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Impact of Protein Surface Denaturation on Droplet Flocculation in Hexadecane Oil-in-Water Emulsions Stabilized by β-Lactoglobulin

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The influence of globular protein denaturation after adsorption to the surface of hydrocarbon droplets on flocculation in oil-in-water emulsions was examined. *n*-Hexadecane oil-in-water emulsions (pH 7.0) stabilized by β-lactoglobulin (1-wt % β-Lg) were prepared by high-pressure valve homogenization. NaCl (0−150 mM) was added to these emulsions immediately after homogenization, and the evolution of the mean particle diameter (*d*) and particle size distribution (PSD) was measured by laser diffraction during storage at 30 °C for 48 h. No change in *d* or PSD was observed in the absence of added salt, which indicated that these emulsions were stable to flocculation. When 150 mM NaCl was added to emulsions immediately after homogenization, *d* increased rapidly during the following few hours until it reached a plateau value, while the PSD changed from monomodal to bimodal. Addition of *N*-ethylmaleimide, a sulfhydryl blocking agent, to the emulsions immediately after homogenization prevented (at 20 mM NaCl) or appreciably retarded (at 150 mM NaCl) droplet flocculation. These data suggests that protein unfolding occurred at the droplet interface, which increased the hydrophobic attraction and disulfide bond formation between droplets. In the absence of added salt, the electrostatic repulsion between droplets was sufficient to prevent flocculation, but in the presence of sufficient salt, the attractive interactions dominated, and flocculation occurred.

KEYWORDS: β-Lactoglobulin; surface denaturation; emulsions; flocculation

INTRODUCTION

Whey proteins are finding increasing usage within the food industry as natural ingredients capable of facilitating the formation and improving the long-term stability of emulsion-based food products (*1*−*6*). The major proteins in whey (β-lactoglobulin, α-lactoalbumin, and bovine serum albumin) are amphiphilic molecules that have compact globular structures in their native state (*7*−*8*). These globular proteins rapidly adsorb to the surface of oil droplets formed during emulsion homogenization, where they facilitate further droplet disruption by lowering the interfacial tension and retard recoalescence within the homogenizer by forming protective membranes around the droplets (*2*, *3*, *9*). The ability of proteins to modulate the colloidal interactions between oil droplets also plays an important role in determining the long-term stability and rheology of oil-in-water emulsions (*9*−*11*).

A major potential drawback of using globular proteins to stabilize oil-in-water emulsions is their tendency to undergo conformational changes after adsorption to the droplet surfaces (*12*) because these changes can lead to emulsion instability (*13*, *14*). In a bulk aqueous solution, a nonadsorbed globular protein is surrounded predominantly by water molecules, but at an oil−water interface, an adsorbed globular protein is partly in contact with water and partly in contact with oil (*2*). This change in the molecular environment of the globular protein on adsorption is the major driving force for surface denaturation (*15*). The protein undergoes a conformational rearrangement to maximize the number of favorable and minimize the number of unfavorable molecular interactions. Studies of various globular proteins adsorbed to surfaces have shown that these conformational rearrangements usually take a few hours to be effectively completed (*16*−*19*).

A variety of experimental techniques have been used to characterize surface denaturation of globular whey proteins and to establish the influence of surface denaturation on the physicochemical properties of emulsions. Calorimetric studies have shown that adsorption of α-lactalbumin and β-lactoglobulin (pH 7) to droplet surfaces in oil-in-water emulsions causes a pronounced change in their thermal transitions, suggesting an appreciable conformational change in the protein (*20*). FTIR studies of β-Lg (pH 7) adsorbed to the surface of droplets in oil-in-water emulsions have shown that there is a slow increase in the amount of disordered secondary structure of the protein with time (*18*). The extent of this change increased when the concentration of protein in the emulsions prior to homogenization was decreased, presumably because there was less protein available for adsorption to the interface so that there was more

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room for conformational changes. Front-surface fluorescence studies of β-Lg and BSA adsorbed to oil droplets have also indicated that globular proteins undergo slow conformational changes with time after homogenization (*16*−*21*). Other experiments suggest that surface denaturation leads to exposure of reactive amino acid residues originally located in the hydrophobic interior of the native protein, e.g., nonpolar groups or sulfur containing groups (*12*, *22*). Exposure of nonpolar groups leads to increased hydrophobic interactions between surface-denatured protein molecules (*13*), whereas exposure of sulfur containing groups leads to disulfide bond formation or disulfide interchange reactions (*12*, *23*). Surface denaturation of globular proteins after adsorption retards desorption kinetics (*15*), thus decreasing the exchange of adsorbed proteins with free proteins or small molecule surfactants in the surrounding aqueous phase (*12*, *24*).

