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Improved Heat Stability of Whey Protein Isolate by Glycation with Inulin

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Abstract: Glycation between proteins and sugars via the Maillard reaction has been shown to improve the heat stability of proteins. In this study, inulin, a healthy dietary fiber, was glycated with whey protein isolate (WPI), and the effects of reaction conditions were investigated. Conjugates were prepared by freeze-drying mixed WPI and inulin solutions at 1:1 to 6:1 WPI-to-inulin weight ratios followed by dry heating at 70, 75, or 80 °C for 12 to 72 h under uncontrolled, 44%, or 80% relative humidity. Heat stability was evaluated by turbidity, particle size, and rheological measurements. Degree of glycation was assessed by quantifying the loss of amino groups and the formation of the Amadori compounds. Results showed that conjugation led to improved heat stability, as shown by decreased turbidity and particle size as well as the ability to maintain the viscosity compared to control samples. Based on the loss of amino groups, the optimum glycation conditions were achieved with WPI–inulin mixtures at 2:1, 4:1, and 6:1 weight ratios and 80 °C temperature for 12 to 72 h without controlling the relative humidity. The improved heat stability could be due to an increase in negative charge as well as increased structural stabilization of the proteins. Under a limited degree of glycation, glycated WPI–inulin conjugates have great potential to be utilized as food ingredients, especially in the beverage industry.

Keywords: whey protein isolate; inulin; glycation; heat stability; Maillard reaction

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1. Introduction

The beverage market related to health and wellness has seen continuous growth in recent years, while the sale of carbonated soft drinks has experienced a decline since 2004 [1,2]. With increasing consumer demand for healthier food products, functional foods including high-protein beverages have seen a surge in sales [1]. However, challenges in developing such products include the loss of heat stability (e.g., increased turbidity and the formation of sediments) at pH values near the isoelectric point (pI) of the protein and astringency at low pH (pH < 4.0). One major goal to improve protein functional properties is to increase the heat stability of the protein at pH values closer to the pI.

Whey protein isolate (WPI), a fractionated whey product with >90% protein, is a common protein ingredient used to manufacture protein beverages due to its exceptional biological value and functional properties [3–5]. The heat aggregation of whey proteins is generally attributed to molecular forces such as Van der Waals, hydrophobic, and electrostatic interactions, and intra- and intermolecular disulfide bonds via sulfhydryl–disulfide interchange [6]. The behavior of the heat-induced denaturation and aggregation of β-lactoglobulin (the major component in whey proteins) was described by a model proposed by Roefs and de Kruif [7]: In the initiation step, the β-lactoglobulin dimer splits into monomers, exposing the free sulfhydryl group and causing the protein to become more reactive. In the propagation step, activated β-lactoglobulin molecules react with non-reactive β-lactoglobulin through thiol/disulfide exchange reaction, and hence build up the aggregates. In the termination step, two active intermediates react and form a larger

disulfide-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 proteins tend to form much larger aggregates under heating. Hoffmann and Van Mil [8] 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 to 1.0%. The pH of the solution affects the heat stability of whey proteins by altering their surface charge and the reactivity and accessibility of the thiol group [9]. A solution with 2.5% protein content will become turbid and form sediments at pH 4–6 because of the low electrostatic repulsion [10]. At the very acidic pH (2–3), the disulfide exchange is very unlikely to occur because the thiol groups are very stable [10].

Maillard reaction between protein and reducing sugar has been shown to improve heat stability of protein [4,11–19]. For example, Liu and Zhong [17] found that a WPI-maltodextrin solution (7% protein concentrate) could remain transparent at pH values from 3 to 7 after being heated at 88 °C for 2 min. The Maillard reaction between proteins and reducing sugars can be achieved by heating them together in "dry" or "wet" conditions. The "dry" condition is achieved by freeze drying or spray drying a solution of mixed protein and reducing sugar before dry-heating of the dried mixture, while the "wet" condition involves heating a solution of mixed protein and sugar without drying [20,21]. The glycation between protein and saccharide is based on the Amadori rearrangement steps in the Maillard reaction. The optimum reaction condition is when the Amadori compounds are formed but are not converted into the advanced Maillard products (AMPs), which are dark colored, insoluble, and may be hazardous to health [20,22]. The ratio between protein and reducing sugar, heating temperature, and relative humidity affect the Maillard reaction rate and the final products. Excessive sugar increases the Maillard reaction rate but also leads to AMP formation due to the higher number of reactive carbonyl groups [22]. Higher temperature increases the rate of Maillard reaction, not only because the increase in reactivity of carbonyl and amino groups, but also due to the greater unfolding of the protein structure. The Amadori compounds formed in the initial stage are rather stable at temperatures lower than 60 °C [23]. Generally, the maximum reaction rate occurs at an intermediate moisture content [24,25]. 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 [23]. Ge Pan and Melton [24] found that at high moisture content, the color formation was highest while the whole Maillard reaction was slow.

