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Evidence that Homogenization of BSA-Stabilized Hexadecane-in-Water Emulsions Induces Structure Modification of the Nonadsorbed Protein

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The structural modification of globular proteins (bovine serum albumin, BSA) in the aqueous phase of emulsions produced by homogenization was studied using front-face fluorescence spectroscopy (FFFS). A series of hydrocarbon oil-in-water emulsions (30 wt % *n*-hexadecane, 0.35 wt % BSA, pH 7.0) were homogenized to differing degrees with a high-speed blender and a high-pressure valve homogenizer. The wavelength of the maximum in the tryptophan emission spectrum (λ_{max}) of serum phases collected from the emulsions by centrifugation was measured and compared to λ_{max} values of BSA solutions subjected to the same homogenization conditions. There was no significant ($p < 0.05$) change in λ_{max} with homogenization conditions for BSA solutions. In contrast, λ_{max} of serum phases from emulsions blended for 2 min in a high-speed blender was significantly smaller ($p < 0.05$) than nontreated BSA solutions ($\Delta\lambda_{max} = 2$ nm). In addition, there was a further significant decrease in λ_{max} of the serum phases with an increasing number of passes of the emulsion through the high-pressure valve homogenizer (e.g., $\Delta\lambda_{max} = 4$ nm for 12 passes). This study shows that globular proteins present in the aqueous phase of a hexadecane-in-water emulsion after homogenization could be altered, which is probably caused by surface modification of the protein structure during temporary adsorption to emulsion droplet surfaces during homogenization.

KEYWORDS: BSA; emulsions; fluorescence; homogenization; adsorption

INTRODUCTION

Proteins are often used as functional ingredients in the food industry, because they facilitate emulsion formation and stability (*1*−*4*). Previous studies of the molecular and physicochemical basis of the functional properties of proteins in emulsions have largely focused on the role of proteins adsorbed to the surfaces of emulsion droplets (*1*−*5*). Nevertheless, nonadsorbed proteins have also been shown to influence the stability and physicochemical properties of oil-in-water emulsions (*6*−*10*). The stability of whey protein stabilized emulsions to droplet flocculation during heating decreased when the concentration of nonadsorbed protein in the aqueous phase was increased (*9*). This decrease was attributed to the ability of the thermally denatured nonadsorbed proteins to act as cross-links between the droplets. The addition of caseinate to the continuous phase of oil-in-water emulsions has been shown to induce droplet flocculation, which was attributed to their ability to form aggregates that promoted depletion flocculation (*7*, *8*, *10*). The extent of droplet flocculation in casein-stabilized emulsions decreased when calcium ions were added to the aqueous phase because the ions promoted additional protein aggregation, thereby reducing the osmotic driving force for depletion flocculation (*6*). Finally, the presence of nonadsorbed proteins influenced the stability of lipids to oxidation in oil-in-water emulsions, because of their ability to act as antioxidants (*11*). It is therefore important to obtain an improved understanding of the role of nonadsorbed proteins on the physicochemical properties of oil-in-water emulsions. In particular, the influence of the type, concentration, and structure of the nonadsorbed proteins on emulsion properties should be better known.

In a recent study using front-face fluorescence spectroscopy (FFFS), we observed that the fluorescence emission spectrum of nonadsorbed BSA in the serum phase of emulsions produced by high-pressure homogenization was different from that of native BSA dissolved in aqueous solution (*12*). This result suggested that the molecular characteristics of the proteins in the aqueous phase of the emulsions had been altered. However, it was not possible to conclude if the alteration occurred during the preparation or storage (1 day) of the emulsions. At least three mechanisms could account for this effect. First, the intense mechanical forces that the proteins experienced during homog-

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enization could have promoted their structure modification and/or aggregation. Second, nonadsorbed native proteins present in the aqueous phase of emulsions immediately after homogenization could have exchanged with surface-modified proteins during emulsion storage. Third, some of the nonadsorbed proteins present in the aqueous phase of the final emulsion may have been temporarily adsorbed to the surfaces of oil droplets during homogenization, where their structures were surface-modified prior to desorption. The objective of the current work is to provide further insights into the influence of the homogenization step on the modification of the structure of nonadsorbed globular proteins in emulsions. In particular, we hope to provide some information about the relative importance of the three mechanisms mentioned above. For this purpose hexadecane-in-water emulsions stabilized by BSA were studied by FFFS.

