IMPROVED HEAT STABILITY OF WHEY PROTEIN ISOLATE BY GLYCATION WITH INULIN

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ABSTRACT

Glycation between protein and sugars has been shown to improve heat stability of protein. In this study, we select inulin, a heathy dietary fiber, to glycate with whey protein isolates (WPI) with the goal to optimize the reaction conditions such that heat stability of WPI was improved without the formation of undesirable advanced Maillard reaction products. Conjugates were prepared by freeze drying the mixture solutions of WPI and inulin at different weight ratios followed by dry-heating at various temperatures, relative humidity levels and incubation times. Visual observation of the conjugate solutions after heating was used to evaluate heat stability and color development. Heat stability was evaluated by turbidity, particle size, and rheological measurements. Degree of glycation was assessed by quantifying the loss of amino groups using TNBS method and the amount of Amadori compounds detected by the absorbance at the 304 nm.

Results showed that optimum glycation can be achieved by dry-heating WPI-inulin mixtures at 2:1, 4:1 and 6:1 weight ratios at 80°C for 12 to 72 h without controlling relative humidity. Glycation was confirmed by an increase in the Amadori compounds and the limited but desirable extent of reaction was demonstrated by a

small loss of available amino groups. Improved heat stability of conjugates was shown by a decrease in turbidity and particle size after heating 6% w/w protein at pH 6.0. In consistent with the limited extent of reaction and improved heat stability, glycated WPI did not show significant change in flow behaviors. Improved heat stability may be due to an increase in negative charge as well as increased stabilization of the protein as shown by increased zeta potential and denaturation temperature, respectively. With limited degree of glycation, low viscosity and improved heat stability, glycated WPI-inulin has a great potential to be utilized as food ingredients, especially in beverage industry.

CHAPTER 1

INTRODUCTION

Health and wellness-related beverage market has continued to grow in the past few years while the sale of carbonated soft drinks has experienced a decline from 2004 to 2014 (Holtz 2014; Beverage-digest 2014). With consumers' increasing demand for healthier food products, functional foods including high protein beverages have seen a surge in sale (Holtz 2014). However challenges in developing such products are the loss of heat stability (e.g., increased turbidity and formation of sediments) at pH near isoelectric point (pI) of the protein and astringency problem at low pH (pH < 4.0). One major goal to improve protein functional properties is to increase heat stability of protein at pH closer to pI.

Whey is a greenish yellow liquid drained after cutting and cooking of the coagulum during cheese making. Whey protein isolate (WPI) is the fractionated whey product with 90-92% protein content and is a common protein ingredient used to manufacture protein beverage (Park and Haenlein 2013; Wang and Zhong 2014). Heat aggregation of whey protein is generally attributed to molecular forces such as Van der Waals, hydrophobic and electrostatic interactions and intra- and inter- molecular disulfide bonds via sulfhydryl-disulfide interchange (Baier and McClements 2005). The behavior of heat induced denaturation and aggregation of β-lactoglobulin (the major component in whey protein) was described by a model proposed by (Roefs and De Kruif

1994): In the initiation step, β-lactoglobulin dimer splits into monomers exposing the free sulfhydryl group and causing the protein to become reactive. In the propagation step, activated β-lactoglobulin molecules react with non-reactive β-lactoglobulin through thiol/disulphide exchange reaction, and hence build up the aggregates. In the termination step, two active intermediates react and form a larger disulphide-linked polymer without an exposed, reactive thiol group. The protein concentration, solution pH and heating temperature affect the behavior of protein denaturation and aggregation. At high protein concentration, whey protein tends to form much larger aggregates under heating. Hoffmann and van Mil (1997) found that when β-lactoglobulin solution was heated at 65°C, the weight fraction of the aggregates shifted toward higher molecular masses with increasing initial β-lactoglobulin from 0.1 - 1.0%. The pH of the solution affects the heat stability of whey protein by altering its surface charge and the reactivity and accessibility of the thiol group. For the solution with 2.5% protein content, it became turbid and form sediments at pH 4 - 6, because of the low electrostatic repulsion (Etzel 2004). At the very acidic pH (2-3), the disulphide exchange is very unlikely to occur also because of the thiol groups are very stable.

Maillard reaction between protein and reducing sugar has been shown to improve heat stability of protein (Aoki and others 2001; Aoki and others 1999; Jiménez-Castaño and others 2007; Jiménez-Castaño and others 2005a; Kato and others 1995; Liu and Zhong 2013; Liu and Zhong 2012; Wang and Zhong 2014; Wang and Ismail 2012; Xu and others 2010). For example, Liu and Zhong (2012) found that WPI-maltodextrin solution (7% protein concentrate) could maintain transparent at pH from 3 to 7 after

heated at 88°C for 2 min. The Maillard reaction between protein and reducing sugars can be achieved by heating them together in “dry” or “wet” conditions. The “dry” condition is achieved by freeze drying or sprays drying a solution of protein and reducing sugar before dry-heating the dried mixture, while the “wet” condition involves heating a solution of mixed protein and sugar without drying (Oliver and others 2006a; Liu and others 2012). The glycation between protein and saccharide is based on the Amadori rearrangement steps in the Maillard reaction and the optimum reaction condition is when the Amadori compound is formed but before it converts into the advanced Maillard products (AMP), which is dark color, insoluble and with potential health hazard (Oliver and others 2006a; Jiménez-Castaño and others 2005b). The ratio between protein and reducing sugar, heating temperature and relative humidity affect the Maillard reaction rate and the final product. Excessive sugar increases the Maillard reaction rate but also the AMP formation, because of the higher number of reactive carbonyl groups (Jiménez-Castaño and others 2005b). Higher temperature increases the rate of Maillard reaction, not only because the increase in reactivity of carbonyl group and amino group, but also because the greater unfolding of the protein structure. The Amadori compounds formed in the initial stage is rather stable under temperature lower than 60°C (Malec and others 2002). Generally, the maximum reaction rate occurs at the intermediate moisture content (Ge Pan and Melton 2007; Ames 1992). In a lactose-casein model system incubated at 50°C, it was found that the rate of lysine loss at A_W 0.52 was roughly 100% higher than that at A_W 0.43 and 0.69 (Malec and others

2002). Ge Pan and Melton (2007) found that at high moisture content, the color formation was highest while the whole Maillard reaction was slow.

As mentioned, glycation of protein and saccharides can lead to improved protein functional properties including heat stability. One challenge in applying glycation in the food industry is the selection of saccharides. Desirable saccharides are those that can offer technological and health benefits. It is also important that selected saccharides are well accepted by the consumers. Inulin is a polymer of fructans consisting of linear chain of fructosyl groups linked by β(2→1) glycosidic bonds with normally one glucopyranose unit at the reducing end (Barclay and others 2010). Ostensibly, inulin is non-reducing sugar, because β(2→1) glycosidic bonds fixing the cyclic structure (Barclay and others 2010). However, the existence of glucose, fructose and the fructans consist exclusively of fructose, whether created by natural enzymatic processes or by hydrolysis, mean that inulin will invariably contain certain reducing capacity (Barclay and others 2010; Oliver and others 2006c). Inulin as a dietary fiber has been shown to increase the growth of beneficial intestinal microflora and the absorption of mineral, modulate the absorption or metabolism of lipids and inhibit the development of cancer (Barclay and others 2010; Kalyani Nair and others 2010; Roberfroid 2005).

The aim of this research was to investigate the effect of heating temperature, incubation time, relative humidity and the reactant's weight ratio on the properties and heat stability of glycated WPI and inulin. The mechanism responsible for improved heat stability was also determined.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Whey protein and its heat stability

Whey is a greenish yellow liquid drained after cutting and cooking of the coagulum during cheese making. The coagulum could be achieved by the addition of rennet or acid and the resulted whey is known as sweet and acid whey respectively. Liquid whey contains about 6% total solids and whey proteins account for 10-12% of them (Marella 2009). Whey protein is a complex mixture with the most common being β-lactoglobulin (∼55%), α-lactolbumin (∼24%), blood serum albumin (∼5%), and immunoglobulins (∼15%) (Damodaran and others 2008).

Liquid whey is traditionally processed into whey powder, whey protein concentrate (WPC) and whey protein isolates (WPI) (Marella 2009). Whey powders are obtained generally using spray drying technology and hence they proportionally contain all the components of liquid whey except water (Park and Haenlein 2013). WPC and WPI are the fractionated whey products, achieved essentially by membrane filtration technology in combination with drying (Park and Haenlein 2013). The protein content of WPC ranged from 34 to 80%. Typically, WPC contains 80-82% protein will also have 4 - 8% lactose, 4 - 8% fat and 3 - 4 % ash. WPI contains 90 - 92% protein, 0.5 - 1% lactose, 0.5 - 1.1% fat and 2 - 3% ash.

Before 1970s, whey was treated as a waste product and was drained if not used as animal feed or applied to fields as liquid fertilizer. Owing to the improvement of filtration, whey products are widely used in the industry (Park and Haenlein 2013). In 2008, about 190,000t of whey powder, 85,000t of WPC and 14,500t of WPI were used in food sectors, majorly being dairy, bakery, dry/ wet blending, functional food application, infant formula, and confectionary (Affertsholt 2009).

2.1.1 Heat stability of whey protein

The majority of the studies on the mechanism of whey protein aggregation have been confined to β-lactoglobulin, since it is the most abundant whey protein in milk (de la Fuente and others 2002). Roefs and De Kruif (1994) proposed a model to describe the aggregation of β-lactoglobulin with initiation step, propagation step and termination step, by analogy with polymer radical chemistry. In the initiation step, β-lactoglobulin dimer splits into monomers, and expose the free sulfhydryl group, which cause the protein to become reactive. In the propagation step, an activated β-lactoglobulin reacts with a non-reactive β-lactoglobulin through thiol/disulphide exchange reaction, and hence builds up the aggregates. In the termination step, two active intermediates react and form a larger disulphide-linked polymer without an exposed, reactive thiol group. Further study showed that the contribution of non-covalent interactions to the overall aggregation mechanism became important at higher temperature (> 90°C) (Galani. and Apenten 1999; Photchanachai and Kitabatake 2001).

2.1.2 Factors affecting heat stability of protein

Higher initial protein concentration leads to the formation of larger aggregates. According to a study conducted by Lametti and others (1996), protein unfolding was independent of protein concentration, whereas the association phenomenon was highly dependent on protein concentration. Hoffmann and van Mil (1997) evaluated the effect of protein concentration in the range 0.1 - 1% on β-lactoglobulin aggregation at 65°C. They found that the weight fraction of the aggregates shifted towards higher molecular masses with increasing initial β-lactoglobulin, and this shift was much stronger than the shift that was observed for one as a function of heating time.

pH has a major effect on the aggregation of β-lactoglobulin by altering its surface charge and the reactivity and accessibility of the thiol groups. For the solution with 2.5% protein content, it became turbid and form sediments at pH 4 - 6, because of the low electrostatic repulsion (Etzel 2004). At pH 8, the thiol group of β-lactoglobulin is readily available for reactions, increasing the rate of polymerization via thiol/disulphide exchange, whereas, at the pH below 7, the molecule has to be heated, or unfolded in another way, to expose the thiol group (de la Fuente and others 2002). Hoffmann and van Mil (1997) studied the aggregation of β-lactoglobulin at 65°C at a range of pH values between 6 and 8. The rate of conversion of native β-lactoglobulin into the aggregates increased with pH but the molecular mass of the aggregates decreased markedly. This was attributed to the fact that a large number of exposed thiol groups in the early stage, formed at higher pH, would increase the probability of termination reactions, resulting in the formation of more, but smaller, disulphide-linked terminated aggregates. At very acidic pH (e.g. pH < 3), the disulphide exchange is very unlikely to occur, because the

thiol groups are very stable at low pH and the repulsion between monomers are large (de la Fuente and others 2002).

Salts have two opposite effects of on protein aggregation. Firstly, the increase in ionic strength reduces the intermolecular repulsion between molecules because of the screening of charged groups, thereby increasing the aggregation rate. Secondly, an increase in ionic strength increases the denaturation temperature of the protein, because screening of charged groups reduces intramolecular repulsion, therefore increasing conformational stability.

Although it is often accepted that the characteristics of β-lactoglobulin dominate the behavior of the whey protein aggregates, the other whey proteins also play an important roles. α-Lactalbumin does not polymerize by itself when heated above 70°C, whereas it could interact with denatured β-lactoglobulin. The composition of the heated whey protein solution is dependent on the ratio of the two proteins: at high concentration of β-lactoglobulin, complexes rich in β-lactoglobulin are formed, while large aggregates lack in high concentration of α-lactalbumin, indicating efficient chain termination in this system. α-lactalbumin could also interact with BSA when heated together.

