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Nanoscale Understanding of Thermal Aggregation of Whey Protein Pretreated by Transglutaminase

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Supporting Information

ABSTRACT: Nanoscale structures of whey protein isolate (WPI) pretreated by microbial transglutaminase (mTGase) and subsequent heating were studied in this work and were correlated to zeta-potential, surface hydrophobicity, thermal denaturation properties, and macroscopic turbidity and viscosity. Dispersions of 5% w/v WPI were pretreated by individual or sequential steps of preheating at 80 °C for 15 min and mTGase, used at 2.0−10.2 U/g WPI for 1−15 h, before adjustment of the pH to 7.0 and to 0−100 mM NaCl for heating at 80 °C for 15 and 90 min. The zeta potential and surface hydrophobicity of WPI increased after all pretreatment steps. Preheating increased cross-linking reactivity of WPI by mTGase, corresponding to significantly increased denaturation temperature. Particle size analysis and atomic force microscopy revealed that structures of sequentially pretreated WPI remained stable after heating at 100 mM NaCl, corresponding to transparent dispersions. Conversely, WPI pretreated by one step aggregated at only 100 mM NaCl and resulted in turbid dispersions. Besides reporting a practical approach to produce transparent beverages, nanoscale phenomena in the present study are important for understanding whey protein structures in relevant applications.

KEYWORDS: *whey protein, thermal aggregation, enzymatic cross-linking, rheology, nanostructure, surface properties*

INTRODUCTION

Whey proteins are among the most studied biopolymers because of their properties to form emulsions and nanoemulsions,[1] nanofibers,[2] hydrogels,[3] and nanostructures for delivering nutraceuticals and pharmaceuticals.[4] Cheese whey is the major source for commercial production of whey protein ingredients, and whey protein isolates (WPI) refer to products purified to a protein content of >90%. WPI are composed of a mixture, with β-lactoglobulin, α-lactalbumin, and bovine serum albumin being the major components.[5] WPI have been used to fabricate a variety of structures by engineering their aggregation induced by acidity, electrolytes, and temperatures.[5,6] Particularly, heating is a common approach to form fibrillar or particulate aggregates of whey proteins by controlling repulsive and attractive intra/intermolecular physical forces such as electrostatic interactions, hydrophobic interactions, and hydrogen bonding and intra- and intermolecular disulfide bonds via sulfhydryl−disulfide interchange.[6] The property of concern in the present study is the dispersibility of whey proteins after heating. In a concentrated dispersion, thermal aggregation of whey protein into a three-dimensional network is important to the formation of hydrogels but is problematic for systems requiring maintained fluidity and low viscosity. Furthermore, because micrometer-sized structures deflect light to cause turbidity and precipitate during storage, fabricating nanostructures of whey proteins after thermal processing is needed to maintain the visual transparency of dispersions, commonly referred to as heat stability.

Thermal aggregation of native whey protein at neutral pH has been well-studied.[6] At low ionic strength, WPI dispersions typically remain transparent after heating because the strong repulsive electrostatic interactions correspond to reaction-limited aggregation and the formed fibril structures are too small to reflect visible light. At high ionic strength, the shortened Debye length favors protein aggregation that is diffusion-limited, forming particulates that result in turbid dispersions or gels. Reducing thermal aggregation of whey proteins has been studied after incorporation of small molecule cosolutes such as sucrose,[7] glycerol,[8] and sorbitol[8b] or chaperones.[9] These compounds increase denaturation temperatures of whey proteins. However, it remains a challenge to maintain dispersion transparency after heating at neutral pH and increased ionic strength. We recently applied microemulsions as templates to produce whey protein nanoparticles by preheating, with and without prior pretreatment by microbial transglutaminase (mTGase), and observed that nanoparticles had much improved heat stability at 100 mM NaCl.[10] However, the capacity of producing nanoparticles in microemulsions is limited and is an obstacle for practical applications.

TGase (EC 2.3.2.13, protein-glutamine γ-glutamyltransferase) catalyzes the cross-linking reaction between protein molecules through acyl transfer reactions to form inter- or intramolecular ε-(γ-glutamyl)lysine isopeptidic bonds.[11] Although the enzyme is commonly used to cross-link proteins to strengthen gel networks, mTGase has been reported to improve the heat stability of whey protein.[11] The specificity of mTGase cross-linking varies with the type of proteins. Native β-lactoglobulin is not an active substrate of mTGase but can become active after prior thermal denaturation, whereas native α-lactalbumin can be cross-linked by the enzyme directly.[12] The

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direct treatment of WPI by mTGase was observed to increase the thermal denaturation temperature of whey proteins,[13] possibly due to formation of covalent cross-links among reactive proteins.[14] The 5% β-lactoglobulin[15] and WPI solutions reduced by 10 mM dithiothreitol (DTT)[16] no longer formed gels at pH 7.5 after extensive cross-linking by mTGase. Intra- and intermolecular bonds formed by mTGase were proposed to affect the unfolding of whey proteins during heating.[16] Tanimoto and Kinsella[15] separated β-lactoglobulin, with prior reduction by DTT and addition of $CaCl_2$ and cross-linking by guinea pig liver TGase, into two fractions based on molecular weight (MW) and evaluated the stability of fractions constituted to 5% protein by heating at 90 °C for 30 min. The fraction bigger than 100 kDa was stable, but a soft gel was observed for the other smaller MW fraction. Moderate mTGase pretreatment conditions (10 U/g protein, 6−9 h at 37 °C, and pH 7.4) were observed to reduce the heat stability of β-lactoglobulin,[17] but mTGase cross-linking for 23 h or longer improved the heat stability, at which conditions the prior denaturation by DTT was not required.[17] Despite these related studies, much work is needed to technologically improve the heat stability of WPI solutions with 5% or more protein, at neutral acidity and increased NaCl concentration, and without using toxic DTT. Scientifically, the formation of supramolecular structures of whey proteins as affected by mTGase pretreatment is to be studied and interpreted by integration with information of protein denaturation properties and colloidal chemistry.

The objective of this work was to study the thermal aggregation of WPI after being pretreated by mTGase at different enzyme levels and durations, with and without prior preheating. Preheating was studied because of the improvement in reactivity of whey protein as a substrate for mTGase.[18] Physicochemical changes were interpreted using several complementary techniques illustrating rheological, surface-charge, and thermal denaturation properties of whey proteins. Particularly, nanoscale structures, studied using particle size measurements by dynamic light scattering (DLS) and atomic force microscopy (AFM), provide missing information about supramolecular structures formed due to mTGase pretreatment and subsequent heating.

MATERIALS AND METHODS

Materials. WPI were a product from Hilmar Ingredients (Hilmar, CA, USA). The powdered mTGase sample (product of Activa TG-TI) was a gift from Ajinomoto Food Ingredients LLC (Chicago, IL, USA). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Determination of mTGase Activity. The freshly prepared mTGase solution was quantified for activity using the literature method,[19] with modification of pH and temperature. One unit (U) of activity was defined as the grams of powdered enzyme able to produce 1 μmol of hydroxamate per minute at pH 7.0 and 50 °C when substrates of *N*-carbobenzoxy-glutaminyl-glycine and hydroxylamine were catalyzed by the enzyme.

Pretreatment Protocol. The WPI powder was hydrated at 5% w/v in 20 mL of a 50 mM sodium phosphate buffer overnight at room temperature (21 °C). For sequential pretreatments of preheating and enzymatic cross-linking, samples were adjusted to pH 7.5 using 1 N NaOH and preheated for 15 min in a water bath maintained at 80 °C, followed by cooling immediately in a room temperature water bath. The mTGase powder was dissolved at 2, 5, and 10% mass of WPI. If necessary, the pH was readjusted to 7.5[16] before incubation in a 50 °C shaking water bath for 1−15 h. After immersion in a room temperature water bath, samples were adjusted to pH 7.0, and 2.0 mL of solution was transferred into 4.0 mL vials. The vials were supplemented with 0, 20, or 40 μL of a 5.0 M NaCl solution (in distilled water), corresponding to a final NaCl concentration of 0, 50, or 100 mM.

