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Glycation of Whey Protein To Provide Steric Hindrance against Thermal Aggregation

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ABSTRACT: Thermal processing is required for a variety of products and remains a problem for whey proteins that undergo denaturation and aggregation above the denaturation temperature. This causes challenges to maintain clarity and dispersibility of protein dispersions, particularly at acidity near the isoelectric point of the protein and increased ionic strength. This work reports for the first time that glycation of whey protein with a sufficient number of maltodextrins prevented protein aggregation before and after heating at 88 °C for 2 min at pH 3.0−7.0 and 0−150 mM NaCl or $CaCl_2$. The mechanism of maintaining protein dispersion clarity during heating was illustrated by several complementary analytical techniques that elucidated primary, secondary, and tertiary structures, as well as thermal denaturation and surface charge properties of glycated whey proteins. Steric hindrance was concluded to be the major mechanism responsible for transparent dispersions with protein structures smaller than 12 nm after heating.

KEYWORDS: *whey protein, glycation, heat stability, analytical ultracentrifugation, atomic force microscopy, steric repulsion*

INTRODUCTION

Whey protein isolate (WPI) is among the major ingredients used in the rapidly growing market of protein beverages.[1] To maintain clarity and a single-phase fluid system, whey proteins must remain dispersed after the thermal processing required for pasteurization and sterilization of beverages. Thermally induced denaturation, above ∼65 °C, and aggregation of whey protein mixture and the most abundant whey protein, *β*-lactoglobulin, have been extensively investigated.[2] Denaturation causes the exposure of thiol groups and nonpolar amino acid residues that may cause the formation of irreversible aggregates due to covalent intermolecular disulfide bonds and noncovalent hydrophobic attraction, respectively.[2] The possibility of protein aggregation also is dependent on the magnitude of long-range electrostatic repulsion. The interplay of repulsive and attractive intermolecular forces and the extent of protein denaturation are affected by pH, temperature, and types and concentrations of ions, which, along with protein concentration, determine the functionality of protein dispersions after heating.[3] Formation of protein aggregates not only causes turbid appearance and precipitation during storage but also possibly induces the formation of protein network and nonfluidic gels, which are problematic for beverage manufacturing.

Generally, protein aggregation is favored near the isoelectric point (p*I*) of proteins, because of the close-to-zero overall net charge, and an increased strength and valence of ions due to reduced effective distance of electrostatic repulsion and/or ionic bridging.[4] Conversely, many beverages are formulated to a wide range of acidity and ionic strength, many of which have acidity near the p*I* values of major whey proteins, which are 5.2, 4.8−5.1, and 4.8−5.1 for *β*-lactoglobulin, *α*-lactalbumin, and bovine serum albumin, respectively.[2] Therefore, it has been a technological challenge in the food industry to maintain the stability of whey protein dispersions during heating at acidity near p*I* and increased ionic strength.

In addition to providing important nutrients, whey proteins have been extensively studied as surfactants[5] and delivery systems of bioactive compounds such as nutraceuticals[6] and pharmaceuticals.[7] For these applications, a large number of attempts have been made to improve functional properties of whey proteins. The formation of covalent bonding between reducing poly- or oligosaccharides and free amino groups of proteins by heating, the Maillard reaction, is one of a few chemical cross-linking methods applicable to food production. Improved solubility, particularly at pH 5.0, and emulsifying and stabilizing properties of WPI were observed after glycation with maltodextrin by dry-heating at 90 °C for 2 h.[8] Similar results were also reported for *β*-lactoglobulin glycated with dextran, dextran sulfate, and propylene glycol alginate by dry-heating at 60 °C for up to 3 weeks.[9] Aoki et al.[10] glycated ovalbumin with glucose or glucuronic acid at 50 °C and 65% relative humidity for up to 3 days and observed the much improved heat stability of 0.1% glycated protein at pH 7.0, evaluated at 60−95 °C for 10 min. Whey proteins are highly charged at pH 7.0 and are typically heat stable at low ionic strength. Much work is needed to stabilize concentrated whey protein dispersions at a wide range of acidity and ionic conditions.

In this work, we report glycation conditions enabling transparent whey protein dispersions after heating at pH 3.0−7.0 and 0−150 mM NaCl or $CaCl_2$. The Maillard reaction is not a single reaction but produces a vast array of poorly characterized products.[11] We applied several complementary analytical techniques to understand structures that enabled transparent dispersions of whey proteins. Particularly, for the first time, we applied analytical ultracentrifugation (AUC) to

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analyze molecular weight distributions of glycated whey proteins.

MATERIALS AND METHODS

Materials. WPI was obtained from Hilmar Ingredients (Hilmar, CA, USA). Maltodextrin (MD) with a dextrose equivalent of 18 was acquired from Grain Processing Corp. (Muscatine, IA, USA). Polyacrylamide gel and protein markers were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). β-Mercaptoethanol was procured from Sigma-Aldrich Corp. (St. Louis, MO, USA). Other chemicals were from Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA).

Preparation of Glycated Whey Protein. WPI and MD were dissolved in distilled water at a mass ratio of 2:1. After hydration overnight, the solution was adjusted to pH 7.0 and spray-dried (model B-290 mini-Spray Dryer, Büchi Laboratoriums-Technik, Flawil, Switzerland) at an inlet air temperature of 160 °C, an air flow rate of 30 m^3/h, an outlet temperature of ca. 65 °C, and a feed flow rate of about 2 mL/min. The resultant powder was collected in a container placed on a perforated plate, with a saturated KBr solution underneath, of an emptied desiccator. The desiccator was pre-equilibrated in an oven at 60 °C, corresponding to a relative humidity of 80%, and the incubation of protein powder was allowed for 24, 48, and 72 h. The resultant samples are referred to as 24, 48, and 72 h conjugates, hereafter. To study the impact of glycation conditions on WPI structure, a WPI control was prepared by incubation at 60 °C and 80% relative humidity for 72 h.