As mentioned, the glycation of proteins 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 consumers. Inulin is a polymer of fructans consisting of a linear chain of fructosyl groups linked by β(2→1) glycosidic bonds, with normally one glucopyranose unit at the reducing end [26]. Ostensibly, inulin is a non-reducing sugar, because β(2→1) glycosidic bonds fix the cyclic structure [26]. However, glucose, fructose, and fructans are naturally present in inulin or are produced by enzymatic processes or hydrolysis during inulin production. Thus, inulin will invariably have a certain level of reducing capacity [26,27]. As a dietary fiber, inulin has been shown to increase the growth of beneficial intestinal microflora and the absorption of minerals, modulate the absorption or metabolism of lipids, and inhibit the development of cancer [26,28,29]. With continuing demand of functional foods and beverages, it would be beneficial to determine the effect of the Maillard-induced glycation of whey proteins and inulin.

Therefore, the aim of this research was to investigate the effect of heating temperature, incubation time, relative humidity, and the reactants' weight ratio on the properties and heat stability of glycated WPI and inulin. Heat stability was evaluated by determining the turbidity, aggregate size, and rheological properties [30]. The mechanism responsible for improved heat stability was also determined.

2. Materials and Methods

2.1. Materials

Whey protein isolate (WPI) was provided by Davisco Foods International, Inc., currently AgroPur (Le Sueur, MN, USA). WPI consisted of 97.5% protein, 2.1% ash, and 0.3% fat (dry weight basis). Inulin with low viscosity (TIC Pretested® Inulin LV 110 Powder) was obtained from TIC Gum (White Marsh, MD, USA). Deionized (DI) water with a resistivity of 18.2 MΩ-cm was used to prepare all solutions.

2.2. Conjugate Preparation

WPI and inulin powders at WPI–inulin weight ratios of 1:1, 2:1, 4:1, 5:1, and 6:1 were dissolved in DI water to a total concentration 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 24 h before freeze drying (Genesis 25 Super ES Freeze Dryer, SP Scientific, Warminster, PA, USA).

The freeze-dried mixtures were incubated in an oven (Thermo Scientific™ Precision™ Oven, St. Louis, MO, USA) at 70, 75, and 80 °C for 12, 24, 48, and 72 h with or without controlling the relative humidity. The relative humidity (RH) was controlled to 44% or 80% using saturated solutions of K_2CO_3 (Millipore Sigma, St. Louis, MO, USA) or KCl (Millipore Sigma, St. Louis, MO, USA) in sealed desiccators. For samples with no RH control, the RH values were 6% and 4% at 60 and 80 °C, respectively. 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.

2.3. 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 HCl, and the final protein concentration was 6% *w/w*. A 6 mL sample was then transferred to a 10 mL vial and heated in a temperature-controlled water bath at 85 °C for 15 min. The visual appearance of the samples was compared by photographing.

2.4. 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 was adapted from the method outlined by Zhu et al. [31]. Conjugates and unheated WPI were dissolved in DI water at 2 mg/mL and centrifuged at 6000 RPM for 15 min at 4 °C (Allegra X12, Beckman Coulter, Fullerton, CA, USA). The absorption of the supernatant was measured at 304 nm (Cary 50 Scan, Varian, Palo Alto, CA, USA) in a 1 cm quartz cell.

