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Microemulsions as Nanoreactors To Produce Whey Protein Nanoparticles with Enhanced Heat Stability by Sequential Enzymatic Cross-Linking and Thermal Pretreatments

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Sequential enzymatic cross-linking and heat pretreatments were used in this work to enhance the heat stability of whey protein isolate (WPI). In the first route, WPI was cross-linked by transglutaminase before incorporation in microemulsions for heat pretreatment at 90 °C for 20 min. In the second route, WPI was cross-linked by transglutaminase within microemulsions before thermal pretreatment. Particles produced from the two routes were different in dimension and heat stability and were also affected by the ratio of WPI and enzyme and cross-linking duration. At appropriate conditions, for example, 10 h of cross-linking by transglutaminase equivalent to 5% mass of WPI using the first route, a 5% dispersion (pH 6.8 and 100 mM NaCl) of the produced nanoparticles remained clear after heating at 90 °C for 20 min. In comparison, nanoparticles produced by thermal pretreatment only in a microemulsion corresponded to a translucent, flowable dispersion, whereas native WPI formed a gel. This novel approach can be used to manufacture heat-stable whey protein ingredients for clear beverage applications.

KEYWORDS: Whey protein; heat stability; enzymatic cross-linking; thermal pretreatment; microemulsion; nanoparticles; transparency

INTRODUCTION

Proteins recovered from cheese whey have been researched extensively because of their nutritional values and functional properties. Whey protein concentrates are commercial products when the recovered product has a protein content of 25−80%, whereas whey protein isolates (WPI) usually have >90% protein (*1*). The recovered whey proteins are a mixture of proteins, with the majority being β-lactoglobulin, α-lactalbumin, and bovine serum albumin (*2*−*5*). These major whey proteins undergo denaturation upon heating to a temperature above ca. 60 °C and possibly form inter- and intramolecular bonds that are either physical (hydrophobic, electrostatic, etc.) or chemical (disulfide bonds). Formation of physical and chemical bonds may lead to the aggregation of whey proteins. At appropriate solvent conditions and a sufficient amount of proteins, formation of a network of thermally denatured whey proteins may result in a gel that can no longer flow. Therefore, improvement of the heat stability of whey proteins has been a research topic targeting applications in beverage products, for which thermal pasteurization or sterilization is required to ensure safety and quality.

Several cosolutes were observed to enhance heat stability of whey proteins. Sucrose was found to increase the gelation temperature and gel strength of bovine serum albumin (*6*) and WPI (*7*). The heat stability of WPI was improved after the addition of glycerol (*8*). No gels were formed when a 10% β-lactoglobulin solution was added with <10% glycerol. Conversely, when glycerol was added to >10%, gels formed and were stronger at a higher glycerol concentration (*9*). Sorbitol was observed to be more effective than glycol to increase thermal denaturation temperatures of WPI (*9*).

Recently, caseins have been studied for the role of molecular chaperones—compounds that can stabilize proteins from unfolding, aggregation, and precipitation (*10*−*12*). The α- and β-caseins were compared for the ability to stabilize β-lactoglobulin (6% w/v, pH 6.0, without salt addition) during heating (*13*). α-Casein was unable to stabilize β-lactoglobulin at a temperature above 75 °C. High-purity (>90%) β-casein at a concentration of 2% w/v effectively maintained the clarity of the β-lactoglobulin solution after heating at 90 °C for the longest reported time of 90 min. In contrast, lower purity β-casein preparations were less effective. This interesting study may provide a molecular approach to stabilize whey proteins. However, high-purity β-casein is expensive, and its stabilizing ability at the presence of salt is unknown.

Extensive cross-linking by transglutaminase (TGase) was observed to increase the heat stability of whey proteins in a few studies (*14*−*17*). Extensive intra- and intermolecular cross-linking was hypothesized to produce whey protein structures that "were too large for effective unfolding for network development" (*17*). Generally, food-unfriendly dithiothreitol or preheating is used to denature whey proteins before TGase treatments, and proteins show enhanced heat stability at a low protein concentration (<5% w/v) and a low ionic strength. Conversely, it is well-known that whey protein aggregation during heating is dramatically

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enhanced at an increased ionic strength, particularly with divalent metallic ions such as those of calcium. It remains a technological challenge to produce whey protein ingredients that are clear after heating when the protein content is > 5% and the ionic strength is significant.

In recent years, nanotechnology has attracted much interest for the production of nanoscale food materials with unique functional properties. This has been seen in the adaptation of many nanotechnologies established in other disciplines to food science. This work is an example in which microemulsions were used as nanoreactors to produce nanometer-sized particles of whey proteins. Microemulsions are thermodynamically stable systems in which the dispersed phase is "dissolved" in and exchangeable among self-assembled surfactants, in contrast to regular emulsions in which the dispersed phase is usually not exchangeable (*18*, *19*). The droplets of dispersed phase in microemulsions are usually smaller than 100 nm, and microemulsions are usually transparent (*18*, *19*). The nanoscale dispersed phase in microemulsions has been used as a nanoreactor for syntheses of nanomaterials, for example, by sequentially dissolving reactant solutions in microemulsions for the production of inorganic nanomaterials (*20*−*26*). There is currently no report of protein nanomaterials produced using microemulsions as nanoreactors.