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) Spectroscopy. The ATR-FTIR spectra were obtained by using a Thermal Nicolet Nexus 670 FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) under ambient conditions. Each spectrum was averaged over 64 scans with a 4 cm^{-1} resolution. The protein sample was dissolved in deuteroxide at a concentration of 5% protein. Ten microliters of a protein solution was placed on the ATR accessory for acquisition of IR spectra. The original spectrum was smoothed within a certain number of smoothing points (11, 21.213 cm^{-1}) with the aid of OMNIC software. Spectra in the wavenumber range from 1200 to 1800 cm^{-1}, which covers the typical amide I and II peaks, were selected for analysis.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed using a precast 4–20% gradient polyacrylamide gel from Bio-Rad Laboratories, Inc. One part of a protein solution was diluted in 4 parts of a SDS-PAGE sample buffer (catalog no. MB01015, GenScript Corp., Piscataway, NJ, USA), followed by heating at 95 °C for 5 min. Ten microliters of each sample was loaded onto the gel for electrophoresis at 150 V until the indicator dye reached the gel bottom. Afterward, the gel was fixed and stained with Coomassie Blue and destained until bands became visible.

Structures of Conjugates Analyzed by AUC. Principles of applying AUC to characterize size distribution of polymers and particles were detailed by Schuck.[12] During centrifugation, a concentration gradient is formed toward the bottom of a centrifuge cell over time, and the concentration distribution is used to determine the molecular weight (MW) by the Lamm equation that includes sedimentation (s) and diffusion coefficients (D) of the polymer. Both s and D are a function of MW and are correlated by the well-known Svedberg equation[12]

$$s(\mathrm{MW}) = D(\mathrm{MW})[\mathrm{MW}(1 - \bar{\nu}_M\rho)/RT] \quad (1)$$

where $\bar{\nu}_M$ is the specific volume of polymer, ρ is the solvent density, R is the gas constant (R = 8.32441 J K^{-1} mol^{-1}), and T is the absolute temperature.

Experiments were performed using a Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA, USA). Solutions with an overall protein concentration of 1.2 mg/mL were prepared in deionized water and adjusted to target pH values. The absorbance at 280 nm was recorded every 4 min using the standard double-sector cell operated at 50000 rpm and 25 °C. Data were analyzed using the continuous $c(s)$ distribution model in SEDFIT software of the instrument based on an estimated anhydrous frictional ratio (f/f_0) of 1.2.

The average MW determined from AUC was used to estimate the number of MD molecules (N) glycated to whey proteins by comparing the average MW of WPI (MW_1), WPI–MD conjugates (MW_2), and MD (MW_3, 1 kDa in this work):

$$N = (\mathrm{MW}_2 - \mathrm{MW}_1)/\mathrm{MW}_3 \quad (2)$$

Zeta Potential. The zeta potentials of native WPI and the conjugate samples were calculated from the measured electrophoretic mobility (model Delsa Nano C, Beckman Coulter, Inc., Brea, CA, USA) at 25 °C. The dispersions were prepared in deionized water at a protein concentration of 5 mg/mL adjusted to pH 3.0–7.0 using 0.1 M HCl and filtered by using a 0.45 μm syringe membrane (EMD Millipore Corp., Billerica, MA, USA) before the analysis. The conjugate dispersions after heating at 88 °C for 2 min were also measured for the zeta-potential. Average values of three measurements were reported for each sample.

Determination of Surface Hydrophobicity (H_o). Determination of H_o using the fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS) was carried out according to the method of Haskard and Li-Chan.[13] A stock solution with 2% w/w protein at pH 7.0 was diluted to 0.005–0.02% w/w protein using 0.01 M phosphate buffer at pH 7.0. The ANS was dissolved at 8.0 mM in the same phosphate buffer. Twenty microliters of ANS solution was mixed with 4 mL of each diluted sample before the fluorescence intensity was measured at an excitation wavelength of 390 nm using an RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The emission spectra were recorded between 400 and 700 nm. The slope obtained from linear regression of the fluorescence intensity–protein concentration (mg/mL) plots was used as an index of H_o.

Heat Stability Test. Heat stability at 88 °C was evaluated for samples constituted of 7% w/v protein, pH 3.0–7.0, and 0–150 mM NaCl or $CaCl_2$. The 1 or 2 mL of final dispersions were contained in 4 mL glass vials for heating in an 88 °C water bath for 2 min, simulating a hot-fill process in the beverage industry.[14]

Differential Scanning Calorimetry (DSC). Thermal denaturation properties of whey protein were monitored using a VP-DSC Micro calorimeter (MicroCal, Inc., Northampton, MA, USQ) from 20 to 120 °C at a scan rate of 60 °C/h. Samples were prepared in 10 mM sodium phosphate buffer at 25 mg/mL protein and adjusted to pH 5.0. The sodium phosphate buffer was used to generate the baseline scan for analysis.[15] All solutions were thoroughly degassed prior to loading into the cell in the instrument, and the cell was pressurized to 0.2 MPa to prevent the formation of gas bubbles during heating. The same sample was scanned twice to study the completeness and reversibility of thermal denaturation. Evaluation of the data was carried out with the manufacturer's software (MicroCal Origin 5.0).

Conformational Changes Studied by Circular Dichroism (CD) Spectroscopy. Far-UV CD spectra were collected using an AVIV model 202 CD spectrometer (AVIV Instrument, Inc., Lakewood, NJ, USA). Samples were diluted to a protein concentration of 0.1 mg/mL, adjusted to pH 7.0, and centrifuged at 18000g for 20 min prior to loading the supernatant to a quartz cuvette of a path length of 2 mm. The samples were scanned in a wavelength range from 190 to 250 nm at a scanning rate of 50 nm/min. Data were expressed as the mean residue ellipticity (θ) in deg cm^2 $dmol^{-1}$ using a mean reference value of 115 for the amino acid residues of WPI in all calculations.[16] Recorded spectra were corrected by subtracting the spectrum of a protein-free buffer. MD solutions had no signals, and a separate baseline subtraction was thus not performed. The secondary structure compositions were calculated using the CONTIN/LL program in the CDPro software of the instrument.