An increased propensity for protein−protein interactions to occur in protein-stabilized emulsions has been shown to influence the physicochemical properties of the interfacial membranes surrounding the droplets and of the emulsion as a whole. An increase in interfacial dilational modulus has been observed after globular proteins adsorb to a planar oil−water interface, which has been attributed to increased hydrophobic and disulfide interactions between neighboring proteins adsorbed to the interface (*25*, *26*). Alterations in the rheology of the interfacial membrane surrounding the oil droplets in an emulsion may have a pronounced influence on the bulk physiochemical properties of an emulsion, e.g., stability and rheology (*6*, *25*−*28*). An increase in droplet flocculation has been observed in oil-in-water emulsions stabilized by globular proteins after homogenization, which has been attributed to increased interactions between surface-denatured proteins adsorbed on different emulsion droplets (*14*, *22*). Droplet flocculation is undesirable in many food emulsions because it leads to an increase in creaming and emulsion viscosity (*11*, *29*).

In this study, we examine the influence of aqueous phase composition (NaCl, NEM, protein) on the flocculation stability of *n*-hexadecane droplets stabilized by β-lactoglobulin. This protein was used as a model globular protein because its molecular structure and functional properties are well established. The overall objective of this study was to obtain further insight into the factors that determine the long-term stability of globular protein stabilized emulsions.

MATERIALS AND METHODS

Materials. Analytical-grade sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium azide (NaN_3), *N*-ethylmaleimide (NEM), 2-mercaptoethanol, and *n*-hexadecane were purchased from the Sigma Chemical Company (St. Louis, MO). Powdered β-lactoglobulin was obtained from Davisco Foods International (LOT # JE 001-1-922, Le Sueur, MN). As stated by the manufacturer, the β-Lg content of the powder determined by electrophoresis was 98% (the remainder being mostly globulins). The decrease in mass of the protein powder upon drying was 2.6%, and the nitrogen content of the powder was 15.6%. Distilled and deionized water was used for the preparation of all solutions.

Solution Preparation. Emulsifier solutions with 0.5, 1.0, or 2.0 wt % protein were prepared by dispersing powdered β-Lg into deionized and distilled water containing 0.04 wt % NaN_3 (as an antimicrobial agent) and stirring for at least 2 h to ensure complete dispersion. Solutions containing different NaCl and NEM concentrations were prepared by dispersing weighed amounts of the powdered material into 5 mM phosphate buffer (pH 7.0).

Emulsion Preparation. An oil-in-water emulsion was prepared by homogenizing 10-wt % *n*-hexadecane oil and 90-wt % emulsifier solution at room temperature. The oil and emulsifier solution were blended using a high-speed blender for 2 min (Model 33BL79, Warring Inc., New Hartford, CT) and then passed through a high-pressure valve homogenizer five times at 7500 psi (Rannie High Pressure, APV-Gaulin, Model Mini-Lab 8.30H, Wilmington, MA). The pH of this emulsion was adjusted to 7.0 using HCl solution (pH Meter 320, Corning Inc., Corning, NY). The emulsions were then diluted with phosphate buffer (5 mM, pH 7) containing NaCl and/or NEM to obtain emulsions with a final composition of 5 wt % hexadecane, 2.5 mM phosphate buffer, 0−200 mM NaCl, and 0−4.4 mM NEM. The emulsions were then stored in a temperature controlled water bath at 30 °C with constant swirling and samples were selected periodically for analysis.

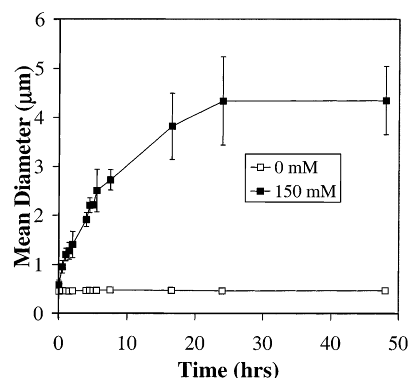

Figure 1. Mean particle diameter (d_{43}) of 5-wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % BSA, pH 7.0) stored at 30 °C as a function of time after homogenization.

Particle Size Determination. The particle size distribution of the emulsions was measured using a laser diffraction instrument (LA900, Horiba Inc, CA). This instrument measures the angular dependence of the intensity of light scattered from a stirred dilute emulsion and then indicates the particle size distribution that gives the closest fit between theoretical calculations and experimental measurements. A refractive index ratio of 1.08 was used in the particle size calculations. To avoid multiple scattering effects, the emulsions were diluted with pH adjusted distilled water (pH 7) prior to making the measurements. The emulsions were stirred continuously throughout the measurements to ensure the samples were homogeneous. Dilution and stirring may have partially disrupted weakly flocculated droplets, although it is unlikely that they will have disrupted any strongly flocculated droplets. The theory used to calculate the particle size distribution assumes that the particles are spherical and homogeneous, and therefore the data obtained on emulsions that contained flocs should be treated with caution because they are nonspherical and nonhomogeneous. Particle size measurements are reported as either full particle size distributions or as weight-average mean diameters, d_{43} ($=\Sigma n_i d_i^4/\Sigma n_i d_i^3$, where n_i is the number of particles with diameter d_i). Mean particle diameters were calculated as the average and standard deviation of measurements made on at least two freshly prepared samples.