2.2 Protein glycation and its effects on protein properties

2.2.1 Maillard Reaction

The conjugation between protein and polysaccharides is based on the Amadori rearrangement steps in the Maillard reaction (Oliver and others 2006a). The Maillard reaction can be summarized into three stages (Kato 2002). In the early stage, the

carbonyl group of a reducing sugar condenses with an unprotonated amine group, forming a Schiff base with the release of water. The Schiff base subsequently cyclizes to the corresponding N-glycosylamine, which then undergoes an irreversible Amadori rearrangement to produce the Amadori compounds; while with ketoses, such as fructose, the Heyns rearrangement takes place instead (Ames 1992). The intermediate stage begins with the degradation of the Amadori/Heyns Products which can undergo numerous transformations involving various divergent pathways. At the final stage, water-insoluble, nitrogen-containing polymeric compounds, known as “melanoidins” which are responsible mostly for the brown color, are produced (Friedman 1996). Since its complexity, the details of the advanced Maillard reaction are not well understood (Oliver and others 2006a)

By attaching several polysaccharides to the protein, conjugation is an effective and safe way to modify the functionality of protein (Oliver and others 2006a). Ideally, glycation should be stopped at the early stage, in order to limit the formation of the advanced Maillard products, which are insoluble, dark and potentially toxic (Jiménez-Castaño and others 2007; Oliver and others 2006a).

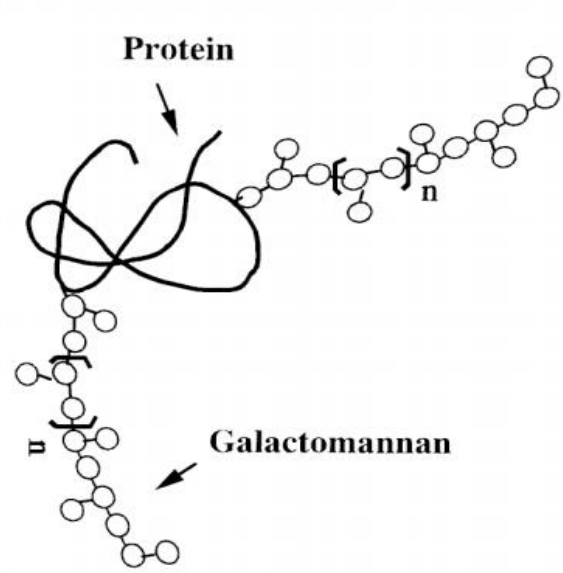

Figure 1. Schematic presentation of Protein-polysaccharides conjugate(Kato 2002)

2.2.2 Factors affecting protein/polysaccharide glycation

The conjugation of protein-polysaccharides could take place in both “wet” and “dry” conditions. Under the “wet” conditions, protein and reducing sugars are dispersed in water and heated at mild temperature. The advantage of this method is that Maillard reaction could be terminated at the initial stage by rapidly decreasing the temperature, owing to the low reaction rate in aqueous solution (Morgan and others 1998). However, the disadvantages of this method prevent it from becoming industrial favorable. Firstly, the protein concentration has to be low so that they will not aggregate during heating, which results in low yield. Secondly, because of the long incubation time and relatively low heating temperature, antimicrobial agent is usually added to prevent the spoilage (Zhu and others 2008). Finally, the final product might need further removal of the excessive water, in order to get good storage and handling (Li and others 2009). “Dry” reaction can be carried out by freeze drying or spray drying a solution of protein and

reducing sugar, followed by heating under controlled temperature and relative humidity for a given time (Liu and Zhong 2013; Liu and others 2012; Liu and Zhong 2012; Oliver and others 2006a). Compared to “wet” method, “dry” method requires less space and time and the resulted products have longer-term stability and greater ease of handling and storage (Oliver and others 2006a). However, under the “dry” condition, it is not easy to control the extent of the reaction and the final products are usually a mixture of intermediate and/or advanced Maillard reaction products that have a yellow or brown color (Zhu and others 2008). Also, in some cases, insoluble aggregates with poor surface wetting properties will formed (Li and others 2009).

The conformation and composition of both protein and sugars have significant effects on the reaction rate and their binding mode. Because of the smaller steric hindrance, the exposed lysyl residues in unfolded protein could react with the reducing sugars at much faster rate (Kato 2002). For example, the typical unfolded protein α-Casein could bind four polysaccharides per mole within 24h, while lysozyme, a folded protein, needs more than 2 weeks to bind 2 polysaccharides per mole (Nakamura and others 1992; Kato and others 1992). Similarly, reducing sugar with smaller molecules weight could react with exposed lysyl residues at much faster rate, owing to their smaller steric hindrance (Kato 2002). Much more lysyl residues of β-lactoglobulin were blocked by dextran with 10 kDa than the dextran with 20 kDa, under same incubation condition and time (Jiménez-Castaño and others 2007). Also more color development was found for 10 kDa than 20KDa. Since the excessive reaction lead to the formation of

side products, reducing sugars with higher molecular weight were more desirable in modifying the protein functionalities (Kato 2002).

Moisture content exerts a major influence on the Maillard reaction and the maximum reaction rate generally occurs at the intermediate moisture content (Ames 1992; Ge Pan and Melton 2007). In a lactose-casein model system that incubated at 50°C, it was found that the lysine loss rate at A_W 0.52 is roughly 100% higher than A_W 0.43 and 0.69 (Malec and others 2002). In another study about whey protein isolates and maltodextrin conjugates, the degree of glycation (indicated as blocked amino acids group) increased when increasing the relative humidity form 50 to 80% (Martinez-Alvarenga and others 2014). At A_W values below 0.3, the decrease in reaction rate is attributed to diffusional limitations between the reactants and at A_W values above 0.8, the decrease is related to dilution effects and inhibition by water. Since the Maillard reaction is very complex, more measurements will be necessary to find out how the moisture content affects the whole reaction process. Ge Pan and Melton (2007) studied the effects of moisture content on the lactose-casein mixture by using Fluorescence Intensity, color, lysine loss, lactose loss and furosine formation. The results showed that the maximum value for each indicator was obtained at different Moisture content. For example, the color formation was fast at 95% relative humidity, while the formation of furosine is slow. Martinez-Alvarenga and others (2014) also found that the relative humidity had the most important effect on the color formation.

It is well known that an increase in temperature increases the rate of Maillard reaction (Jiménez-Castaño and others 2005b). Broersen and others (2004) reported that

an increase of 15 °C (form 45°C to 60°C) resulted in a 56% increase in blocked amino groups in conjugates formed between β-lactoglobulin and glucose. Similarly, an increase of 10°C (from 40 °C to 50°C) showed an increase in 62% of blocked amino groups in conjugates created between β-lactoglobulin and tagatose (Corzo-Martínez and others 2008). Zhu and others (2008) found little Schiff base formation in WPI-dextran conjugates at temperatures between 40 and 50°C. However, a significant increase was observed at temperatures between 50 and 60°C. This increase can be attributed not only to an increase in reactivity between the carbonyl group and amino group, but also to the greater unfolding of the protein structure, which is favored with increasing temperature (Martinez-Alvarenga and others 2014). It has been reported that under the temperature lower than 60°C, the Amadori product formed in the initial stage is rather stable (Malec and others 2002).

The maximum rate of condensation was considered to occur at weakly acidic pH (Martins and others 2000). Theoretically, high concentration of protonated carbonyl group and unprotonated amino group leads to high condensation rate (Namiki 1988). The concentration of protonated carbonyl group is higher at a lower pH, while that of the unprotonated carbonyl group is higher at a higher pH (Martins and others 2000). In a study conducted by Wang and Zhong (2014), WPI and maltodextrin also had the highest conjugation rate at weakly acidic pH (e.g., pH 6). Different from the condensation reaction, the formation of melanoidins is favored under more alkaline conditions (Ajandouz and others 2001). Wang and Zhong (2014) also found that conjugates prepared at pH 6 had a lighter color than that at pH 7. During conjugation,

pH decreased due to the formation of acids, the conversion of basic amines and the condensation reaction between free amines of protein and carbonyl groups of reducing carbohydrates (Beck and others 1990; Matmaroh and others 2006).

2.2.3 Effects of glycation on the heat stability of Protein

Many studies have indicated the improved heat stability of protein after glycation with saccharides via the Maillard reaction (Aoki and others 2001; Aoki and others 1999; Kato and others 1995; Jiménez-Castaño and others 2007; Jiménez-Castaño and others 2005a; Liu and Zhong 2013; Liu and Zhong 2012; Wang and Zhong 2014; Wang and Ismail 2012; Xu and others 2010). Kato and others (1995) found that the ovalbumin glycated with glucose 6-phosphate had very good heat stability that its solution with 5% protein concentrate did not aggregate after heated at 100°C for 10min, while the ovalbumin solution under the same condition already became very turbid. Another study conducted by this lab (1999) also showed significantly improved heat stability by conjugating the ovalbumin with glucuronic acid. However, these two conjugates showed considerable brown color development and protein polymerization. Later in another paper (2001), they found that the similar heat stability could be achieved by conjugating ovalbumin with oligogalacturonic acid, but with much less color development and protein polymerization. By conjugating WPI with Maltodextrin, Liu and Zhong (2012) found these conjugates solution (7% protein concentrate) could maintain transparent at all pH from 3 to 7 after Heated at 88°C for 2 min.

The increased steric hindrance, brought by the grafted polysaccharides, plays an important role in the improved heat stability of glycated protein. There is a positive

correlation between the heat stability of glycated protein and the molecular weight of the sugars that drafted. Lysozyme glycated with galactomannan (6000 - 24000 KDa) were much more heat stable than the one glycated with xyloglucan (1400 KDa) (Shu and others 1996). Similarly, WPI glycated with lactose and maltodextrin were much more heat stable than the one glycated with glucose (Liu and Zhong 2013).

The other changes accompanying with the conjugation might also help to explain the improved heat stability. After glycation with saccharides, the denaturation temperature of protein increased (Broersen and others 2004; Hattori and others 1997; Liu and Zhong 2013; Liu and Zhong 2012); the static repulsion was higher at certain pH, owing to the lysine reduction and the charge brought by the sugars (Aoki and others 1999; Hattori and others 1997; Liu and Zhong 2013; Liu and Zhong 2012; Wang and Zhong 2014); the surface hydrophobicity increased (Achouri and others 2005; Broersen and others 2004; Liu and Zhong 2013; Liu and Zhong 2012; Li and others 2009; Wang and Zhong 2014; Wang and Ismail 2012).

2.3 Inulin

Inulin is a naturally occurring carbohydrate used as energy reserve for more than 36,000 plants (Niness 1999; van Loo and others 1995). Among them, the main sources of inulin are chicory, dahlia and jerusalem artichoke (Dan and others 2009). Inulin is a polymer of fructans consisting of linear chain of fructosyl groups linked by β (2→1) glycosidic bonds with normally one glucopyranose unit at the reducing end, which can be symbolized as GFn, where G is the gucosyl moiety, F is the fructosyl moiety and n is

the number of fructosyl moiety (Kalyani Nair and others 2010; Barclay and others 2010). The degree of polymerization (DP) of inulin ranges from 2 to 60 (Franck 2002).

Inulin is biosynthesized from a starting molecule of sucrose. Then the fructose is attached to the relatively reactive primary hydroxyl group linked to the anomeric carbon through the methylene group of C1 of the fructose group in the sucrose substrate (Barclay and others 2010; van Loo and others 1995; Wack and Blaschek 2006). The fructose at the terminal end of inulin can be cleaved, when energy is required for the plants. When the fructose concentration is high, the enzyme can add fructose to fructose monomers creating fructose only chains (Fn) (Barclay and others 2010). So, the inulin from the natural source contains glucose, fructose, sucrose, oligosaccharides and polysaccharides with both GFn and Fn type.

Inulin is nearly exclusively processed from chicory whose inulin content can be as high as 70% at dry base (Franck 2002). The production process involves extraction, refining, evaporation and spray-drying (Franck 2002). The final product could be classified as standard inulin, high performance inulin and oligofructose. Standard inulin is a non-fractionated inulin that contain glucose, fructose, sucrose and small oligosaccharides with an average DP of 12 (Franck 2002). Among them, about 10% have a DP between 2 and 5 (Roberfroid 2005). High performance inulin is obtained by removing the oligofructose and sugars. Their DP ranges from 10 to 60 with an average of 60. While, oligofructose is obtained by partial enzymatic hydrolysis of inulin using an endo-inulinase, and its DP is from 2 to 7.