Specifically in this work, we were interested in utilizing microemulsions to pretreat whey proteins to modify the ability of proteins to form intermolecular bonds. In a separate paper (*27*), we applied thermal pretreatment to improve the heat stability of WPI, on the hypothesis that irreversible physical and chemical bonds formed during pretreatment would not reoccur when the pretreated WPI is reheated. Furthermore, we dispersed WPI solutions in swollen micelles of water/oil (W/O) microemulsions for thermal pretreatment (at 90 °C for 20 min) to limit the space available for protein aggregation, so that the thermally formed particles may be smaller than the 5−100 nm diameters of swollen micelles. We observed the formation of nanoparticles after thermal pretreatment that gave a transparent appearance after dispersion at a 5% w/v concentration in a pH 6.8 buffer with 100 mM NaCl. After 20 min of heating at 80 °C, no gelation was observed for the dispersion (in contrast, the control 5% native WPI formed a gel). The dispersion after heating, however, was slightly turbid, which, compared to the transparent appearance before heating, indicated the aggregation of the formed nanoparticles. In this paper, we investigated if the sequential pretreatments of enzymatic cross-linking and heat would further enhance the heat stability of whey proteins. Different TGase concentrations were used to cross-link WPI for different durations before being pretreated by heat in microemulsions, followed by recovering protein particles for characterization. The thermal pretreatment step is expected to inactivate TGase used in the first pretreatment of enzymatic cross-linking because the activity of TGase is lost at 70 °C within a few minutes (*28*).

MATERIALS AND METHODS

Materials. The WPI sample was from Davisco Foods International, Inc. (Le Sueur, MN). Limonene, 1-butanol, polyoxyethylene 20 sorbitan monostearate (Tween 60), and ethanol were procured from Acros Organics (Morris Plains, NJ). The TGase (product Activa TG-TI) was a gift from Ajinomoto Food Ingredients LLC (Chicago, IL). The TGase had a specific activity of 1100 U/g of powder, where 1 unit is defined as the formation of 1 μmol of hydroxamate/min from substrates of *N*-carbobenzoxy-glutaminyl-glycine and hydroxylamine at pH 6.0 and 37 °C (*17*). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

Protocol of Pretreatments. *Overall Design*. Two sequential pretreatment routes were studied. In the first route, WPI was cross-linked by TGase for different durations before being incorporated in W/O microemulsions for thermal pretreatment at 90 °C for 20 min. In the second route, the solutions of WPI with codissolved TGase were dispersed in W/O microemulsions and incubated at enzymatic cross-linking conditions for different durations before thermal pretreatment at 90 °C for 20 min. Because microemulsions were used as nanoreactors to form WPI nanoparticles, the first route was referred to as "ex situ cross-linking" because cross-linking was not performed in the microemulsions. In contrast, the second route was named "in situ cross-linking" because the enzymatic cross-linking was completed within the microemulsions. Variables of TGase concentrations (relative to WPI mass) and cross-linking durations were studied for the effect on the dimension and heat stability of the produced particles.

Preparation of Oil Phase and Surfactant. A literature microemulsion system suitable for food processing was used (*29*, *30*). The oil phase of microemulsions was composed of limonene and 1-butanol at a mass ratio of 1:1. The surfactant Tween 60 was then mixed at the same mass as the oil phase until a transparent solution was obtained. This mixture was used in the following pretreatment of WPI.

Pretreatment Protocol Involving ex Situ Cross-Linking. The WPI solution was prepared to a 5% w/v concentration, and the TGase was then added at different mass ratios to WPI. After adjustment of the pH to 7.5 using 1 N NaOH, the solution was incubated in a shaking water bath maintained at 50 °C for different durations. The WPI solution was then dispersed in the above mixture of oil phase and surfactant at a ratio of 0.3 mL/10 g of the mixture. The microemulsion was heated for 20 min in a water bath maintained at 90 °C, immediately followed by cooling in a room temperature water bath. After centrifugation of the cooled sample at 5000*g* for 2 min (model MiniSpin Personal, Eppendorf, Westbury, NY), the supernatant was decanted and the pellet (thermally aggregated whey protein) was repeatedly washed with fresh ethanol four times. The slurry was flushed with nitrogen before drying for 1 h in a vacuum oven (model N7595-1, Lab-line Instruments, Inc., Melrose Park, IL) set at 180 mmHg underpressure and 90 °C. The dried powders were stored in a −20 °C freezer until analyses.

Pretreatment Protocol Involving in Situ Cross-Linking. For the in situ cross-linking, the above 5% WPI solution, with different amounts of added TGase and adjusted to pH 7.5, was dispersed at the same ratio as above to the mixture of oil phase and surfactant. The microemulsion was then placed in a shaking water bath at 50 °C for enzymatic cross-linking for different durations. Afterward, the microemulsion was placed in another 90 °C water bath for heat pretreatment for 20 min. The heat-pretreated microemulsions were processed as above to recover the thermally aggregated whey protein particles.

Characterization of Whey Protein Particles. The following conventional techniques were used to characterize the produced whey protein particles, briefly described below.

Scanning Electron Microscopy (*SEM*). The morphology of the whey protein particles was imaged with a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany).

Sodium Dodecyl Sulfate−*Polyacrylamide Gel Electrophoresis* (*SDS-PAGE*). The SDS-PAGE experiments were performed at the reducing condition using a Mini Protean Tetra Cell (Bio-Rad, Hercules, CA) at a constant voltage of 200 V. The 18% Tris-HCl gel (Ready Gel Precast Gel) was a product from Bio-Rad (Hercules, CA). The gel was stained with Coomassie Blue.

Protein Content Analysis. The nitrogen content of powdered products was measured using the Dumas method (AOAC method 992.23) (*31*) by Galbraith Laboratories, Inc. (Knoxville, TN). The protein content was estimated using a conversion factor of 6.38 based on the nitrogen content (*32*).