Characterization of Protein Structures by Atomic Force Microscopy (AFM). The tapping mode images were collected using a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA, USA). A silicon-etched FESPA cantilever of 200–250 μm length with a spring constant of 1–5 N/m (Bruker Nanoprobe, Camarillo, CA, USA), a typical resonant frequency of about 71.0 kHz, and a scan rate of 1.0 Hz were applied under ambient conditions.

Aliquots (2 μL) of protein samples previously diluted to 10 ppm using deionized water were spread on freshly cleaved mica disks and air-dried for >2 h prior to imaging. Images were analyzed using the software (version 5.30r3, Digital Instruments Inc., Tonawanda, NY, USA) of the AFM instrument.

Statistical Analysis. Tests were repeated in triplicate, each with a new batch of glycated sample. Analysis of variance (ANOVA) was performed using the SPSS 13.0 statistical analysis system (SPSS Inc., Chicago, IL, USA), and a least significant difference (LSD) test with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Structure of WPI−MD Conjugate Characterized by FTIR, SDS-PAGE, AUC, CD, and AFM. *FTIR.* Figure 1 shows

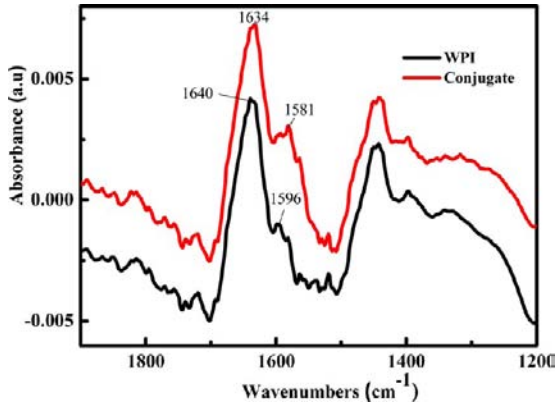

Figure 1. Comparison of FTIR spectra of WPI and the WPI−MD conjugate prepared by 72 h of glycation.

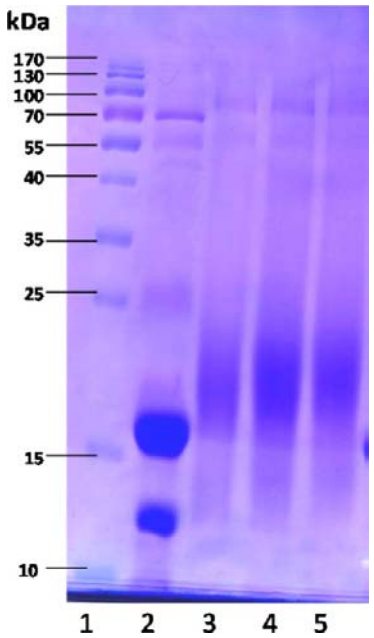

Figure 2. SDS-PAGE results of WPI and WPI−MD conjugates. Lanes: 1, markers; 2, native WPI; 3, 4, and 5, conjugate samples prepared by glycation for 24, 48, and 72 h, respectively.

the FTIR spectra of WPI and the 72 h conjugate. Characteristic absorption bands around wavenumbers of 3480−3440, 3260−3270, 2960−2878, and 1350−1300 cm^{-1} were assigned to vibrations of −OH, −NH, −CH, and C−N stretching, respectively.[17] The peaks located around 1641 and 1532

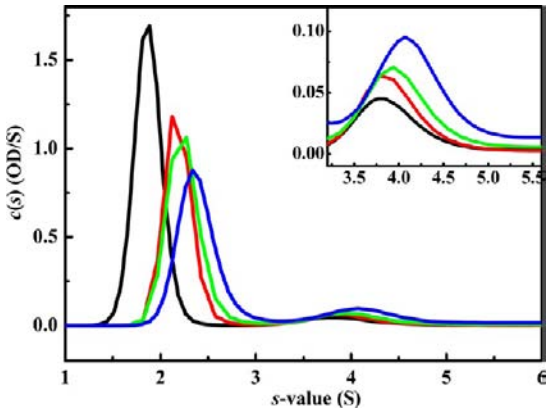

Figure 3. Best-fit sedimentation coefficient (s, in Svedberg unit, S) distributions ($c(s)$) from analytical ultracentrifugation of WPI (black) and WPI−MD conjugate solutions. The conjugate samples were prepared by dry-heating at 60 °C and 80% relative humidity for 24 (red), 48 (green), and 72 (blue) h. The protein concentration in all samples was 1.2 mg/mL and adjusted to pH 3.0. (inset) Zoom-in view of curves at s values of 3.3−5.6 S.

Table 1. Molecular Weight Characteristics of WPI and WPI−MD Conjugates Determined by AUC

	peak 1		peak 2		
compound	area %	av MW (kDa)	area %	av MW (kDa)	no. of MD glycated to protein
native WPI	94.4	15.7	5.6	47.5	
WPI incubated at glycation conditions for 72 h	93.5	15.7	6.5	47.5	
24 h conjugate	88.8	19.8	11.2	48.4	4.1
48 h conjugate	86.4	20.6	13.6	50.0	4.9
72 h conjugate	80.1	22.8	19.9	52.1	7.1

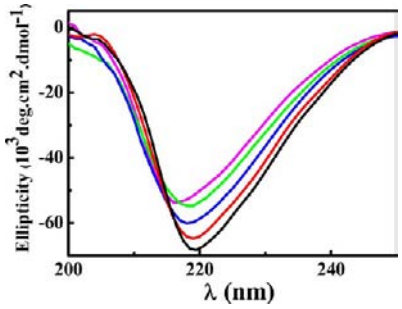

Figure 4. Far-UV CD spectra of WPI (black), WPI incubated for 72 h at glycation conditions (red), and WPI−MD conjugate prepared by glycation for 24 (blue), 48 (green) and 72 h (pink). Dispersions were adjusted to pH 7.0 before analysis.

