Effect of Thermal Treatment, Ionic Strength, and pH on the Short-Term and Long-Term Coalescence Stability of β-Lactoglobulin Emulsions

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We present experimental results about the effects of thermal treatment, ionic strength, and pH on the protein adsorption and coalescence stability of freshly prepared (2 h after emulsification) and 6-day-stored emulsions, stabilized by the globular protein β-lactoglobulin (BLG). In all emulsions studied, the volume fraction of the dispersed soybean oil is 30% and the mean drop diameter is $d_{32} \approx 40$ μm. The protein concentration, C_{BLG}, is varied between 0.02 and 0.1 wt %, the electrolyte concentration, C_{EL}, between 1.5 mM and 1 M, and pH between 4.0 and 7.0. The emulsion heating is performed at 85 °C, which is above the denaturing temperature of BLG. The results show that, at $C_{BLG} \geq 0.04$ wt %, $C_{EL} \geq 150$ mM, and pH ≥ 6.2, the heating leads to higher protein adsorption and to irreversible attachment of the adsorbed molecules, which results in enhanced steric repulsion between the protein adsorption multilayers and to higher emulsion stability. At low electrolyte concentration, $C_{EL} \leq 10$ mM, the emulsion stability is determined by electrostatic interactions and is not affected significantly by the emulsion heating. The latter result is explained by electrostatic repulsion between the adsorbed protein molecules, which keeps them separated from each other and thus precludes the formation of disulfide covalent bonds in the protein adsorption layer. The coalescence stability of heated and nonheated emulsions is practically the same and does not depend on C_{EL}, when pH is around the isoelectric point (IEP) of the protein molecules. This is explained with the adsorption of uncharged BLG molecules, in compact conformation, which stores the reactive sulfhydryl groups hidden inside the molecule interior, thus preventing the formation of covalent intermolecular bonds upon heating. We studied also the effect of storage time on the stability of heated and nonheated emulsions. The stability of nonheated emulsions ($C_{BLG} = 0.1$ wt %, $C_{EL} \geq 150$ mM, and pH = 6.2) significantly decreases after 1 day of storage (aging effect). In contrast, no aging effect is observed after emulsion heating. FTIR spectra of heated and nonheated, fresh and aged emulsions suggest that the aging effect is caused by slow conformational changes of the protein molecules in the adsorption layer, accompanied with partial loss of the ordered secondary structure of the protein and with the formation of lateral noncovalent bonds (H-bonds and hydrophobic interactions) between the adsorbed molecules. After thermal treatment of the BLG emulsions, the molecules preserve their original secondary structure upon storage, which eliminates the aging effect.

1. Introduction

Proteins are used as emulsifiers in the food, cosmetic, and pharmaceutical industries. The efficiency of protein molecules to stabilize emulsions depends strongly on the density and structure of the protein adsorption layers on drop surface.[1−15] The adsorption layers prevent the drop−drop coalescence by stabilizing the emulsion films, which form between two neighboring drops. The detailed molecular mechanisms of emulsion stabilization by adsorbed proteins are still poorly understood and under discussion in the literature.[12−16]

The ability of protein molecules to stabilize emulsion films is usually attributed to (1) formation of adsorption layers with certain mechanical stability, which protect the film against rupture,[2,15] or (2) creation of a barrier in the disjoining pressure, Π (force per unit area of the film), which opposes the film thinning and thus prevents the direct contact of the two opposite film surfaces.[3−6,11,12] Different factors are expected to play a decisive

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role in these two mechanisms. The rheological properties of the adsorption layers (such as interfacial elasticity and viscosity) are often deemed to be important for mechanism 1, whereas the electrostatic, van der Waals, and steric interactions are expected to govern mechanism 2.

In previous studies[10−12] we quantified the coalescence stability of β-lactoglobulin (BLG) emulsions in terms of the critical emulsion osmotic pressure leading to emulsion decay, P_{OSM}^{CR}, and compared the experimental results for the effect of several factors (protein concentration, pH, and electrolyte concentration) with theoretical estimates of the barrier in the disjoining pressure isotherm, Π_{MAX}. This comparison allowed us to distinguish[11] three qualitatively different cases of emulsion stabilization against coalescence: (1) electrostatically stabilized emulsions with monolayer protein adsorption; (2) sterically stabilized emulsions, in which the drop surfaces are covered by protein adsorption multilayers; and (3) sterically stabilized emulsions with a monolayer adsorption on the drop surface.

The coalescence stability of emulsions type 1 (obtained at low electrolyte concentration and away from the protein isoelectric point) was reasonably well described[11] by the DLVO theory, which accounts for the long-ranged electrostatic and van der Waals interactions between the emulsion droplets. In emulsions type 2 (obtained at high electrolyte and protein concentrations), the steric repulsion is created by overlapping protein adsorption multilayers. The stability of these emulsions was described by considering the steric + DLVO interactions, with the steric repulsion being modeled similarly to the case of dispersions stabilized by adsorbed polymers (the role of the polymer chains being played by adsorbed protein aggregates). In emulsions type 3 (obtained at low protein concentration and high electrolyte concentration), the coalescence stability of two neighboring drops is ensured by adsorption monolayers of protein molecules, which are in direct contact with each other, because the long-ranged electrostatic and steric repulsions are suppressed under these conditions. One could speculate that the stability of these emulsions is mostly governed by the rheological properties of the adsorption monolayers, but we are not aware of experimental data (neither ours nor those of other authors) that could support or reject unambiguously this idea.

Our previous studies of the emulsion coalescence stability (refs 10 and 11) were performed with nonheated, freshly prepared emulsions (their stability was evaluated ≈1 h after emulsion preparation). On the other hand, it is known from the literature that the conformation of the protein molecules and the structure of the adsorption layer change significantly upon heating and/or shelf-storage of the emulsions.[17−25] For example, in a series of systematic studies, McClements and co-workers[13,24,26−30] showed that the heating and aging significantly affect the flocculation stability of BLG emulsions, and several nontrivial trends were observed and explained by considering the structure and changes in the protein adsorption layers.[13,26−30]

The major goal of the present paper is to complement the previous studies[10−13,26−30] by performing a detailed investigation of the effects of emulsion heating and aging on the coalescence stability of BLG-containing emulsions. The specific aims are to determine the main factors that affect the coalescence stability of heated (at 85 °C) and 6-day-aged emulsions and to relate the measured stability with the structure of the protein adsorption layers. The emulsification procedure is chosen to provide emulsions with fixed oil volume fraction (30%) and practically constant mean drop diameter ($d_{32} \approx 40$ μm), thus focusing the study on the effects related to the changes in the adsorbed protein upon emulsion heating and storage. The experiments are made at several BLG concentrations (varied between 0.02 and 0.1 wt %) corresponding to a monoloyer and multilayer BLG adsorption, in a wide range of electrolyte concentrations (between 1.5 mM and 1 M), with pH varied between 4.0 and 7.0, which includes the BLG isoelectric point, IEP = 5.2. The critical osmotic pressure for drop coalescence, P_{OSM}^{CR}; the protein adsorption on drop surface, Γ; the mean drop size, d_{32}; and FTIR spectra of the heated and aged emulsions were measured. The obtained results are discussed from the viewpoint of the structure of the protein adsorption layers and possible explanations for the observed trends are proposed.

The paper is organized as follows: In section 2 we describe the materials and experimental methods used. In section 3 we present the main experimental results about the effect of emulsion heating on protein adsorption and on short-term coalescence stability, both measured 2 h after heating. The experimental results for the effect of storage time on drop size, protein adsorption, and coalescence stability for nonheated and heated emulsions are presented in section 4. The FTIR spectra of BLG solutions and emulsions are presented and discussed in section 5. The main results and conclusions are summarized in section 6.

2. Materials and Methods

2.1. Materials. β-Lactoglobulin (BLG) from bovine milk was used as received from Sigma (Cat. # L-0130, Lot # 052K7018 for the results shown in sections 3, 4.2, 4.4, and 5, and Lot No. 124H7045 for the results described in sections 4.1 and 4.3). Commercial grade soybean oil was purified from polar contaminants by multiple passes through a glass column filled with Florisil adsorbent.[31] The interfacial tension of purified oil was 29.5 ± 0.5 mN/m.

