

Thermal aggregation properties of whey protein glycosylated with various saccharides

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ABSTRACT

Aggregation of whey proteins during thermal processing is a challenge to maintain the dispersibility, particularly at pH near isoelectric point and increased ionic strength. We report glycation of whey protein isolate (WPI) with lactose and maltodextrin enabled transparent dispersions after heating at 88 °C for 2 min, when tested at 7%w/v protein, pH 3.0–7.0 and 0–150 mM NaCl or CaCl₂. Transparent dispersions corresponded to particles smaller than 14 nm based on atomic force microscopy. In comparison, WPI glycosylated with glucose behaved similarly to WPI control, forming turbid dispersions or gels at many conditions. Based on analytical ultracentrifugation, reducing saccharide with a smaller molecular weight was glycosylated at a larger number on each protein molecule, agreeing with changes in amino acid composition. Glycation lowered the isoelectric point and increased denaturation temperatures of WPI. The results suggest that repulsive steric interactions are the dominant mechanism controlling whey protein aggregation and thus transparency of dispersions.

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1. Introduction

Whey proteins have high contents of essential amino acids and versatile functionalities, and whey proteins isolate (WPI), with a protein content higher than 90%, is a category of commercially available ingredients suitable for production of transparent beverages (Vardhanabhuti, Yucel, Coupland, & Foegeding, 2009). Thermal processing is required to ensure microbiological safety and quality. For refrigerated dairy products, thermal pasteurization is achieved at 63 °C for 30 min, 72 °C for 15 s, or 89 °C for 1 s (CFR, 2008), while ultra-high temperature (UHT) processing at 280 °F (138 °C) for at least 8 s is required for shelf-stable products (McGarrahan, 1982). Conversely, the three major whey proteins, β -lactoglobulin, α -lactalbumin, and bovine serum albumin denature at around 78, 62, and 64 °C, respectively (Bryant & McClements, 1998). Denaturation facilitates protein aggregation that involves physical forces such as van der Waals, hydrophobic, electrostatic, and hydrogen bonding interactions and covalent disulfide bonds, and the significance of each force is a function of solution pH, concentration and type of electrolytes, presence of co-solutes, and the extent of thermal treatment (Baier & McClements, 2005; Bryant et al., 1998; Foegeding, Davis, Doucet, & McGuffey, 2002; de la

Fuente, Singh, & Hemar, 2002). When solution pH is near the isoelectric point (pI) of a protein, protein aggregation is favored because of the close-to-zero overall net charge (Golovanov, Hautbergue, Wilson, & Lian, 2004). The pI of β -lactoglobulin, α -lactalbumin, and bovine serum albumin is 5.2, 4.8–5.1, and 4.8–5.1, respectively (Hickstein & Peuker, 2008; Nakano & Ozimek, 2000), which is within the acidity range of many beverages (Etzel, 2004; Mettler, Rusch, & Colombani, 2006).

Because aggregation results in turbidity, dispersion instability, and even formation of non-flowable gels, attempts in improving heat stability of whey proteins have been studied by addition of co-solutes such as sucrose, glycerol, sorbitol (Baier & McClements, 2001; Chanasattru, Decker, & McClements, 2007; Chantapornchai & McClements, 2002; Kulmyrzaev, Bryant, & McClements, 2000), β -casein with >90% purity (Yong & Foegeding, 2008), preformation of nanoparticles using microemulsion templates (Zhang & Zhong, 2009, 2010), and pretreatment using transglutaminase (Agyare & Damodaran, 2010; Tang & Ma, 2007; Tanimoto & Kinsella, 1988; Truong, Clare, Catignani, & Swaisgood, 2004). These approaches mostly increase the denaturation temperature (T_d) of whey protein, showing different degrees of improved heat stability at neutral acidity. Technologies are still needed to stabilize whey protein during heating at varied acidity and ionic conditions.

Glycating whey proteins with reducing saccharides via the Maillard reaction is one of few chemical cross-linking methods

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to alter biopolymer structure and has been demonstrated in several studies to improve functional properties of food proteins (Ledesma-Osuna, Ramos-Clamont, & Vázquez-Moreno, 2008; Li et al., 2012; Oliver, Melton, & Stanley, 2006). The Maillard reaction for glycation of food proteins can be achieved by the wet (Li et al., 2012; Zhu, Damodaran, & Lucey, 2008) or dry (Akhtar & Dickinson, 2003, 2007) heating method. The former involves heating an aqueous solution or dispersion with protein and saccharide and typically requires a reaction time of several hours to several days that may have microbiological spoilage issue. In the dry heating method, a powdered mixture of protein and saccharide is heated, with a higher reaction rate at an increased temperature and humidity. The dry heating method typically has a higher reaction efficiency than the wet method and is studied more extensively (Oliver et al., 2006). The saccharides glycosylated on protein molecules theoretically provide steric hindrance against protein aggregation (Akhtar et al., 2007), but there is little information about the thermally-formed structure of glycosylated whey protein, especially at acidity near pI and increased ionic strength, at which conditions electrostatic repulsion is weakened. From the application perspective, much work is needed to prepare whey protein dispersions that remain transparent after heating at varied pH and ionic conditions, especially at a concentration higher than 10 g protein/240 mL when a “high protein” label can be used (Etzel, 2004). Lastly, glycation reduces the content of lysine, an essential amino acid (Liu, Ru, & Ding, 2012), and conflicting results have been reported for digestibility of whey proteins after glycation (Bouhallab, Morgan, Henry, Mollé, & Léonil, 1999; Luz Sanz, Corzo-Martínez, Rastall, Olano, & Moreno, 2007; Moreno, Quintanilla-López, Lebrón-Aguilar, Olano, & Sanz, 2008; Yeboah et al., 2004). These nutritional quality parameters are to be studied for glycosylated WPI, in addition to structure and heat stability.

The first objective of this work was to study molecular structure of WPI after glycation with mono-, di-, and oligosaccharides. In addition to conventional techniques of amino acid composition and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), analytical ultracentrifugation (AUC) was used to quantitatively characterize changes of primary structure of WPI after glycation. Circular dichroism (CD) spectroscopy was used to study secondary structures of WPI before and after glycation. The second objective was to understand thermal denaturation properties and colloidal interactions of glycosylated whey proteins, and the relevance to stability and structure formation after heating at pH 3.0–7.0 and 0–150 mM NaCl or CaCl₂. Surface charge (zeta-potential) and denaturation properties were correlated to macroscopic heat stability and nanoscopic structures of WPI, studied using atomic force microscopy (AFM). The third objective was to compare *in vitro* digestibility of WPI before and after glycation.

