Characterization of β-Lactoglobulin A Gelation in the Presence of Sodium Caprate by Ultrasound Spectroscopy and Electron Microscopy

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The effect of sodium caprate (a fatty acid salt) on the formation of β-lactoglobulin A gels was studied at constant temperature (30 or 35 °C) using ultrasonic spectroscopy. During incubation at these temperatures, ultrasonic attenuation increased with the addition of sodium caprate, and reached a plateau after 5−7 h of incubation. Comparing β-lactoglobulin A with and without sodium caprate, a decrease in net ultrasonic velocity was observed. These results suggested that aggregation occurred during incubation with sodium caprate, and the sample showed an increase in compressibility. Transmission electron microscopy with negative staining showed the formation of filamentous aggregates of β-lactoglobulin A at around 3−4.5 h of incubation with sodium caprate. These results demonstrated that sodium caprate induced the formation of structures with unique gel properties compared to those formed by heating β-lactoglobulin in the presence of NaCl alone.

Introduction

It has been previously reported that fatty acid salts with more than 10 carbon atoms can induce transparent protein gels with high water-holding properties.[1,2] Sodium caprate, being easily solubilized in water at ambient temperature, is often employed to study the thermodynamic analysis of a protein complex with fatty acid salts as a kind of anionic surfactant[3,4] and has been compared with other types of surfactants in its ability to interact with proteins.[4] In particular, we have studied on the formation of protein gels induced by fatty acid salts and demonstrated that the addition of sodium caprate to ovalbumin and β-lactoglobulin solutions results in the formation of transparent gels at room temperature.[5,6] Circular dichroism (CD) spectroscopy demonstrated that the presence of fatty acid salts causes an immediate structural change of ovalbumin to a molten globule state, inducing the formation of a gel network.[5] The fatty acid salt-induced gels that formed at room temperature (around 25 °C) have unique gel textures compared to that of heat-induced gels.[5] Rheological measurements carried out at 25 °C have also demonstrated that the gelation time of β-lactoglobulin decreased with increasing concentrations of sodium caprate.[6] For example, a 12% β-lactoglobulin solution shows a sol−gel transition at room temperature after 916, 198, and 158 min of incubation, with 2.4, 3.6, and 4.8% sodium caprate, respectively. Under comparable conditions, fatty acid salt-induced ovalbumin gels[7] are formed at a faster rate than β-lactoglobulin gels.[6] In both protein systems, a marked conversion from intramolecular to intermolecular β-sheet is observed during structure formation.[6,7] These results, together with previous data[8] on the heat-induced gelation of ovalbumin in the presence of sodium caprate, suggest that fatty acid salts modify the protein structure, cause a reduction in the thermal stability of the protein, and induce aggregation at lower temperatures.

On the other hand, there are also some contrasting findings; for example, the presence of an excess of palmitic acid stabilized β-lactoglobulin against heat denaturation.[9,10] In these studies, rheological measurements during heating at 82 °C also demonstrated that, in the presence of palmitic acid, β-lactoglobulin forms gels with a lower storage modulus and longer gelation times than those without palmitic acid.[10] Furthermore, concerning bovine serum albumin (BSA) with sodium dodecyl sulfate (SDS), detailed analysis was performed.[11] These reports indicated that the thermal stabilities of both β-lactoglobulin and BSA were increased by the addition of a large amount of fatty acid derivatives to protein.

Kerstens et al. investigated the evolution of the microstructure during the thermal gelation of β-lactoglobulin with Tween 20, which is a kind of nonionic surfactant.[12] β-Lactoglobulin has a central hydrophobic calyx similar to retinol binding protein, in which small hydrophobic ligands can bind.[9] It seems that the behavior of ionic surfactant is different from that of nonionic surfactant.[12] Nonionic surfactants tend to interact by means of their alkyl chains with the hydrophobic domain of the protein, whereas, in the ionic surfactants, the charged headgroup may also be involved in the attractive interactions with oppositely charged groups on the protein. Therefore, in the case of sodium caprate in our study, there are both ionic and hydrophobic interactions between surfactants and proteins. We also have a previous study on the effects of fatty acid derivatives including SDS on the protein gel characteristics (appearance, water-holding property), the gel containing fatty acid salts formed a very transparent and high water-holding gel compared to those of Tween 20 or sucrose monolaurate.[4] These data suggest that not only hydrophobic interaction but also ionic interaction is required for giving a high transparency and hydrophilicity to the mixed gel.

The mechanism of gelation of β-lactoglobulin has been widely studied (see, for example, ref 13) and can be described as a

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three-step process, namely, unfolding of the protein, the formation of intermediate soluble aggregates, and finally, the formation of a three-dimensional network. Heat-induced changes in the secondary and tertiary structure of the proteins can be observed using CD spectroscopy; however, very dilute protein solutions need to be used for an accurate determination of the changes, and this is quite limiting, as processes such as gelation are concentration-dependent. Previous Fourier transform infrared (FT-IR) studies carried out with no dilution on gelling systems containing >10% β-lactoglobulin, demonstrated that the addition of sodium caprate induces changes in the secondary structure of β-lactoglobulin.[6] The dissociation of β-lactoglobulin from dimer to monomer is the first step in the process after the addition of sodium caprate to the protein solution. These results suggested that the addition of fatty acid salt modifies the structure of β-lactoglobulin, inducing a more flexible state (initial unfolding state) of the protein and causing protein−protein interactions, mainly characterized by an intermolecular β-sheet. High-resolution ultrasonic spectroscopy has recently been employed to elucidate structural changes and aggregation states of β-lactoglobulin during heating at gelling concentrations.[14] By using ultrasonic spectroscopy, it is possible to measure structural changes during the pre-gelation and gelation stages of protein systems in situ, with minimal sample disruption and no need for dilution. For this reason, ultrasonic spectroscopy is the ideal tool for the study of protein gelation induced by changes in environmental conditions. The objective of the current study was to examine the changes occurring in β-lactoglobulin A in the presence of sodium caprate under isothermal conditions (30 or 35 °C), using ultrasonic spectroscopy. Transmission electron microscopy (TEM) studies were also performed on mixtures of β-lactoglobulin A and sodium caprate to complement the ultrasonic spectroscopy data.