MATERIALS AND METHODS

Three different experiments were carried out to elucidate the influence of homogenization on the properties of nonadsorbed protein in oil-in-water emulsions. First, we examined the influence of the severity of the homogenization conditions on the FFFS spectra of BSA in aqueous solutions and in serum phases collected from emulsions. Second, we examined the influence of storage time on the FFFS spectra of BSA in serum phases collected from emulsions. Third, we examined the influence of contaminating oil droplets (which were present in the serum phases collected from the emulsions) on the FFFS signal of BSA solutions.

Materials. Analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium azide (NaN_3), polyoxyethylene sorbitan monolauarate (Tween 20), and *n*-hexadecane were purchased from the Sigma Chemical Co. (St. Louis, MO). Powdered BSA (Lot 7602E, Fraction V) was obtained from ICN Chemicals Inc. (Aurora, OH). Deionized water was used for the preparation of all solutions (18 MΩ resistivity, MilliQ Water Purification System).

Influence of Homogenization Conditions on FFFS Spectra of BSA. An emulsifier solution was prepared by dispersing 5 g/L of BSA and 0.2 g/L of sodium azide (as an antimicrobial) in deionized water. The resulting solution was stirred for 3 h to ensure complete dissolution of the protein, and then the pH was adjusted to 7.0 with 1 M NaOH solution. The protein solution was then stored at 4 °C for 20 h before use and considered as the control native BSA solution. Hexadecane oil-in-water emulsions were prepared from 30 wt % (45 g) *n*-hexadecane and 70 wt % (105 g) emulsifier solution at room temperature. Hexadecane oil and emulsifier solution were blended using a high-speed blender at 20 000 rpm for 2 min (Polytron PT 6100, Kinematica, Littau, Switzerland), and then the resulting emulsion was passed through a one-stage high-pressure valve homogenizer (Stansted Fluid Power, UK) at 7 MPa for a determined number of times. Aliquots of the emulsion were collected for analysis immediately after high-speed blending (0 passes) and after 1, 2, 4, 8, and 12 passes of the emulsion through the homogenizer. The temperature of the emulsions never exceeded 30 °C during the homogenization process, as determined by a digital thermometer dipped into the emulsion. All the emulsions were stored for 1 h at 20 °C prior to centrifugation and analysis. Duplicate experiments were carried out.

Emulsifier solutions (5.0 g/L of BSA, 0.2 g/L of sodium azide, pH 7.0) were subjected to the same homogenization conditions as the emulsions and were also stored for 1 h at 20 °C prior to analysis.

We observed that the serum phases collected from the emulsions (see below) were slightly turbid. We therefore measured the size distribution of the particles (see below) in the serum phase collected from an emulsion that had been passed through the high-pressure valve homogenizer 12 times: $d_{32} = 0.2$ μm (Malvern, Worcs, U.K.). In addition, a centrifugation of the serum phase was carried out for 30 min at 15 000*g* in an attempt to separate the contaminating particles from the continuous phase.

Influence of Storage Time on FFFS Spectra of BSA. To study the effect of storage time on the FFFS spectra of nonadsorbed proteins, a hexadecane oil-in-water emulsion was prepared as described above by blending followed by 12 passes through the high-pressure valve homogenizer. This emulsion was then diluted with native BSA solution (0.5 wt %) immediately after homogenization to produce a final emulsion containing 20 wt % *n*-hexadecane, 0.23 wt % BSA added before homogenization, and 0.17 wt % BSA added after homogenization. The final emulsion was divided into three portions, which were stored at room temperature for 1 h, 24 h, or 1 week. Duplicate experiments were carried out.

Influence of Droplet Concentration on FFFS Spectra of BSA Solutions. The influence of contaminating hexadecane droplets on the front-face fluorescence spectra of protein solutions was examined by adding known quantities of hexadecane droplets to BSA solutions. A 10 wt % *n*-hexadecane oil-in-water emulsion stabilized by Tween 20 (2 wt %) with a mean droplet diameter as close as possible to the value of the droplets in the serum phase was prepared: $d_{32} = 0.3$ μm (Malvern, Worcs, U.K.). The nonionic surfactant Tween 20 was used to prepare these emulsions, because it was not possible to make emulsions with mean droplet diameters less than about 1 μm using BSA with the one-stage homogenizer. A series of solutions containing 5 g/L of BSA and different concentrations of hexadecane droplets (0−0.08 vol %) was then prepared by adding different concentrations of hexadecane oil-in-water emulsions to a 15 g/L BSA solution.