In the food industry, Inulin was usually used as fat replacer, because of its gelling properties. For instance, in low-fat dairy products such as milk drinks, fresh cheese, creams, the addition of a small amount of inulin imparts a better-balanced round flavor and a creamier mouthfeel (Franck 2002). Inulin could also give more crispness and expansion to extruded snacks and cereal (Franck 2002).

2.3.1 Inulin properties

Polymerizing by attaching fructose via β (2→1) glycosidic bonds with sucrose as the starting group, inulin is a non-reducing sugar, for the glycosidic bonds fixing the cyclic structure and sucrose it is non-reducing either (Barclay and others 2010). However, the existence of glucose, fructose and F_n type fructans, whether created by natural enzymatic processes or by hydrolysis, mean that inulin will invariably contain certain reducing capacity (Barclay and others 2010; Oliver and others 2006c). de Gennaro and others (2000) found the dextrose equivalent (DE) of standard inulin was 1.67. Oliver and others (2006c) found the total percentage of reducing sugar in inulin was around 4.1%. By removing the mon- and disaccharides, Stevens and others (2001) found the native inulin has a residual reducing power of 0.5%-2.5%.

During the hydrolysis process, inulin undergoes cleavage of the glycosidic bond. The rate of hydrolysis is high under high temperature and/or extreme pH with the presence of water. The terminal fructose units are cleaved more easily than internal ones, most likely due to the smaller change of conformation required during hydrolysis (Barclay and others 2010).

Standard inulin can solubilize in water up to 10% at room temperature, while the high performance inulin and oligofructose can solubilize up to 2.5% and 80% respectively (Franck 2002).

2.3.2 Inulin nutrition

Because of the β (2→1) configuration, inulin resists digestion in the upper gastrointestinal tract but is fermented in the colon (Roberfroid 2005). Being utilized by the bacteria in the large bowel, inulin can selectively stimulates the growth of the beneficial flora (Roberfroid 2005). In a study to determine the effects of inulin of fecal bifidobacteria in human subjects, the presence of inulin significantly increased bifidobacteria from 9.8 to 11.0 $\log_{10}$/g dry feces (Kruse and others 1999). In an ex vivo study, an increase in both bifidobacteria and lactobacilli count in the mucosa was found for the fifteen healthy volunteers who asked to supplement their diet with inulin (15 g/d) for 2 weeks (Langlands and others 2004).

Inulin was thought to increase the absorption of mineral, particularly calcium and magnesium (Roberfroid 2005). The absorption of calcium and magnesium increased about 60% for the growing rats fed diet with inulin (Delzenne and others 1995). Coudray and others (1997) found that the nine middle-aged men had significantly increased calcium absorption when they received 40 g of inulin per day. The effects of inulin on mineral absorption are likely due to its mediation towards the lower part of gut (Roberfroid 2005). Inulin transfer water into large intestine, due to its osmotic effect, and hence enhance the solubility of mineral (Mb 1998). It also reduces the colon pH,

due to the short chain carboxylic acids fermented by the microflora, and forms soluble calcium and magnesium salts of these acids (Kalyani Nair and others 2010).

Modulation of absorption or the metabolism of lipids that affect triglyceride and cholesterol is another nutritional benefit of inulin (Delzenne and others 2002). When inulin was added into the diet of saturated fat fed rats, their triglyceride content of blood and liver significantly reduced (Kok and others 1996). Another study indicated that both the VLDL production and the plasma total cholesterol were reduced for male golden Syrian hamsters that fed 16% inulin diet for five weeks (Trautwein and others 1998). The triglyceride-lowering effects of inulin were attributed to the short chain fatty acid that inhibits the hepatic fatty acid synthesis in liver via the inhibition of lipogenic enzymes in animals (Kaur and Gupta 2002). The effect of inulin toward cholesterol in animal was thought to be achieved by enhancing secretion of bile acid and reducing hepatic cholesterol synthesis (Kim and Shin 1998; Kalyani Nair and others 2010).

Dietary inulin inhibits development of cancer, especially colon cancers in animal models (Kalyani Nair and others 2010; Barclay and others 2010). Male weaning rats, fed with a diet containing 10% inulin, had significantly reduced aberrant crypt foci per colon than the control rats (Reddy and others 1997). The breast cancer growth of intramuscularly transplanted mouse tumors was significant inhibited for the one fed with inulin or oligofructose added diet (Taper and Roberfroid 1999). Several hypothetical mechanisms were proposed to explain the inhibitory effect of inulin on tumor growth. The proportions of healthier colonic microflora which compete with putrefactive and pathogenic bacteria increased and hence reduce the levels of toxin and

carcinogenic producing enzymes (Rumney and Rowland 1995). By binding with carcinogen, bifidobacteria can help to excrete it from the body (Kaur and Gupta 2002). The short chain fatty acids, the fermentation product of inulin, were also thought to attribute to its tumor inhibition (Kaur and Gupta 2002).

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials

Whey protein isolate (WPI) was provided by Davisco Foods International, Inc (Le Sueur, MN). WPI consisted of 97.5% protein (dry weight basis). Inulin with low viscosity (TIC Pretested® Inulin LV 110 Powder) was obtained from Tic Gum (White Marsh, MD).Deionized (DI) water with a minimum of 18.2 MΩ-cm was used to prepare all solutions.

3.2 Conjugates preparation

WPI and inulin powders at weight ratios of 1:1, 2:1, 4:1, 5:1 and 6:1 WPI to inulin were dissolved in DI water (with a total concentrate of 15%) with continuous stirring for at least 2 h at room temperature (22 ± 2°C) and stored at 4 °C overnight to allow for full hydration. The solutions were adjusted to pH 7 using 1 N HCl and frozen at -18°C for 24h before freeze drying (Genesis 25 Super ES Freeze Dryer, SP Scientific, PA).

The freeze-dried mixtures were incubated at 70, 75 and 80°C for 12, 24, 48 and 72h with or without controlling the relative humidity. The relative humidity was controlled to 44% or 80% using saturated solutions of K_2CO_3 or KCl in sealed desiccators. As control, WPI was individually dry-heated in a similar manner. After incubation, all samples were stored at -18°C until further used in the following measurements.

3.3 Visual observation

Conjugate solutions were prepared in deionized water and hydrated overnight at 4°C. The pH of solutions was adjusted to 6.00 ± 0.02 using 0.1 N and 0.01 N and the final protein concentration was 6% w/w.6 ml sample was then transferred to 10 ml vial and heated in a temperature-controlled water batch at 85°C for 15 minutes. The visual appearance of samples was compared by photographing.

3.4 Amadori compounds and available free amino groups

The degree of conjugation of WPI-inulin mixtures was determined by measuring the contents of Amadori compounds and free amino groups. The measurement of Amadori compounds adapted from the method outlined by Zhu and others (2008). Conjugates and unheated WPI were dissolved in DI water at 2 mg/ml and centrifuged at 6,000 RPM for 15 min and at 4°C (Allegra X12, Beckman Coulter, Fullerton, CA). The absorption of the supernatant was measured at 304 nm (Cary 50 Scan, Varian, Palo Alto, CA) in a 1 cm quartz cell.

The quantification of free amino groups was determined using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method (Tang and others 2011) with modification. Sample (2.5 mg/ml) was prepared in 50 mM phosphate buffer (pH 8.5) containing 50 NaCl and mixed with 0.1% TNBS solution at 1:1 volume ratio. The resultant mixtures were incubated in a 60°C water bath for 2 h and cooled to room temperature. One ml of 10% SDS and 0.5 ml of 1.0 M HCl were added to stop the reaction. The absorbance of the final mixtures was measured at 335 nm (Cary 50 Scan, Varian, Palo Alto, CA) against the reagent blank. The absorbance of unheated WPI-Inulin mixture was defined as 100%

free amino groups, and the degree of glycation was calculated by percent decrease in absorbance relative to that of unheated WPI-Inulin Mixture. All experiments were replicated at least three times.

3.5 Particle size and turbidity

Heat stability was determined by measuring the turbidity and particle size of heated solutions. The turbidity was measured at 630 nm using an ultra-microplate reader (BioTek Instruments Inc., Winooski, USA). The particle size of heated samples (0.3% w/w diluted with 10 mM phosphate buffer at pH 6.0) was measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with 633 nm laser and 173° detection optics. Each measurement was the average of three measurements, and the whole experiment was replicated three times.

3.6 Zeta Potential

Zeta potentials of unheated WPI and conjugates samples were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). WPI or conjugate powders were prepared in DI water at a protein concentration of 5 mg/ml and pH 6.00 ± 0.02. Average values of six measurements were reported for each sample, and three replicates were tested for each sample.

3.7 Differential Scanning Calorimetry (DSC)

Thermal denaturation properties of conjugates were monitored using a differential scanning calorimeter (DSC) (Model DSC 7, Perkin-Elmer Instruments, Shelton, CT, USA) from 30 to 110°C at a scan rate of 5°C/min. Conjugates and WPI were dissolved in 100 mM phosphate buffer (pH 7) at 20% W/W protein. Samples (15 ml) was

pipetted into the aluminum pans (Perkin-Elmer) and hermetically sealed. An empty crimped aluminum pan was used as control. Three replicates were tested for each sample.

3.8 Viscosity measurement

The viscosity of heated WPI and conjugate solutions was measured by a Kinexus Pro rheometer (Malvern Instruments Ltd., Worcestershire, United Kingdom). The flow curve was obtained by monitoring shear stress as a response to the shear rate increased from 0.1 - 200 S^{-1}. All tests were performed at 25°C using cone-plate geometry (40 mm) and a constant gap of 0.05mm. Flow behavior was described using the Power Law model:

$$\tau = K \cdot \dot{y}^n$$

Where K is the consistency coefficient ($pa.S^n$) and the exponent n is the flow behavior index. Apparent viscosity values between different samples were compared at a shear strain rate of 50 S^{-1}.

3.9 Statistical Analysis

All statistical analyses were performed using SPSS software (SPSS Inc., VER. 20, Chicago, IL). One-way ANOVA was conducted to compare the mean values and determine the differences between treatments. Significant differences were obtained by the Turkey-Kramer test at a significance level of 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Visual observation

The effects of temperature (T), relative humidity (RH), weight ratio (WR) of whey protein to inulin, and incubation time (t) were systematically investigated, in order to determine the appropriate conditions. The visual appearances of the conjugate solutions heated at 85°C for 15 minutes were evaluated (Figure 2). Ideally, the optimum glycosylation condition is when glycation between protein and saccharide occurs (e.g., formation of Amadori compounds) but the glycated protein does not undergo subsequent reaction in order to prevent dark color formation, flavor change and loss of solubility (Jiménez-Castaño and others 2005b). In this study, the optimum condition refers to the one that has significantly increased heat stability without extensive browning.

As shown in Figure 2, all the control samples (WPI-inulin mixtures at respective weight ratios without incubation) became opaque after heating at pH 6.0, while the conjugates were more transparent with slight yellowish to dark brownish color. There is a clear trend of the effects of WPI-inulin ratio, temperature, incubation time and relative humidity. Decreasing WPI-inulin ratio (e.g. increasing inulin content) and increasing temperature, incubation time, as well as relative humidity resulted in conjugates with increased heat stability and degree of browning, indicating a correlation

between Maillard reaction and improved heat stability. Dry heating at temperature below 70°C did not improve heat stability of the WPI-inulin mixture within the time range we used (data not shown). The minimum dry heating temperature was in agreement with the results reported by Oliver and others (2006c) that glycation between caseinate and inulin (1:1 weight ratio) was slow at 60°C and 80% RH. Glycation between whey protein and other polysaccharides, i.e., dextran or maltodextrin, were reported to require lower temperature or shorter time (Liu and Zhong 2013; Liu and Zhong 2012; Zhu and others 2008). This is probably due to the low content of reducing sugar, high themostability and low solubility of inulin, compared to other saccharides (Oliver and others 2006c; van Boekel 2001). As the Inulin content increased, the color of the conjugates and their heated solutions became much darker because of the higher number of reactive carbonyl groups (Kroh and others 1996). Similarly, formation of brown pigments was greatly accelerated when the relative humidity was controlled at 44% and 80%. These results were consistent with the early report that the Maillard reaction rate increased gradually with increased water activity from 0.3 to 0.8 (Ames 1992). In addition, the reducing sugars created during the hydrolysis of inulin, which happens when heating in the presence of water, could also contribute to the increased reaction rate. However, since the RH-controlled conjugates became very dark before achieving similar heat stability as no RH-controlled samples, the increased moisture content seems to favor the advanced Maillard reaction (majorly responsible for the color development) more than the early stage. A similar finding was discovered by Ge Pan and Melton (2007) that the color formation was high while the formation of

furosine (derivative of Amadori compounds formed during HPLC analysis) is slow at high RH. At the 80% RH, heated conjugate solutions either turned very dark and viscous or showed phase separation. The insoluble compounds formed at 80% RH were probably due to the excessive polymerization of proteins during the advanced Maillard reaction (Oliver and others 2006b).