Differential Scanning Colorimetry (*DSC*). The 5% w/v samples (in pH 6.8, 100 mM NaCl) of comparable whey proteins were analyzed by using a temperature ramp from 30 to 95 at 1.5 °C /min (model DSC Q2000, TA Instruments, New Castle, DE). The ramp was repeated twice using the same sample to test the reversibility of thermal denaturation.

Particle Sizing. The hydrodynamic radius of particles was determined by dynamic light scattering using a Delsa Nano C particle analyzer (Beckman Coulter, Fullerton, CA). Mean diameters of particles dispersed at a concentration of ca. 7 mg/mL in a buffer (pH 6.8, 100 mM NaCl) were reported on the basis of the results from 30 scans for each sample.

Evaluation of the Heat Stability of Whey Protein Nanoparticles. The samples were dispersed at a 5% w/v concentration, with 100 mM NaCl added and the pH adjusted to 6.8. The ionic strength was chosen because WPI formed the strongest gel when studied at different NaCl concentrations and neutral pH (*33*). The dispersions were heated at 80 or 90 °C for 20 min. Native WPI was used as a control and processed similarly. The vials prior to and after heating were compared for visual appearance by photographing or for turbidity by measuring the absorbance at 500 nm using a UV−vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA).

Statistical Analysis. Statistical differences among comparable data points from the same set were analyzed with a least-significant difference ($p < 0.05$) mean separation method (LSD) using SAS software (version 9.2, SAS Institute, Cary, NC). Analysis of variance (ANOVA) for data sets from comparable in situ and ex situ treatments was performed using a statistical analysis package (version 15.0, SPSS Inc., Chicago, IL) at a significance level of 0.05.

RESULTS AND DISCUSSION

Effect of TGase Concentration. The concentration of TGase was studied at 0−10% mass of WPI for both ex situ and in situ cross-linking. The produced whey protein particles were dispersed in a pH 6.8 buffer with 100 mM NaCl, and the mean diameters of dispersions are presented in **Figure 1A**. Overall, mean diameters of particles produced from the in situ cross-linking route were bigger than those produced by the ex situ route (**Figure 1A**), and the results from the two routes were significantly different ($p = 0.038$). Without TGase (at 0%), the produced particles were larger than 1000 nm for both cross-linking methods. Generally speaking, the TGase concentration significantly influenced the diameter of resultant particles, and particles produced using a higher concentration of TGase during ex situ cross-linking corresponded to smaller particles. When the TGase was used at 2 or 4% mass of WPI, the produced particles were smaller than 200 nm. When the TGase usage was increased to 6−10% mass of WPI, the particles produced were smaller than 100 nm (**Figure 1A**). The smallest particles were produced at a TGase level corresponding to 6% mass of WPI. In contrast, the trend was not so obvious for the particles produced by in situ cross-linking using different TGase concentrations, where the smallest particles were produced at a TGase level corresponding to 4% mass of WPI. **Figure 1B** shows an exemplary SEM image for particles produced by in situ cross-linking with TGase equivalent to 10% mass of WPI. Some particles are spherical and smaller than 100 nm, but some particles are larger than 100 nm and are nonspherical. Some nonspherical particles appear as "partially coalesced spheres," whereas others are rod-shaped. The nonspherical structures may have been caused by the interfacial activities of proteins, as discussed below. In addition, globular whey proteins cross-linked by TGase may be a string of several monomers, and the strings may aggregate with each other into nonspherical structures after thermal pretreatment.

When the WPI solution is dissolved in reverse micelles of microemulsions, two implications may occur. First, whey proteins may adsorb to the W/O interface due to their interfacial activity. The adsorption of whey proteins onto the interface is followed by unfolding of proteins that may take >24 h to reach equilibrium (*34*). Second, one characteristic of microemulsions is the interexchange of dispersed phase dissolved in surfactant micelles, which may be complicated by the present proteins. As a result, whey proteins may protrude from the interfacial film, leading to nonspherical liquid droplets or even coalescence of the water droplets. Furthermore, because proteins are present in swollen micelles for a longer time during 24 h of in situ cross-linking, the possibility of proteins adsorbing onto and subsequently complicating the interface may be higher, resulting in particles larger

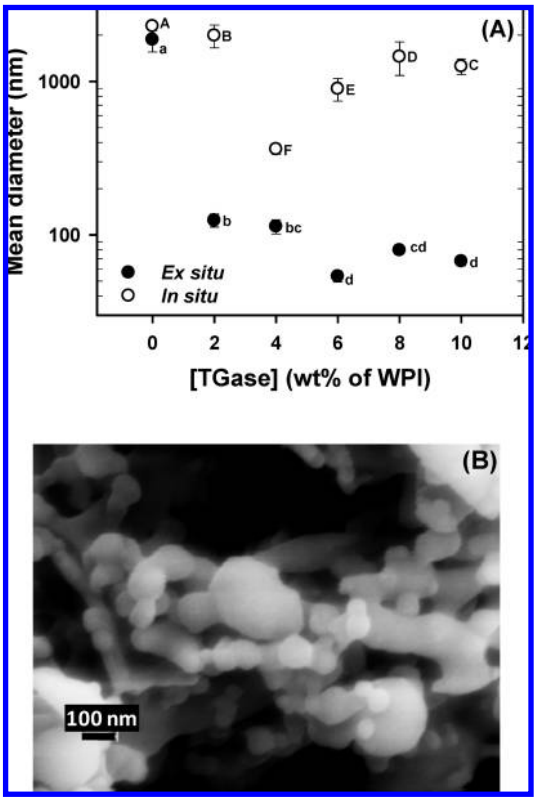

Figure 1. Mean diameters of whey protein particles produced by cross-linking with different transglutaminase (TGase) concentrations for 24 h followed by heat pretreatment at 90 °C for 20 min in a microemulsion. The TGase was used at 0−10% mass of whey protein isolate (WPI); the concentration of WPI was 5% w/v. For ex situ cross-linking, WPI was cross-linked before being dispersed in microemulsions for thermal pretreatment. For in situ cross-linking, the 5% WPI solution with dissolved TGase was incorporated in microemulsions, followed by thermal treatment after 24 h of cross-linking. (**B**) is a sample produced by in situ cross-linking for 24 h with a TGase concentration corresponding to 10% mass of WPI. Error bars represent standard deviations of results from 30 scans during dynamic light scattering. Different letters next to symbols represent statistical differences among the data points from the same (in situ or ex situ) enzymatic cross-linking route ($p < 0.05$).