Table 2. Secondary Structure Compositions of WPI and Conjugates at pH 7.0

sample	α-helix (%)	β-strand (%)	turns (%)	aperiodic structure (%)
WPI	23.8	35.1	12.0	32.2
WPI incubated at glycation conditions for 72 h	22.6	34.0	11.2	33.8
24 h conjugate	21.4	32.5	10.8	34.9
48 h conjugate	17.6	30.7	9.7	36.7
72 h conjugate	15.7	29.0	9.0	40.6

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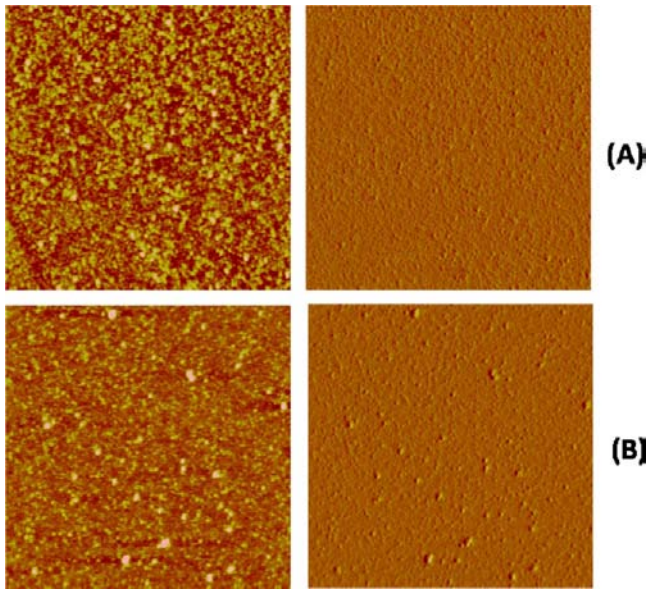

Figure 5. Tapping mode AFM images of native WPI (A) and glycated WPI (B), prepared by 72 h of conjugation. Height (left) and phase (right) images are shown for each sample scanned at a size of 2 × 2 μm.

Table 3. AFM Section Analysis of Particle Heights for WPI and WPI−MD Conjugates before Heating and Conjugates after Heating at 88 °C for 2 min at pH 3.0-6.0 and 0 mM NaCl[a]

sample	height (nm)
native WPI	3.2 ± 0.2 c
conjugate, before heating	3.6 ± 0.70 c
conjugate, after heating at pH 3.0	5.8 ± 0.24 b
conjugate, after heating at pH 4.0	7.1 ± 0.40 b
conjugate, after heating at pH 5.0	12.0 ± 1.58 a
conjugate, after heating at pH 6.0	6.2 ± 0.60 b

[a]The WPI−MD conjugate was prepared by 72 h of glycation. Values are the mean ± standard error from height measurements of at least 10 particles on AFM images. Means with different letters differ significantly ($P < 0.05$).

cm^{-1}, which belong to amide I and amide II, respectively, are characteristics of proteins.[18] The amide I absorption band at 1639 cm^{-1} was reported to represent the spectral overlap of the C═O group, coupled with in-plane −NH bending and C═N linkage.[19,20] The absorption band at 1520 cm^{-1} was attributed to a protonated amine group.[21] The main absorption bands of WPI at 1640 and 1596 cm^{-1} shifted to 1634 and 1581 cm^{-1}, respectively, after glycation with MD, suggesting the consumption of amino groups and the formation of Schiff bases due to the Maillard reaction.[17] Decreases in the absorption bands at 1639, 1518, and 1441 cm^{-1} and increases in the bands at 1594 and 1561 cm^{-1} indicate the conversion of the primary amino groups to the secondary amine groups during the Maillard reaction.

SDS-PAGE. Figure 2 shows the time course of the glycation reaction. The WPI showed three major bands at around 13.8, 16.0, and 70.0 kDa that correspond to α-lactalbumin, β-lactoglobulin, and bovine serum albumin monomers, respectively.[22] After glycation, bands corresponding to α-lactalbumin and β-lactoglobulin dissapeared and a smearing zone appeared at a MW range higher than that of β-lactoglobulin, whereas the band corresponding to bovine serum albumin shifted to a higer MW band. A longer heating time generally resulted in products with higher MW. The band corresonding to α-lactalbumin almost completely disappeared after 24 h of conjugation. The observation that α-lactalbumin is easier to be conjugated with MD than other whey proteins has previously been reported.[22] The impact of glycation time on SDS-PAGE patterns generally agrees with literature studies.[8]

AUC. The glycoconjugates formed via the Maillard reaction are not a single species and are poorly characterized,[11] particularly if the saccharide is a polyelectrolyte.[23] AUC is a practical and effective method to determine the average MW of water-soluble polymers as well as interactions and self-assembly of protein molecules.[23] Although AUC is accurate, simple, and rapid, it has not been used to characterize the MW distribution of glycated whey protein.