ζ-Potential Measurements. The electrical charge (ζ-potential) on the particles was measured using a particle electrophoresis instrument (ZEM5003, Zetamaster, Malvern Instruments, Worcs., U.K.). Samples of 10-wt % *n*-hexadecane oil-in-water emulsions were diluted 500-fold with 20 mM NaCl solution adjusted to pH 7 prior to measurements. The diluted emulsion was mixed thoroughly and then injected into the measurement chamber of the instrument. ζ-Potential is reported as the average and standard deviation of measurements made on two freshly prepared samples, with five readings made per sample. The ζ-potential of the emulsion droplets measured under these conditions was -41.8 ± 0.8 mV.

RESULTS AND DISCUSSIONS

Influence of NaCl on Droplet Aggregation. Initially, we measured the evolution in the mean particle diameter (*d*) and particle size distribution (PSD) of 5 wt % *n*-hexadecane oil-in-water emulsions stored at 30 °C containing either 0 or 150 mM NaCl added immediately after homogenization (**Figures 1**

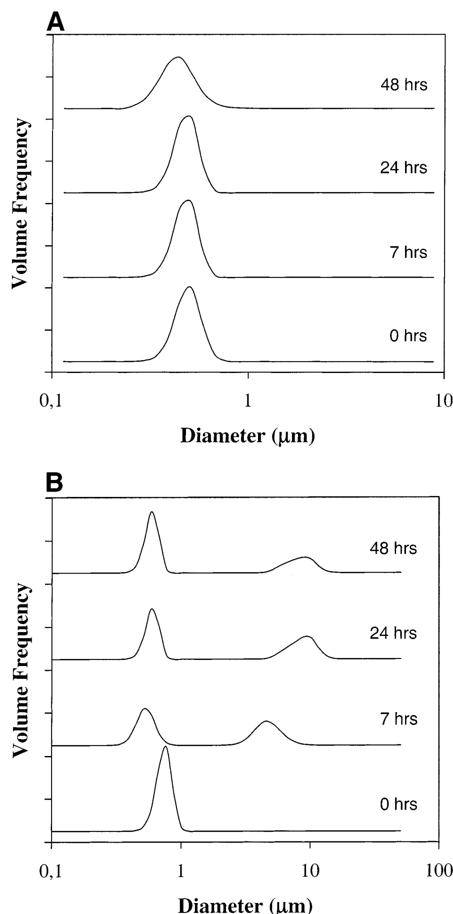

Figure 2. Evolution of particle size distribution of 5-wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % BSA, pH 7.0) at 30 °C for 48 h after homogenization: A, 0 mM NaCl; B, 150 mM NaCl.

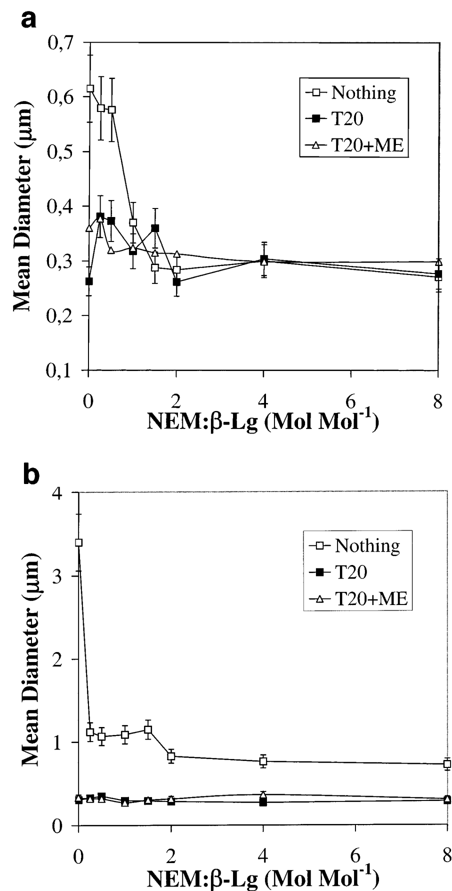

Figure 3. Mean particle diameter (d_{43}) of 5-wt % *n*-hexadecane oil-in-water emulsions (0.5 wt % BSA, pH 7.0) containing different NEM-to-β-Lg molar ratios measured 24 h after homogenization: (a) 20 mM NaCl; (b) 150 mM NaCl. Tween 20 or Tween 20 + 2-mercaptoethanol were added to some of the emulsions to disrupt flocs.

and **2**). In the absence of added salt, no significant changes were observed in *d* or PSD of the emulsions, which suggested that droplet aggregation did not occur. In the presence of added salt, there was a steep rise in *d* during the first 24 h following homogenization, after which the particle diameter reached a relatively constant value. In addition, the PSD changed from monomodal to bimodal during the initial stages of storage (**Figure 2b**). These results indicated that the emulsion droplets became increasingly aggregated during the first few hours after homogenization. In light of previous studies, we postulate that the progressive surface denaturation of β-Lg molecules after adsorption to oil droplet surfaces played an important role in droplet aggregation (*18*−*21*). These conformation changes led to an increase in the number of nonpolar and sulfhydryl amino acids exposed to the aqueous phase, which increased the hydrophobic attraction and disulfide bond formation between protein molecules adsorbed on different droplets (*13*, *14*). Support for this hypothesis comes from studies of the susceptibility of β-Lg to enzyme hydrolysis, which have shown that amino acid sequences capable of forming disulfide bonds are exposed to the aqueous phase after the protein has adsorbed to an oil droplet surface (*21*). We postulate that no droplet aggregation was observed in the absence of added salt because there was a strong electrostatic repulsion between the droplets, which prevented them from coming into close contact (see below).

