Food Chemistry 119 (2010) 1318–1325

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Microemulsions as nanoreactors to produce whey protein nanoparticles with enhanced heat stability by thermal pretreatment

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ARTICLE INFO

Article history:
Received 3 June 2009
Received in revised form 18 August 2009
Accepted 31 August 2009

Keywords:
Whey protein
Heat stability
Nanoparticles
Microemulsions
Thermal pretreatment

ABSTRACT

Native whey proteins (NWPs) may form gels or aggregates after thermal processing. The goal of this work was to improve heat stability of NWPs by incorporating protein solutions in nanoscalar micelles of water/oil microemulsions to form whey protein nanoparticles (WPNs) by thermal pretreatment at 90 °C for 20 min. The produced WPNs smaller than 100 nm corresponded to a transparent dispersion. The WPNs produced at NWP solution pH of 6.8 had a better heat stability than those produced at pH 3.5. The salt concentration (0–400 mM NaCl) in NWP solutions did not significantly change the size of corresponding WPNs. Compared to NWPs, the 5% (w/v) dispersion of WPNs at pH 6.8, 100 mM NaCl did not form a gel after heating at 80 °C for 20 min. The improved heat stability and reduced turbidity of WPNs may enable novel applications of whey proteins in beverages.

1. Introduction

Whey proteins are a group of proteins recovered from cheese manufacturing, with β-lactoglobulin and α-lactalbumin being the most abundant. Functionality of whey proteins as food ingredients has been studied extensively (Bryant & McClements, 1998; Foegeding, Davis, Doucet, & McGuffey, 2002). Many methods have been developed to modify whey protein functionality, including conjugation with carbohydrates for improved interfacial properties (Dickinson & Galazka, 1991), cross-linking (via thermal aggregation or enzymatic reaction) and hydrolysis (Foegeding et al., 2002).

Whey proteins, including β-lactoglobulin, α-lactalbumin, and bovine serum albumin, form aggregates or gels (at high concentrations) when being heated above ~60 °C, which is a problem for beverages requiring thermal pasteurisation or sterilisation. For example, thermal pasteurisation of milk products with less than 10% fat may be achieved by treatment at 63 °C for 30 min, 72 °C for 15 s, or 89 °C for 1 s (CFR, 2008). Aggregates formed during thermal treatments may cause undesirable qualities such as a turbid appearance and compromised dispersibility (precipitation during storage); gels formed at certain conditions result in undrinkable (non-flowable) products. These negative effects are more significant at high protein concentrations, e.g., greater than 5%.

Several studies researched heat stability of whey proteins with added co-solutes. Addition of sucrose was observed to increase the gelation temperature and gel strength of bovine serum albumin (Baier & McClements, 2001) and whey protein isolate (WPI – commercial whey protein preparation with a protein content higher than 90%) (Kulmyrzaev, Bryant, & McClements, 2000). Glycerol was also observed to improve heat stability of WPI, delay protein gelation, decrease turbidity, and increase the gel strength (Chantrapornchai & McClements, 2002). For 10% β-lactoglobulin, addition of less than 10% glycerol did not enable gel formation; above 10% glycerol, gels formed and were more solid-like at a higher glycerol concentration (Chanasattru, Decker, & McClements, 2007). Both glycerol and sucrose were speculated to enhance protein–protein interactions. Sorbitol was found to be more effective than glycerol to increase the thermal denaturation temperatures of whey proteins, and no gels formed after heating 10% β-lactoglobulin with added 0–55% sorbitol (Chanasattru et al., 2007). These important studies illustrated the significance of the above co-solutes in relevant products. Effects of these co-solutes on the aggregate size were not directly reported, but the indirect turbidity data suggested smaller aggregates after addition of co-solutes.

Increased heat stability of pre-heated whey protein concentrate (pH 6.7, no salt addition) was recently observed after extensive cross-linking by transglutaminase (Lorenzen, 2007), which has been conventionally used to cross-link whey proteins for enhanced gel strength (Jaros, Partschefeld, Henle, & Rohm, 2006). The loss of gelation properties was observed after extensive cross-linking of native β-lactoglobulin (5%, in 50 mM sodium phosphate, pH 7.5) (Tanimoto & Kinsella, 1988) and WPI (pH 7.5, reduced by 10 mM

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doi:10.1016/j.foodchem.2009.08.043

dithiothreitol) (Truong, Clare, Catignani, & Swaisgood, 2004) by transglutaminase. Truong et al. (2004) hypothesised that extensive intra- and intermolecular cross-linking may have resulted in whey protein structures that "were too large for effective unfolding for network development".

Our interest was to apply material science approaches to produce heat-stable whey protein nanoparticles (WPNs). Further, if the size of WPNs can be controlled to be smaller than ca. 100 nm, the dispersion of WPNs may not scatter light extensively so that a transparent appearance may be enabled for clear beverage applications. To control the particle size, we used water-in-oil (W/O) microemulsions that have been used as nanoreactors for syntheses of inorganic nanoparticles (Lee et al., 2005). The rationale of using microemulsions as nanoreactors is that the aqueous phase can be dissolved in reverse micelles, forming "swollen micelles" with a dimension of ca. 5–100 nm (Flanagan & Singh, 2006). Therefore, if whey protein solutions can be dissolved in reverse micelles, WPNs can be formed within swollen micelles by an appropriate method.