To compare with sequentially pretreated samples, a set of samples was prepared by heating the pH 7.5 solutions at 80 °C for 15 min only, whereas another set of samples was directly pretreated by dissolving the enzyme powder in the pH 7.5 solutions, followed by incubation at 50 °C for cross-linking. These samples were adjusted to pH 7.0 and NaCl concentrations as above. The control without pretreatment was the 5% w/v WPI solution directly adjusted to pH 7.0 and 0, 50, or 100 mM NaCl.

Evaluation of Heat Stability. The vials prepared as above were heated for 15 min in a water bath pre-equilibrated to 80 °C. After cooling to room temperature in a water bath, the vials were compared for visual appearance by photography or for absorbance acquired at 400 and 600 nm[9] using a UV−vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA, USA).

Differential Scanning Calorimetry (DSC). Thermal denaturation properties of WPI after different pretreatments were studied using a differential scanning calorimeter (VP-DSC, MicroCal, Northamption, MA, USA). The WPI dispersions after different pretreatments were diluted to 2% w/v using the pH 7.0 phosphate buffer. Samples were degassed at 20 °C for 10 min before injection into the sample cell, with deionized water used as a reference. Scanning was conducted from 25 to 105 °C at a rate of 1.0 °C/min. The total calorimetric apparent enthalpy change (ΔH) and the denaturation temperature (T_d) were determined via analysis of the thermographs, which was performed by using a two-state model (supplied by MicroCal).

Surface Hydrophobicity. The surface hydrophobicity of WPI was determined using fluorescence probe 1-anilino-8-naphthalenesulfonate (ANS) according to a literature method,[20] with modification. Each sample was diluted to five concentrations from 0.005 to 0.2% w/v using a 10 mM phosphate buffer at pH 7.0. The ANS solution was prepared at 8 mM in the same buffer. Ten microliters of ANS solution was added to 2 mL of each protein solution. The fluorescence intensity was measured at an excitation wavelength of 365 nm and an emission wavelength of 484 nm using an RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The background fluorescence was calibrated using distilled water. Surface hydrophobicity was calculated by linear regression analysis, using the initial slope of the fluorescence intensity−protein concentration plot as an index of surface hydrophobicity (S_0).

Particle Size Analysis (DLS). The native WPI dispersion with 50 mM sodium phosphate and 100 mM NaCl formed a gel after heating at 80 °C for 15 min. To overcome this difficulty, this 5% w/v WPI dispersion was prepared without sodium phosphate but with 100 mM NaCl to produce a flowable sample after heating. Other samples were prepared as in the pretreatment protocol and adjusted to 100 mM NaCl. Protein dispersions before and after heating at 80 °C for 15 min were measured using a Delsa Nano particle size/zeta potential analyzer from Beckman Coulter, Inc. (Brea, CA, USA). The volume−length mean particle diameter was calculated using eq 1

$$d_{4,3} = \frac{\sum_{i=1} n_i d_i^4}{\sum_{i=1} n_i d_i^3} \quad (1)$$

where n_i is the number of particles with a diameter d_i.

Zeta Potential. WPI dispersions were diluted to 0.5% w/v using a 50 mM sodium phosphate buffer adjusted to pH 7.0 before zeta-potential measurements using the Delsa Nano instrument (Beckman Coulter Inc.). Samples containing 100 mM NaCl also were desalted by 40 h of dialysis in deionized water using a membrane with a molecular weight cutoff of 3500 Da (Fisher Scientific, Fair Lawn, NJ, USA). The bulk deionized water was replaced every 8 h. After dialysis, WPI dispersions were diluted to 0.5% w/v using the phosphate buffer and adjusted to pH 7.0 for zeta-potential analysis. Two replicates were tested three times each, and the averages from six measurements were reported.

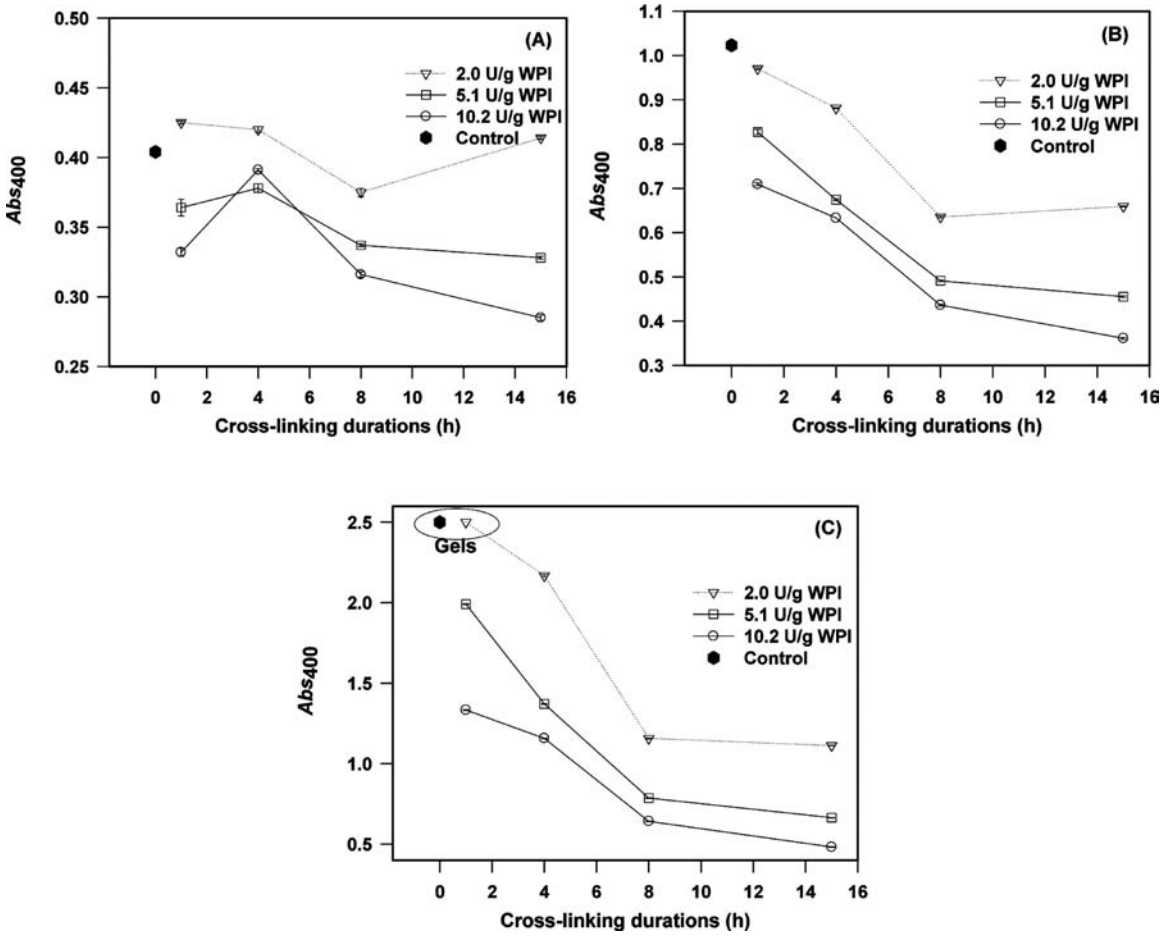

Figure 1. Absorbance of 5% w/v WPI dispersions at 400 nm (Abs_{400}) after heating at 80 °C for 15 min. Before heating, samples were adjusted to pH 7.5 and cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1−15 h at 50 °C before adjustment of the pH to 7.0 and to (A) 0, (B) 50, and (C) 100 mM NaCl. Control samples were heated directly after adjustment of the pH and NaCl concentration. Error bars are standard deviations from duplicate samples.

Atomic Force Microscopy (AFM). The WPI dispersions were diluted in deionized water to an overall protein concentration of 10 ppm. For a gelled sample, the gel was added with deionized water and vortexed overnight. Four microliters of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA, USA). The samples were scanned using a rectangular cantilever probe (FESPA, Bruker Corp.) with aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m. Imaging was conducted using a Multimode microscope (Veeco Instruments, Inc., Plainview, NY, USA) operated using the tapping mode. Both topography and phase images with a preset scan area of 5 × 5 or 2 × 2 μm were generated. Topography images were used to measure heights of individual particles using the instrument software, and the averages of particle heights were reported.