2. Materials and methods

2.1. Materials

WPI was kindly provided by Hilmar Ingredients (Hilmar, CA). Maltodextrin (MD) with a dextrose equivalent of 18, corresponding to a molecular weight (MW) of 1000 Da or approximately 5.6 glucose, was acquired from Grain Processing Corp. (Muscatine, IA). Polyacrylamide gel and prestained protein markers were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). β -Mercaptoethanol, pepsin with an activity of 3300 units/mg-protein, and pancreatin were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Other chemicals, including D-glucose and D-lactose monohydrate, and bile salt, were from Thermo Fisher Scientific Inc. (Pittsburgh, PA).

2.2. Preparation of glycosylated whey protein

WPI and saccharides were hydrated overnight in distilled water at a mass ratio of 1:1, adjusted to pH 7.0, and spray-dried (model B-290 Mini-Spray Dryer, Büchi Laboratories-Technik, Flawil, Switzerland). The spray drying parameters were as follows: inlet temperature = 160 °C, outlet temperature = ca. 65 °C, air flow rate = 30 m³/h, and feed rate = 2 mL/min. The collected powder was incubated for 2 h in an 80 °C oven, with the relative humidity controlled at 80% using a saturated KBr solution.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A precast 4–20% gradient polyacrylamide gel was used in the reducing SDS-PAGE. The protein solution diluted 5 times in an SDS-PAGE sample buffer (GenScript Corp., Piscataway, NJ) was heated at 95 °C for 5 min, and 10 μ L of the sample was loaded onto the gel for electrophoresis at 150 V. After fixing, the gel was stained with Coomassie® brilliant blue G-250. The destained wet gel was scanned (model Perfection 3490, Seiko Epson Corp., Nagano, Japan), and the composition of the bands was analyzed by ImageJ software (National Institutes of Health; Bethesda, MD) using the gel analysis function.

2.4. Analytical ultracentrifugation (AUC)

AUC is a well-established method to characterize size distribution of polymers and particles (Schuck, 2000) but has not been applied to analyze glycosylated whey protein. AUC experiments were performed on a Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA). The solution adjusted to an overall protein concentration of 1.2 mg/mL was loaded to the centrifuge cell, and the UV absorbance at 280 nm was recorded every 4 min using the standard double sector cell operated at 50,000 rpm and 25 °C. Data were analyzed under the continuous *c(s)* distribution model in SEDFIT software of the instrument based on an estimated anhydrous friction ratio (*f/f*₀) of 1.2 for protein. The average molecular weight (MW) representing monomeric proteins was used to estimate the number of saccharide molecules (*N*) glycosylated to one whey protein molecule by comparing average MW of WPI (MW₁), conjugate (MW₂) and saccharide (MW₃):

$$N = (MW_2 - MW_1)/MW_3 \quad (1)$$

2.5. Amino acid composition

Amino acid compositions are commonly studied for the impact of glycation (Li et al., 2012; Ogasawara, Katsumata, & Egi, 2006; Verzijl et al., 2000). The analysis was conducted by the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, CT), with the procedures provided as follows. A 1–13 mg portion of protein powder was weighed into a rimless 10 × 75 mm Pyrex™ tube and mixed with 0.3 mL of hydrolysis acid (6N HCl, 0.2% v/v phenol, 1 nmole norvaline as internal standard). After flame-sealing the tube under vacuum, the sample was hydrolyzed at 115 °C for 16 h. After cooling, the sample was opened and the hydrolyzate was evaporated to dryness in a heated vacuum-centrifuge. The dried sample was dissolved in 0.5 mL of 0.02 M HCl (pH 1.5) and serially diluted so that a 20 μ L injection would be 0.1% of the hydrolyzate. Sample was filtered through a 0.22 μ m centrifugal-filter and placed in the 0.3 mL limited volume insert of an 11 mm Snap-Cap glass vial and loaded onto the Hitachi L-8900

PH amino acid analyzer (Tokyo, Japan) equipped with a Hitachi 2622SPH ion-exchange column (4.6 mm ID $\times$ 60 mm) for amino acid composition analysis. Data was collected using “EZChrom Elite for Hitachi” software. It should be noted that the HCl hydrolysis partially destroys tryptophan and cysteine, so those values are not included in the results. Duplicate samples were tested.

2.6. Circular dichroism (CD) spectroscopy

Far-UV CD spectra were recorded between 200 and 260 nm on an AVIV model 202 CD spectrometer (AVIV Instrument, Inc., Lakewood, NJ) at 25 °C under a nitrogen flow. Samples were prepared at a protein concentration of 0.1 mg/mL, adjusted to pH 7.0, and loaded into a quartz cuvette of a pathlength of 2 mm before being centrifuged at 18,000 g for 20 min. The scanning was operated at a rate of 50 nm/min. Data were displayed as the mean residue ellipticity (θ) in unit of deg cm² dmol⁻¹. Recorded spectra were corrected by subtracting the spectrum of a protein-free buffer and the amino acid residue value of 115 for WPI was used in all calculations (Zhu & Damodaran, 1994). The secondary structure compositions were obtained using the CONTIN/LL program in the CDPro software of the instrument (Sreerama & Woody, 2000).

2.7. Zeta potential

Zeta potential of protein dispersions at 25 °C was measured using a model Delsa Nano C analyzer (Beckman Coulter, Inc., Brea, CA). 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 N HCl, and filtered through a 0.45 μ m syringe membrane (EMD Millipore Corp., Billerica, MA) before analysis. Average values of three measurements were reported for each sample.

2.8. Heat stability test

Heat stability was evaluated for samples constituted to 7%w/v protein, pH 3.0–7.0, and 0–150 mM NaCl or CaCl₂. The 1 mL of final dispersions was contained in 4 mL glass vials that were heated for 2 min in an 88 °C water bath, simulating a hot-fill process in the beverage industry for acidic products with pH below 4.6 (Etzel, 2004).

2.9. Differential scanning calorimetry (DSC)

DSC analysis of protein samples was performed by using a VP-DSC Micro calorimeter (MicroCal, Inc., Northampton, MA). Samples were prepared in 10 mM sodium phosphate buffer at a protein concentration of 25 mg/mL and adjusted to pH 3.0 or 5.0 using 1 N HCl. All solutions were thoroughly degassed prior to loading into the cell of the instrument, and the cell was pressurized to 0.2 MPa to prevent the formation of gas bubbles during heating from 20 to 120 °C at a rate of 60 °C/h. The samples were cooled from 120 to 20 °C at the same rate and scanned for another time using the same thermal profile. The sodium phosphate buffer was used to generate the baseline scan for analysis (McGuffey, Epting, Kelly, & Foegeding, 2005). Analysis of the data was carried out using the manufacturer's software (MicroCal Origin 5.0).