Figure 3 presents AUC data for WPI−MD conjugates prepared at different glycation durations. Samples were adjusted to pH 3.0 because it has been shown that the most abundant whey protein, β-lactoglobulin, is present predominately as monomers at pH 3.0 or below but has significant presence of dimers and trimers at other pH conditions.[24,25] The MW distribution of native WPI consisted of two portions with 94.4 and 5.6% of total areas under the curve, which corresponded to average MWs of 15.7 and 47.5 kDa, respectively (Table 1). The results confirmed that untreated whey proteins are primarily monomeric at pH 3.0. The MW distribution was not statistically different ($P > 0.05$) for WPI with and without incubation at glycation conditions for 72 h. After conjugation with MD for a longer time, the smaller MW peak became smaller, whereas the bigger MW peak became bigger, both shifting to a larger average MW (Table 1). Furthermore, the AUC data were used to determine the numbers of MD attached to whey proteins, which were 4.1, 4.9, and 7.1 after glycation for 24, 48, and 72 h, respectively (Table 1). The estimation agrees with a previous report that each α-lactalbumin molecule can be glycated with multiple mono- and oligosaccharides, which was estimated to be about three or four using ultraperformance liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry.[26] The area corresponding to the bigger MW peak increased by 2-fold after 24 h of glycation and by >3-fold after 72 h of glycation, which suggests that the Maillard reaction causes not only glycation but also cross-linking of protein.[26] Overall, the quantitative assessment from AUC agrees with qualitative SDS-PAGE results under reducing conditions (Figure 2).

CD. The far-UV CD spectra (Figure 4) were characterized for the WPI with and without 72 h of incubation at glycation conditions and WPI−MD conjugates prepared at different durations. The broad negative peak in the region of 210−220 nm is characteristic of proteins rich in β-sheet.[16,22,27] The calculated secondary structure compositions are listed in Table 2. WPI was reported to have an average of 20.5% α-helix, 42.5% β-sheet, 1.5% turns, and 34.5% aperiodic structure at pH 7.0,[16] whereas our results demonstrated 23.8% α-helix, 35.1% β-sheets, 12.0% turns, and 32.2% aperiodic structure (Table 2). The discrepancy may be due to different sources of WPI. After glycation for a longer time, the ellipticity of conjugates became significantly less negative (Figure 4), corresponding to decreased percentages of α-helix, β-sheet, and turns and increased content of aperiodic structure.

When WPI alone was incubated at glycation conditions, secondary structure changes were less pronounced than those

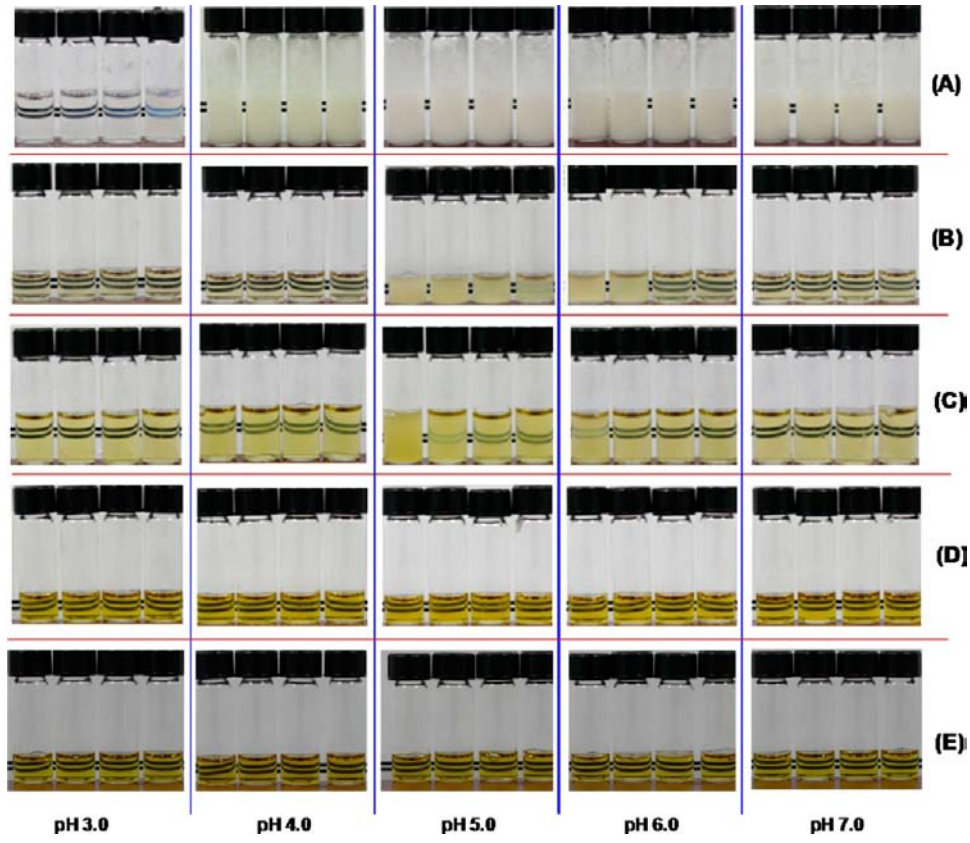

Figure 6. Photographs of aqueous solutions containing 7% w/v WPI after heating at 88 °C for 2 min: a simple mixture of WPI and MD at a mass ratio of 2:1 (A) and WPI−MD conjugate prepared by glycation for 24 (B), 48 (C), and 72 h (D). Samples were adjusted to pH 3.0−7.0 and 0, 50, 100, and 150 mM NaCl (vials from left to right in each image) before heating. Samples in row E were similar to those in row D, except that NaCl was substituted by the same molar concentration of $CaCl_2$.

of the comparable conjugate, which indicates the significance of glycation on protein denaturation. The results contrast with no distinct changes in secondary structure compositions of pea 7S globulin and phaseolin after glycation with glucose,[28,29] which may be due to differences in protein variety and degree of glycation (<13.4% in the literature vs nearly 100% in our work, based on the AUC and SDS-PAGE results).

AFM. Native WPI demonstrated particles (Figure 5A) with an average height of 3.2 nm (Table 3). The 72 h conjugate remained particulate structures (Figure 5B), but the average height increased to 3.6 nm (Table 3). The increased dimension directly observed from AFM agrees with the increased MW estimated in AUC.