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Protein solutions were prepared with deionized water, purified by a Milli-Q Organex system (Millipore). These solutions always contained 0.01 wt % (1.5 mM) of the antibacterial agent NaN_3 (Riedel-de Haën, Seelze, Germany). The solution ionic strength was adjusted between 1.5 mM (only NaN_3) and 1 M, by using NaCl. The desired pH value was adjusted by addition of small aliquots of 0.1 M NaOH or 0.1 M HCl into the protein solution. In several series of experiments no acid or base was added and the obtained "natural" pH of the respective protein solutions was equal to 6.2 ± 0.1. At pH = 5.0, small fibrilar aggregates were observed to form in the nonheated protein solution. The heating of BLG solutions leads to formation of protein precipitate, at high electrolyte concentration and natural pH (C_{EL} = 1 M, pH = 6.2) and at low pH = 4.0 or 5.0 for arbitrary C_{EL}. The other solutions were clear.

2.2. Emulsion Preparation and Heating Protocol. Oil-in-water emulsions were prepared by stirring 35 mL of protein solution and 15 mL of soybean oil (30 vol. %) for 3 min with a rotor-stator homogenizer Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA-Labortechnik), operating at 13 500 rpm. This procedure ensures formation of emulsions with a mean volume−surface diameter of around 40 μm.[10−12]

Immediately after emulsification, the emulsions were loaded in centrifugal tubes and stored at room temperature. Some of the emulsions were heated in a water bath by using the following protocol: the heating always started 30 min after emulsion preparation; the temperature was raised for 15 min up to the desired temperature (85 °C in most of the experiments) and then the sample was kept at this heating temperature for 10 min, taken out of the bath, and stored at room temperature for different periods of time, varying from 2 h to 6 days. At the end of the storage period, the emulsions were centrifuged for characterization of their coalescence stability (section 2.5). In parallel experiments, the protein concentration in the aqueous phase was determined in other emulsions, prepared under equivalent conditions, to determine the protein adsorption on the drop surface (section 2.4).

2.3. Determination of Mean Drop Size. The mean drop size in the studied emulsions was determined by optical microscopy.[32−34] Specimens were taken at different stages of emulsion evolution—immediately after emulsification, after heating, and after shelf-storage for different periods of time. The oil drops were observed under transmitted light with a microscope (Axioplan, Zeiss, Germany), equipped with an objective (Epiplan, ×50) and connected to a CCD camera (Sony) and video recorder (Samsung SV-4000). The diameters of the drops were afterward measured (one by one) from the recorded video frames, by using custom-made image analysis software, operated with a Targa+ graphic board (Truevision). The diameters of at least 10 000 drops from two to five independently prepared emulsions were measured for each system. The mean volume−surface diameter, d_{32}, was calculated from the measured drop diameters. The mean drop size in all studied emulsions was measured to be 42 ± 4 μm.

2.4. Determination of Protein Adsorption. Protein adsorption on the surface of the emulsion drops, Γ, was determined from the specific surface area of the drops, *S*, and from the decrease of the protein concentration in the aqueous phase, as a result of the emulsification process. The following mass balance, relating Γ with the decrease of protein concentration was used:

$$\Gamma = \frac{V_C(C_{INI} - C_{SER})}{SV_{OIL}} = \frac{(1-\Phi)d_{32}}{6\Phi}(C_{INI} - C_{SER}) \qquad (1)$$

where V_C and V_{OIL} are the volumes of the continuous and oil phases, Φ is the oil volume fraction, C_{INI} is the initial BLG concentration in the aqueous solution, and C_{SER} is the BLG concentration in the aqueous phase after emulsification (in the serum).

C_{SER} was determined by the BCA method, which involves reduction of Cu^{2+} ions to Cu^{+} by the protein (biuret reaction) and colorimetric detection of the obtained Cu^{+} ions by a reagent containing bicinchoninic acid (BCA).[35] Formation of a purple complex causes a strong light absorbance at 562 nm, which allows one to determine protein concentration by a spectrophotometer.

The following procedure for determination of C_{SER} was applied: Samples from the serum remaining below the emulsion cream were taken 30 min after emulsion preparation, after emulsion heating, and after emulsion shelf-storage. The original serum was slightly turbid, because it contained a small fraction of tiny dispersed oil drops. To remove these drops, the serum was centrifuged for 1 h at 5500 rpm; the lower half of the serum (deprived of drops) was used for further analysis. A fraction of the centrifuged serum was diluted to a protein concentration falling in the range between 20 and 2000 μg/mL. Then, 100 μL of this sample was mixed with 2 mL of BCA reagent (product of Sigma) in a test tube. The test tubes were incubated for 2 h at 25 °C. For samples with protein concentration below 20 μg/mL, the test tubes were incubated for 1 h at 37 °C. After incubation, the tubes were stored at room temperature (25 ± 2 °C) for 10 min and then the light absorbance was measured at 562 nm by a UV−vis spectrophotometer (UNICAM 5625). From the measured absorbance we calculated C_{SER} by using a calibration curve. Different calibration curves were prepared for the various conditions studied (electrolyte concentrations, pH, thermal treatment). To determine the protein concentration in the serum of heated emulsions, we used calibration curves prepared with protein solutions, which were heated under the same conditions, as the emulsions studied.

2.5. Evaluation of the Emulsion Stability by Centrifugation. The emulsions were centrifuged at 20 °C in a 3K15 centrifuge (Sigma Laborzentrifugen). Preliminary experimental checks, performed at different durations of the centrifugation time (ranging from 30 min up to 6 h), showed that for these emulsions 30 min is sufficient for completion of the process of water drainage from the emulsion cream and for reaching mechanical equilibrium at a given acceleration.[10−12] Therefore, all emulsions in the systematic series of experiments were centrifuged for 1 h at a given acceleration. The emulsion stability was characterized by the critical osmotic pressure, P_{OSM}^{CR}, at which the continuous oil layer was released on top of the emulsion cream in the centrifuge tube.[10−12] P_{OSM}^{CR} was calculated from the experimental data by using the equation:[10−12]

$$P_{OSM}^{CR} = \Delta\rho g_k(V_{OIL} - V_{REL})/A \qquad (2)$$

where $\Delta\rho$ is the difference between the mass densities of the aqueous and oil phases, g_k is the centrifugal acceleration, V_{OIL} is the total volume of oil used for emulsion preparation, V_{REL} is the volume of released oil on top of the cream at the end of centrifugation, and *A* is the cross-sectional area of the centrifuge test tube. Equation 2 implies that P_{OSM}^{CR} is the osmotic pressure of the emulsion at the top of the emulsion column, where the latter is in mechanical equilibrium with a continuous layer of oil. The principle of the method and the used procedures are described in ref 10.

2.6. Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectroscopy was used to detect changes in the secondary structure of the protein molecules upon adsorption, heating, and shelf-storage of solutions and emulsions. For these experiments, soybean oil-in-D_2O emulsions were prepared by using a rotor-stator homogenizer, as described in section 2.2. The FTIR spectra were recorded on a Bruker IFS113 FT-IR spectrophotometer, at 20 °C, by using a six-

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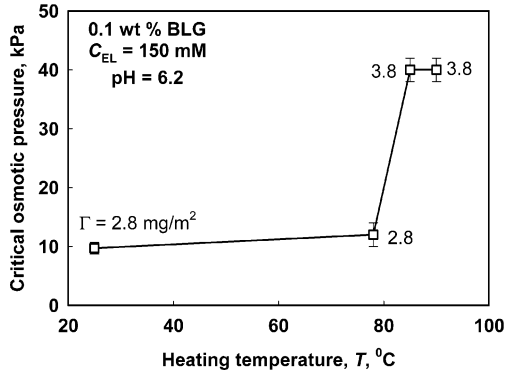

Figure 1. Critical osmotic pressure for emulsion destruction, P_{OSM}^{CR}, as a function of the heating temperature, *T*, for emulsions stabilized by 0.1 wt % BLG at C_{EL} = 150 mM and natural pH = 6.2. The numbers associated with the experimental points are the measured values of the protein adsorption, Γ.

reflection ATR cell with a TlBr/TlI crystal. Hundred-scan spectra were collected between 4000 and 400 cm^{-1}, in single-beam mode, with 2 cm^{-1} resolution. The spectra of the aqueous electrolyte solution and of the oil, as well as the bands originating from the water vapors in the 1720 and 1580 cm^{-1} amide I region, were subtracted from the spectra of the protein-containing samples to remove all bands unrelated to the protein. The corrected spectra were smoothed using a 13-point Savitsky−Golay method, and the second-derivative spectra were calculated and analyzed.

