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Heat Stability Improvement of Whey Protein Isolate by Glycation with Maltodextrin without control of the relative humidity

running title：Glycation without control of the relative humidity

Xinguang Qin[1]，Gang Liu[*2]，Wenjie Zhen[1], Jingren He[2]，Zhenzhou Zhu[2] and Sola Lamikanra[2]

[1]Department of Chemistry, Jinan University, Guangzhou, 510632, China
[2]College of Food Science and Engineering, Wuhan Polytechnic University, Wuhan, 430023，China

Please send correspondence to

*Gang Liu, Associate Professor

College of Food Science and Engineering

Wuhan Polytechnic University

Wuhan, Hubei province,430023

E-mail:lg820823@163.com

Abstract

Reduction of protein aggregation and their improved heat stability in solutions is often achieved through their glycation. While controlled relative humidity (RH) is a standard practice in the glycation process, its requirement, and associated cost, as a condition for the treatment has not been demonstrated. The improved heat stability of whey protein isolate (WPI) at pH 3-7 and 0-150 mM NaCl after glycation with maltodextrin (MD) by way of the Maillard reaction in the dry state without the need to control of RH is reported in this study. Dispersions of glycated WPI remained transparent after heating at 88 °C for 2 min at pH values that are close to whey protein pI values, including pH 5.0. Transparent dispersions were enabled as indicated by circular dichroism by WPI-MD conjugates heated in aqueous solutions that underwent secondary structure changes, and AFM images that indicated globular aggregates smaller than 40 nm.

Keywords: Whey protein isolate, glycation, heat stability, atomic force microscopy, zeta-potential, steric interactions

1. Introduction

Whey protein isolates (WPI), a byproduct of cheese manufacturing, is a rich source of high quality protein. WPI contain over 90% protein. Considerable interest and research on WPI and WP products have been mainly driven by the quality, functionality and relative abundance(Foegeding, Davis, Doucet, & McGuffey, 2002). Acidity of the main WPI proteins, β-lactoglobulin, α-lactalbumin, and bovine serum albumin, present in WPI (pI = 5.2, 4.8-5.1, and 4.8-5.1, respectively) (Hickstein & Peuker, 2008; Nakano & Ozimek, 2000), however, causes these molecules to aggregate during processing of low pH products as a consequence of the low electrostatic repulsion due to comparable charges on the protein molecules and the aqueous medium(Golovanov, Hautbergue, Wilson, & Lian, 2004). This effect is enhanced with increased ionic strength of the solution (Zhang & Zhong, 2009) . Compounding this is the fact that thermal pasteurization denatures proteins with a consequent increase in hydrophobic interactions and increased and increased disulfide bond formation (Bryant & McClements, 1998). Low pH beverages in particular are more susceptible to undesirable turbidity and protein precipitation as a result of the combined effects of these interactions.

Induction of Maillard reaction through glycation of dry protein products at various relative humidity values (RH) has been reported to improve their stability in processed products (Gang Liu & Zhong, 2012, 2013; Visser & Thomas, 1987). The effectiveness of the treatment, however, is dependent on the properties of the saccharides used including their emulsifying and stabilizing capabilities (Dunlap & Côté, 2005; Neirynck, Van Der Meeren, Bayarri Gorbe, Dierckx, & Dewettinck, 2004) and molecular weights (Dickinson & Euston, 1991; K. Fujiwara, Oosawa, & Saeki, 1998; D. Zhu, Damodaran, & Lucey, 2010). Protein solutions (7% w/v; pH 5) were reported to be transparent and fluxible after heating at 88 °C for 2 min(Gang Liu & Zhong,

2012, 2013). While protein glycation for use in protein stabilization all appear to have been conducted under controlled RH(Gang Liu & Zhong, 2012, 2013), there seems to be no study to be performed without controlled RH for the process. The ability to efficiently glycate proteins for this purpose without the need to control RH would significantly reduce processing cost required. Most reports also do not address the heat-induced structural changes in glycated WPI, especially as these relate to controls in which RH was not controlled during the glycation process. Hydrodynamic radius of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, the main whey proteins are 2.6-4.9 (Parker, Noel, Brownsey, Laos, & Ring, 2005), 2.0 (Molek & Zydney, 2007), and 3.7 nm (Brownsey, Noel, Parker, & Ring, 2003), respectively. Consequently, their colloidal properties will be expected to impact structural changes and stability of their glycated products in heated solutions.

The choice of maltodextrins in the present study was made due to its commercially available, aqueous solubility and low viscosity (Marchal, Beeftink, & Tramper, 1999), and it has been extensively used in the glycation between the whey protein under the control of relative humidity(Gang Liu & Zhong, 2012). Nonuniform MW distribution of maltodextrin (MD), a dextrose equivalent of 18 corresponding to a molecular weight (MW) of 1000 Da, which is produced by acid or enzymatic hydrolysis of starch(Avaltroni, Bouquerand, & Normand, 2004).

In this study, we report the Maillard reaction-mediated glycation of WPI at different pH values and ionic strengths with maltodextrin (MD) in an uncontrolled RH environment. The effects of glycation and conditions under which they were carried out on WPI structure were determined. Nanoscale structures of glycated WPI as they relate to colloidal interactions and heat stability are also discussed.

2. Materials and Methods

2.1. Materials

WPI was obtained from Hilmar Ingredients (Hilmar, CA). MD with a dextrose equivalent of 18, corresponding to an average molecular weight of ca. 1000 Da, was acquired from Grain Processing Corporation (Muscatine, IA). Polyacrylamide gel, Coomassie Blue, and protein markers were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). β-Mercaptoethanol (2-ME) and deuterium oxide were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffer and deionized water were bought from Thermo Fisher Scientific Inc. (Pittsburgh, PA). All other chemicals were of analytical grade.