Particle Size Measurements. The particle size distribution of the emulsions was measured just after their preparation and after storage using a laser diffraction instrument (Malvern, Worcs, U.K.), as described previously (*12*). Particle size measurements are reported as volume-surface mean diameters, d_{32} ($=\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of particles with diameter d_i). The measured mean particle diameters were highly reproducible (<5% difference) and are reported as the average of measurements made on two samples.

Collection of Serum Phases. Serum phases of emulsions were collected by centrifugation of the BSA emulsions. Centrifugation was carried out by placing aliquots of emulsions in 16 mL plastic tubes (Nalgen) and centrifuging for 60 min at 1000*g* to separate them into a creamed layer and a serum layer. The serum layers were then collected by making small holes in the bottom of each of the plastic tubes and decanting off the lower transparent phase.

Free Protein Concentration Measurements. The free protein concentration of the serum phases was determined using a modified Lowry method (*13*), as described in a previous article (*12*), and was used to calculate the adsorbed protein concentration.

Turbidity Measurements. Turbidity measurements were made at 600 nm (UV−visible spectrophotometer) on serum phases of emulsion homogenized under different conditions, on homogenized BSA solutions and on BSA solutions with different concentrations of hexadecane droplets.

Front-Face Fluorescence Measurements. Fluorescence emission spectra were collected using a spectrofluorometer (4800C, SLM Instruments, Urbana, IL). The spectra were recorded from 300 to 380 nm with a 1 nm step, with the excitation wavelength set at 290 nm using the conditions described previously (*12*). The spectra were then smoothed using the Loess procedure (5% smoothing). Quadratic interpolation was used to obtain a wavelength increment of 0.2 nm between successive points (Peakfit software, 4th version, Jandel Scientific, Chicago, IL). Spectra were measured on two or three different aliquots of each sample at 20 ± 1 °C. The height (I_{max}) and wavelength (λ_{max}) of the fluorescence emission maximum were determined as described previously (*12*). The spectra of serum phases, homogenized BSA solutions, native BSA solutions, and BSA solutions with different droplet concentrations were recorded. One-way analysis of variance was performed on I_{max} and λ_{max} to determine whether the effect of homogenization on protein structure was statistically significant (StatGraphics Software, 3rd edition, Statistical Graphics Corp., Rockville, MD). Means were compared with the least-squares difference (LSD) test.

RESULTS AND DISCUSSION

Influence of Homogenization on Droplet Size and Nonadsorbed Protein Concentration. The influence of homogenization conditions on mean droplet diameter and on the droplet

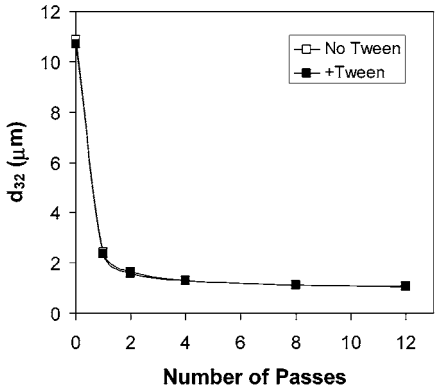

Figure 1. Influence of homogenization conditions on the mean droplet diameters of diluted *n*-hexadecane-in-water emulsions stabilized by BSA (pH 7.0). Measurements were made before and after 1 wt % Tween 20 was added to the emulsions to disrupt any flocs formed.

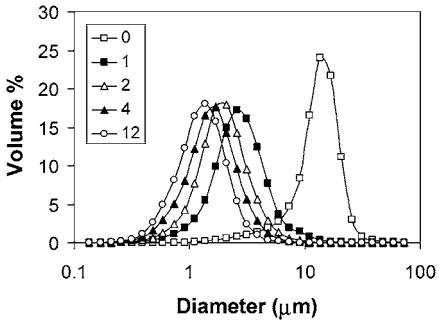

Figure 2. Influence of homogenization conditions on the particle size distribution of diluted *n*-hexadecane-in-water emulsions stabilized by BSA (pH 7.0). Numbers in the annotation box refer to the number of passes of the emulsion through a high-pressure valve homogenizer.