Materials and Methods

Purification of β-Lactoglobulin A. Purified β-lactoglobulin A was prepared from whey protein isolate (Alacen 895) donated by New Zealand Milk Protein (NZMP, Mississauga, Canada). The protein was purified using ion-exchange chromatography on Sepharose Q (Amersham Biosciences, Baie d'Urfe, Canada) with 20 mM Tris-HCl (pH 7.0) and a linear gradient of 0−0.35 M NaCl as previously reported.[15] Fractions containing β-lactoglobulin A were collected, dialyzed extensively against water, and lyophilized.

Ultrasonic Spectroscopy Measurements. Ultrasonic spectroscopy measurements were carried out using an HR-US102 instrument (Ultrasonic-Scientific, Dublin, Ireland), and the data were collected with the manufacturer's software (version 4-50-25-0). The equipment passes transverse sound waves through sample and reference cells and measures both the velocity and attenuation of the transmitted sound wave.[16] Solutions of β-lactoglobulin A (12%) were prepared in MilliQ water containing 0.2 M NaCl with either 2.4 or 3.6% sodium caprate (from a 30% stock in MilliQ water). The final pH of the mixture was 7.5. Both sample and reference solutions were degassed[17] and equilibrated to each testing temperature. Sample solutions (1 mL) were loaded, and the instrument was tuned to measure five frequencies corresponding to 3.1, 5.1, 7.8, 11.6, and 14.7 MHz for water at 25 °C. The velocity and attenuation of the sound waves at the selected frequencies were measured continuously in both the sample and reference cells. The internal temperature of the cell was controlled using a programmable Haake F8 water bath (Thermo-Haake, Georgetown, Canada), and the temperature of the spectrometer cell was recorded using a thermocouple installed in the instrument. Samples were subjected to isothermal measurements at 30 or 35 °C. In addition, some measurements were conducted with samples containing 0.025% NaN_3 (an antimicrobial reagent) to prevent spoilage during the long isothermal runs (48 h). At least two independent experiments were carried out for each concentration and ratio of protein/fatty acid salt. All runs were analyzed and plotted using routines written in Microsoft Excel. Gradient changes were calculated by separately summing five points before and five points after a given point, determining the difference, and dividing the result by the time gradient in the same data range.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis. A 1.5 mL sample of 12% β-lactoglobulin A containing 0.2 M NaCl and 3.6% sodium caprate (pH 7.5) was divided into 1 and 0.5 mL samples. The 1 mL solutions were immediately placed into the ultrasonic spectrometer, kept at constant temperature, and subjected to a heating/cooling program (30 °C constant for 8 h followed by linear temperature ramps from 30 to 85 °C at 0.46 °C/min, and kept for 30 min at 85 °C, and cooling to 30 °C at 0.46 °C/min). As a control, a sample of 12% β-lactoglobulin A containing 0.2 M NaCl (pH 7.5) was also prepared using ultrasonic spectrometer.

The 0.5 mL sample solution was incubated at room temperature and an aliquot was taken off every time period. An aliquot (50 μL) was removed just after sample mixing (0 h), and, at 3, 4, and 8 h of incubation, each aliquot was diluted in water (1:10) to stop the reaction and then refrigerated. If the sample had already gelled, then 10 mg of the sample was added to 90 μL of water. Samples were analyzed under nonreducing conditions by SDS-PAGE to determine differences in protein−protein interactions before and after heating. The 100 μL samples were added to 200 μL of 20% SDS and 100 μL of 0.01% bromophenol blue. Samples were heated at 95 °C with vigorous stirring for 5 min. SDS-PAGE samples (3 μL each) were loaded on a 20% Phast-gel (nongradient) (Amersham Pharmacia) and electrophoresed on a Phast-system (Amersham Pharmacia). The gels were automatically stained with Coomassie brilliant blue (Phast-gel blue-R, Amersham Pharmacia) and destained with methanol and acetic acid according to the manufacturer's instructions.

TEM Analysis. Negative staining observation was performed using TEM (H7100, Hitachi High Technologies, Tokyo, Japan). A solution containing 12% β-lactoglobulin A and 3.6% sodium caprate in 0.2 M NaCl (pH 7.5) was diluted 10,000 times at 0, 2.6, 4.5, and 7 h of incubation using a 0.2 M NaCl solution to stop the aggregation. The control 12% protein sample in 0.2 M NaCl (lacking sodium caprate) was also diluted with 0.2 M NaCl (10,000-fold dilution). Each diluted sample was stored at 4 °C until analysis. Aliquots (4 μL each) were placed on an electron microscopy nickel grid (200Hex) (Canemco, St. Laurent, Canada) precoated with Formvar, and the β-lactoglobulin A was precipitated onto the grid by soaking for 30 s at room temperature. Excess sample was wicked off with filter paper and then immediately stained for 1 min with one drop of saturated uranyl acetate in 50% ethanol. After removing the excess staining material, the microstructure of each sample was observed in the TEM at an acceleration voltage of 75 kV and a magnification of 50 and 100 K. Digital images were captured using AnalySIS software (AnalySIS Pro, version 3.0, Soft Imaging System Corp., USA).