2.2. Preparation of glycated WPI

WPI and MD were dissolved in distilled water at a mass ratio of 2:1, adjusted to pH 7.0, and spray-dried using a model 290 Mini-Spray Dryer (Büchi Laboratoriums-Technik, Flawil, Switzerland). The inlet air temperature and the rate of aspiration were set to be 160 °C and 30 m^3/h, respectively. The outlet temperature was controlled to be approximately 65 °C by adjusting the pump flow rate to be 2 mL/min. The resultant powder was collected and placed in an oven for heating at 90 and 115 °C for 2 h or 135 °C for 10, 30, and 60 min without control of the relative humidity.

2.3. Structures of conjugates analyzed by analytical ultracentrifugation (AUC)

AUC was used to study structure and aggregation properties of conjugates at different pH conditions by estimating sedimentation profiles. Solutions were prepared at a protein concentration of 1.2 mg/mL in deionized water and adjusted to target pH values. Sedimentation velocity (SV) runs were performed on a Beckman XL-I analytical ultracentrifuge using an An50-Ti rotor (Beckman Coulter, Palo Alto, CA). The 400 µL sample and reference solutions were placed in an Epon charcoal-filled double sector cell with an optical pathlength of 12 mm.

The absorbance at 280 nm was recorded every 4 min at 50,000 rpm and 25 °C. Data were analyzed using the continuous $c(s)$ distribution model in SEDFIT which uses solutions to the Lamm equation to produce size distribution profiles (Schuck, 2000). Additional analysis of the AUC data for WPI-MD conjugates was performed using the continuous $c(s, f/f_0)$ and continuous $c(s)$ fixed f/f_0 variable $\bar{\upsilon}$ models.

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

SDS-PAGE was used to qualitatively assess molecular weight distributions of conjugates produced at different conditions. The protein solutions were diluted 5 times in an SDS-PAGE sample buffer (GenScript Corp., Piscataway, NJ). After heating at 95 °C for 5 min, 10 μL of each sample was loaded onto a precast 4–20% gradient polyacrylamide gel purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). The electrophoresis at 150 Volts was terminated after the indicator dye reached the gel bottom, followed by fixing, staining with Coomassie Blue, and destaining until satisfactory band visibility.

2.5. Heat stability test

The heat stability was evaluated for dispersions prepared at 5%w/v protein, pH 3.0-7.0, and 0-150 mM NaCl. A 1 or 2 mL volume of aqueous dispersion was placed in sealed vials for heating in a 88 °C water bath for 2 min, simulating a hot-fill process in the beverage industry (Etzel, 2004). The vials were inverted and photographed after cooling to room temperature.

2.6. Conformational changes studied by using circular dichroism (CD) spectroscopy

Far-UV CD spectra were collected using an AVIV model 202 CD spectrometer (AVIV Instrument, Inc.). Untreated and glycated WPI samples were diluted to a protein concentration of 0.1 mg/mL in a phosphate buffer solution, adjusted to pH 3.0, 5.0 or 7.0, heated at 88 °C for 0.5, 1, 1.5, and 2 min, and centrifuged at 18,000 *g* for 20 min prior to loading to a quartz cuvette of a

pathlength of 2 mm. The scanning at a wavelength range from 190 to 250 nm was performed at a rate of 50 nm/min. Data were expressed as the mean residue ellipticity (θ) in deg cm^2 $dmol^{-1}$ using a mean value of 115 for the amino acid residues of WPI as a reference in all calculations (H. Zhu & Damodaran, 1994). The recorded spectra were corrected by subtracting the spectrum of a protein-free buffer.

2.7. Characterization of conjugate structures by AFM

The dimension and morphology of glycated WPI molecules and their aggregates were imaged using AFM that has been successfully used to study proteins (G. Liu, Li, Shi, Wang, Chen, Liu, et al., 2009), polysaccharides (Kirby, Gunning, & Morris, 1996; Rief, Oesterhelt, Heymann, & Gaub, 1997), and DNA (Hansma, Laney, Bezanilla, Sinsheimer, & Hansma, 1995; Mao, Sun, & Seeman, 1999). The glycated WPI was dissolved in deionized water, adjusted for pH, heated at 88 °C for 2 min, and diluted to be a protein concentration of 10 ppm with deionized water. Aliquots (2 μL) of the diluted samples were spread on freshly cleaved mica disks and air-dried for more than 2 h. Topographical images were collected at room temperature using a NanoScope IIIA Multimode microscope (Veeco Instruments Inc., Santa Barbara, CA) operated in the tapping mode. The 200-250 μm long silicon-etched FESPA cantilever had a nominal spring constant of 1-5 N/m and was operated at a typical resonant frequency of 71.0 kHz (Bruker Nanoprobe, Camarillo, CA) and a scan rate of 1.0 Hz. Images were analyzed using the software (version 5.30r3, Digital Instruments, Veeco) provided by the manufacturer.

3. Results and Discussion

3.1. Structure changes of whey protein after glycating with MD

Structural changes of WPI glycated by MD were studied by complementary techniques of

SDS-PAGE, AUC and FTIR. All treatments were analyzed using SDS-PAGE, while FTIR and AUC were studied only for the sample showing the highest extent of glycation.