After stored at 4°C for more than 24 h, conjugate solution with high inulin content (e.g. 1:1 WPI-inulin weight ratio) formed a white precipitate (not shown in pictures). White precipitate was also found in the caseinate-inulin conjugate dispersion during storage and was attributed to the poor solubility of inulin (Oliver and others 2006c). In another study, white precipitate formed after the WPI-dextran conjugate solution being centrifuged and this was thought to be the self-association of dextran (Zhu and others 2008). Since the inulin had both relatively low solubility and self-associate behavior (Dan and others 2009), it is reasonable to consider the white precipitate was due to the aggregation of Inulin.

Conjugates prepared at 80°C, 2:1, 4:1 and 6:1 WR without controlling the relative humidity were closest to our requirement for improved heat stability without significant color development and loss of solubility. Thus, these conjugates were selected for further analysis to determine properties of the conjugates and their mechanism in improving het stability

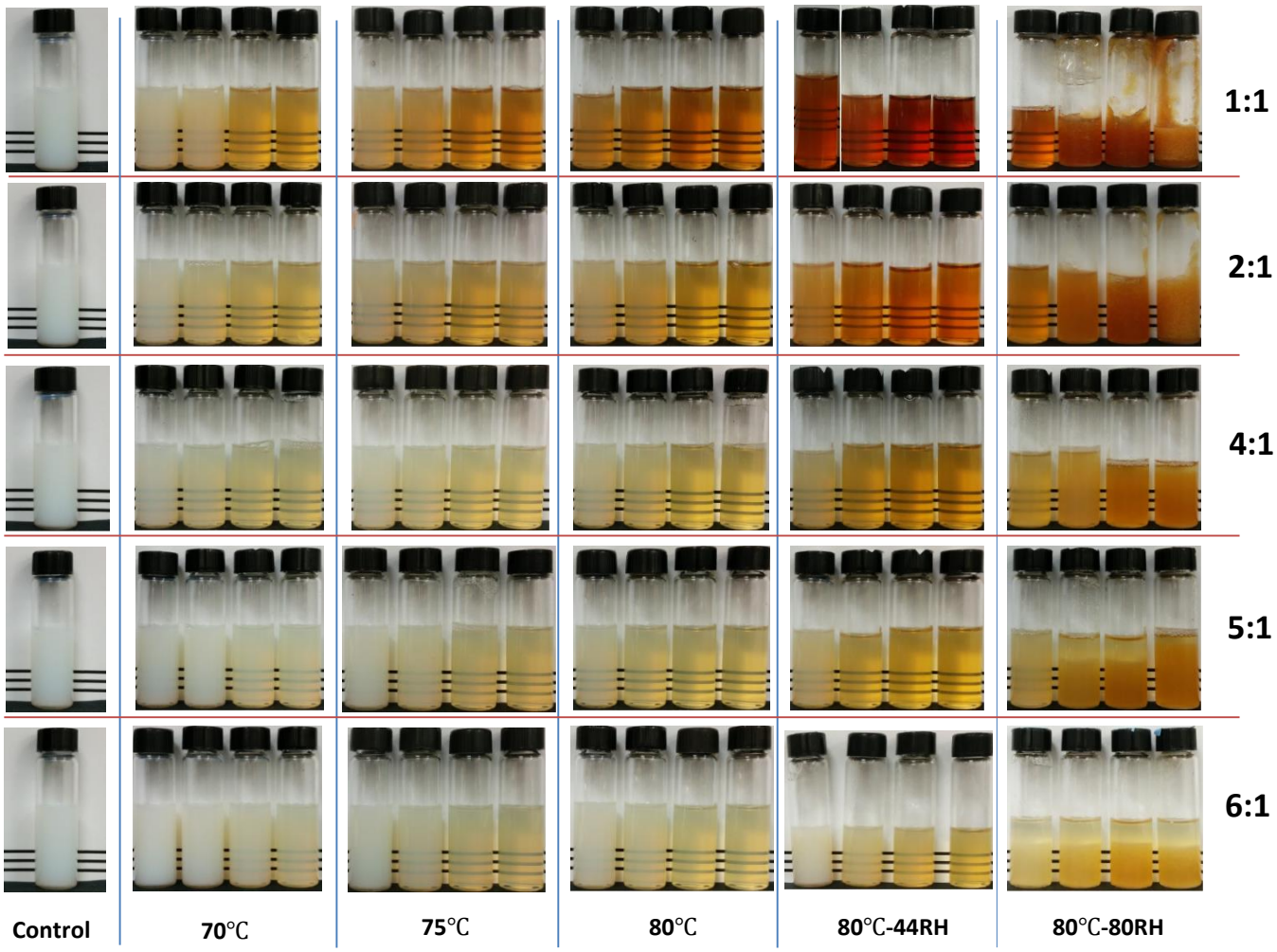

Figure 2. WPI-inulin conjugate solutions (6% w/w protein, pH 6.00) prepared at different dry-heating conditions after heating at 85°C for 15 min. Left: WPI-inulin mixtures at respective weight ratios. Vials from left to right in each image refer to 12, 24, 48, 72h incubation time.

4.2 Degree of glycation

Amadori compounds

During the initial stage of the Maillard reaction, reducing carbonyl groups condenses with free amino groups, forming a Schiff base and the Schiff base then undergoes an irreversible Amadori rearrangement to from the Amadori compounds (Oliver and others 2006a; Liu and others 2012). The peak at 304 nm was presumably assigned to the Schiff base formation (Heinert and Martell 1963; Blonski and others 1997). Absorbance at 304 nm has been utilized to detect the Schiff base formed in WPI-dextran conjugates and bovine lactoferrin-glucose or fructose conjugates (Zhu and others 2008; Wang and Ismail 2012; Joubran and others 2013).

As shown in Figure 3, all the unheated WPI and WPI-inulin mixture had similar absorbance values, which indicated that inulin had very little or no absorption at 304 nm. After dry-heating, WPI did not show significant change in the absorbance ($p > 0.05$). For the conjugates, the amount of Amadori compounds gradually increased during the incubation. At the first 24 h, the conjugates prepared at 6:1, 4:1 and 2:1 weight ratio (CJ 6:1, CJ 4:1, and CJ 2:1) all had significantly higher content of Amadori compounds than the unheated ones. The amount of the Amadori compounds of CJ 2:1 continued to increase significantly with incubation time up to 72h, while those of CJ 4:1 and CJ 6:1 did not increase after 24 h ($P > 0.05$). The Amadori compounds were unstable at the temperature as high as 80°C, and will degrade to form more advanced Maillard product (Malec and others 2002). No significant increase in Amadori compounds of CJ 4:1 and 6:1 after 24h was probably due to the limited carbonyl groups and the conversion of

Amadori compounds into advanced Maillard product began to be equal to its formation (Jiménez-Castaño and others 2005b; Wang and Ismail 2012).

Available amino groups

The percentage of available amino groups in WPI-inulin conjugates is shown in Figure 4. The content of available amino groups in unheated WPI was used as reference (100% available amino groups). Unheated WPI and WPI-inulin mixtures at different weight ratios showed similar absorbance value at 335 nm (data not shown), indicating that their initial available amino groups were similar as expected. The available amino groups began to decrease as the incubation time increased, and higher inulin content led to faster reduction. Similar to the results of Amadori compounds, conjugates prepared at 2:1 WR and 72 h dry-heating had the lowest available amino groups (81.8%). This level of available amino groups was relatively high compared to the results reported in the literatures (only 30-65.6% available amino acids) after WPI or β-Lactoglobulin glycation with saccharides (Chevalier and others 2002; Jiménez-Castaño and others 2005b; Martinez-Alvarenga and others 2014).

The relatively high residual amino groups shown in Figure 4 could be due to the uncontrolled relative humidity and the low content of reducing sugar in inulin. Typically, high relative humidity leads to higher diffusion of reactants and accelerates protein-saccharide conjugation and protein polymerization, which lead to higher amino group blockage (Jiménez-Castaño and others 2005b; Malec and others 2002; Martinez-Alvarenga and others 2014). By controlling the relative humidity to 80%, WPI and maltodextrin mixture (1:3 weight ratios) had only 48% available amino groups after

heated at 60°C for 48h (Martinez-Alvarenga and others 2014). In another study, conjugation between β-lactoglobulin and dextran at 55°C and 65% RH for 14 d had only 30% available amino groups left (Jiménez-Castaño and others 2005b). Being consistent with their results, the samples we prepared at 44% and 80% relative humidity had very dark color and some even became insoluble, which were the indicator of advanced Maillard reaction and protein polymerization. Thus, the relatively low amino group blockage could be partially explained by the non-controlled relative humidity during conjugate preparation, which limit the reaction rate and prevents further polymerization. The ratio between protein and sugars is another important factor for the blockage of amino groups. As a polymer of fructans consisting of β (2→1) fructosyl fructose units with one glucopyranose unit at the reducing end (Stevens and others 2001), inulin is ostensibly a non-reducing sugar (Barclay and others 2010; Stevens and others 2001). However, the existence of glucose, fructose and Fn type fructans, created whether by natural enzymatic process or partially hydrolysis, means inulin has certain reducing capacity (Barclay and others 2010). The total percentage of reducing sugars in inulin was about 4.1% (Guan and others 2006). Owing to its low chemical reactivity and steric hindrance, inulin was even added in the caseinate-fructose mixture to lower the Maillard reaction (Oliver and others 2006c; Oliver and others 2006b). So, the low reducing of inulin also contributed to the relative low amino blockage.

Based on the results of Amadori compounds and the available amino groups, it can be concluded that conjugation at 80°C led to a quick formation of the initial products of Maillard reaction and the no RH-controlled condition was able to reduce the

protein polymerization. One major concern about glycation has been the loss of lysine which is the main amino group lost during the reaction, thus reaction condition that leads to limited amino blockage is highly desirable. Owing to the low content of reducing sugars in inulin, its Maillard reaction between whey proteins could be well manipulated.

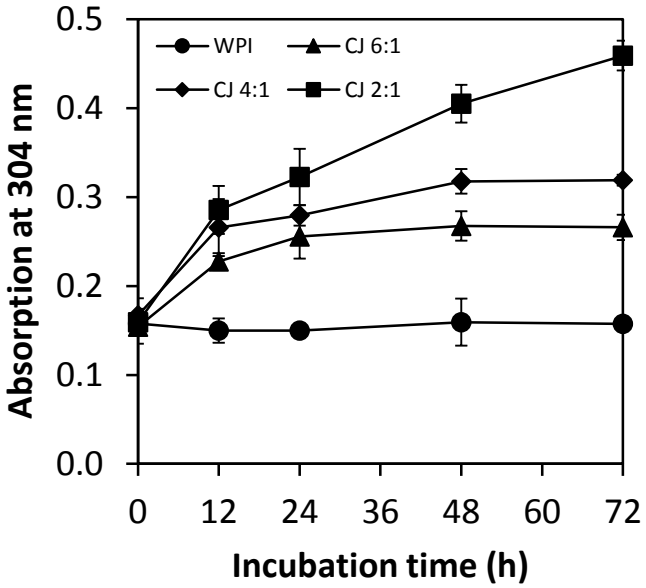

Figure 3. Formation of Amadori compounds monitored at 304 nm in whey protein isolate (WPI) and WPI-inulin conjugates (CJ) incubated at 80°C for 0-72h. 6:1, 4:1 and 2:1 refers to the mass ratio of WPI to Inulin. Error bars are standard deviations from triplicate measurements.