Rheology. Fifteen milliliter samples were contained in 20 mL vials and pretreated using the above protocols. After heating at 80 °C for 15 min, samples were transferred into the sample cup of a Searle setup (bob outer diameter = 28 mm and cup inner diameter = 30 mm) of an AR2000 rheometer (TA Instruments, New Castle, DE, USA). Shear rate ramps were performed from 0.1 to 1000 s^{-1} at 20 °C.

Statistical Analysis. Results were presented as averages and standard deviations from replicates. All statistical analyses were performed using SAS software (version 9.2, SAS Institute, Cary, NC, USA). A two-way analysis of variance (ANOVA) was carried out to test the significance between treatments. Statistical differences among comparable data points for various treatments were calculated by post hoc comparison of means according to the least significant difference (LSD) mean separation method at a p level of 0.05.

RESULTS AND DISCUSSION

The mTGase activity was measured to be 102.1 U/g powder. The levels of mTGase were thus 2.0, 5.1, and 10.2 U/g WPI when the mTGase powder was used at 2, 5, and 10% mass of WPI. Absorbance values at 400 and 600 nm for heated samples followed similar trends after pretreatment at various conditions. The absorbance at 600 nm was smaller than that at 400 nm, and only the results at 400 nm are presented here. The results are first presented for heat stability in two groups for samples with and without preheating, followed by properties probed by DLS, AFM, zeta potential, surface hydrophobicity, DSC, and rheology.

Heat Stability of Samples Pretreated by mTGase without Preheating. Heat stability was compared for absorbance because bigger structures formed during thermal treatment cause higher absorbance/turbidity. Samples pretreated by three levels of mTGase without preheating were heated at 80 °C for 15 min, and the absorbance values are shown in Figure 1, panels A, B, and C for pH 7.0 samples at 0, 50, and 100 mM NaCl, respectively. A higher level of mTGase and a longer treatment time generally resulted in a lower absorbance value. Samples at 0 mM NaCl were all clear after heating, corresponding to an absorbance value lower than 0.45 at 400 nm (Figure 1A) and below 0.1 at 600 nm (not shown). The heat stability of WPI at a low ionic strength and neutral pH

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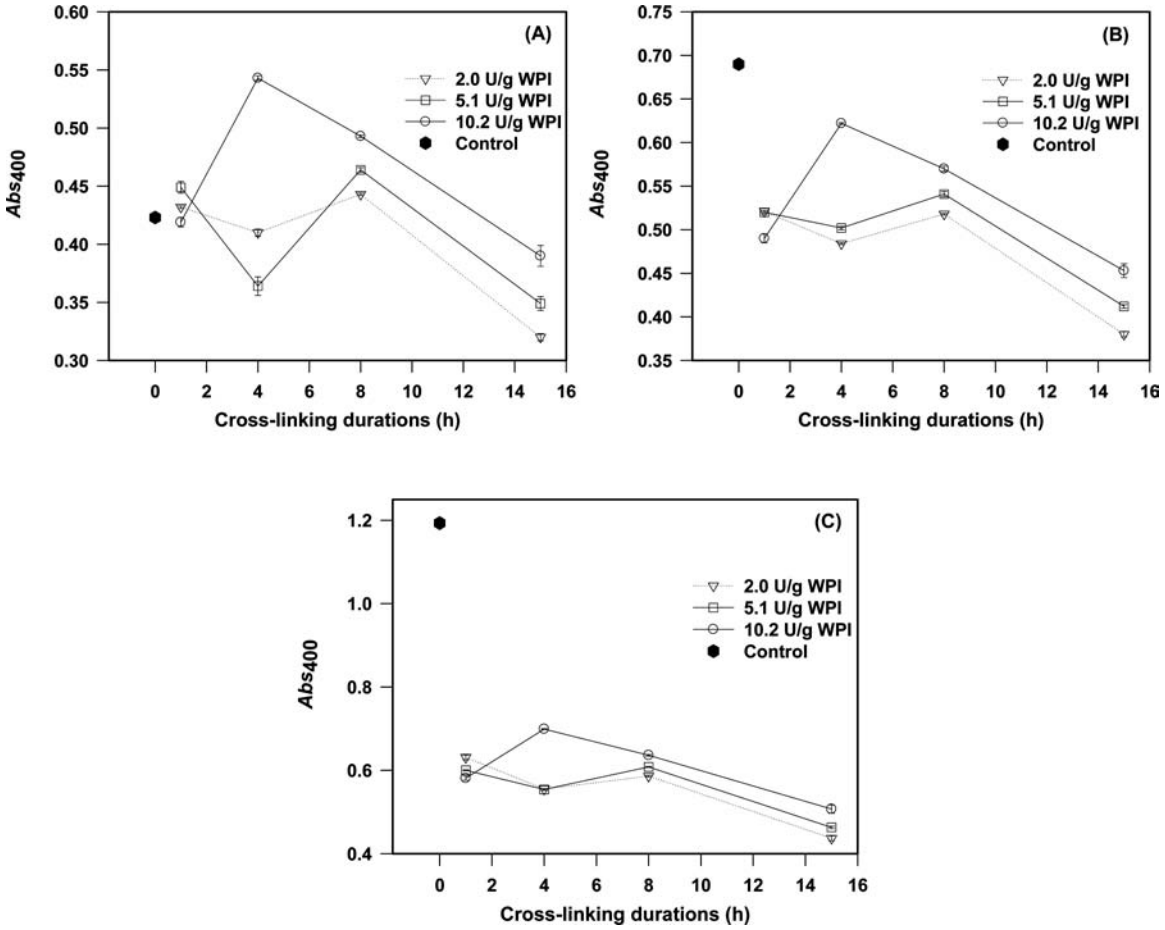

Figure 2. Absorbance of 5% w/v WPI dispersions at 400 nm (Abs_{400}) after heating at 80 °C for 15 min. Before heating, samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, and then cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1−15 h at 50 °C before adjustment of the pH to 7.0 and to (A) 0, (B) 50, and (C) 100 mM NaCl. Control samples were preheated at the same conditions before the pH and NaCl concentration were adjusted. Error bars are standard deviations from duplicate samples.

is well-known, due to strong electrostatic repulsion.[6] At 50 mM NaCl, samples pretreated by mTGase to a greater extent had lower absorbance (Figure 1B), consistent with improved visual clarity of dispersions. At 100 mM NaCl, the control sample and the sample treated with the least amount of mTGase for the shortest duration (at 2.0 U/g WPI for 1 h, Figure 1C) formed gels after heating, whereas other samples were fluidic, with improved clarity after being pretreated by a higher level of mTGase for a longer time. The statistical analysis showed the significances ($p < 0.05$) of mTGase level and incubation time during pretreatments and significant impacts ($p < 0.05$) of NaCl concentration on sample turbidity.

Heat Stability of Samples Sequentially Pretreated by Preheating and mTGase. After preheating at 80 °C for 15 min at pH 7.5 and 0 mM NaCl, samples were pretreated by mTGase before adjustment to pH 7.0 and 0−100 mM NaCl for a second heating at 80 °C for 15 min. The control with preheating only was transparent at 0 mM NaCl, slightly turbid at 50 mM NaCl, and turbid but flowable at 100 mM NaCl. Preheating alone thus improved heat stability when compared to the gel formation at 100 mM NaCl for native WPI (Figure 1C). All sequentially pretreated samples were visually clear, with absorbance values presented in Figure 2. At 0 mM NaCl, the absorbance values of samples (Figure 2A) were similar to those without preheating (Figure 1A). Opposite to observations from samples directly pretreated by mTGase (Figure 1), samples preheated and pretreated by a higher mTGase level overall had a higher absorbance value (Figure 2), but the absorbance difference between the control (with preheating only) and sequentially pretreated samples was much smaller than the difference for treatments without preheating.