Visual Observation of Dispersion Appearance after Heating. Photographs of dispersions after heating at 88 °C for 2 min are presented in Figure 6. After heating, dispersions with WPI and MD mixture remained transparent only at pH 3.0 and 0−100 mM NaCl (Figure 6A), similar to dispersions of WPI with and without incubation for 72 h at glycation conditions (results not shown). Conjugate samples were yellowish, with an increased color intensity after a longer glycation duration (Figure 6B−D), which is characteristic of nonenzymatic browning due to the Maillard reaction.[8] After heating, the 24 h conjugate was stable at pH 3.0, 4.0, and 7.0 and all NaCl conditions and at pH 6.0 and >100 mM NaCl (Figure 6B). For the sample glycated for 48 h, samples were stable except at pH 5.0 and 0 mM NaCl (Figure 6C). Dispersions of the 72 h conjugate remained transparent at all tested solvent conditions (Figure 6D), even after substitution of NaCl by an equal molarity of $CaCl_2$ (Figure 6E). Physical properties of glycated whey proteins were further studied using several complementary techniques, detailed in the following sections.

Zeta Potential and Surface Hydrophobicity. After glycation, the magnitude of the zeta potential decreased and the p*I* shifted slightly to lower pH (Figure 7). The shift of p*I* toward lower values after glycation had been observed previously.[28,30] Conjugates prepared at three durations were not significantly different in zeta potential. The magnitude of the zeta potential of conjugates decreased further after heating. Because MD is nonionic and long-range electrostatic repulsion between whey proteins is weakened as the pH approaches the p*I* and the ionic strength increases,[31] the improved thermal stability can be attributed to steric effects provided by the glycated MD, as suggested previously.[9,32,33] This agrees with the well-established theory that polymers grafted on a colloidal particle provide steric hindrance against aggregation if solvent conditions allow the extension of polymer chains to the continuous phase.[34] The nonionic MD adopted in this work had good water solubility at all pH conditions and provided steric repulsion as a dominant mechanism responsible for transparent dispersions of glycated whey protein after heating. Furthermore, because steric hindrance is a function of polymer chain density on colloidal particle surface,[34] the larger number of MD attached to whey protein prepared at a longer glycation time (Table 1) agrees with the improved heat stability (Figure 6).

The H_o values determined with the fluorescence probe ANS at pH 7.0 are given in Table 4. The H_o of WPI increased after

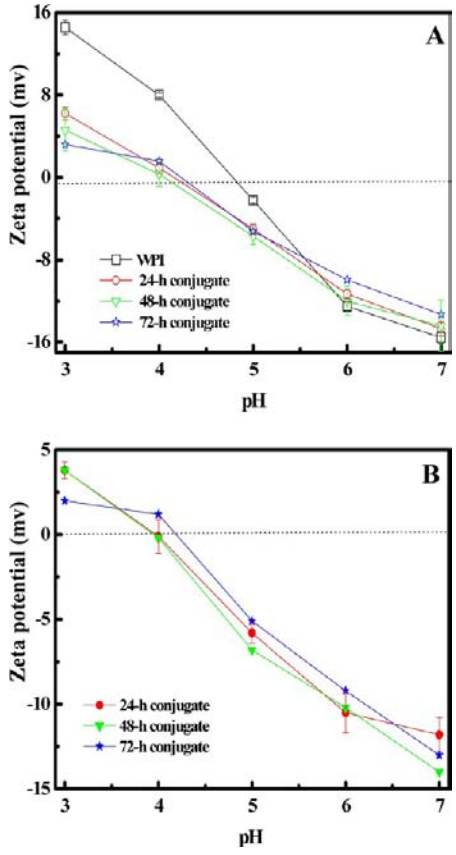

Figure 7. Zeta potential profiles WPI−MD conjugates before (A) and after (B) heating at pH 3.0−7.0, with comparison to WPI before heating. Error bars are standard errors from triplicate measurements.

Table 4. Surface Hydrophobicity (H_o) of WPI−MD Conjugates before and after Heating at 88 °C for 2 min at pH 7.0 and 0 mM NaCl, in Comparison to WPI with and without 72 h of Incubation at Glycation Conditions

sample	H_o $(10^3)^a$	
	before heating	after heating
native WPI	1.82 ± 0.028 b	2.19 ± 0.042 a
WPI incubated at glycation conditions for 72 h	1.90 ± 0.056 a	2.24 ± 0.084 a
24 h conjugate	1.24 ± 0.058 b	1.84 ± 0.085 b
48 h conjugate	1.21 ± 0.071 b	1.48 ± 0.057 c
72 h conjugate	1.18 ± 0.11 c	1.42 ± 0.014 c

[a]Numbers are the mean ± standard deviation from triplicates. Different letters in the same column indicate statistical difference ($P < 0.05$).

the powder had been incubated at glycation conditions for 72 h (Table 4) or heated in aqueous dispersions. After glycation, H_o reduced to a greater extent after glycation for a longer duration, which is in agreement with a larger number of MD molecules estimated in AUC (Table 1) and less-ordered structures assessed in CD (Figure 4; Table 2). The reduced H_o after glycation is consistent with the literature.[30,35] Similar to WPI, the H_o of conjugates increased after heating. Furthermore, because charged amino acid residues are mostly on the surface, glycation of ε-amino groups of the lysyl residues by saccharides is expected to reduce the amount of net surface charge and surface hydrophobicity,[28] as confirmed in the present work (Figure 7; Table 4).