Influence of NEM, 2-Mercaptoethanol, and Surfactant on Droplet Aggregation. To gain insight into the relative importance of hydrophobic interactions and disulfide bonds in promoting droplet aggregation, we added varying concentrations of a sulfhydryl blocking agent (0−4.4 mM NEM) to emulsions containing salt (0, 20, or 150 mM NaCl) immediately after homogenization. This NEM concentration range corresponded to a molar ratio (*R*) of NEM-to-β-Lg of 0−8. The emulsions were then incubated at 30 °C for 24 h, and their mean particle diameters were measured by laser diffraction (**Figure 3**). Prior to making the laser diffraction measurements, nonionic surfactant (1 wt % Tween 20), reducing agent (1 wt % 2-mercaptoethanol), or nonionic surfactant + reducing agent (1 wt % Tween 20 + 1 wt % 2-mercaptoethanol) was stirred into some of the emulsions for 1 h.

At 0 mM NaCl, no droplet aggregation was observed in any of the emulsions (data not shown). At 20 mM NaCl (in the absence of surfactant or reducing agent), an appreciable decrease in the mean particle diameter occurred when *R* was increased from 0 to 1, and at higher molar ratios, the mean particle diameter was similar to that of the original nonaggregated emulsion (**Figure 3a**). When surfactant or surfactant + reducing agent were added to these emulsions prior to the laser diffraction measurements, the measured mean particle diameters were similar to that of the original emulsion. At 150 mM NaCl (in the absence of surfactant or reducing agent), there was a rapid decrease in the mean particle diameter when *R* was increased from 0 to 0.25, followed by a more gradual decrease when *R* was increased from 1.5 to 2 (**Figure 3b**). At higher molar ratios, the mean particle diameter remained fairly constant, but it was considerably higher (~0.7 μm) than that of the original emulsion

(~0.3 μm). When surfactant or surfactant + reducing agent were incorporated into these emulsions prior to the laser diffraction measurements, the mean particle diameters were similar to that of the original emulsion. When reducing agent alone was added to emulsions containing either 20 or 150 mM NaCl prior to making the particle size measurements, the degree of droplet aggregation increased (data not shown).

These data can be explained in terms of the influence of NEM, 2-mercaptoethanol, and Tween 20 on the interactions between droplets. The origin of droplet aggregation in our emulsions was obviously flocculation, rather than coalescence, since the aggregates could be disrupted and made to release their original droplets by adding surfactant or surfactant in combination with reducing agent. The surfactant molecules (Tween 20) displace the protein molecules from the droplet surfaces and form interfacial membranes that render the droplets stable to flocculation (*1*). The fact that flocs could be disrupted equally well by surfactant as by surfactant + reducing agent suggested that disulfide bond formation at the droplet surface was not so extensive that it prevented the proteins from being desorbed by surfactant, as is the case in emulsions heated above the thermal denaturation temperature of the adsorbed proteins (*30*). The fact that the emulsions were flocculated, rather than coalesced, was confirmed by optical microscopy measurements, which showed that emulsion droplets became increasingly aggregated into clusters as the time after homogenization increased (data not shown).

The reason that droplet aggregation was not observed in the emulsions containing 0 mM NaCl was presumably because the electrostatic repulsive interactions between the droplets was strong enough to overcome any attractive interactions (see below). The fact that floc formation could be prevented in emulsions containing 20 mM NaCl by adding NEM ($R > 1$) immediately after homogenization suggests that disulfide bond formation played an important role in holding the flocs together (**Figure 3a**). In the absence of disulfide bond formation, hydrophobic interactions alone were not sufficiently strong to promote droplet flocculation (or at least to hold flocs together during dilution and stirring in the laser diffraction instrument). As will be shown below, there is still a large electrostatic repulsion between the droplets at this salt concentration, which would be expected to prevent the droplets from coming close enough together to strongly flocculate. For disulfide bonds to form between proteins adsorbed onto different droplets, it is necessary for the droplets to come into close proximity. It therefore seems likely that disulfide bonds are formed between emulsion droplets that have been brought closer together due to electrostatic screening in combination with relatively long-range hydrophobic interactions associated with protein surface denaturation.