2.10. Characterization of protein structures by atomic force microscopy (AFM)

Samples were diluted to 10 ppm protein using deionized water and adjusted to pH 3.0–7.0 using 0.1 N HCl or NaOH. Two μ L of a sample was spread evenly onto a freshly-cleaved mica disk and air-dried for more than 2 h prior to imaging. The instrument was

a NanoScope IIIA Multimode AFM (Veeco Instruments Inc., Santa Barbara, CA). A silicon-etched FESPA cantilever of 200–250 μ m length with a spring constant of 1–5 N/m (Bruker Nanoprobe, Camarillo, CA) was applied at a scan rate of 1.0 Hz and a typical resonant frequency of about 71.0 kHz, and the tapping mode images were collected under ambient conditions. Image analysis was conducted by using the software (version 5.30r3, Digital Instruments Inc., Tonawanda, NY) of the AFM instrument.

2.11. In vitro gastrointestinal digestion

The *in vitro* digestibility of WPI before and after glycation was compared for SDS-PAGE patterns after simulated gastric and intestinal digestions following the literature method (Jovaní, Barberá, Farré, & de Aguilera, 2001; Miquel et al., 2005). For simulated gastric digestion, protein powder with 400 mg net protein was dissolved in 10 mL of a simulated gastric fluid constituted by dissolving 0.04 g pepsin in 10 mL of 0.1 N HCl and adjusted to pH 2.0. The solution was incubated in a shaking water bath at 37 °C for 2 h. The pH was checked every 15 min and readjusted to 2.0 with 1 N HCl if necessary. After terminating pepsin digestion by cooling in ice for 10 min, samples were centrifuged at 10,000 g for 10 min at 4 °C, and the supernatant was collected for SDS-PAGE using the procedures as detailed previously.

To simulate intestinal digestion, the pH of the sample after the simulated gastric digestion was increased to 5.2 by drop-wise adding 1 N NH₄CO₃, followed by mixing with 1.25 g of a fresh pancreatin-bile extract mixture that was prepared by dissolving 0.04 g pepsin and 0.25 g pancreatin and bile salt in 10 mL of 1 N NaHCO₃. The final mixture was incubated in a 37 °C shaking water bath for 2 h, and the reaction was terminated by immersing samples in ice water for 10 min and adjusted to pH 7.2. The supernatant after centrifugation at 10,000 g for 10 min at 4 °C was transferred for SDS-PAGE.

2.12. Statistical analysis

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

3. Results and discussion

3.1. Structure of glycated WPI analyzed by SDS-PAGE, AUC, amino acid composition, and CD

Fig. 1 shows the SDS-PAGE results of WPI before and after dry heating with glucose, MD, sucrose, or lactose. 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 (Agyare et al., 2010). The content of α -lactalbumin appeared to be lower than the composition expected from dairy chemistry, about 1/3 of β -lactoglobulin (Bryant et al., 1998). The sample treated with sucrose showed an almost identical pattern as WPI, indicating the insignificance of glycation. This agrees with the fact that sucrose is a non-reducing disaccharide. For the other three saccharides, the band corresponding to α -lactalbumin disappeared and those of β -lactoglobulin and bovine serum albumin shifted to higher MW bands, with smearing zones at an MW range higher than that of β -lactoglobulin. When compared to WPI, there was a band with MW of $\sim$ 40–50 kDa for conjugate samples, which is likely caused by the covalent cross-linking of protein during the Maillard reaction, as reported in the literature

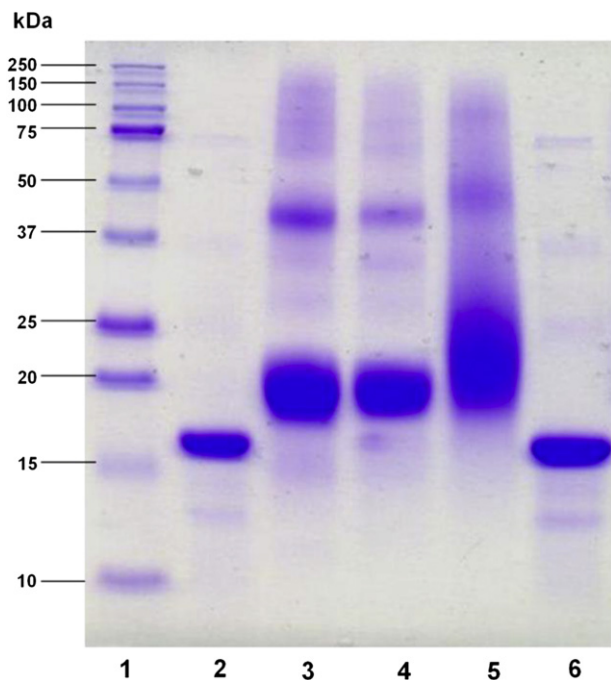


Fig. 1. SDS-PAGE results of WPI before and after dry heating with different saccharides. Lane 1: markers; Lane 2: native WPI; Lanes 3, 4, 5 and 6: WPI after heating with equal mass of glucose, lactose, MD and sucrose, respectively, at 80 °C and 80% relative humidity for 2 h. Fifteen μ g protein was loaded in each lane.

(Jiménez-Castaño, Villamiel, Martín-Álvarez, Olano, & López-Fandiño, 2005; Ter Haar, Westphal, Wierenga, Schols, & Gruppen, 2011). The bands of MD conjugate are less distinct, with much broader smearing zones, which likely is caused by the nonuniform MW distribution of MD that is produced by acid or enzymatic hydrolysis of starch (Avaltroni, Bouquerand, & Normand, 2004).

Because SDS-PAGE only provides qualitative information, AUC was used to study protein structural changes after glycation with various saccharides. Fig. 2 shows AUC data of WPI and its conjugate at pH 3.0, with the analyses summarized in Table 1. Samples were

Table 1

AUC analysis of molecular weight (MW) characteristics of WPI after glycation with glucose, lactose and MD.