than those produced by ex situ cross-linking (**Figure 1A**). The long incubation of samples during in situ cross-linking may have also complicated the effects of TGase concentration on the size of produced particles. On the other hand, the hydrodynamic radii of β-lactoglobulin, α-lactalbumin, and bovine serum albumin are 2.6−4.9 (*35*), 2.0 (*36*), and 3.7 nm (*37*), respectively, and these three proteins are known to form aggregates upon heating. The nanostructures shown in **Figure 1B** may be aggregates from individual or mixed whey proteins during thermal pretreatment and/or enzymatic cross-linking.

When the produced particles were dispersed at a concentration of 5% w/v in a pH 6.8 buffer with 100 mM NaCl, the absorbances of the dispersions before and after heating at 90 °C for 20 min are presented in **Figure 2**. For samples produced by the ex situ cross-linking route (**Figure 2A**), the sample produced with 0% TGase had higher absorbance before heating than those treated with other enzyme concentrations, which may have been caused by the much larger particles (>1000 vs 200 nm or smaller, **Figure 1A**). For samples produced with a TGase concentration equivalent to 6−8% mass of WPI, the absorbances were all <0.1, corresponding to transparent dispersions. After heating, the absorbance of all samples increased, and the absorbance was generally

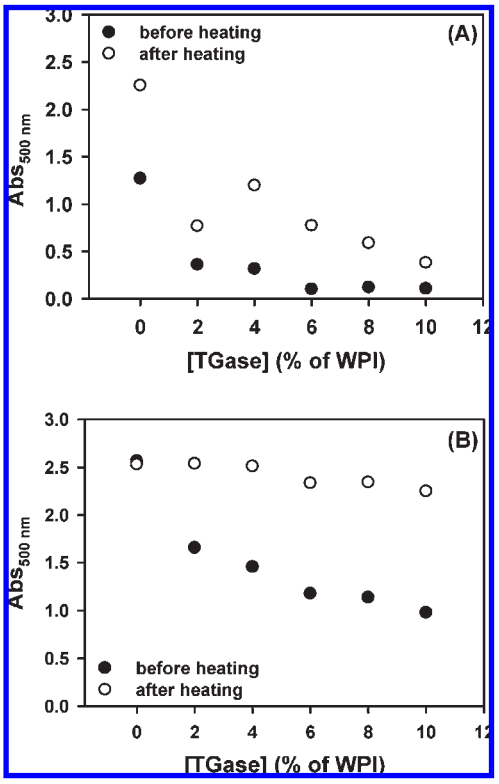

Figure 2. Turbidity of 5% w/v dispersions (in pH 6.8, 100 mM NaCl) of the whey protein particles produced using the conditions described in the caption of **Figure 1**: (**A**) ex situ cross-linking before thermal pretreatment; (**B**) in situ cross-linking before thermal pretreatment. Solid and open circles present the turbidity of dispersions before and after heating at 90 °C for 20 min, respectively.

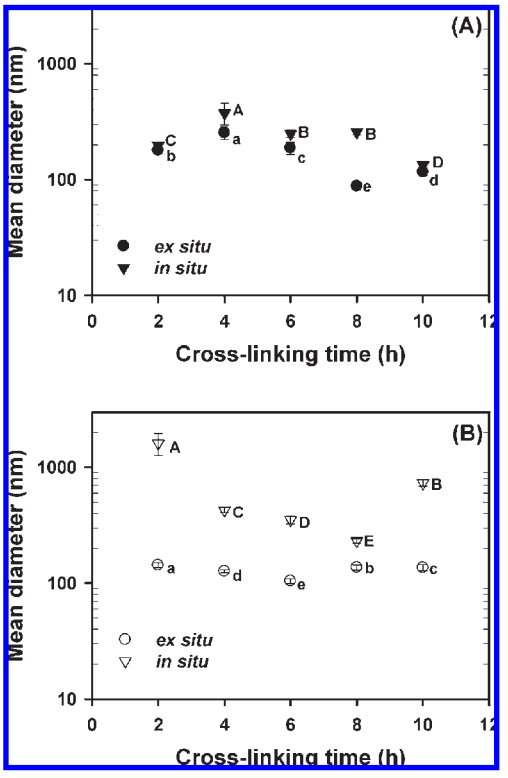

Figure 3. Mean diameters of whey protein particles produced after different durations of ex situ cross-linking by TGase before thermal pretreatment in microemulsions at 90 °C for 20 min or of in situ cross-linking by TGase in microemulsions before thermal pretreatment. The amounts of TGase were (**A**) 5% and (**B**) 10% mass of WPI. Error bars represent standard deviations of results from 30 scans during dynamic light scattering. Different letters next to symbols represent statistical differences among the data points from the same (in situ or ex situ) enzymatic cross-linking route ($p < 0.05$).

smaller for the sample treated with a higher concentration of TGase, except for the treatment with a TGase concentration equaling 2% mass of WPI. The exact reason of this exception is unknown.