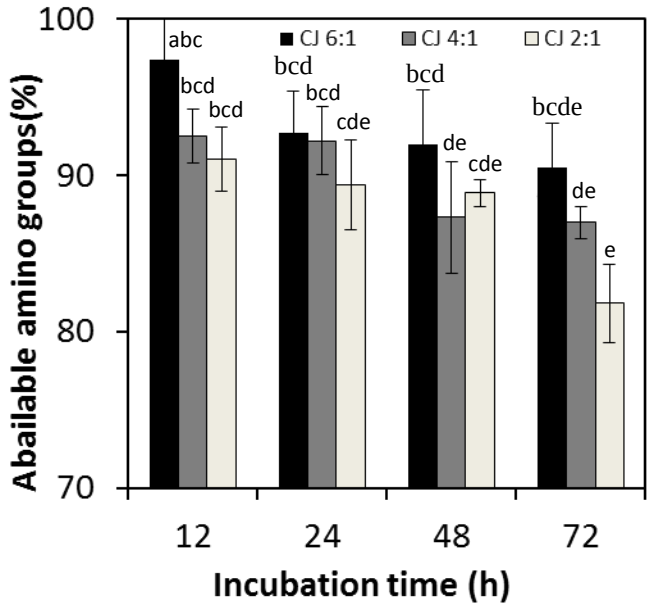

Figure 4. Available amino groups (%) of conjugates after heating at 80℃ for 12 to 72h. Different letters above each column represent significant difference in mean ($p < 0.05$)

4.3 Heat stability

Heat stability of WPI and conjugates were quantified by measuring their turbidity and particle size after heated 6% w/w protein solutions (pH 6) at 85°C for 15 minutes. As shown in Figures 5 and 6, without dry-heating, WPI and WPI-inulin mixtures turned turbid with large particle sizes after heating. With 12 h dry-heating, all conjugate samples showed a drastic decrease in both turbidity and particle size, especially when compared to WPI. Turbidity of the heated conjugate solutions continued to decrease after 24 h dry-heating ($p < 0.05$); however, prolonged incubation (48 and 72 h) did not show significant difference. A630 values were similar ($p > 0.05$) among different conjugates when compared at the same incubation time. Similar to the turbidity results, 12 h dry-heating resulted in a drastic decrease in particle sizes of heated conjugate solutions. Particle sizes continued to decrease with increasing incubation time up to 48 h. There appeared to be an effect of weigh ratio with CJ 2:1 showing the smallest particle sizes at 24, 48 and 72 h incubation. The z-average diameters of heated solutions of CJ 2:1, 4:1, and 6:1 were 86, 66, and 63 nm smaller than those of heated WPI solutions after 24 h dry-heating and 115, 85, and 85 nm after 72 h. The difference between the turbidity and particle size results after 24 h dry-heating was probably due to the lack of sensitivity in differentiating samples having particle size smaller than 94 nm. When plotting the Amadori compounds or available amino groups against particle size, linear relationships could be established as followed (R^2=0.73 and 0.81 respectively) (Figure 7):

$$\text{Particle size} = -422.46(\text{Amadori compounds}) + 223.12 \quad (1)$$

Particle size =7.52 (available amino groups)-590.52 (2)

There are several changes that could possibly explain the improved heat stability of protein after glycation with saccharides: increased steric hindrance, changes of conformation and surface charge. The grafted polymer can provide steric hindrance against aggregation if it could extent in the continuous phase (Israelachvili 2011). Steric hindrances were regarded as the dominant mechanism enabling the improvement in heat stability of glycated protein (Liu and Zhong 2013; Liu and Zhong 2012). In their studies, the net surface charge of conjugates increased at certain pH and the denaturation temperature increased from 79 to 92°C, however, the WPI-maltodextrin conjugates remained clear when heated at pH 3 to 7 and temperature well above the denaturation temperature, which indicated the trivial effects of the changes of surface charge and the increased denaturation temperature. The effects of steric hindrance could also be indirectly demonstrated by the positive correlation between the length of grafted saccharides and the heat stability of the conjugates (Liu and Zhong 2013; Shu and others 1996). Lysozyme had much better heat stability when they conjugated with galactomannan (3.5-24 KDa) than with xyloglucan (1.4 KDa)(Shu and others 1996). In another study, conjugates of WPI and maltodextrin (1 KDa) were more heat stable than glucose and lactose (Liu and Zhong 2013).

In some studies, improved heat stability of conjugates was mostly attributed to the conformational changes (Wang and others 2013; Wang and Ismail 2012). As shown by the measurements of surface hydrophobicity and sulfhydryl groups, WPI glycated with dextran had much slower rate of exposing their internal -SH and hydrophobic

groups during heating than untreated WPI. They also found Denaturation temperature of β-lactoglobulin and α-lactalbumin increased by 15.5°C and 18.7°C after partially glycation. In some cases, increased surface charge also plays an essential role in improving heat stability of glycated protein (Kato and others 1995; Aoki and others 1999). By glycating with glucuronic acid, ovalbumin had much better improved heat stability compared to ovalbumin glycated with glucose due to the ability of replacing the lysine residues with the anionic carboxyl groups.

In the next sections, we will test the zeta potential and Denaturation properties of conjugates in order to find the reasons for the improved heat stability.

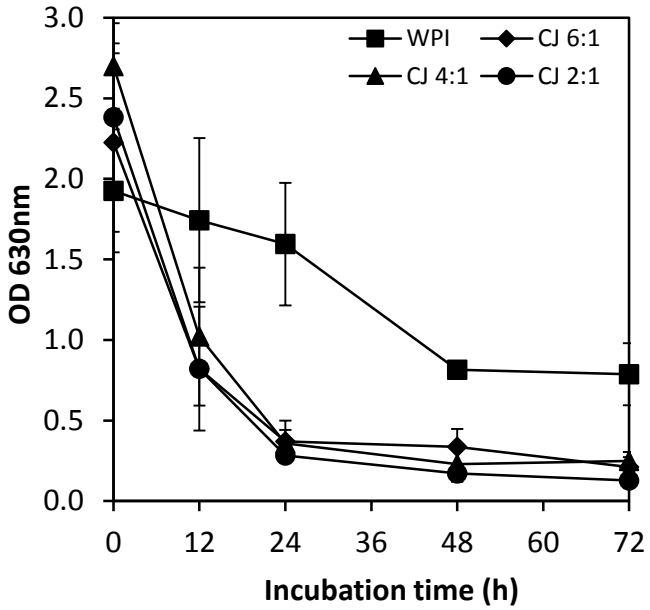

Figure 5.Turbidity of whey protein isolates (WPI) and WPI-inulin conjugates (CJ) solutions (6% w/w protein concentrate, pH 6.00) after heated at 85°C for 12min. WPI and CJ were incubated at 80°C for 0-72h. 6:1, 4:1 and 2:1 refers to the mass ratio of WPI to Inulin. Error bars are standard errors from duplicate measurements.

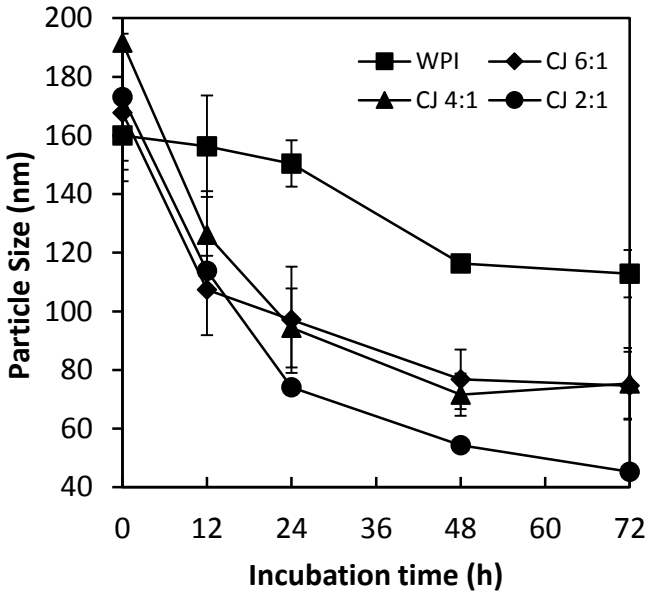

Figure 6. Particle size of whey protein isolates (WPI) and WPI-inulin conjugates (CJ) solution (6% w/w protein concentrate, pH 6.00) after heated at 85°C for 12min. The WPI and CJ were incubated at 80°C for 0-72h. 6:1, 4:1 and 2:1 refers to the mass ratio of WPI to Inulin. Error bars are standard errors from triplicate measurements.

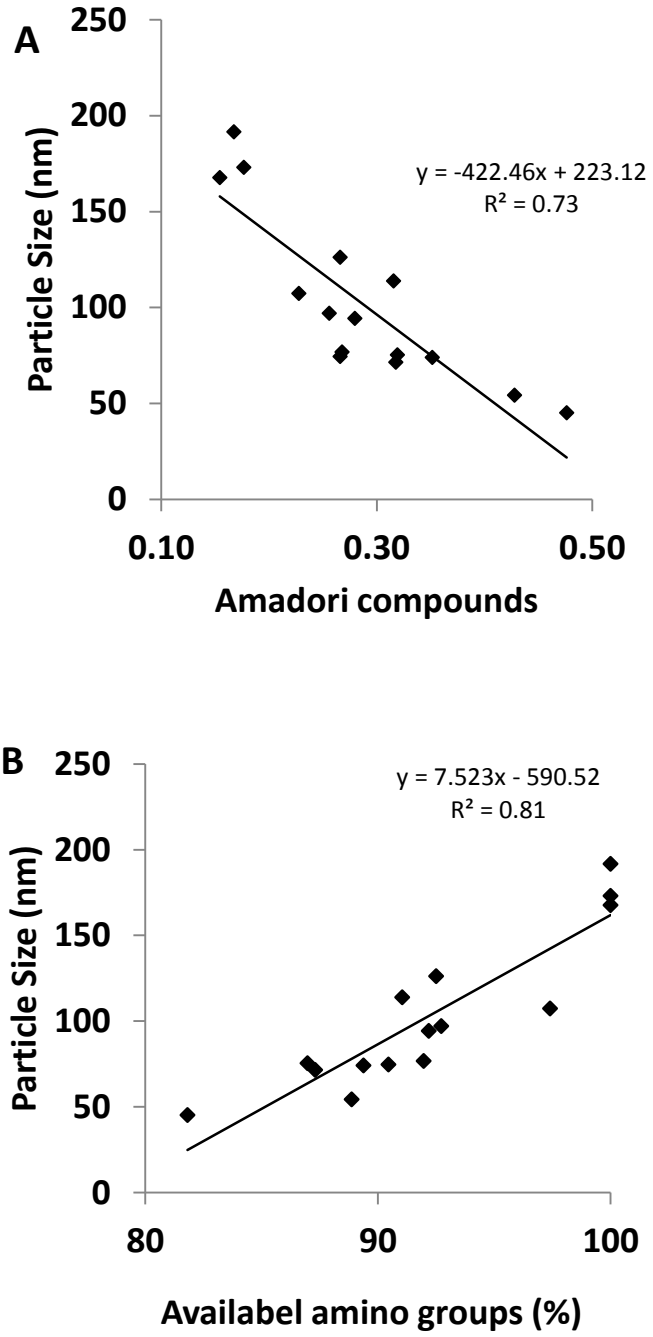

Figure 7. Correlation between particle size and Amadori compounds (A) or available amino groups (B).

4.4 Viscosity

Viscosity of protein solution after heating is another important factor determining heat stability of protein. No or minimal increase in viscosity indicates higher stability and is desirable especially in beverage applications. As shown in Table 1, heated WPI solutions (6% w/w at pH 6) showed Newtonian behavior with the apparent viscosity of 1.7 mPa.s at 50 S^{-1}. Glycation at all weight ratios and incubation time did not significantly affect flow behaviors of heated conjugate solutions as demonstrated by the consistency coefficient (K), flow behavior index (n) and the apparent viscosity (50 S^{-1}).

Proteins were reported to become significantly more viscous and pseudoplastic after glycation with saccharides (Baniel and others 1992; Corzo-Martínez and others 2010; Martinez-Alvarenga and others 2014; Oliver and others 2006b; Oliver and others 2006c; O'Regan and Mulvihill 2009; Paraman and others 2007). For example, after glycation with galactose, sodium caseinate had a 4.9-fold higher consistency factors (K) and its flow behavior index (n) decreased from 0.96 to 0.73 (Corzo-Martínez and others 2010). The increased molecular size, owing to the attached sugars, was thought to be partially responsible for the changes in flow behavior (Baniel and others 1992; Corzo-Martínez and others 2010; O'Regan and Mulvihill 2009; Ge Pan and Melton 2007). According to Paraman and others (2007), rice protein isolate glycated with xanthan gum was significantly more viscous than the one glycated with glucose, indicating the effect of molecular size on the viscosity of conjugates. The advanced Maillard reaction and the accompanying crosslinking of proteins were also thought to attribute to the changes in flow behavior (Corzo-Martínez and others 2010; Oliver and others 2006b). As reported

by Corzo-Martínez and others (2010), sodium caseinate (SC) did not show significant changes in the flow behavior after glycation with dextran, while became more pseudo-plastic and viscous after glycation with lactose and galactose. Although dextran had much larger molecular weight than lactose and galactose, it was proposed that the small effect of dextran on rheological properties of SC was due to the limited extent of the Maillard reaction, and the high reactivity of galactose and lactose accelerated the polymerization of SC and thus changed the flow behavior of SC. We also observe a much increase in viscosity in glycated samples under controlled humidity (data not shown) which could be caused by excessive protein crosslinking.

