5(1), 102–110.

Appendix

Figure 2-1. Absorbance at 600 nm (Abs_{600}) of (A) dispersions with 0.45% w/v pectin, 0.45% w/v native or preheated WP, and their mixtures at a WP:pectin mass ratio of 10:1; (B) dispersions with 0.45% preheated WP and pectin at WP:pectin mass ratios of 1:0, 20:1, 10:1, and 5:1. Error bars are standard deviations ($n=4$).

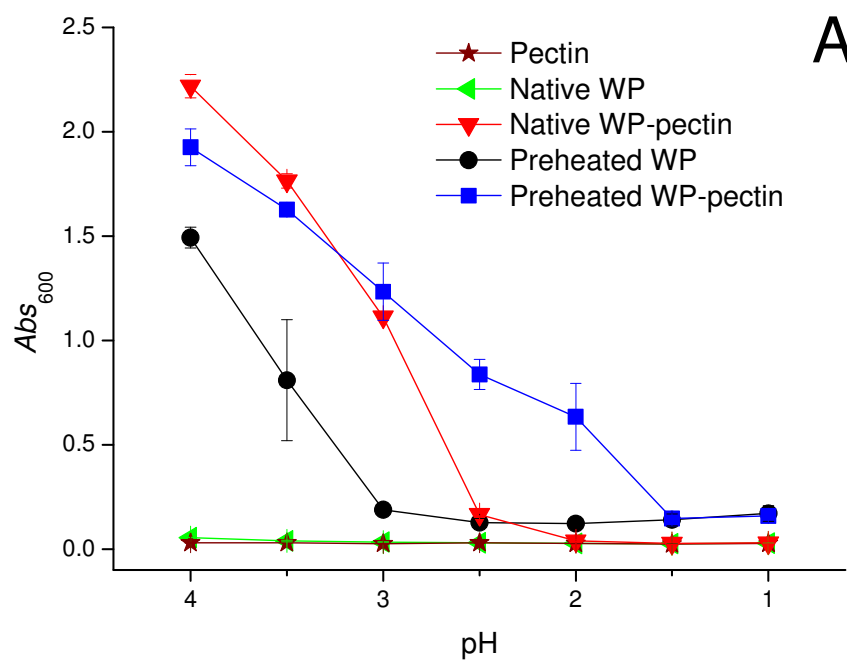


Figure 2-1 continued

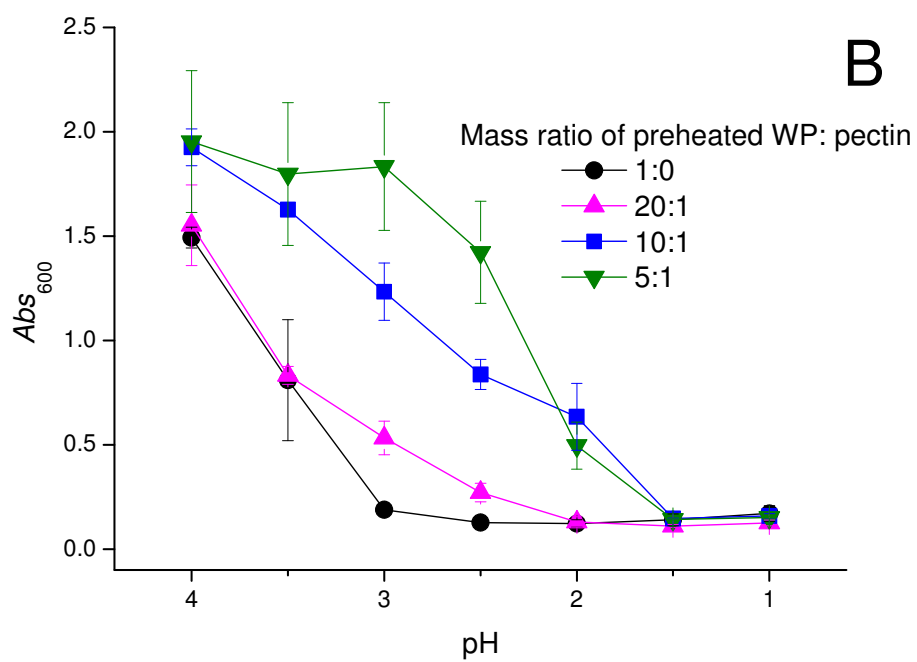


Figure 2-1 continued

Figure 2-2. Abs₆₀₀ of dispersions of (A) native WP and pectin, (B) preheated WP, and (C) preheated WP and pectin with various concentrations of NaCl or CaCl₂. WP content was 0.45% w/v in all treatments, and the WP:pectin mass ratio was 10:1. Error bars are standard deviations (n=2).

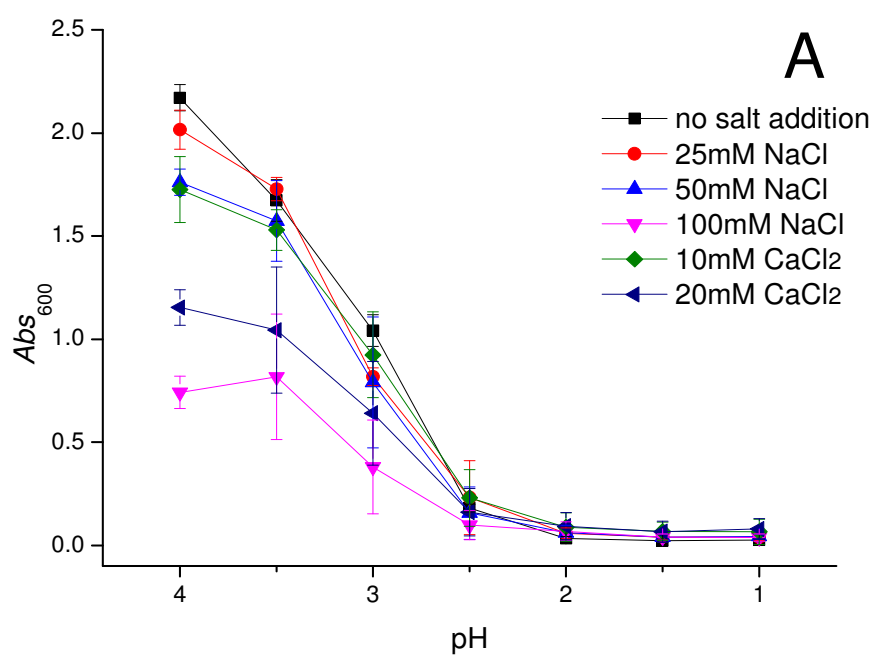


Figure 2-2 continued

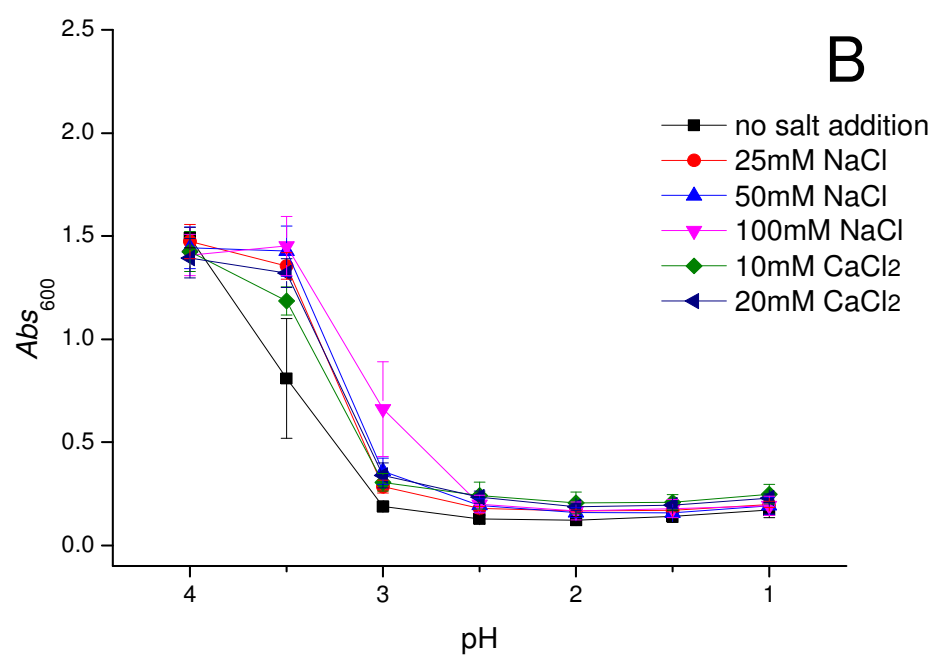


Figure 2-2 continued

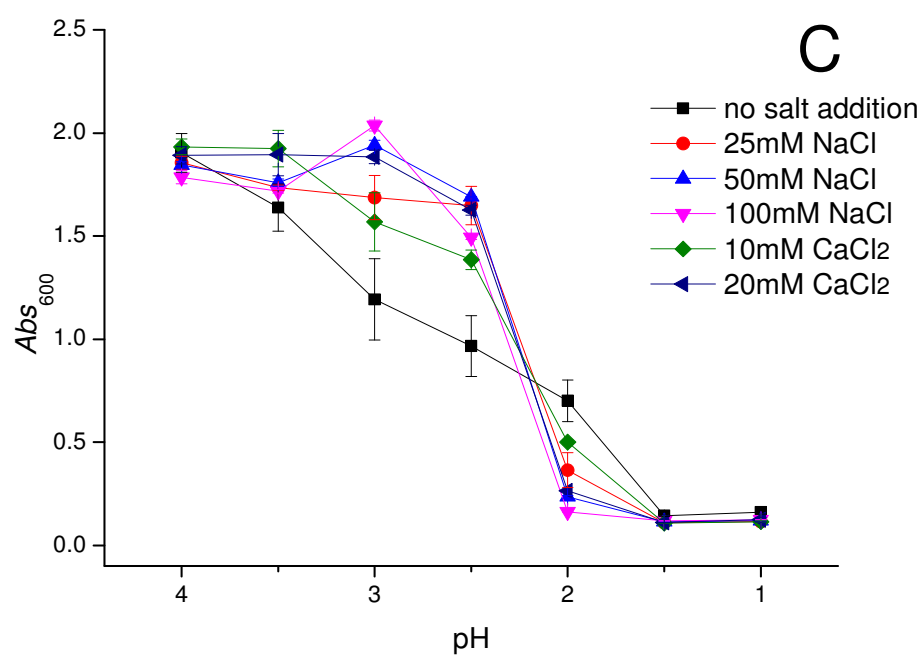


Figure 2-2 continued

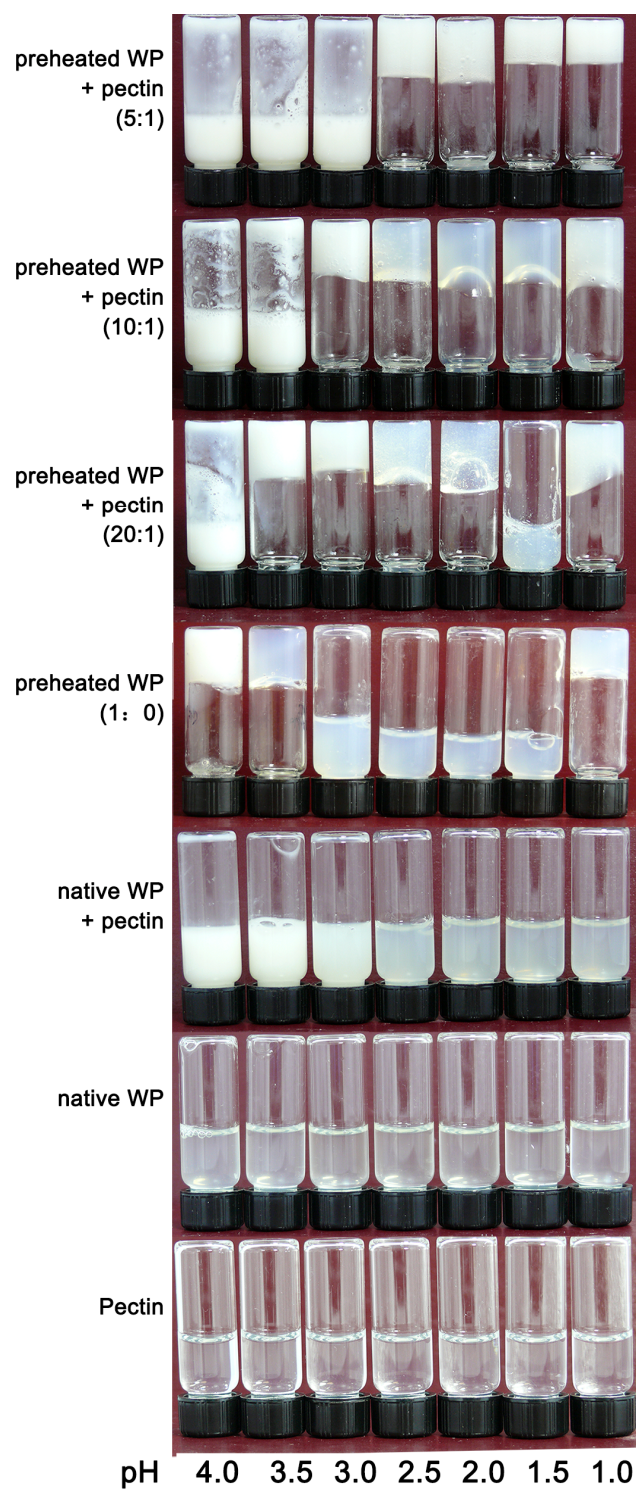


Figure 2-3. Photographs of samples with pectin (0.63% w/v), native or preheated WP, and their mixtures at different WP to pectin mass ratios. WP content was 6.3% w/v in all treatments.