The samples produced by the in situ cross-linking protocol (**Figure 2B**) had absorbance values much higher than the corresponding samples produced by the ex situ cross-linking protocol (**Figure 2A**). This may have been caused by the much bigger particles shown in **Figure 1A**. After heating, the absorbance further increased to a value >2.0, corresponding to more turbid dispersions. The degree of absorbance increase after heating was greater than the corresponding sample in **Figure 2A**. The underlying mechanism leading to the differences needs future examination.

Effect of Cross-Linking Duration. The next variable studied was the duration of cross-linking, studied for up to 10 h for two TGase concentrations: 5 and 10% mass of WPI. Mean diameters of particles after redispersion in a pH 6.8 buffer with 100 mM NaCl are presented in **Figure 3**. At a TGase concentration equivalent to 5% mass of WPI (**Figure 3A**), the particles produced by ex situ cross-linking were mostly between 100 and 300 nm. Particles produced by in situ cross-linking were generally bigger than those produced by ex situ cross-linking. The difference was not significant for the two cross-linking routes ($p = 1.54$). The effect of cross-linking time on particle diameter was significant.

Larger diameters of particles produced by in situ cross-linking than those produced by ex situ cross-linking were also observed when the TGase concentration was 10% mass of WPI (**Figure 3B**). The differences in comparable particle diameters due to the two cross-linking routes were greater than those produced at a lower TGase concentration (**Figure 3A**), possibly due to the presence of more proteins (TGase) in the swollen micelles during in situ cross-linking. For the ex situ treatments, the particle diameters, between 100 and 200 nm, did not show an obvious trend as affected by cross-linking time, although the statistical analysis showed differences, possibly due to the fast kinetics at a high enzyme concentration.

The absorbance of the samples in **Figure 3** is presented in **Figure 4** before and after heating at 90 °C for 20 min. At a TGase concentration of 5% mass of WPI, the absorbance of samples produced by ex situ cross-linking (**Figure 4A**) was much lower than those produced by in situ cross-linking (**Figure 4B**). All samples showed an increase in the absorbance after heating.

For the samples prepared with a TGase concentration equivalent to 10% of WPI, the absorbances of samples produced by ex situ cross-linking were all lower than 0.5 before heating (**Figure 4C**). However, after heating, all absorbance values increased by >1.0, indicating significant aggregation after heating. For the samples produced by in situ cross-linking, the absorbance before heating (**Figure 4D**) was higher than the counterpart product produced by ex situ cross-linking (**Figure 4C**), due to larger particles shown in **Figure 3**. After heating, the absorbance values all increased to >2.0 (**Figure 4D**).

Characteristics of Nanoparticles Produced by Pretreatments. Among all treatments, the sample cross-linked ex situ for 10 h with a TGase concentration of 5% mass of WPI showed the lowest absorbance after heating (**Figure 4A**). The recovery of nanoparticles from the mass of WPI used in pretreatments was about

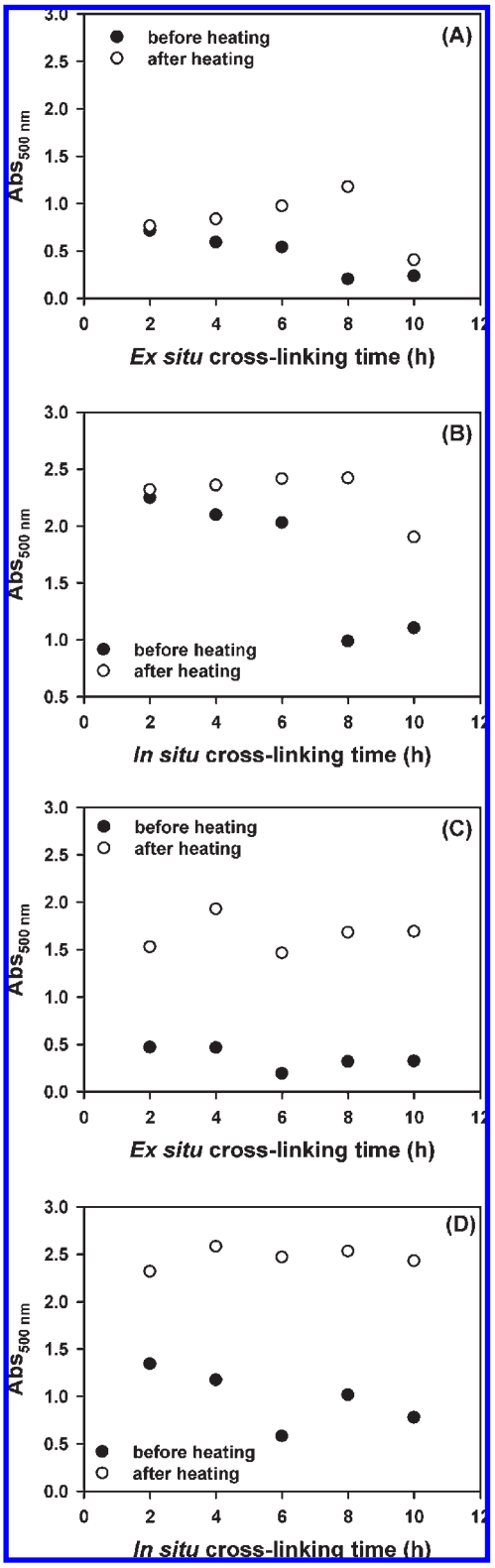

Figure 4. Turbidity of 5% w/v dispersions of whey protein particles (in pH 6.8, 100 mM NaCl) before and after heating at 90 °C for 20 min. Particles were produced by ex situ (cross-linking before dispersion in microemulsions) or in situ cross-linking (cross-linking within microemulsions) using TGase concentrations of 5% (**A**, **B**) or 10% (**C**, **D**) mass of WPI, followed by thermal pretreatment at 90 °C for 20 min.