Overall, the sequentially pretreated WPI had much improved heat stability compared with those with preheating or mTGase pretreatment only, demonstrated in Figure 3 corresponding to an enzyme level of 5.1 U/g WPI and a cross-linking duration of 4 h. It has been shown that native whey proteins are not very susceptible to cross-linking by mTGase,[21] particularly the most abundant β-lactoglobulin,[22] possibly because glutamine and lysine groups involved in the cross-linking reaction are buried within the protein core and inaccessible to the enzyme.[23] The mTGase cross-linking is much facilitated after preheating to a temperature above the denaturation temperatures of whey proteins.[18b,21,24] This was verified in our preliminary experiments using sodium dodecyl sulfate−polyacrylamide gel electrophoresis that demonstrated no, limited, and extensive cross-linking for samples pretreated by preheating only, mTGase only, and sequential steps, respectively. To further understand structural formation as affected by pretreatments, the following sections present findings from treatments using mTGase at 5.1 U/g WPI for 4 h.

Particle Size Changes Measured by DLS. Table 1 shows $d_{4,3}$ of WPI dispersions after various pretreatments. The $d_{4,3}$

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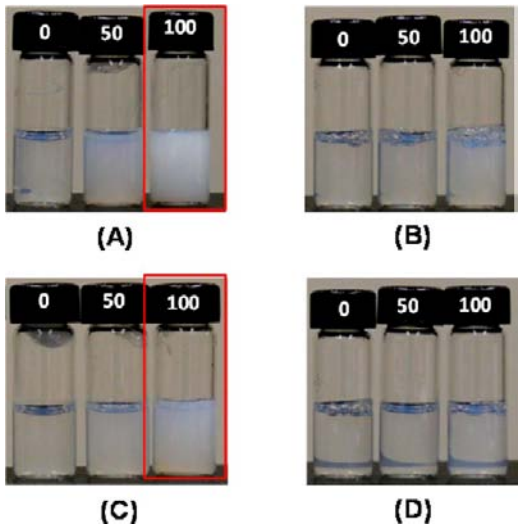

Figure 3. Photographs showing appearance of 5% w/v WPI dispersions, adjusted to pH 7.0 and 0, 50, and 100 mM NaCl (as labeled on vials), after being heated at 80 °C for 15 min. Image A shows samples without pretreatment. Samples in images B–D were pretreated differently before heating. Samples in image B were adjusted to pH 7.5 and preheated for 15 min at 80 °C before pH and NaCl adjustment for a second heating at 80 °C for 15 min. Samples in image C were pretreated by mTGase using a level of 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h before the pH and NaCl concentration were adjusted as in image B. Samples in image D were preheated as in image B and then pretreated by mTGase as in image C before the pH and NaCl concentration were adjuste as in image B. The highlighted vials indicate gel formation.

Table 1. $d_{4,3}$ of Whey Protein Dispersions after Pretreatments and after Further Heating at pH 7.0 and 100 mM NaCl for 15 and 90 min at 80 °C

	$d_{4,3}$[b] (nm)		
pretreatment conditions[a]	after pretreatment	after heating for 15 min with 100 mM NaCl	after heating for 90 min with 100 mM NaCl
none (control)	9.8 ± 2.6G	93.0 ± 3.9A	
preheating only	31.7 ± 1.3F	52.1 ± 3.8D	68.7 ± 2.6C
mTGase only	11.9 ± 2.3G	65.2 ± 3.4C	82.0 ± 5.0B
sequential preheating and mTGase	37.0 ± 3.3EF	36.7 ± 1.0E	36.5 ± 4.4E

[a]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, whereas the mTGase pretreatment was conducted using 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h. [b]Numbers are the mean ± standard deviation from two replicates, each measured three times. Different letters indicate statistical difference in the mean ($p < 0.05$).

increased from ~10 nm of the untreated WPI dispersion to ~32 nm after preheating at 80 °C for 15 min. Results in Table 1 are slightly higher than the hydrodynamic radius of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, that is 2.6–4.9,[25] 2.0,[26] and 3.7 nm,[27] respectively, possibly because the dispersion contained 50 mM sodium phosphate, which causes some flocculation of proteins. The increase in particle size after preheating is expected because whey proteins are known to form filament-type aggregates after heating at neutral or slightly alkaline pH and low ionic strength.[5,6] After pretreatment by mTGase directly, the $d_{4,3}$ increased slightly to ~12 nm, indicating a small degree of cross-linking that resulted in a structure smaller than that after preheating. The sequentially pretreated WPI dispersion had the largest $d_{4,3}$ of ~37 nm, indicating the further formation of structures from preheated whey protein by mTGase cross-linking. When comparing the sequentially pretreated WPI and that directly pretreated by mTGase, the particle size and the net increase in particle size (from preheated sample or control) were much bigger for the sequential pretreatment. After adjustment to 100 mM NaCl and 15 min of heating at 80 °C, the sequentially pretreated dispersion had an insignificant change in $d_{4,3}$, whereas the increase was significant for other samples, following the order of control without pretreatment > mTGase pretreatment > preheating. Because bigger structures scatter light to a greater extent, DLS results are in agreement with visual appearance (Figure 3) and absorbance (Figures 1 and 2).

Structures of Whey Proteins Studied by AFM. Nanoscale structures of whey proteins after various pretreatments were studied using AFM for samples before and after heating at 100 mM NaCl. Images scanned at 2 × 2 μm are presented in Figure 4, whereas those scanned at 5 × 5 μm are shown in supplementary Figure S1 (Supporting Information). Images scanned at 2 × 2 μm were also used to measure average heights of particles that are listed in Table 2. Native whey proteins were mostly monomeric, with some dimer-like structures. The average height of whey proteins was 3.4 nm, which is similar to the reported dimension of individual whey protein molecules, particularly that of β-lactoglobulin.[28] After heating at pH 7.0 and 100 mM NaCl, WPI formed a turbid gel. The vortexed gel revealed coarse particulate aggregates with a height of ~33 nm, consistent with the literature studies.[29]

Figure 4B shows preheated WPI before and after heating at 80 °C for 15 min. Preheating WPI at pH 7.0 in the absence of NaCl corresponded to clusters of granular aggregates with a height of ~7 nm. Aggregates with an increased height of 16 nm were observed after heating for a second time in the presence of 100 mM NaCl, corresponding to translucent appearance (Figure 3B). Ikeda and Morris[29b] previously revealed similar heat-induced structures of WPI and β-lactoglobulin, presumably through steps of the initial formation of granular aggregates with various sizes and subsequent aggregation between these granular aggregates. This results in the coexistence of small particles with varied sizes depending on ionic strength and duration of heat treatment and much larger aggregates of small particles.

After being pretreated with mTGase without preheating, the structure of whey proteins (Figure 4C, left) was similar to that of native WPI (Figure 4A, left), but the particle height increased to 6.5 nm, indicating limited cross-linking. After heating at 100 mM NaCl, aggregates were observed (Figure 4C, right), with an average height of 17 nm (Table 2), which was smaller than the structure formed from native WPI.

After sequential pretreatments, granular aggregates with an average height of 10.7 nm were observed (Figure 4D, left; Table 2; supplementary Figure S1, Supporting Information). Both morphology (supplementary Figure S2, Supporting Information) and height (Table 2) suggest a greater extent of aggregation than a single pretreatment step. This verifies the improved enzymatic reactivity of WPI after preheating[18b,21,24] and cross-linking of structures formed after preheating. After the second heating in the presence of 100 mM NaCl, the morphology of aggregates was similar to that before heating (image D in Figure 4 and supplementary Figure S1, Supporting