Results

Changes in the Ultrasonic Properties of β-Lactoglobulin A with or without Sodium Caprate at Constant Temperature. Figure 1 shows changes in relative ultrasonic velocity over time at 30 °C for samples of β-lactoglobulin A in 0.2 M NaCl with (Figure 1A) or without (Figure 1B) sodium caprate. The difference in the velocity of sound between the protein sample and the respective salt solutions increased at a constant rate over 15 h of measurement, with total velocity change in less than 1.5 m/s. These results are in contrast to the much larger velocity changes reported in the case of β-lactoglobulin heat-induced gels[14] and signify that very little change occurred to the protein hydration shell and structure over time. However, a small decrease in the overall compressibility of the protein solution

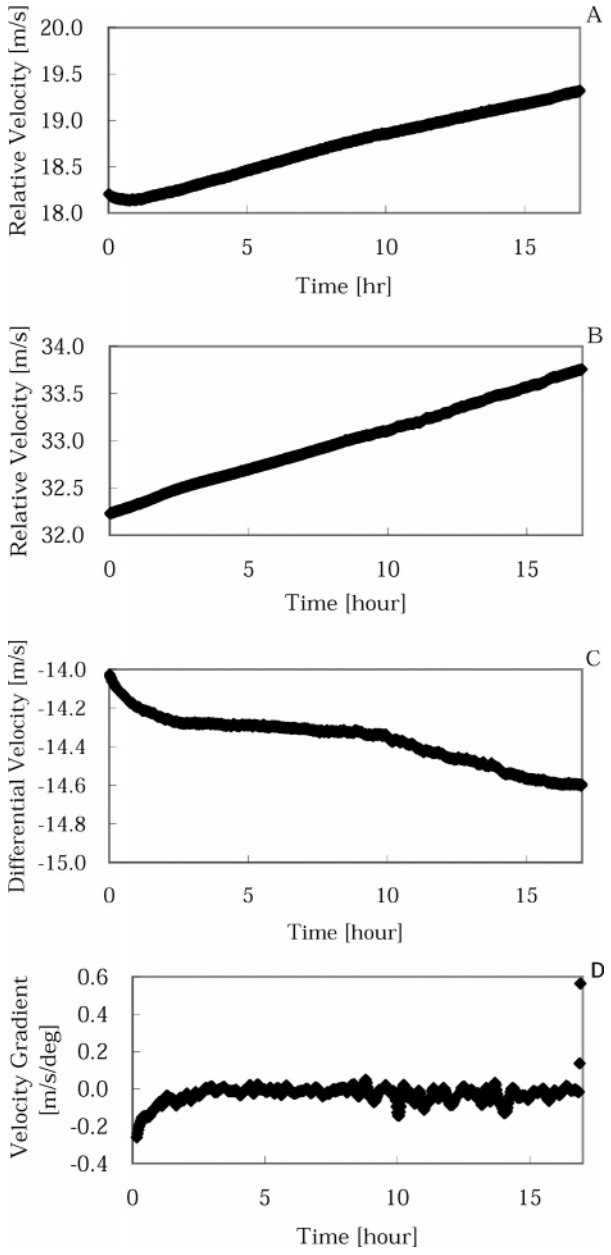

Figure 1. Time dependence of the relative ultrasonic velocity for β-lactoglobulin A (12%) containing 0.2 M NaCl and 3.6% sodium caprate (A) or the protein with 0.2 M NaCl with no sodium caprate (B) measured at 30 °C. The relative ultrasonic velocity is the difference between the velocity measured in the sample cell and that measured in the reference cell (containing the salt solution with no protein). The difference in the relative ultrasonic velocity between panels A and B is shown in panel C. Panel D is the gradient of velocity as a function of time for C. Four independent experiments were carried out, and a typical result is presented.

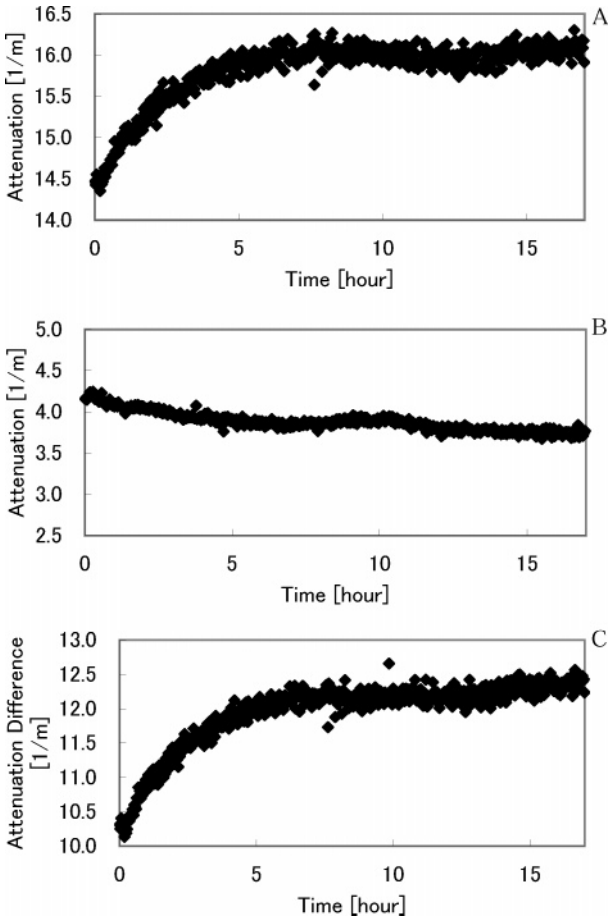

Figure 2. Time dependence of the ultrasonic attenuation for β-lactoglobulin A (12%) containing 0.2 M NaCl and 3.6% sodium caprate (A) or the protein with 0.2 M NaCl with no sodium caprate (B) measured at 30 °C and 5.3 MHz. The difference in values of attenuation between panels A and B is shown in panel C. Four independent experiments were carried out, and a typical result is presented.

was measured. The major changes in ultrasonic velocity shown in Figures 1A were not caused by gelation, but appeared to be gradual over the 15 h of the experiment. An increase in velocity was also observed by incubating protein with NaCl in the absence of fatty acid salt, and indicates an increase in protein hydration (Figure 1B). The effect of sodium caprate on the ultrasonic velocity of a β-lactoglobulin A solution was determined by subtracting the ultrasonic relative velocity of the protein solution without fatty acid salts from the β-lactoglobulin A/sodium caprate solution (Figure 1C). The net difference in velocity at 0.6 m/s indicated that, in the presence of sodium caprate, β-lactoglobulin A was more compressible. The increase compressibility measured in the fatty acid salt-containing solutions may be due to a higher local concentration of nonpolar groups, which contribute to the formation of large void areas. When looking at the gradient of the velocity over time, it is confirmed that most of the structural changes occurred within the first 4 h of incubation by analysis, as mentioned in the Materials and Methods section (Figure 1D).