The quantification of free amino groups was determined using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) method [32] with modification. Sample (2.5 mg/mL protein) was prepared in 50 mM phosphate buffer (pH 8.5) containing 50 mM NaCl and mixed with 0.1% TNBS solution at a 1:1 volume ratio. The resultant mixtures were incubated in a 60 °C water bath for 2 h and subsequently cooled to room temperature. Then, 1 mL of 10% sodium dodecyl sulfate (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, USA) 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 the percent decrease in absorbance relative to that of unheated WPI–inulin mixture. All experiments were replicated at least three times.

2.5. Particle Size and Turbidity

The turbidity was measured at 630 nm using an ultra-microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The particle size of heated samples (0.3% *w/w* 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.

2.6. 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. The refractive index of 1.47 was used. Average values of six measurements were reported for each sample, and three replicates were tested for each sample.

2.7. Differential Scanning Calorimetry (DSC)

The thermal denaturation properties of conjugates were monitored using a differential scanning calorimeter (DSC) (Model DSC 7, Perkin-Elmer Instruments, Shelton, CT, USA) at temperatures 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. Sample (15 µL) 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.

2.8. Viscosity Measurement

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

$$\tau = k\dot{r}^{n}$$

where k is the consistency coefficient (Pa·s^n) and the exponent n is the flow behavior index. Apparent viscosity (η) values between different samples were compared at a shear strain rate of 50 s^{-1}.

2.9. Statistical Analysis

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

3. Results and Discussion

3.1. Visual Observation

The effects of temperature (T), relative humidity (RH), weight ratio (WR) of whey proteins to inulin, and incubation time (t) were systematically investigated in order to determine the suitable glycation conditions. The visual appearance of the conjugate solutions heated at 85 °C for 15 min were evaluated (Figure 1). The optimum glycosylation condition is when glycation between protein and saccharide occurs (e.g., formation of Amadori compounds) but the glycated proteins do not undergo subsequent reaction in order to prevent dark color formation, flavor change, and loss of solubility [22]. In this study, the optimum condition was the one that showed significantly increased heat stability without extensive browning.

As shown in Figure 1, all control samples (WPI–inulin mixtures at respective weight ratios without dry-heating) became opaque after heating at pH 6.0, while the conjugates were more transparent with slight yellowish to dark brownish color. With the exception of 80 °C-80RH, there was a clear trend in the effects of WPI–inulin ratio, temperature, and incubation time. Decreasing WPI to inulin ratio (i.e., increasing inulin content) as well as increasing temperature and incubation time 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 the 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 et al. [27], which indicate that glycation between caseinate and inulin (1:1 weight ratio) was slow at 60 °C and 80% RH. Glycation between whey proteins and other polysaccharides such as dextran or maltodextrin have been reported to require lower temperature or shorter time [16,17,31]. This is probably due to the low content of reducing sugar, high heat stability, and low solubility of inulin compared to other saccharides [27,33]. 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 [34]. Similarly, the formation of brown pigments was greatly accelerated when the relative humidity was 44% and 80%. These results are consistent with the early report that the Maillard reaction rate increased gradually with increasing water activity from 0.3 to 0.8 [25]. In addition, the reducing sugars released during the hydrolysis of inulin, when heated in the presence of water, could also contribute to the increased reaction rate. However, the conjugates prepared at 44% and 80% RH became very dark before achieving similar heat stability to samples with no RH control. 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 reported by Ge et al. [24], who found that the color formation was enhanced, while the formation of furosine (e.g., the derivative of the Amadori compounds) was slow at high RH. At 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 stage [35].