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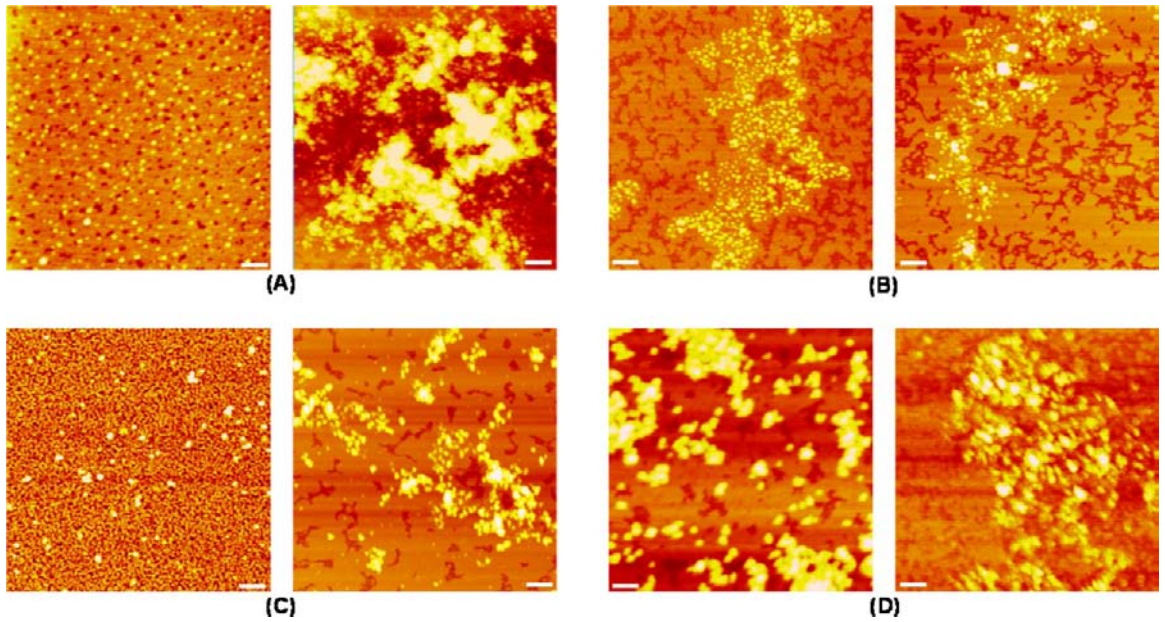

Figure 4. AFM height images of whey protein samples before (left) and after (right) heating at 80 °C for 15 min at pH 7.0 and 100 mM NaCl. Before pH and NaCl adjustments, the 5% w/v WPI dispersions were subjected to (A) no pretreatment, (B) preheating at pH 7.5 for 15 min at 80 °C, (C) pretreatment by mTGase at 5.1 U/g WPI for 4 h, or (D) sequential pretreatments as in panels B and C. Scale bar = 200 nm.

Table 2. Particle Heights Estimated from AFM for WPI after Pretreatments and after Further Heating at pH 7.0 and 100 mM NaCl for 15 and 90 min at 80 °C

	particle height[b] (nm)		
pretreatment conditions[a]	after pretreatment	after heating for 15 min with 100 mM NaCl	after heating for 90 min with 100 mM NaCl
none (control)	3.4 ± 0.3F	32.6 ± 1.1A	
preheating only	7.4 ± 0.7DE	16.5 ± 0.4B	16.3 ± 1.6B
mTGase only	6.5 ± 0.4EF	17.2 ± 3.3B	30.2 ± 1.3A
sequential preheating and mTGase	10.7 ± 2.0CD	12.4 ± 1.8C	12.5 ± 2.0C

[a]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, whereas the mTGase pretreatment was conducted using 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h. [b]Numbers are the mean ± standard deviation from duplicate measurements. Different letters represent significant difference in the mean ($p < 0.05$).

Information). The average height increased to 12 nm, but the increase was not significant (Table 2).

To further study the aggregation ability of WPI at 100 mM NaCl, pretreated samples were heated at 80 °C for 90 min. The samples (supplementary Figure S2, Supporting Information) had an appearance similar to that in Figure 3, except that samples with one pretreatment step only became more turbid than those in Figure 3. Changes in sample appearance were in agreement with $d_{4,3}$ data that showed a constant dimension for the sequentially pretreated sample but increased dimensions at a longer heating time for those with only one pretreatment step (Table 1). The morphology based on AFM generally agreed with the DLS (Figure 5, supplementary Figures S3 and S1, Supporting Information). For the sample pretreated by mTGase only, the morphology (Figures 4 and 5) and particle height (Table 2) after heating for 90 min were similar to those of the native WPI heated for 15 min. This indicates that WPI cross-linked by mTGase to a limited extent aggregates similarly to native WPI, except at a slower rate. In contrast, AFM particle heights (Table 2) of the sequentially pretreated samples were very similar at heating durations of 15 and 90 min, which, together with constant $d_{4,3}$, suggests that cross-linked structures are stable during heating at 100 mM NaCl. For the preheated WPI, the $d_{4,3}$ was bigger at a longer heating duration, but AFM

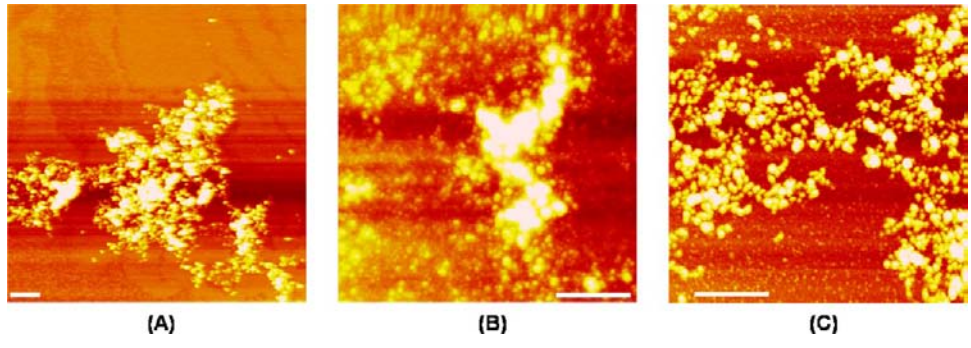

Figure 5. Samples in Figure 4, at pH 7.0 and 100 mM NaCl, after heating at 80 °C for 90 min: (A) preheating only; (B) mTGase pretreatment only; (C) sequential pretreatments. Scale bar = 500 nm.

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Table 3. Zeta Potential of WPI Dispersions at pH 7.0 after Various Pretreatments and Subsequent Heating at pH 7.0 and 0 or 100 mM NaCl for 15 min at 80 °C

	zeta potential[b] (mV)			
pretreatment conditions[a]	after pretreatment	after heating with 0 mM NaCl	after heating with 100 mM NaCl	after heating with 100 mM NaCl, dialyzed[c]
none (control)	−27.0 ± 0.4G	−35.1 ± 4.0CD	−26.9 ± 0.4G	−38.6 ± 0.4AB
preheating only	−33.5 ± 0.8DE	−43.6 ± 2.3A	−30.5 ± 1.6FG	−41.0 ± 1.7AB
mTGase only	−30.4 ± 0.8EFG	−40.2 ± 0.5AB	−29.0 ± 1.5G	−40.3 ± 2.1AB
sequential preheating and mTGase	−32.8 ± 1.5DEF	−40.0 ± 0.3AB	−32.3 ± 0.9DE	−37.8 ± 1.0BC

[a]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, whereas the mTGase pretreatment was conducted using 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h. [b]Numbers are the mean ± standard deviation from two replicates, each measured three times. Different letters indicate statistical difference in the mean ($p < 0.05$). [c]Samples heated at 100 mM NaCl were dialyzed using a membrane with a molecular weight cutoff of 3.5 kDa.

Table 4. Surface Hydrophobicity (S_0) of WPI Measured at pH 7.0 after Pretreatments and Subsequent Heating at 80 °C for 15 min in the Presence of 100 mM NaCl

	S_0[b] (slope ×10^6)	
pretreatment conditions[a]	after pretreatment	after heating with 100 mM NaCl
none (control)	1.85 ± 0.02D	2.10 ± 0.01C
preheating only	3.71 ± 0.01A	
mTGase only	2.29 ± 0.01B	
sequential preheating and mTGase	3.66 ± 0.02A	3.76 ± 0.32A

[a]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, whereas the mTGase pretreatment was conducted using 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h. [b]Numbers are the mean ± standard deviation from duplicate measurements. Different letters represent significant difference in the mean ($p < 0.05$).