The objective of this work was thus to explore the feasibility of forming WPNs in surfactant micelles by simple and purely physical thermal pretreatment. Application of thermal pretreatment was based on the hypotheses that (1) thermal pretreatment causes the irreversible formation of some bonds, e.g., disulphide bonds and (2) the WPNs produced by thermal pretreatment will form much less bonds when the recovered WPNs are redispersed and heated for a second time, i.e., improved heat stability. Although many microemulsions have been developed, we were interested in fully-dilutable, food grade microemulsion systems that are composed of an oil phase of limonene (citrus oil), co-surfactants of short-chain alcohols, a water phase, and nonionic polysorbate (Tween) family surfactants (Garti, Yaghmur, Leser, Clement, & Watzke, 2001; Spernath, Aserin, & Garti, 2006).

2. Materials and methods

2.1. Materials

The WPI sample was kindly provided by Davisco Foods International, Inc. (Le Sueur, MN). Limonene, 1-butanol, polyoxyethylene sorbitan monostearate (Tween 60), ethanol, and toluene were purchased from Acros Organics (Morris Plains, NJ). Other chemicals were products of Fisher Scientific (Pittsburgh, PA).

2.2. Construction of partial phase diagrams

A literature microemulsion system (Garti et al., 2001; Spernath et al., 2006) with the oil phase of limonene, co-surfactant 1-butanol, and surfactant Tween 60 was adopted in this work. For our application, we re-evaluated phase diagrams of the system to define working conditions corresponding to transparent appearance, with an attempt to identify the maximum dissolution of the aqueous phase in W/O microemulsions. Experimentally, solutions were prepared with different mass ratios (1:9 to 9:1) of the oil phase (limonene and 1-butanol at a mass ratio of 1:2, 1:1, or 2:1) and Tween 60. Afterwards, deionised water was drop-wise added into the solution of oil phase and surfactant that was continuously mixed by a magnetic stirring plate at room temperature. The volume of added water was recorded up to a point when the system became visually turbid. The compositions of mixtures were then used to prepare pseudo-ternary phase diagrams to demonstrate regimes with a transparent or turbid appearance under the working conditions. Microemulsions were formed when the system appeared transparent.

2.3. Protocol of particle production

The WPI solutions were prepared at a 5% w/v concentration in a 50 mM potassium phosphate buffer adjusted to pH 3.5 or 6.8 and 0–400 mM sodium chloride. The oil phase (limonene and 1-butanol) was mixed with Tween 60 according to the selected formulation in a 20 mL vial to a total mass of 10 g. Whilst being mixed by a magnetic stirring plate, the 5% WPI solution was drop-wise added into the solution of oil and surfactant. After stirring for 20 min, the vial was placed into a water bath maintained at 90 °C and heat-treated for 20 min, followed by immediate immersion in a room temperature water bath. The cooled sample was centrifuged at 5000×*g* for 2 min (model MiniSpin Personal, Eppendorf, Westbury, NY). After decanting the supernatant, the pellet (thermally aggregated whey protein) was repeatedly washed with fresh ethanol for 4 times. The washed particles were flushed by nitrogen before being dried for 1 h in a vacuum oven at 180 mm Hg under-pressure and 90 °C.

2.4. Characterisation of particles

Scanning electron microscopy (SEM): The dried samples were imaged with a LEO 1525 SEM microscope (LEO Electron Microscopy, Oberkochen, Germany). The sample was sputter-coated with a gold layer for a thickness of ca. 5 nm before imaging.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE): Powders were dispersed to a concentration of 50 μg/mL in deionised water. The dispersions were mixed 1:1 (v/v) with a sample buffer containing 5% β-mercaptoethanol, followed by heating at 100 °C for 5 min. The 30 μL mixture was loaded onto a 18% Tris–HCl gel (Ready Gel Precast Gel from Bio-Rad, Hercules, CA). Electrophoresis was performed with a Protean II xi 2-D Cell (Bio-Rad) at a constant voltage of 200 V. The staining with Coomassie Blue and destaining procedures followed the instruction manual of the Tris–HCl gel.

Differential scanning colorimetry (DSC): The DSC analyses (model DSC Q2000, TA Instruments, New Castle, DE) were performed for native WPI and the produced particles. Approximately 20–30 mg of the 5% sample (pH 6.8, 100 mM NaCl) was used. Thermal analyses followed the following steps: (1) equilibrium at 30 °C for 3 min, (2) a temperature ramp from 30 to 95 °C at 1.5 °C/min, and (3) holding at 95 °C for 10 min. Thermal scans were repeated twice for the same sample.

Residual concentrations of Tween 60, limonene, and butanol in WPNs: The GC–MS was used to quantify residual concentrations of microemulsion constituents. The instrument was a gas chromatograph (model GC-17A) coupled with a mass spectrometer (model GCMS-QP5000, Shimadzu, Tokyo, Japan). The capillary column (model XTI-5, Restek Corp., Bellefonte, PA) had a length of 30 m, an inner diameter of 0.25 mm, and a film thickness of 0.25 μm. Helium was used as a carrier gas, and the injection volume was 2 μL. The split ratio was set at 50:1 and the flow rate in the column was 1 mL/min. Temperatures of the injector and oven of GC and MS were set at 220 and 180 °C, respectively, for detection of Tween 60. To detect limonene and 1-butanol, the oven temperature was firstly kept at 50 °C for 5 min, raised to 200 °C at the rate of 20 °C/min, and then held for at 200 °C 10 min. The data were acquired and processed with the Class-5000 software (ver. 2.23, Shimadzu, Tokyo, Japan). Mass spectra were collected under the TIC (total ion chromatograph) mode at a detector voltage of 1.5 kV, with a scanning rate of 0.5 scan/s in the 35–350 amu range.