size distribution are shown in **Figures 1** and **2**, respectively. As expected, the droplet size decreased as the number of passes through the homogenizer increased, with the largest decrease occurring between the emulsion that had been blended (0 passes) and the one that had been passed once through the homogenizer (**Figure 1**). All the emulsions had monomodal size distributions (**Figure 2**). The particle size distributions of the emulsions that had been passed through the homogenizer had fairly similar shapes, regardless of the number of passes, but they shifted toward smaller droplet sizes as the number of passes was increased (**Figure 2**). The addition of 1 wt % Tween 20 to the emulsions prior to making the particle size measurements had no significant influence on the measured mean particle diameters (**Figure 1**), which indicated that the emulsion droplets were not flocculated.

The concentration of BSA remaining in the aqueous phase of the emulsions after homogenization and storage for 24 h at 20 °C was measured (**Table 1**). Approximately 95% of the BSA remained in the aqueous phase of the emulsion that had only been blended, which was due to the fact that this emulsion only had a relatively low specific interfacial area (**Table 1**). The percentage of protein in the aqueous phase decreased progressively as the number of passes of the emulsion through the homogenizer was increased, indicating that progressively more protein adsorbed to the droplet surfaces as the mean size of the droplets decreased and the specific interfacial area increased (**Table 1**). After 12 passes, about 35% of the BSA remained in the aqueous phase of the emulsion. The specific surface area, that is the surface area of hexadecane−water interface per cubic meter of emulsion, was calculated from the droplet volume

Table 1. Influence of Homogenization Conditions on Serum Phase Concentration (BSA Concentration), Serum Phase as a Percentage of Original Concentration (BSA Percentage), Specific Surface Area (S_A), and Surface Load (Γ) of BSA in *n*-Hexadecane-in-Water Emulsions (pH 7)[a]

passes	BSA concn (g/L)	BSA percentage (%)	S_A (10^6 m^2/m^3)	Γ (mg/m^2)
0	4.76	95.2	0.20	0.78
1	3.65	73.0	0.91	0.97
2	3.18	63.6	1.36	0.88
4	2.56	51.2	1.63	0.96
8	2.05	41.0	1.90	1.00
12	1.73	34.6	2.01	1.05

[a] Values are the mean of two measurements. Deviations were lower than 5% for BSA concentration, BSA percentage, and S_A and lower than 10% for Γ.

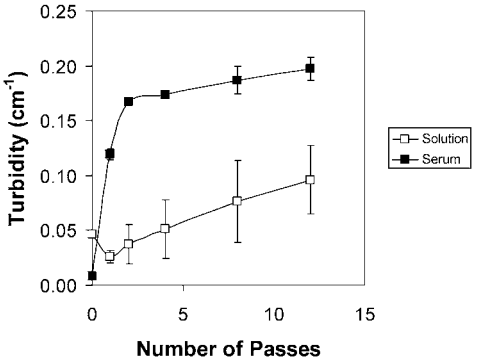

Figure 3. Influence of homogenization conditions on the turbidity of serum phases separated from *n*-hexadecane-in-water emulsions and of BSA solutions.

fraction (ϕ) and mean droplet diameter (d_{32}): $S_A = 6\phi/d_{32}$. The surface load of the droplets (Γ), that is, the mass of protein adsorbed per unit interfacial area, was calculated from the adsorbed protein concentration (C_{ads} in mg m^{-3} of emulsion) and the specific surface area: $\Gamma = C_{ads}/S_A$. The surface load increased from about 0.78 ± 0.03 mg m^{-2} for the emulsion that had been blended to 1.06 ± 0.03 mg m^{-2} for the emulsion that had been passed 12 times through the homogenizer. The emulsions that had been homogenized in the high-pressure valve homogenizer all had fairly similar surface loads, between 0.9 and 1.1 mg m^{-2}, suggesting that the droplet surfaces were saturated with protein. These values are typical for those found in the literature for globular proteins (*3*).