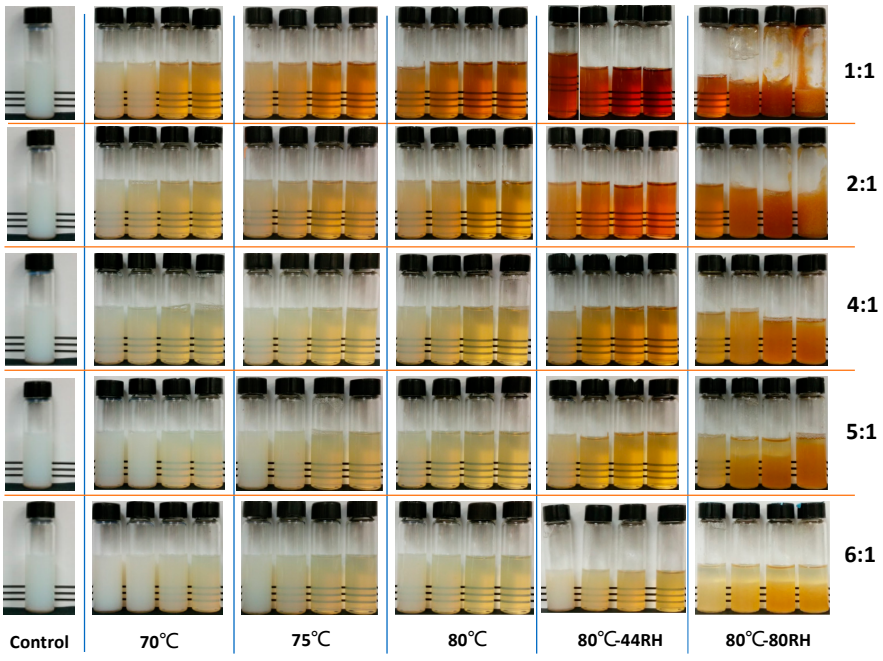

Figure 1. Solutions (6% *w*/*w* protein, pH 6.00) of whey protein isolate (WPI)–inulin mixtures (e.g., control) or conjugates prepared in different dry-heating conditions after heating at 85 °C for 15 min. Samples from top to bottom were at different WPI to inulin ratios. Vials from left to right at the same dry-heating temperature represent conjugates at 12, 24, 48, and 72 h incubation time.

After 24 h storage at 4 °C, conjugate solutions with high inulin content (e.g., 1:1 WPI–inulin weight ratio) formed white precipitates (data not shown). White precipitates were also found in the caseinate–inulin conjugate dispersions during storage and were attributed to the poor solubility of inulin [27]. In another study, white precipitate formed

after a WPI–dextran conjugate solution was centrifuged, and this was thought to be the self-association of dextran [28]. Since the inulin exhibited both relatively low solubility and self-associating behavior [36], it is reasonable to consider that the white precipitate was due to the aggregation of inulin.

Because they had the highest heat stability without excessive color change, 2:1, 4:1, and 6:1 WR conjugates prepared at 80 °C (no RH control) were selected for further analysis to determine properties of the conjugates and their mechanism of improving heat stability.

3.2. Degree of Glycation

3.2.1. Amadori Compounds

During the initial stage of the Maillard reaction, Schiff bases are formed via the condensation between carbonyl groups of reducing sugar and free amino groups of protein. The Schiff bases then undergo an irreversible Amadori rearrangement to form the Amadori compounds [20,21]. The peak at 304 nm is generally assigned to the Schiff base formation [37,38]. Absorbance at 304 nm has been used to detect the Schiff bases formed in WPI–dextran conjugates and bovine-lactoferrin–glucose or fructose conjugates [18,30,39].

As shown in Figure 2, WPI and WPI–inulin mixtures (0 h) exhibited similar absorbance values, indicating very little or no absorption of inulin 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 incubation. After 24 h, the conjugates had significantly higher content of Amadori compounds than the unglycated ones. The amount of the Amadori compounds for the conjugate with a WPI–inulin WR of 2.1 (CJ 2:1) continued to increase significantly with incubation time up to 72 h, 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 80 °C and would degrade to form advanced Maillard products [23]. No significant difference was observed in CJ 4:1 and 6:1 after 24 h incubation which was probably due to the limited carbonyl groups. In addition, it is possible that the formation of Amadori compounds was equal to their conversion into advanced Maillard products [18,22].