To detect the residual concentration of Tween 60, a literature sample preparation method was used (Li, Zhang, & Hui, 2004). The 50–100 mg of WPNs was placed into a 20 mL vial, followed by adding 3 mL of an ether/toluene (1:1, v/v) binary mixture and extraction for 15 min. After supplementation of 2 mL 2% w/v NaOH (in methanol), the slurry was heated in a water bath at 50 °C for

15 min. After cooling back to room temperature, 2 mL deionised water was added, and the sample was centrifuged at 3000×*g* for 5 min. The upper organic solution after centrifugation was transferred for GC analysis. As an external standard, 5–10 mg of the pure Tween 60 was similarly methylated before GC analysis.

To analyse residual concentrations of limonene and 1-butanol, 50–100 mg of WPNs was extracted by 3 mL ethanol for 15 min. After centrifugation as above, the supernatant was used for GC analysis.

Size and distribution of WPNs: The produced WPNs were dispersed at a concentration of approximately 7 mg/mL in a pH 6.8 buffer with 100 mM NaCl. The size and distribution were determined by dynamic light scattering using a Delsa™ Nano C particle analyser (Beckman Coulter, Fullerton, CA). Fifty scans were performed for each sample and the averages were reported.

Evaluation of heat stability of WPNs: The produced WPNs were dispersed at a 5% w/v concentration in 100 mM NaCl, adjusted to pH 6.8. The dispersions were heated at 80 or 90 °C for 20 min. The 100 mM NaCl was chosen because WPI forms the strongest gel at this ionic strength when the pH is neutral (Ikeda, Foegeding, & Hagiwara, 1999). Native WPI was used as a control and processed similarly. The vials prior to and after heating were photographed.

The thermal stability of WPNs was also quantitatively compared by measuring the turbidity. Samples were prepared at 5% w/v in a pH 6.8 buffer with 100 mM NaCl, followed by heating in a water bath at 90 °C for 20 min and then cooling in a water bath at room temperature. The turbidity was evaluated by measuring the absorbance at 500 nm using a UV/Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA).

2.5. Statistical analysis

Results from replicates were presented as averages ± standard deviations. Analysis of variance was performed using the *t*-test.

3. Results and discussion

3.1. Principle of formation of whey protein nanoparticles in W/O microemulsions

The W/O microemulsions are composed of a continuous oil phase with dissolved surfactants and/or co-surfactants and a dispersed water phase that is dissolved in reverse micelles (Fig. 1). Water droplets in W/O microemulsions are smaller than 100 nm, which gives a transparent appearance of microemulsions. If protein solutions are dissolved in surfactant micelles, microemulsions can be used as nanoreactors (Fig. 1, top). Further, the size of aggregates is difficult to control when a protein solution is heated; in contrast, the size of protein aggregates may be confined within the nanoscalar micelles if microemulsions are used (Fig. 1, right bottom). The thermally-formed WPNs recovered from microemulsions may have much improved heat stability when heating the redispersed WPNs during the pasteurisation step of manufacturing protein drinks, and the nanometre-sized WPNs may also have much improved transparency and dispersibility.

However, there are several unknowns to thermally form WPNs in microemulsions. The first one is the need of food grade microemulsions that can incorporate certain amounts of aqueous solutions. The second one is the ability of microemulsions to incorporate protein solutions and the stability of microemulsions during thermal pretreatment. The third one is the recovery and purity of the produced WPNs. The last one is the dispersibility and heat stability of the recovered WPNs. The rest of this paper addresses these questions.

3.2. Partial phase diagrams

The microemulsion system adopted was reported to be fully dilutable in that at certain dilution lines, a transparent appearance was observed at all proportions of the oil and water phases (Garti et al., 2001). The fully-dilutable property was confirmed for an oil phase with a limonene:butanol ratio of 1:2 when the starting ratio of oil phase and Tween 60 was 7:3 (Fig. 2A). A decrease of butanol content (at a higher ratio of limonene:butanol) in the oil phase generally reduced the system's ability to incorporate water, indicated by a smaller area of the one-phase regime (Fig. 2B and C). This is due to the role of co-surfactant for butanol that reduces the interfacial tension and increases the fluidity of interfacial films (Flanagan & Singh, 2006). The phase diagrams were different from the original references, possibly due to the different evaluation methods used. Strictly speaking, phase diagrams should be defined at a fixed temperature and pressure at equilibrium conditions. In this work, our intent was to establish "phase diagrams" at typical