Influence of Homogenization on Turbidity. The turbidity of BSA solutions (5 g/L protein) and on serum phases collected from emulsions (1.5−4.8 g/L protein) is shown in **Figure 3**. There was a slight increase in the turbidity of the BSA solutions with increasing number of passes through the homogenizer. On the other hand, there was a steep increase in the turbidity of the serum phases collected from the emulsions on going from 0 passes (blended only) to 2 passes through the homogenizer, but only a slight increase in turbidity from 2 to 12 passes (**Figure 3**). This increase in turbidity with homogenization severity suggests that there were some particles formed that were large enough to scatter light. In the case of BSA solutions, these particles were probably a small fraction of the protein molecules that had associated with each other during homogenization, possibly due to some foaming of the solutions leading to surface modification of the protein conformation. In the case of the serum phases, these particles could have been due to protein aggregates or due to emulsion droplets. It is possible that the particles could interfere with the subsequent FFFS analysis, and

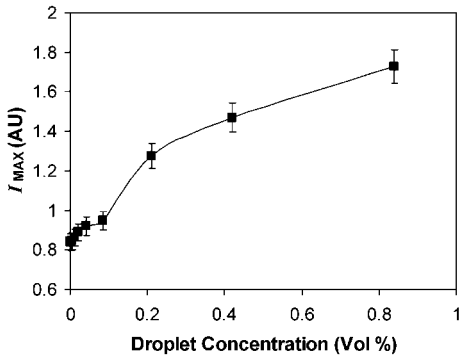

Figure 4. Influence of added droplet concentration on the height of the maximum in the FFFS tryptophan emission spectrum (I_{max}) of BSA solutions (5 g/L). Added droplets were stabilized by Tween 20.

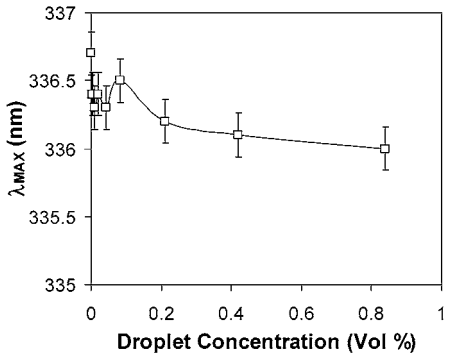

Figure 5. Influence of added droplet concentration on the wavelength of the maximum in the tryptophan emission spectrum (λ_{max}) of BSA solutions (5 g/L) measured by FFFS. Added droplets were stabilized by Tween 20.

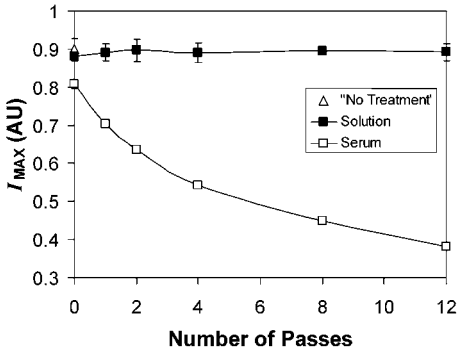

Figure 6. Influence of homogenization conditions on the height of the maximum in the tryptophan emission spectrum (I_{max}) of serum phases separated from emulsions and of BSA solutions (5 g/L) measured by FFFS. "No treatment": value obtained for the native BSA solution.

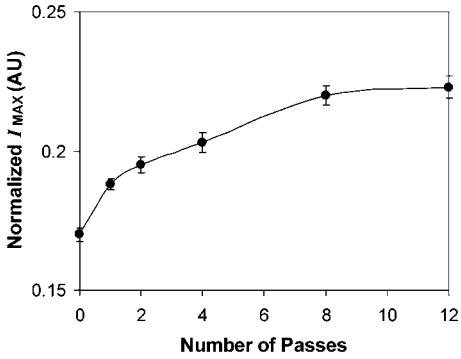

Figure 7. Influence of homogenization conditions on the normalized height of the maximum in the tryptophan emission spectrum (normalized I_{max}) of serum phases separated from emulsions. Normalized I_{max} corresponds to I_{max} divided by the concentration of protein in the serum phase.

therefore we carried out further studies to identify the origin of the particles and to determine their effect on the fluorescence measurements.

After centrifugation of the serum phases at 15000*g*, a thin white layer was observed at the top of the tubes. This layer was attributed to the upward movement of hexadecane droplets, since hexadecane has a lower density than water, whereas protein has a higher density than water and so protein aggregates should move downward and form pellets at the bottom of the tubes, which was not observed. Thus, the turbidity of the serum layers collected from the emulsions was due to the presence of hexadecane droplets. These droplets were probably so small that they did not cream appreciably during the initial centrifugation used to collect the serum phases from the emulsions. This would also explain why the turbidity of the serum layers was considerably higher for 2−12 passes through the homogenizer, since the droplets were smaller in these emulsions (**Figure 1**).