Compound	Peak 1		Peak 2		Number of saccharides per protein ^a
	Area%	Average MW (kDa)	Area%	Average MW (kDa)	
WPI	94.4	15.7	5.6	47.5	—
WPI glycated with glucose	91.6	17.5	8.4	49.5	10.0
WPI glycated with lactose	88.7	18.4	11.3	49.6	7.9
WPI glycated with MD	92.5	20.6	7.52	50.7	4.9
WPI dry-heated with sucrose	94.1	15.7	5.9	47.5	—

^a Estimated from peak 1.

studied at pH 3.0 because β -lactoglobulin, the most abundant whey protein, is present mostly as monomers at pH 3.0 or below (Adams, Anderson, Norris, Creamer, & Jameson, 2006; Haug, Skar, Vegarud, Langsrud, & Draget, 2009). The majority of native WPI mass (94.4%) corresponded to an average MW of 15.7 kDa, with a minor (5.6% of total area under the curve) higher molecular weight portion averaging 47.5 kDa. The major peak likely is composed of α -lactalbumin and β -lactoglobulin that have an MW of 13.8 and 16.0 kDa, respectively (Agyare et al., 2010). The AUC result of the sucrose treatment was similar to that of WPI, which agrees with the SDS-PAGE pattern in Fig. 1. After glycation with glucose, lactose, and MD, two peaks were still observed, and both showed an increase in MW, with the higher MW peak growing to a bigger portion than that of WPI. The quantitative assessment of MW changes of WPI using AUC thus agrees with qualitative results from SDS-PAGE.

The quantified MW of glycated WPI also enabled the estimation of the number of glucose, lactose and MD molecules attached to each whey protein according to eq. (1), which was 10.0, 7.9, and 4.9, respectively (Table 1). Estimations from AUC agree with studies reporting multiple reducing saccharides glycated on one β -lactoglobulin (Morgan et al., 1999) or α -lactalbumin molecule (Ter Haar et al., 2011). Because glycation was conducted at the same WPI:saccharide mass ratio, a larger amount of saccharide molecules with a smaller MW theoretically enables the better access of aldehyde groups of reducing saccharide to amino groups of amino acid residues at identical glycation conditions. Reducing saccharides smaller MW were also observed to have higher reactivity during glycation with whey proteins (Jiménez-Castaño, Villamiel, & López-Fandiño, 2007). Furthermore, the significant increase of the bigger MW peak area (Table 1) suggests that the Maillard reaction also causes cross-linking of protein, as observed in SDS-PAGE (Fig. 1) and reported previously (Jiménez-Castaño et al., 2005; Lillard, Clare, & Daubert, 2009; Ter Haar et al., 2011).

To further study the impacts of saccharide structure on the structure of glycated whey protein, the amino acid composition of WPI before and after glycation was analyzed. Table 2 shows that lysine is the dominating amino acid residue reacting with reducing saccharides, with the extent varying with saccharide structure. The extent of overall reduction in the amino acid agrees with the number of reacted saccharides estimated in AUC (Table 1). Results in Table 2 agree with a report showing better reactivity of D-glucose than D-lactose during glycation with bovine serum albumin (Ledesma-Osuna et al., 2008).

Secondary structures of WPI before and after glycation were also characterized. The far-UV CD spectra (Fig. 3) showed broad negative bands at 210–220 nm, which is characteristic of proteins rich in β -sheet (Agyare et al., 2010; Zhu et al., 1994; Zirwer, Gast, Welfle, Schlesier, & Dieter Schwenke, 1985). Table 3 presents secondary

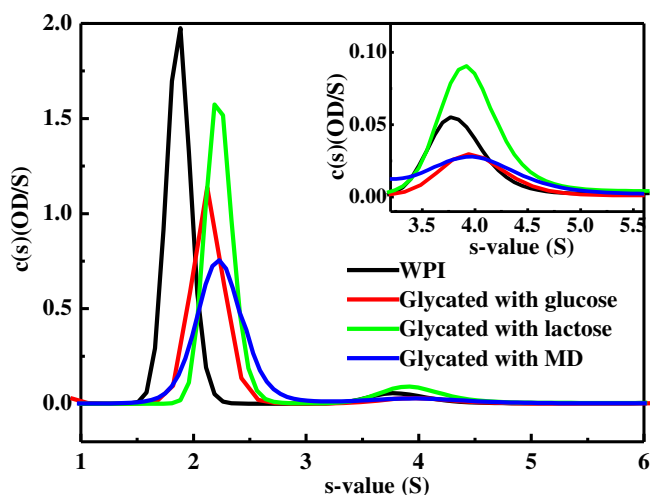


Fig. 2. The best-fit sedimentation coefficient (s , in Svedberg unit, S) distributions ($c(s)$) from analytical ultracentrifugation of WPI before and after glycation with MD (black), lactose (red) and glucose (green). The insert figure is a zoom-in view of curves at s values of 3.2–5.6 S. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2

Amino acid composition of WPI after glycation with different saccharides.

Amino acid	Composition (mole%) ^a				Normalized by mole% of WPI		
	WPI	Lactose conjugate	Glucose conjugate	MD conjugate	Lactose conjugate	Glucose conjugate	MD conjugate
Asx	9.9 ± 0.6a	10.1 ± 0.4a	10.4 ± 0.5a	10.3 ± 0.3a	1.03	1.05	1.05
Thr	6.5 ± 0.3a	6.5 ± 0.6a	6.7 ± 0.2a	6.9 ± 0.4a	1.00	1.03	1.06
Ser	4.6 ± 0.2a	4.1 ± 0.4a	4.5 ± 0.2a	4.6 ± 0.5a	0.91	0.99	1.00
Glx	14.8 ± 0.8a	14.9 ± 0.7a	15.4 ± 0.5a	16.1 ± 0.9a	1.00	1.04	1.08
Gly	2.4 ± 0.2a	2.6 ± 0.2a	2.7 ± 0.4a	2.4 ± 0.1a	1.08	1.09	1.01
Ala	9.1 ± 0.5a	9.9 ± 0.4a	9.9 ± 0.6a	9.4 ± 0.3a	1.08	1.08	1.03
Val	6.7 ± 0.2a	7.0 ± 0.3a	7.2 ± 0.5a	6.9 ± 0.2a	1.04	1.06	1.02
Met	2.4 ± 0.3a	2.5 ± 0.1a	2.5 ± 0.2a	2.6 ± 0.4a	1.07	1.06	1.08
Ileu	6.9 ± 0.4a	7.3 ± 0.2a	7.4 ± 0.4a	7.1 ± 0.3a	1.05	1.06	1.02
Leu	13.0 ± 0.8a	13.4 ± 0.5a	13.6 ± 0.9a	13.2 ± 0.7a	1.03	1.05	1.02
Tyr	2.1 ± 0.4a	1.6 ± 0.3a	1.7 ± 0.4a	2.1 ± 0.3a	0.79	0.84	1.00
Phe	2.4 ± 0.3a	2.5 ± 0.2a	2.7 ± 0.5a	2.5 ± 0.2a	1.05	1.11	1.01
Lys	9.3 ± 0.5a	7.0 ± 0.3b	5.2 ± 0.4c	6.6 ± 0.4b	0.75	0.56	0.70
His	1.4 ± 0.3a	1.4 ± 0.1a	1.1 ± 0.3a	1.3 ± 0.4a	0.98	0.79	0.93
Trp	—	—	—	—	—	—	—
Arg	1.8 ± 0.4a	1.9 ± 0.5a	1.3 ± 0.4a	1.8 ± 0.5a	1.03	0.71	1.00
Pro	6.6 ± 0.5a	7.3 ± 0.6a	7.8 ± 0.9a	6.3 ± 0.6a	1.10	1.19	0.96

