Sulfhydryl−Disulfide Interchange-Induced Interparticle Protein Polymerization in Whey Protein-Stabilized Emulsions and Its Relation to Emulsion Stability

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Polymerization of proteins via sulfhydryl−disulfide interchange occurs at the oil−water interface. However, it is not known if this polymerization reaction takes place only within the protein film of an emulsion particle or also occurs between the protein films of emulsion particles. To elucidate this, emulsions made with pure β-lactoglobulin and pure α-lactalbumin were mixed at 1:1 ratio and the time-dependent intermolecular sulfhydryl−disulfide interchange between the protein films of β-lactoglobulin-stabilized and α-lactalbumin-stabilized emulsion droplets were studied. In pure protein emulsions, polymerization of β-lactoglobulin occurred whereas it did not occur in α-lactalbumin. However, disulfide cross-linked β-lactoglobulin−α-lactalbumin polymers formed in the cream phase but not in the serum phase of the emulsion-mix emulsions of pure β-lactoglobulin and pure α-lactalbumin emulsions. The extent of polymerization increased with storage time, indicating the occurrence of interparticle polymerization in this emulsion system. The interparticle polymerization promoted aggregation of emulsion particles and decreased the kinetic stability of the emulsion.

Keywords: *Emulsion; protein polymerization; β-lactoglobulin; α-lactalbumin; sulfhydryl−disulfide interchange*

INTRODUCTION

Proteins play a vital role as macromolecular surfactants in emulsion and foam-type food products. It is well recognized that the stability of protein-stabilized emulsions critically depends on the protein's ability to readily undergo interfacial denaturation and, more importantly, its ability to form a viscoelastic film at the oil−water interface. Formation of a viscoelastic film depends on both noncovalent interactions, hydrogen bonding, hydrophobic and electrostatic interactions, and covalent interactions, viz., sulfhydryl−disulfide interchange reaction, between the adsorbed protein molecules. It is known that heat-induced polymerization of β-lactoglobulin occurs predominantly via sulfhydryl−disulfide interchange reaction (Zhu and Damodaran, 1994; Monahan et al., 1995; Shimada and Cheftel, 1988). Several pieces of evidence indicate that sulfhydryl−disulfide interchange reaction also occurs at the air−water and oil−water interfaces of protein-stabilized foams and emulsions (Kitabatake and Doi, 1987; Dickinson and Matsumura, 1991). Dickinson and Matsumura (1991) have shown that the sulfhydryl−disulfide interchange reaction occurs in β-lactoglobulin- and a mixture of β-lactoglobulin plus α-lactalbumin-stabilized emulsions but not in pure α-lactalbumin-stabilized emulsion. Similar observations also have been made by Monahan et al. (1995). This interchange reaction has been implicated as being responsible for the time-dependent increase in surface viscosity of β-lactoglobulin at the oil−water interface (Dickinson et al., 1990). The initiation and time-dependent propagation of the sulfhydryl−disulfide interchange reaction at the oil−water interface is attributed to interfacial denaturation of β-lactoglobulin, which exposes and activates the buried free sulfhydryl group.

The above studies were mainly concerned with elucidating the formation of sulfhydryl−disulfide interchange reaction-induced polymerization within the protein film (i.e., intrafilm) of emulsion particles. What is not known is that whether such sulfhydryl−disulfide interchange reaction-induced cross-linking and polymerization also occurs between the protein films (i.e., interfilm) of emulsion particles. Such cross-linking between protein films of emulsion particles may lead to flocculation during storage. Since flocculation is the first critical step in coalescence and eventual phase separation of emulsions, the occurrence of sulfhydryl−disulfide interchange reaction-induced interparticle polymerization may adversely impact the kinetic stability of emulsions.

The purpose of the present study is to obtain direct evidence for the involvement of a sulfhydryl−disulfide interchange reaction between the protein films of emulsion particles. To achieve this, oil−water emulsions were made with purified β-lactoglobulin and α-lactalbumin; these emulsions were mixed in a 1:1 (v/v) ratio, and the formation of high molecular weight polymers through sulfhydryl−disulfide interchange reactions between the adsorbed films of β-lactoglobulin and α-lactalbumin were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) under nonreducing conditions. The impact of interparticle polymerization on the kinetic stability of these emulsions has also been studied.

MATERIALS AND METHODS

Materials. β-Lactoglobulin, α-lactalbumin, and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade. Commercial corn oil was purchased from a local store and used as such.

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Emulsion Preparation. Oil−water emulsions were prepared by using a Branson-450 sonifier equipped with a microtip horn. Identical energy input conditions were used for making emulsions as the geometry of the sample container, the solution volume, the position of the microtip horn, and the time of emulsification were invariant for all prepared emulsions. During emulsification, the temperature of the emulsion was kept constant at 25 °C by circulating water from a thermostated water bath. The volume fraction of the oil phase (corn oil) in all emulsions was fixed at 20% (v/v). The emulsions were prepared in two different ways: In one case, equal volumes of β-lactoglobulin and α-lactalbumin solutions (in 20 mM phosphate-buffered saline solution, $I = 0.1$, pH 7.0) of 1% concentration were mixed first and then emulsified with the oil. This emulsion was labeled as the "protein-mix" emulsion. In the other case, emulsions of each protein were made separately and then equal volumes of the emulsions were mixed. This emulsion was labeled as "emulsion-mix" emulsion. Sodium azide (0.02%) was included in the aqueous phase of all emulsions to inhibit microbial growth during storage.