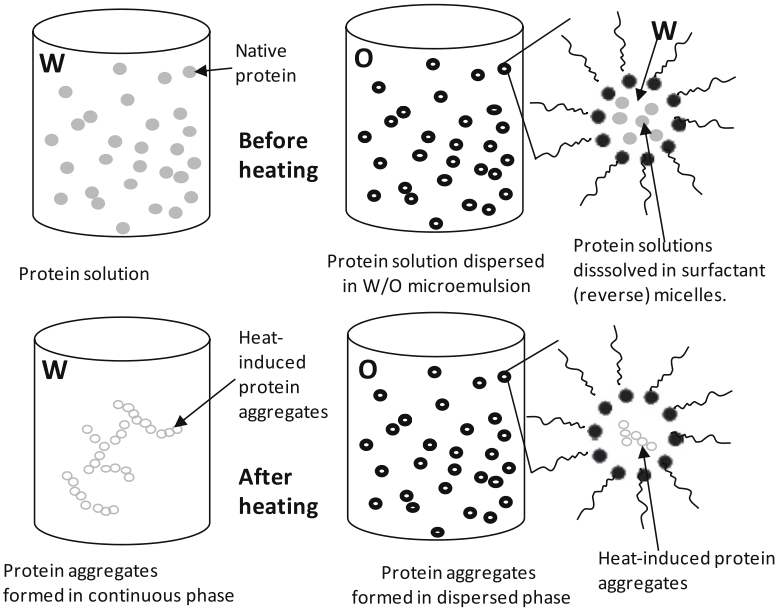

Fig. 1. Principle of using W/O microemulsions as nanoreactors to form whey protein nanoparticles.

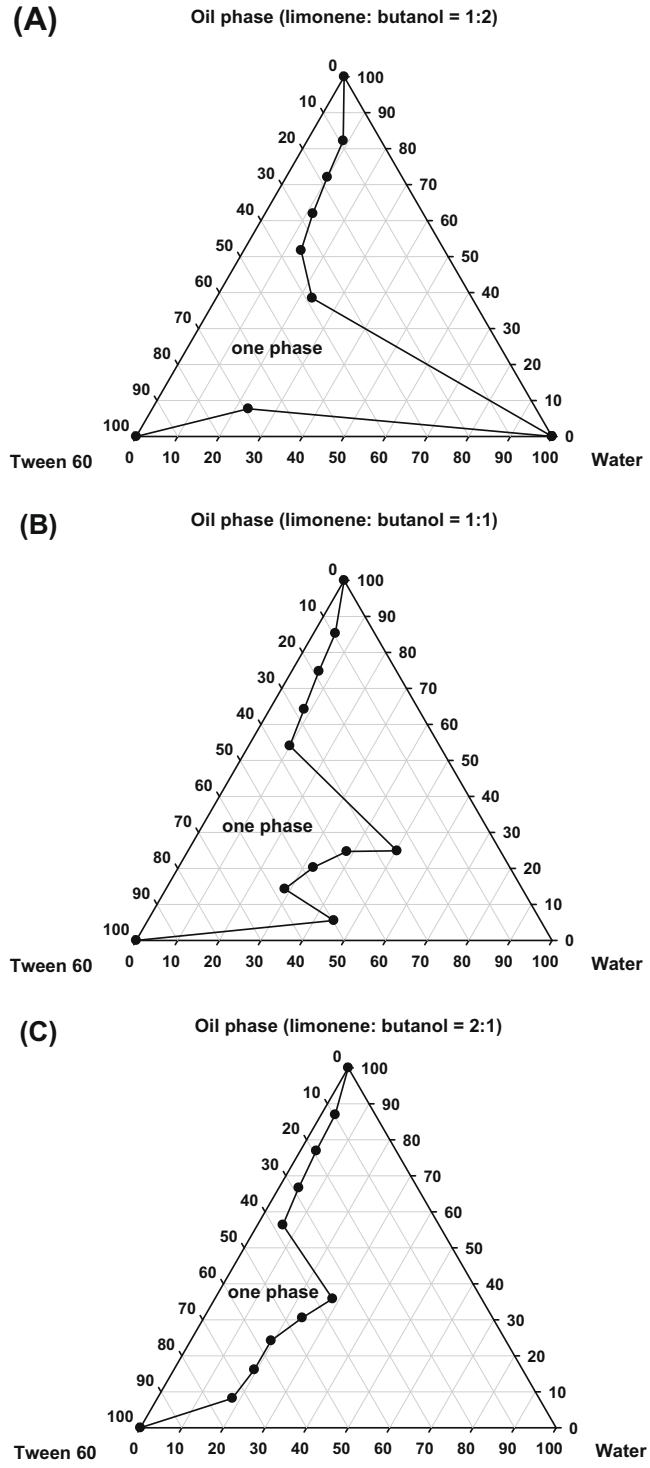

Fig. 2. Partial pseudo-ternary phase diagrams of the microemulsion systems for the oil phase with a limonene:butanol ratio of: (A) 1:2, (B) 1:1 and (C) 2:1. The one-phase regime indicates that the system with the corresponding formulation is transparent.

lab-working conditions (typically 21 °C in our laboratory) so that the information can be used for production. It should, however, be reminded that phase diagrams are strongly affected by temperature, as well as constituents of the aqueous phase, as presented below when the WPI solution was dispersed as the aqueous phase. These fundamentals are to be researched in the future.

At a limonene:butanol ratio of 1:2 (Fig. 2A), there was no clear indication of a transition from a W/O microemulsion to an O/W one when the water content was gradually increased. This ratio was not used in later studies because of the uncertainty of forming W/O microemulsions. Instead, we used the formulation in Fig. 2B where the oil phase was composed of limonene and butanol at a mass ratio of 1:1 and the Tween 60 was incorporated at the same mass as the oil phase. The selected formulation was capable of dissolving up to the same mass of water.