SDS-PAGE. Figure 1 shows results from SDS−PAGE analysis of untreated WPI and WPI-MD conjugates prepared by heating under various conditions. The presence of high molecular weight products is more evident for conjugates prepared at a higher conjugation temperature and a longer heating time. For example, the products glycated at 135 °C show prominent bands with molecular weights greater than 170 kDa (lanes 3 and 4 of Figure 1). Heating for 10 min (lane 3) resulted in both modified monomeric proteins and high molecular weight species, while extending the heating time to 1 h led to most protein present in the high molecular weight band (lane 4). For reactions using a shorter heating time (lane 3) or lower temperatures (lanes 5 and 6), the major protein band corresponding to β-lactoglobulin demonstrated an increase of MW by 2000-5000 Da. The SDS-PAGE results are consistent with previous studies that reported the higher molecular weight products of whey protein and saccharide mixtures heated for a longer time (Akhtar & Dickinson, 2003, 2007; Boratyński & Roy, 1998; Lillard, Clare, & Daubert, 2009).

AUC. AUC is a non-destructive method to study molecular weight and self-assembled structures of water-soluble polymers, including proteins (Hao, Ryan, Bailey, & Smith, 2009; Laue & Stafford III, 1999). The sedimentation coefficient (s) distributions, $c(s)$, of WPI and WPI-MD conjugates are presented in Figure 2. The weight-average molecular masses of untreated WPI were determined to be 18.0, 38.2, 29.1, and 18.0 kDa at pH 3.0, 4.0, 5.0, and 6.0, respectively (Figure 2). At each pH, the major peak comprised more than 95% of the total signal, and higher order species were insignificant. These results indicate that untreated whey proteins are primarily monomeric at pH 3.0 and 6.0, form some dimers at pH 5.0, and are predominately dimers at pH

4.0. After conjugation with MD by heating at 135 °C for 60 min, the sedimentation profile showed both a change in *s* of the major peak relative to untreated WPI and the appearance of higher order species (Figure 2). At pH 3.0, 4.0 and 6.0 the major peak is shifted to a higher *s* value reflecting the added mass after conjugation. At pH 5.0, the major peak shifts to a lower *s* value. This change results from a shift to mostly monomeric species from the mixture of monomers and dimers observed in untreated WPI. Thus, MD attachment inhibits dimer formation at pH 5.0. This is likely caused by the pI shift of whey protein towards a lower pH after conjugation (Figure 4D below). It is also possible that WPI-MD conjugate is unable to form dimers at this pH due to steric hindrance provided by MD molecules attached to WPI. At each pH condition tested, greater than one half of the protein sedimented as a higher order (larger than dimer) species. The WPI-MD conjugates showed a lower degree of polymerization with increasing pH (Table 1). At pH 6.0, 44.2% of the loading concentration was in the monomer/dimer range. This value decreased with a decrease in pH, dropping to 34.7% at pH 3.0.

Molecular weight calculations for the WPI-MD conjugates are complicated by not having prior knowledge of the exact number of modifications per protein molecule. Increasing the number of modifications will both increase the frictional coefficient (f/f_0) and decrease the partial specific volume ($\bar{\upsilon}$) of the glycated protein (Durchschlag, 1989). Both of these properties are variables in the molecular weight calculation from sedimentation velocity data. Therefore, only a range of molecular weight values could be determined for the WPI-MD data using a combination of continuous $c(s, f/f_0)$ and continuous $c(s)$ with fixed (f/f_0) analysis. The values are shown in Figure 2B. At pH values of 3.0 and 5.0, the major peak contains monomeric protein with 3-5 MD molecules attached. This calculation uses the molecular weight of 18.0 kDa determined by AUC of untreated WPI and the molecular weight of 1.0 kDa for MD. At pH 6.0, this calculation shows

198 2-4 MD per protein. At pH 4.0, major peak appears to contain 2-8 MD molecules per dimer (or
199 1-4 MD per monomer).

200 *3.2. Heat stability of glycated WPI*

201 The heat stability of WPI-MD conjugates was evaluated by heating aqueous solutions
202 containing 5%w/v protein at 88 °C for 2 min, shown in Figure 3 for a simple mixture of WPI and
203 MD, and WPI-MD conjugates prepared by heating at 115 °C for 2 h and 135 °C for 30 min and 1
204 h (Figure 3). All samples at pH 3.0 remained flowable and clear after heating at all NaCl
205 concentrations. At pH 7.0, unconjugated WPI became more turbid at a higher ionic strength,
206 which contrasts with transparent appearance of all conjugate samples. At pH 4.0-6.0, near the pI
207 of whey proteins, unconjugated WPI formed turbid weak gels that flew upon inverting vials. The
208 conjugate prepared by heating at 115 °C had similar heat stability as unconjugated WPI, with
209 improvement only at pH 7. The conjugate prepared by heating at 135 °C for 30 min remained
210 transparent at pH 3.0, 6.0 and 7.0 and that glycated at 135 °C for 1 h remained transparent at pH
211 3.0 and 5.0-7.0. For both conjugate samples prepared at 135 °C for 30 min and 1 h, no
212 remarkable effects of salt concentration were noticed. The darker color generated by glycation at
213 a higher temperature is a drawback to be studied in future research, this unfavorable change in
214 color was also existed in the glycation under controlled RH conditions(Gang Liu & Zhong, 2012;
215 Spotti, Perduca, Piagentini, Santiago, Rubiolo, & Carrara, 2013). Approaches of reducing color
216 generation during the Maillard reaction include adoption of high pressure (Guan, Yu, Yu, Xu,
217 Shi, & Sun, 2011) and incorporation of catechol-containing compounds (Y. Fujiwara, Kiyota,
218 Tsurushima, Yoshitomi, Mera, Sakashita, et al., 2011) and ferulic acid (Silván, Assar, Srey, del
219 Castillo, & Ames, 2011).