Table 1

Viscosity measurements on WPI and Conjugates solution (6% w/w protein concentrate, pH 6.00) after heated at 85°C for 15min.

Treatment	n[a]	K (Pa s^n)[a]	Apparent Viscosity($50S^{-1}$)
WPI-0h[b]	1.00429 ± 0.03928 a	0.00175 ± 0.00015 a	0.00173 ± 0.00015 a
WPI-12h	0.94983 ± 0.06189 a	0.00194 ± 0.00062 a	0.00153 ± 0.00028 a
WPI-24h	0.94299 ± 0.09083 a	0.00199 ± 0.00065 a	0.00154 ±0.00009 a
WPI-48h	0.97875 ± 0.06691 a	0.00183 ± 0.00019 a	0.00159 ±0.00021 a
WPI-72h	0.98177 ± 0.12655 a	0.00203 ± 0.00070 a	0.00172 ± 0.00019 a
CJ 6:1-0h	0.94349 ± 0.05309 a	0.00199 ± 0.00026 a	0.00156 ± 0.00007 a
CJ 6:1-12h	0.95501 ± 0.05248 a	0.00193 ± 0.00035 a	0.00158 ± 0.00003 a
CJ 6:1 -24h	0.98002 ± 0.05971 a	0.00179 ± 0.00043 a	0.00158 ± 0.00005 a
CJ 6:1-48h	0.94422 ± 0.04945 a	0.00229 ± 0.00084 a	0.00175 ± 0.00034 a
CJ 6:1-72h	0.97305 ± 0.04910 a	0.00184 ± 0.00032 a	0.00162 ± 0.00012 a
CJ 4:1-0h	0.92468 ± 0.03013 a	0.00209 ± 0.00023 a	0.00154 ± 0.00004 a
CJ 4:1-12h	0.91144 ± 0.03548 a	0.00227 ± 0.00035 a	0.00160 ± 0.00008 a
CJ 4:1-24h	0.95520 ± 0.05664 a	0.00181 ± 0.00048 a	0.00147 ± 0.00012 a
CJ 4:1-48h	0.90523 ± 0.02008 a	0.00241 ± 0.00044 a	0.00166 ± 0.00025 a
CJ 4:1-72h	0.91273 ± 0.00903 a	0.00251 ± 0.00045 a	0.00177 ± 0.00022 a
CJ 2:1-0h	0.94307 ± 0.03628 a	0.00207 ± 0.00032 a	0.00163± 0.00008 a
CJ 2:1-12h	0.98134 ± 0.02235 a	0.00199 ± 0.00020 a	0.00181 ± 0.00010 a
CJ 2:1-24h	0.93484 ± 0.01350 a	0.00213 ± 0.00009 a	0.00163 ± 0.00003 a
CJ 2:1-48h	0.94967 ± 0.04534 a	0.00227 ± 0.00058 a	0.00180 ± 0.00025 a
CJ 2:1-72h	0.95565 ± 0.04462 a	0.00218 ± 0.00042 a	0.00178 ± 0.00016 a

[a] n and K are the powder law model parameters: flow behavior index and consistency coefficient

[b] Each value is an average of three samples ± standard deviation. The means followed by different online letters in the same column are significantly different ($p < 0.05$) by Turkey-Kramer test. WPI means whey protein isolate. Numbers of CJ correspond to mass ratio between WPI and inulin, and dry heating time at 80°C, respectively.

4.5 Zeta potential

Zeta potential measurement was used to investigate the surface charge properties of the particles. The surface charge of proteins has very important effect on their functional properties including heat stability. As shown in Figure 8A, the zeta potential of WPI solution at pH 6 was -14.8 mV, and addition of inulin (no dry-heating) did not affect the zeta potential except at 2:1 weight ratio which showed lowered negative charge for unclear reason. Dry-heated WPI did not affect its zeta potential ($p > 0.05$). Glycation with inulin led to a significant increase in zeta potential, and the conjugates became significantly more negative as the incubation time increased. After 72 h incubation, the zeta potentials of CJ 2:1, CJ 4:1, and CJ 6:1 were -16.8, -17.8, and -19.0 mV which corresponded to the net increase of 5.6, 3.3, and 4.1 mV, respectively. During glycation, saccharides will attach to the lysyl residues of the proteins. Since lysine with pK of 10.6 is a major contributor of positive charge, the glycated protein will have increased net negative charge. As shown in Figure 8B, the difference in zeta potential of untreated WPI at pH 6 and 7 was about 9 mV. This difference in zeta potential results in very different properties of the heated protein solutions with clear solution at pH 7 and very turbid solution at pH 6. Therefore, even though the differences in zeta potential between unheated WPI-inulin mixtures and conjugates were small, the increased net negative charge could be part of the mechanisms behind increased heat stability of the conjugates.

Zeta potentials of WPI and conjugates were also measured from pH 3 to 7 to investigate the change in isoelectric point (pI) (Figure 8B). Compared to WPI, conjugates

after 72 h incubation were more negatively charges across the pH values studied. As discussed, this could be due to reduced lysyl content from glycation. Studies have reported that slight shift of pI of glycated protein were due to the blockage of lysine, which are otherwise protonated at a pH lower than their pKa values (Liu and Zhong 2013; Liu and Zhong 2012; Martinez-Alvarenga and others 2014; Tang and others 2011; Xu and others 2010).

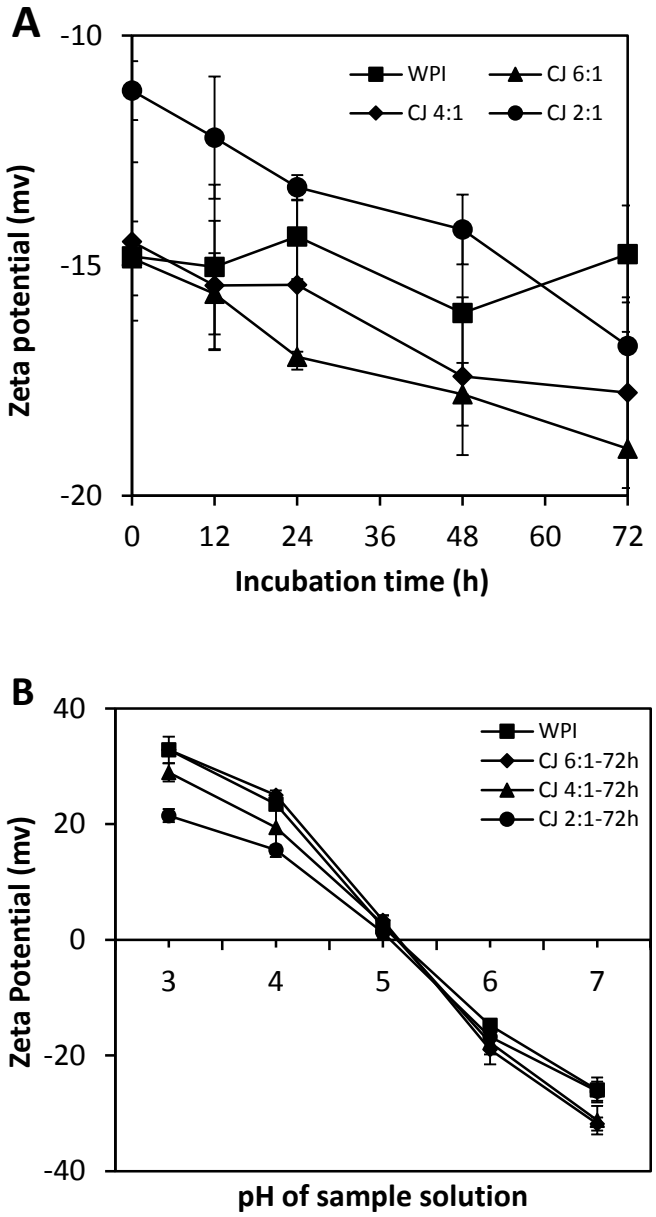

Figure 8. Zeta potential of Whey protein isolate (WPI) and WPI-inulin conjugates (CJ) at PH 6 after incubated at 80°C for 0-72h (A) and zeta potential of WPI and CJ at PH 3-7 after incubated at 80°C for 72h (B) . 6:1, 4:1 and 2:1 refers to the mass ratio of WPI to Inulin. Error bars are standard errors from triplicate measurements.

4.6 Differential Scan Calorimetry (DSC)

Thermal denaturation properties are important to understand the aggregation of whey protein during heating and are usually measured by DSC (McGuffey and others 2005). The area under the curve of denaturation profiles (figures not shown) was used to determine the denaturation enthalpy (ΔH), whereas the temperature corresponding to the peak center of the curve was referred to as the denaturation temperature (T_d) after the polynomial baseline fitting. The determined ΔH and Td values are listed in Table 2.

The Td of untreated WPI was around 71.2°C, which was in the range reported in the literatures (Medrano and others 2009; Wang and Ismail 2012). The T_d of WPI increased after glycation with inulin with CJ 2:1 having higher Td than CJ 6:1, indicating increased stabilizing effect of the protein from conjugation at higher inulin content. Similar results were observed in WPI glycation with maltodextrin which showed increased T_d (Liu and others 2012). However, compared to previous studies, the increase in T_d shown in this study was relatively small (Broersen and others 2004; Liu and Zhong 2013; Liu and Zhong 2012; Medrano and others 2009; Wang and Zhong 2014; Wang and Ismail 2012; Xu and others 2010). For example, Wang and Ismail (2012) found the denaturation temperature of WPI increased from 68°C to 85°C at pH 7 after glycation with dextran. Wang and Zhong (2014) observed 5-7°C increase in the denaturation temperature of WPI after Glycation with maltodextrin. Other than the differences between the material and methods, the relatively low degree of glycation could partially explain the small increase of T_d. Glycation with inulin led to a decrease in ΔH from 6.9 to

2.8 J/g, which was consistent with the results reported by Medrano and others (2009), Wang and Ismail (2012) and Wang and Zhong (2014). The reduction of ΔH was thought to be caused by partially unfolding of tertiary structure induced by glycation (Wang and Ismail 2012). However, some studies reported an increase in ΔH of proteins after glycation with sugars (Liu and Zhong 2013; Liu and Zhong 2012; Xu and others 2010).

Table 2
Denaturation temperature (Td) and enthalpy change (ΔH) of WPI and conjugates dispersion at pH 7.0

Sample	T_d (°C)	ΔH (J/g)
WPI	71.21 ± 0.04^a	6.90 ± 0.54^a
CJ 6:1-72h	71.82 ± 0.03^b	3.60 ± 0.54^b
CJ 2:1-72h	73.60 ± 0.08^c	2.85 ± 0.86^b

Numbers are mean ± standard deviation from triplicate measurements. Different superscript letters in each column represent significant difference in mean ($p < 0.05$)

CHAPTER 5

CONCLUSION AND FUTURE WORK

Improved heat stability of whey protein at pH closer to pI is highly desirable in protein applications, especially in beverage industry. This research demonstrated that heat stability of WPI can be improved by glycating with inulin. Consistent with previous results, degree of glycation increased as the heating temperature, weight ratio between WPI and inulin, incubation time and relative humidity increased. When the relative humidity was controlled at 44 or 80% during incubation at 80°C, the WPI-inulin conjugates had excessive browning and some became insoluble particulates. However, the reaction can be optimized such that heat stability was achieved at early stages of the Maillard reaction without proceeding to form undesirable polymerized products. Significant improvement in heat stability can be achieved by incubating the freeze-dried mixtures at 80°C for 12 - 72 h without controlling relative humidity. Improved heat stability was shown by a reduction in turbidity (e.g., 62 to 95 % reduction in A630) and 36 to 74% decrease in particle size without significant change in flow behavior. Maillard reaction was also evident from an increased amount of Amadori compounds and a slight change in color. However, under controlled reaction, the loss of available amino group ranged from 3 to 18% which was considerably low. Improved heat stability of glycated WPI could be explained by increased net negative charge and increased denaturation temperature of WPI. Increased net negative charge can be due to the blockage of

lysine, while increased denaturation temperature could be attributed to the alterations of secondary and tertiary structures of WPI.