3. Effect of Emulsion Heating on Short-term Coalescence Stability

3.1. Effect of Heating Temperature on the Coalescence Stability of Emulsions. In this series of experiments, the critical osmotic pressure, P_{OSM}^{CR}, and protein adsorption, Γ, were measured as functions of the heating temperature for emulsions stabilized by 0.1 wt % BLG, at C_{EL} = 150 mM and natural pH = 6.2 (see Figure 1). As one can see, the emulsion heating at 78 °C leads to only ≈30% increase of P_{OSM}^{CR}, whereas the heating at 85 °C increases the emulsion stability more than 4 times. The emulsions heated at 85 and 90 °C have virtually the same stability, $P_{OSM}^{CR} = 40 \pm 4$ kPa. These results show that the emulsion coalescence stability increases stepwise in the range between 78 and 85 °C, in which the denaturing of the BLG molecules is known to occur.[36,37]

To check whether the increased emulsion stability after heating is related to increased protein adsorption, Γ, we measured the adsorption, and the results are shown as numbers (in mg/m^2) associated with the points in Figure 1. We found that Γ also increases in a stepwise manner with the increase of the heating temperature—from $\Gamma = 2.8 \pm 0.4$ mg/m^2 for the nonheated emulsions and those heated to 78 °C, up to 3.8 mg/m^2 for the emulsions heated to 85 and 90 °C. The observed increase of Γ after heating is in agreement with the results reported in refs 17, 21, 29.

These results indicate that the increased emulsion stability after heating at 85 °C is at least partially due to reinforced steric repulsion between the protein adsorption layers, caused by the increased protein adsorption. However, similar adsorption, Γ = 3.8 mg/m^2, was measured in nonheated emulsions at high electrolyte concentration, C_{EL} = 1 M (see Figure 3), while the respective coalescence stability of the emulsion was only 14 kPa

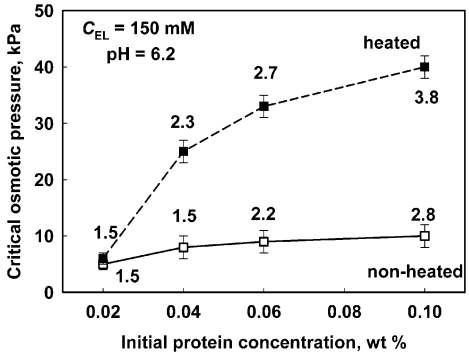

Figure 2. Critical osmotic pressure as a function of protein concentration, C_{BLG}, for heated (full squares) and nonheated (empty squares) emulsions prepared in aqueous solution with C_{EL} = 150 mM and pH = 6.2. The heating is performed at 85 °C. The numbers associated with the experimental points are the measured values of the protein adsorption, Γ.

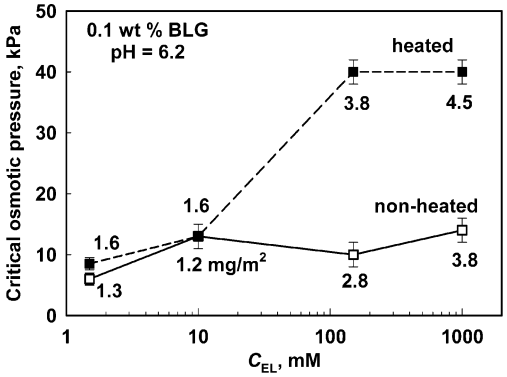

Figure 3. Critical osmotic pressure as a function of electrolyte concentration, C_{EL}, for heated (full squares) and nonheated (empty squares) emulsions stabilized by 0.1 wt % BLG at pH = 6.2. The heating is performed at 85 °C. The numbers associated with the experimental points are the measured values of the protein adsorption, Γ.

(vs 40 kPa for the heated emulsions). This comparison shows that the enhanced coalescence stability after heating could not be explained by the increased protein adsorption only.

The most probable explanation for the enhanced stability is the irreversible attachment of all BLG molecules in the adsorption multilayer after heating. As known from the literature, the molecules in the first adsorption layer, which are in direct contact with the oil−water interface, are irreversibly adsorbed on the drop surface, whereas the molecules adsorbed over the first layer (which for brevity will be termed below "the second adsorption layer") are usually adsorbed reversibly and can desorb upon rinsing of the emulsion with electrolyte solution.[10−12,38−39] Thus for nonheated fresh emulsions prepared with 0.1 wt % BLG, we showed that ≈1.6 mg/m^2 of the adsorbed protein is irreversibly attached (presumably this is the protein in the first adsorption layer), whereas the remaining 1.2 mg/m^2 are reversibly attached and desorb after rinsing the emulsion with 150 mM NaCl solution.[10]

To check whether the fraction of irreversibly adsorbed BLG molecules increases after heating, we rinsed the emulsion heated at 85 °C (Γ = 3.8 mg/m^2, Figure 1) with 150 M NaCl solution. The following procedure was used:[10,39] First, the heated emulsion

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was stored undisturbed for 3 h at room temperature and a cream of closely packed drops formed in the upper part of the emulsion, under the effect of buoyancy. Afterward, by using a syringe, the serum remaining below the cream was removed and replaced by the same volume of 150 mM NaCl and 0.1 wt % NaN_3 solution (without dissolved protein). This sample was gently agitated by hand, until the emulsion drops completely dispersed in the entire volume, and then left undisturbed for another 1 h. A sample from the rinsing electrolyte solution, which remained below the cream, was taken and the protein concentration was determined by the BCA method. By applying a mass balance to the protein used in emulsion preparation, from the average drop size (d_{32}) and protein concentrations in the initial solution (C_{INI}), in the serum after emulsification (C_{SER}), and in the rinsing solution, (C_{RIN}), we calculated the protein adsorption before and after rinsing of the emulsion. This calculation showed that no protein desorbed from the drop surface upon rinsing of the heated emulsion, which means that a multilayer of irreversibly adsorbed protein molecules (probably connected with disulfide covalent bonds) is formed during heating. As mentioned above, exactly the same procedure leads to desorption of almost a half of the adsorbed BLG from the drop surface, when applied to nonheated emulsions.[10]

To check whether the possible flocculation of the neighboring drops during heating affects the coalescence stability of the emulsions, in one series of experiments we studied emulsions that were heated at 85 °C under continuous mild stirring at 350 rpm. The mean drop size of these emulsions was measured to be the same as in the emulsions prepared without stirring ($d_{32} = 38 \pm 2\,\mu$m). The optical observation of heated emulsion showed that in both types of heated emulsions (with and without stirring) most of the emulsion drops were collected into flocs. However, after introducing these flocs into 30 mM sodium dodecyl sulfate (SDS) solution, most of them disintegrated into individual oil drops, except for the small drops ($d < 10\,\mu$m), a significant fraction of which remained flocculated. The fact that most of the flocs disrupted in SDS solution (which is not expected to break the disulfide bonds) shows that the observed flocculation in the heated emulsions was due mostly to formation of noncovalent bonds. Interestingly, the degree of flocculation in the stirred and nonstirred samples was different, while the coalescence stability of these samples was measured by centrifugation to be practically the same, $P_{OSM}^{CR} \approx 40$ kPa. The latter result shows that the interdroplet interactions, which govern the drop−drop flocculation during heating, were not important for the coalescence stability of the emulsions studied. On the other hand, the fact that the coalescence stability significantly increases after emulsion heating (with and without stirring) evidences that the S−S bonds formed between the molecules inside the protein adsorption layer have an important impact on the emulsion coalescence stability.