^a Numbers are averages ± standard deviations from two replicates. Different letters next to numbers in the same row indicate significant ($p < 0.05$) differences.

structure compositions calculated using CDPro. Our results demonstrated that WPI had an average of 16.6% α -helix, 36.1% β -sheet, 15.0% turns, and 31.8% aperiodic structure (Table 3). After glycation, the ellipticity became significantly less negative (Fig. 3), corresponding to decreased contents of ordered structures (α -helix, β -sheet, and turns) and increased content of aperiodic structure. Changes in protein secondary structures after glycation were speculated to result from the reduced hydrophobicity of whey proteins (Monahan, German, & Kinsella, 1995), which may have been responsible for variations in secondary structure changes after glycation with different saccharides.

3.2. Visual observation of dispersion appearance after heating

Before heating, WPI samples were transparent except at pH 5.0, near the pI of major whey proteins, while all conjugate samples were transparent. Photographs of dispersions after heating at 88 °C for 2 min are presented in Fig. 4. Dispersions of WPI without glycation remained transparent after heating only at pH 3.0 and 0–150 mM NaCl, and pH 7.0 and 0 mM NaCl (Fig. 4A). Dispersions of WPI dry-heated with sucrose had similar colors and heat stability as those of WPI (data not shown). Dispersions of WPI glyated with lactose had the darkest colors, while those with WPI glyated with MD had light yellow color (Fig. 4), which indicates different degrees

of the Maillard reaction when these saccharides reacted with WPI for identical temperature and duration (Akhtar et al., 2007). After heating, the sample glyated with glucose showed improvement in stability at pH 4.0 and 0–150 mM NaCl, and pH 5.0 and >50 mM NaCl (Fig. 4B). For WPI glyated with lactose and MD, samples remained transparent at all pH and NaCl conditions (Fig. 4C, D), even after substituting NaCl by equal molarity of CaCl₂ (not shown).

3.3. Zeta potential changes of WPI after glycation

After glycation, the pH corresponding to zero zeta potential (pI) of WPI shifted from ca. 4.8 to slightly lower pH (Fig. 5). At pH above pI, the zeta-potential of WPI was within the range of glyated WPI. At pH below pI, the zeta-potential of glyated WPI was consistently lower than that of WPI. After glycation, the amount of lysine, with pK of 10.6 (Walstra, 2003), significantly reduced (Table 2). Because lysine residues are contributors of positive charges, the reduced content after glycation causes the lowered positive zeta-potential at pH below 5.0. Overall, the WPI glyated with a smaller saccharide showed greater electrophoretic mobility (larger magnitude of zeta-potential) likely because of smaller MW (Table 1), in addition to differences in surface amino acid compositions that were not determined.

Whey proteins are globular proteins whose aggregation is an overall impact of various types of colloidal interactions (eq. (2)).

$$W_{\text{total}} = W_{\text{vdW}} + W_{\text{hydrophobic}} + W_{\text{electr}} + W_{\text{steric}} \quad (2)$$

where subscripts from left to right indicate total, van der Waals, hydrophobic, electrostatic, and steric interactions, respectively.

Because WPI glyated with lactose and MD still has pH-dependent zeta-potential (Fig. 5) but is stable after heating at all

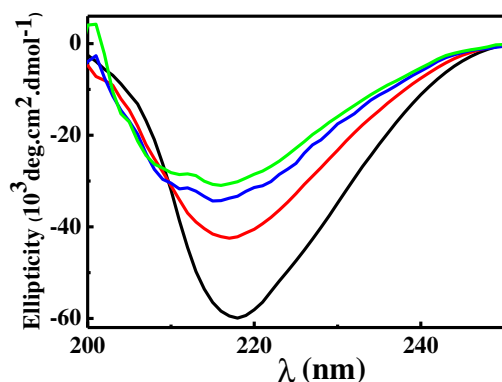


Fig. 3. Far-UV CD spectra of WPI (black) and WPI glyated with MD (red), glucose (blue), and lactose (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3

Secondary structure compositions of WPI and conjugates at pH 7.0.

Sample	α -Helix (%)	β -Strand (%)	Turns (%)	Aperiodic structure (%)
WPI	16.6	36.1	15.0	31.8
WPI glyated with MD	12.2	34.2	14.4	36.4
WPI glyated with glucose	14.1	32.5	14.8	38.7
WPI glyated with lactose	13.7	33.0	11.0	40.6