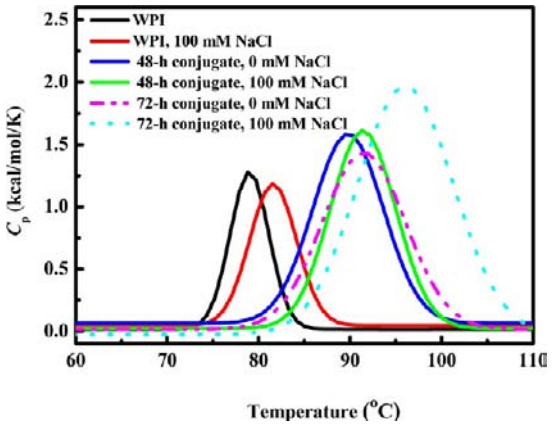

Figure 8. DSC thermograms demonstrating specific heat (C_p) of native WPI and glycated WPI at pH 5.0. The conjugate sample was prepared by glycation for 48 or 72 h.

Table 5. DSC Assessment of Denaturation Temperature (T_d) and the Associated Enthalpy Change (ΔH) for Native WPI and WPI−MD Conjugate at pH 5.0 and 0 and 100 mM NaCl[a]

sample	T_d (°C)	ΔH (kcal/mol)
native WPI, 0 mM NaCl	79.1 ± 0.32 e	13.81 ± 0.48 e
native WPI, 100 mM NaCl	81.4 ± 0.40 d	15.04 ± 0.46 d
48 h conjugate, 0 mM NaCl	89.5 ± 0.50 c	28.21 ± 0.37 b
48 h conjugate, 100 mM NaCl	91.5 ± 0.90 b	24.44 ± 0.26 c
72 h conjugate, 0 mM NaCl	91.6 ± 0.40 b	29.10 ± 0.82 b
72 h conjugate, 100 mM NaCl	95.7 ± 1.00 a	35.53 ± 0.63 a

[a]The WPI−MD conjugate was prepared by 48 or 72 h of glycation. Values are the mean ± standard error from triplicate measurements. Means with different letters differ significantly ($P < 0.05$).

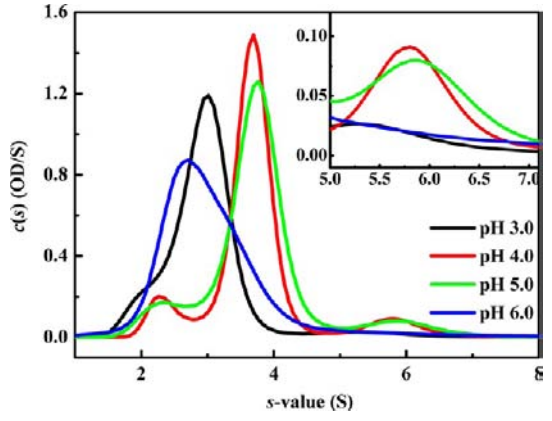

Figure 9. Best-fit sedimentation coefficient (s, in Svedberg unit, S) distributions ($c(s)$) from analytical ultracentrifugation of heated WPI−MD conjugate solutions. The conjugate sample, prepared by 72 h of glycation, was hydrated at an overall protein concentration of 1.2 mg/mL, adjusted to pH 3.0−6.0, and heated at 88 °C for 2 min. (Inset) Zoom-in view of curves at s values of 5.0−7.2 S.

Denaturation Profiles of WPI and WPI−MD Conjugate. DSC is commonly used to characterize thermal properties of proteins as thermal denaturation is an important characteristic related to the stability during heating.[15] The analysis in this work was focused on pH 5.0 because the higher tendency of whey protein aggregation during heating at a higher ionic strength is well-known,[1,36] but the conjugate

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Table 6. AUC Determination of Molecular Weight (MW) Characteristics of WPI−MD Conjugate[a] after Heating at 88 °C for 2 min at pH 3.0−6.0 and 0 mM NaCl

	peak 1		peak 2		peak 3	
pH	area %	av MW (kDa)	area %	av MW (kDa)	area %	av MW (kDa)
3.0	100	29.8				
4.0	11.1	22.3	81.2	43.1	7.7	86.5
5.0	10.5	21.2	78.9	44.0	10.0	90.5
6.0	100	32.8				

[a]The conjugate was prepared by 72 h of glycation.