Emulsion Stability. Aliquots (5 mL) of each of the protein-mix and emulsion-mix emulsions taken in capped glass vials were kept in constant gentle agitation in a Specimixer placed inside an incubator maintained at 25 °C. The Specimixer speed was set at about 10 cycles/min. Aliquots (0.5 mL) were withdrawn at different time intervals over a period of 12 days. These aliquots were accurately diluted with 0.1% SDS to give an absorbance value of less than 0.7 at 350 nm. The turbidity spectra were recorded in the visible range from 350 to 800 nm in a Beckman DU-60 spectrophotometer. The emulsion stability was analyzed as follows: Coalescence of emulsions generally follows a first-order kinetics (Reddy and Fogler, 1981)

$$N_t/N_0 = \exp(-k_r t) \quad (1)$$

where N_0 and N_t are the initial number concentrations of emulsion droplets at zero time and at time t, respectively, and k_r is the first-order rate constant. Reddy and Fogler (1981) showed that, for a polydispersed emulsion system in which the turbidity is strongly dependent on the wavelength, the change in the ratio N_t/N_0 can be determined from

$$N_t/N_0 = \frac{1}{r_0^3}\left[\frac{\lambda^{m_t - m_0} r_0^{m_0 - 1}}{T_0/T_t}\right]^{3/(m_t - 1)} \quad (2)$$

where T_0 and T_t are turbidities at $t = 0$ and $t = t$, respectively; m_0 and m_t are the slopes of a plot of ln T versus ln λ of an emulsion at zero time and at time t, respectively; and r_0 is the initial average droplet radius. An approximate value of r_0 can be obtained from (Pearce and Kinsella, 1978)

$$r_0 = 3\Phi_0/2T_0 \quad (3)$$

where Φ_0 is the initial volume fraction of the dispersed phase (oil). The changes in the number concentration of emulsion droplets in the protein-mix and emulsion-mix emulsions as a function of time were determined using eq 2.

Analysis of Adsorbed and Unadsorbed Proteins. The relative amounts of β-lactoglobulin and α-lactalbumin adsorbed to emulsion droplets, and detection of inter- and intradroplet polymerization of these proteins in the protein-mix and emulsion-mix emulsions, were determined by SDS−PAGE as follows: Following storage for specific time periods, to each emulsion sample was added an aliquot of a 0.2 M stock solution of NEM (final NEM concentration was 20 mM) to block the free sulfhydryl groups and thus stop further sulfhydryl−disulfide interchange reaction. Each emulsion sample was then centrifuged at 12000g for 30 min at room temperature. The bottom aqueous phase was removed, and the cream phase was redispersed in 20 mM phosphate buffer containing 20 mM NEM and centrifuged again. The procedure was repeated three times to remove unadsorbed proteins from the emulsion droplets. The washings were pooled and made up to a known volume; the proteins were analyzed by SDS−PAGE in the presence and absence of 0.3 M β-mercaptoethanol. The proteins adsorbed on emulsion droplet surface in the washed emulsion cream were extracted by adding an equal amount of double-strength SDS−PAGE sample buffer (4% SDS, 20% glycerol in 0.125 M Tris-HCl buffer, pH 6.8) containing 20 mM NEM and stirring at 25 °C for 24 h. The resulting solution was then centrifuged at 12000g for 15 min. The adsorbed protein fraction was then analyzed by SDS−PAGE in the presence and absence of 0.3 M β-mercaptoethanol. The relative intensities of the stained protein bands were quantified using a computerized Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) system, and the pixel densities of digitized protein bands were analyzed using Image Quant software (Molecular Dynamics, Sunnyvale, CA), version 3.1.

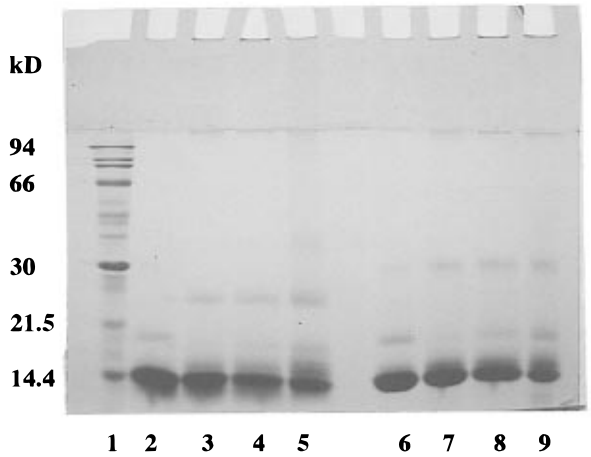

Figure 1. SDS−PAGE of the cream phase of α-lactalbumin-stabilized emulsions. Lane 1 is molecular weight markers. Lanes 2−5 were not treated with β-mercaptoethanol, and lanes 6−9 were β-mercaptoethanol treated. Lanes 2 and 6, 3 and 7, 4 and 8, and 5 and 9 correspond to storage times of 24, 48, 120, and 288 h, respectively.

Electrophoresis. SDS−PAGE was performed as described by Laemmli (1970) using 10−20% linear gradient slab gels. The molecular weight marker proteins were from Pharmacia (Piscataway, NJ). The gels were stained with Coomassie Brilliant Blue (R-250).

Light Microscopy. Aggregation of emulsion particles as a function of storage time was determined by light microscopy using a Lazer Zee meter (Model 501, Pen Kem, Inc., Bedford Hills, NY) equipped with a sample holder for zeta-potential measurement and a video camera. The diluted emulsion sample was placed directly in the sample holder, and the image of the emulsion particles was photographed. According to the manufacturer, the overall magnification of the video image on the monitor was about 2440-fold.

RESULTS AND DISCUSSION

α-Lactalbumin Emulsion. The SDS−PAGE pattern of adsorbed α-lactalbumin in the cream phase (adsorbed) of the α-lactalbumin-stabilized emulsion is shown in Figure 1. Under nonreducing conditions, no high molecular weight protein polymers formed during the 12-day storage period, indicating that no sulfhydryl−disulfide interchange reaction between adsorbed proteins occurred in the α-lactalbumin-stabilized emulsion. This is expected because α-lactalbumin contains only four disulfide bonds and no free sulfhydryl group. It should be noted, however, that a faint band corresponding to α-lactalbumin dimer appeared after 2 days of storage (lane 3, Figure 1), and its intensity slightly increased with storage time. This dimer formation might have been caused by small amount of β-lactoglobulin contaminant in the α-lactalbumin sample (see lane 2). It is likely that the free sulfhydryl group of β-lactoglobulin could have created a free sulfhydryl group in a few α-lactalbumin molecules via a sulfhydryl−