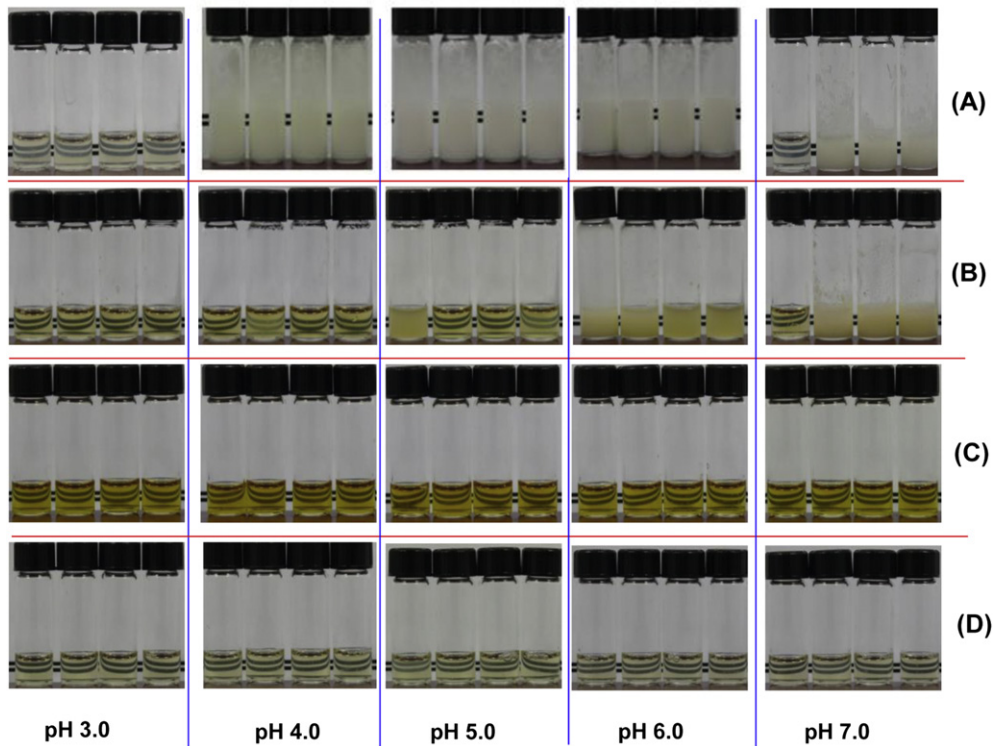


Fig. 4. Photographs of aqueous solutions containing 7% w/v protein after heating at 88 °C for 2 min: WPI (A) and WPI dry-heated with equal mass of glucose (B), lactose (C), or maltodextrin (D). Samples were adjusted to pH 3.0–7.0 and 0, 50, 100, and 150 mM NaCl (vials from left to right in each image) before heating.

pH and ionic conditions (Fig. 4), electrostatic repulsion shall not be the primary colloidal force responsible for the improved heat stability of glycosylated WPI. The improved thermal stability can then be attributed to repulsive steric interactions provided by the glycosylated saccharides (Akhtar et al., 2003; Dickinson & Galazka, 1991; Lillard et al., 2009). Specifically, steric interactions are a function of the number and effective chain length of saccharides grafted on whey protein molecules, as described in eqs. (3) and (4) (Tuinier & de Kruif, 2002). In the present study, the saccharides adopted are all non-ionic and have good water solubility at studied conditions. Although the number of glucose attached to whey protein was higher than that of lactose and MD treatments (Table 1), the chain height of glucose on proteins (H in eq. (4)) was smaller than that of lactose and MD. Therefore, the WPI glycosylated with glucose was the least stable (Fig. 4), indicating that the repulsive steric interactions resulting from the monosaccharide are not stronger than attractive

van der Waals and hydrophobic interactions during heating. It should however be noted that specific locations of glycosylated saccharides and possible implication of thermal treatment on the stability and locations of glycosylated saccharides are to be elucidated to completely understand the exact mechanism.

$$\begin{aligned} \frac{W_{\text{steric}}(h)}{k_B T} &= \infty & \text{for } h < 0 \\ &= f(H, h, \theta) & \text{for } 0 < h < 2H \\ &= 0 & \text{for } h > 2H \end{aligned} \quad (3)$$

where H is the height of grafted polymers on a colloidal particle, h is the separation distance between two particle surfaces, θ is the density of grafted polymers, k_B is the Boltzman constant, and T is the absolute temperature (Tuinier et al., 2002).

$$H = Nb \left(\frac{8 v_{\text{eff}} \theta}{\pi^2} \right)^{1/3} \quad (4)$$

where N is the number of polymer segments, b is the segment length, and v_{eff} is the effective excluded volume (Tuinier et al., 2002).

3.4. Denaturation properties of WPI as impacted by glycation

Thermal denaturation properties are important to understand the aggregation of whey protein during heating and are commonly analyzed using DSC (McGuffey et al., 2005). The impacts of glycation on denaturation of WPI were characterized at pH 3.0 because all samples were transparent after heating (Fig. 4) and β -lactoglobulin is present mostly as monomers at pH 3.0 (Adams et al., 2006; Haug et al., 2009). Tests also were conducted at pH 5.0 as it is close to pI of WPI (Fig. 5). Fig. 6 shows the DSC denaturation profiles during the first scan, and there was no peak observed

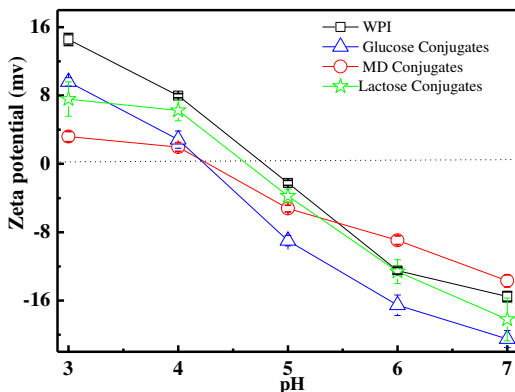


Fig. 5. Zeta potential profiles of WPI before and after glycation with glucose, MD or lactose. Error bars are standard errors from triplicate measurements.

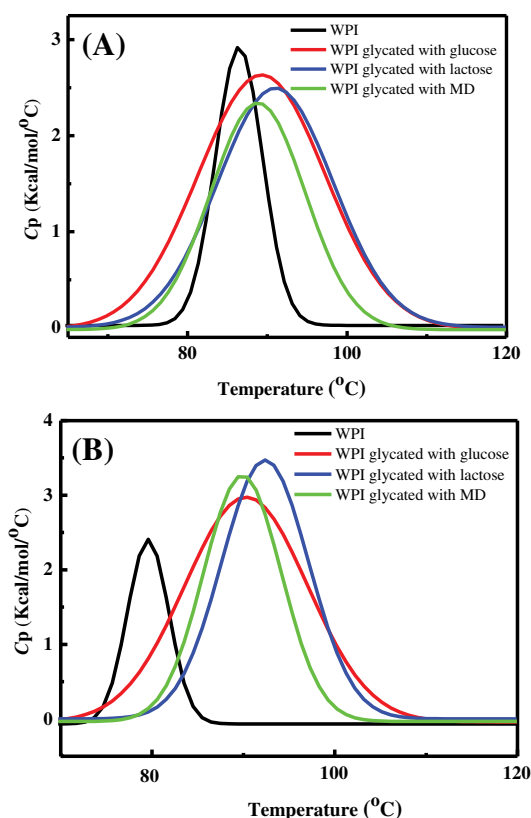


Fig. 6. DSC thermograms demonstrating specific heat (C_p) of native and glycated WPI at pH 3.0 (A) and 5.0 (B).

during the second scan. The calorimetric enthalpy (ΔH), which measures the energy needed to overcome non-covalent interactions during protein denaturation (Boye & Alli, 2000), was obtained from the area under the curve in Fig. 6 after polynomial baseline fitting. The determined ΔH and the denaturation temperature (T_d) are listed in Table 4. The T_d and ΔH of native WPI were estimated to