3.3. Effect of WPI solution volume, pH, and ionic strength on particle formation

When the 5% WPI solution was drop-wise added in 10 g of the mixture of oil phase and surfactant, the system became slightly turbid when the WPI solution volume exceeded approximately 0.2 mL. The turbidity, based on visual inspection, increased with an increased amount of WPI solution. In comparison to Fig. 2B where the mixture became turbid when 10 mL water was used, the ability of micelles in W/O microemulsions to dissolve the (~0.2 mL) WPI solution is much compromised. In W/O microemulsions, the flexibility and fluidity of interfacial films are much improved by co-surfactants (butanol in this work), which allows the dissolving and exchange of the aqueous phase in reverse micelles. Conversely, whey proteins are known for their ability to adsorb onto and stabilise interfaces and thus might compete for adsorption at the oil and water interface of microemulsions. The competitive adsorption by whey proteins may cause the protrusion of proteins out of interfacial films, which may have caused a slightly turbid appearance (instead of a transparent appearance when water was used). In addition, much bigger molecular weights (than water) and surface activity of proteins may compromise their ability to diffuse through the interfacial films during dissolution into micelles and cause some interexchange between aqueous droplets become irreversible, increasing the size of water droplets thus the turbidity of the entire system.

After thermal pretreatment, WPNs were recovered easily by simple centrifugation, and the yield of WPNs, i.e., ratio of the mass of recovered WPNs to the mass of WPI used in preparation, was about 90%. An example of the recovered WPNs is illustrated in Fig. 3A for those produced by thermal pretreatment of a microemulsion with 2% mass of the dissolved WPI solution (pH 6.8). These WPNs are mostly smaller than 100 nm; however, most particles are not perfectly spherical. The size distribution of the corresponding WPNs after dispersion in a pH 6.8 buffer with 100 mM NaCl is presented in Fig. 3B, showing WPNs are smaller than 100 nm. Similar dimensions from SEM and dynamic light scattering indicate the good dispersibility of WPNs. Further, because most WPNs were smaller than 100 nm, the results indicate that most WPI solutions were dissolved in micelles and the thermal aggregation was limited in the swollen micelles, as hypothesised in Fig. 1. Non-spherical WPNs (some bigger than 100 nm) shown in Fig. 3A may have resulted from the protrusion of proteins from interfacial films and the irreversible exchange between aqueous droplets, as disused above.

Approximately 80% whey proteins are β-lactoglobulin and α-lactalbumin, which, along with bovine serum albumin, are known to form aggregates during thermal treatments. β-lactoglobulin has a hydrodynamic radius of 2.6–4.9 nm at pH 6–8 (Parker, Noel, Brownsey, Laos, & Ring, 2005); α-lactalbumin has a hydrodynamic radius of 2.0 nm at pH 7 (Molek & Zydney, 2007); and bovine serum albumin has a hydrodynamic radius of ~3.7 nm at pH 4–8 (Brownsey, Noel, Parker, & Ring, 2003). The WPNs observed in Fig. 3A therefore could be aggregates of single or blend whey proteins.

Fig. 3C shows mean diameters of whey protein particles prepared by dispersing different amounts of WPI solutions (at pH 6.8 or 3.5) in microemulsions, measured by dynamic light scatter-