Disulfide bonds also played an important role in stabilizing the flocs formed in the emulsions containing 150 mM NaCl, since there was a large decrease in the extent of droplet flocculation when NEM was added to the emulsions immediately after homogenization (**Figure 3b**). On the other hand, an appreciable amount of droplet flocculation still occurred in the emulsions at NEM concentrations where all the disulfide groups should have been blocked ($R > 1$). We propose that the droplets are strongly flocculated at this relatively high salt concentration because the hydrophobic attraction arising between droplets due to protein surface denaturation is sufficiently strong to overcome the electrostatic energy repulsion (see below). Strongly flocculated droplets are not easily disrupted by dilution

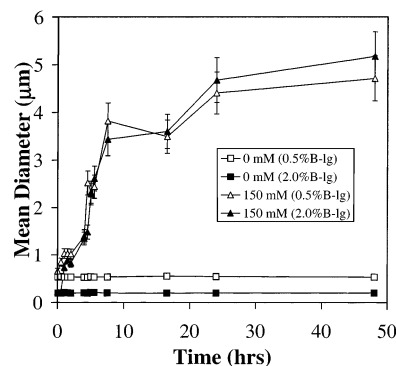

Figure 4. Effect of protein concentration added before homogenization and NaCl concentration on the time-dependence of the mean particle diameter (d_{43}) of 5-wt % *n*-hexadecane oil-in-water emulsions.

or stirring in a laser diffraction instrument, and therefore there is an increase in the measured particle size.

The increase in droplet flocculation observed when 2-mercaptoethanol alone was added to the emulsions probably occurred because this strong reducing agent cleaves a disulfide bond in β-Lg, which causes an increase in the protein's surface hydrophobicity (*31*). Consequently, there is a stronger hydrophobic attraction between the emulsion droplets, which promotes droplet flocculation.

Influence of Protein Concentration on Droplet Flocculation. Previous studies of the extent of surface denaturation of globular proteins at oil–water interfaces have shown that the degree of protein unfolding depends on the protein concentration in the aqueous phase during the adsorption process (*18*, *32*). At sufficiently low protein concentrations, globular proteins can undergo extensive unfolding after adsorption because there are no physicochemical constraints imposed by neighboring protein molecules (*32*). On the other hand, at sufficiently high protein concentrations, the extent of globular protein surface denaturation is reduced because there is less space available for them to unfold into due to the presence of all the other adsorbed proteins in their immediate vicinity. For this reason, we examined the influence of aqueous phase protein concentration during homogenization on the degree of droplet flocculation in oil-in-water emulsions.

Varying amounts (0.5–2 wt %) of β-Lg were added to the aqueous solution (150 mM NaCl, pH 7.0) used to prepare the emulsions prior to homogenization. The oil and aqueous phases were then homogenized and the change in mean particle diameter of the emulsions was measured over 48 h at 30 °C using laser light scattering. We observed no appreciable difference in the rate or extent of droplet flocculation in the emulsions as the protein concentration was increased from 0.5 to 2 wt % (**Figures 1** and **4**). A possible explanation of these results is that surface denaturation of the β-Lg was independent of protein concentration and that the free protein concentration in the aqueous phase prior to homogenization had little impact on the kinetics or extent of protein unfolding (under the conditions used in our experiments). This would suggest that even when β-Lg was fairly densely packed at the interface it was still capable of undergoing conformation changes that led to exposure of nonpolar and sulfhydryl groups, thereby increasing droplet–droplet interactions. An alternative explanation of our data is that the electrostatic repulsion between the droplets was sufficiently screened at 150 mM NaCl that all of the emulsions were unstable to flocculation, regardless of the droplet surface hydrophobicity. Theoretical predictions were carried out

to provide further insight into the origin of droplet flocculation in the emulsions (see below).

The results reported in this section have important implications for the development of protein-stabilized emulsions that have relatively high salt concentrations. It seems that it is not possible to prevent droplet flocculation by increasing the protein concentration in the aqueous phase prior to homogenization so as to retard protein unfolding at the droplet interface. Nevertheless, further experiments are required using a wider range of protein concentrations and adding the additional protein both before and after homogenization.

Theoretical Prediction of Colloidal Interactions. Considerable insight into the relative importance of different colloidal interactions on the aggregation stability of protein coated emulsion droplets can be obtained by calculating the droplet−droplet interaction potential ($w(h)$) as a function of droplet surface-to-surface separation (h) (*11*). We assumed that the overall interaction between two emulsion droplets stabilized by globular proteins could be described by the sum of the van der Waals (w_{VDV}), electrostatic (w_E), steric (w_S), and hydrophobic interactions (w_H):