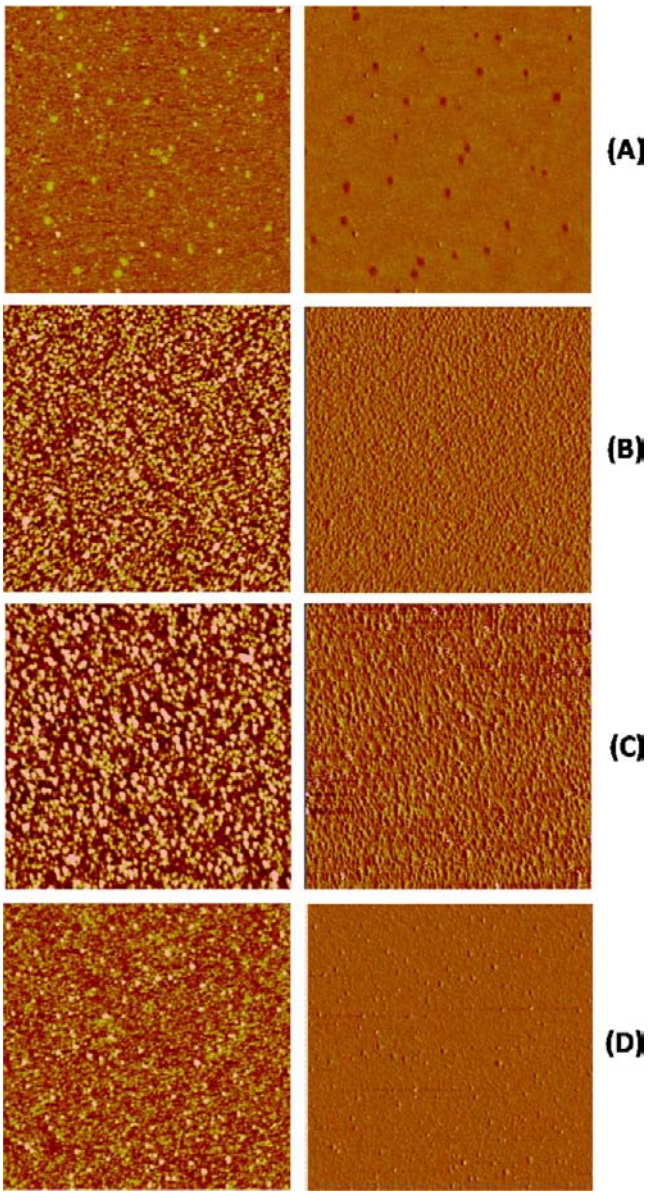

Figure 10. Tapping mode AFM images of glycated WPI, prepared by 72 h of conjugation, after heating at pH 3.0 (A), 4.0 (B), 5.0 (C), and 6.0 (D). Glycated WPI was prepared at 7% w/v protein and heated at 88 °C for 2 min before dilution to 10 ppm protein for AFM. Height (left) and phase (right) images are shown for each sample scanned at a size of 2 × 2 μm.

glycated for 48 h became turbid only at pH 5.0 and 0 mM NaCl (Figure 6). Figure 8 shows the DSC denaturation profiles of WPI and the 48 and 72 h conjugates at 0 and 100 mM salt during the first scan. Curves during the second scan did not show any peak (figure not shown), indicating the denaturation was completed during the first scan and was mostly irreversible. The area under the curve in Figure 8 was used to determine the calorimetric 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 ΔH is contributed by the energy for overcoming noncovalent interactions during protein denaturation.[37] The determined ΔH and T_d values are listed in Table 5. The T_d of native WPI was estimated to be 79.1 and 81.4 °C at pH 5.0 and 0 and 100 mM NaCl, respectively, while ΔH was 13.81 and 15.04 kcal/mol at 0 and 100 mM, respectively. The T_d is lower than the ∼83 °C reported for β-lactoglobulin at pH 5.0,[38] possibly because WPI contains α-lactalbumin and bovine serum albumin that denature at lower temperatures than β-lactoglobulin.[2]

The T_d and ΔH of glycated WPI were higher than those of native WPI and higher for the 72 h conjugate than the 48 h one (Table 5), which agrees with improved heat stability (Figure 6). The impacts of ionic strength on T_d and ΔH can be attributed by changes in intramolecular hydrogen bonds and interactions between nonpolar amino acid residues.[22] For the 48 h conjugate, the T_d at 0 and 100 mM NaCl was 89.5 and 91.5 °C (Table 5), respectively, which may have resulted in the turbid sample with 0 mM NaCl and the clear sample with 100 mM NaCl after heating at 88 °C for 2 min (Figure 6).

The DSC results indicate that protein aggregation due to thermal treatment at 88 °C can be prevented by steric hindrance originating from the glycated MD and/or increased T_d. Separate tests using the 72 h conjugate demonstrated transparent dispersions after heating at 138 °C for 1 min (not shown), and this further suggests that steric hindrance is sufficiently strong to prevent protein aggregation caused by thermal denaturation.

Impacts of Heating on Conjugate Structures Studied by AUC and AFM. AUC was used to study the MW of 72 h conjugate after heating at 88 °C for 2 min at pH 3.0−6.0. The distributions of sedimentation coefficients and estimated average MW are presented in Figure 9 and Table 6, respectively. When compared to the bimodal distribution at pH 3.0 before heating (Figure 3; Table 1), a single peak was observed after heating at pH 3.0 and 6.0 (Figure 9), corresponding to an average MW of 29.8 and 32.8 kDa, respectively. Before heating, the conjugate had ca. 20% mass with an average MW of 52.1 kDa and the rest with 22.8 kDa (Table 1), corresponding to an overall average MW of 28.6 kDa. This indicates that the large MW portion of conjugate dissociated during heating and that a small portion of low MW conjugate aggregated, which was more significant at pH 6.0 than at pH 3.0. After heating at pH 4.0 and 5.0, three peaks were observed (Figure 9), with the majority (∼80%) having a MW (∼44 kDa) of about twice the low MW portion (22.8 kDa) before heating (Table 6 vs Table 1), and the largest MW portion had a MW of ca. 90 kDa (Table 6) that likely resulted from aggregation of multiple conjugate molecules. The AUC results were supported by AFM data, showing coarser and bigger structures formed after heating at pH 4.0 and 5.0 than at pH 3.0 and 6.0 (Figure 10; Table 3), which is due to the weakened electrostatic repulsion at pH 4.0 and 5.0 (Figure 7). The AUC and AFM data further confirmed that steric hindrance due to MD glycated on whey protein molecules

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was the major mechanism enabling transparent dispersions after heating at all studied acidity and ionic conditions (Figure 6). Although aggregation is possible at pH 4.0 and 5.0, the overall structures formed after heating are too small (<12 nm, Table 3) to scatter visible light to cause turbidity.

In summary, glycation of whey protein with MD increased the MW, lowered the p*I*, reduced the zeta potential and H_o, caused reductions of ordered secondary structures, and increased T_d. After glycation with a sufficient number of MD, transparent dispersions were obtained after heating at acidity and ionic conditions expected in most consumer products. The steric hindrance provided by the glycated MD was the dominant mechanism enabling the improvement in heat stability of whey protein. Although aggregation was still possible at acidity near the protein p*I*, the steric hindrance minimized intermolecular aggregation to structures smaller than about 12 nm or several whey protein molecules that enabled the visual transparency of dispersions after heating. Because of the wide range of applications of whey proteins, findings from this study can be applied in industrial production of transparent high protein beverages and adapted for preparation of emulsions and delivery systems relevant to food, cosmetic, environmental, and pharmaceutical products.

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