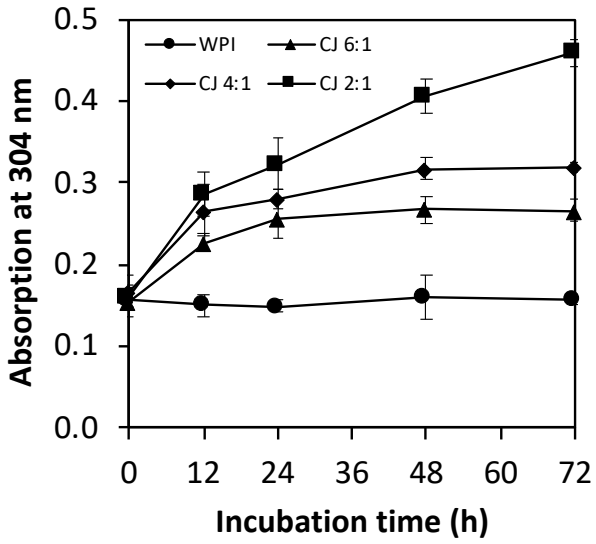

Figure 2. Formation of Amadori compounds monitored at 304 nm in WPI and WPI–inulin conjugates (CJ). WPI and CJ (6:1, 4:1 and 2:1 weight ratio (WR) of WPI to inulin) were incubated at 80 °C (no relative humidity (RH) control) for 0–72 h. All samples were prepared to contain 2.5 mg/mL protein. Error bars are standard deviations from triplicate measurements.

3.2.2. Available Amino Groups

The available amino groups (%) in WPI–inulin conjugates are shown in Figure 3. The content of available amino groups in untreated WPI sample (at 0 h) 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 ($p > 0.05$, 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. Similarly, 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 literature (only 30–65.6% available amino acids) after the glycation of WPI or β-lactoglobulin with saccharides [14,40].

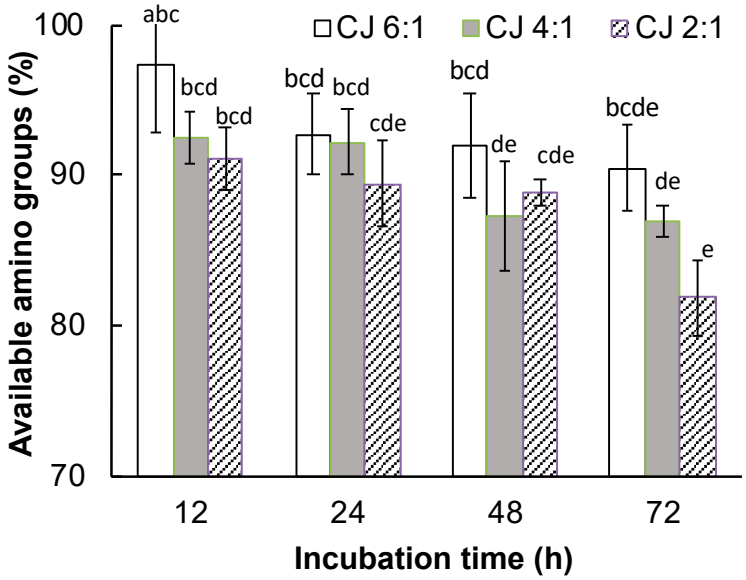

Figure 3. Available amino groups (%) of conjugates after heating at 80 °C (no RH control) for 12 to 72 h. Data represent means ± standard deviations. Different letters above each column represent significant differences in means ($p < 0.05$).

The relatively high residual amino groups shown in Figure 3 could be due to the uncontrolled relative humidity and the low content of reducing sugars in inulin. Typically, high relative humidity leads to a higher diffusion of reactants and accelerates protein–saccharide conjugation and protein polymerization, which results in higher amino group blockage [22,23,40]. By controlling the relative humidity at 80%, WPI and maltodextrin mixture (1:3 weight ratios) had only 48% available amino groups after heating at 60 °C for 48 h 40]. In another study, only 30% of available amino groups were left after conjugation between β-lactoglobulin and dextran at 55 °C and 65% RH for 14 d [22]. Here, samples prepared at 44% and 80% RH exhibited very dark color and some even became insoluble, which indicates the occurrence of the advanced Maillard reaction and protein polymerization. Thus, the relatively low amino group blockage could be partially explained by the uncontrolled relative humidity during conjugate preparation, which limits the reaction rate and prevents further polymerization. The ratio between proteins 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 [41], inulin is ostensibly a non-reducing sugar [26,41]. However, the existence of glucose, fructose, and Fn-type fructans, created whether by natural enzymatic process or partial hydrolysis, means that inulin has a certain reducing capacity [26]. The total percentage of reducing sugars in inulin was about 4.1% [42]. Owing to its low chemical reactivity and steric hindrance, inulin was even added in a caseinate–fructose mixture to lower the Maillard reaction [27,35]. So, the low reducing ability of inulin also contributed to the relatively low amino blockage in our system.