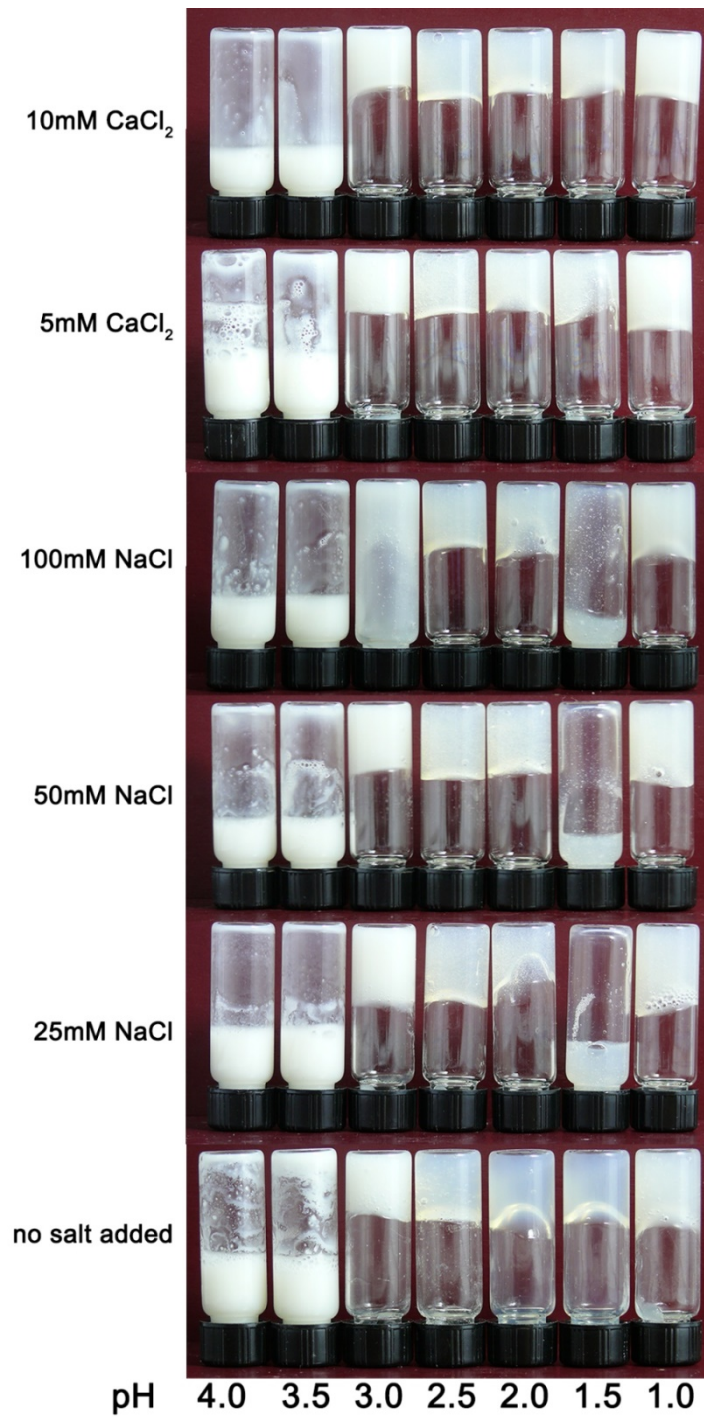


Figure 2-4. Photographs of mixtures with 6.3% w/v preheated WP and 0.63% w/v pectin supplemented with various concentrations of NaCl or CaCl_2 .

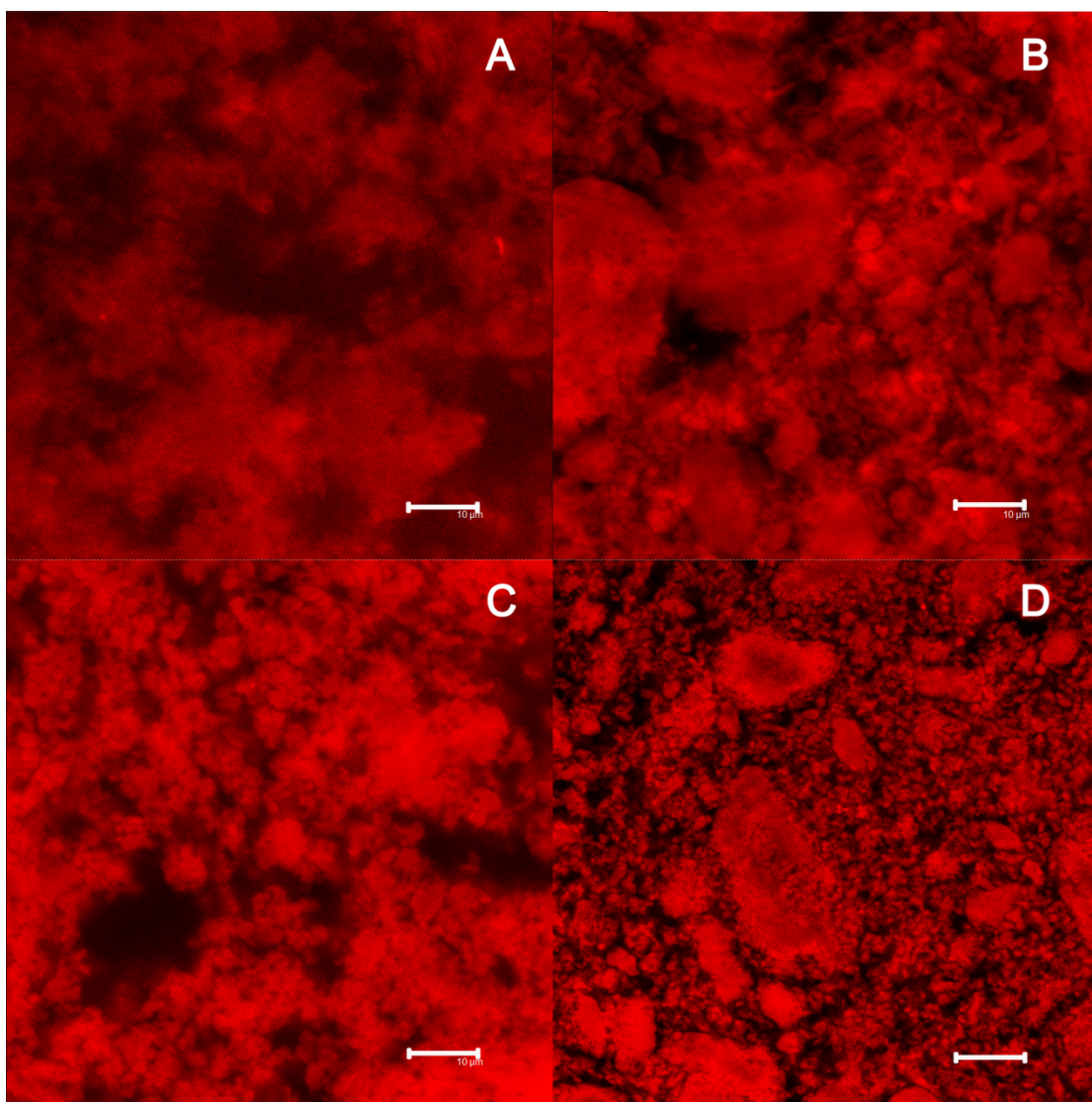


Figure 2-5. CLSM images of (A) preheated WP at pH 3.5, (B) preheated WP at pH 4.0, (C) preheated WP and pectin at pH 2.5, and (D) preheated WP and pectin at pH 3.0. WP (stained by red Rhodamine B) and pectin concentrations were 6.3% and 0.63% w/v, respectively. Bar = 10 μm .

Figure 2-6. Storage modulus of samples with 6.3% w/v preheated WP adjusted to pH 3.5 (A and C) or 4.0 (B and D) during time sweep (A and B) at 37 °C or temperature ramp (C and D) from 37-80 °C at 5 °C/min. Samples were also added with 10% w/v urea or 5 mM NEM. Two parallel tests were performed and representative curves are shown.

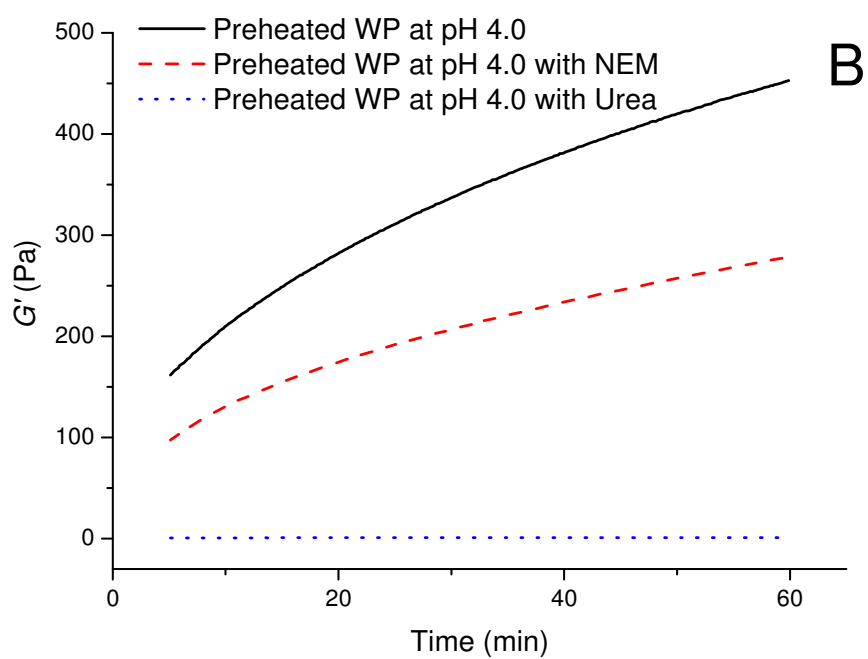
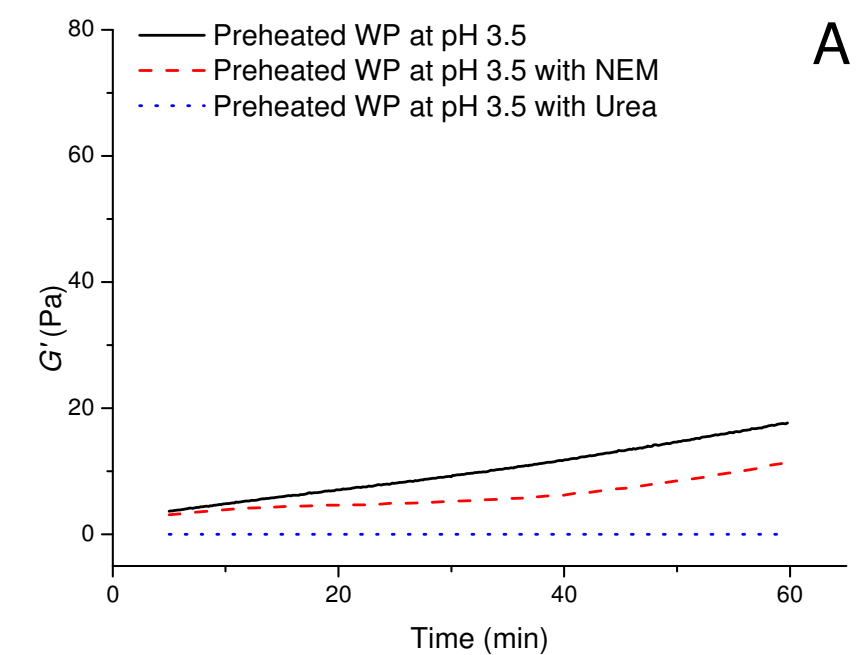