In conclusion, the heating at $T \geq 85$ °C of emulsions prepared at $C_{BLG} = 0.1$ wt %, $C_{EL} = 150$ mM, and pH = 6.2 leads to 4-fold increase of emulsion coalescence stability, which is explained by the increased protein adsorption and the formation of mulitilayers of irreversibly attached protein molecules. These molecules are probably bound by disulfide bonds, which are known to form between adsorbed BLG molecules upon heating at such high temperatures.[40,41]

3.2. Effect of Protein Concentration on the Coalescence Stability of Heated Emulsions. The results for the effect of the initial protein concentration, C_{BLG}, on the stability of heated and nonheated emulsions are presented in Figure 2. All emulsions were prepared with aqueous solutions of $C_{EL} = 150$ mM and pH = 6.2, and were heated at 85 °C.

One sees that emulsion stability remains almost the same after heating at the lowest protein concentration studied ($C_{BLG} = 0.02$ wt %), whereas the emulsion stability increases more than 3 times after heating at higher BLG concentrations. Note that at $C_{BLG} = 0.02$ wt %, the protein adsorption after heating remains almost the same and corresponds to a monolayer (see ref 10). At higher protein concentration, the heating leads to additional adsorption and irreversible attachment of the protein molecules in the second adsorption layer, which is due to formation of strong (probably covalent) intermolecular bonds between the molecules in the first and second adsorption layers.

These results support the hypothesis that the increased emulsion stability after heating is mainly due to enhanced steric repulsion between the drops (and possible reinforcement of the adsorption layer), caused by additional adsorption and irreversible attachment of protein molecules in the adsorption multilayers.

3.3. Effect of Electrolyte Concentration on the Coalescence Stability of Heated Emulsions. The experimental results for the effect of electrolyte concentration, C_{EL}, on the stability of heated and nonheated emulsions are presented in Figure 3. All emulsions were prepared at $C_{BLG} = 0.1$ wt % and pH = 6.2 and were heated at 85 °C. One sees that the stability of heated and nonheated emulsions is almost the same for $C_{EL} \leq 10$ mM, whereas at high electrolyte concentration, $C_{EL} \geq 150$ mM, the heating increases emulsion stability by about 4 times.

The protein adsorption, Γ, increases slightly from 1.3 to 1.5 mg/m^2 after heating of the emulsions prepared at $C_{EL} = 1.5$ mM, whereas a significant increase of Γ is observed after heating for emulsions prepared at $C_{EL} = 150$ mM (from 2.8 to 3.8 mg/m^2) and $C_{EL} = 1$ M (from 3.8 to 4.5 mg/m^2).

The fact that the emulsion stability and protein adsorption remain almost the same after heating of emulsions prepared at $C_{EL} = 1$ and 10 mM (corresponding to monolayer adsorption of BLG) means that the conformational changes in the adsorbed protein molecules, occurring during heating, are of secondary importance for these systems. We suppose that significant electrostatic repulsion between the adsorbed protein molecules keeps them separated from each other, so that no intermolecular bonds are formed during heating (though some conformational changes and bond formation within the BLG molecules are possible). At such low electrolyte concentrations, the coalescence stability is governed mainly by electrostatic and van der Waals interactions,[11] which are not affected significantly by the emulsion heating.

As explained in the previous subsection, multilayers of covalently bound molecules are formed when $C_{EL} \geq 150$ mM and the heating temperature is $T \geq 85$ °C, which leads to enhanced emulsion stability.

To check whether the suppressed electrostatic repulsion between the adsorbed BLG molecules is of crucial importance for the formation of covalent bonds and enhancing the emulsion stability, we performed a series of experiments, in which the electrolyte concentration was changed at different stages of emulsion evolution, see Figures 4 and 5.

One sees from the results shown in Figure 4 that the stability of all emulsions *heated* at $C_{EL} \geq 150$ mM is practically the same, $P_{OSM}^{CR} = 40$ kPa, irrespective of the electrolyte concentration during emulsification, the emulsion coalescence stability being evaluated by centrifugation. For example, the emulsion prepared at $C_{EL} = 10$ mM and heated at $C_{EL} = 150$ mM had the same

(40) Kitabatake, N.; Wada, R.; Fujita, Y. Reversible conformational change in β-lactoglobulin A modified with *N*-ethylmaleimide and resistance to molecular aggregation on heating. *J. Agric. Food Chem.* **2001**, *49*, 4011.

(41) Hoffmann, M.; Sala, G.; Olieman, G.; Kruif, K. G. Molecular mass distribution of heat-induced β-lactoglobulin aggregates. *J. Agric. Food Chem.* **1997**, *45*, 2949.

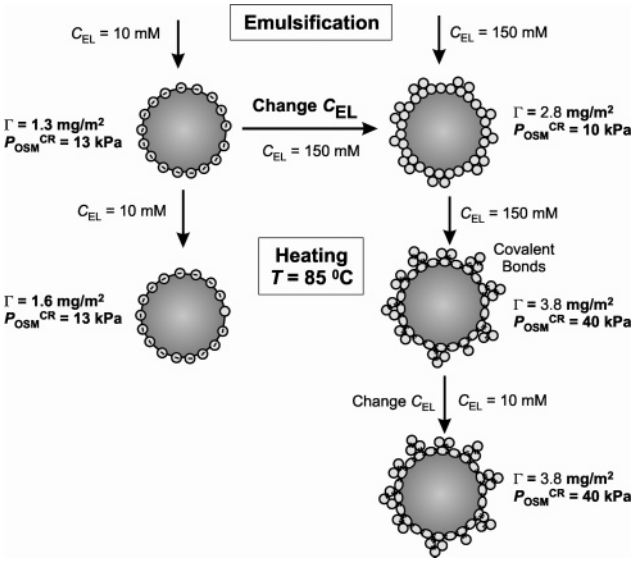

Figure 4. Schematic presentation of the effects of electrolyte concentration on the protein adsorption, Γ, and emulsion coalescence stability assessed by the critical osmotic pressure for emulsion destruction, P_{OSM}^{CR}.

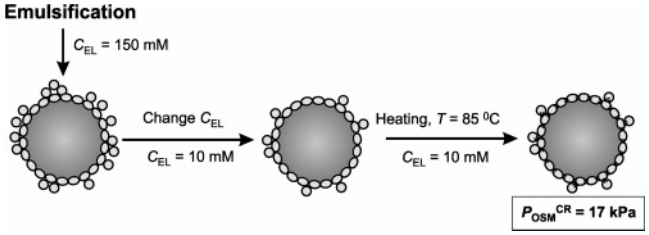

Figure 5. Schematic presentation of the effects of electrolyte concentration on emulsion coalescence stability, P_{OSM}^{CR}, for a system in which the emulsification is performed at C_{EL} = 150 mM, followed by a heating at C_{EL} = 10 mM.

stability as the emulsion that was both prepared and heated at C_{EL} = 150 mM. Furthermore, if the emulsion is heated at C_{EL} = 150 mM and afterward C_{EL} is decreased down to 10 mM, the emulsion stability remains the same, P_{OSM}^{CR} = 40 kPa (the same result was obtained with the emulsions heated at C_{EL} = 1 M). Therefore, the emulsion stability after heating is determined exclusively by the irreversible attached protein molecules in the formed adsorption multilayers during heating.

Let us discuss briefly the stability of emulsions prepared at C_{EL} = 150 mM and then heated and centrifuged at C_{EL} = 10 mM (see Figure 5). During emulsification, the protein molecules formed an adsorption multilayer on the drop surface with Γ = 2.8 mg/m² (see Figure 4). As shown in our previous study, a fraction of the adsorbed molecules (Γ = 1.6 mg/m²) is irreversible attached in the first adsorption layer (directly contacting with the OW interface), whereas the remaining protein is reversibly attached onto the first layer, thus forming a second adsorption layer that is not in a direct contact with the OW interface. The decrease of C_{EL} after emulsification down to 10 mM most probably leads to some desorption of the protein molecules from the second adsorption layer, which however could not be detected by the method used (the expected change of the protein concentration as a result of desorption is only 0.005 wt %, which cannot be detected when the total protein concentration is around 0.1 wt %). The subsequent heating of this emulsion at C_{EL} = 10 mM also does not lead to detectable change in the protein adsorption, whereas the emulsion stability increases from 13 to 17 kPa. Probably, the electrostatic repulsion between the protein molecules at C_{EL} = 10 mM suppresses the additional adsorption of protein during heating. However, covalent bonds could be formed between the adsorbed protein molecules, as illustrated schematically in Figure 5.