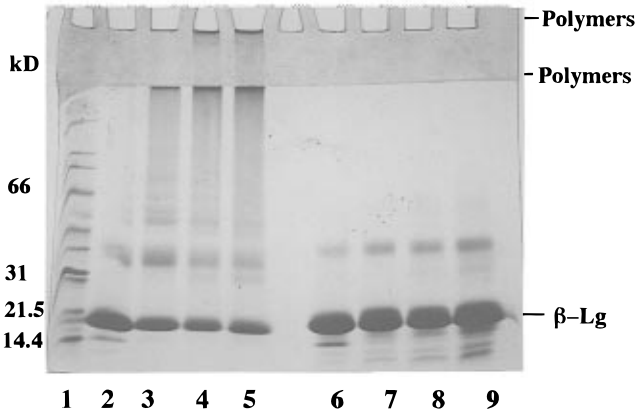

Figure 2. SDS−PAGE of the cream phase of β-lactoglobulin-stabilized emulsions. Lanes 2−5 were not treated with β-mercaptoethanol, and lanes 6−9 were β-mercaptoethanol treated. Lanes 2 and 6, 3 and 7, 4 and 8, and 5 and 9 correspond to storage times of 24, 48, 120, and 288 h, respectively.

disulfide interchange reaction, and these free sulfhydryl groups could have initiated limited sulfhydryl−disulfide interchange between α-lactalbumin molecules. The α-lactalbumin dimer disappears when the sample is treated with a reductant (lanes 6−9). However, it is interesting to note that a faint band corresponding to a molecular weight of about 32 000 (possibly a α-lactalbumin−β-lactoglobulin dimer) appears in the electrophoresis pattern of samples treated with β-mercaptoethanol (lanes 6−9). Apparently, this dimer species, which did not show up in the nonreduced samples, must have been present as a part of the disulfide cross-linked polymers. However, the fact that the dimer itself is not dissociated by β-mercaptoethanol indicates that it contains a covalent cross-link other than the disulfide bond. It is very likely that this might be related to free radical-catalyzed protein−protein cross-linking as a result of lipid oxidation during storage of the emulsion at room temperature as shown.

$$LH + O_2 \rightarrow LOOH$$

$$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$$

$$P + LO^{\bullet} \rightarrow P^{\bullet} + LOH$$

$$P + P^{\bullet} \rightarrow P{-}P^{\bullet}$$

$$P^{\bullet} + P^{\bullet} \rightarrow P{-}P$$

where LH is an unsaturated lipid, LOOH is lipid peroxide, P is protein, $P^{\bullet}$ and $P{-}P^{\bullet}$ are protein free radicals, and P−P is protein dimer. Nevertheless, the electrophoretic profile clearly indicates that sulfhydryl−disulfide interchange reaction-induced polymerization among adsorbed α-lactalbumin molecules does not occur to a significant extent in oil−water emulsions.

***β*-Lactoglobulin Emulsion.** The electrophoretic profile of the cream phase of β-lactoglobulin-stabilized emulsion is shown in Figure 2. With storage time, increasing amounts of high molecular weight polymers appeared at the cost of monomeric β-lactoglobulin (lanes 2−5). At 24 h after emulsion preparation, β-lactoglobulin dimer appeared as the major species along with small amounts of high molecular weight polymers. At longer storage time, increasing amounts of polymer species that could not penetrate the stacking and separating gels appeared. In the presence of β-mercaptoethanol, these polymers were mostly reduced to monomeric β-lactoglobulin (lanes 6−9), indicating that the polymerization occurred essentially via a sulfhydryl−disulfide interchange reaction between adsorbed β-lactoglobulin molecules. It should be noted, however, that the electrophoretic profile in the presence of β-mercaptoethanol showed a minor band corresponding to a molecular weight of about 36 000, possibly a dimer of β-lactoglobulin cross-linked by a covalent bond other than the disulfide bond. The intensity of this band increased with storage time. As in the case of β-lactalbumin emulsion, it is very likely that this non-disulfide cross-linking might be the result of lipid oxidation and free radical-induced oxidation of β-lactoglobulin during emulsion storage at room temperature. Moreover, the electrophoretic profile of the 12th day sample shows low molecular weight protein bands that were not present either in the control (not shown) or in the 24 h sample (lanes 1 and 5). These must be polypeptides formed as a result of free radical-induced cleavage of β-lactoglobulin. Nevertheless, the results clearly indicate that sulfhydryl−disulfide interchange reaction-induced polymerization among adsorbed β-lactoglobulin molecules occurs extensively in oil−water emulsions.

Emulsion-Mix Emulsion. The results shown in Figures 1 and 2 demonstrate that while no sulfhydryl−disulfide interchange-induced polymerization occurs in pure α-lactalbumin-stabilized emulsion, it does occur to a high degree in β-lactoglobulin-stabilized emulsion. Thus, in an emulsion-mix emulsion containing equal volumes of β-lactoglobulin-stabilized and α-lactalbumin-stabilized emulsions, one should find only β-lactoglobulin polymers and neither β-lactoglobulin-α-lactalbumin polymers nor α-lactalbumin polymers. This will be so only if polymerization occurs within the protein film of each emulsion particle (i.e., intrafilm polymerization) and no polymerization occurs via sulfhydryl−disulfide interchange reaction between the protein films of emulsion particles (i.e., interfilm polymerization). If the latter occurs, then the polymers formed in the emulsion-mix emulsion must contain α-lactalbumin as a protein component.

The electrophoretic profile of the cream phase of the emulsion-mix emulsion under nonreducing conditions is shown in Figure 3A. The relative concentrations of α-lactalbumin and β-lactoglobulin monomers in the adsorbed phase decreased gradually up to about 72 h followed by a rapid decrease beyond 72 h (Figures 3A and 3C). This was accompanied by formation of an increasing amount of high molecular weight polymers with time. When the samples were run after reduction with β-mercaptoethanol, the high molecular weight polymers disappeared and the band intensities of monomeric α-lactalbumin and β-lactoglobulin in each of the samples increased (Figure 3B) compared to those of the corresponding nonreduced samples (Figure 3A), suggesting that both α-lactalbumin and β-lactoglobulin were present in the disulfide cross-linked polymers. For instance, it is notable that the relative concentration of monomeric α-lactalbumin in the 24 and 48 h emulsion samples is almost 2-fold higher in the reduced samples than in the unreduced samples (Figure 3C), indicating that a substantial amount of α-lactalbumin was present in the form of disulfide cross-linked polymers. Polymerization of α-lactalbumin in the emulsion-mix emulsion is possible only if there was a sulfhydryl−disulfide interchange reaction between the protein films of α-lactalbumin and β-lactoglobulin emulsion particles. That