Figure 2 shows the changes in the attenuation of the sound wave propagating through the β-lactoglobulin A sample containing sodium caprate and NaCl (Figure 2A) and only NaCl (Figure 2B) measured at 5.3 MHz. The flat spectrum indicates that the attenuation of sound of β-lactoglobulin A was not affected by the presence of NaCl (Figure 2B). Higher values of attenuation (>10 m^{-1}) were observed from the beginning for protein samples containing sodium caprate and NaCl (approximately 14.5 m^{-1}, Figure 2A) compared to those obtained for a β-lactoglobulin A solution in 0.2 M NaCl (approximately 4 m^{-1}, Figure 2B). The details of the difference in attenuation between the protein solution containing sodium caprate and NaCl and that containing only NaCl as a function of time are shown in Figure 2C. Protein solutions containing sodium caprate showed an increase in attenuation of sound, over time, showing a plateau after about 7 h of incubation (Figure 2A,C). The same behavior was apparent for all the other frequencies measured (data not shown). Samples of β-lactoglobulin A and sodium caprate under these conditions formed very transparent gels,

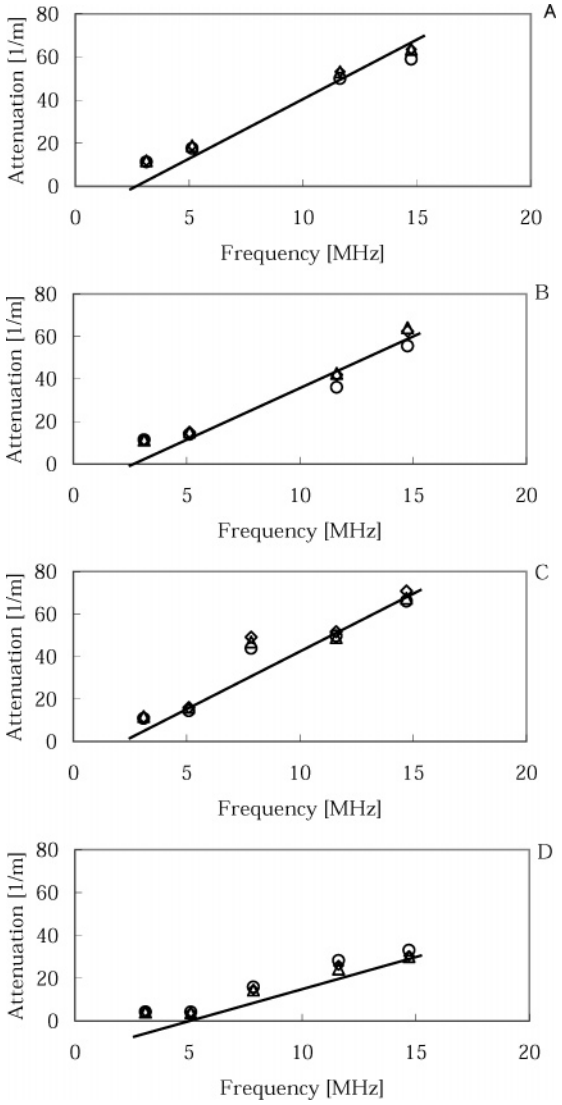

Figure 3. Frequency dependence of ultrasonic attenuation as a function of incubation time for β-lactoglobulin A (12%) solutions in 0.2 M NaCl under various conditions: (A) protein solution containing 2.4% sodium caprate at 35 °C. (B) 3.6% sodium caprate at 35 °C. (C) 3.6% sodium caprate at 30 °C. (D) no sodium caprate at 30 °C. (○) 0 h; (◇) 10 h; (△) 17 h. At least two independent experiments were carried out for each concentration and temperature, and a typical result is presented.

and the results indicated that most changes occurred within the first 5−7 h of incubation.

Effects of Sodium Caprate Concentration. Earlier research investigated the effect of sodium caprate concentration on the rheological properties of a β-lactoglobulin AB type (AB) at 25 °C.[6] It was demonstrated that the gelation of the β-lactoglobulin AB containing 3.6% sodium caprate occurred at a faster rate compared with the solution containing 2.4% sodium caprate. For this reason, the effect of the addition of sodium caprate at two different concentrations on the ultrasonic properties of β-lactoglobulin solutions was tested. Figure 3 illustrates the frequency dependence of the ultrasonic attenuation while incubating β-lactoglobulin A at 35 and 30 °C over time under various conditions to determine the particle size distribution of the colloidal dispersion.[17] As shown in Figure 3A,B, no differences were noted as a function of time of incubation between 2.4 and 3.6% sodium caprate. The results also indicate that the presence of sodium caprate caused a change in acoustic energy, as the values of attenuation were higher in samples with