$$w(h) = w_{VDV}(h) + w_E(h) + w_S(h) + w_H(h) \quad (1)$$

where

$$w_{VDV}(h) = -rA_H/12h \quad (2)$$

$$w_S(h) = (2\delta/h)^{\infty} \quad (3)$$

$$w_E(h) = -2\pi\epsilon_0\epsilon_R r\Psi^2 \ln[1 - e^{-\kappa h}] \quad (4)$$

$$w_H(h) = -2\pi r\gamma\phi\lambda e^{-h/\lambda} \quad (5)$$

Here, r is the droplet radius (in m), A_H is the Hamaker function (in J), δ is the thickness of the adsorbed protein layer (in m), ϵ_0 is the dielectric constant of a vacuum (8.85×10^{-12} C^2 J^{-1} m^{-1}), ϵ_R is the relative dielectric constant of the medium separating the droplets, Ψ is the surface potential of the droplets (in V), κ is the reciprocal of the Debye screening length ($3.29 \times 10^9 \sqrt{I}$ m^{-1}), I is the ionic strength (in M), γ is the interfacial tension at the oil−water interface (in J m^{-2}), ϕ is the fractional hydrophobicity of the droplet surfaces, and λ is the decay length of the hydrophobic interactions (in m). We have assumed that the van der Waals, electrostatic, and hydrophobic interactions begin at the outer surface of the adsorbed protein layer, whereas the steric interaction begins at the oil droplet surface. The above equations only give a rough approximation of the actual droplet−droplet pair potential because they ignore phenomenon such as retardation and interfacial layer effects on van der Waals interactions, ion binding and charge regulation effects on electrostatic interactions and the precise molecular details of steric interactions (*11*). In addition, we have ignored the influence of covalent (disulfide) bonds on droplet interactions. Even so, the above equations do provide some valuable insights into the influence of solution conditions on colloidal interactions, e.g., pH, surface charge, and droplet hydrophobicity.

Initially, we examined the influence of ionic strength of the aqueous solution surrounding the droplets on their colloidal interactions in the absence of any hydrophobic interactions ($\phi = 0$). The variation of the overall droplet−droplet pair potential with sodium chloride concentration (50−250 mM) is shown in **Figure 5**. The pair potential was calculated using

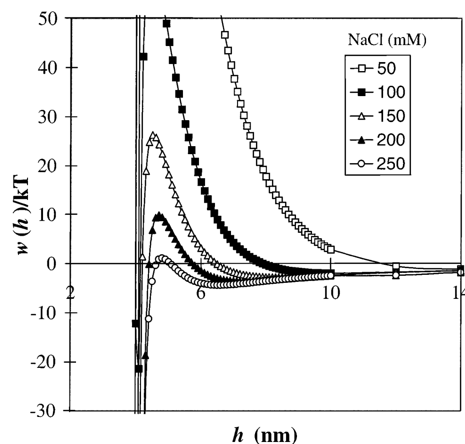

Figure 5. Theoretical prediction of the influence of ionic strength on droplet–droplet interactions in a protein stabilized oil-in-water emulsion. Calculations were performed using eq 1 and the following parameters: $r = 0.3$ μm, $A_H = 5.33 \times 10^{-21}$ J, $\delta = 2$ nm, $\epsilon_R = 80$, $\Psi = -42$ mV, $I = 0$–250 mM, $\lambda = 1$ nm, $\gamma = 10$ mJ m^{-2}. The Hamaker function was corrected for electrostatic screening effects as described by McClements (1999): $A_H = (5.33 \times 10^{-21}$ J$) \times (0.52e^{-2\kappa h} + 0.48)$. The change in ζ-potential with NaCl concentration was taken into account by assuming constant surface charge density conditions (McClements, 1999). Droplets were assumed to have no hydrophobic surface character: $\phi = 0$.

physicochemical parameters (r, δ, T, A_H, etc.) that were representative of the emulsions used in our experimental study (see figure caption). At close separations ($h < 2\delta$) there is an extremely strong steric repulsion between the droplets, which accounts for the steep positive increase in the droplet−droplet pair potential at close droplet separations ($h < 4$ nm). This strong short-range repulsion would be expected to prevent the droplets from coming close enough together to coalesce. At intermediate separations ($4 < h < 15$ nm), the overall pair potential is a balance between attractive van der Waals interactions and repulsive electrostatic interactions. At low ionic strengths (<150 mM), the electrostatic repulsion is sufficiently larger than the van der Waals attraction, so that there is a relatively high energy barrier that prevents the droplets from falling into the deep primary minimum that occurs at $h \sim 4$ nm. Under these solution conditions, we would not expect the droplets to aggregate into strong flocs, although they may form weak flocs due to the presence of the shallow secondary minimum to the right of the energy barrier. At higher ionic strengths, the van der Waals attraction dominates the electrostatic repulsion, so the energy barrier either disappears or is no longer high enough to prevent the droplets from falling into the deep primary minimum. Under these solution conditions, we would expect the droplets to aggregate into strong flocs. Recent experimental studies have shown that droplet flocculation occurs in whey protein stabilized emulsions at pH 7 when the salt concentration is increased above about 200 mM for KCl (*33*), which gives empirical support to our theoretical predictions.

The influence of surface denaturation of β-Lg on droplet flocculation was ascertained by examining the effect of increasing the droplet surface hydrophobicity ($\phi = 0-2\%$) on the droplet−droplet pair potential in the presence of 150 mM NaCl (**Figure 6**). The various physical parameters used to calculate the pair potential are given in the figure caption. In the absence of exposed hydrophobic groups ($\phi = 0\%$), the electrostatic repulsion dominates the van der Waals attraction, so there is a relatively high energy barrier that prevents the droplets from falling into the deep primary minimum ($h \sim 4$ nm). Under these