Table 4

DSC assessment of denaturation temperature (T_d) and the associated enthalpy change (ΔH) for WPI and conjugates at pH 3.0 and 5.0 and 0 mM NaCl.^a

	Sample	T_d (°C)	ΔH (kcal/mol)
pH 3.0	WPI	86.2 ± 0.7b,c	19.7 ± 0.6e
	WPI glycated with glucose	88.8 ± 1.3c	35.4 ± 0.5b
	WPI glycated with lactose	91.5 ± 1.6a	29.8 ± 1.1c
	WPI glycated with MD	88.0 ± 1.0c	23.6 ± 0.8d
pH 5.0	Native WPI	79.1 ± 0.5d	13.6 ± 0.8f
	WPI glycated with glucose	89.2 ± 0.7c	38.3 ± 0.7a
	WPI glycated with lactose	92.3 ± 1.1a	35.2 ± 1.0b
	WPI glycated with MD	89.2 ± 0.8c	30.8 ± 0.9c

^a Values are means ± standard errors from triplicate measurements. Means with different letters next to numbers in the same column differ significantly ($p < 0.05$).

be 86.2 °C and 19.7 kcal/mol at pH 3.0, respectively. After glycation, T_d increased for all conjugates, which indicates the enhanced resistance against thermal denaturation after glycation. When pH was increased from 3.0 to 5.0, the T_d and ΔH of native WPI reduced, consistent with the better thermal stability of β -lactoglobulin at the lower pH (Boye et al., 2000). The T_d of conjugates was similar at pH 3.0 and 5.0, while ΔH was higher at pH 5.0. Furthermore, because all glycated WPI had the significantly increased T_d but the WPI glycated with glucose aggregated at various conditions, the increase in T_d alone was not a primary contributor to the limited aggregation of WPI glycated by lactose and MD.

3.5. Nanoscale structures of glycated WPI after heating at varied acidity conditions

AFM was used to study nanostructures of glycated WPI at pH 3.0 before heating, and those at pH 3.0–7.0 after heating at 88 °C for 2 min. Representative AFM images are shown in Fig. 7 for WPI glycated with lactose. Although all samples were transparent (Fig. 4), coarser and bigger structures formed after heating at pH 4.0–6.0 than at pH 3.0 and 7.0 (Fig. 7). Because zeta-potential is lower at pH 4.0–6.0 (Fig. 5), AFM images indicate that steric interactions are not sufficiently strong to completely inhibit aggregation during heating when electrostatic repulsion is weakened. Particle heights of structures were measured based on AFM

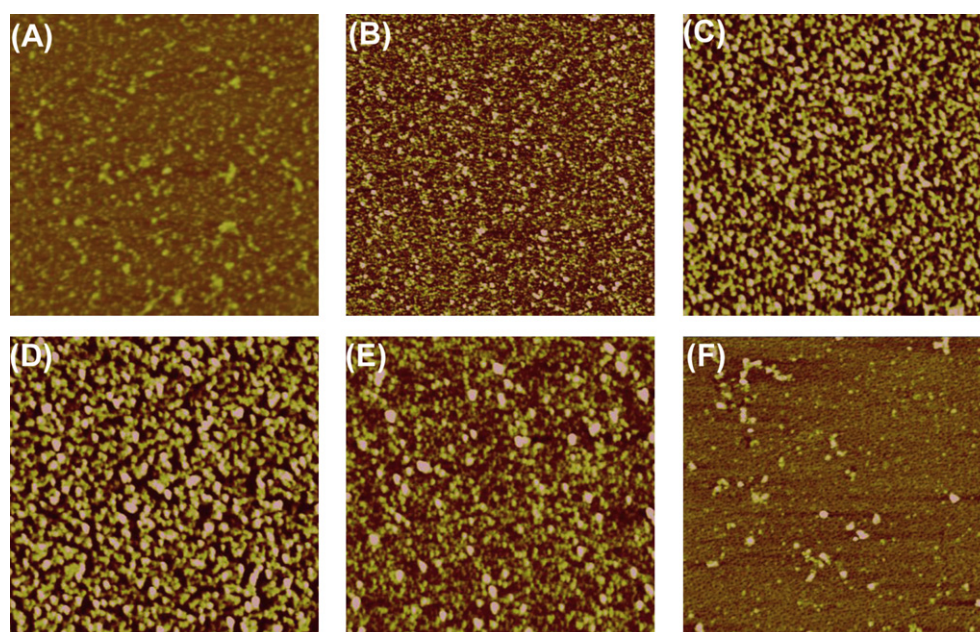


Fig. 7. Tapping mode AFM height images of whey protein glycated by lactose before heating at pH 3.0 (A), and after heating at pH 3.0 (B), 4.0 (C), 5.0 (D), 6.0 (E), and 7.0 (F). The scanned area is 2 μm × 2 μm .

Table 5

AFM section analysis of particle heights for WPI conjugated with glucose, lactose and MD after heating at 88 °C for 2 min at pH 3.0–7.0 and 0 mM NaCl.^a

Processing	Height (nm)		
	Glucose conjugate	Lactose conjugate	MD conjugate
Before heating, at pH 3.0	4.9 ± 0.5d	3.4 ± 0.3e	3.3 ± 0.4e
After heating, at pH 3.0	7.7 ± 0.2c	4.3 ± 0.4d	7.9 ± 0.2d
After heating, at pH 4.0	11.3 ± 0.5a	8.3 ± 0.3bc	9.2 ± 0.2c
After heating, at pH 5.0	>200	13.8 ± 0.5a	13.7 ± 1.0a
After heating, at pH 6.0	>200	9.1 ± 0.1b	10.5 ± 0.8b
After heating, at pH 7.0	8.9 ± 0.3b	7.6 ± 0.6c	9.1 ± 0.2c

^a Values are means ± standard errors from height measurements of particles on AFM images. Means with different letters next to numbers in the same column differ significantly ($p < 0.05$).

images, and the averages are summarized in Table 5. At pH 3.0, WPI glycosylated with glucose was significantly bigger than the other two treatments. At pH 5.0, near pI of WPI (Fig. 5), the particles were the biggest for all treatments, while the smallest structures were observed at pH 3.0. Although thermal aggregation still occurred for glycosylated WPI, an overall structure smaller than 14 nm (Table 5) corresponded to transparent dispersions (Fig. 4), because of the inability of these nanostructures to scatter visible light to cause any turbidity. In contrast, particle heights bigger than 200 nm (WPI glycosylated by glucose, after heating at pH 5.0 and 6.0) resulted in turbid dispersions (Fig. 4).