Influence of Contaminating Droplets on FFFS Spectra. The solutions containing different concentrations of hexadecane droplets were analyzed by FFFS. As the droplet concentration increased in the emulsions, there was an appreciable increase in I_{max} (the means were distributed into nine groups with an LSD statistical test) (**Figure 4**) and a slight decrease in λ_{max} (the means were distributed into three groups with an LSD test) (**Figure 5**). The slight decrease in λ_{max} can be attributed to the presence of Tween 20 in the emulsions, since this surfactant has previously been shown to cause a blue shift in λ_{max} when it interacts with BSA (*12*). The same authors also observed a decrease in I_{max} with increasing Tween 20 concentration in BSA solutions. An increase in I_{max} with increasing droplet (and Tween 20) concentration was observed in the present study (**Figure 4**), which suggests that the influence of the droplets on I_{max} is greater than the influence of Tween 20−BSA interactions. The turbidity (τ) of the protein solutions increased approximately linearly with droplet concentration from 0 to 0.08 vol % hexadecane: $\tau = 3.25\phi\% + 0.05\ cm^{-1}$, $r^2 = 0.997$, where $\phi\%$ is the volume percentage of droplets present (data not shown). This would suggest that the concentration of hexadecane droplets in the serum phases collected from the emulsions (from 2 to 12 passes) was between 0.04 and 0.05 vol %, since their turbidities were between 0.17 and 0.2 (**Figure 3**). At these low droplet concentrations, we would therefore expect that the presence of the hexadecane droplets would influence the I_{max} measurements (means were found different with the LSD test) but would have little influence on the λ_{max} measurements (means were not found different with the LSD test). Moreover, at these low droplet concentrations the amount of any BSA adsorbed to the emulsion droplets would be expected to be so small (<0.001 wt %) that it had no significant influence on λ_{max} (*14*).

Influence of Homogenization on FFFS Spectra. Fluorescence spectra of serum phases collected by centrifugation of BSA-stabilized emulsions that had been subjected to increasingly severe homogenization conditions were measured, and the values of I_{max} and λ_{max} were determined. I_{max} decreased ($p < 0.05$) with increasing degree of homogenization (**Figure 6**), primarily because there was a decrease in BSA concentration in the serum phases as the number of passes of the emulsion through the homogenizer was increased (**Figure 3**). Nevertheless, we found that there was actually an increase in the normalized height of the fluorescence spectra (**Figure 7**). This increase in normalized intensity could have occurred because of the presence of contaminating emulsion droplets. This hypothesis is consistent

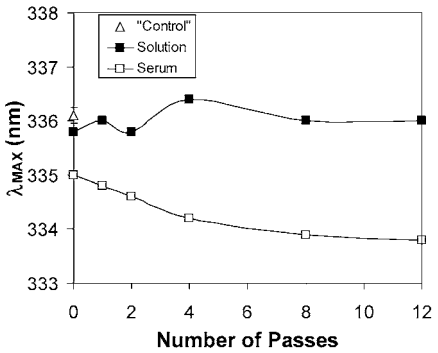

Figure 8. Influence of homogenization conditions on the wavelength of the maximum in the tryptophan emission spectrum (λ_{max}) of serum phases separated from emulsions and of BSA solutions (5 g/L) measured by FFFS. "Control": value obtained for the native BSA solution.

Table 2. Influence of Homogenization Conditions on λ_{max} of BSA in Solution and in the Serum Phase Collected by Centrifugation of Emulsions[a]

passes	serum phase	soln
control	336.1 (a)	336.1 (a)
0	335.0 (b)	335.8 (a)
1	334.8 (b)	336.0 (a)
2	334.6 (b, c)	335.8 (a)
4	334.2 (c, d)	336.4 (a)
8	333.9 (d)	336.0 (a)
12	333.8 (d)	336.0 (a)

[a] Values are the mean of two measurements. Samples marked with different letters are different at the 5% confidence level. Control: native BSA solution.