Figure 2-6 continued

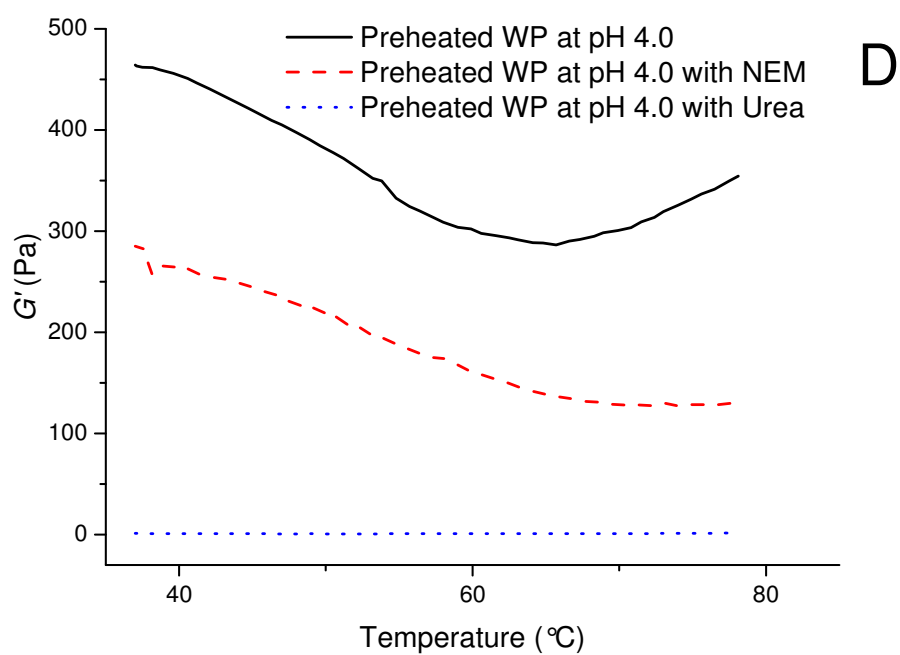
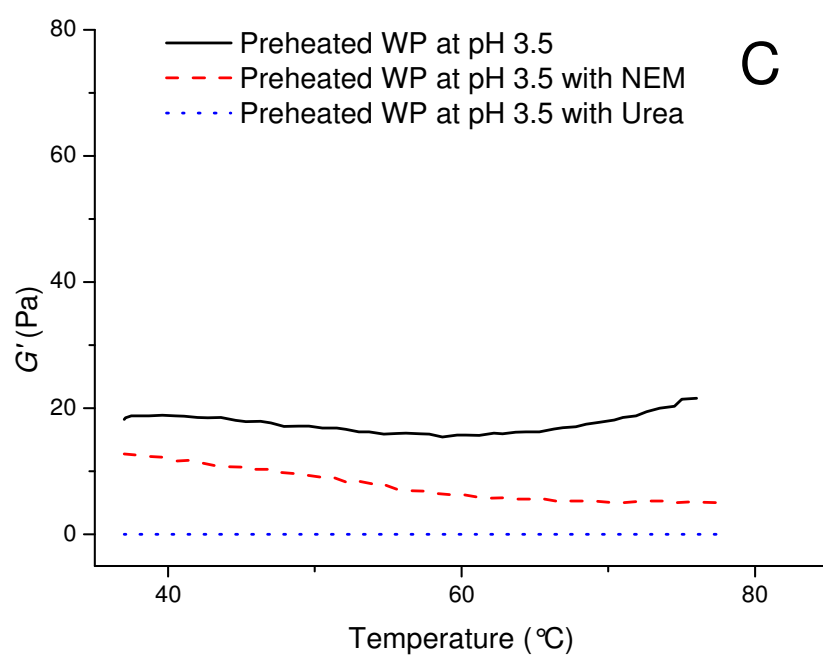


Figure 2-6 continued

Figure 2-7. Storage modulus of mixtures with 6.3% w/v preheated WP and 0.63% w/v pectin adjusted to pH 3.5 (A and C) or 4.0 (B and D) during time sweep at 37 °C (A and B) or temperature ramp from 37-80 °C at 5 °C/min (C and D). Samples were added with 10% w/v urea or 5 mM NEM. Two parallel tests were performed and representative curves are shown.

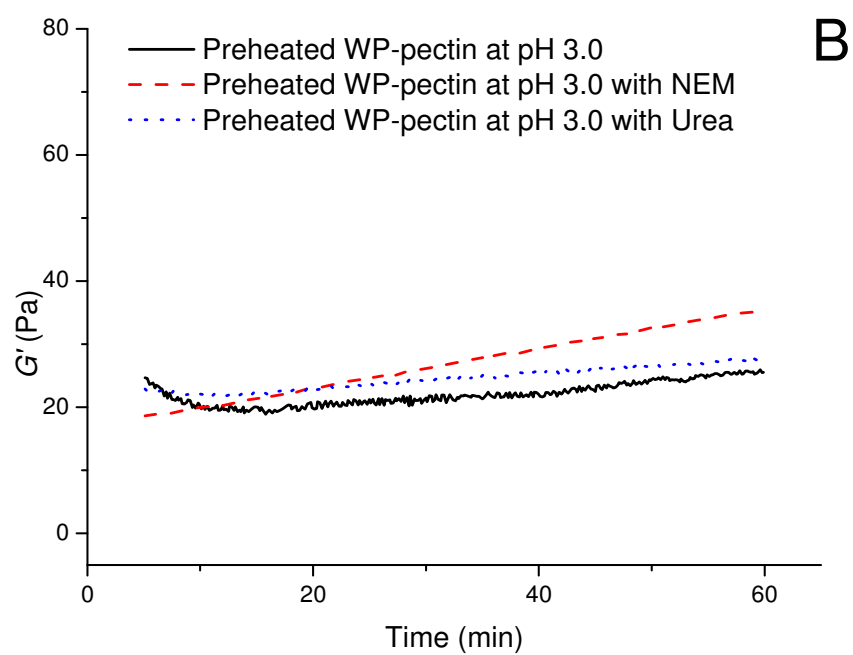
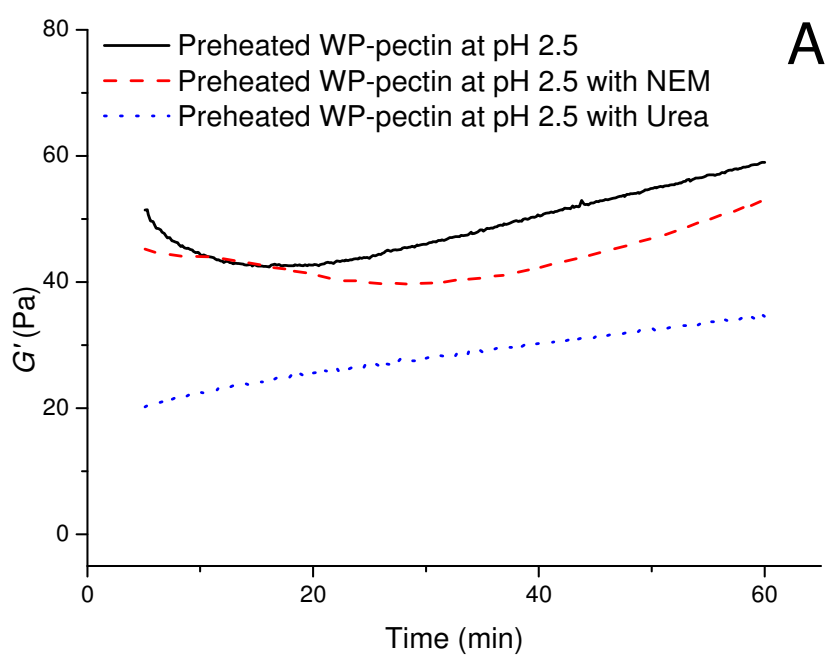


Figure 2-7 continued

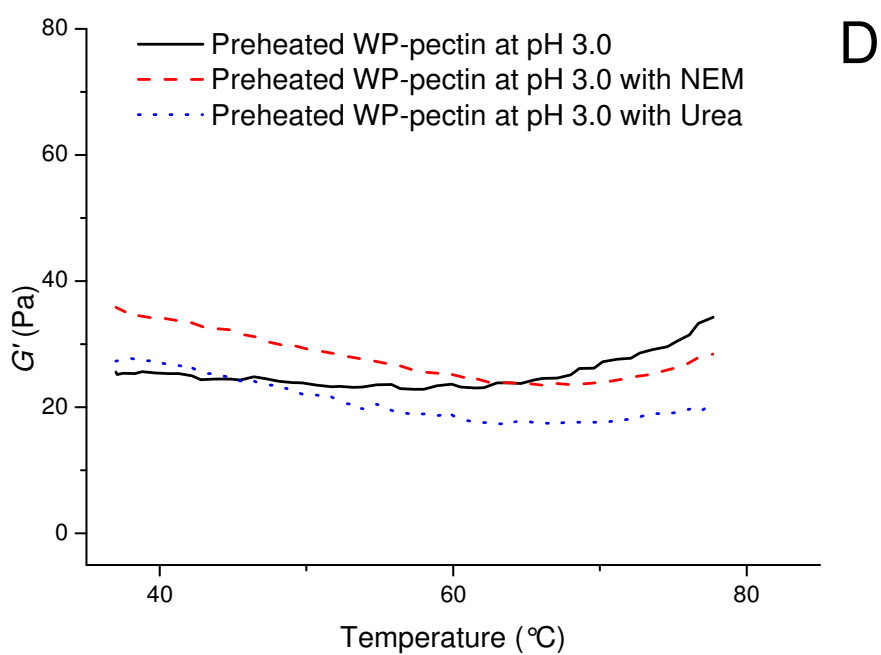
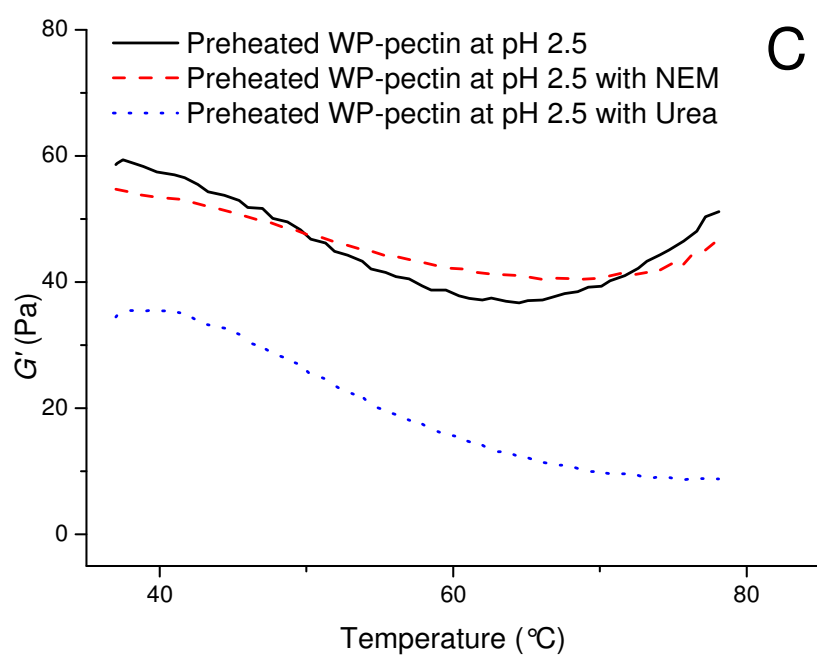