3.6. *In vitro* digestibility of native and glycosylated WPI

Native β -lactoglobulin was reported to resist hydrolysis by pepsin in the gastric environment (Astwood, Leach, & Fuchs, 1996;

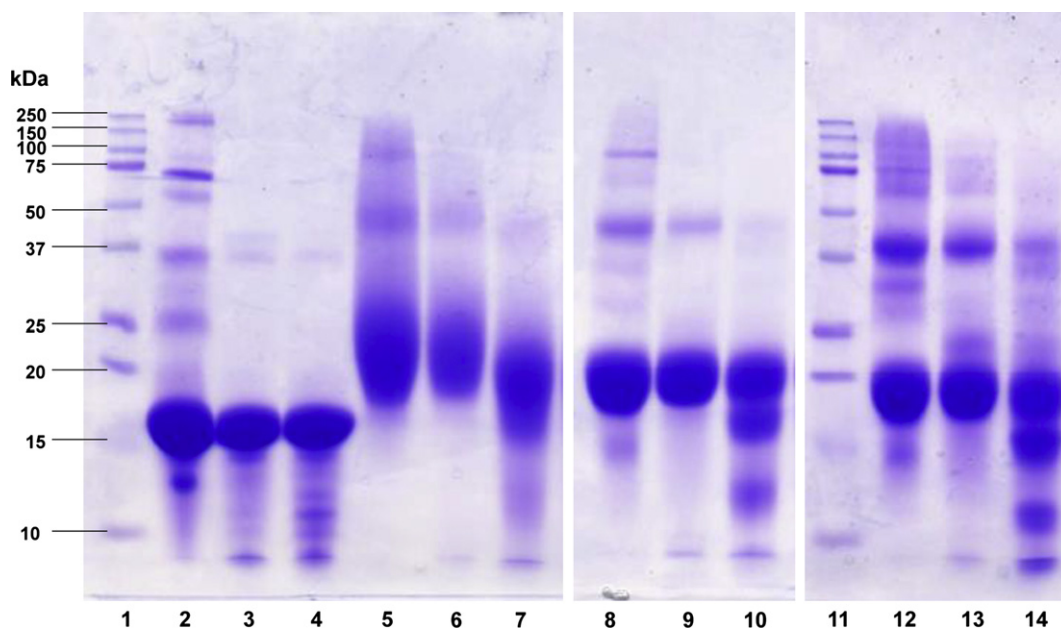


Fig. 8. SDS-PAGE showing impacts of simulated digestions on WPI (lanes 2–4) and its maltodextrin (lanes 5–7), lactose (lanes 8–10), and glucose (lanes 12–14) conjugates. For each sample, lanes from left to right are samples before and after simulated gastric and intestinal digestions at 37 °C for 2 h. Lanes 1 and 11 are markers. Twenty-five μ g protein was loaded in each lane.

Table 6

SDS-PAGE analysis of WPI and conjugates after simulated gastric and intestinal digestions.

Sample	MW of protein bands (kDa)	% Of band intensity	MW of protein bands after gastric digestion (kDa)	% Of band intensity	MW of protein bands after intestinal digestion (kDa)	% Of band intensity
WPI	250	1.9	—	—	—	—
	72	2.7	—	—	—	—
	60	1.9	—	—	—	—
	36	5.5	36	3.9	36	2.7
	26	3.6	—	—	—	—
	18	72.7	18	65.2	18	56.2
	13	11.7	<18	30.9	<18	41.1
WPI glycosylated with MD	>28	9.5	>28	3.8	>28	3.0
	18–28	90.5	18–28	96.2	18–28	54.6
	<18	—	<18	0	<18	43.4
	—	—	—	—	—	—
WPI glycosylated with lactose	100	4.4	—	—	—	—
	40	12.2	40	10.5	40	2.5
	20	75.0	20	79.2	20	55.1
	18	8.3	<18	10.3	<18	42.4
WPI glycosylated with glucose	>50	20.2	>50	2.6	—	—
	40	10.6	40	12.3	40	6.6
	19.4	64.3	22	6.2	22	4.0
	15	4.9	19.4	75.0	19.4	44.0
	—	—	<19	3.9	<19	45.4

Luz Sanz et al., 2007; Morgan et al., 1999) and thus may remain intact after reaching the upper portion of the small intestine (Kitabatake & Kinekawa, 1998). Both reduced (Luz Sanz et al., 2007; Moreno et al., 2008) and increased digestibility (Bouhallab et al., 1999; Yeboah et al., 2004) has been reported for whey protein after glycation. The reduced trypsin digestion has been proposed to be caused by the lowered reactivity of trypsin on lysine and arginine residues after glycation, while the improved digestion has been attributed to the conformational changes of proteins (Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010). In the present study, SDS-PAGE was used to qualitatively compare the digestibility of WPI before and after glycation (Fig. 8), and the relative intensities of protein bands were quantified to provide a direct comparison (Table 6). Based on the intensity of bands smaller than 18 kDa (MW of β -lactoglobulin), the digestion of WPI in the simulated gastric fluid decreased significantly after glycation, while the digestibility slightly improved after the second step simulated intestinal digestion (Table 6). This agrees with the reduced content of ordered secondary structures after glycation (Table 3). The presence of other digestive enzymes in pancreatin such as amylases may also impact the digestion of glycated WPI. While more comprehensive studies are required to evaluate the digestibility and also allergenicity (Corzo-Martínez et al., 2010), our study indicates that glycation is a promising method to improve heat stability of WPI for transparent beverage application and study whey protein-based delivery systems for nutraceuticals (Chen, Remondetto, & Subirade, 2006) and pharmaceuticals (Livney, 2010).

4. Conclusions

Results from several complementary techniques in this work demonstrated that the structure of saccharides greatly impacted the amount of saccharides attached to whey protein and both the number and length of saccharides were critical to the aggregation of glycated WPI during heating. Glycation increased T_d and modified charge characteristics of whey protein, but the thermal aggregation of glycated WPI still occurred, especially at pH near pI. The repulsive steric interactions provided by the glycated saccharides minimized inter-molecular aggregation. When WPI was glycated with a sufficient number of di- or oligosaccharides, the aggregate structures were smaller than about 14 nm or several whey protein molecules after heating at pH 3.0–7.0, corresponding to transparent dispersions. Glycation did not seem to reduce the digestibility of WPI after sequential steps of simulated gastric and intestinal digestions, and the findings from this study are promising for the application of WPI in manufacturing products involving thermal processing.

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