Maillard reaction between protein and polysaccharides has a great potential in improving protein heat stability. By manipulating the incubation conditions, such as heating temperature, incubation time, weight ratio between reactants and relative humidity, and selecting appropriate saccharides, the Maillard reaction can be controlled to increase the heat stability of protein without the formation of advanced Maillard reaction products. Having relatively higher molecular weight and limited reducing capacity, inulin is an appropriate saccharide to optimize the reaction. In addition, health benefits of inulin as prebiotic are well accepted. Thus, WPI-inulin conjugate has great potential to be utilized as important food ingredient that provides both technological and health benefits. For future study, the digestion of WPI-inulin conjugates should be investigated to evaluate the effects of glycation on prebiotic properties of inulin and digestibility of WPI. Other functional properties including foaming, gelation and emulsifying properties of WPI-inulin conjugates could also be studied to expand their potential applications in food products.

REFERENCES

Achouri A, Boye JI, Yaylayan VA, Yeboah FK. 2005. Functional Properties of Glycated Soy 11S Glycinin. Journal of Food Science 70(4):C269-C74.

Affertsholt T. 2009. International Whey Market Overview. 2009 ADPI/ABI Annual Conference. Chicago.

Ajandouz Eh, Tchiakpe Ls, Ore FD, Benajiba A, Puigserver A. 2001. Effects of pH on Caramelization and Maillard Reaction Kinetics in Fructose-Lysine Model Systems. Journal of Food Science 66(7):926-31.

Ames JM. 1992. The Maillard Reaction. In: Hudson BJF, editor. Biochemistry of Food Proteins. Boston, MA: Springer US. p. 99-153.

Aoki T, Hiidome Y, Kitahata K, Sugimoto Y, Ibrahim HR, Kato Y. 1999. Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. Food Research International 32(2):129-33.

Aoki T, Hiidome Y, Sugimoto Y, Ibrahim HR, Kato Y. 2001. Modification of ovalbumin with oligogalacturonic acids through the Maillard reaction. Food Research International 34(2–3):127-32.

Baier SK, McClements DJ. 2005. Influence of Cosolvent Systems on the Gelation Mechanism of Globular Protein: Thermodynamic, Kinetic, and Structural Aspects of Globular Protein Gelation. Comprehensive Reviews in Food Science and Food Safety 4(3):43-54.

Baniel A, Caer D, Colas B, Gueguen J. 1992. Functional properties of glycosylated derivatives of the 11S storage protein from pea (Pisum sativum L.). Journal of Agricultural and Food Chemistry 40(2):200-5.

Barclay T, Ginic-Markovic M, Cooper P, Petrovsky N. 2010. Inulin-a versatile polysaccharide with multiple pharmaceutical and food chemical uses. International Pharmaceutical Excipients Council.

Beck J, Ledl F, Sengl M, Severin T. 1990. Formation of acids, lactones and esters through the Maillard reaction. European Food Research and Technology 190(3):212-6.

Special Issue: U.S. Beverage Results for 2013. BEVERAGE-DIGEST.COM; 2014 [Accessed 2014 31 March] Available from: http://www.beverage-digest.com/pdf/top-10_2014.pdf.

Blonski C, De Moissac D, Perie J, Sygusch J. 1997. Inhibition of rabbit muscle aldolase by phosphorylated aromatic compounds. Biochemestry Journal 323:71-7.

Broersen K, Voragen AGJ, Hamer RJ, de Jongh HHJ. 2004. Glycoforms of β-lactoglobulin with improved thermostability and preserved structural packing. Biotechnology and Bioengineering 86(1):78-87.

Corzo-Martínez M, Moreno FJ, Olano A, Villamiel M. 2008. Structural Characterization of Bovine β-Lactoglobulin−Galactose/Tagatose Maillard Complexes by Electrophoretic, Chromatographic, and Spectroscopic Methods. Journal of Agricultural and Food Chemistry 56(11):4244-52.

Corzo-Martínez M, Moreno FJ, Villamiel M, Harte FM. 2010. Characterization and improvement of rheological properties of sodium caseinate glycated with galactose, lactose and dextran. Food Hydrocolloids 24(1):88-97.

Coudray C, Bellanger J, Castiglia-Delavaud C, Rémésy C, Vermorel M, Rayssignuier Y. 1997. Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. European Journal of Clinical Nutrition 51(6):375-80.

Damodaran S, Parkin K, Fennema OR. 2008. Fennema's food chemistry, 4th ed. Boca Raton: CRC Press/Taylor & Francis.

Dan A, Ghosh S, Moulik SP. 2009. Physicochemical studies on the biopolymer inulin: A critical evaluation of its self-aggregation, aggregate-morphology, interaction with water, and thermal stability. Biopolymers 91(9):687-99.

de Gennaro S, Birch GG, Parke SA, Stancher B. 2000. Studies on the physicochemical properties of inulin and inulin oligomers. Food Chemistry 68(2):179-83.

de la Fuente MA, Singh H, Hemar Y. 2002. Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. Trends in Food Science & Technology 13(8):262-74.

Delzenne N, Aertssens J, Verplaetse H, Roccaro M, Roberfroid M. 1995. Effect of fermentable fructo-oligosaccharides on mineral, nitrogen and energy digestive balance in the rat. Life Sciences 57(17):1579-87.

Delzenne NM, Daubioul C, Neyrinck A, Lasa M, Taper HS. 2002. Inulin and oligofructose modulate lipid metabolism in animals: review of biochemical events and future prospects. British Journal of Nutrition 87(S2):S255-S9.

Etzel MR. 2004. Manufacture and Use of Dairy Protein Fractions. The Journal of Nutrition 134(4):996S-1002S.

Franck A. 2002. Technological functionality of inulin and oligofructose. British Journal of Nutrition 87(S2).

Friedman M. 1996. Food Browning and Its Prevention: An Overview. Journal of Agricultural and Food Chemistry 44(3):631-53.

Galani. D, Apenten RKO. 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):467-76.

Ge Pan G, Melton LD. 2007. Nonenzymatic Browning of Lactose and Caseinate during Dry Heating at Different Relative Humidities. Journal of Agricultural and Food Chemistry 55(24):10036-42.

Guan J-J, Qiu A-Y, Liu X-Y, Hua Y-F, Ma Y-H. 2006. Microwave improvement of soy protein isolate–saccharide graft reactions. Food Chemistry 97(4):577-85.

Hattori M, Ogino A, Nakai H, Takahashi K. 1997. Functional Improvement of β-Lactoglobulin by Conjugating with Alginate Lyase-Lysate. Journal of Agricultural and Food Chemistry 45(3):703-8.

Heinert D, Martell AE. 1963. Pyridoxine and Pyridoxal Analogs. VI. Electronic Absorption Spectra of Schiff Bases. Journal of the Ameriacian Chemical Society 85(2):183-8.

Hoffmann MAM, van Mil PJJM. 1997. Heat-Induced Aggregation of β-Lactoglobulin: Role of the Free Thiol Group and Disulfide Bonds. Journal of Agricultural and Food Chemistry 45(8):2942-8.

Consumers alternately demand wellness and big tastes, and they're willing to pay for both. 2014 [Accessed 2014 Feb] Available from: http://www.cspnet.com/sites/default/files/magazine-files/F1-BevReport.pdf.

Israelachvili JN. 2011. Intermolecular and surface forces. Burlington, MA: Academic Press.

Jiménez-Castaño L, López-Fandiño R, Olano A, Villamiel M. 2005a. Study on β-lactoglobulin glycosylation with dextran: effect on solubility and heat stability. Food Chemistry 93(4):689-95.

Jiménez-Castaño L, Villamiel M, López-Fandiño R. 2007. Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. Food Hydrocolloids 21(3):433-43.

Jiménez-Castaño L, Villamiel M, Martín-Álvarez PJ, Olano A, López-Fandiño R. 2005b. Effect of the dry-heating conditions on the glycosylation of β-lactoglobulin with dextran through the Maillard reaction. Food Hydrocolloids 19(5):831-7.

Joubran Y, Mackie A, Lesmes U. 2013. Impact of the Maillard reaction on the antioxidant capacity of bovine lactoferrin. Food Chemistry 141(4):3796-802.

Kalyani Nair K, Kharb S, Thompkinson DK. 2010. Inulin Dietary Fiber with Functional and Health Attributes—A Review. Food Reviews International 26(2):189-203.

Kato A. 2002. Industrial Applications of Maillard-Type Protein-Polysaccharide Conjugates. Food Science and Technology Research 8(3):193-9.

Kato A, Mifuru R, Matsudomi N, Kobayashi K. 1992. Functional Casein-Polysaccharide Conjugates Prepared by Controlled Dry Heating. Bioscience, Biotechnology, and Biochemistry 56(4):567-71.

Kato Y, Aoki T, Kato N, Nakamura R, Matsuda T. 1995. Modification of Ovalbumin with Glucose 6-Phosphate by Amino-Carbonyl Reaction. Improvement of Protein Heat Stability and Emulsifying Activity. Journal of Agricultural and Food Chemistry 43(2):301-5.

Kaur N, Gupta AK. 2002. Applications of inulin and oligofructose in health and nutrition. Journal of Biosciences 27(7):703-14.

Kim M, Shin HK. 1998. The water-soluble extract of chicory influences serum and liver lipid concentrations, cecal short-chain fatty acid concentrations and fecal lipid excretion in rats. The Journal of Nutrition. 128(10):1731-6.

Kok N, Roberfroid M, Robert A, Delzenne N. 1996. Involvement of lipogenesis in the lower VLDL secretion induced by oligofructose in rats. British Journal of Nutrition 76(06):881-90.

Kroh LW, Jalyschko W, Häseler J. 1996. Non-volatile Reaction Products by Heat-induced Degradation of α-Glucans. Part I: Analysis of Oligomeric Maltodextrins and Anhydrosugars. Starch/Stärke 48(11-12):426-33.

Kruse H-P, Kleessen B, Blaut M. 1999. Effects of inulin on faecal bifidobacteria in human subjects. British Journal of Nutrition 82(05):375-82.

Lametti S, de 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):106-12.

Langlands SJ, Hopkins MJ, Coleman N, Cummings JH. 2004. Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. Gut 53(11):1610-6.

Li Y, Lu F, Luo C, Chen Z, Mao J, Shoemaker C, Zhong F. 2009. Functional properties of the Maillard reaction products of rice protein with sugar. Food Chemistry 117(1):69-74.

Liu G, Zhong Q. 2012. Glycation of Whey Protein To Provide Steric Hindrance against Thermal Aggregation. Journal of Agricultural and Food Chemistry 60(38):9754-62.
Liu G, Zhong Q. 2013. Thermal aggregation properties of whey protein glycated with various saccharides. Food Hydrocolloids 32(1):87-96.

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):170-83.

Malec LS, Pereyra Gonzales AS, Naranjo GB, Vigo MS. 2002. Influence of water activity and storage temperature on lysine availability of a milk like system. Food Research International 35(9):849-53.

Marella C. 2009. Whey protein fractionation using membrane separation technology. [Ph.D.]: South Dakota State University.

Martinez-Alvarenga MS, Martinez-Rodriguez EY, Garcia-Amezquita LE, Olivas GI, Zamudio-Flores PB, Acosta-Muniz CH, Sepulveda DR. 2014. Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate – Maltodextrin conjugates. Food Hydrocolloids 38:110-8.

Martins SIFS, Jongen WMF, van Boekel MAJS. 2000. A review of Maillard reaction in food and implications to kinetic modelling. Trends in Food Science & Technology 11(9–10):364-73.

Matmaroh K, Benjakul S, Tanaka M. 2006. Effect of reactant concentrations on the Maillard reaction in a fructose–glycine model system and the inhibition of black tiger shrimp polyphenoloxidase. Food Chemistry 98(1):1-8.

Mb R. 1998. Prebiotics and synbiotics: concepts and nutritional properties. Btritish Journal of Nutrition 80(4):S197-202.

McGuffey MK, Epting KL, Kelly RM, Foegeding EA. 2005. Denaturation and Aggregation of Three α-Lactalbumin Preparations at Neutral pH. Journal of Agricultural and Food Chemistry 53(8):3182-90.

Medrano A, Abirached C, Panizzolo L, Moyna P, Añón MC. 2009. The effect of glycation on foam and structural properties of β-lactoglobulin. Food Chemistry 113(1):127-33.

Morgan F, Saïd, Mollé D, Henry G, Maubois J-L, Léonil J. 1998. Lactolation of β-Lactoglobulin Monitored by Electrospray Ionisation Mass Spectrometry. International Dairy Journal 8(2):95-8.

Nakamura S, Kato A, Kobayashi K. 1992. Bifunctional lysozyme-galactomannan conjugate having excellent emulsifying properties and bactericidal effect. Journal of Agricultural and Food Chemistry 40(5):735-9.