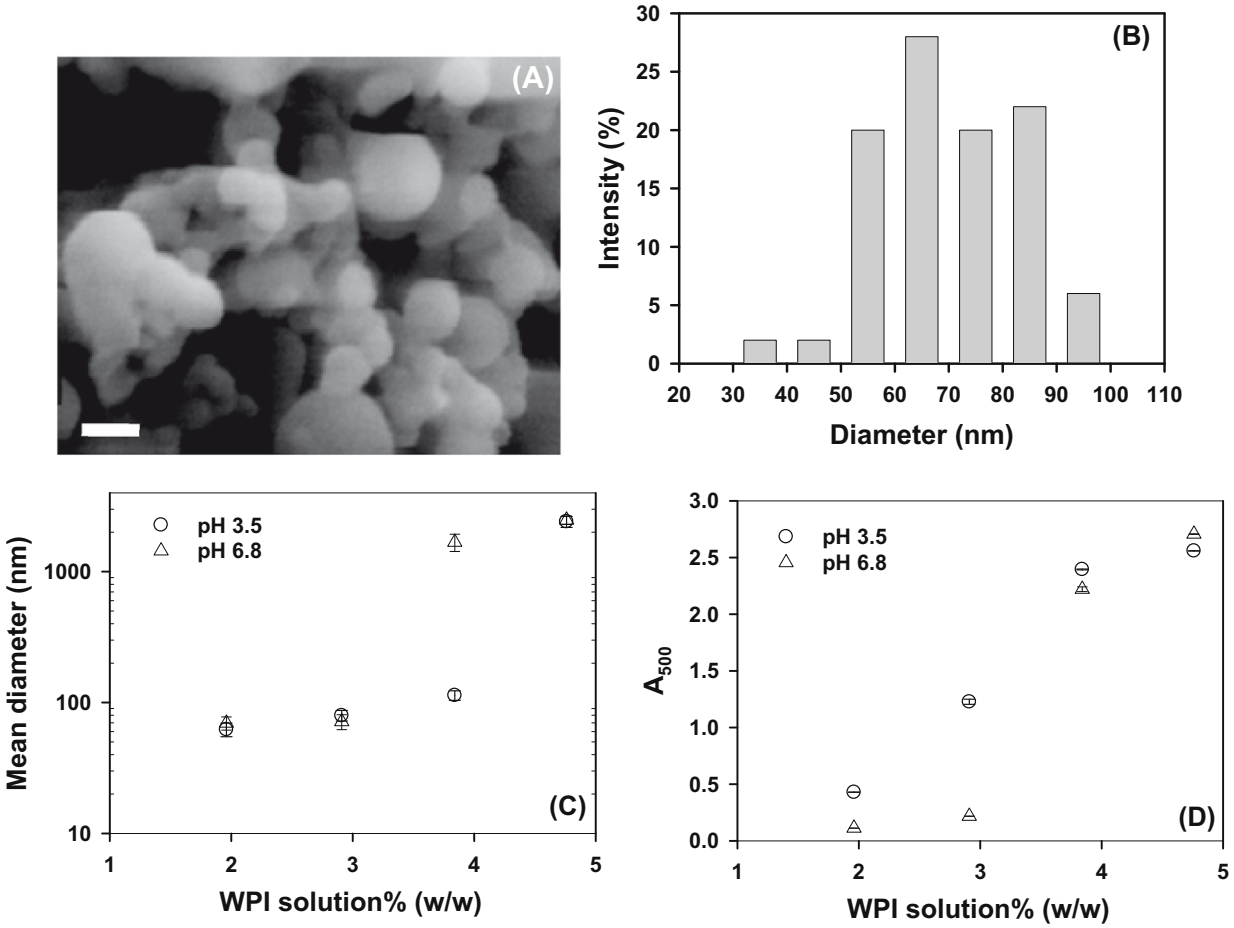

Fig. 3. Whey protein nanoparticles produced by thermal pretreatment (90 °C for 20 min) of a 5% WPI solution dispersed in microemulsions: (A) exemplary SEM image of nanoparticles prepared by dispersing a 5% WPI solution (pH 6.8) to overall 2% mass in the microemulsion; (B) size distribution of the nanoparticles corresponding to the sample in (A); (C) mean diameters of the particles produced by adding different amounts of the 5% WPI solution (pH 6.8 or 3.5) in microemulsions; and (D) turbidity of the 5% dispersions of particles in picture (C) after heating at 90 °C for 20 min.

ing after dispersion in a buffer at pH 6.8 with 100 mM NaCl. Overall, the more turbid appearance when dispersing a larger amount of the WPI solution in microemulsions, as described above, corresponded to bigger particles after thermal pretreatment. When the mass of WPI solution in microemulsion was ca. 4%, the mean diameter of the produced particles was 1679 nm when the WPI solution pH was 6.8, whilst the mean diameter was 113 nm when the WPI solution pH was 3.5. When the mass of WPI solution in microemulsions was increased to ca. 5%, the particles were greater than 2400 nm for both WPI solution pH conditions.

When the particles in Fig. 3 were dispersed at 5% w/v in a pH 6.8 buffer with 100 mM NaCl, the absorbance of dispersions after heating at 90 °C for 20 min is shown in Fig. 3D. The particles produced by adding a larger amount of WPI solution in microemulsions corresponded to a higher absorbance value of the dispersion, possibly due to the corresponding bigger particle sizes (Fig. 3C). When the WPI solution was incorporated at 2% or 3% mass of microemulsions, the absorbance of heated dispersions of particles prepared at the WPI solution pH 3.5 was higher than those prepared at the WPI solution pH of 6.8. For samples prepared from microemulsions containing 4% or 5% of the aqueous phase (WPI solution), the absorbance of heated dispersions was higher than 2.0 for both pH 6.8 and 3.5 treatments. Because a better clarity was our goal, the sample giving the lowest turbidity after heating, i.e., the WPI solution at pH 6.8 was used at an overall 2% mass of microemulsion, was chosen for further studies.

When the WPI solution (pH 6.8) during thermal pretreatment was adjusted to 0–400 mM NaCl, the mean diameters of WPNs formed were not significantly ($P < 0.005$) affected by the salt concentration during particle formation (Fig. 4). Presumably, when the WPI solution is dispersed in microemulsions, aggregation of

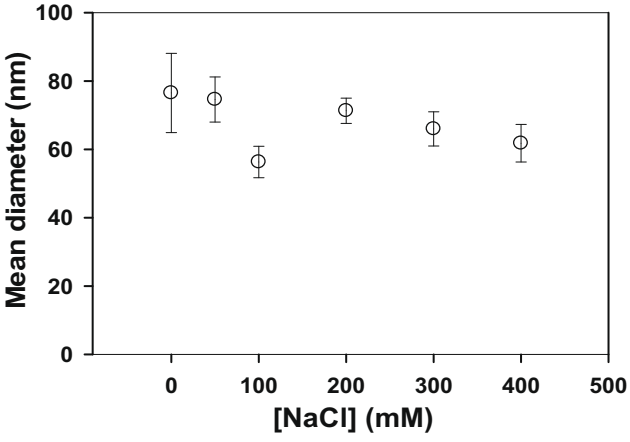

Fig. 4. Mean diameters of whey protein nanoparticles produced by dispersing 5% native WPI solutions (pH 6.8, 0–400 mM NaCl) in microemulsions to 2% mass of the entire microemulsion, followed by thermal pretreatment at 90 °C for 20 min.