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 the Maillard reaction, and the RH-controlled conditions were unable to reduce the protein polymerization. One major concern about glycation is the loss of lysine, which is the main amino group lost during the reaction; thus, reaction conditions that lead to limited amino blockage is highly desirable. Owing to the low content of reducing sugars in inulin, its Maillard reaction with whey proteins could be well controlled.

3.3. Heat Stability

Heat stability of WPI and conjugates was determined by measuring their turbidity and particle sizes after heating of 6% *w/w* protein solutions (pH 6) at 85 °C for 15 min. As shown in Figure 4a,b, 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 sizes, especially when compared to WPI. The 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 lead to significant differences. Absorbance at 630 nm (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 the 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 weight 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 were 115, 85, and 85 nm smaller 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 with particle size smaller than 94 nm.

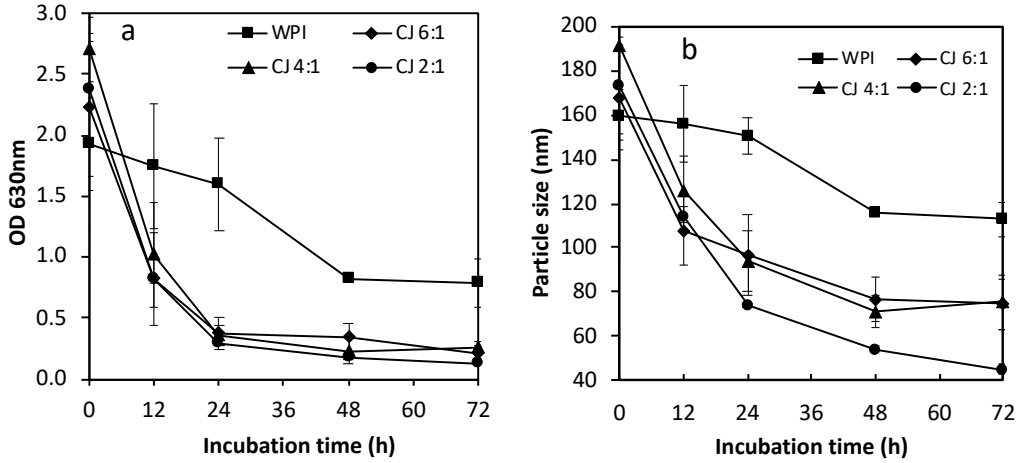

Figure 4. Turbidity (**a**) and particle size (**b**) of WPI and WPI–inulin conjugate (CJ) solutions (6% *w/w* protein concentrate, pH 6.00) after heating at 85 °C. WPI and CJ were incubated at 80 °C (no RH control) for 0–72 h. Here, 6:1, 4:1 and 2:1 refer to the weight ratios of WPI to inulin. Error bars are standard deviations.

There are several factors that could possibly explain the improved heat stability of protein after glycation with saccharides, including increased steric hindrance, changes of conformation, and surface charge. The grafted polymer can provide steric hindrance against aggregation if it can extend in the continuous phase [43]. Steric hindrances were regarded as the dominant mechanism enabling the improvement in heat stability of glycated protein in [16,17]. Increased surface charge and structural stability were not the major factors influencing the heat stability of the conjugates. The effects of steric hindrance could also be indirectly linked with the positive correlation between the length of the grafted saccharides and the heat stability of the conjugates [16,44]. Lysozyme demonstrates

much better heat stability when conjugated with galactomannan (3.5–24 kDa) than with xyloglucan (1.4 kDa) [44]. In another study, conjugates of WPI and maltodextrin (1 kDa) were more heat stable than those containing glucose and lactose [16].