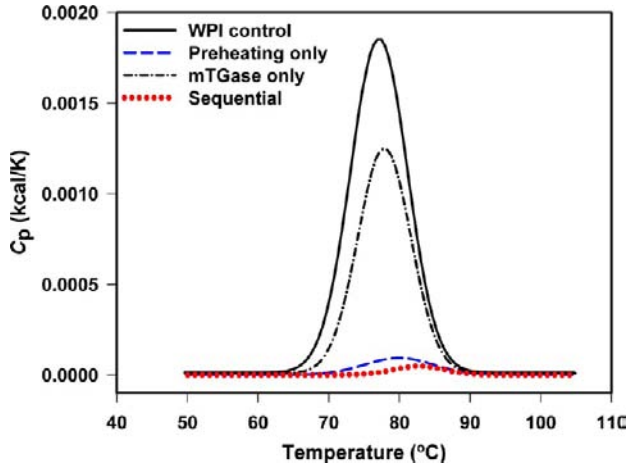

Figure 6. Comparison of DSC thermograms of WPI at pH 7.0 and 100 mM NaCl before and after pretreatments as in Figure 4.

particle heights remained unchanged for the two heating durations. Therefore, denatured whey proteins after preheating at studied conditions can still aggregate at 100 mM NaCl, with possible interpretation given in a later section.

Overall, the measured particle heights from AFM (Table 2) are much smaller than the dimensions measured in DLS (Table 1). In a dispersion, the measured dimension (hydrodynamic diameter) in DLS indicates the overall space occupied by the aggregated particles and is expected to be bigger than the height of granular aggregates (Table 2), which is smaller than the dimension suggested by aggregate morphology (Figures 4 and 5). Furthermore, the 5% w/v dispersion used in particle size measurements was diluted to 10 ppm in AFM, and weakly flocculated aggregates may be measured in DLS but not AFM. The drying process also can reduce the dimension of aggregates, resulting in smaller particle heights measured in AFM.

Table 5. Denaturation Temperature (T_d) and Enthalpy Change (ΔH) at pH 7.0 and 100 mM NaCl for WPI Dispersions Pretreated at Different Conditions

pretreatment conditions[a]	T_d[b] (°C)	ΔH[b] (kcal/g)
none	77.45 ± 0.53C	12.40 ± 0.77A
preheating only	79.98 ± 0.40B	3.58 ± 0.08B
mTGase only	77.76 ± 0.11C	11.10 ± 0.38A
sequential preheating and mTGase	82.76 ± 0.06A	2.71 ± 0.10B

[a]Preheating was conducted at pH 7.5 and 0 mM NaCl by heating at 80 °C for 15 min, whereas the mTGase pretreatment was conducted using 5.1 U/g WPI at pH 7.5 and 50 °C for 4 h. [b]Numbers are the mean ± standard deviation from duplicate measurements. Different letters in each column represent significant difference in the mean ($p < 0.05$).

Zeta Potential and Surface Hydrophobicity. The long-range electrostatic repulsion and shorter range hydrophobic attraction are critical for the stability of whey protein.[6,30] Table 3 shows the zeta potential of WPI samples at pH 7.0 after various pretreatments and subsequent heating at pH 7.0 and 0 or 100 mM NaCl for 15 min at 80 °C. The zeta potential of native WPI dispersion was −27 mV at pH 7.0 and became more negative after pretreatments, with the magnitude increased further after heating at 0 mM NaCl. The results in Table 3 are higher than the −24.6 mV of native WPI and −26.6 mV of WPI heated at 90 °C for 10 min at pH 6.8,[31] and the difference can be due to different supplies of WPI, pH, and heating conditions. For the cross-linking catalyzed by mTGase, the reaction between an acyl donor (glutamine) and an acyl acceptor (lysine) results in the loss of an amino group,[11] which reduces the number of positive charges. Cysteine, with a pK_a of 8.5, is an amino acid with free thiol groups available for formation of intra- and intermolecular disulfide bonds when native whey proteins are heated,[6] which may reduce the surface positive charges. Furthermore, denaturation causes the redistribution of surface amino acids, and the number of negatively charged surface amino acids can increase after heating and/or mTGase cross-linking. Because the zeta potential increased more significantly after heating than after mTGase treatments (Table 3), whey protein structure changes due to thermal denaturation are the major cause of increased

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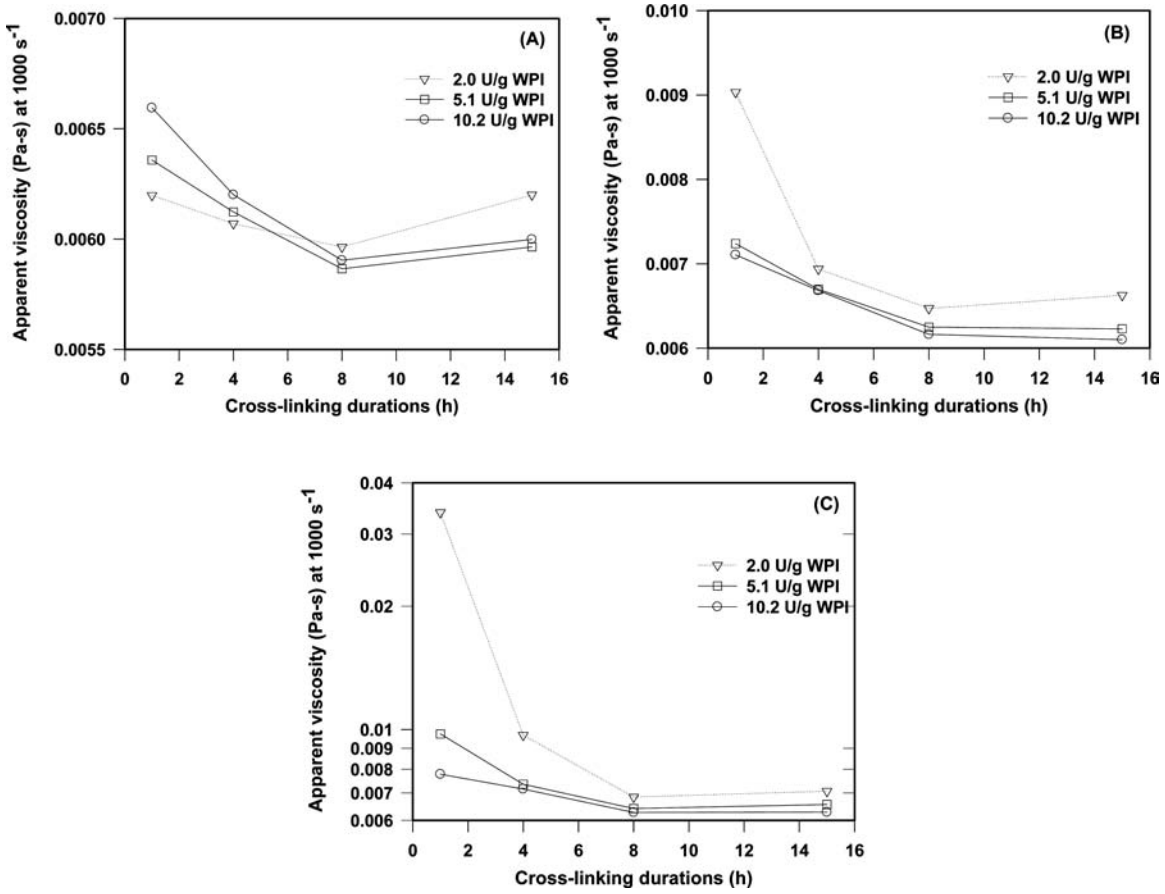

Figure 7. Apparent viscosities at 20 °C and 1000 s^{-1} for 5% w/v WPI dispersions heated at 80 °C for 15 min. Before heating, WPI samples were adjusted to pH 7.5 and cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1−15 h at 50 °C before adjustment of the pH to 7.0 and to (A) 0, (B) 50, and (C) 100 mM NaCl.

zeta potential. The increased zeta potential, that is, electrostatic repulsion, of pretreated WPI can reduce the aggregation during heating but is not the major cause of the improvement in heat stability, because the preheated WPI (with zeta potential of −33.5 mV) formed bigger structures (Tables 1 and 2) and more turbid dispersion (Figure 3) than the sequentially pretreated WPI (with zeta potential of −32.8 mV).