220 As discussed previously, the weakened electrostatic repulsion at pH approaching pI and

increased ionic strength favors protein aggregation as a result of short-range hydrophobic and long-range van der Waals attractions (Xiong, Dawson, & Wan, 1993). Hydrophobic attraction is further strengthened after thermal denaturation (Bryant & McClements, 1998). This was demonstrated for unconjugated WPI in Figure 3A. For conjugates, the glycated MD creates a molecular layer on protein molecules to provide steric hindrance against protein aggregation, as demonstrated in Figure 3B, C and D and previously discussed (Akhtar & Dickinson, 2003; Dickinson & Galazka, 1991; Lillard, Clare, & Daubert, 2009). Because MD is nonionic and much bulkier than ions, the steric repulsion is not impacted by NaCl concentration, as shown for samples glycated at 135 °C. After glycation, the pI of WPI shifted to ~pH 4.0 (Figure 5D), agreeing with the literature (Wang & Ismail, 2012). Therefore, the steric repulsion is strong enough to prevent extensive aggregation before heating but the thermal denaturation of whey protein strengthens hydrophobic attraction that is the strongest at pI, still causing turbid gels after heating at pH 4.0.

3.3. Secondary structure changes of glycated whey protein after heating in solutions at different pH conditions

Dispersions of WPI and WPI-MD conjugates prepared by heating at 135 °C for 1 h were adjusted to pH 3.0, 5.0, and 7.0 for heating at 88 °C up to 2 min, and the resultant samples were characterized for far-UV CD spectra (Figure 4 and Table 2). The negative peak near 210-220 nm was observed for WPI and is characteristic of a β-sheet-type secondary structure (H. Zhu & Damodaran, 1994; Zirwer, Gast, Welfle, Schlesier, & Dieter Schwenke, 1985). The secondary structure compositions of WPI and WPI-MD conjugates calculated using the CONTIN/LL program in the CDPro software (Sreerama & Woody, 2000) are tabulated in Table 2. Before heating, WPI was composed of 24.5% α-helix, 35.4% β-sheets, 12.5% turns, and 30.8%

aperiodic structure at pH 7.0. These numbers are a little different with an earlier study reporting 20.5% α-helix, 42.5% β-sheet, 1.5% turns, and 34.5% aperiodic structure at pH 7.0 (H. Zhu & Damodaran, 1994), the difference between them may be probably attributed to processing and the variety of the WPI. When WPI was heated at pH 3.0, the CD spectra remained practically unchanged (Figure 4). At pH 5.0, the ellipticity became less negative after heating for a longer time and the peak shifted to a longer wavelength (Figure 4), corresponding to significant increases in the content of aperiodic structure (from 31.2 to 47.3%) and decreases in β-sheet structures (from 37.2 to 20.9%) and α-helix (from 24.7 to 10.4%) (Table 2). At pH 7.0, the shift of CD spectra to a longer wavelength after heating for a longer time was also observed, which was in general agreement of a study where WPI was heated at 70 °C and pH 7.0 (H. Zhu & Damodaran, 1994). Increases of aperiodic structure contents after heating were the most significant at pH 5.0 (Table 2), indicating protein denaturation after heating is more pronounced near pI.

After glycation with MD, the percentage of aperiodic structure significantly decreased (Table 2). When WPI-MD conjugates were heated at 88 °C for 2 min, the magnitudes of negative ellipticity decreased significantly at all examined pH conditions, corresponding to significant decreases in β-sheet and α-helix structures and increases in aperiodic structure contents (Table 2). It can be noted that major changes in the CD spectra occurred within 1 min after heating at 88 °C for both WPI and WPI-MD conjugate (Table 2), indicating that heating for 2 min was long enough to compare heat stability characteristics of WPI treatments.

3.4. Morphology of glycated WPI after heating in solutions at pH 3.0 and 5.0

AFM was applied to further investigate structures of glycated WPI after heating at pH 3.0 and 5.0 without NaCl. AFM has the unique ability to study topographical images of both

separated and aggregated proteins without complicated sample preparations (G. Liu, et al., 2009). At an identical mass concentration (10 ppm), a greater number of ellipsoidal particles were observed at pH 5.0 than at pH 3.0 (Figure 5A,and B), indicating a greater extent of protein aggregation at pH 5.0. Representative height profiles of protein particles are shown in Figure 5C. Particles formed at pH 5.0 were generally taller than those at pH 3.0, with the maximum heights of 34.5 and 20.2 nm at pH 5.0 and 3.0, respectively. Therefore, it can be concluded that the conjugated sample had a greater tendency to aggregate at pH 5.0 than at pH 3.0, resulting in increased turbidity at pH 5.0 (Figure 3). The structures smaller than 40 nm were not sufficiently big to cause turbidity, enabling mostly transparent dispersions after heating.

4. Conclusions

It has been demonstrated that the heat stability of whey protein can be improved by glycation with MD even without control of the relative humidity, with more pronounced improvement at a greater degree of glycation. Glycation at a higher temperature for a longer time increased the MW to a greater extent, as previously reported based on SDS-PAGE With AUC, the increase in MW was estimated to be about 4 MD molecules glycated onto each whey protein molecule. The percentage of ordered secondary structures of WPI decreased after glycation and decreased further after heating in aqueous solutions. Glycation lowered the pI of WPI from pH 4.5 to ~ pH 4.0, similar to the literature. The glycated MD provided steric hindrance that reduced protein aggregation during heating at wide ranges of acidity and ionic strength, except at pH near pI when hydrophobic interactions are stronger than repulsive steric interactions. The AFM revealed that glycated WPI aggregated to a greater extent at pH 5.0, neary pI of WPI, than at pH 3.0, but the aggregated protein particles were small enough to prevent turbidity. The present

study provides helpful guidelines for the development of transparent beverages containing relatively high contents of whey proteins.