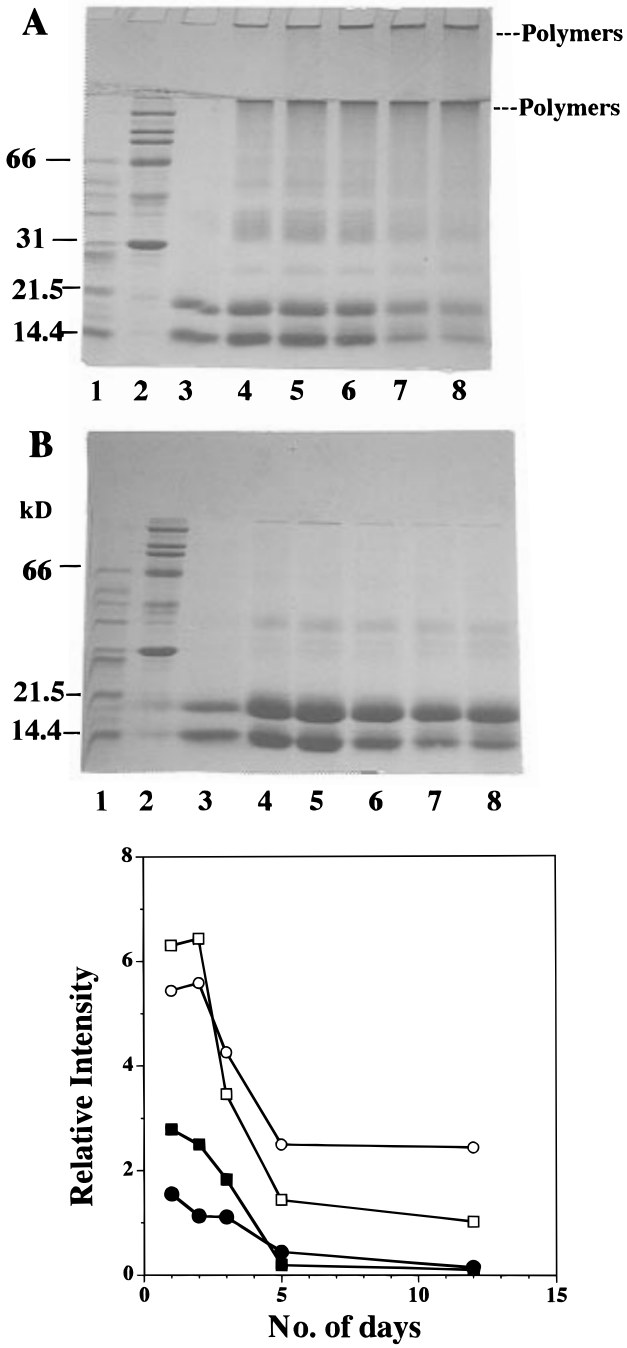

Figure 3. (A) SDS−PAGE of the cream phase of the emulsion-mix emulsions without β-mercaptoethanol treatment. Lanes 1 and 2 are molecular weight markers. Lanes 3−8 correspond to storage times of 0, 24, 48, 72, 120, and 288 h, respectively. (B) SDS−PAGE of the cream phase of the emulsion-mix emulsions with β-mercaptoethanol treatment. Lanes 1 and 2 are molecular weight markers. Lanes 3−8 correspond to storage times of 0, 24, 48, 72, 120, and 288 h, respectively. (C) Relative changes in the concentrations of α-lactalbumin (□, ■) and β-lactoglobulin (○, ●) monomers with storage time in the cream phase of the emulsion-mix emulsion. The open symbols correspond to samples reduced with β-mercaptoethanol, and the filled symbols correspond to samples not treated with β-mercaptoethanol. Note that the zero-time band intensities were less than the subsequent lanes. This was because, at zero time, the emulsions were very stable and we had difficulty in separating the cream from the serum phase.

is, to cause polymerization of α-lactalbumin in the α-lactalbumin-stabilized emulsion particle, the free sulfhydryl group of the adsorbed β-lactoglobulin of the β-lactoglobulin-stabilized emulsion particle must initiate a sulfhydryl−disulfide interchange reaction with the

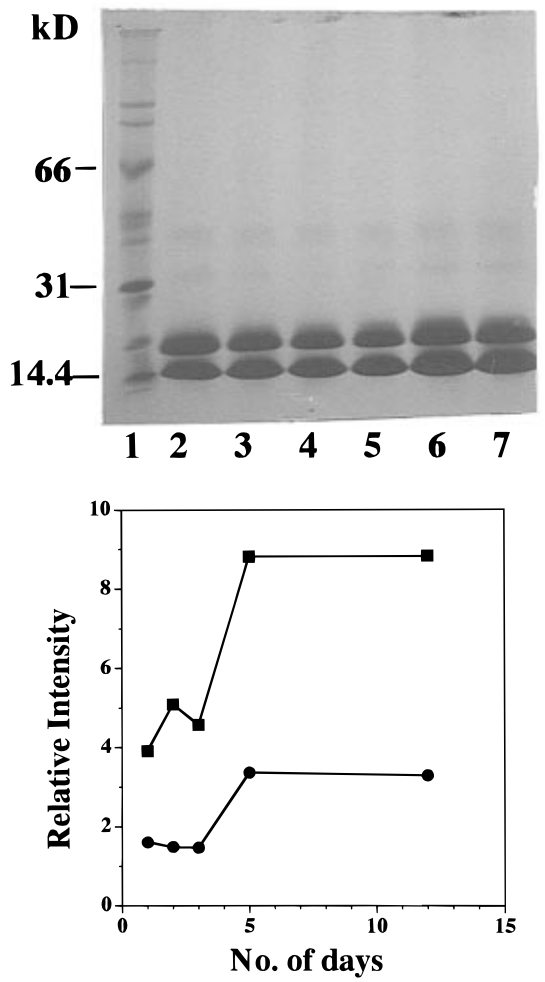

Figure 4. (A) SDS−PAGE of the serum phase of emulsion-mix emulsion without treatment with β-mercaptoethanol treatment. Lane 1 is molecular weight markers. Lanes 2−7 correspond to storage time of 24, 48, 72, 120, and 288 h, respectively. (B) Relative changes in the concentrations of α-lactalbumin (■) and β-lactoglobulin (●) monomers with storage time in the cream phase of emulsion-mix emulsions.

disulfide bonds of α-lactalbumin. This would result in interparticle cross-linking. Once the disulfide bonds of a few of the α-lactalbumin molecules are cleaved, the newly liberated sulfhydryl groups of α-lactalbumin molecules may propagate a sulfhydryl−disulfide interchange chain reaction within the α-lactalbumin film, leading to polymerization.