For the samples heated at 100 mM NaCl, the zeta potential was significantly lower than that before heating when samples were measured directly. After dialysis, the measured zeta potential was significantly higher than that before dialysis and that before heating and was similar to that heated at 0 mM NaCl. The increased zeta potential measured after dialysis is expected because colloidal particles have shortened Debye length at an increased ionic strength and, therefore, reduced mobility in an electric field.[32]

As for surface hydrophobicity, the S_0 increased after all pretreatment steps (Table 4). Heating caused a greater increase in S_0 than mTGase, and the increase in S_0 was greater when heated at 0 mM NaCl (the preheated sample) than at 100 mM NaCl (the control sample). Thermal denaturation causes the exposure of initially embedded hydrophobic amino acid residues, and therefore the increase in S_0.[33] The protection of NaCl against thermal denaturation has been reported for a higher extent of whey protein denaturation at 0 mM NaCl (∼100%) than at 100 mM NaCl (∼10%).[34] The overall trend in Table 4 is consistent with the results of Ryan et al.[31] Because an increase in S_0 favors protein aggregation, changes in S_0 are not responsible for the observed heat stability of WPI after pretreatment.

Thermal Denaturation Properties of Whey Proteins after Pretreatments. All DSC thermograms of samples at pH 7.0 and 100 mM NaCl showed an endothermic peak (Figure 6), with the estimated ΔH and T_d summarized in Table 5. Thermal denaturation of whey proteins involves both endothermic and exothermic processes, with the former attributed by protein unfolding due to mechanisms such as disruption of intramolecular hydrogen bonds and the latter resulting from hydrophobic attraction and formation of covalent (disulfide) bonds during the aggregation of denatured protein.[34,35] The overall thermogram depends on dispersion pH, ionic strength, protein concentration, and heating rate.[35b] The T_d of native WPI at 100 NaCl was at 77 °C, which is in good agreement with the literature.[34,35b,36] The center of the endothermic peak of preheated WPI shifted to 80 °C, with a much reduced intensity when compared to native WPI, corresponding to increased T_d and decreased ΔH. Preheating WPI increases electrostatic repulsion and reduces the formation of covalent disulfide bonds during the second heating,[37] corresponding to a greater resistance against salt-induced aggregation.[31,38] The T_d and ΔH of WPI cross-linked by mTGase directly were not statistically different from those of native WPI, suggesting limited improvement of heat stability. This agrees with studies showing that native β-lactoglobulin is not a very reactive substrate during mTGase cross-linking.[21] Preheating partially unfolds whey protein and increases the

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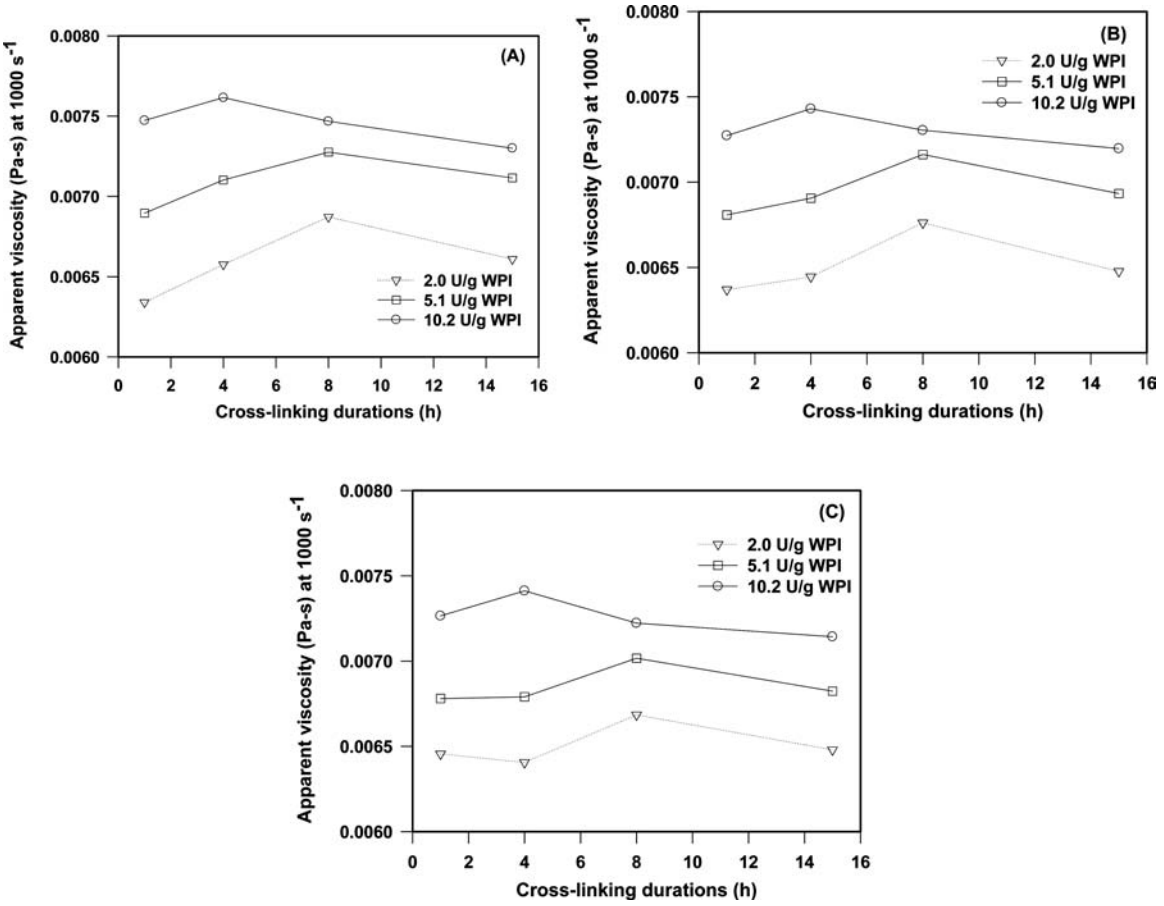

Figure 8. Apparent viscosities at 20 °C and 1000 s^{-1} for 5% w/v WPI dispersions heated at 80 °C for 15 min. Before heating, WPI samples were adjusted to pH 7.5, preheated at 80 °C for 15 min, cross-linked by mTGase at 2.0, 5.1, and 10.2 U/g WPI for 1−15 h at 50 °C, and then adjusted to pH 7.0 and to (A) 0, (B) 50, and (C) 100 mM NaCl.

susceptibility to cross-linking by mTGase,[10b,18a,24a] corresponding to significantly increased T_d and decreased ΔH of sequentially pretreated WPI. Because the zeta potential alone is insufficient to interpret the most improved heat stability after sequent pretreatments, the denaturation properties of whey protein are responsible for thermal stability observed in Figure 3.

Viscosity of Samples Heated at 80 °C for 15 min after Various Pretreatments. Shear rate ramps of WPI samples after heating at 80 °C for 15 min showed typical shear-thinning properties, that is, a reduced viscosity at a higher shear rate, shown in supplementary Figure S4 (Supporting Information) for those with 0 mM NaCl. Whey proteins are globular proteins, and the aggregation of whey proteins during heating has been described in the theoretical framework of colloidal interactions.[6] For colloidal dispersions, the dependence of apparent viscosity on shear rate measured using a mechanical rheometer typically shows a plateau regimen at the low shear rate regime, called zero-shear viscosity (η_0), and another plateau at the high shear rate regimen, called infinite-shear viscosity (η_∞), illustrated in supplementary Figure S4 (Supporting Information). There was no identifiable η_0 for all samples tested, possibly because the lowest shear rate used in tests, 0.1 s^{-1} (10 times of the instrument limit of 0.01 s^{-1}), was not sufficiently low.

The impact of various pretreatment conditions on whey protein structures is best indicated by η_0, which is not interfered with by external shear force. The apparent viscosity of samples at 0.1 s^{-1} did not show any trend as affected by the studied pretreatment conditions and is thus not reported here. This may have been caused by two factors. First, the apparent viscosity at 0.1 s^{-1} was not η_0, as discussed above. Second, samples were heated in vials before being loaded in the sample cup of the rheometer because of the need to adjust solvent conditions during sample preparation. Transferring samples during the process may interrupt some structures formed during pretreatments and the final heating step. On the other hand, η_∞ is another indicator of sample characteristics because weaker interactions are disrupted by shear, which also can overcome the errors resulting from sample preparation.