Acknowledgments

This work was supported by National Natural Science Foundation for Young Schloars of China (31401640), the Scienctific Research Foundation for the Returned Overseas Chinese Schlolars, State Education Ministry, Chen Guang Project supported by the Science Technology Foundation for Young Scientist of HuBei Province, China (2014072704011258). We also appreciate the funding from Science and Technology Support Program of Hubei Province, China (2015BBA167).

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Zhu, H., & Damodaran, S. (1994). Heat-Induced Conformational Changes in Whey Protein Isolate and Its Relation to Foaming Properties. *J. Agric. Food Chem., 42*, 846.

Zirwer, D., Gast, K., Welfle, H., Schlesier, B., & Dieter Schwenke, K. (1985). Secondary structure of globulins from plant seeds: a re-evaluation from circular dichroism measurements. *Int. J. Biol. Macromol., 7*, 105.

398 Table 1. Size-distribution of WPI-MD conjugate determined by sedimentation velocity obtained
399 in analytical ultracentrifugation.

pH	Parameters	Major peak	Multimeric species ≤ 200 kDa	Multimeric species > 200 kDa
3.0	*c*(*s*) range	1.03–3.51	3.61-10.38	≥ 10.40
	Percentage of sample	34.7%	33.3%	32.0%
	Weight average *c*(*s*)	2.27	6.13	14.40
4.0	*c*(*s*) range	2.69-5.23	5.23-10.51	≥ 10.52
	Percentage of sample	40.9%	33.0%	26.1%
	Weight average *c*(*s*)	3.51	7.59	13.89
5.0	*c*(*s*) range	1.01-4.02	4.02-10.00	≥ 10.01
	Percentage of sample	42.3%	28.6%	29.1%
	Weight average *c*(*s*)	2.41	6.32	13.8
6.0	*c*(*s*) range	1.09-3.52	3.52-10.00	≥ 10.01
	Percentage of sample	44.2%	33.4%	22.4%
	Weight average *c*(*s*)	2.19	5.96	13.47

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402 Table 2. Secondary structure compositions (%) of WPI and conjugate* before and after heating at
403 88 °C for different durations.

		Duration (min)	α-Helix	β-Strand	Turns	Aperiodic structure
WPI	pH 3.0	0	25.7	35.2	10.6	31.4
		0.5	24.5	34.3	11.5	31.1
		1.0	23.4	34.0	11.1	32.1
		1.5	23.2	32.6	11.3	32.6
		2.0	22.6	32.1	10.9	32.7
	pH 5.0	0	24.7	37.2	11.9	31.2
		0.5	21.6	34.5	16.1	33.8
		1.0	18.2	30.1	16.1	34.5
		1.5	15.3	22.4	19.4	45.4
		2.0	10.4	20.9	18.8	47.3
	pH 7.0	0	24.5	35.4	12.5	30.8
		0.5	23.3	34.6	9.5	31.4
		1.0	22.6	32.5	9.2	29.7
		1.5	21.0	31.8	10.6	32.2
		2.0	19.7	30.4	9.9	33.6
Conjugate	pH 3.0	0	30.3	29.9	9.2	21.5
		0.5	26.2	22.7	4.3	22.8
		1.0	21.5	21.3	4.9	28.6
		1.5	20.7	20.9	5.6	29.2
		2.0	20.3	20.6	6.5	33.9
	pH 5.0	0	26.8	35.0	12.8	24.2
		0.5	25.9	29.2	7.6	26.8
		1.0	22.9	27.5	11.3	30.2
		1.5	22.4	23.0	10.1	32.5
		2.0	21.3	20.4	8.6	33.3
	pH 7.0	0	28.5	38	11.9	22.7
		0.5	26.6	34.8	19.2	26.7
		1.0	22.9	30.3	15.8	29.2
		1.5	22.7	28.9	12.9	30.5
		2.0	22.1	27.8	15.7	32.9

404 * The conjugate was prepared by heating at 135 °C for 1 h.