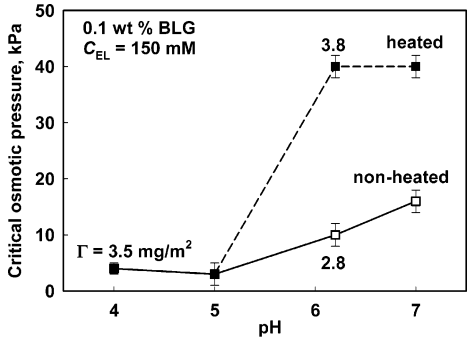

Figure 6. Critical osmotic pressure for emulsion destruction as a function of pH, for heated (full squares) and nonheated (empty squares) emulsions stabilized by 0.1 wt % BLG at C_{EL} = 150 mM. The heating is performed at 85 °C. The numbers associated with the experimental points are the measured values of the protein adsorption, Γ.

From these series of experiments we can conclude that (1) the heating of emulsions at $C_{EL} \geq$ 150 mM leads to additional adsorption of protein and to formation of covalent bonds between the adsorbed protein molecules. Both processes enhance the emulsion stability. (2) The heating of emulsions at $C_{EL} \leq$ 10 mM (prepared at the same C_{EL}) does not change their stability, due to significant electrostatic repulsions between the protein molecules, which suppresses the additional protein adsorption and precludes the formation of intermolecular bonds during heating. (3) The heating of emulsions at $C_{EL} \leq$ 10 mM, initially prepared at C_{EL} = 150 mM, leads to formation of intermolecular bonds between the adsorbed protein molecules, without significant additional adsorption of protein during heating. As a result, the emulsion stability in this case moderately increases after heating and is between those of cases 1 and 2.

3.4. Effect of pH on the Coalescence Stability of Heated Emulsions. This series of experiments is aimed at clarifying the effect of pH on the protein adsorption and coalescence stability of heated emulsions. All emulsions are prepared with 0.1 wt % BLG solution, at C_{EL} = 150 mM, and heating at 85 °C.

The protein adsorption for heated and nonheated emulsions at pH = 4.0 is almost the same and indicates formation of protein multilayers, Γ ≈ 3.5 mg/m² (see Figure 6). Significant protein precipitation was induced by the heating at pH ≈ 5 ≈ IEP, which did not allow us to measure the protein adsorption after heating at this pH. As discussed in section 3.1, Γ increases significantly after heating at pH ≥ 6.2.

As seen from the results in Figure 6, the heating of emulsions at pH ≥ 6.2 (C_{BLG} = 0.1 wt %, C_{EL} = 150 mM) leads to significant increase of emulsion stability. In contrast, the emulsion stability remains almost the same for emulsions prepared at pH between 4.0 and 5.0. Most probably, at pH ≤ 5, the BLG molecules do not unfold upon adsorption and during heating, so that the reactive sulfhydryl groups remain hidden in the molecule interior, which precludes the possibility for formation of intermolecular covalent bonds. This explanation is in qualitative agreement with the experimental results reported in ref 30, in which the flocculation in heated emulsions at pH = 3.0 and 7.0 are compared and it is found that no disulfide bonds are formed at pH = 3.0.

To compare the impact of pH on the emulsion stability at different stages of emulsion treatment (during emulsification, during heating, and after heating) we performed a series of

Table 1. Critical Osmotic Pressure for Emulsion Destruction, P_{OSM}^{CR}, for Emulsions Prepared at C_{BLG} = 0.1 wt %, C_{EL} = 150 mM and Heated at 85 °C, for Which the pH Is Changed during the Emulsion Treatment as Indicated

pH			
during emulsification	during heating	during centrifugation	P_{OSM}^{CR}, kPa
4	4	4	4 ± 1
6.2	4.5	4.5	4 ± 1
5	5	5	3 ± 1
4	5.6	5.6	13 ± 4
5	5.9	5.9	15 ± 4
6.2	6.2	6.2	40 ± 4
6.2	6.2	4.5	40 ± 4
6.2	6.2	5.2	40 ± 4

experiments in which the pH of the emulsion aqueous phase was changed after emulsion preparation (see Table 1). One sees that the emulsion coalescence stability correlates with the pH values at which the heating is performed; for example, all emulsions heated at pH = 6.2 have a coalescence barrier of $P_{OSM}^{CR} \approx 40$ kPa, despite the various pH values during emulsification and centrifugation. Furthermore, when the emulsion is prepared and heated at pH = 6.2, the subsequent lowering of pH does not affect emulsion stability. These results confirm the hypothesis that the covalent bonds formed between the protein molecules in the adsorption layer during heating are most important for the emulsion stability of the heated emulsions.

4. Effect of Storage Time on the Emulsion Coalescence Stability

4.1. Effect of Storage Time on Emulsion Stability. In this series of experiments we studied the emulsion coalescence stability as a function of the shelf-storage period at room temperature, for emulsions stabilized with 0.1 wt % BLG, C_{EL} = 150 mM, pH = 6.2 (for similar experiments at lower BLG concentration, 0.02 wt %, corresponding to adsorption monolayer, see ref 12). The following protocol was used: A series of BLG emulsions was prepared under equivalent conditions. These emulsions were stored undistributed at room temperature for different periods of time, from 1 min (i.e., immediately after emulsion preparation) up to 14 days. The critical osmotic pressure for drop coalescence, P_{OSM}^{CR}, was periodically determined during this storage period by centrifugation. The respective drop size distribution and protein adsorption were determined in parallel emulsion samples.

The results for P_{OSM}^{CR} as a function of storage time, shown in Figure 7, indicate several stages of emulsion evolution: (1) a slight increase of emulsion stability for storage times between 1 and 30 min, (2) a subsequent plateau in the interval between ca. 5 and 180 min, and (3) a significant decrease of emulsion stability by more than 4 times during the following 6 days of storage.

Remarkably, we did not detect any tendency for increase or decrease of Γ at storage times longer than 30 min. The measured drop size distributions and mean volume-surface diameters were virtually the same for all emulsions. Therefore, the decreased emulsion stability observed at storage times longer than several hours (called hereafter "the aging effect") could not be explained by changes in the amount of adsorbed protein or by changes in drop size. These results mean that the observed aging effect is due primarily to changes in the structure of the protein adsorption layer, which make it less efficient in emulsion stabilization. To check whether the storage time leads to formation of intermolecular bonds between the adsorbed molecules, we measured the

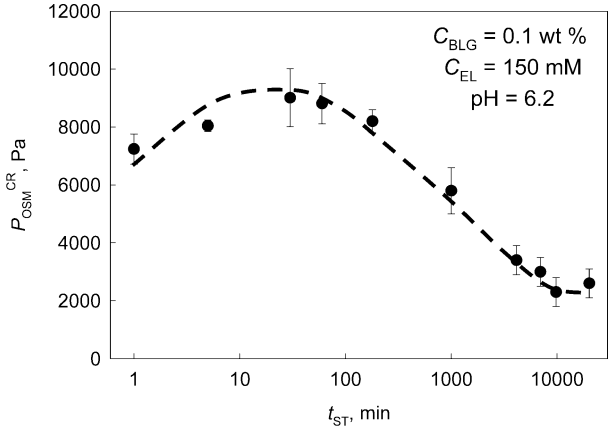

Figure 7. Critical osmotic pressure for emulsion destruction, P_{OSM}^{CR}, as a function of storage time for emulsions stabilized by 0.1 wt % BLG, C_{EL} = 150 mM, pH = 6.2.