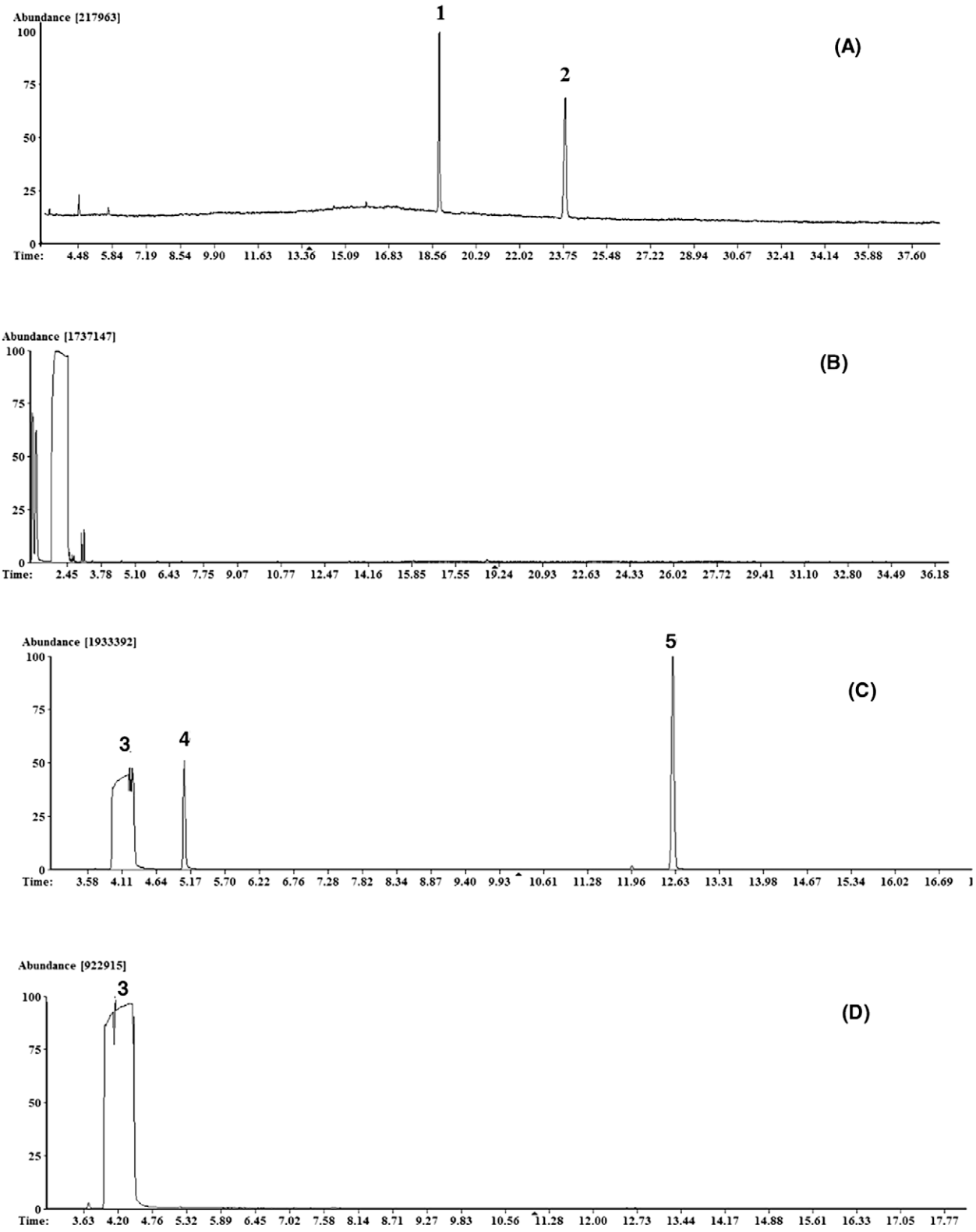

Fig. 5. GC/MS analyses of residual microemulsion components in whey protein nanoparticles. Residual Tween 60 is compared for (A) pure Tween 60 and (B) whey protein nanoparticles. The samples were methylated before injection to GC, detailed in the experiment section. Peaks 1 and 2 are methyl stearate and palmitate, respectively. Residual limonene and butanol are compared for (C) pure solvents and (D) an ethanol extract of whey protein nanoparticles. Peaks 3, 4, and 5 are ethanol, 1-butanol and limonene, respectively. Nanoparticles were produced using the conditions in Fig. 3A.

whey proteins occurs within the dispersed droplets, whose dimension may be similar at the studied conditions. Aggregation of whey proteins in swollen micelles thus resulted in WPNs with a dimension independent on the salt concentration.

3.4. Characteristics of the produced WPNs

The WPNs discussed in this section were prepared for the system where the WPI solution (pH 6.8, without additional NaCl)

was composed of 2% mass of the overall microemulsion. Fig. 5 shows results of detecting residual concentrations of microemulsion constituents in WPNs. Two peaks were detected for the pure Tween 60 sample (Fig. 5A), as expected due to the two fatty acids (stearic and palmitic acids) used in the production of the surfactant. In contrast, the WPNs did not have any peaks at the elution times corresponding to Tween 60 (Fig. 5B). Fig. 5C and D also illustrated no residual limonene and butanol in WPNs. The washing step (four times using fresh ethanol) was thus sufficient to remove possible residual constituents of microemulsions.