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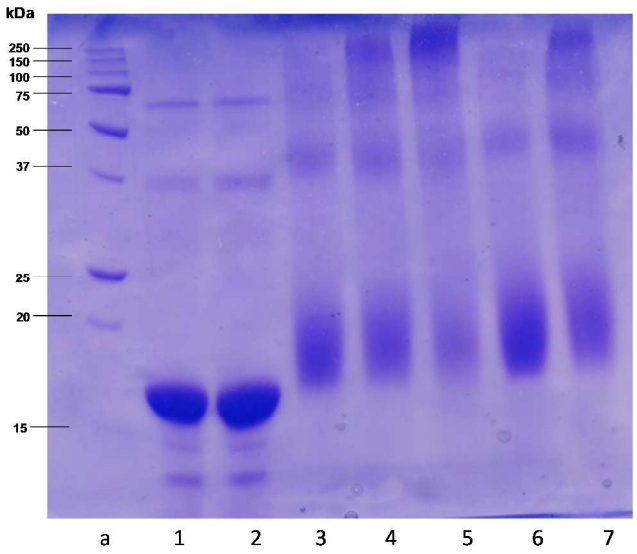
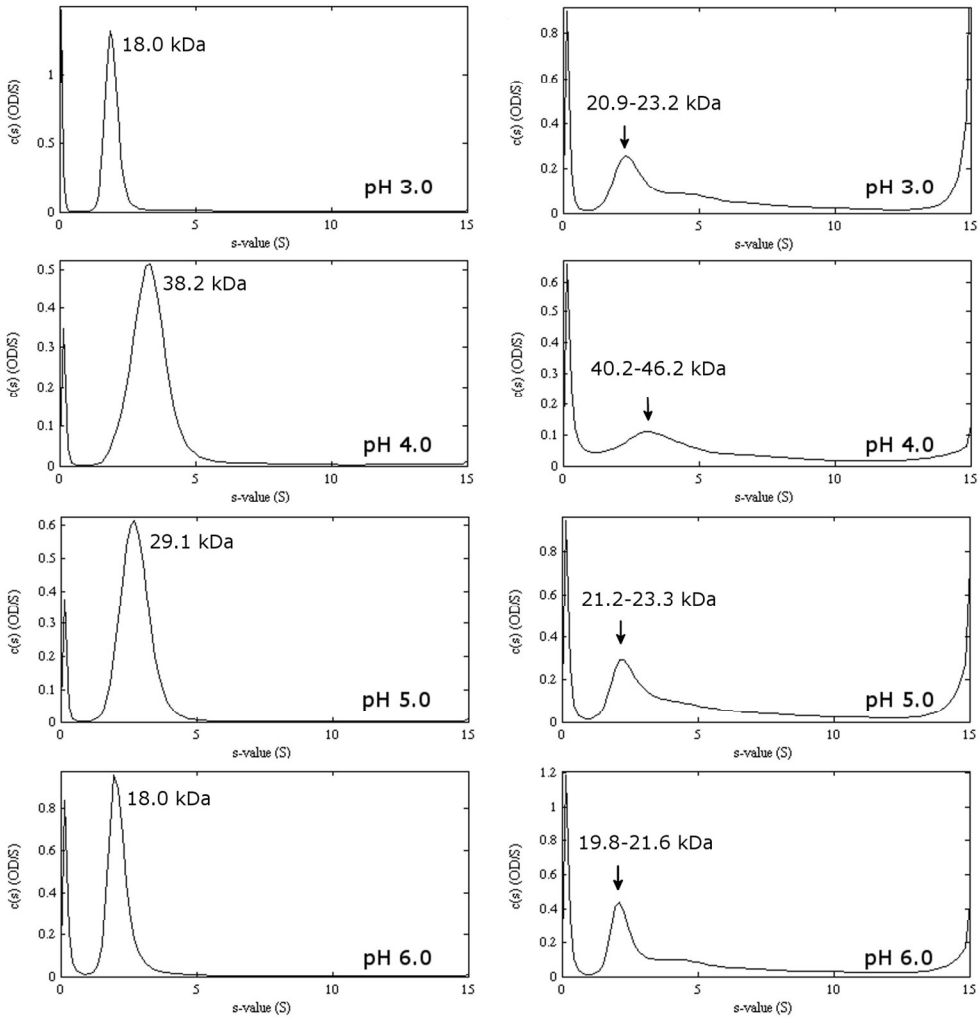


Figure 1. SDS-PAGE of WPI and WPI-MD conjugates. Lane a: molecular weight markers; Lane 1: WPI, Lane 2: mixture of WPI and MD; Lane 3, 4, and 5: WPI–MD conjugate prepared by heating at 135 °C for 10, 30, and 60 min, respectively; Lane 6: WPI-MD conjugate prepared by heating at 90 °C for 2 h; Lane 7: WPI- MD conjugate prepared by heating at 115 °C for 2 h.



(A) (B)

Figure 2. The best-fit distributions from analytical ultracentrifugation of WPI (A) and WPI-MD conjugates (B) at pH 3.0-6.0.