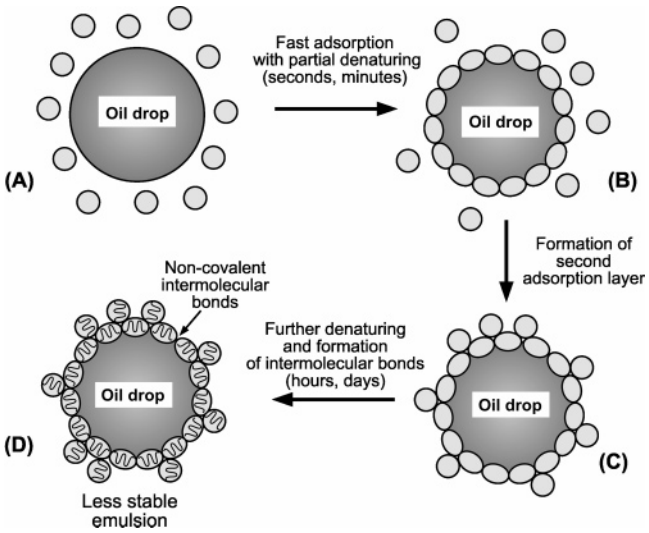

Figure 8. Schematic presentation of the processes that occur in the adsorption layer during emulsion shelf storage.

protein adsorption after rinsing of the emulsions with electrolyte solution 1 h after their preparation and after 6 days. We found that the molecules in the second adsorption layer are reversible attached after 1 h and they become irreversible attached after 6 days of storage. Hence, strong intermolecular bonds are formed between the protein molecules adsorbed in the first and the second adsorption layers during the storage period.

The most probable explanation of the aging effect is the formation of noncovalent intermolecular bonds (H-bonds and hydrophobic interactions) between the adsorbed protein molecules (see Figure 8), which transform the adsorption layer into a brittle shell, which ruptures upon surface extension. As explained in ref 10, the formation of an emulsion film (or the expansion in area of such film) in the point of contact between two drops is accompanied with an extension of the drop surfaces. If the adsorption layer is brittle, then bare (deprived of protein) oil−water spots could appear on the film surfaces upon their expansion, leading to film destabilization and drop−drop coalescence.

The above explanation is in qualitative agreement with the results by Murray et al.,[42] who studied the rheological properties of BLG adsorption layers on air/water interface, as a function of aging time. It was shown[42] that the elasticity of the BLG adsorption layer increased with time, and the consequent

(42) Murray, B. S.; Cattin, B.; Schuler, E.; Sonmez, Z. O. Response of adsorbed protein films to rapid expansion. *Langmuir* **2002**, *18*, 9476.

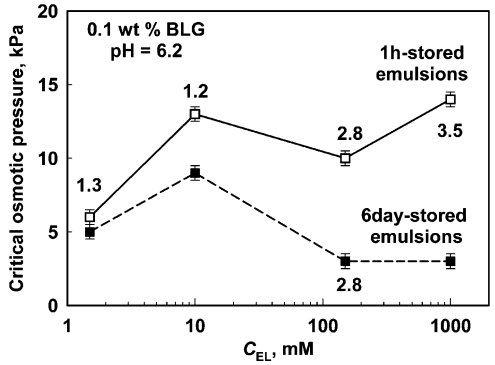

Figure 9. Critical osmotic pressure for emulsion destruction as a function of electrolyte concentration, C_{EL}, for 1-h-stored (empty squares) and 6-day-stored (full squares) emulsions stabilized by 0.1 wt % BLG at pH = 6.2.

expansion of the surface led to slower relaxation of the surface tension. This result was explained in ref 42 with the formation of robust surface aggregates, which suppressed the further adsorption of the protein molecules on the interface.[42] One should note that such brittle adsorption layers are expected to exhibit rather complex rheological behavior, for example, exhibiting high elastic modulus upon small lateral deformations and low "effective" elastic modulus upon larger deformation, which could rupture the layers.

4.2. Effect of Electrolyte Concentration on the Long-Term Emulsion Stability. In this series of experiments we measured the critical osmotic pressure, P_{OSM}^{CR}, for 6-day-stored emulsions, as a function of electrolyte concentration. As seen from Figure 9, the stability of the aged emulsions is lower than the stability of the respective fresh emulsions (measured 1 h after preparation) for all studied electrolyte concentrations. This aging effect is more pronounced at moderate and high electrolyte concentrations.

The smaller aging effect, observed at lower C_{EL}, is probably related to the larger distance between the protein molecules in the adsorption layer, which leads to slower buildup of the presumed brittle protein shell on the drop surface. We recall that $\Gamma \approx 1.3$ mg/m^2 at $C_{EL} = 10$ mM, which is noticeably lower than the adsorption $\Gamma \approx 2.8$ mg/m^2 measured at $C_{EL} = 150$ mM.

In conclusion, we suppose that the observed aging effect is due to formation of a brittle protein shell on the drop surface, which is built through formation of strong lateral noncovalent bonds (H-bonds and hydrophobic interactions) between the adsorbed protein molecules. This hypothesis is checked below with several additional series of experiments.

4.3. Effect of Additives on the Aging Effect. To check for the effect of possible formation of intermolecular bonds in the adsorption layer on emulsion aging, we performed additional experiments in the presence of additives. First, we determined the role of the possible covalent intermolecular disulfide S−S bonds by performing centrifugation experiments for evaluating the emulsion stability (immediately after formation and after shelf-storage) in the presence of 10 mM dithiothreitol (DTT), a reducing reagent that is known to block the formation of S−S bonds between protein molecules.[41] We found that DTT had no significant effect on emulsion stability, which means that the aging effect is not related to the possible formation of disulfide bonds in the adsorption protein layer.

The role of the noncovalent interactions was tested by addition to the aqueous phase of urea, which is known to break the hydrogen bonds and to suppress the hydrophobic interaction between the protein molecules. The results for the emulsion stability at three different concentrations of urea, presented in Table 2, show that the addition of 4 M of urea only is sufficient to prevent completely the aging effect. As shown by Pace and Tanford,[43] the addition of 4.4 M urea has little effect on the BLG molecules in the bulk protein solution at room temperature—no significant denaturing of the dissolved protein is observed. This means that in our experiments, in which 3 or 4 M urea is added to the protein solutions, the urea acts exclusively on the protein molecules incorporated in the adsorption layer. Even at 3 M urea, which is certainly insufficient to cause a detectable protein denaturing in the bulk solution,[43] the aging effect of the studied emulsions is strongly suppressed, which means that the urea strongly affects the bond formation between the partially unfolded adsorbed molecules (see also the discussion of the FTIR spectra in the next section).

Table 2. Critical Osmotic Pressure for Emulsion Destruction, P_{OSM}^{CR}, for Nonheated Emulsions (0.1 wt % BLG, C_{EL} = 150 mM, pH = 6.2) in the Presence of Urea

storage time	P_{OSM}^{CR}, kPa			
	no urea	3 M urea	4 M urea	6 M urea
30 min	9.7 ± 1.3	20 ± 1.5	26.7 ± 1.5	25 ± 1.5
24 h	2.5 ± 0.5	16 ± 1.5	25.7 ± 1.5	25 ± 1.5
6 days	2.0 ± 0.3	not measured	25.7 ± 1.5	25 ± 1.5

Table 3. Critical Osmotic Pressure for Emulsion Destruction, P_{OSM}^{CR} (kPa), for Nonheated Emulsions and Those Heated at 85 °C, Stabilized by 0.1 wt % BLG at Three Electrolyte Concentrations, C_{EL} (pH = 6.2)

	nonheated		heated	
C_{EL}, mM	3 h	6 days	3 h	6 days
1.5	6 ± 1	5 ± 1	8 ± 2	5 ± 2
10	13 ± 2	9 ± 2	13 ± 2	10 ± 2
150	10 ± 1	3 ± 0.5	40 ± 4	40 ± 4

Thus, we can conclude that the aging effect is related to formation of noncovalent hydrogen and hydrophobic bonds between the adsorbed protein molecules, which probably transform the adsorption protein layer into a brittle shell, which is inefficient in stabilizing the emulsion drops against coalescence (see also section 5 below). Although some disulfide bonds could be formed upon shelf-storage of the emulsions, the performed experiments with DTT and urea show that these bonds have no important impact on the aging effect.