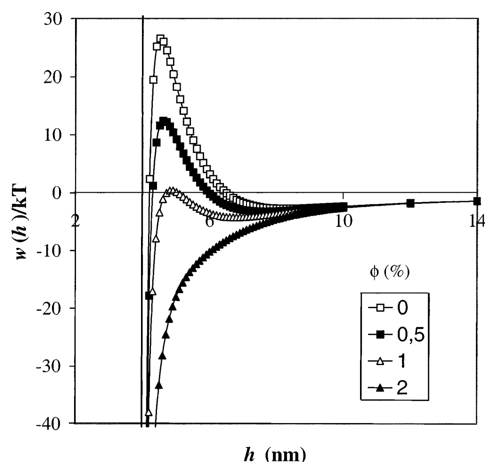

Figure 6. Theoretical prediction of the influence of surface hydrophobicity on droplet–droplet interactions in a protein stabilized oil-in-water emulsion. The parameters used in the calculations are the same as those for **Figure 6**; however, the ionic strength was assumed to be 150 mM, and the fractional hydrophobicity was varied (see text box).

solution conditions, we would not expect the droplets to aggregate into strong flocs, although they may form weak flocs due to the presence of the shallow secondary minimum to the right of the energy barrier. When the adsorbed β-Lg molecules unfolded, the surface hydrophobicity of the droplets increased, which meant that the hydrophobic attraction became increasingly strong and eventually dominated the electrostatic repulsion. Hence, the energy barrier either disappears or is no longer sufficiently high to prevent the droplets from falling into the deep primary minimum. Under these solution conditions, we would expect the droplets to aggregate into strong flocs, which was supported by our experimental measurements (**Figure 1**). These theoretical calculations suggest that only a small increase in the surface hydrophobicity of the droplets is required to promote droplet flocculation (<2% of the droplet surface), provided that the electrostatic repulsion is sufficiently small. The increase in surface hydrophobicity of nonadsorbed β-Lg upon thermal denaturation is well established in the literature (*34*−*36*). Nevertheless, changes in the surface hydrophobicity of emulsion droplets coated with β-Lg upon surface denaturation have not so far been measured, and so we cannot assess if the increase in surface hydrophobicity that we propose is reasonable.

CONCLUSIONS

We have proposed that surface denaturation of globular proteins after adsorption to oil droplet surfaces leads to extensive flocculation in oil-in-water emulsions. It is postulated that surface denaturation increases the surface hydrophobicity of emulsion droplets, which increases the hydrophobic attraction between droplets. In addition, surface denaturation exposes protein sulfhydryl groups to the aqueous phase, which promotes disulfide bond formation between proteins adsorbed to different emulsion droplets. When the electrostatic repulsion between the droplets is sufficiently low (relatively high salt concentrations), the increased hydrophobic attraction and disulfide bond formation is enough to promote droplet flocculation. Additional insights into the origin of droplet flocculation in globular protein-stabilized emulsions will require the application of analytical techniques that can provide information about conformation changes and interactions of globular proteins after adsorption to emulsion droplet surfaces. The data obtained in this study is important for improving the functionality of globular proteins as emulsifiers in food, health care, and pharmaceutical products.

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LITERATURE CITED

(1) Dickinson, E. *Introduction to Food Colloids*; Oxford University Press: Oxford, England, 1992.
(2) Damodaran, S. Amino acids, peptides and proteins. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Marcel Dekker: New York, 1996; p 321.
(3) Dalgleish, D. G. Food emulsions. In *Emulsions and Emulsion Stability*; Sjoblom, J. Ed; Marcel Dekker: New York, 1996.
(4) Nakai, S.; Modler, H. W. *Food Proteins: Properties and Characterization*; VCH Publishers: New York, 1996.
(5) Euston, S. R.; Hirst, R. L. The emulsifying properties of commercial milk protein products in simple oil-in-water emulsions and in a model food system. *J. Food Sci.* **2000**, *65*, 934−940.
(6) Wilde, P. J. Interfaces: their role in foam and emulsion behaviour. *Curr. Opin. Colloid Int.* **2000**, *5*, 176−181.
(7) Kinsella, J. E.; Whitehead, D. M.. Proteins in whey: Chemical, physical and functional properties. *Adv. Food Nutr. Res.* **1989**, *33*, 343−438.
(8) Phillips, L. G.; Whitehead, D. M.; Kinsella, J. E. *Structure−Function Properties of Food Proteins*; Academic Press: San Diego, CA, 1994.
(9) Dickinson, E. Adsorbed protein layers at fluid interfaces: interactions, structure and surface rheology *Colloids Surf., B* **1999**, *15*, 161−176.
(10) Bergensthanl, B. A.; Claesson, P. M. Surface forces in emulsions. In *Food Emulsions*, 3rd ed.; Friberg S. E., Larsson, K., Eds.; Marcel Dekker: New York, 1997; pp 57−109.
(11) McClements, D. J. *Food Emulsions*: *Principles, Practice and Techniques;* CRC Press: Boca Raton, FL, 1999.
(12) 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*, 26−32.
(13) McClements, D. J.; Monahan, F. J.; Kinsella, J. E. Disulfide bond formation affects stability of whey-protein isolate emulsions. *J. Food Sci.* **1993**, *58*, 1036−1039.
(14) Damodaran, S.; Anand, K. Sulfhydryl-disulfide interchange-induced interparticle protein polymerization in whey protein-stabilized emulsions and its relation to emulsion stability. *J. Agric. Food Chem.* **1997**, *45*, 5, 3813−3820.
(15) MacRitchie, F. Reversibility of protein adsorption. In *Proteins at Interfaces*; Mobius, D., Miller, R., Eds.; Elsevier Science: London, 1998; pp 149−177.
(16) Castelain, C.; Genot, C. Partition of adsorbed and nonadsorbed bovine serum albumin in dodecane-in-water emulsions calculated from front-face intrinsic fluorescence measurements. *J. Agric. Food Chem.* **1996**, *44*, 4, 1635−1640.
(17) Castelain, C.; Genot, C. Conformational-changes of bovine serum albumin upon its adsorption in dodecane-in-water emulsions as revealed by front-face steady-state fluorescence. *Biochim. Biophys. Acta* **1994**, *1199*, 59−64.
(18) Fang, Y.; Dalgleish, D. G. Conformation of β-lactoglobulin studied by FTIR: Effect of pH, temperature, and adsorption to the oil−water interface. *J. Colloid Interface Sci.* **1997**, *196*, 292−298.
(19) Rampon, V.; Lethuaut, L.; Mouhous-Riou, N.; Genot, C. Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by front-surface fluorescence. *J. Agric. Food Chem.* **2001**, *49*, 4046−4051.
(20) Corredig, M., & Dalgleish, D. G. A differential microcalorimetric study of whey proteins and their behaviour in oil-in-water emulsions. *Colloids Surf., B* **1995**, *4*, 411.