Apparent viscosities at 1000 s^{-1} are treated as η_∞ for samples heated at 80 °C for 15 min after pretreatment at various conditions. For samples without preheating (Figure 7), the general trend was similar to absorbance values as affected by pretreatments (Figure 1): lower η_∞ at a greater extent of enzymatic treatment. For preheated samples (Figure 8), samples treated by a higher enzyme level showed higher η_∞. At each NaCl concentration, the maximum η_∞ was observed for WPI pretreated by mTGase for 4 h at the highest level of enzyme and for 8 h at two other enzyme levels, and the overall trend is similar to the impact of pretreatment on sample absorbance (Figure 2).

The agreement between viscosity and absorbance data and the different trends observed for the treatments with and without preheating are to be interpreted carefully. A higher extent of aggregated structures is correlated to a higher viscosity

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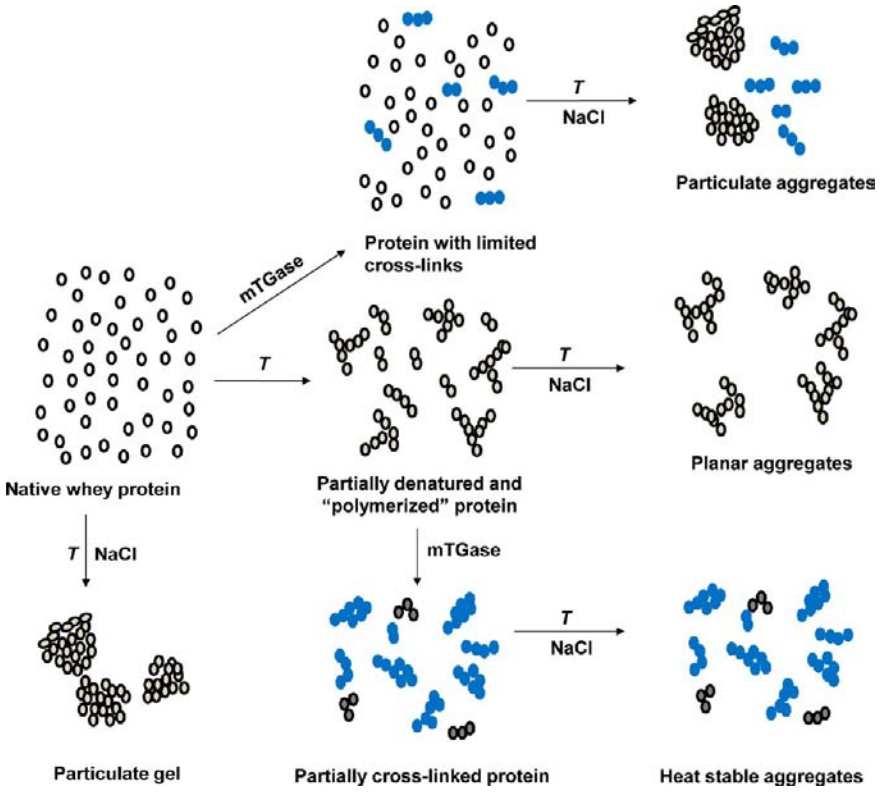

Figure 9. Proposed mechanisms of whey protein aggregation during heating at 80 °C, pH 7.0, and 100 mM NaCl as affected by individual or sequential preheating and mTGase pretreatments.

because of increased inertia against fluid flow.[39] Because WPI without preheating is not an active substrate of mTGase, the extent of cross-linking is expected to follow a reaction kinetics that is a function of reaction time and mTGase concentration. Because cross-linked whey proteins are stable against aggregation during heating at 80 °C for 15 min, thermal aggregation is less extensive for samples subjected to a higher degree of enzyme treatment, corresponding to a lower absorbance value (Figure 1) and viscosity after heating (Figure 7). In contrast, the preheated WPI is cross-linked by mTGase easily and becomes resistant to aggregation during the final heating step at 80 °C for 15 min. The extent of mTGase cross-linking then determines absorbance and viscosity, which shows a higher absorbance value and viscosity at a higher mTGase level at the same ionic strength. At the same level of mTGase, the highest absorbance and viscosity were observed at an intermediate cross-linking duration. It is possible that individual aggregates formed during preheating are cross-linked initially, corresponding to an increase in viscosity, followed by cross-linking between initially cross-linked structures, which reduces viscosity if cross-linking is not on the perimeter. This corresponds to the maximum space occupied by aggregates at an intermediate cross-linking duration, which is shorter when mTGase is used at a higher level, as indicated by absorbance and viscosity data (Figures 2 and 8).

Physical Interpretation of Thermal Aggregation of Whey Protein at 100 mM NaCl and Neutral pH As Affected by mTGase Pretreatment. On the basis of the nanoscale structural information reported in this work, the impacts of pretreatments on thermal aggregation of whey protein at 100 mM NaCl can be summarized in Figure 9. Aggregation between colloidal particles is either reaction- or diffusion-limited.[40] For native WPI, proteins are denatured during heating and become more hydrophobic, and the weakened electrostatic repulsion at an increased ionic strength (100 mM NaCl) favors protein aggregation. This corresponds to diffusion-limited aggregation, resulting in turbid gels formed of particular aggregates with much increased $d_{4,3}$ and AFM particle height (Tables 1 and 2). Heating whey proteins at low ionic strength is known to be diffusion-limited, forming filament-type aggregates.[6] In this work, preheating was conducted at pH 7.5 with 50 mM sodium phosphate for limited duration, forming flake-like aggregates (Figure 4 and supplementary Figure S1, Supporting Information) with mostly two to three layers of denatured proteins (Table 2). The remaining protein molecules can still be denatured and join the aggregates during the second heating with 100 mM NaCl, corresponding to both increased $d_{4,3}$ and AFM particle height (Tables 1 and 2) when heated for 15 min. At an elongated heating duration, the initially formed aggregates form bigger structures laterally due to excluded volume, corresponding to increased $d_{4,3}$ and similar AFM particle height (Tables 1 and 2). Compared to native WPI, the increased zeta potential and T_d after preheating reduce the extent of aggregation, corresponding to fluidic translucent dispersion.

For samples directly pretreated by mTGase, the aggregation characteristics are similar to native WPI, except to a less extent due to a portion of cross-linked structures that are stable against thermal aggregation. For the sequentially pretreated sample, it can be deduced that mTGase cross-linking occurs mostly between proteins within flakes formed during preheating and those between flakes and individual smaller particles, corresponding to bigger AFM particle height and $d_{4,3}$ than the preheated sample. The cross-linked whey proteins have

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much higher T_d, corresponding to practically constant particle heights and $d_{4,3}$ during heating with 100 mM NaCl (Tables 1 and 2) and transparent dispersions (Figure 3 and supplementary Figure S2, Supporting Information). It however should be noted that not all preheated proteins are cross-linked by mTGase under the studied conditions. These proteins can remain as separate structures or become a part of the cross-linked structures upon second heating.

In conclusion, individual or combined pretreatment methods of preheating and mTGase improved heat stability of WPI to different degrees. The sequential pretreatment showed the best improvement as transparent dispersions were observed for the three NaCl concentrations tested. When compared to increased zeta potential and surface hydrophobicity after different pretreatments, the reduced protein aggregation resulted from the increased T_d. The excellent agreement between absorbance and viscosity results revealed the structure formation kinetics as catalyzed by mTGase. Nanostructures assessed by DLS and AFM revealed nanoscale phenomena underlying macroscopic properties of whey protein aggregation as affected by prior pretreatments. Native WPI and that pretreated by mTGase directly, to a limited extent, aggregated similarly, forming particulate structures. Preheating increased the resistance of whey protein against thermal aggregation, and the subsequent heating at increased ionic strength involved the addition of free proteins and lateral aggregation of initially formed structures. Sequential pretreatments induced the formation of aggregates resistant to aggregation, enabling transparent dispersions regardless of heating duration and ionic strength. The findings of this work may be used to produce transparent beverages with added salts such as sports drinks.

■ ASSOCIATED CONTENT

Supporting Information

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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