Results from DSC showed a peak between ca. 70–80 °C during the first scan of native WPI (figure not shown), similar to literature (Fitzsimonsa, Mulvihilla, & Morris, 2007). The peak corresponds to the denaturation of whey proteins (Abd El-Salam, El-Shibiny, & Buchheim, 1996; Heeboll-Nielsen, Justesen, & Thomas, 2004; Nakano & Ozimek, 2000; Zydney, 1998). In contrast, the curve during the second scan did not show any peak, indicating the denaturation of whey proteins was completed during the first scan and the denaturation was mostly irreversible. The DSC curves of the WPNs did not show any peaks during the first and second scans (figure not shown), which may have resulted from the thermal pretreatment step during the production of WPNs.

Heat stability of the WPNs was compared with native WPI after dispersion to a mass concentration of 5% w/v in a buffer with 100 mM NaCl, adjusted to pH 6.8. Both samples were transparent before heating (Fig. 6A), indicating the good dispersibility of WPNs and the significance of nanoscalar particle dimension to the transparent appearance. After heating at 80 °C for 20 min, the native WPI formed a strong gel that did not flow after inverting the vial, but the WPNs sample gave a translucent appearance and flowed easily (Fig. 6B). The visual difference of the WPNs sample before and after heating indicated that some WPNs were still able to aggregate during heating. After further heating at 90 °C for 20 min, the turbidity of the WPNs sample further increased, but no gel was formed (Fig. 6C). Because complete aggregation of WPI at pH 7.0 may take more than 1 h at 90 °C (Ikeda et al., 1999), the pretreatment condition (90 °C for 20 min) may not be sufficient for complete denaturation and aggregation of whey proteins in microemulsions. When analysed by SDS–PAGE, similar bands were observed for WPNs and native WPI (Fig. 6D).

Nevertheless, because WPNs were produced at 90 °C for 20 min, the dispersion of WPNs may not need further thermal pasteurisation and may be used for clear beverage applications directly. If additional heating is required, future research will be needed in order to prepare novel protein ingredients for clear beverage applications. Our ongoing efforts are to study how thermal pretreatment conditions (combination of temperature and duration, WPI solution properties) can be further optimised to enhance heat stability of WPNs. Additionally, we studied the sequential enzymatic cross-linking and thermal pretreatments that performed better than thermal pretreatment alone, to be reported elsewhere.

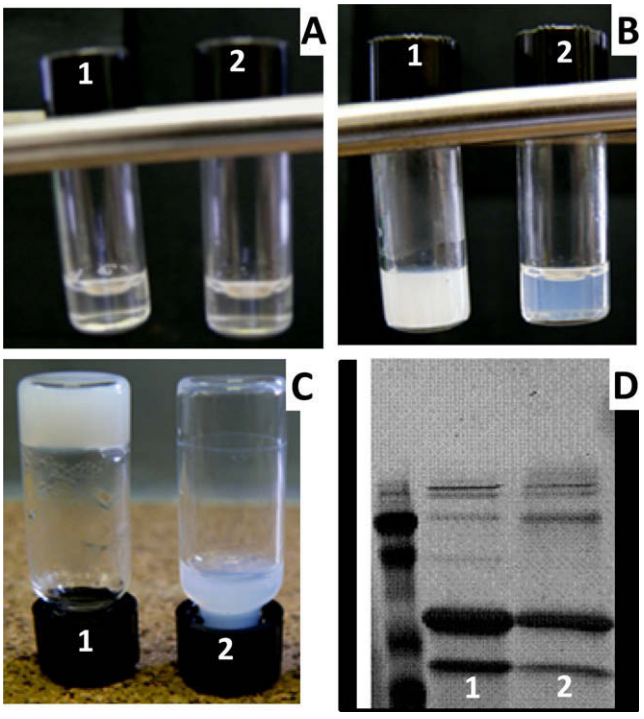

Fig. 6. Appearance of 5% w/v whey protein in a pH 6.8 buffer with 100 mM NaCl: (A) before and (B) after heating at 80 °C for 20 min. Samples in picture (C) are the same samples in the picture (B) after additional heating at 90 °C for 20 min. Image D shows samples analysed by SDS–PAGE. Sample 1 has 5% native whey protein isolate, and Sample 2 is a dispersion of 5% whey protein nanoparticles produced by conditions described in Fig. 3A.

4. Conclusions

Reverse micelles in W/O microemulsions were capable of dissolving WPI solutions to an amount much smaller than water. Aggregation of whey proteins in swollen micelles during thermal pretreatment enabled the formation of WPNs that no longer had gelation properties. The diameters of formed WPNs were independent on the salt concentration during thermal pretreatment, and no difference in SDS–PAGE patterns was observed for native WPI and WPNs. The dispersion of WPNs was transparent and may be directly used as clear beverages. The WPNs however were still able to aggregate at the studied conditions and novel strategies are needed to further enhance heat stability of the WPNs.

Acknowledgements

Funding for this project was provided by the Tennessee Agricultural Experiment Station. The authors are thankful for the SEM experiment performed by Minfeng Jin.

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