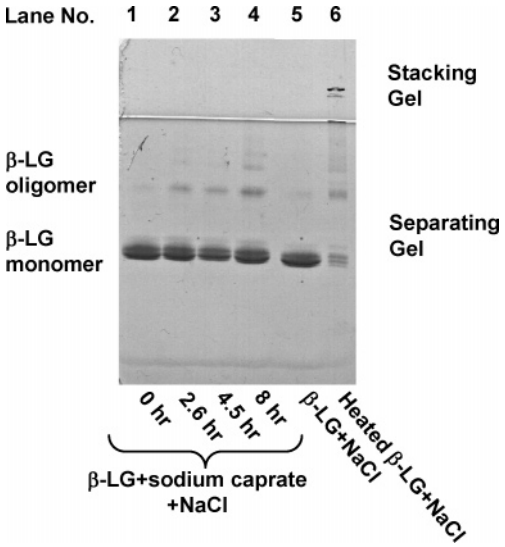

Figure 4. SDS-PAGE analysis under nonreducing conditions (no β-mercaptoethanol) of soluble aggregates and solubilized materials during the formation of sodium caprate-induced gels during incubation at 30 °C of 12% β-lactoglobulin A in 0.2 M NaCl. Lanes 1−4: solutions containing 3.6% sodium caprate right after mixing (lane 1), after 2.6 h of incubation (lane 2), after 4.5 h of incubation (lane 3), and after 8 h of incubation (lane 4). Lanes 5 and 6: protein solution with no sodium caprate, before (lane 5) and after heating (lane 6). β-LG = β-lactoglobulin A.

3.6% sodium caprate and NaCl (Figure 3C) than in control samples containing only NaCl (Figure 3D). The increase in attenuation may be caused by the oscillation of the proteins interconnected within a network.[18] However, these changes in attenuation are specific to the system structured with the fatty acid salts and are almost completed within 5−7 h as no changes were evident after 10 or 17 h of incubation. The lack of change in the attenuation spectra after incubation indicates that the formation of the β-lactoglobulin A network in the presence of 3.6% fatty acid salt does not show the appearance of particles after 10 or 17 h of incubation.[19] It is important to note that, in the present experiments, the range of frequency tested was much smaller than that reported previously [17−19], because of the limitations in the capability of our instrument. However, even at low frequencies (5 and 7 MHz), the changes in the present data are much smaller than those that showed a large increase in attenuation for heated β-lactoglobulin AB or whey protein solutions.[14] In other words, these much smaller and early changes in attenuation are specific for sodium caprate-induced gels.

Electrophoretic Analysis of the Sodium Caprate-Induced Aggregates. SDS-PAGE was carried out under nonreducing conditions to identify changes in the type of aggregates formed in β-lactoglobulin A gels with or without sodium caprate (Figure 4). In addition, heated samples were also tested to determine differences in the type of aggregates formed. Samples at 0 and 2.6 h were still liquid, and the solutions became viscous after 4.5 h of incubation. Aliquots were removed at various times of incubation and quenched by extensive dilution with water. The sample was then stored in the refrigerator. The samples after 8 h of incubation were stored at 5 °C without dilution, because it was already in a soft transparent gel state. As a control, β-lactoglobulin A in 0.2 M NaCl without sodium caprate was subjected to a heating and cooling program in the ultrasound spectrometer as mentioned in the Materials and Methods section and analyzed by SDS−PAGE. This sample (β-lactoglobulin A without sodium caprate) formed a white turbid gel.