90%. The protein content of nanoparticles was 95.7% (dry base), comparable with 95.2% of the native WPI. This sample was further characterized as below.

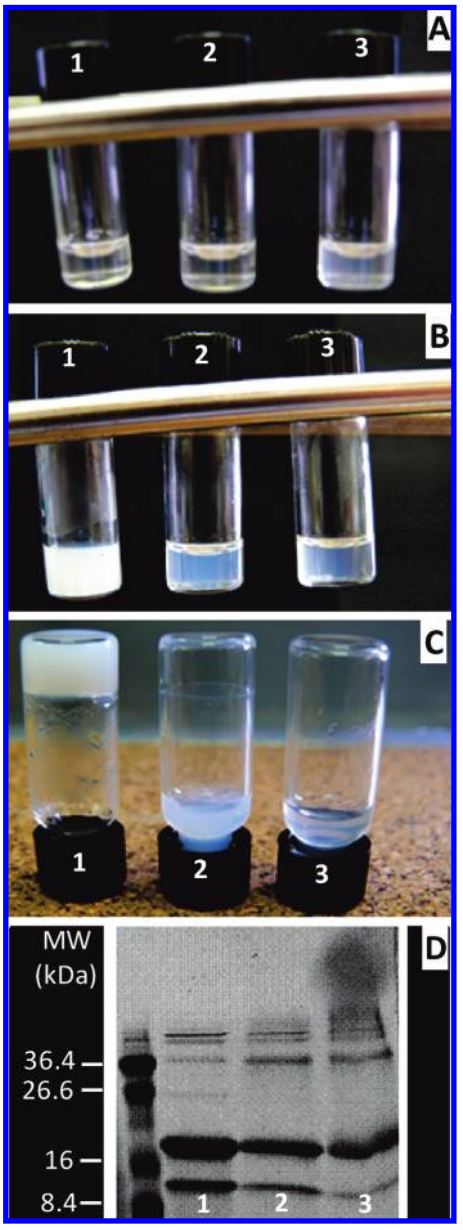

Figure 5. Appearance of 5% w/v whey protein (in pH 6.8, 100 mM NaCl): (**A**) before heating; (**B**) after heating at 80 °C for 20 min. Samples in picture **C** are the same samples as in the picture **B** after additional heating at 90 °C for 20 min. Image **D** shows samples analyzed by SDS-PAGE: sample 1, 5% native WPI; sample 2, 5% whey protein particles produced by thermal pretreatment at 90 °C for 20 min in a microemulsion; sample 3, 5% whey protein particles produced by enzymatic cross-linking with transglutaminase (5% mass of WPI) for 10 h followed by dispersion in a microemulsion for thermal pretreatment at 90 °C for 20 min. Samples 1 and 2 are identical to those reported in a separate paper (*27*) and are used here for comparison purposes only.

After dispersion of nanoparticles at 5% w/v in a pH 6.8 buffer with 100 mM NaCl, the visual appearance of the dispersion is shown in **Figure 5** before and after heating. The sample from our separate paper (*27*), that is, by thermal pretreatment only (at 90 °C for 20 min), is also presented for comparison, along with a 5% native WPI control. The samples were mostly clear before heating (**Figure 5A**), with the enzymatically cross-linked sample showing slight turbidity, which may be due to slightly larger particles (mean diameter of 56.3 nm for particles produced by thermal pretreatment only vs 116.3 nm for particles

produced by sequential ex situ cross-linking and thermal pretreatments).

After heating at 80 °C for 20 min, the 5% native WPI sample formed a gel, the sample with 5% particles produced by thermal pretreatment only had a significant increase in turbidity, whereas the sample with 5% particles produced by sequential treatments had no appreciable visual change in turbidity (**Figure 5B**). After further heating at 90 °C for 20 min, the dispersion of particles produced by thermal pretreatment only became more turbid, but the turbidity of the sequentially pretreated sample had turbidity similar to that after the first heating step (**Figure 5C**). Because physical and chemical bonds are formed during heating of native WPI, the pretreatments reduced the formation of bonds when pretreated WPI samples were heated, which was more apparent for the sequentially pretreated sample.

At neutral pH and a low ionic strength, whey proteins form filament-like aggregates (also called polymers in some papers) during heating that do not precipitate or form gels. This unique property has been used to preheat whey proteins (typically at 80−90 °C for 15 min or longer) to form gels from denatured proteins induced by salt (sodium or calcium chloride) or acid, known as cold-set gels (*1*, *38*−*42*). It was also shown that cross-linking of β-lactoglobulin (reduced by dithiothreitol) by TGase enhanced the protein heat stability (*16*). To demonstrate the much improved heat stability of whey proteins shown in this work, WPI solutions were pretreated by three methods, comparable to pretreating WPI solutions in microemulsions (samples 2 and 3 in **Figure 5**). In pretreatment method A, 5% WPI was rehydrated in a 50 mM sodium phosphate buffer for 24 h at room temperature, adjusted to pH 7.5, and pretreated at 90 °C for 20 min. After pretreatment, the sample was immediately cooled in a room temperature water bath, followed by adjustment of the pH to 6.8 and supplementation of NaCl to a concentration of 100 mM. In pretreatment method B, the WPI solution in pretreatment A (pH 7.5) was added with TGase to an amount equivalent to 5% mass of WPI, followed by cross-linking at 50 °C for 10 h. After cooling to room temperature in a water bath, the TGase-pretreated sample was adjusted to pH 6.8 and 100 mM NaCl. In pretreatment method C, the sample in pretreatment method B after enzymatic cross-linking was pretreated at 90 °C for 20 min, cooled to room temperature in a water bath, and adjusted to pH 6.8 and 100 mM NaCl. Samples from the three pretreatment methods after heating at 80 °C for 20 min are shown in **Figure 6**. The sample from pretreatment method A formed a turbid gel that did not flow after inverting the vial, whereas samples after pretreatment methods B and C formed weak gels that flowed after the vials were inverted. Whey protein nanoparticles produced by pretreatment in microemulsions, especially by sequential enzymatic and thermal pretreatments, thus had much improved heat stability.