In some studies, improved conjugate heat stability was mostly attributed to conformational changes [18,45]. As shown by the measurements of surface hydrophobicity and sulfhydryl groups, WPI glycated with dextran exposed their internal -SH and hydrophobic groups at a much slower rate during heating than untreated WPI. The authors of these studies also found that the denaturation temperature of β-lactoglobulin and α-lactalbumin increased by 15.5 °C and 18.7 °C after partial glycation. In some cases, increased surface charge also plays an essential role in improving the heat stability of glycated protein [12,15]. By glycating with glucuronic acid, ovalbumin had a much better improved heat stability compared to ovalbumin glycated with glucose due to the ability of replacing the lysine residues with anionic carboxyl groups.

3.4. Flow Behavior

The viscosity of a protein solution after heating is another important indicator of the protein's heat stability. No or minimal increase in viscosity indicates a higher stability, and is especially desirable in beverage applications. Heated WPI solutions (6% *w/w* at pH 6) showed Newtonian behavior with a consistency coefficient (k), flow behavior index (n), and apparent viscosity (at 50 s^{-1}) of 0.0017 ± 0.0001 ($Pa.s^n$), 1.0042 ± 0.0392, and 0.0017 ± 0.0001 (Pa.s), respectively. There was no significant difference ($p > 0.05$) in n, k, or apparent viscosity among samples, indicating that glycation at all conditions did not affect the flow behaviors of heated conjugate solutions.

Proteins were reported to become significantly more viscous and pseudoplastic after glycation with saccharides [27,35,40,46–49]. For example, after glycation with galactose, sodium caseinate had a 4.9-fold higher k and its flow behavior index (n) decreased from 0.96 to 0.73 [46]. The increased molecular size, owing to the attached sugars, was thought to be partially responsible for the changes in flow behavior [24,46–48]. According to [49], a rice protein isolate glycated with xanthan gum was significantly more viscous than 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 suggested to be attributable to the changes in flow behavior in [35,47]. As reported by Corzo-Martínez et al. [47], sodium caseinate (SC) did not show significant changes in the flow behavior after glycation with dextran, while it became more pseudo-plastic and viscous after glycation with lactose and galactose. Although dextran had a much larger molecular weight than lactose and galactose, it was assumed that the minor effect of dextran on the 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. A significant increase in the viscosity of glycated samples under controlled humidity was observed in the present study (data not shown), which could be caused by excessive protein crosslinking.

3.5. Zeta Potential

Zeta potential measurement was used to investigate the surface charge properties of the particles. The surface charge of proteins has a very important effect on their functional properties, including heat stability. As shown in Figure 5a, the zeta potential of heated WPI solution at pH 6 was −14.8 mV. The addition of inulin (0 h or no dry-heating) did not affect the zeta potential except at the 2:1 weight ratio, which showed a less-negative charge. No effect of dry heating on the zeta potential of WPI was observed ($p > 0.05$). Glycation with inulin led to a significant increase in zeta potential, and the conjugates became significantly more negatively charged 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 a net increase of 5.6, 3.3, and 4.1 mV, respectively. During glycation, saccharides 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 could increase in net negative charge. As shown in Figure 5b, the difference in zeta potential of untreated WPI at pH 6 and 7 was about 9 mV. This difference in zeta potential resulted 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 mechanism behind the increased heat stability of the conjugates.