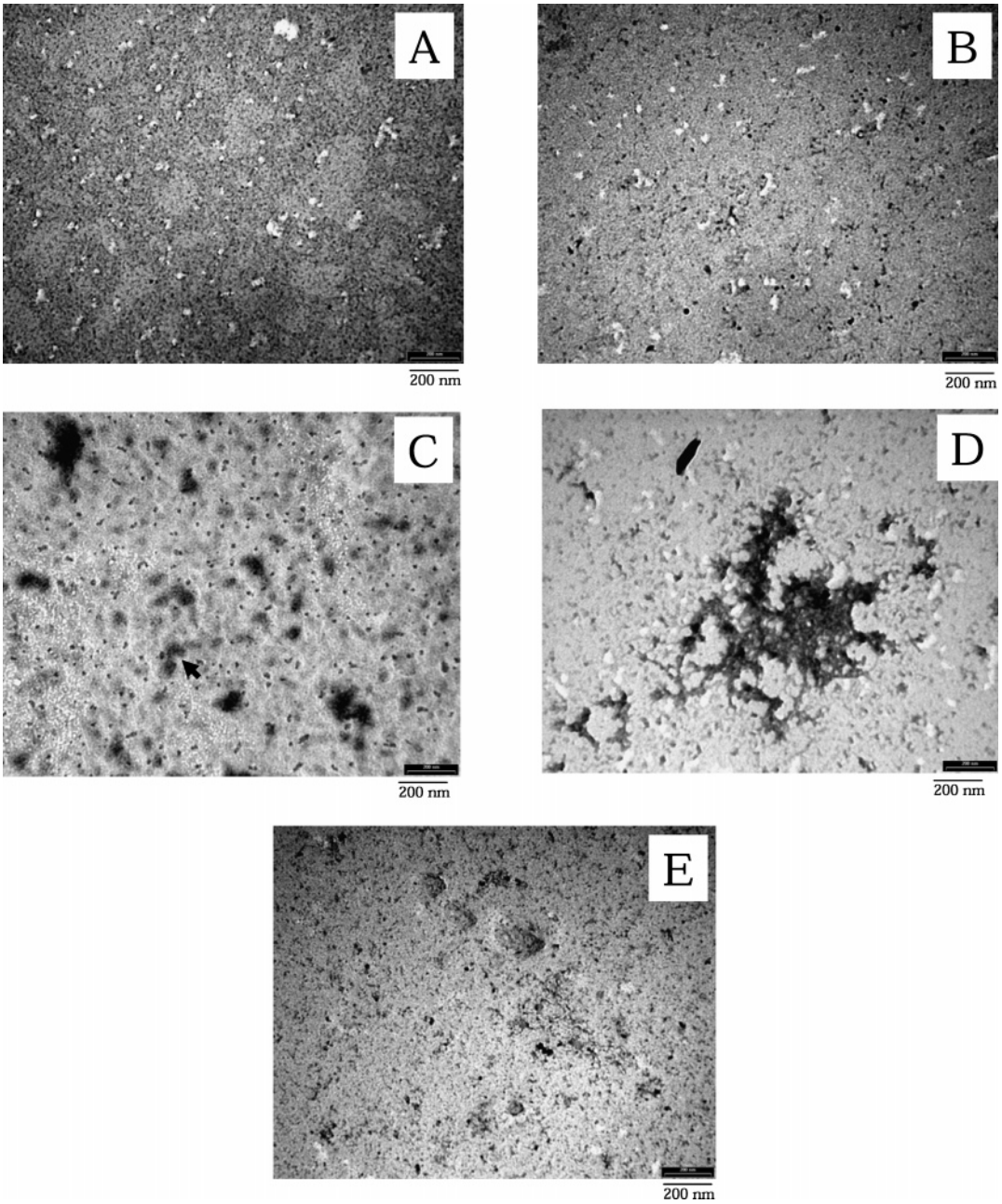

Figure 5. Negative staining micrographs of soluble aggregates prepared from 12% β-lactoglobulin A solutions in 0.2 M NaCl at various stages of incubation at 30 °C in the presence of 3.6% sodium caprate: (A) 0 h, (B) 2.6 h, (C) 3 h, and (D) 4.5 h of incubation. (E) β-lactoglobulin A (control solution without sodium caprate). The black arrow in panel C indicates the area magnified in Figure 6A.

Only one β-lactoglobulin A band was visible for samples containing β-lactoglobulin A and salts before heat treatment (Figure 4, lanes 1 and 5). SDS-PAGE analysis under nonreducing conditions indicated that, although aggregation occurred after a few hours of incubation at 30 °C, very little covalent interactions occurred (Figure 4, lanes 2, 3, and 4). Some oligomers were present, but it was concluded that most proteins aggregated by noncovalent bonds. This result was in contrast to that of samples subjected to heating and cooling. The β-lactoglobulin A solution containing NaCl and sodium caprate sample showed no protein migration into the gel, indicating that disulfide interactions occurred during heating, forming large aggregates (data not shown). Similarly, very little protein migrated into the gel when β-lactoglobulin A solution was heated in the presence of NaCl without fatty acid salts (Figure 4, lane 6).

Relationship between the Ultrasonic Properties and the Microstructure during Gel Formation. Ultrasonic measurements indicated that during incubation of the protein with sodium caprate, the values of attenuation increased over time reaching a plateau (Figure 2A). The refraction points in the ultrasonic attenuation curves yield important information regarding the formation of gel networks. The microstructure changes prior to the completion of gelation were investigated during the early stages at room temperature up to 4.5 h after β-lactoglobulin A and sodium caprate were mixed. Figure 5 shows the microstructure of the protein solutions incubated at various times obtained using TEM with negative staining. The images were also compared to those obtained from a control sample containing β-lactoglobulin A in 0.2 M NaCl. The sample containing 12% β-lactoglobulin A and 3.6% sodium caprate obtained right after mixing (Figure 5A) showed some excess

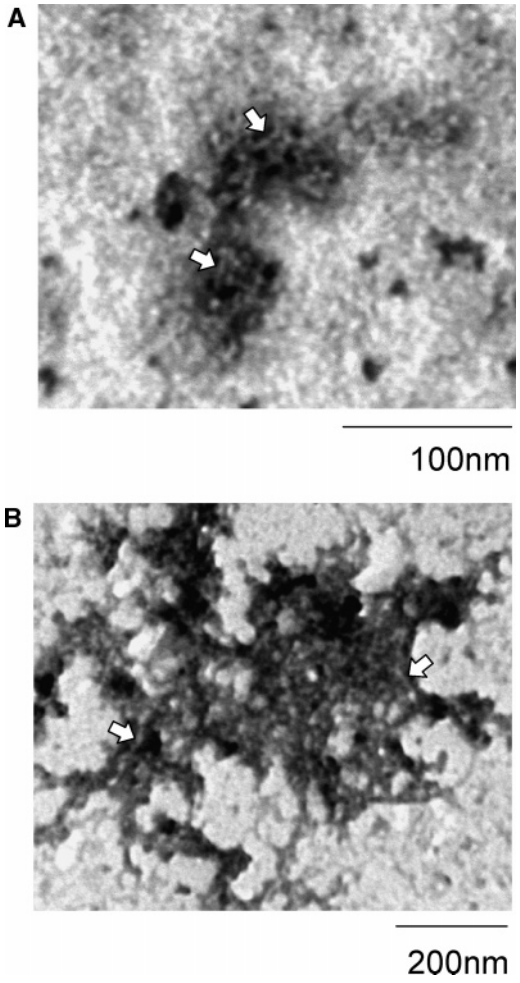

Figure 6. Magnified images for 3 or 4.5 h incubated β-lactoglobulin A with sodium caprate. Detailed conditions are the same as those described in Figure 5C,D. The arrows indicate a white continuous structure (filamentous network).

staining material (black particles) with no evidence of a network, similar to the TEM images for the protein without sodium caprate (Figure 5E). The white stained particles shown in the TEM images are sodium caprate or sodium chloride crystals, as determined by control experiments (data not shown). After 2.6, 3, and 4.5 h of incubation at ambient temperature, the reaction was stopped by extensive dilution with water as previously described. While after 2.6 h of incubation, the samples looked similar to those at time 0, which were observed right after mixing (Figure 5B), after 3 h, the black area contained very small white particles in the micrograph (Figure 5C). The images obtained after 4.5 h of incubation implied that some network was developed (Figure 5D). To clarify these in Figure 5C,D, Figure 6 shows the magnified networks from the images after 3 h (Figure 6A) and 4.5 h (Figure 6B), respectively. The fibrils width ranged from about 30 to 100 Å in Figure 6A,B. It has been previously reported that fatty acid salt-induced ovalbumin gels have a very fine and homogeneous structure composed of filaments of about 80 Å wide, as determined by field emission scanning electron microscope and ultrathin section observations by TEM.[5] The present results suggest that the fibrous structure was observed in the early stages of incubation up to about 4.5 h, and the network is almost comparable to transparent gel from ovalbumin in the fibrous widths.