with the measurements presented in the previous section (**Figure 4**). I_{max} was independent of homogenization treatment ($p > 0.05$) for the 5 g/L BSA solutions (**Figure 6**). This was partly because the protein concentration did not change in this case, but it also suggests that there was no appreciable change in the fluorescence characteristics of the BSA in solution induced by homogenization. There was a significant decrease of λ_{max} ($p < 0.05$) for the serums collected from the emulsions when the number of passes through the homogenizer increased, whereas λ_{max} was independent of the severity of homogenization treatment ($p > 0.05$) for the BSA solutions (**Figure 8**; **Table 2**). The above changes in λ_{max} suggest that the protein structure was not influenced by homogenization conditions when the BSA was dispersed in water in the absence of hexadecane droplets but that it was partially modified when the BSA was contained in an emulsion. Rampon et al. (*15*) did not observe differences in λ_{max} when they compared native BSA solutions with BSA in serum phases collected from emulsions made with sunflower oil. It is possible that the dispersed phase (hexadecane) used in the present experiment had a greater impact on the structure of the protein than sunflower oil because of its greater hydrophobicity.

Changes in FFFS Spectra during Storage. The serum phase of the emulsion diluted with native BSA solution showed a value of λ_{max} between the λ_{max} of native protein solution and that of the serum phase of the emulsion without addition of native protein (data not shown). This was due to an increase in the proportion of native protein relative to the adsorbed surface-modified protein in the continuous phase of the emulsion. Nevertheless, no significant change in serum phase fluorescence spectra with storage time was observed (data not shown). This suggests that there was no exchange of free native protein with adsorbed surface-modified protein during storage; otherwise, we would have expected a significant blue shift in λ_{max}.

Origin of Protein Structure Modification Caused by Homogenization. In this section, we use the above data to provide some insight into the molecular origin of the modification of the BSA structure in the aqueous phase of emulsions produced by homogenization. In the introduction we postulated three possible mechanisms that could lead to structure modification of the nonadsorbed proteins in the emulsions.

1. The intense mechanical forces experienced by globular proteins during homogenization promoted structure modification and/or aggregation.

2. Native nonadsorbed proteins present in the aqueous phase of the emulsions immediately after homogenization exchanged with surface proteins, whose structure was modified during emulsion storage.

3. During homogenization proteins are adsorbed to the surface of freshly formed droplets, where they undergo conformational changes. During homogenization some droplets are being disrupted while others are coalescing; hence, some of the surface-modified proteins adsorbed to the droplets may leave the droplet surfaces and end up in the aqueous phase.

The fact that we did not see any significant change in the fluorescence spectra of BSA solutions that were homogenized using the same conditions as those used to homogenize the emulsions suggests that these conditions were not sufficient in themselves to promote protein structure modification. This is not surprising, since previous studies of static-hydrostatic-pressure treatment of BSA solutions have shown that pressures exceeding hundreds of megapascals are needed to irreversibly denature the protein at ambient temperature and neutral pH (*16*, *17*). In addition, in a high-pressure homogenizer, the pressure is only applied for a very short time (probably not long enough to induce thermodynamic changes) and the globular proteins are too small to be influenced by the high local spatial and temporal gradients that cause droplet disruption. Hence, we can eliminate the first mechanism listed above. The fact that we did not see any significant change in serum phase fluorescence spectra of emulsions containing added native protein after 1 h, 1 day, or 1 week suggests that native proteins in the aqueous phase did not exchange with surface-modified proteins adsorbed to the droplet surfaces. It therefore seems likely that the second mechanism listed above is also not responsible for the presence of structure-modified proteins in the aqueous phase of the emulsions.

Previous studies have shown that the structure of globular proteins becomes modified after adsorption to oil−water interfaces (*18*−*20*), which has been referred to as "surface denaturation". In our experiment, we preferred to speak about "protein structure modification", because our fluorescence data did not indicate direct evidence of protein unfolding. It has also been demonstrated that repeated homogenization of oil-in-water emulsions in high-pressure valve homogenizers leads to a steady-state balance between droplet disruption and droplet coalescence (*21*−*24*). It is therefore possible for some of the proteins that were adsorbed to the surfaces of emulsion droplets to become desorbed when droplets coalesce, releasing them into the aqueous phase. We therefore propose that the third mechanism listed above is the one that is most likely to account for the presence of structurally modified proteins in the aqueous phase of the emulsions.