Upon analysis by SDS-PAGE, the thermally pretreated sample (lane 2, **Figure 5D**) had a band pattern similar to that of the native WPI (lane 1, **Figure 5D**), whereas the sample produced by sequential pretreatments showed some portion of proteins that did not enter the gel (lane 3, **Figure 5D**), typical for proteins cross-linked by TGase (*17*). The results from DSC are presented in **Figure 7** for the three samples in **Figure 5**. For native WPI, the curves corresponding to the first and second scans were significantly departed. A peak centered on ca. 75 °C was observed during the first scan, whereas the second scan showed a straight line. For the thermally and sequentially pretreated samples, the curves looked similar (**Figures 7B**,**C**). Curves corresponding to the first scan were in the vicinity of those from the second scan and were not straight, which indicates that the denaturation of whey

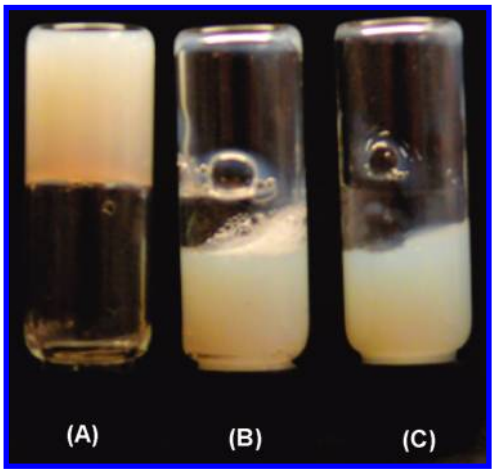

Figure 6. Visual appearance of samples with 5% WPI (in 50 mM sodium phosphate, with 100 mM NaCl, adjusted to pH 6.8) after heating at 80 °C for 20 min. The 5% WPI samples were pretreated for different conditions before adjustment of the pH and supplementation with NaCl for heat stability tests: (**A**) preheating at 90 °C for 20 min; (**B**) cross-linking for 10 h using transglutaminase equivalent to 5% mass of WPI; (**C**) cross-linking for 10 h using transglutaminase equivalent to 5% mass of WPI followed by preheating at 90 °C for 20 min.

proteins was not complete after the thermal pretreatment (at 90 °C for 20 min).

Similar curves of the thermally and sequentially pretreated samples during the first scan are likely due to the identical thermal pretreatments (90 °C for 20 min) used. Enzymatic cross-linking at 50 °C most likely did not induce noticeable denaturation of whey protein, as indicated by the straight portions of the three curves at a temperature below 50 °C during the first scan (**Figure 7**). This also corresponded to the known fact that whey proteins are held at the globular state at a temperature below ca. 65 °C predominantly by hydrophobic interactions before becoming the molten globule state at higher temperatures (*1*, *38*, *43*, *44*). The additionally enhanced thermal stability of whey protein nanoparticles after sequential pretreatments, compared to thermal pretreatment alone, was thus due to enzymatic cross-linking.

Relevance of Thermodynamic State to Pretreated WPI. Although molecular structures of α-lactalbumin and β-lactoglobulin suggest that both are suitable substrates for TGase (*45*), it is generally recognized that native whey proteins are not active substrates of TGase (*46*). Pre-heat-treatment (*46*−*48*) or addition of a reducing agent such as dithiothreitol (*17*, *47*, *49*, *50*) increases the susceptibility of whey proteins to cross-linking by TGase. The WPI sample used in this work was prepared by spray-drying, according to the manufacturer's brochure. The exposure to heat during drying of a whey protein solution by hot air likely denatures whey proteins to some extent, which may have caused the ability of TGase to cross-link the WPI used in this study, as shown in the SDS-PAGE (**Figure 5**). Additionally, the bottom band (corresponding to α-lactalbumin, the second most abundant whey protein) of the enzymatically cross-linked sample (lane 3) in the SDS-PAGE was depleted more than the second-to-the-bottom band (corresponding to β-lactoglobulin, the most abundant whey protein). This may be explained by the literature findings showing α-lactalbumin was more susceptible to cross-linking by TGase than β-lactoglobulin at the native state and after pre-heat-treatment at 85 °C for 15 min (*51*). The degree of cross-linking of individual proteins at the studied conditions in this work, however, requires future investigation, for example, using capillary electrophoresis (*48*). The limited susceptibility of the