Kavanagh et al.[20] investigated the effects of NaCl on the microstructure of soluble β-lactoglobulin aggregates at acidic pH upon heating and cooling treatment. In contrast to our study using a fatty acid salt, their results showed that NaCl has an inhibitory effect on the formation of long linear aggregates. These observations suggest that fatty acid salt-induced gels of β-lactoglobulin A formed at ambient temperature follow a unique aggregation mechanism, distinct from that in the presence of NaCl.

Discussion

The ultrasonic velocity is the distance the ultrasonic wave moves through the sample per unit time.[17] As ultrasonic velocity is related to the chemical composition of the solution and the compressibility of the system, the decrease in the relative velocity observed during the incubation of the β-lactoglobulin solution with sodium caprate may be due to an increase in the number of participating molecules in the conformational changes as well as an increase in hydrophobic interactions. Gekko and Noguchi[21] reported that an increase in compressibility occurs during protein denaturation because of the high local concentration of nonpolar groups. Our results indicate that the velocity changes seen during the formation of β-lactoglobulin A gel with fatty acid salts are much smaller than those observed during heat-induced gel formation of β-lactoglobulin without fatty acid salts;[14] the relative ultrasonic velocity changes with temperature during heating from 20 to 70 °C were about 10 m/s for β-lactoglobulin AB without fatty acid salts,[14] about 7 times larger when compared with the 1.5 m/s change observed in our study during isothermal denaturation of the protein in the presence of fatty acid salts (Figure 1A). It is possible to conclude that the gel formed by fatty acid salts is more hydrophilic than the gel induced by β-lactoglobulin A without fatty acid salt, and thus no drastic changes occurred in ultrasound velocity for β-lactoglobulin A in the presence of fatty acid salts during gelation because of the high level of hydration.

Ultrasonic attenuation is determined by measuring the reduction in amplitude of the ultrasonic wave propagating through a material.[17−18] Attenuation is determined by absorption and scattering of the ultrasonic wave such as the energy of dissipation caused by solute−solute interactions or particle diffusion. Buckin and Smith[16] stated that the scattering contribution occurred when the liquid is nonhomogeneous and contains particles with a size comparable to the ultrasonic wavelength. In these samples, most of the acoustic energy losses were caused by viscous losses, thermal losses, scattering, intrinsic absorption, and structure-dependent losses.[17−18,22]

This study suggests that ultrasonic attenuation is strongly affected by the type of network, including the fineness, roughness, regularity, or irregularity of the gel network. It has been previously reported[5] that the microstructure of protein gels induced by fatty acid salts has a more homogeneous network composed of filamentous structures with larger pores filled with water. The gel formed with a mixture of β-lactoglobulin A and sodium caprate is very transparent, similar in appearance to the sodium caprate-induced ovalbumin gel (data not shown). It appears that these filamentous structures are closely related to the structural rearrangement of the fluid system, and the structures do not induce large changes in the ultrasonic attenuation and velocity, especially when compared to the case of a particle gel.[14,19]

With regards to the frequency dependence of attenuation, it has been also reported that the evaluation of frequency spectra is a means for determining the concentration and size distribution of particles suspended in a fluid.[17] Generally, for each sample, attenuation values increase with an increase in frequency, and

this increase is most evident at frequencies greater than 10 MHz.[18] In the present study, the gradient of attenuation with frequency for β-lactoglobulin A with fatty acid salt was about 2 times that in β-lactoglobulin A alone at 30 °C (Figure 3C,D). It appears that larger gradients result from the appearance of soluble aggregates in the fluid containing β-lactoglobulin A and sodium caprate. The gradient of β-lactoglobulin A after heating without fatty acid salts showed attenuation spectra similar to those of the protein solutions with sodium caprate (data not shown). These results indicate that incubation of the β-lactoglobulin A/fatty acid salt mixture at ambient temperature (about 30 °C) caused the formation of gel networks with frequency spectra comparable to that of β-lactoglobulin A heat-induced gels.

In addition to the structural changes due to the presence or absence of fatty acid salts, we investigated the effect of the concentration on gel formation. There was a slight difference between 2.4 and 3.6% in each frequency sweep of attenuation of the sound (Figure 3A,B), in spite of the marked difference in texture of these gels.[6] In these studies, ultrasound spectroscopy was not able to show the differences between samples with different gel strength (varying salt concentrations), confirming previous reported data on heat-induced gelation.[14]

Microstructure observation carried out by TEM with a negative staining method without chemical fixation increased the understanding of the step preceding gelation, at the point when ultrasonic attenuation reaches a plateau. In addition, the time when the values of attenuation show a peak corresponded to the point when linear soluble aggregates were observed by TEM.