It may be argued that polymerization of α-lactalbumin in the adsorbed film might have been caused by β-lactoglobulin in the serum phase and not necessarily by the adsorbed β-lactoglobulin. If this is the case, then it is reasonable to expect that the serum phase β-lactoglobulin also should be able to induce polymerization of α-lactalbumin in the serum phase of the emulsion-mix emulsion. However, the electrophoretic profile of nonreduced serum phase showed only monomeric α-lactalbumin and β-lactoglobulin bands and no polymers (Figure 4A). The faint bands in Figure 4A corresponding to β-lactoglobulin dimer and α-lactalbumin/β-lactoglobulin heterodimer were also present in the electrophoresis profile of reduced samples (data not shown), suggesting that these dimers are nondisulfide cross-linked dimers produced as a result of lipid oxidation. Thus, the results demonstrate that interparticle cross-linking via sulfhydryl−disulfide interchange reaction

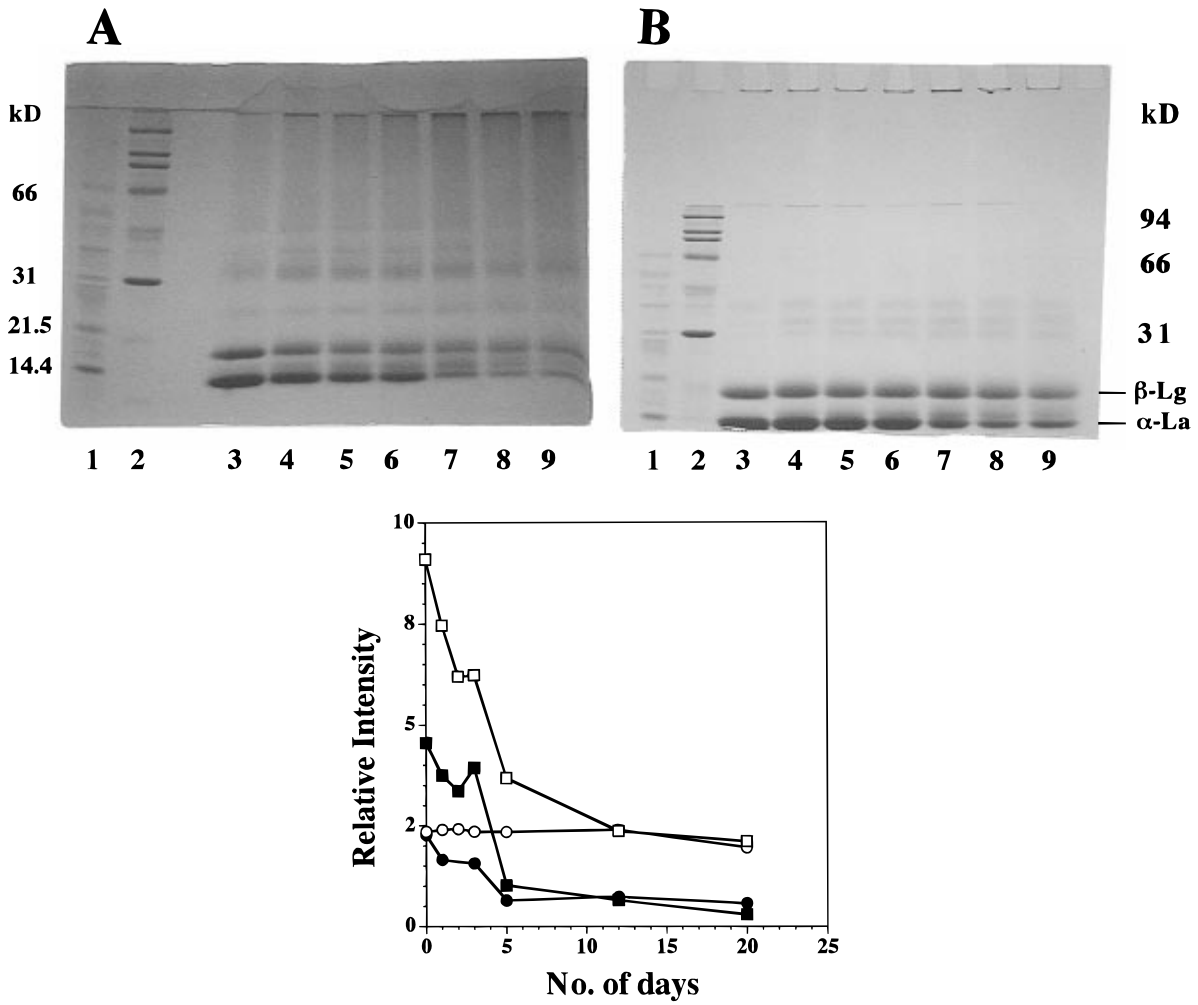

Figure 5. (A) SDS−PAGE of the cream phase of the protein-mix emulsions without β-mercaptoethanol treatment. Lanes 1 and 2 are molecular weight markers. Lanes 3−9 correspond to storage times of 0, 24, 48, 72, 120, 288, and 480 h, respectively. (B) SDS−PAGE of the cream phase of the protein-mix emulsions with β-mercaptoethanol treatment. Lanes 1 and 2 are molecular weight markers. Lanes 3−9 correspond to storage times of 0, 24, 48, 72, 120, 288, and 480 h, respectively. (C) Relative changes in the concentrations of α-lactalbumin (□, ■) and β-lactoglobulin (○, ●) monomers with storage time in the cream phase of the protein-mix emulsion. The open symbols correspond to samples reduced with β-mercaptoethanol, and the filled symbols correspond to samples not treated with β-mercaptoethanol.

between β-lactoglobulin-stabilized and α-lactalbumin-stabilized emulsion particles occurs in the emulsion-mix emulsion.