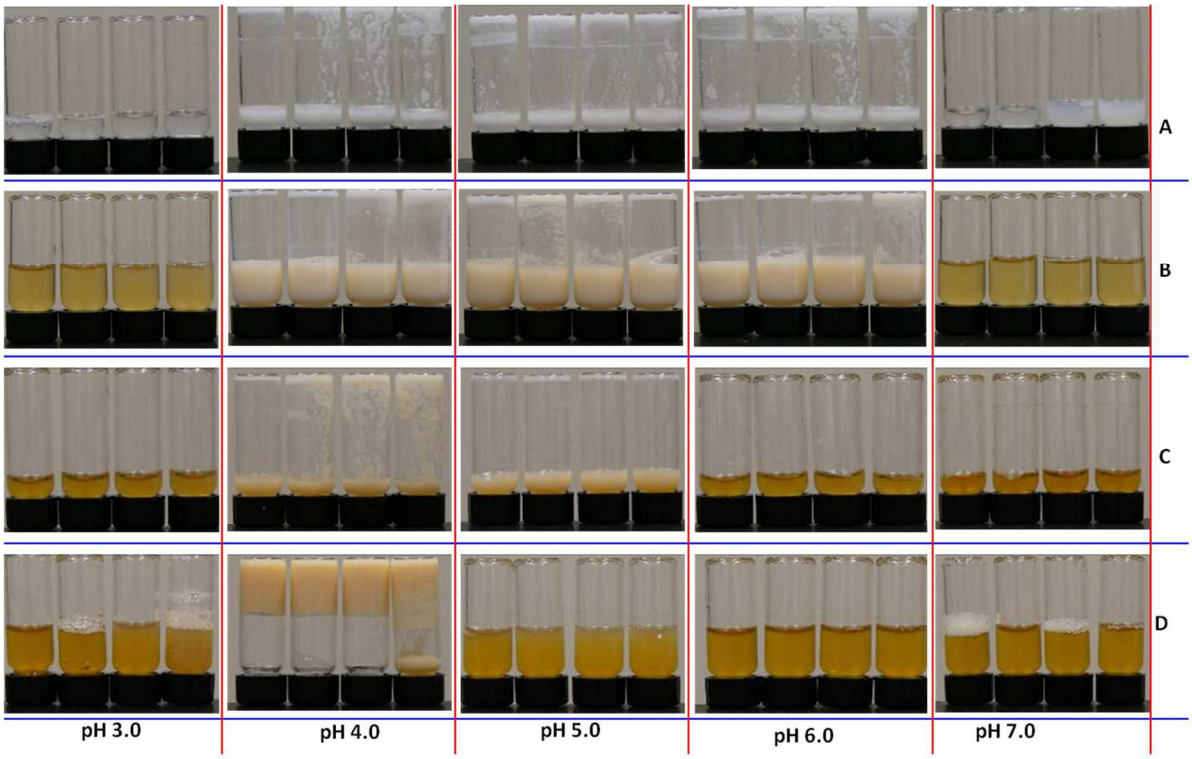


Figure 3. Photographs of aqueous dispersions containing 5%w/v WPI after heating at 88 °C for 2 min: a simple mixture of WPI and MD at a mass ratio of 2:1 (A), WPI-MD conjugate prepared by heating at 115 °C for 2 h (B), 135 °C for 0.5 h (C), and 135 °C for 1 h (D). All samples were adjusted to pH 3.0-7.0 and 0, 50, 100, and 150 mM NaCl (vials from left to right in each image) before heating.