Figure 2-7 continued

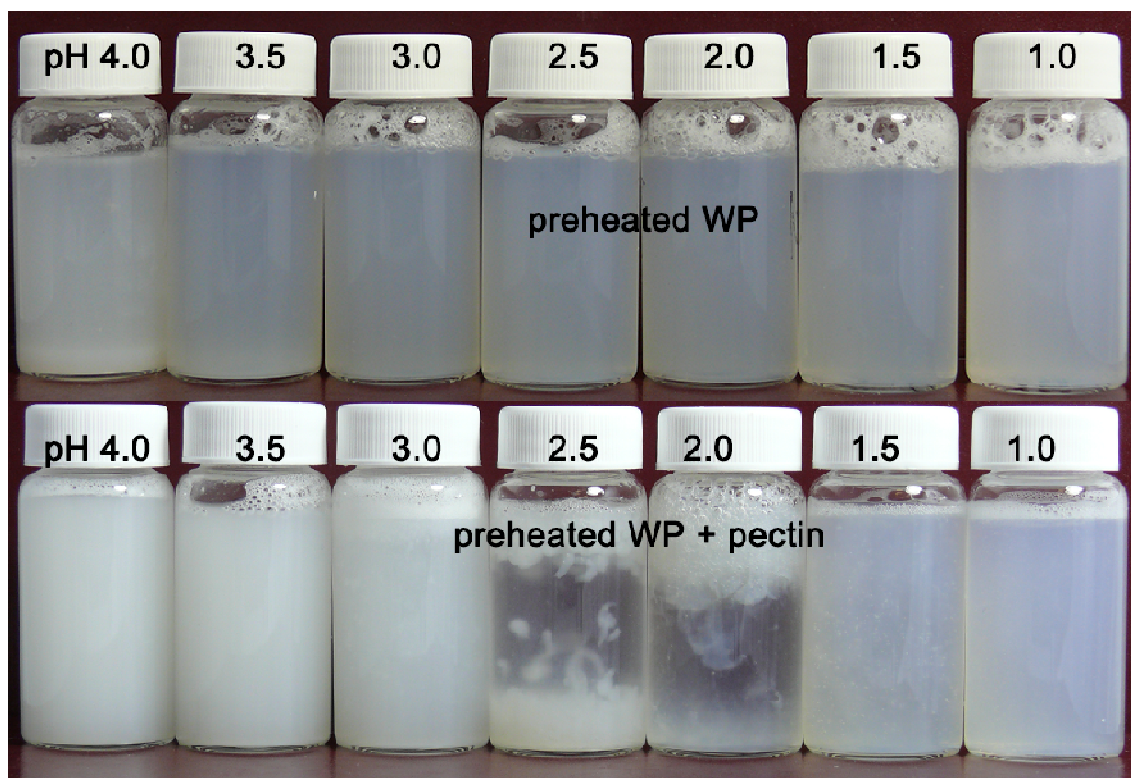


Figure 2-8. Photographs of 5 mL 6.3% w/v preheated WP (top) and 6.3% w/v preheated WP and 0.63% w/v pectin mixtures (bottom) with 10mM CaCl_2 , after mixing with 15 mL deionized water.

Chapter 3. Sodium Stearoyl Lactylate Improves Thermal Stability of Whey Protein at pH 5.5-6.0

3.1. Abstract

Whey protein (WP) is preferred in manufacturing protein beverages. However, thermal sterilization and pasteurization in beverage manufacturing cause protein aggregation to increase sample turbidity and even form gels. The objective of this chapter was to improve thermal stability of WP by a combination of preheating treatment and binding with sodium stearyl lactylate (SSL). With an increase in SSL content from 0 to 1.0%, thermal stability of native WP at pH 5.5-6.0 was improved. Particle size and zeta-potential results indicated the promoted binding between SSL and WP after heating. Circular dichroism spectroscopy indicated that preheated WP with a more ordered secondary structure was formed by SSL. Preheated WP had higher a magnitude of zeta-potential, and reduced denaturation, exposure of sulfhydryl groups, and surface hydrophobicity by preheating WP with SSL. These properties may enhance electrostatic repulsion and reduce hydrophobic attraction and formation of disulfide bonds during subsequent heating at pH 5.5-6.0. As a result, thermal stability of preheated WP was improved by SSL that transparent fluid containing 4.5% WP and 0.2% SSL was obtained at pH 5.5. In addition, preheating conditions impacted thermal stability of preheated WP with SSL.

Keywords: Sodium stearyl lactylate, whey protein, thermal stability, denaturation

3.2. Introduction

Ready-to-drink beverages offer advantages of convenience and portability to fast-paced consumers (Burrington, 2012). Whey protein (WP) ingredients are preferred in protein beverages because of high contents of essential amino acids, bland flavor, digestibility, and health benefits (Kinsella & Morr, 1984). However, thermal sterilization and pasteurization during beverage manufacturing cause protein aggregation to increase sample turbidity and even form gels, especially around pH 5.2 which is the isoelectric point of WP (Bryant et al., 1998). Thermal aggregation of WP mainly results from hydrophobic attraction and formation of intramolecular disulfide bonds (Nicolai et al., 2011).

Preheating WP is a method to improve thermal stability of WP at neutral acidity (Ryan et al 2012). After preheating at neutral acidity, soluble WP aggregates are formed and are more stable during the second heating than heating native WP directly. Thermal stability of WP can be further improved by crosslinking preheated WP with transglutaminase (Wang et al., 2012; Zhong, Wang, Hu, & Ikeda, 2013). Preheating WP in individual solution droplets dispersed in nanometer-sized water-in-oil microemulsions was studied to form nanoparticles with improved stability after heating at pH 6.8 and 100 mM NaCl (Zhang & Zhong, 2010).

Binding surfactants on WP is another method to improve thermal stability of WP. As an anionic surfactant, sodium dodecyl sulfate (SDS) binds with protein through hydrophobic attraction between the alkyl chain of SDS and hydrophobic groups of proteins and electrostatic attraction between anionic head group of SDS and positively charged amino acid residues of proteins (Jones, 1975; Zaidi, Nusrat, Zaidi, & Khan, 2014). The relative importance of hydrophobic and electrostatic contributions to binding, and the exact mechanism by which SDS induces conformational changes of proteins are not completely understood despite extensive studies (Goddard & Ananthapadmanabhan, 1993; Jones, 1992). SDS, sodium stearoyl lactylate

(SSL), diacetyl tartaric acid ester of monoglyceride, lysophosphatidylcholine, Tween 20/80, Brij 78 and sodium laurate inhibited thermal denaturation of native WP at pH 7.05 or 6.55 (Giroux & Britten, 2004; Le et al., 2011). However, interactions between surfactants and preheated WP and the subsequent impacts on thermal stability of preheated WP have not been studied. We hypothesize that unfolding WP during preheating facilitates the binding with surfactants on amino acid patches initially embedded at the native state and the enhanced binding with surfactants improves thermal stability of preheated WP.

Stearoyl-lactylates are a group of anionic surfactants that can bind proteins by hydrophobic attraction and ionic bonds similar to other anionic surfactants (Whitehurst, 2008). Both sodium and calcium salts of stearoyl lactylates are commonly used in the food industry. Because SSL has a higher solubility in water than the calcium ones (Whitehurst, 2008), it was chosen in this study. The calculated hydrophile-lipophile balance (HLB) value of SSL is 21, but its experimentally determined HLB value is close to 12 (Bortnowska et al., 2015; Giroux & Britten, 2004; Miller et al., 2000). SSL has a pKa of about 3.5 (FoodDB, n.d.), indicating most lactyl groups are ionized at pH 5.25-6.0. Commercial SSL typically contains about 50% stearoyl-1-lactylate, 20% stearoyl-2-lactylate, 5% stearoyl-3-lactylate and trace amounts of stearoyl-4-lactylate (Whitehurst, 2008). SSL is an FDA-approved food additive, and its first application was to improve bread quality (Thompson & Buddemeyer, 1954). Nowadays, SSL is extensively applied in major food applications including bakery, pet foods and emulsions (Whitehurst, 2008).

In this chapter, mixtures of WP and SSL were heated to test thermal stability at pH 5.25-6.0. Fluorescence spectroscopy, circular dichroism (CD) spectroscopy, particle size, zeta-potential, sulfhydryl group contents, and surface hydrophobicity of mixtures of WP and SSL were characterized to study molecular interactions between SSL and WP, and the influence of binding on aggregation and conformation of WP.

3.3. Materials and methods

3.3.1. Materials

WP isolate (WPI) was obtained from Hilmar Ingredients, Inc (Hilmar, CA). The product contains 91.0% w/w protein, 0.2% w/w lactose, 0.2% w/w fat, 4.0% w/w moisture, and 1.0% w/w ash according to the manufacturer. SSL was purchased from Modernist Pantry (York, ME). Ellman's reagent (5,5'-Dithiobis-(2-nitrobenzoic acid)) was purchased from Acros Organic (Morris Plains, NJ). Unless otherwise stated, all chemicals were of the analytical grade.

3.3.2. Sample preparation

Fifty grams of WPI powder was dissolved in 500 mL deionized water and adjusted to a total volume of 550 mL and a protein concentration of about 9%. After adjusting to pH 7.0 and hydration overnight at 4 °C, the obtained protein stock solution was used in later studies. Two grams of SSL powder was mixed with 100 mL of 0.04 M NaOH, stirred on a plate and heated in a water bath at 90 °C until it became translucent. Protein solution was mixed with SSL dispersion to form mixtures containing 4.5% w/v WP and 0, 0.05, 0.1, 0.2, 0.5 and 1% w/v SSL. To test thermal stability, these mixtures were adjusted to pH 5.25-6.0 using 1.0 M HCl and heated at 95°C for 1 min in a water bath.

In another set of samples, dispersions with 4.5% w/v WP with or without 0.2% w/v SSL were adjusted to pH 7.0 using 1.0 M HCl and heated at 80-95 °C for 20 min to produce preheated WP (dWP) and dWP+SSL, respectively. The dWP dispersion was also mixed with 0.2% w/v SSL, denoted as dWP-SSL hereafter to distinguish from the dWP+SSL treatment with both preheated together. Treatments with dWP, dWP+SSL, and dWP-SSL were formulated with a WP concentration of 4.5% w/v and a SSL concentration of 0.2% w/v and adjusted to pH 5.25-6.0 using 1.0 M HCl for heat stability test at 95°C for 1 min.

3.3.3. Analysis of particle size and zeta-potential

Particle sizes and zeta-potentials of dispersions were measured using a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK) at a WP

concentration of 5 mg/mL. All measurements were carried out at 21 °C.

3.3.4. Circular dichroism (CD) spectroscopy

The instrument used in the far-UV CD spectroscopy was an AVIV model 202 CD spectrometer (AVIV Instrument, Inc., Lakewood, NJ). Samples were diluted to a WP concentration of 1 mg/mL with deionized water, before loading to a quartz cuvette with a path length of 0.2 cm. The spectra were collected at 190–250 nm using a scanning interval of 1 nm and a rate of 60 nm/min.

3.3.5. Intrinsic fluorescence intensity

The fluorescence spectra were recorded using an RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). Each sample was diluted to 0.15% w/v WP using a 10 mM phosphate buffer at pH 7.0. The excitation wavelength was 285 nm. Both the excitation and emission slit widths were set at 10 nm. The emission spectra were recorded between 300 and 450 nm, with the background fluorescence calibrated using 10 mM phosphate buffer.

3.3.6. Determination of sulfhydryl group (-SH) contents

The –SH group content of WP samples was determined by the method of Shimada and Cheftel (1989) using the Ellman's reagent (Ellman, 1959). Each sample was diluted to 0.09% w/v WP using a 10 mM phosphate buffer at pH 8.0 or a buffer containing 0.086 M Tris, 0.09 M glycine, 4 mM ethylenediaminetetraacetic acid, and 8 M urea at pH 8.0. One mL of the diluted sample was added with 0.01 mL of the Ellman's reagent (10 mM), vortexed for 5 s, and measured for absorbance at 412 nm using a UV–vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA, USA). The –SH content was calculated using a molar extinction coefficient of 13,600 /M-cm. The protein content of WPI in the label was used in calculations. The –SH content of samples diluted without urea was defined as surface -SH content that is reactive (Hongprabhas & Barbut, 1997) or accessible (Patrick & Swaisgood, 1976). The –SH content of samples diluted with 8 M urea was defined as the total -SH content. Tests were carried out with two independent replicates.