There are closely related reports on the interaction between protein and surfactant in aqueous solution at ambient temperature[3,23] or the gelation step with heat treatment.[11−12] It has been reported that the formation of some complex between sodium caprate and the proteins in an aqueous medium was induced by mainly hydrophobic interactions;[3] the complex induced a drastic decrease in the protein stability. Furthermore, Semenova et al.[23] also investigated the interaction between surfactant and protein using different types (e.g., ionic or nonionic) of surfactant and different concentrations (below and above the critical micelle concentration (cmc)), and found that, although there are slight differences in the radii of gyration, discriminative enthalpy for heat endothermic flow was observed upon a heating temperature sweep depending on the kind of anionic surfactant. From the data, which was a sharp increase in endothermic heat effect on the interaction with protein, they indicated that a commercial anionic surfactant, CITREM, which is a mixture of stearic and palmitic acid with citric acid, led to the formation of a large protein aggregation with much higher hydrophilicity and more compact structure and spherical architecture compared with protein alone above the cmc. They furthermore suggested that CITREM has a similar effect with SDS on the interaction with protein along with other previous reports.[24] In our study, the concentration of sodium caprate was higher than the cmc. We previously studied the effects of fatty acid derivatives on rice globulin, and SDS showed an effect on the gel similar to that seen with sodium caprate.[4] Furthermore, Roversi and Mesa investigated the thermodynamics of BSA with SDS.[11] Their differential scanning calorimetry analysis indicated the occurrence of two thermal transitions (two endothermic peaks appeared) in the system with SDS, one at a temperature close to the gelation threshold, and another one at a much higher temperature, which seems to be related to the thermal denaturation of the protein. It suggests that the BSA−SDS complex has two step changes prior to heat-induced gelation. The first transition at lower temperature may be a particularly important step to induce mild structural changes. Their system is close to our system since BSA has a lipophilic compound binding site as does β-lactoglobulin, and SDS is an ionic surfactant with similar structure except for the sulfate moiety. The result for the BSA−SDS complex matches with our study where β-lactoglobulin interacted with fatty acid salt even at around 30 °C and formed a soft gel.

From reports mentioned above, our data (very small change in velocity) using ultrasound spectroscopy in a mixture composed of β-lactoglobulin A and sodium caprate suggests that sodium caprate induces mild structural changes in proteins such as CITREM or SDS, which has also been supported by CD[5] and FT-IR[6] analyses.

It is possible to conclude that ultrasonic spectroscopy is a very useful method for investigating the changes of macromolecular structures in a fluid system in situ and real time, even with small changes of velocity and attenuation in the system.

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References and Notes

(1) Yuno-Ohta, N.; Maeda, H; Okada, M.; Hasegawa, K. *J. Food Sci.* **1992**, *57*, 86−90.
(2) Yuno-Ohta, N. *Nippon Shokuhin Kagaku Kokagu Kaishi* **2004**, *51*, 281−287.
(3) Antipova, A. S.; Semenova M. G.; Belyakova, L. E. *Colloids Surf., B* **2001**, *21*, 217−230.
(4) Yuno-Ohta, N.; Ohta, H.; Maeda, H.; Okada, M.; Hasegawa, K. In *Food Hydrocolloids: Structures, Properties, and Functions*; Nishinari, K., Doi, E., Eds.; Plenum Press: New York, 1994; pp 371−374.
(5) Yuno-Ohta, N.; Higasa, T.; Tatsumi, E.; Sakurai, H.; Asano, R.; Hirose, M. *J. Agric. Food Chem.* **1998**, *46*, 4518−4523.
(6) Yuno-Ohta, N.; Murata, K.; Miyahara M.; Ohta, H. *J. Am. Oil Chem. Soc*. **2004**, *81*, 225−230.
(7) Yuno-Ohta, N. *Food Hydrocolloids* **2006**, *20*, 357−360.
(8) Yuno-Ohta, N.; Toryu, H.; Higasa, T.; Maeda, H.; Okada, M.; Ohta, H. *J. Food Sci.* **1996**, *61*, 906−910, 920.
(9) Puyol, P.; Perez, M.D.; Peiro, J.M.; Calvo, M. *J. Dairy Sci.* **1994**, *77*, 1494−1502.
(10) Puyol, P.; Perez, M.D.; Burgos, J.; Calvo, M. *Int. Dairy J.* **1998**, *8*, 119−123.
(11) Roversi, M.; La Mesa, C. *J. Colloid Interface Sci.* **2005**, *284*, 470−476.
(12) Kerstens, S.; Murray, B. S.; Dickinson, E. *Food Hydrocolloids* **2005**, *19*, 625−633.
(13) Roefs, S. P.; De Kruif, K. G. *Eur. J. Biochem*. **1994**, *226*, 883−889.
(14) Corredig, M.; Verespej, E.; Dalgleish, D. G. *J. Agric. Food Chem.* **2004**, *52*, 4465−4471.
(15) Andrews, A. T.; Taylor, M. D.; Owen, A. J. *J. Chromatogr.* **1985**, *348*, 177−185.
(16) Buckin, V.; Smith, C. *Semin. Food Anal.* **1999**, *4*, 113−130.
(17) McClements, D. J. In *Encyclopedia of Analytical Chemistry*; Meyers, R. A., Ed.; John Wiley & Sons, Ltd: Chichester, England, 2000; pp 1−8.
(18) Dukhin, A. S.; Goetz, P. J.; Wines, T. H.; Somasundaran, P. *Colloids Surf., A* **2000**, *173*, 127−158.
(19) Bryant, C. M.; McClements, D. J. *Food Hydrocolloids* **1999**, *13*, 439−444.
(20) Kavanagh, G. M.; Clark, A. H.; Ross-Murphy, S. B. *Int. J. Biol. Macromol.* **2000**, *28*, 41−50.
(21) Gekko, K.; Noguchi, H. *J. Phys. Chem.* **1979**, *83*, 2706−2713.
(22) Riebel, U.; Loffler, F. *Part. Part. Syst. Charact.* **1989**, *6*, 135−143.
(23) Semenova, M. G.; Belyakova, L. E.; Polikarpov, Y. N.; II'in, M. M.; Istarova, T. A.; Anokhina, M. S.; Tsapkina, E. N. *Biomacromolecules* **2006**, *7*, 101−113.
(24) Nakai, S.; Ho, L.; Tung, M. A. *Can. Inst. Food Sci. Technol.* **1980**, 13, 14−22.

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