(21) Dufour, E.; Dalgalarrondo, M.; Adam, L. Conformation of β-lactoglobulin at an oil/water interface as determined from proteolysis and spectroscopic methods. *J. Colloid Interface Sci.* **1998**, *207*, 264−272.
(22) Monahan, F. J.; McClements, D. J.; Kinsella, J. E. Polymerization of whey proteins in whey protein stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826−1831.
(23) Apenten, R. K. O.; Galani, D. Protein stability function relations: native beta-lactoglobulin sulphhydryl-disulphide exchange with PDS. *J. Sci. Food Agric.* **2000**, *80*, 447−452.
(24) Bentaleb, A.; Abele, A.; Haikel, Y.; Schaaf, P.; Voegel, J. C. FTIR-ATR and radiolabeling study of the adsorption of ribonuclease A onto hydrophilic surfaces: Correlation between the exchange rate and the interfacial denaturation. *Langmuir* **1998**, *14*, 6493−6500.
(25) Murray, B. S. Interfacial rheology of mixed food protein and surfactant adsorption layers with respect to emulsion and foam stability. In *Proteins at Interfaces*; D. Mobius, R. Miller, Eds.; Elsevier Science: London, England, 1998; pp 179−220.
(26) Patino, J. M. R.; Nino, M. R. R.; Sanchez, C. C. Adsorption of whey protein isolate at the oil−water interface as a function of processing conditions: A rheokinetic study. *J. Agric. Food Chem.* **1999**, *47*, 2241−2248.
(27) Langevin, D. Influence of interfacial rheology on foam and emulsion properties. *Adv. Colloid Interface Sci.* **2000**, *88*, 209−222.
(28) Bos, M. A.; van Vliet, T. Interfacial rheological properties of adsorbed protein layers and surfactants: a review. *Adv. Colloid Interface Sci.* **2001**, *91*, 437−471.
(29) Demetriades, K.; Coupland. J. N.; McClements, D. J. Physicochemical properties of whey protein-stabilized emulsions as affected by heating and ionic strength. *J. Food Sci.* **1997**, *62*, 462−467.
(30) Monahan, F. J.; McClements, D. J.; German, J. B. Disulfide-mediated polymerization reactions and physical properties of heated WPI-stabilized emulsions. *J. Food Sci.* **1996**, *61*, 504−510.
(31) Poon, S.; Clarke, E.; Schultz, C. J. Effect of denaturants on the emulsifying activity of protein. *J. Agric. Food Chem.* **2001**, *49*, 281−286.
(32) Stuart. M. A. C. Marcomolecular adsorption: A brief introduction. In *Biopolymers at Interfaces*; Malmsten, M., Ed.; Marcel Dekker: New York, 1998; pp 1−25.
(33) Keomaneechai, E.; McClements, D. J. Effect of calcium and potassium ions on physicochemical properties and stability of model nutritional beverages. *J. Food Sci.* **2002**, *67*, 665−671.
(34) Iametti, S.; DeGregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat treatment of beta-lactoglobulin. *Eur. J. Biochem.* **1996**, *237*, 106−112.
(35) Relkin, P. Reversibility of heat-induced conformational changes and surface exposed hydrophobic clusters of beta-lactoglobulin: their role in heat-induced sol−gel state transition. *Int. J. Biol. Macromol.* **1998**, *22*, 59−66.
(36) Manderson, GA.; Hardman, MJ.; Creamer, LK. Effect of heat treatment on bovine beta-lactoglobulin A, B and C explored using thiol availability and fluorescence. *J. Agric. Food Chem.* **1999**, *47*, 3617−3627.

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