3.3.7. Surface hydrophobicity (H_0)

The H_0 of WP was determined using a fluorescence probe (1-anilino-8-naphthalenesulfonate, (ANS)) according to a literature method (Alizadeh-Pasdar & Li-Chan, 2000) with modification. Each sample was diluted to five concentrations from 0.03 to 0.09 mg/mL WP using a 10 mM phosphate buffer at pH 7.0. The ANS solution was prepared at 8 mM in the same buffer. Ten microliters of the ANS solution was added to 3 mL of each protein solution. The fluorescence intensity was measured at an excitation wavelength of 365 nm and an emission wavelength of 484 nm using the above spectrofluorometer. The background fluorescence was calibrated using the phosphate buffer. The initial slope of the fluorescence intensity–protein concentration plot after linear regression was treated as H_0 .

3.3.8. Statistical analysis

Statistical analyses were performed using the SAS Enterprise Guide program (version 6.1, SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using Tukey's test. The significance level was set at 0.05.

3.4. Results and discussion

3.4.1. Influence of SSL on thermal stability of native WP (nWP)

Mixtures with 4.5% w/v nWP and 0-1.0% w/v SSL at pH 5.25-6.0 after heating at 95 °C for 1 min are shown in Fig. 3-1. Without SSL, heating resulted in a translucent fluid at pH 6.0, opaque fluids at pH 5.5 and 5.75, and a gel at pH 5.25, as a result of the weakening electrostatic repulsion as pH being closer to the pI of WP (Nicolai et al., 2011). With 0.2% w/v SSL, the sample at pH 6.0 and 5.75 became clear after heating. With 1.0 % w/v SSL, the gelation at pH 5.5 was inhibited and a fluid sample was obtained. In addition, samples with 0.5-1.0% w/v SSL adjusted to pH 5.75-6.0 became turbid after heating. SSL is only slightly soluble in cold water (EFSA, 2013), and excess SSL can cause turbidity after heating and cooling. Therefore, SSL inhibited thermal

aggregation of nWP at pH 5.5-6.0.

3.4.2. Binding between SSL and nWP

The binding of SSL with nWP was studied at a series of SSL concentrations using particle size, zeta-potential, and CD spectroscopy. Diameters of the mixtures with various WP:SSL mass ratios are shown in Fig. 3-3. nWP and dWP dispersions (1:0 mass ratio) had particles smaller than 100 nm, while the SSL dispersion (0:1 mass ratio) had big particles (around 900 nm) that indicates the poor solubility of SSL at pH7 and 21 °C. The mixtures of nWP and SSL had particles with a size of 160-390 nm, while the mixtures of dWP-SSL had particles of 40-100 nm. These observations indicate that a greater extent of excess SSL in the mixtures of nWP and SSL than in dWP-SSL, resulting from the enhanced binding of SSL with dWP than nWP. Since most non-polar groups of β -Lg are located in the interior of the globular protein (De Wit, 1981), the exposure of hydrophobic groups favors the binding of SSL on dWP when compared with nWP. After heating the mixtures of nWP and SSL, particle size decreased at each mass ratio (Fig. 3-2), which agreed with the improvement of heat stability after adding SSL (Fig. 3-1). During heating, SSL aggregates dissociates to monomers (Bhuyan, 2010) that are now active in binding on non-polar groups of WP after thermal denaturation.

Zeta-potentials of the mixtures of WP and SSL at pH 7.0 are shown in Fig. 3-3. With an increase of SSL content, the zeta-potential magnitude of mixtures increased, as a result of ionization of lactyl groups of SSL. After heating mixtures of nWP and SSL, the magnitude of zeta-potential increased at each WP:SSL mass ratio, which confirmed the previous results about the enhanced binding of SSL on WP after heating.

The far-UV CD spectra of mixtures of nWP and SSL before and after heating at 90 °C and pH 7.0 for 20 min are presented in Fig 3-4. Significant dips at 217 nm were observed for all samples, which are an indicator of a β -type secondary structure of β -Lg (Kuwata et al., 1998). The magnitudes of the dips at 217nm are shown in Fig 3-5. With an increase in SSL content, there is no

significant change in the magnitude for the mixtures of nWP and SSL before heating, indicating the insignificant extent of binding. Conversely, the magnitude of samples after heating increased significantly with an increase in SSL content. The CD data indicate that binding of SSL on dWP during heating resulted in the formation of more ordered structures (Liu & Zhong, 2013).

3.4.3. Influence of SSL on properties of preheated WP (dWP)

Thermal aggregation of WP is mainly caused by hydrophobic attraction among non-polar groups of WP and formation of disulfide bonds (Nicolai et al., 2011). The influence of SSL on exposures of non-polar and –SH groups of dWP was studied in this section after preheating WP at 80-95 °C for 20 min. The concentration of SSL mixing with 4.5% w/v WP was fixed at 0.2% w/v, considering the limitation of SSL as a food additive (Department of health and human services, 2014) and its solubility. The intrinsic fluorescence intensities of nWP, dWP, and dWP+SSL are shown in Fig. 3-6. The fluorescence intensities of both dWP and dWP+SSL increased with an increase of preheating temperature, as a result of an increased extent of denaturation. The fluorescence intensities of dWP+SSL were significantly lower than dWP, especially after preheating at 80 °C. Intrinsic fluorescence of WP is used as an indicator of a degree of β -Lg unfolding (Creamer, 1995; Iametti et al., 1995). During unfolding, the Trp 19 located at the bottom of the central hydrophobic calyx of native β -lactoglobulin (β -Lg) is exposed and Trp-61 becomes more distant from a fluorescence quencher Cys-66-Cys-160 disulfide bridge, which increases fluorescence intensity (Cowgill, 1967; Creamer, 1995; Mills & Creamer, 1975; Papiz et al., 1986; Renard et al., 1998). This phenomenon indicates the dWP+SSL had a difference conformation from dWP, which was evident in CD spectra (Fig. 3-4,5). The binding of SSL on hydrophobic patches on or near fluofores (Trp) can also cause the reduction of fluorescence intensity.

The formation of disulfide bonds requires at least one exposed –SH. The major –SH source (cys121) of β -Lg is buried and not reactive at the native

status (Larsen et al., 1949; Livney et al., 2003). In this study, contents of surface and total –SH groups were defined as those reactive and accessible without and with the unfolding and dissociation of dWP by 8 M urea, respectively. As presented in Fig. 3-7, the total –SH content of nWP was about 37 nmol/mg protein that was not affected by the presence of SSL. The total –SH content nWP decreased significantly after preheating at a higher temperature from 80 to 95 °C, as a result of oxidation of –SH (Nicolai et al., 2011). The surface –SH content of nWP with and without SSL was undetectable. With the increase of preheating temperature from 80 to 95 °C, the surface –SH content of dWP increased from 25.4 to 28.6 nmol/mg protein, and that of dWP+SSL also increased from 15.0 to 23.5 nmol/mg protein. The dWP+SSL had a lower surface –SH content than dWP at each temperature, and the differences were significant at 80 and 85°C. This phenomenon confirmed the results of fluorescence intensity that the degree of denaturation increased with an increase in preheating temperature and the binding of SSL with dWP inhibited the denaturation.

Particle size distributions of SSL, dWP, dWP-SSL (mixing dWP with SSL), and dWP+SSL (preheating nWP and SSL together) prepared by preheating at 90 °C and pH7 for 20 min are shown in Fig 3-8A. The major peak of SSL was centered on about 1000 nm. dWP had a unimodal distribution with the peak center at about 40 nm. dWP-SSL and dWP+SSL also had a peak centered at about 40 nm representing the dWP and another peak centered at above 500 nm representing SSL aggregates. The diameter of the first peak in each distribution are summarized in Fig. 3-8B and were not significantly different among dWP, dWP-SSL and dWP+SSL after preheating at 80, 85 and 90 °C. This phenomenon indicates that SSL had an insignificant effect on the particle size of WP aggregates after interacting with WP either before or after preheating at 80-90 °C. After preheating at 95 °C, dWP had a significantly smaller dimension (53 nm) than the treatments of dWP-SSL (63 nm) and dWP+SSL (66 nm), which suggests the enhanced extent of denaturation

resulted in a sufficient number of SSL binding with WP to impact particle dimension.

H_0 is an important indicator of the quantity of exposed non-polar groups of protein available to bind with ANS. H_0 of dWP, dWP-SSL and dWP+SSL is shown in Fig. 3-9 after preheating at 80-95 °C. Because WP aggregates in these samples had comparable particle sizes and therefore surface areas to bind with ANS (Fig. 3-8B), H_0 of these samples can be compared. No significant difference in H_0 of dWP was observed after different preheating conditions, which can result from two competing phenomena. A greater extent of thermal denaturation at an increased temperature is expected to increase the extent of exposed hydrophobic amino acid residues, while the aggregation of WP via hydrophobic surfaces during preheating reduces the total sites available for binding with ANS (Ryan et al., 2013). The H_0 of dWP-SSL and dWP+SSL was significantly lower than that of dWP for preheating treatments of 80, 85 and 90 °C, indicating SSL binds with hydrophobic patches on the surface of dWP. There is no significance in H_0 of dWP-SSL and dWP+SSL, except for the 80 °C preheating treatment where SSL largely inhibited the unfolding of WP during preheating (Fig. 3-6 and Fig. 3-7).

3.4.4. Thermal stability of preheated WP-SSL

Thermal stability of dWP, dWP-SSL, and dWP+SSL adjusted to pH 5.25-6.0 was evaluated at 95 °C for 1 min (Fig. 3-10). After heating, dWP was a clear fluid at pH 6.0 and a slightly turbid fluid at pH 5.75, but formed gels at pH 5.25 and 5.5, which shows the improved thermal stability when compared to nWP (Fig. 3-1). The possible mechanisms of the improved thermal stability after preheating WP include the increased surface charge, slowed aggregation kinetics (aggregates vs. monomers), and formation of disulfide bonds within aggregates (Ryan et al., 2012; Schmitt et al., 2007). The dWP-SSL was a clear fluid at pH 5.75 and 6.0, a turbid and opaque fluid or gel at pH 5.5 depending on preheating temperature, but formed gels at pH 5.25. This observation indicates the improvement of thermal stability of dWP by SSL at pH 5.5, which

can be a result from a higher magnitude of zeta-potential (Fig. 3-3) and lower H_0 (Fig. 3-9). The dWP+SSL was also a clear fluid at pH 5.75-6.0, and a transparent or opaque fluid at pH 5.5. The improved heat stability of dWP+SSL than d-WP-SSL at pH 5.5 may be attributed to the promoted binding during preheating the mixture of WP and SSL (Fig. 3-2 and Fig. 3-3) that, weakened hydrophobic attraction after preheating at 80 °C (Fig. 3-9), enhanced the electrostatic repulsion (Fig. 3-3), lowered the exposure of reactive groups (Fig. 3-6), and inhibited the formation of disulfide bonds (Fig. 3-7).

Preheating temperature had a significant impact on thermal stability of dWP at pH 5.5 (Fig. 3-10). An intermediate preheating temperature, 85 °C for dWP-SSL and 90 °C for dWP+SSL, resulted in the best improvement in heat stability at pH 5.5. This can be caused by the increased overall aggregate hydrophobicity after a greater amount of SSL binds with WP denatured to a greater extent during/after preheating at a higher temperature.

3.5. Conclusions

In conclusion, SSL improved thermal stability of both native and preheated WP at pH 5.5-6.0. The thermal stability improvement resulted from the strengthened electrostatic repulsion and lowered hydrophobic attraction after binding SSL on denatured WP. The binding appeared to be a function of SSL concentration and preheating conditions, which resulted in differences in surface properties as measured for zeta-potential, surface –SH content, and H_0 among treatments of dWP-SSL and dWP+SSL. Direct addition of 0.2% SSL in 4.5% nWP improved the heat stability at pH 5.5 and 6.0, while the same formulation after preheating at 90°C for 20 min appeared to be effective in improving heat stability at pH 5.5. In addition, preheating conditions impacted thermal stability of preheated WP with SSL. These observations may be adopted to manufacture beverages formulated to these acidity conditions.