CONCLUSION

The molecular characteristics of nonadsorbed proteins are known to alter the bulk physicochemical properties of oil-in-

water emulsions; hence, the fact that the nonadsorbed proteins may be modified during emulsion preparation may have important implications for the creation of stable oil-in-water emulsions.

LITERATURE CITED

(1) Dickinson, E. Adsorbed protein layers at fluid interfaces: interactions, structure, and surface rheology. *Colloids Surf. B* **1999**, *15*, 161−176.

(2) Dickinson, E. Properties of emulsions stabilized with milk proteins: overview of some recent developments. *J. Dairy Sci.* **1997**, *80*, 2607−2619.

(3) Dickinson, E. *An Introduction to Food Colloids*; Oxford Science Publishers: Oxford, England, 1992.

(4) McClements, D. J. *Food Emulsions*: *Principles, Practice and Techniques*; CRC Press: Boca Raton, FL, 1998.

(5) Dickinson, E. Milk protein interfacial layers and the relationship to emulsion stability and rheology. *Colloids Surf. B* **2001**, *20*, 197−210.

(6) Dickinson, E.; Golding, M. Influence of calcium ions on creaming and rheology of emulsions containing sodium caseinate. *Colloids Surf. A* **1998**, *144*, 167−177.

(7) Dickinson, E.; Ritzoulis, C.; Povey, M. J. W. Stability of emulsions containing both sodium caseinate and Tween 20. *J. Colloid Int. Sci.* **1999**, *212*, 466−473.

(8) Euston, S. R.; Hirst, R. L. Comparison of the concentration-dependent emulsifying properties of protein products containing aggregated and nonaggregated milk protein. *Int. Dairy J.* **1999**, *9*, 693−701.

(9) Euston, S. R.; Finnigan, S. R.; Hirst, R. L. Aggregation kinetics of heated whey protein-stabilized emulsions. *Food Hydrocolloids* **2000**, *14*, 155−161.

(10) Srinivasan, M.; Singh, H.; Munro, P. A. Creaming stability of oil-in-water emulsions formed with sodium and calcium caseinates. *J. Food Sci.* **2001**, *66*, 441−446.

(11) Tong, L. M.; Sasaki, S.; McClements, D. J.; Decker, E. A. Mechanisms of the antioxidant activity of a high molecular weight fraction of whey. *J. Agric. Food Chem.* **2000**, *48*, 1473−1478.

(12) Rampon, V.; Genot, C.; Riaublanc, A.; Anton, M.; Axelos, M. A. V.; McClements, D. J. Front-face fluorescence spectroscopy study of globular proteins in emulsions: Displacement of BSA by a nonionic surfactant. *J. Agric. Food Chem.* **2003**, *51*, 2482−2489.

(13) Markwell, M. A.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206−210.

(14) 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*, 1635−1640.

(15) 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.

(16) Galazka, V. B.; Ledward, D. A.; Sumner, I. G.; Dickinson, E. Influence of high pressure on bovine serum albumin and its complex with dextran sulfate. *J. Agric. Food Chem.* **1997**, *45*, 3465−3471.

(17) Hosseini-Nia, T.; Ismail, A. A.; Kubow, S. Effect of high hydrostatic pressure on the secondary structures of BSA and apo- and holo-alpha-lactalbumin employing Fourier transform infrared spectroscopy. *J. Food Sci.* **2002**, *67*, 1341−1347.

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

(19) 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.

(20) MacRitchie, F. Reversibility of protein adsorption. In *Proteins at Interfaces*; Mobius, D., Miller, R., Eds., Elsevier Science: London, 1998; pp 149−177.

(21) Taisne, L.; Walstra, P.; Cabane, B. Transfer of oil between emulsion droplets. *J. Colloid Interface Sci.* **1996**, *184*, 378−390.

(22) Narsimhan, G.; Goel, P. Drop coalescence during emulsion formation in a high-pressure homogenizer for tetradecane-in-water emulsion stabilized by sodium dodecyl sulfate. *J. Colloid Interface Sci.* **2001**, *238*, 420−432.

(23) Lobo, L.; Svereika, A.; Nair, M. Coalescence during emulsification − 1. Method development. *J. Colloid Interface Sci.* **2002**, *253*, 409−418.

(24) Walstra, P. Principles of emulsion formation. *Chem. Eng. Sci.* **1993**, *48*, 333−350.

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