4.4. Effect of Storage Time on the Coalescence Stability of Heated Emulsions. This series of experiments was aimed at comparing the short-term (3 h after preparation) and long-term stability (after 6 days of storage) of heated and nonheated emulsions. The experiments were performed with emulsions containing 0.1 wt % BLG and 1.5, 10, or 150 mM electrolyte, at heating temperature of 85 °C. As seen in Table 3, a weak aging effect (≈30% decrease in emulsion stability) is observed at low electrolyte concentration, 1.5 and 10 mM, for the heated emulsions. In contrast, at high electrolyte concentration (150 mM), the coalescence stability of the heated emulsions does not change with the storage time. The latter result indicates that the conformational changes in the protein molecules occurring during heating at high C_{EL} suppress the subsequent formation of noncovalent hydrogen and hydrophobic bonds, thus preventing the aging effect described in section 4.1. This hypothesis is supported by the FTIR spectra, which are presented and discussed in the following section.

(43) Pace, N. C.; Tanford, C. Thermodynamics of the unfolding of β-lactoglobulin A in aqueous urea solutions between 5 and 55 °C. *Biochemistry* **1968**, *7*, 198.

5. FTIR Spectra

As discussed in section 4, the pronounced aging effect observed at high electrolyte and protein concentrations (C_{EL} = 150 mM, C_{BLG} = 0.1 wt %) is not related to changes in the mean drop size or protein adsorption during emulsion storage. The obtained results suggest that the emulsion aging is primarily caused by buildup of intralayer noncovalent bonds between the adsorbed protein molecules, which led to formation of a brittle protein shell on the drop surface. In contrast, the thermal treatment of BLG emulsions significantly increases their short-term stability and the aging effect disappears (see Table 3), which is explained with the formation on the drop surface of a network of denatured BLG molecules, which are cross-linked by disulfide bonds.

To investigate the conformational changes in the secondary structure of the protein molecules after their adsorption and upon heating, we recorded FTIR spectra of protein solutions and emulsions prepared at 0.1 wt % BLG and C_{EL} = 150 mM, by following the procedure of Fang and Dalgliesh.[20]

5.1. Effects of Storage Time and Heating on the FTIR Spectra of BLG Solutions. The FTIR spectra of heated and nonheated BLG solutions, stored for different periods of time, are compared in Figure 10. From these spectra one can deduce the following.

(1) The FTIR spectra of freshly prepared BLG solutions (see Figure 10A) are similar to those reported in the literature, with six major characteristic bands at 1694 cm^{-1} (β-type structure), 1680 cm^{-1} (β-sheet), 1668 cm^{-1} (small band for β-turns), 1652 cm^{-1} (α-helix), and 1634 and 1623 cm^{-1} (β-sheet).[20,44−46]

(2) The shelf-storage of nonheated solution leads to significant changes in the spectra (Figure 10A), which can be interpreted[20,45] as a result of partial denaturing of the molecules, disintegration of initially present protein aggregates in the fresh solution (see the reduction of the peak at 1623 cm^{-1}), and formation of a more hydrated, less compact protein structure—see the shifts of the β-sheet band from 1634 to 1629 cm^{-1} and of the α-helix band from 1650 to 1648 cm^{-1}.

(3) The heating of the protein solutions leads to strong local perturbation of the protein structure (the peaks are wider and smaller in height, Figure 10B), but the original secondary structure of the dissolved protein remains stable upon long-term storage (Figure 10C).

It is worthwhile noting that we do not report in the current study results about the stability of emulsions that are prepared with preheated protein solutions. Preliminary experiments showed that the heating of the protein solutions before emulsification had rather different effect on emulsion stability, as compared to the effect of heating of a preformed emulsion. One obvious reason for this difference is that the protein solution heating (prior to emulsification) could not directly induce formation of intermolecular bonds inside the adsorption layer, which is the main effect of heating on preformed emulsions. On the other hand, the effect of solution heating on the rheological properties of postformed protein adsorption layers was studied in the literature and a complex time evolution was observed (e.g., refs 47, 48).

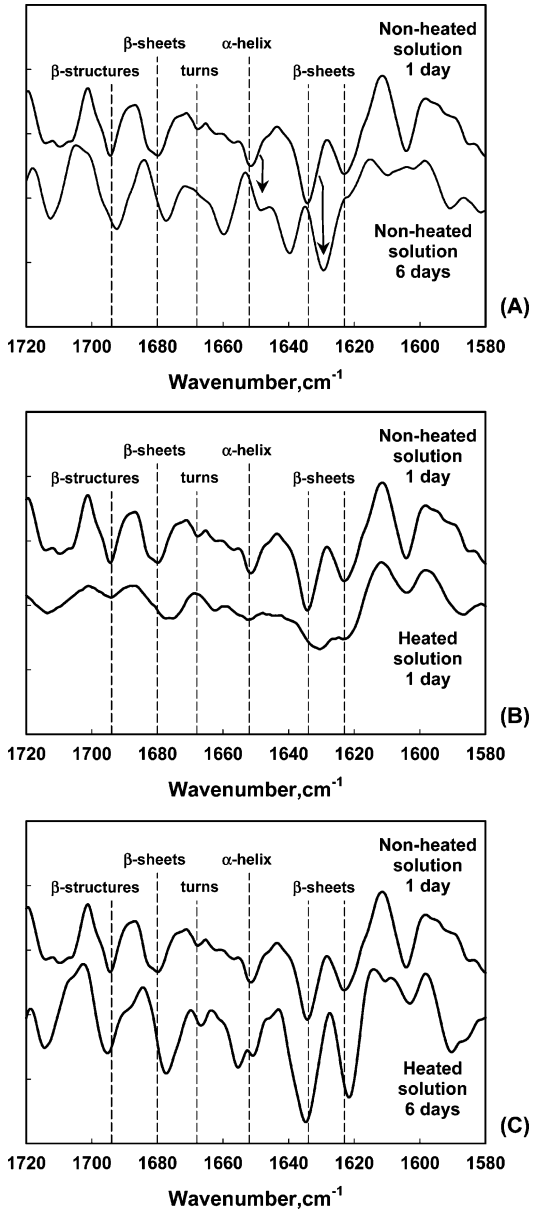

Figure 10. Comparison of the FTIR spectra for (A) nonheated BLG solution 1 day and 6 days after solution preparation, (B) nonheated BLG and heated BLG solutions 1 day after preparation, and (C) nonheated BLG and heated BLG solutions after 6 days of storage at room temperature.

Therefore, the effect of solution heating on emulsion stability deserves a detailed investigation, not attempted in the current paper.

5.2. Effects of Protein Adsorption and Emulsion Heating. The comparison of the FTIR spectra obtained from nonheated BLG solution and from BLG-stabilized emulsion (several hours after emulsification) (Figure 11A) shows that the secondary structure of the BLG molecules remains well preserved for several hours after the adsorption—see the main bands for β-sheets, turns, and α-helix, which are similar in the solutions and in the emulsions. This result is in agreement with the data of Fang and Dalgleish.[20]

The BLG spectra obtained from freshly prepared and from 6-day-stored (aged) emulsions are compared in Figure 11B. A

(44) Boye, J. I.; Ma, C. Y.; Ismail, A.; Harwalkar, V. R.; Kalab, M. Molecular and microstructural studies of thermal denaturation and gelation of β-lactoglobulins A and B. *J. Agric. Food Chem.* **1997**, *45*, 1608.

(45) Dong, A.; Huang, P.; Caughey, W. S. Protein secondary structures in water from second-derivative amide I infrared spectra. *Biochemistry* **1990**, *29*, 3303.

(46) Qi, X.; Holt, C.; Mcnulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochem. J.* **1997**, *324*, 341.

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(48) Wang, Z.; Narsimhan, G. Interfacial dilatational elasticity and viscosity of β-lactoglobulin at air−water interface using pulsating bubble tensiometry. *Langmuir* **2005**, *21*, 4482.