References

- Alizadeh-Pasdar, N., & Li-Chan, E. C. Y. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry*, 48(2), 328–334.
- Bhuyan, A. K. (2010). On the mechanism of SDS-induced protein denaturation. *Biopolymers*, 93(2), 186-199.
- Bortnowska, G., Juśkiewicz, J., Król, B., Kosmala, M., Milala, J., Zduńczyk, Z., Niedźwiedzka, E. (2015). Multilayer oil-in-water emulsions: formation, characteristics and application as the carriers for lipophilic bioactive food components—a review. *Polish Journal of Food and Nutrition Sciences*, 2015, Vol. 65, No. 3, pp. 0-0 .
- Burrington, K. (2012). Technical report: whey protein heat stability. Retrieved from <http://www.usdairy.com/~media/usd/public/technicalreportwheyproteinheatstability.pdf.pdf>
- Cowgill, R. W. (1967). Fluorescence and protein structure: XI. Fluorescence quenching by disulfide and sulfhydryl groups. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 140(1), 37–44.
- Creamer, L. K. (1995). Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine beta-Lactoglobulin. *Biochemistry*, 34(21), 7170–7176.
- De Wit, J. N. (1981). Structure and functional behaviour of whey proteins. *Netherlands Milk and Dairy Journal (Netherlands)*, 35(1).
- Department of health and human services. (2014). Code of Federal Regulations. 21CFR172.846
- EFSA Panel on Food Additives and Nutrient Sources added to Food. (2013). Scientific Opinion on the re-evaluation of sodium stearoyl-2-lactylate (E 481)

- and calcium stearyl-2-lactylate (E 482) as food additives. parma, Italy.
Retrieved from <http://www.efsa.europa.eu/en/efsajournal/doc/3144.pdf>
- Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, 82(1), 70–77.
- FoodDB. (n.d.). Compound Sodium stearyl 2-lactylate (FDB010802).
Retrieved from <http://foodb.ca/compounds/FDB010802>
- Giroux, H. J., & Britten, M. (2004). Heat treatment of whey proteins in the presence of anionic surfactants. *Food Hydrocolloids*, 18(4), 685–692.
- Goddard, E. D., & Ananthapadmanabhan, K. P. (1993). Interactions of surfactants with polymers and proteins. CRC Press, Boca Raton, FL, 1993
- Hongsprabhas, P., & Barbut, S. (1997). Structure-forming processes in Ca²⁺-induced whey protein isolate cold gelation. *International Dairy Journal*, 7(12), 827–834.
- Iametti, S., Cairolì, S., De Gregori, B., & Bonomi, F. (1995). Modifications of high-order structures upon heating of beta-lactoglobulin: dependence on the protein concentration. *Journal of Agricultural and Food Chemistry*, 43(1), 53–58.
- Jones, M. N. (1975). A theoretical approach to the binding of amphipathic molecules to globular proteins. *Biochem. J*, 151, 109–114.
- Jones, M. N. (1992). Surfactant interactions with biomembranes and proteins. *Chem. Soc. Rev.*, 21(2), 127–136.
- Kinsella, J. E., & Morr, C. V. (1984). Milk proteins: physicochemical and functional properties. *Critical Reviews in Food Science & Nutrition*, 21(3), 197–262.
- Kuwata, K., Hoshino, M., Era, S., Batt, C. A., & Goto, Y. (1998). $\alpha \rightarrow \beta$ transition of β -lactoglobulin as evidenced by heteronuclear NMR. *Journal of Molecular Biology*, 283(4), 731–739.
- Larsen, R. A., Jenness, R., & Geddes, W. F. (1949). Effect of heat treatment on the sulfhydryl groups of milk serum proteins. *Cereal Chemistry*, 26(4), 287–297.

- Le, T. T., Sabatino, P., Heyman, B., Kasinos, M., Dinh, H. H., Dewettinck, K., Van Der Meeren, P. (2011). Improved heat stability by whey protein–surfactant interaction. *Food Hydrocolloids*, 25(4), 594–603.
- Liu, G., & Zhong, Q. (2013). Dispersible and thermal stable nanofibrils derived from glycated whey protein. *Biomacromolecules*, 14(7), 2146–2153.
- Livney, Y. D., Verespej, E., & Dalglish, D. G. (2003). Steric effects governing disulfide bond interchange during thermal aggregation in solutions of β -lactoglobulin B and α -lactalbumin. *Journal of Agricultural and Food Chemistry*, 51(27), 8098–8106.
- Miller, M. S., Surber, K. J., Lis, D. G., Martin Jr, R. W., & Hasenhuettl, G. L. (2000, March 7). Mouthfeel and lubricity enhancing composition. Google Patents.
- Mills, O. E., & Creamer, L. K. (1975). A conformational change in bovine β -lactoglobulin at low pH. *Biochimica et Biophysica Acta (BBA)-Protein Structure*, 379(2), 618–626.
- Nicolai, T., Britten, M., & Schmitt, C. (2011). β -Lactoglobulin and WPI aggregates: Formation, structure and applications. *Food Hydrocolloids*, 25(8), 1945–1962.
- Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., Kraulis, P. J. (1986). The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* 324, 383 – 385.
- Patrick, P. S., & Swaisgood, H. E. (1976). Sulfhydryl and disulfide groups in skim milk as affected by direct ultra-high-temperature heating and subsequent storage. *Journal of Dairy Science*, 59(4), 594–600.
- Renard, D., Lefebvre, J., Griffin, M. C. A., & Griffin, W. G. (1998). Effects of pH and salt environment on the association of β -lactoglobulin revealed by intrinsic fluorescence studies. *International Journal of Biological Macromolecules*, 22(1), 41–49.
- Ryan, K. N., Vardhanabhuti, B., Jaramillo, D. P., van Zanten, J. H., Coupland, J. N., & Foegeding, E. A. (2012). Stability and mechanism of whey protein

- soluble aggregates thermally treated with salts. *Food Hydrocolloids*, 27(2), 411–420.
- Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of whey protein soluble aggregates for thermal stability—a hypothesis paper. *Journal of Food Science*, 78(8), R1105–R1115.
- Sakurai, K., Oobatake, M., & Goto, Y. (2001). Salt-dependent monomer-dimer equilibrium of bovine beta-lactoglobulin at pH 3. *Protein Science*, 10(11), 2325-2335.
- Schmitt, C., Bovay, C., Rouvet, M., Shojaei-Rami, S., & Kolodziejczyk, E. (2007). Whey protein soluble aggregates from heating with NaCl: physicochemical, interfacial, and foaming properties. *Langmuir*, 23(8), 4155–4166.
- Shimada, K., & Cheftel, J. C. (1989). Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *Journal of Agricultural and Food Chemistry*, 37(1), 161–168.
- Thompson, J. B., & Buddemeyer, B. D. (1954). Improvement of flour mixing characteristics by a stearyl lactic acid salt. *Cereal Chemistry*, 31(4), 296–302.
- Wang, W., Zhong, Q., & Hu, Z. (2012). Nanoscale understanding of thermal aggregation of whey protein pretreated by transglutaminase. *Journal of Agricultural and Food Chemistry*, 61(2), 435–446.
- Whitehurst, R. J. (2008). *Emulsifiers in food technology*. John Wiley & Sons.
- Zaidi, N., Nusrat, S., Zaidi, F. K., & Khan, R. H. (2014). pH-dependent differential interacting mechanisms of sodium dodecyl sulfate with bovine serum fetuin: A Biophysical Insight. *The Journal of Physical Chemistry B*, 118(46), 13025–13036.
- Zhang, W., & Zhong, Q. (2010). Microemulsions as nanoreactors to produce whey protein nanoparticles with enhanced heat stability by thermal pretreatment. *Food Chemistry*, 119(4), 1318–1325.

Zhong, Q., Wang, W., Hu, Z., & Ikeda, S. (2013). Sequential preheating and transglutaminase pretreatments improve stability of whey protein isolate at pH 7.0 during thermal sterilization. *Food Hydrocolloids*, 31(2), 306–316.

Appendix

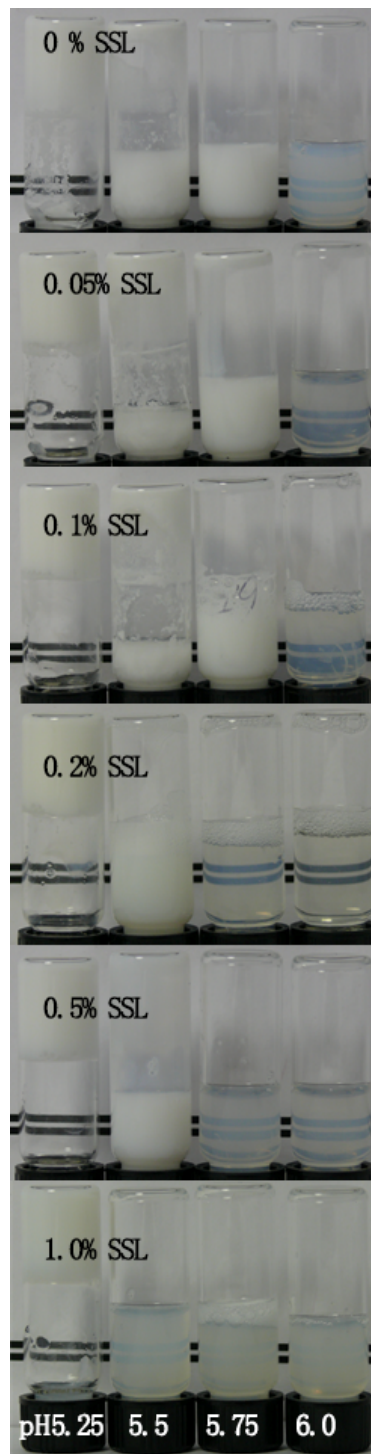


Figure 3-1. Photographs of native WP (4.5%) and SSL (0-1.0%) mixtures heated at pH 5.25-6.0 and 95 °C for 1 min.

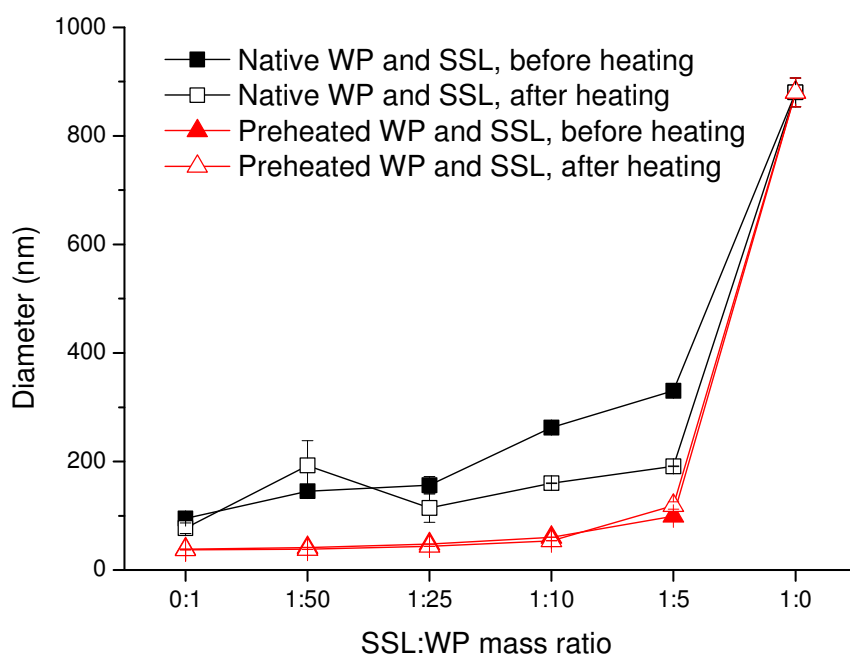


Figure 3-2. Diameters of mixtures with SSL and native or preheated WP at various mass ratios, before and after heating at 90 °C and pH 7.0 for 20 min. Preheated WP was prepared by heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n = 2).