The amount of β-lactoglobulin and α-lactalbumin monomers in the cream phase of the emulsion-mix emulsion (Figure 3C) remained constant up to about 48 h and decreased thereafter with storage time. On the other hand, the concentrations of unadsorbed β-lactoglobulin and α-lactalbumin in the serum phase increased (Figure 4B), indicating desorption of proteins from the oil−water interface during storage. Apparently, α-lactalbumin is desorbed more than β-lactoglobulin, probably because of its limited extent of polymerization in the adsorbed film.

Protein-Mix Emulsion. The electrophoretic profile of the cream phase of protein-mix oil−water emulsions made with a protein solution containing a 1:1 mixture of β-lactoglobulin and α-lactalbumin is shown in Figure 5A. The amount of protein polymers that could not penetrate the separating gel increased, and the amount of monomeric β-lactoglobulin and α-lactalbumin decreased with storage time (Figure 5A). The involvement of a sulfhydryl−disulfide interchange reaction between β-lactoglobulin and α-lactalbumin was evident by the higher amounts of β-lactoglobulin and α-lactalbumin monomers in the presence of β-mercaptoethanol than in its absence (Figure 5B). These results are in agreement with those of Monahan et al. (1993), who also observed involvement of both β-lactoglobulin and α-lactalbumin in the time-dependent polymerization at the oil−water interface of whey protein emulsions. Comparison of the electrophoretic profiles of the cream phases of emulsion-mix (Figure 3A) and protein-mix (Figure 5A) emulsions suggests that the amount of polymers formed is relatively higher in the emulsion-mix emulsion than in the protein-mix emulsion. It is likely that heterologous polymers between α-lactalbumin and β-lactoglobulin are formed in the protein-mix film, and this may impact the extent of polymerization as well as the size of the polymers formed. α-Lactalbumin, being rigid, may not readily undergo a sulfhydryl−disulfide interchange reaction with β-lactoglobulin and thus may interfere with the extent of polymerization in the film. Much of the β-lactoglobulin in the protein-mix film may exist as filler molecules in a disulfide cross-linked α-lactoglobulin matrix. It should be noted that the total amount of β-lactoglobulin in the protein-mix film remained constant, whereas the concentration of α-lactalbumin decreased during the 20-day storage period (Figure 5C). In the case of the emulsion-mix emulsion however, the concentrations of both α-lactalbumin and β-lactoglobulin of the cream

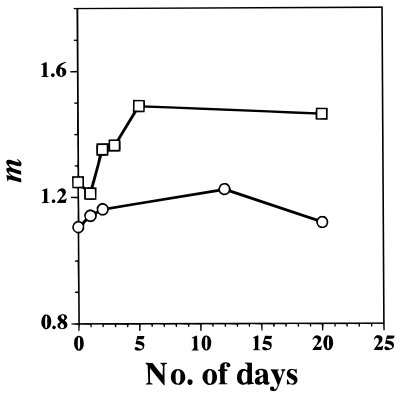

Figure 6. Changes in the value of *m* with storage time of emulsion-mix (□) and protein-mix (○) emulsions. See text for details.

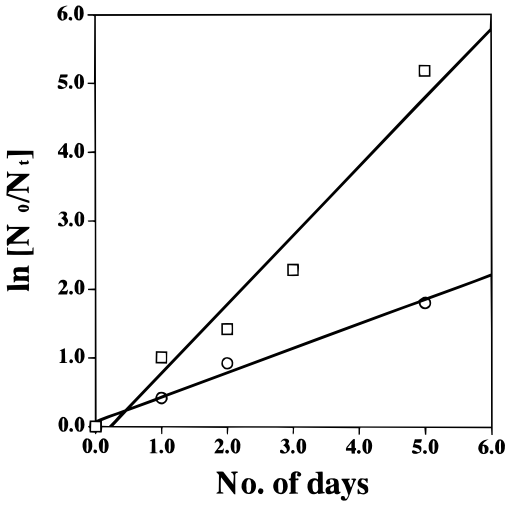

Figure 7. First-order kinetics of coalescence of emulsion-mix (□) and protein-mix (○) emulsions.

phase decreased with time (Figure 3C). This suggests that almost all of β-lactoglobulin molecules in the protein-mix film exist in the polymerized state, whereas this is not the case in the pure β-lactoglobulin film in the emulsion-mix emulsion.

Emulsion Stability. The kinetic stability of the emulsions was analyzed according to eq 2. As strong wavelength dependence was observed for the turbidity of both emulsion-mix and protein-mix emulsions, the wavelength exponent *m* was obtained from the slopes of ln *T* versus ln λ plots. The wavelength exponent *m* is directly related to the average size of droplets; the higher the value of *m* (i.e., strong wavelength dependence), the smaller is the droplet size. In order to determine the change in droplet size of emulsions as a function of storage time, the values of *m* were plotted as a function of time, as shown in Figure 6. At zero time, the values of *m* were about the same for both emulsion-mix and protein-mix emulsions. However, the value of *m* increased with storage time for the emulsion-mix emulsion. This indicates that this emulsion contained mixtures of two distinct populations of droplet sizes, and as the coalescence proceeded mainly among the large droplets, the emulsion was left only with small droplets. For the protein-mix emulsion, however, the value of *m* remained almost constant for 12 days of storage, indicating that this emulsion contained droplets of narrow size distribution.

Assuming first-order kinetics for the coalescence of emulsions, the coalescence rates of the emulsion-mix and protein-mix emulsions were determined according to eq 2 in combination with eq 3, and the results are shown in Figure 7. The plot of $\ln(N_0/N_t)$ versus time show good linearity up to 5 days. The first-order rate constants, calculated from the slopes, were 11.6×10^{-6} and 4.1×10^{-6} s^{-1} for the emulsion-mix and protein-mix emulsions, respectively, indicating that the protein-mix emulsion was more stable than the emulsion-mix emulsion. It is often assumed that protein polymerization in emulsions enhances the stability of emulsions. If this is the case then, since the extent of SH−SS-induced polymerization is greater in the emulsion-mix emulsion than in the protein-mix emulsion, one should expect the emulsion-mix emulsion to be more stable than the protein-mix emulsion. The results presented here apparently show that this is not the case. It is likely that the type of polymerization, i.e., intrafilm versus interfilm protein polymerization, may impact the stability of emulsions. That is, whereas the intrafilm polymerization may promote emulsion stability, interfilm polymerization, which leads to aggregation and coalescence, may actually promote emulsion instability.