Namiki M. 1988. Chemistry of Maillard reactions: recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. Advances in Food Research 32:115-84.

Niness KR. 1999. Inulin and Oligofructose: What Are They? J. Nutr. 129(7):1402S-6s.
O'Regan J, Mulvihill DM. 2009. Preparation, characterisation and selected functional properties of sodium caseinate–maltodextrin conjugates. Food Chemistry 115(4):1257-67.

Oliver CM, Melton LD, Stanley RA. 2006a. Creating Proteins with Novel Functionality via the Maillard Reaction: A Review. Critical Reviews in Food Science and Nutrition 46(4):337-50.

Oliver CM, Melton LD, Stanley RA. 2006b. Functional properties of caseinate glycoconjugates prepared by controlled heating in the 'dry' state. Journal of the Science of Food and Agriculture. 86(5):732-40.

Oliver CM, Melton LD, Stanley RA. 2006c. Glycation of caseinate by fructose and fructo-oligosaccharides during controlled heat treatment in the 'dry' state. Journal of the Science of Food and Agriculture 86(5):722-31.

Paraman I, Hettiarachchy NS, Schaefer C. 2007. Glycosylation and Deamidation of Rice Endosperm Protein for Improved Solubility and Emulsifying Properties. Cereal Chemistry Journal 84(6):593-9.

Park YW, Haenlein GFW. 2013. Milk and Dairy Products in Human Nutrition: Production, Composition and Health: John Wiley & Sons.

Photchanachai S, Kitabatake N. 2001. Heating of β-Lactoglobulin A Solution in a Closed System at High Temperatures. Journal of Food Science 66(5):647-52.

Reddy BS, Hamid R, Rao CV. 1997. Effect of dietary oligofructose and inulin on colonic preneoplastic aberrant crypt foci inhibition. Carcinogenesis 18(7):1371-4.

Roberfroid MB. 2005. Introducing inulin-type fructans. British Journal of Nutrition 93(Supplement S1):S13-S25.

Roefs SPFM, De Kruif KG. 1994. A Model for the Denaturation and Aggregation of β-Lactoglobulin. European Journal of Biochemistry 226(3):883-9.

Rumney C, Rowland I. 1995. Non-digestible oligosaccharides — potential anticancer agents? Nutrition Bulletin 20(3):194-203.

Shu Y-W, Sahara S, Nakamura S, Kato A. 1996. Effects of the Length of Polysaccharide Chains on the Functional Properties of the Maillard-Type Lysozyme−Polysaccharide Conjugate. Journal of Agricultural and Food Chemistry 44(9):2544-8.

Stevens CV, Meriggi A, Booten K. 2001. Chemical Modification of Inulin, a Valuable Renewable Resource, and Its Industrial Applications. Biomacromolecules 2(1):1-16.

Tang C-H, Sun X, Foegeding EA. 2011. Modulation of Physicochemical and Conformational Properties of Kidney Bean Vicilin (Phaseolin) by Glycation with Glucose: Implications for Structure–Function Relationships of Legume Vicilins. Journal of Agricultural and Food Chemistry 59(18):10114-23.

Taper HS, Roberfroid M. 1999. Influence of Inulin and Oligofructose on Breast Cancer and Tumor Growth. Journal of Nutrition 129(7):1488S-91s.

Trautwein EA, Rieckhoff D, Erbersdobler HF. 1998. Dietary Inulin Lowers Plasma Cholesterol and Triacylglycerol and Alters Biliary Bile Acid Profile in Hamsters.Journal of Nutrition 128(11):1937-43.

van Boekel MaJS. 2001. Kinetic aspects of the Maillard reaction: a critical review. Nahrung 45(3):150-9.

van Loo J, Coussement P, de Leenheer L, Hoebregs H, Smits G. 1995. On the presence of inulin and oligofructose as natural ingredients in the western diet. Critical Reviews in Food Science and Nutrition 35(6):525-52.

Wack M, Blaschek W. 2006. Determination of the structure and degree of polymerisation of fructans from Echinacea purpurea roots. Carbohydrate Research 341(9):1147-53.

Wang Q, He L, Labuza TP, Ismail B. 2013. Structural characterisation of partially glycosylated whey protein as influenced by pH and heat using surface-enhanced Raman spectroscopy. Food Chemistry 139(1–4):313-9.

Wang Q, Ismail B. 2012. Effect of Maillard-induced glycosylation on the nutritional quality, solubility, thermal stability and molecular configuration of whey proteinv. International Dairy Journal 25(2):112-22.

Wang W, Zhong Q. 2014. Properties of whey protein–maltodextrin conjugates as impacted by powder acidity during the Maillard reaction. Food Hydrocolloids 38:85-94.

Xu C-h, Yang X-q, Yu S-j, Qi J-r, Guo R, Sun W-W, Yao Y-J, Zhao M-M. 2010. The effect of glycosylation with dextran chains of differing lengths on the thermal aggregation of β-conglycinin and glycinin. Food Research International 43(9):2270-6.

Zhu D, Damodaran S, Lucey JA. 2008. Formation of Whey Protein Isolate (WPI)−Dextran Conjugates in Aqueous Solutions. Journal of Agricultural and Food Chemistry 56(16):7113-8.

Appendix

Table 1 Raw data of Amadori measurements

Samples	Batch1	Batch2	Batch 3
WPI-0h	0.16	0.15	0.17
WPI-12h	0.14	0.15	0.16
WPI-24h	0.14	0.15	0.15
WPI-48h	0.14	0.14	0.19
WPI-72h	0.15	0.16	0.16
CJ 6:1-0h	0.15	0.14	0.18
CJ 6:1-12h	0.22	0.22	0.24
CJ 6:1 -24h	0.27	0.23	0.27
CJ 6:1-48h	0.26	0.26	0.29
CJ 6:1-72h	0.26	0.25	0.28
CJ 4:1-0h	0.17	0.15	0.18
CJ 4:1-12h	0.24	0.30	0.26
CJ 4:1-24h	0.28	0.27	0.29
CJ 4:1-48h	0.32	0.30	0.33
CJ 4:1-72h	0.32	0.31	0.33
CJ 2:1-0h	0.16	0.19	0.19
CJ 2:1-12h	0.29	0.32	0.34
CJ 2:1-24h	0.32	0.35	0.39
CJ 2:1-48h	0.41	0.43	0.45
CJ 2:1-72h	0.46	0.48	0.49

Table 2 Raw data of available amino groups

Samples	Batch1	Batch2	Batch 3
WPI-0h	0.4174	0.4012	0.4031
CJ 6:1-0h	0.4099	0.4094	0.4120
CJ 6:1-12h	0.4155	0.3695	0.4034
CJ 6:1 -24h	0.3996	0.3889	0.3894
CJ 6:1-48h	0.3998	0.3875	0.3850
CJ 6:1-72h	0.3828	0.3944	0.3839
CJ 4:1-0h	0.4255	0.4077	0.4123
CJ 4:1-12h	0.4051	0.3942	0.3899
CJ 4:1-24h	0.4094	0.3909	0.3869
CJ 4:1-48h	0.3992	0.3807	0.3708
CJ 4:1-72h	0.3943	0.3762	0.3777
CJ 2:1-0h	0.4017	0.4160	0.4175
CJ 2:1-12h	0.3824	0.3879	0.3988
CJ 2:1-24h	0.3843	0.3847	0.3874
CJ 2:1-48h	0.3741	0.3908	0.3882
CJ 2:1-72h	0.3645	0.3645	0.3717
Empty	0.1649	0.1674	0.1651

Table 3 Raw data of turbidity measurements

Samples	Batch1	Batch2	Batch 3
WPI-0h	2.34	1.85	1.59
WPI-12h	2.30	1.30	1.63
WPI-24h	1.50	2.02	1.27
WPI-48h	0.81	0.83	0.81
WPI-72h	0.99	0.76	0.61
CJ 6:1-0h	1.75	2.09	2.83
CJ 6:1-12h	0.71	0.50	1.25
CJ 6:1 -24h	0.51	0.34	0.26
CJ 6:1-48h	0.36	0.21	0.43
CJ 6:1-72h	0.28	0.20	0.16
CJ 4:1-0h	2.76	2.41	2.93
CJ 4:1-12h	0.69	0.87	1.50
CJ 4:1-24h	0.42	0.40	0.26
CJ 4:1-48h	0.20	0.35	0.14
CJ 4:1-72h	0.21	0.31	0.23
CJ 2:1-0h	2.69	1.86	2.60
CJ 2:1-12h	0.85	0.82	0.79
CJ 2:1-24h	0.29	0.28	0.29
CJ 2:1-48h	0.16	0.16	0.19
CJ 2:1-72h	0.13	0.13	0.14

Table 4 Raw data of particle size

Samples	Batch1(nm)	Batch2(nm)	Batch 3(nm)
WPI-0h	173.30	154.90	151.77
WPI-12h	173.60	139.00	155.00
WPI-24h	145.50	155.37	139.67
WPI-48h	117.20	114.90	116.73
WPI-72h	121.20	112.10	105.13
CJ 6:1-0h	146.17	164.50	192.47
CJ 6:1-12h	107.10	92.06	123.10
CJ 6:1 -24h	93.61	81.03	116.77
CJ 6:1-48h	80.60	65.33	84.55
CJ 6:1-72h	72.49	64.23	87.17
CJ 4:1-0h	195.83	167.00	212.10
CJ 4:1-12h	109.23	136.03	133.33
CJ 4:1-24h	88.40	84.87	109.73
CJ 4:1-48h	64.56	78.88	71.27
CJ 4:1-72h	63.93	74.46	87.93
CJ 2:1-0h	178.03	149.33	191.77
CJ 2:1-12h	114.07	108.70	118.83
CJ 2:1-24h	74.66	73.21	74.49
CJ 2:1-48h	55.00	52.65	55.27
CJ 2:1-72h	45.21	43.24	47.27

Table 5 Raw data of Zeta poetical as a function of incubation time

Samples	Batch1(mv)	Batch2(mv)	Batch 3(mv)
WPI-0h	-14.82	-14.90	-14.68
WPI-12h	-16.48	-13.03	-15.57
WPI-24h	-14.10	-15.40	-13.60
WPI-48h	-14.48	-15.65	-17.97
WPI-72h	-14.13	-14.15	-15.97
CJ 6:1-0h	-15.30	-15.32	-13.92
CJ 6:1-12h	-14.60	-16.07	-16.18
CJ 6:1 -24h	-16.97	-16.88	-17.10
CJ 6:1-48h	-17.07	-17.92	-18.42
CJ 6:1-72h	-16.05	-20.47	-20.42
CJ 4:1-0h	-16.35	-14.12	-12.97
CJ 4:1-12h	-16.77	-13.97	-15.57
CJ 4:1-24h	-16.80	-16.13	-13.33
CJ 4:1-48h	-19.27	-17.07	-15.88
CJ 4:1-72h	-18.03	-19.68	-15.57
CJ 2:1-0h	-11.83	-10.56	-11.20
CJ 2:1-12h	-13.63	-11.00	-12.03
CJ 2:1-24h	-13.55	-13.02	-13.33
CJ 2:1-48h	-13.47	-14.20	-14.98
CJ 2:1-72h	-16.80	-16.78	-16.67

Table 6 Raw data of zeta poetical as a function of pH

Samples	Batch1 (mv)	Batch2(mv)	Batch 3(mv)
WPI-0h	32.53	30.72	35.28
WPI-12h	23.45	23.82	23.08
WPI-24h	2.91	1.30	2.58
WPI-48h	-14.82	-14.90	-14.68
WPI-72h	-28.28	-24.05	-25.55
CJ 6:1-0h	33.02	33.50	32.08
CJ 6:1-12h	24.75	24.35	25.90
CJ 6:1 -24h	3.29	2.57	4.26
CJ 6:1-48h	-16.05	-20.47	-20.42
CJ 6:1-72h	-30.77	-31.77	-33.00
CJ 4:1-0h	27.17	29.42	30.23
CJ 4:1-12h	13.52	22.43	22.35
CJ 4:1-24h	1.20	2.91	4.16
CJ 4:1-48h	-18.03	-19.68	-15.57
CJ 4:1-72h	-33.78	-30.93	-28.90
CJ 2:1-0h	22.65	21.43	20.35
CJ 2:1-12h	15.72	16.20	14.77
CJ 2:1-24h	2.34	1.17	0.29
CJ 2:1-48h	-16.80	-16.78	-16.67
CJ 2:1-72h	-25.62	-24.92	-28.05