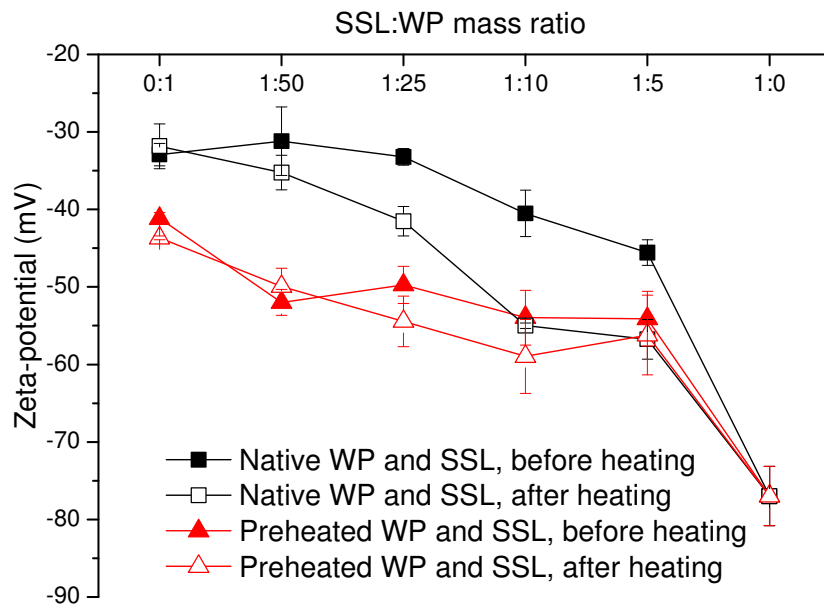


Figure 3-3. Zeta-potential of native and preheated WP with SSL before and after heating at 90 °C and pH 7.0 for 20 min. Preheated WP was prepared by heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n=2).

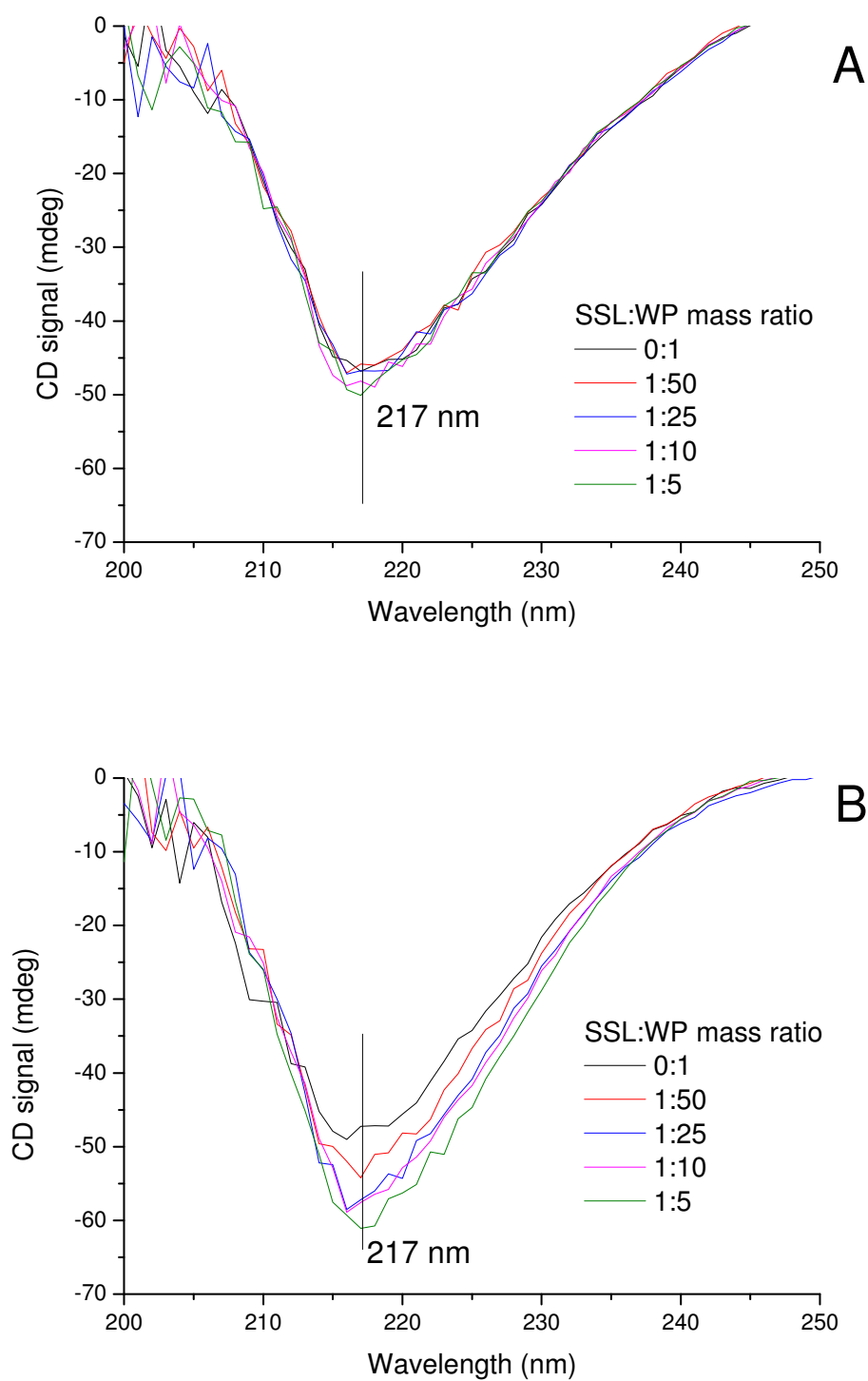


Figure 3-4. CD signals of mixtures with various mass ratios of native WP and SSL (A) before or (B) after heating at 90 °C and pH 7.0 for 20 min.

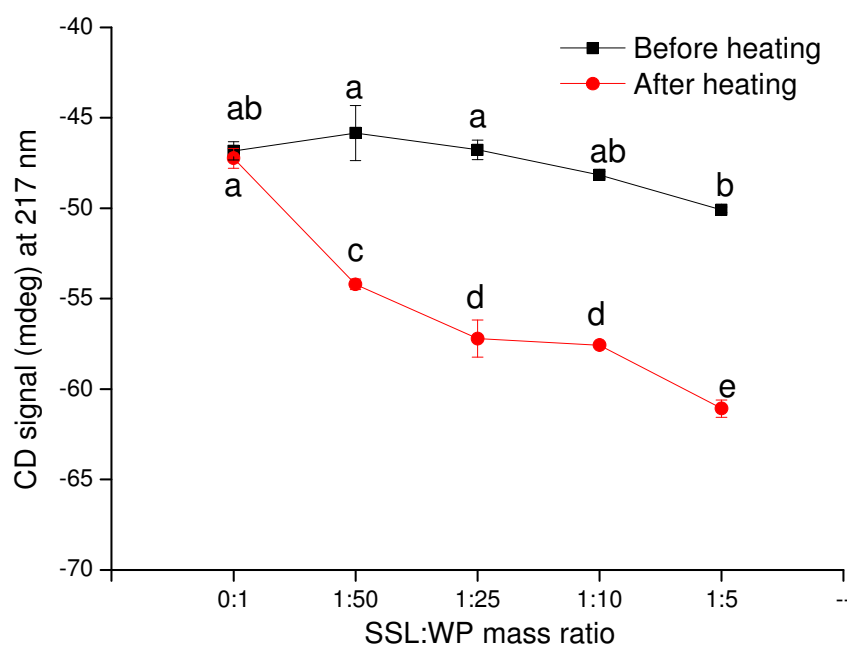


Figure 3-5. CD signals of mixtures with various mass ratios of native WP and SSL at 217 nm before and after heating at 90 °C and pH 7.0 for 20 min. Error bars are standard deviations (n=2). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).

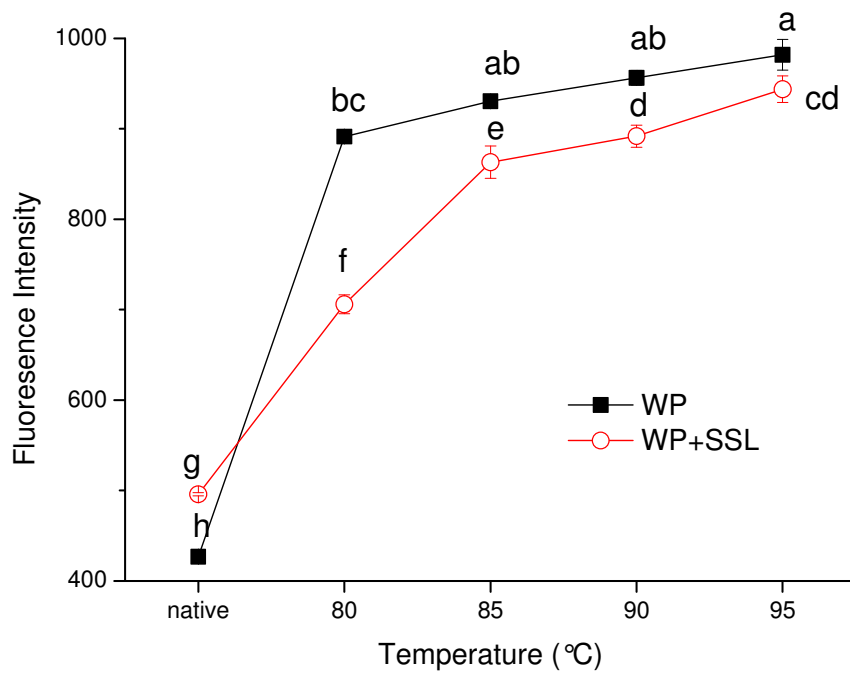


Figure 3-6. Intrinsic fluorescence intensities of WP before and after heating at 80-95 °C for 20 min, in comparison to a mixture with WP and SSL after heating together at identical conditions (WP+SSL). Error bars are standard deviations ($n = 2$). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).

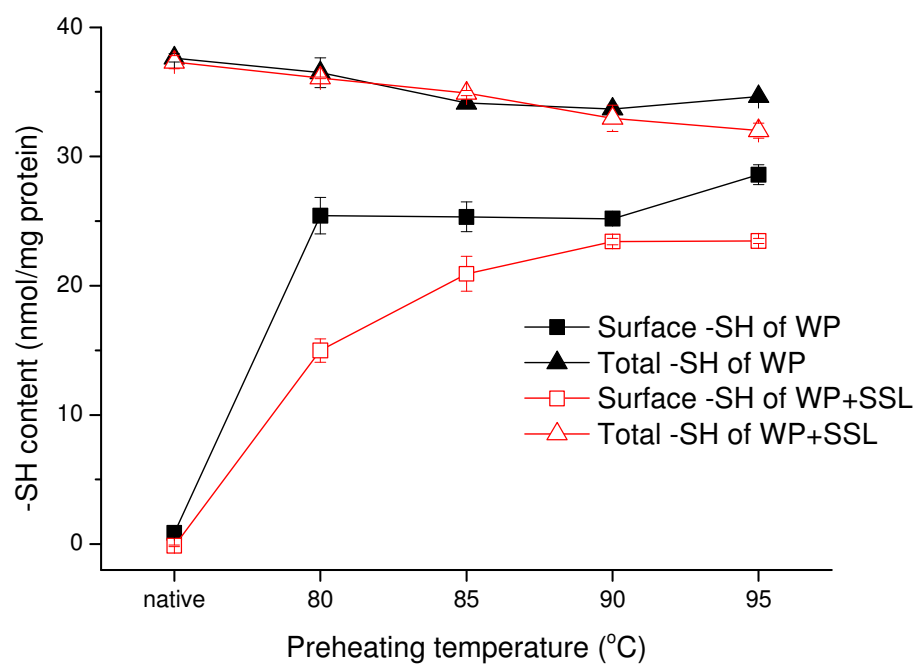


Figure 3-7. Surface and total –SH contents of WP before and after heating at 80-95 °C for 20 min, in comparison to a mixture with WP and SSL after heating together at identical conditions (WP+SSL). Error bars are standard deviations (n = 2).

Figure 3-8. (A) Particle size distributions of SSL and preheated WP (at 90 °C and pH 7.0 for 20 min) without and with SSL (dWP-SSL), in comparison to a mixture with WP and SSL after heating together at identical conditions (dWP+SSL). Plot B shows diameters of the highlighted peak in A of these three samples preheated at 80-95 °C and pH 7.0 for 20 min (B). Error bars are standard deviations ($n = 2$). The data point labeled with an asterisk represents significant differences ($P < 0.05$) from the other two samples at the same preheating temperature.