To understand the role of interfilm polymerization on emulsion stability, the extent of formation of aggregates

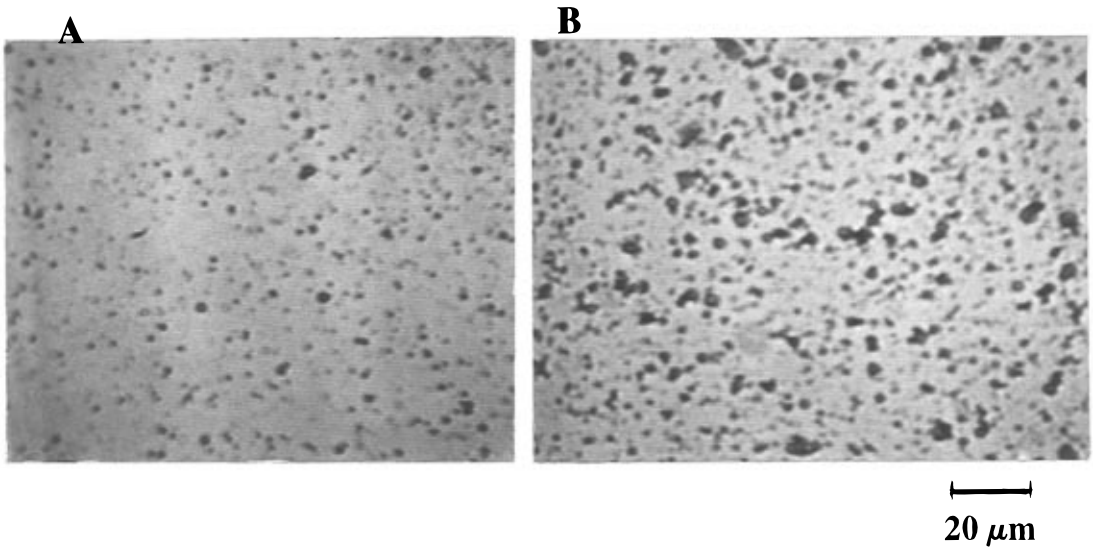

Figure 8. Light microscopic images of α-lactalbumin-stabilized emulsion after 24 (left) and 98 h (right) storage at room temperature

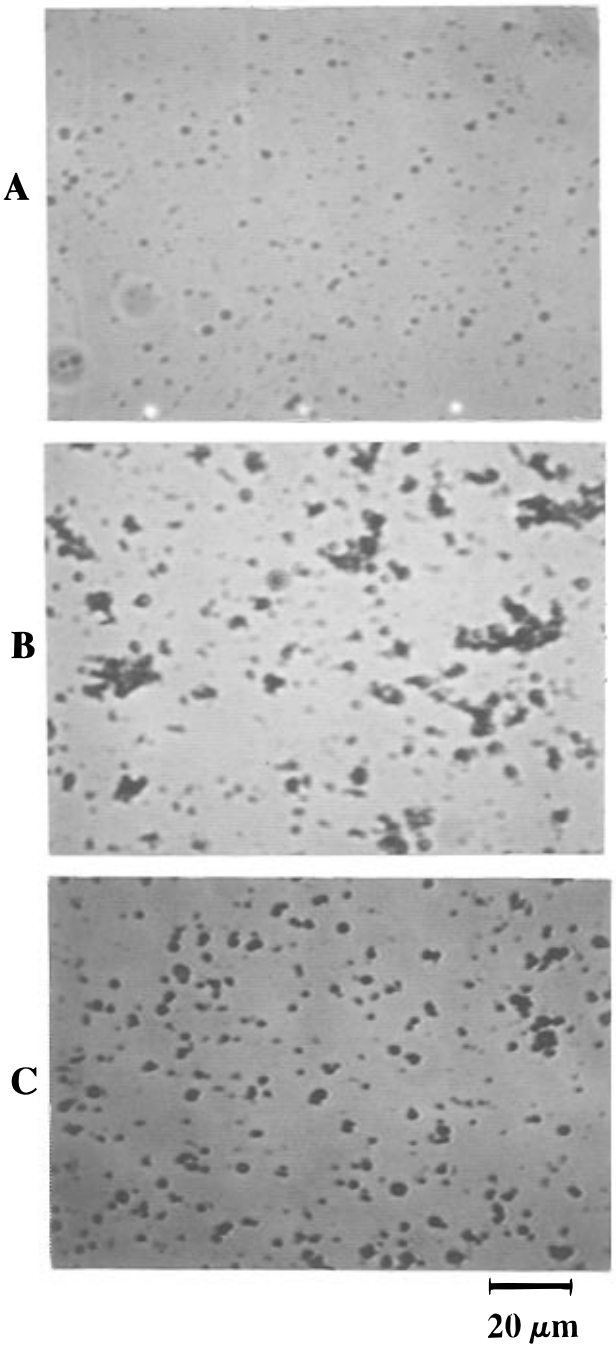

Figure 9. Light microscopic images of β-lactoglobulin-stabilized emulsion as a function of storage time at room temperature: (A) emulsion after 24 h with no NEM; (B) after 98 h with no NEM; (C) after 98 h with 20 mM NEM.

as a function of storage time was studied using light microscopy. Figure 8 shows the size of droplets of α-lactalbumin-stabilized emulsion after 24 and 98 h of storage. At 98 h there is some degree of aggregation. Since the SH−SS interchange-induced polymerization is absent in pure α-lactalbumin-stabilized emulsion (Figure 1), these aggregates must be predominantly noncovalent aggregates. Figure 9 shows light microscopy of β-lactoglobulin-stabilized emulsions after 24 and 98 h storage. At 24 h, the emulsion contained no aggregates (Figure 9A), implying that the polymers observed at 24 h (Figure 2) were mainly intrafilm polymers. After 98 h storage, however, the emulsion contained large nonspherical aggregates (Figure 9B). These aggregates might have formed as a result of both noncovalent (hydrophobic) as well as SH−SS interchange-induced cross-linking among emulsion droplets. To determine if interparticle disulfide cross-linking is involved in these aggregates, to an aliquot of the original emulsion was added 20 mM NEM; the resultant mixture was stored under identical conditions as for Figure 9B, and the results are shown in Figure 9C. In the presence of NEM, the size of the aggregates were much smaller and more spherical than those formed in the absence of NEM, suggesting that the formation of large nonspherical aggregates in the absence of NEM was mainly driven by interparticle SH−SS interchange-induced cross-linking.