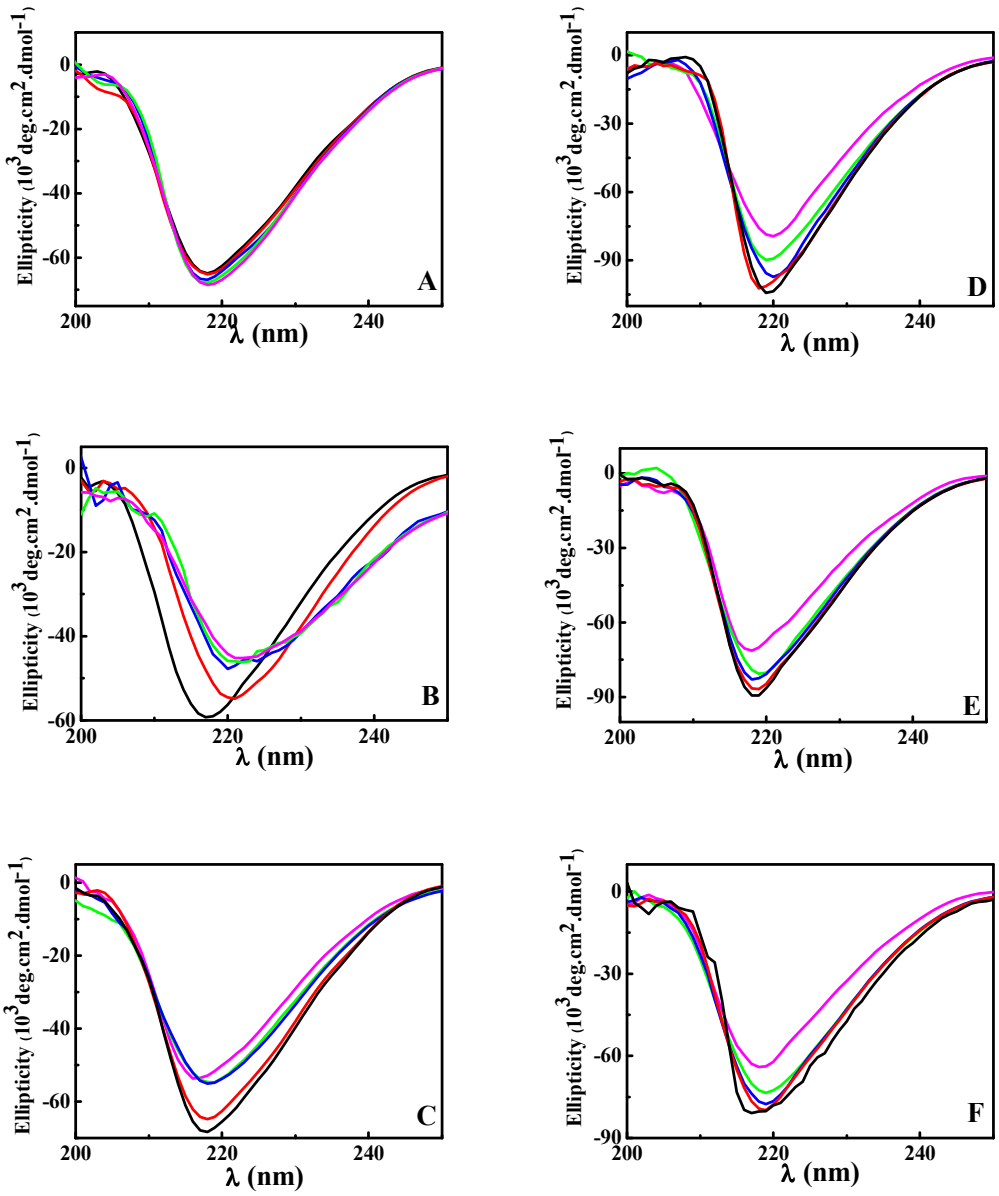
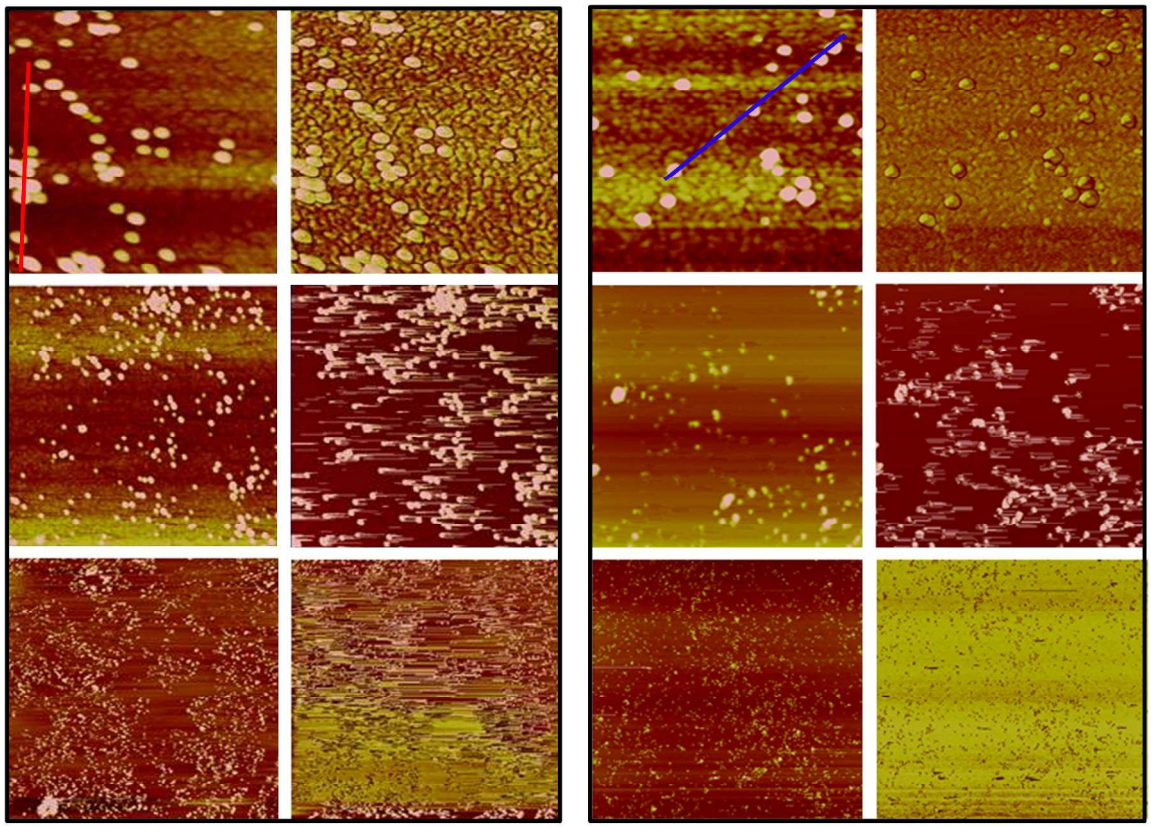
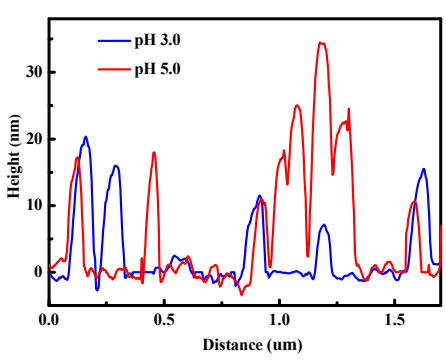
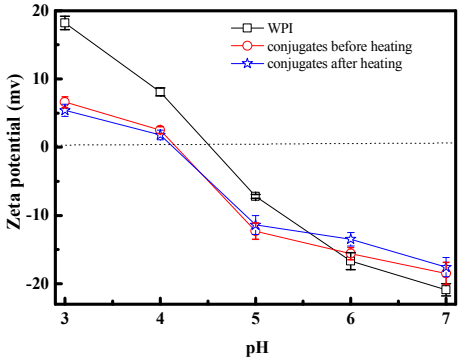


Figure 4. Far-UV CD spectra of aqueous solutions containing WPI (A, B, C) and WPI-MD conjugate prepared by heating at 135 °C for 1 h (D, E, F). Samples were adjusted to pH 3.0 (A, D), 5.0 (B, E), and 7.0 (C, F) and heated at 88 °C for 0 (black), 0.5 (red), 1 (blue), 1.5 (green), and 2 min (pink).







(C) (D)

Figure 5. Topographical (left) and error (right) AFM images of WPI-MD conjugate, prepared by heating at 135 °C for 1 h, after heating at 88 °C for 2 min in aqueous solutions at pH 5.0 (A) and (B) 3.0. Particle heights at the drawn lines are shown in (C)，Scan sizes: 2 × 2 μm (top); 5× 5 μm (middle); and 20 × 20 μm (bottom). (D) Zeta potential profiles of WPI and WPI-MD conjugate before and after heating at 88 °C for 2 min. The conjugate sample was prepared by 1-h glycation at 135 °C. Error bars are standard errors from triplicate measurements.

Graphical Abstract