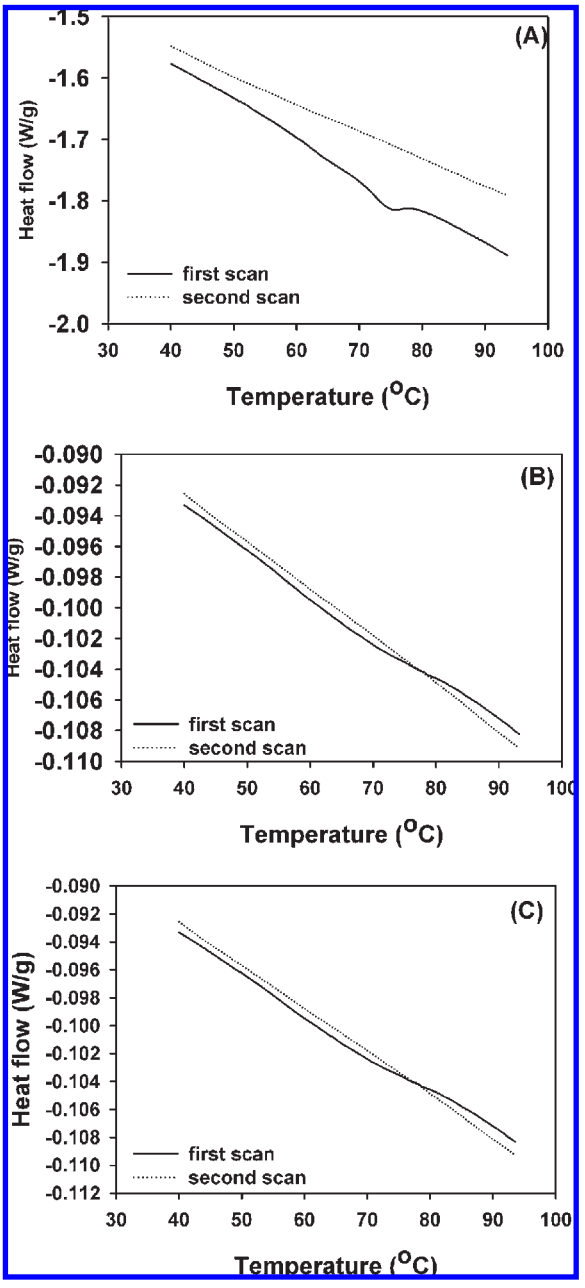

Figure 7. DSC profiles of (**A**) native WPI, (**B**) WPI nanoparticles produced by thermal pretreatment only, and (**C**) WPI nanoparticles produced by sequential ex situ cross-linking and thermal pretreatments. Conditions of producing particles are detailed in the caption of **Figure 5**. Whey protein samples for DSC were dispersed in a pH 6.8 buffer with 100 mM NaCl. Plots **A** and **B** were discussed in a separate paper but not presented (*27*); they are listed here for comparison.

WPI sample used in this study during cross-linking by TGase may also have contributed to some observations from this work. If proteins are readily cross-linked by TGase, a long reaction time and a high enzyme concentration would likely create "supra-polymer" structures for the ex situ cross-linking treatment where the reactor volume is an entire container, in contrast to the nanoscale dispersed phase in microemulsions. These supra-polymers may further aggregate into larger structures after thermal pretreatment. However, most protein particles produced by the ex situ route were smaller than 200 nm (**Figures 1** and **3**), which may have resulted from limited cross-linking of whey proteins by TGase due to the (only partially denatured) thermodynamic state of the WPI used in this work. It should also be remembered that particle sizes presented in **Figures 1** and **3**, also the corresponding absorbance data before heating, are collectively affected by the structures resulting from both enzymatic cross-linking and thermal pretreatment steps. Whey proteins subjected to an intermediate degree of cross-linking, that is, with an intermediate enzyme concentration at the same cross-linking time in **Figure 1** or an intermediate cross-linking time at the same enzyme concentration in **Figure 3**, corresponded to smaller particles after thermal pretreatment. Enzymatic cross-linking changes the unfolding properties of whey proteins during heating because of the formed inter- and intramolecular divalent bonds, as discussed above. The intermediately cross-linked whey proteins may have an intermediate size and intermediate ability to aggregate during the subsequent thermal pretreatment, which may have resulted in smaller particles in **Figures 1** and **3**. The "intermediate" cross-linking conditions may not be identical for the two cross-linking routes studied, possibly because of the interfacial activity of proteins in microemulsions. Future experiments are needed to elucidate how each pretreatment step fundamentally changes molecular and physical structures of whey proteins and how these structures are correlated to the observations from heat stability tests.

Finally, the TGase sample is composed of 99% maltodextrin and 1% enzyme (*52*), and the role of TGase sample constituents needs further study. Cosolutes such as sucrose, glycerol, and sorbitol affect the thermal stability of whey protein (*7*,*8*,*44*,*53*−*56*), but the impact of maltodextrin on the formation of whey protein particles and the subsequent heat stability is unknown. The similar protein contents of native WPI and whey protein nanoparticles formed from sequential pretreatments indicate that maltodextrin may not be present in the whey protein nanoparticles. As for TGase, because the reducing SDS-PAGE was used and a band corresponding to TGase [molecular mass of ~40 kDa (*15*)] was not observed in the regime corresponding to bands of native whey proteins, it is likely that TGase is not a component of the whey protein nanoparticles. However, experiments are needed to show if TGase is a part of the large molecules that did not enter the polyacrylamide gel (**Figure 5**), assuming the possibility of self-cross-linking of TGase (*57*) or cross-linking itself with whey proteins.

Conclusions. This work demonstrated that the heat stability of whey proteins was further enhanced by enzymatic cross-linking before thermal pretreatment. The enhancement was a function of the TGase concentration and the cross-linking duration. With appropriate sequential ex situ cross-linking and thermal pretreatment conditions, the produced whey protein nanoparticles had a good thermal stability suitable for unique applications in protein beverages. This work also leads to several interesting directions for fundamental studies, including interfacial phenomena of microemulsions with dissolved protein solutions, detailed structures from different enzymatic cross-linking conditions (duration, kinetics, and available substrate concentrations), structural changes of enzymatically cross-linked proteins during further heating, and physicochemical properties of the eventual protein nanoparticles as correlated to sequential pretreatment conditions.

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