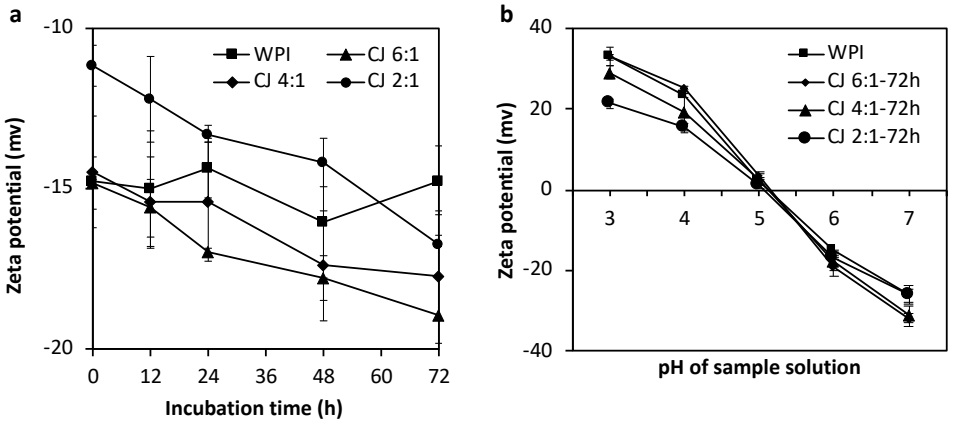

Figure 5. (**a**) Effect of incubation time on zeta potentials of WPI and CJ (2:1, 4:1, and 6:1 WPI–inulin) solutions (6% *w*/*w*, pH 6) after heating at 85 °C. (**b**) Zeta potentials of heated WPI and CJ solutions as a function of pH. Error bars are standard deviations from triplicate measurements.

Zeta potentials of WPI and conjugates were also measured from pH 3 to 7 to investigate the change in isoelectric point (pI) (Figure 5b). Compared to WPI, 2:1 conjugates after 72 h incubation were more negatively charged. As discussed, this could be due to a reduced lysyl content from glycation. Studies have reported that slight shifts of the pI of glycated proteins were due to the blockage of lysine residues, which are otherwise protonated at a pH values lower than their pKa [16,17,19,32,40]. However, from Figure 5b, a change in pI was not observed. Another possible explanation for the decreased absolute zeta potential of 2:1 conjugates could be that the less-charged inulin was also scattering some light, thus the average zeta potential became reduced. These results indicate that the effect of glycation on charge properties should be further investigated.

3.6. Differential Scanning Calorimetry

Thermal denaturation properties are important to understand the aggregation of whey proteins during heating, and are commonly measured by DSC, Raman spectroscopy, fluorescence spectroscopy, or electrophoresis [4,50,51]. In DSC, 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 T_d values are listed in Table 1.

Table 1. Denaturation temperature (T_d) and enthalpy change (ΔH) of WPI and conjugate solutions. Numbers are means ± standard deviations from triplicate measurements. Different superscript letters (e.g., a, b, c) in each column represent significant differences in means ($p < 0.05$).

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

The T_d of untreated WPI was around 71.2 °C, which was in the range reported in the literature [18,51]. The T_d of WPI increased after glycation with inulin, with CJ 2:1 having higher T_d than CJ 6:1, indicating an increased stabilizing effect of the protein from conjugation at higher inulin content. Similar results were observed in the glycation of WPI with maltodextrin, which showed increased T_d [21]. However, compared to previous studies, the increase in T_d shown in this study was relatively small [4,16–19,52,53]. For example, Wang et al. [18] found the denaturation temperature of WPI increased from 68 to 85 °C at pH 7 after glycation with dextran. Wang and Zhong [4] observed a 5–7 °C increase in the denaturation temperature of WPI after glycation with maltodextrin. Other than the differences between the materials and methods, the relatively low degree of glycation could partially explain the small increase in T_d. Glycation with inulin led to a decrease in ΔH from 6.9 to 2.85 kJ/g, which is consistent with previous reports [4,18,52]. The reduction of ΔH was thought to be caused by the partial unfolding of the tertiary structure induced by glycation [18]. However, some studies reported an increase in ΔH of proteins after glycation with sugars [16,17,19].

4. Conclusions

An improved heat stability of whey proteins at pH values closer to the protein pI is highly desirable in protein applications, especially in the beverage industry. This research demonstrated that heat stability of WPI can be improved by glycating with inulin. Consistent with previous results, the degree of glycation increased as heating temperature, WPI–inulin weight ratio, incubation time, and relative humidity increased. By varying glycation conditions, limited glycation could be achieved such that WPI–inulin conjugates exhibited improved heat stability without excessive browning. The optimal glycation condition appeared to be at 6:1 WR and 48 h incubation at 80 °C (no RH control). The resulting conjugates exhibited <10% loss of available amino groups. Results from this study can be applied in the development of functional high-protein beverages. Future work could focus on how WPI–inulin conjugates can be used as ingredients in other applications, as well as how glycation influences the gut microbiome.

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