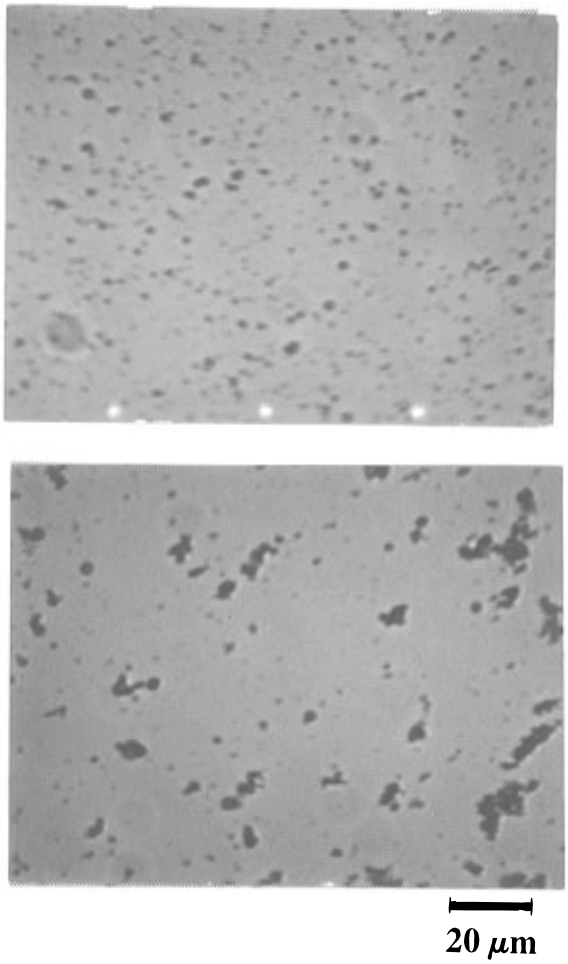

Figure 10. Light microscopic images of emulsion-mix emulsion after 24 (top) and 98 h (bottom) storage at room temperature.

Figure 10 shows light microscopic images of the emulsion-mix emulsion after 24 and 98 h storage, and Figure 11 shows that of the protein-mix emulsion after 98 h. In both cases, the emulsion after 98 h storage contained large nonspherical aggregates. However, the extent of formation was greater and the size of the aggregates was larger in the emulsion-mix emulsion than in the protein-mix emulsion. Qualitative examination of the results suggest that intrafilm disulfide cross-linking might be greater in the protein-mix emulsion than in the emulsion-mix emulsion, and this contributes to the greater storage stability of the protein-mix emulsion. Conversely, the storage instability of the emulsion-mix emulsion might be partly related to the greater extent of formation of interparticle cross-linking, which promotes aggregation, coalescence, and phase separation.

The results presented here demonstrate that both intrafilm and interfilm protein polymerizations occur via

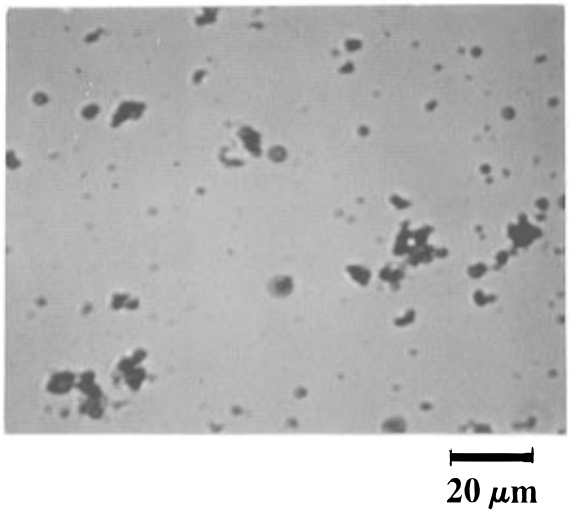

Figure 11. Light microscopic image of protein-mix emulsion after 98 h storage at room temperature.

a sulfhydryl−disulfide interchange reaction in protein-stabilized emulsions. While the intrafilm protein polymerization may increase the viscoelastic properties of the adsorbed protein film in emulsion particles, and thus may positively contribute to the storage stability of emulsions, interfilm protein polymerization promotes aggregation and coalescence and, thus, promotes storage instability in emulsions. In proteins containing both cysteine and cystine residues, the balance between intrafilm and interfilm protein polymerization may influence the storage stability of the emulsion.

LITERATURE CITED

Dickinson, E.; Matsumura, Y. Time-dependent polymerization of β-lactoglobulin through disulphide bonds at the oil−water interface in emulsions. *Int. J. Biol. Macromol.* **1991**, *13*, 26−30.

Dickinson, E.; Rolfe, S. E.; Dalgleish, D. G. Surface shear viscometry as a probe of protein−protein interactions in mixed milk protein films adsorbed at the oil−water interface. *Int. J. Biol. Macromol.* **1990**, *12*, 189−194.

Kitabatake, N.; Doi, E. Conformational changes of hen egg ovalbumin during foam formation detected by 5,5′-dithiobis-(2-nitorbenzoic acid). *J. Agric. Food Chem.* **1987**, *35*, 954−957.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T_4. *Nature (London)* **1970**, *227*, 680.

Monahan, F. J.; McClements, D. J.; Kinsella, J. E. Polymerization of whey proteins in whey protein-stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826−1829.

Monahan, F. J.; German, J. B.; Kinsella, J. E. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem.* **1995**, *43*, 46−52.

Pearce, K. N.; Kinsella, J. E. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *J. Agric. Food Chem.* **1978**, *26*, 716−723.

Reddy, S. R.; Fogler, H. S. Emulsion stability: Determination from turbidity. *J. Colloid Interface Sci.* **1981**, *79*, 101−104.

Shimada, K.; Cheftel, J. C. Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 1018−1025.

Zhu, H.; Damodaran, S. Heat-induced conformational changes in whey protein isolate and its relation to foaming properties. *J. Agric. Food Chem.* **1994**, *42*, 846−855.

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