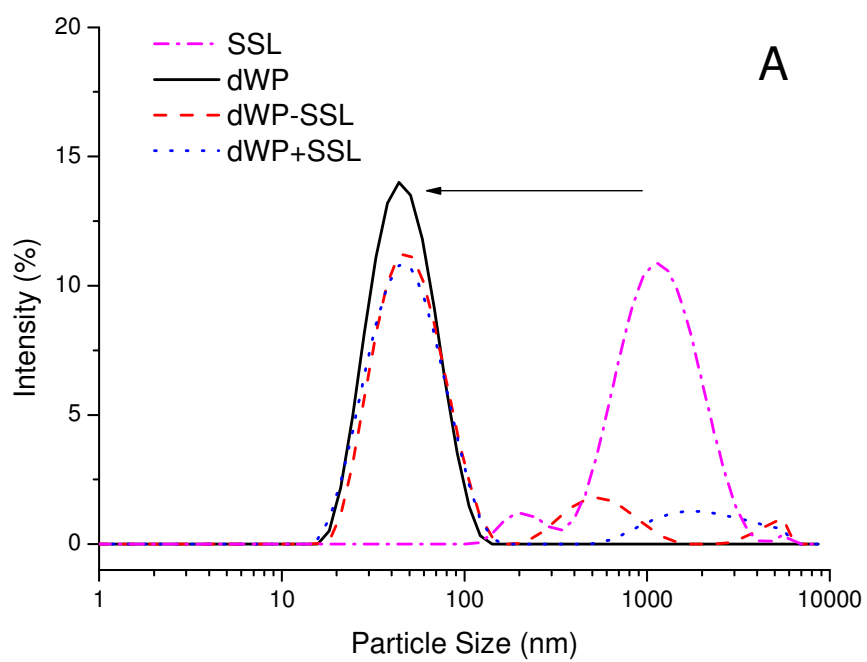


Figure 3-8 continued.

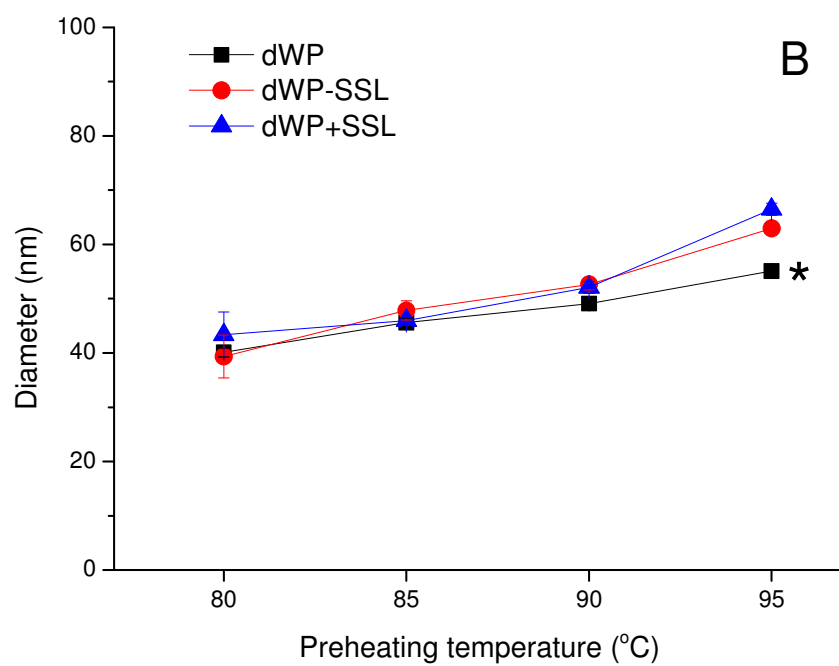


Figure 3-8 continued.

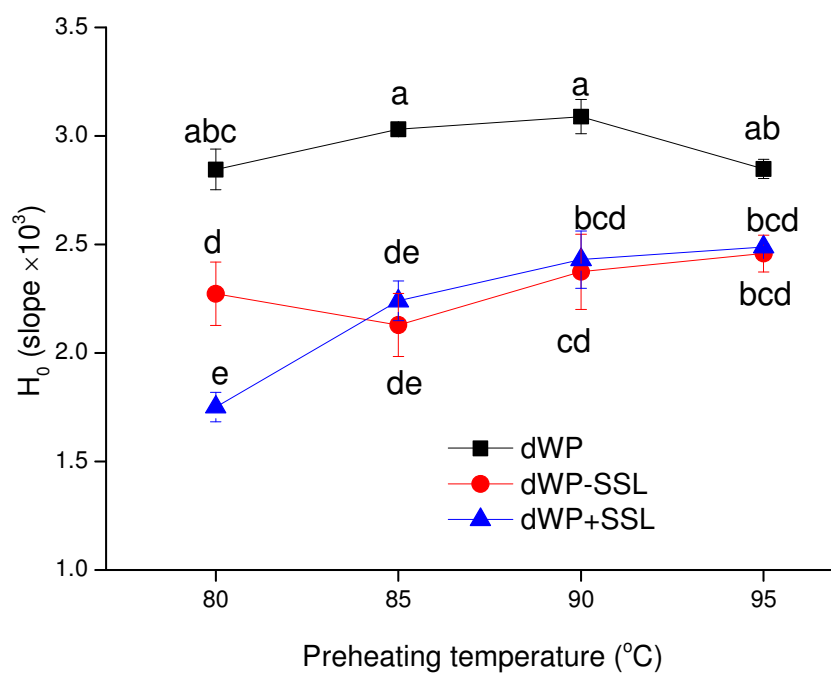


Figure 3-9. Surface hydrophobicity (H_0) of WP after heating at 80-95 $^{\circ}\text{C}$ and pH 7.0 for 20 min without and with 0.2% SSL (dWP+SSL), in comparison to treatments after adding SSL in WP preheated at same conditions (dWP-SSL). Error bars are standard deviations ($n = 2$). Different letters next to symbols indicate significant differences in the mean ($P < 0.05$).

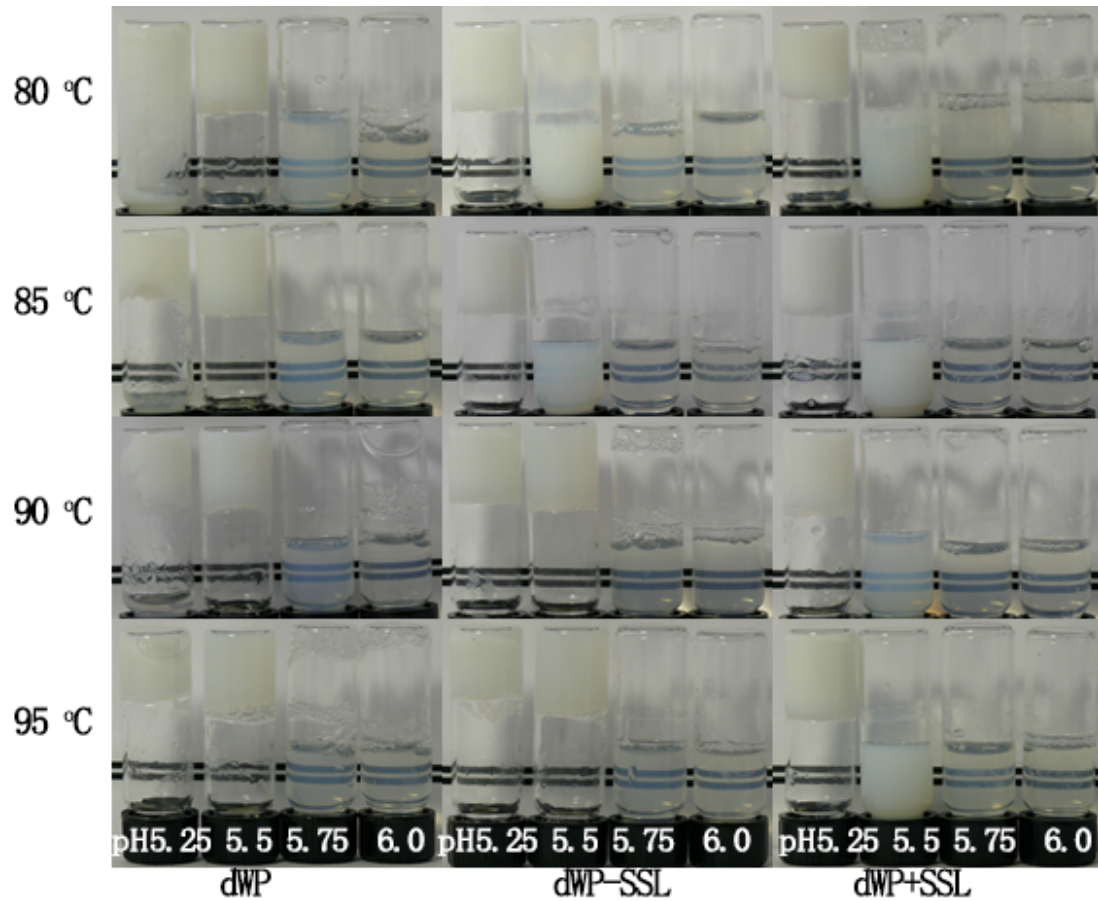


Figure 3-10. Photographs of WP after heating at 80-95 °C and pH 7.0 for 20 min without and with 0.2% SSL (dWP+SSL), in comparison to treatments after adding SSL in WP preheated at same conditions (dWP-SSL), after adjusting to pH 5.25-6.0 and heating at 95 °C for 1 min. Concentrations of WP and SSL were 4.5 % and 0.2 % w/v, respectively.

Chapter 4. Concluding Remarks and Future Work

4.1. Conclusions

Thermal aggregation of WP can be either enhanced or inhibited to broaden its application in the food industry. To provide satiety, aggregation of preheated WP was enhanced to form gels by charge neutralization between positively charged groups of WP and negatively charged pectin at gastric pH 1.0-4.0. All gels prepared by fast acidification to simulate mixing in the stomach had different structures and properties from GDL-induced gels. Intense agitation affected the strength and homogeneity of WP gels, and fast acidification limited the formation of disulfide bonds. Formation of WP gels involved hydrophobic attraction and disulfide bonds, while electrostatic attraction was critical in the gel network formation of mixtures with WP and pectin. Preheated WP gels were dissolved in water while preheated WP-pectin gels with CaCl_2 at pH 2.5 and 3.0 remained partially undissolved.

To improve thermal stability of WP, SSL was studied to inhibit thermal aggregation of WP at pH 5.5-6.0. With an increase in SSL content from 0 to 1%, thermal stability of native WP at pH 5.5-6.0 was improved. Particle size and zeta-potential indicated that heating promoted binding of SSL on WP. Circular dichroism spectra indicated the formation a more ordered secondary structure promoted by SSL. The binding resulted in differences in surface properties as measured for zeta-potential, surface $-\text{SH}$ content, and H_0 among treatments of dWP-SSL and dWP+SSL. These properties may enhance electrostatic repulsion and reduce hydrophobic attraction and formation of disulfide bonds. As a result, thermal stability of preheated WP was improved by SSL to enable a transparent fluid containing 4.5% WP and 0.2% SSL at pH 5.5. In addition, preheating conditions impacted thermal stability of preheated WP with SSL.

Overall, this thesis provides scientific understanding of aggregation properties of WP and possible methods to enhance or control thermal aggregation. The information will be useful in future studies illustrating interactions between native and preheated WP and anionic polysaccharides and surfactants and developments of WP-based satiety foods and shelf-stable

ready-to-drink beverages.

4.2. Future work

To extend the study of satiety gel, the *in vitro* and *in vivo* digestion properties of WP-pectin complexes can be studied to evaluate the improvement of satiety of animals or human. In addition, other polysaccharides including alginate can be studied.

The scientific basis of the improved thermal stability of WP by SSL can be extended to measure the exact number of SSL binding on native and preheated WP, and the conformation of preheated WP changed by SSL can be studied for parameters of pH, ionic strength, heating temperature and duration. Other surfactants can also be studied in the future.

Vita

KangKang Li entered College of Food Science and Technology, Jiangnan University (Wuxi, China) in 2007 to pursue a Bachelor's degree in Food Quality and Safety. After graduating from Jiangnan University, he continued his study for a Master of Engineering degree, focusing on the food proteins at the South China University of Technology (Guangzhou, China). His Master's thesis project focused on zein colloidal particles. In August 2013, he began his master 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. His thesis research focused on thermal aggregation of whey protein to extend its application for protein-rich foods.