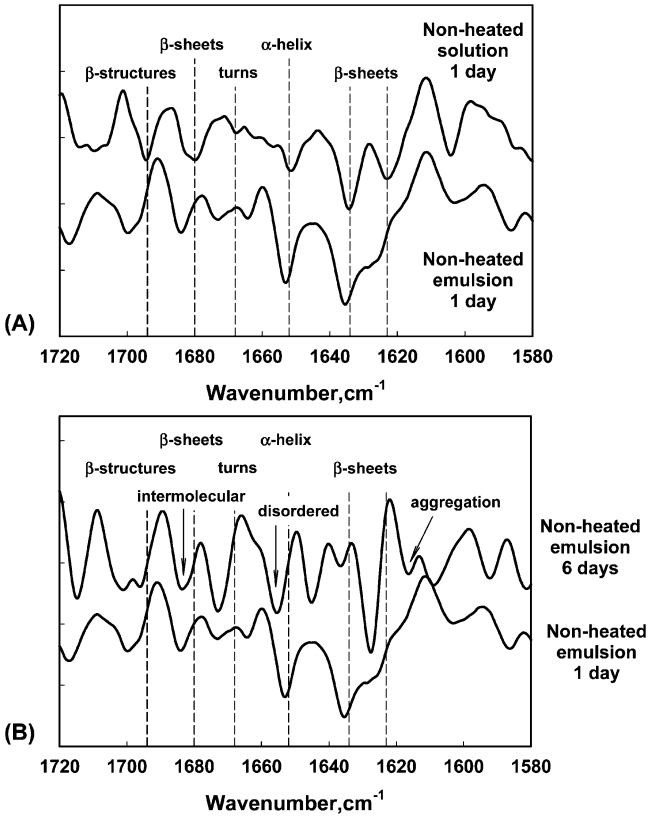

Figure 11. Comparison of the spectra for (A) nonheated solution and emulsion 1 day after preparation and (B) nonheated emulsion 1 day and 6 days after preparation.

large peak from disordered protein domains is seen at 1645 cm^{-1} in the spectrum of the aged emulsion, whereas this peak is almost missing in the spectrum of freshly adsorbed BLG. The intensity of the β-sheet bands at 1637 and 1628 cm^{-1} noticeably decreases, which indicates that a fraction of the initial β-sheets transforms into disordered structure. A new band appeared in the spectrum of the aged emulsion at 1616 cm^{-1}, which is attributed in the literature to formation of protein aggregates.[46]

Let us compare now the spectra of 6-day-stored emulsion and 6-day-stored BLG solution (cf. Figures 10A and 11B). This comparison allows one to distinguish the conformational changes upon storage, which are related to protein adsorption. The main difference between the spectra of aged emulsion and solution could be summarized as follows: In the spectrum of the aged emulsion we see bands at 1684 and 1616 cm^{-1} from intermolecular bonds and a band at 1645 cm^{-1} from disordered structures, which are missing in the spectrum of the aged BLG solution. Hence, the adsorption of the protein facilitates the disordering of the molecule's structure and the formation of intermolecular bonds (these two processes are probably interrelated).

The comparison between the FTIR spectra of heated emulsion after 6 days of storage and of freshly prepared BLG solution is presented in Figure 12. Interestingly, the main bands related to the β-sheets at 1694, 1680, 1634, and 1623 cm^{-1} had almost the same intensity and width as those of the spectrum of the stored emulsion, which indicates that the main features of the secondary structure of the protein in heated emulsion are preserved during shelf-storage. Note that the structure of the heated BLG solutions is also preserved upon shelf-storage (Figure 10C).

Let us compare now the spectra of heated and nonheated emulsions that had been stored for 6 days (Figures 11B and 12). The main β-structure bands remained almost the same in the heated emulsion after 6 days of storage. In contrast, the protein

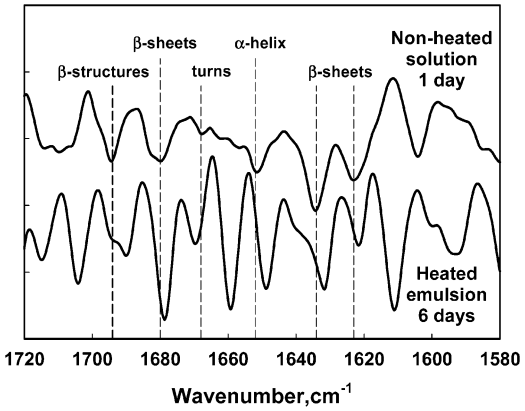

Figure 12. Comparison of the spectra of heated emulsion (6 days after preparation) and nonheated fresh BLG solution.

in the nonheated emulsion significantly changed its secondary structure during shelf-storage, and many disordered regions and intermolecular bonds were formed. These differences in the spectra of the two emulsions correlate well with the different emulsion stability: a large aging effect is observed for the nonheated emulsions and no aging effect is found for the heated ones.

In conclusion, no significant difference is observed between the spectra of heated and nonheated emulsions within several hours after their preparation. However, after 6 days of storage, the secondary structure of the adsorbed protein in heated emulsion remained similar to that of the initially dissolved protein, whereas the adsorbed molecules in the nonheated emulsion significantly changed their structure; i.e., more disordered structure and surface aggregates were formed. These conformational changes correlate well with the observed changes in the emulsion stability upon shelf-storage and after heating.

6. Summary of the Main Results and Conclusions

A combination of experimental methods was applied to assess the effects of several factors (heating, shelf storage, pH, electrolyte concentration, protein adsorption) on the coalescence stability of BLG-containing emulsions and to explain the observed trends:

(A) Effect of Emulsion Heating on Protein Adsorption and Emulsion Coalescence Stability. The heating of emulsions at $C_{BLG} \geq 0.04$ wt %, $C_{EL} \geq 150$ mM, and pH ≥ 6.2 leads to additional protein adsorption and irreversible attachment of the protein molecules in the formed adsorption multilayers. As a result, the emulsion coalescence stability increases more than 3 times. The stability of emulsions, heated under these conditions, does not change after subsequent decrease of pH, change of C_{EL}, or upon shelf storage (up to 6 days).

The increased adsorption and the irreversible attachment of the protein molecules in the adsorption layer (observed at $C_{BLG} \geq 0.04$ wt %, $C_{EL} \geq 150$ mM, and pH ≥ 6.2) are most probably due to formation of disulfide bonds upon heating.

The heating of emulsions at $C_{EL} \leq 10$ mM (prepared at the same C_{EL}) does not change emulsion stability, due to significant electrostatic repulsion between the protein molecules, which suppresses the additional protein adsorption and the formation of intermolecular bonds during heating.

The heating of emulsions at pH around IEP does not change their stability, which indicates that no intermolecular covalent bonds are formed in this case. Probably, the protein molecules do not unfold after adsorption, which keeps the reactive sulfhydryl groups hidden inside the molecular interior.

(B) Change of Emulsion Coalescence Stability during Shelf-Storage (Aging Effect). The stability of BLG-containing emulsions may significantly decrease after 1 day of shelf storage. This phenomenon, termed "the aging effect", is not related to changes in the mean drop size or protein adsorption.

The aging effect is caused by conformational changes in the protein adsorption layer, accompanied with formation of non-covalent bonds (H-bonds and hydrophobic interactions) between the adsorbed molecules. Probably, these bonds transform the adsorption layer into a brittle shell, which is inefficient in protecting the drops against coalescence.

The FTIR spectra show that the emulsion heating preserves the initial conformation of the adsorbed BLG molecules upon shelf-storage, which correlates very well with the disappearance of the aging effect after heating.

The observed effects of emulsion heating and aging are much stronger when protein multilayers are formed on the surface of the oil drops. Remarkably, both heating and aging lead to formation of strong intermolecular bonds inside the adsorption layers and to irreversible attachment of the adsorbed protein molecules (including those that are not in direct contact with the oil−water interface), while the effect on emulsion coalescence stability is the opposite: the heating increases emulsion stability, whereas the aging strongly decreases the emulsion stability.

Most of the explanations given above are in a very good agreement with the explanations given by McClements and coauthors[13,24,26−30] for the flocculation stability of BLG emulsions (a detailed comparison of the main conclusions from our studies and those by McClements et al. is presented in a review article[12]). Although the results are obtained with one specific protein, BLG, the proposed physicochemical and molecular explanations imply that the observed trends might be typical for other globular proteins that partially unfold upon adsorption and form intermolecular S−S bonds upon heating.

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