JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Effect of Thermal Behavior of β-Lactoglobulin on the Oxidative Stability of Menhaden Oil-in-Water Emulsions

Pui Yeu Phoon,[†,§] Ganesan Narsimhan,[‡] and Maria Fernanda San Martin-Gonzalez*[,†]

[†]Department of Food Science, 745 Agricultural Mall Drive, Purdue University, West Lafayette, Indiana 47907, United States

[‡]Department of Agricultural and Biological Engineering, 225 South University Street, Purdue University, West Lafayette, Indiana 47907, United States

ABSTRACT: This study reports how emulsion oxidative stability was affected by the interfacial structure of β-lactoglobulin due to different heat treatments. Four percent (v/v) menhaden oil-in-water emulsions, stabilized by 1% (w/v) β-lactoglobulin at pH 7, were prepared by homogenization under different thermal conditions. Oxidative stability was monitored by the ferric thiocyanate peroxide value assay. Higher oxidative stability was attained by β-lactoglobulin in the molten globule state than in the native or denatured state. From atomic force microscopy of β-lactoglobulin adsorbed onto highly ordered pyrolytic graphite in buffer, native β-lactoglobulin formed a relatively smooth interfacial layer of 1.2 GPa in Young's modulus, whereas additional aggregates of similar stiffness were found when β-lactoglobulin was preheated to the molten globule state. For denatured β-lactoglobulin, although aggregates were also observed, they were larger and softer (Young's modulus = 0.45 GPa), suggesting increased porosity and thus an offset in the advantage of increased layer coverage on oxidative stability.

KEYWORDS: *β-lactoglobulin, emulsion, oxidation, nanorheology, atomic force microscopy (AFM)*

INTRODUCTION

There has been ongoing great attention given to the use of milk protein β-lactoglobulin and its complexes with other ingredients in the encapsulation of oil to control flocculation and creaming in emulsions.[1−5] From time to time, there were also research works reported on the lipid oxidative stability of emulsions with β-lactoglobulin dispersed in the continuous aqueous phase, whereby the focus had been on the antioxidant activity of the protein.[6−8] In comparison, the relationship between the lipid oxidative stability of β-lactoglobulin emulsions with the protein structure at the oil/water interface, however, is scarcely reported. Hu et al.[9] studied the impact of β-lactoglobulin, sweet whey, whey protein isolate, and α-lactalbumin on the oxidative stability of salmon oil-in-water emulsions and found that, at pH 3, the greatest oxidative stability could be engineered using β-lactoglobulin.

The aim of this work was to understand how the thermal behavior of the globular protein β-lactoglobulin affects its interfacial structure when adsorbed onto an oil−water interface and how this in turn determines oxidative stability. The thermal behavior of β-lactoglobulin, at a neutral pH and a 1% w/v concentration, proceeds along different phases. When heating is up to 60 °C, it has been reported that the native dimer of β-lactoglobulin dissociates into monomers.[10] On further heating to 70 °C, there is an increase in the exposure of the buried hydrophobic residues and thiol groups, the latter being described as a precursor group for initiating polymerization.[11] The tertiary structure becomes looser and less defined, but the native secondary structure is largely retained, leading to what is known as a "molten globule state".[12] The molten globule has a strong tendency to aggregate into spherical particulates,[13] which form the obligatory building blocks of larger aggregates during heating between 70 and 80 °C.[13,14] Aggregation at this high temperature range is caused by thiol/disulfide exchange and nonspecific noncovalent bonding.[15]

Not much investigation has been reported on the interfacial structural properties of protein in an emulsion system, due to the paramount challenge of emulsion turbidity. Very recently, synchrotron radiation circular dichroism (SRCD) spectroscopy has been reported to be useful in following the conformational changes of β-lactoglobulin during adsorption to a hexadecane/water interface in emulsions.[16] In other cases, the investigation involves disruptive analysis of interfacial protein separated from emulsions, as in some FT-IR studies reported,[17,18] or centers around a simplified bilayer system with a planar interfacial film, using techniques such as interfacial dilatational rheometry[19] and Raman microspectroscopy.[20] In this study, the oxidative stability of a fish oil-in-water emulsion that was made by high-pressure homogenization (HPH) and stabilized by β-lactoglobulin subjected to different heat treatments was investigated. The secondary and tertiary conformational changes as well as aggregation of β-lactoglobulin were characterized. Atomic force microscopy (AFM) was used to study the force properties of β-lactoglobulin adsorbed onto a hydrophobic surface in an aqueous environment. Highly ordered pyrolytic graphite (HOPG) substrate was used to simulate the hydrophobicity of an oil surface. Although actual oil was not used and the dynamic effects of emulsification could not be simulated, this work provides a reference, from an alternative nanorheological perspective, of the interfacial behavior of β-lactoglobulin subjected to different thermal treatments. The observed differences in oxidative stability

Received: November 13, 2012
Revised: January 23, 2013
Accepted: January 28, 2013
Published: January 28, 2013

under different thermal treatments were explained in terms of differences in the structure of protein adsorbed layer at the oil–water interface.

MATERIALS AND METHODS

Chemicals. Bovine β-lactoglobulin powder was supplied by Davisco Foods International, Inc. (Le Sueur, MN, USA). The protein was considered to be in native state, through verification of its circular dichroism spectra—at ranges of wavelength pertaining to secondary and tertiary structures—against the reference signature spectra in the literature. The following were purchased from Sigma-Aldrich (St. Louis, MO, USA): 1-anilino-8-naphthalene (ANS), 2-propanol, 30% hydrogen peroxide, ammonium thiocyanate, barium chloride dehydrate, disodium phosphate, ferric chloride hexahydrate, ferrous sulfate heptahydrate, isooctane, menhaden fish oil, and sodium chloride. The following were purchased from Mallinckrodt Chemicals Inc. (St. Louis, MO, USA): butanol, hydrochloric acid, and methanol. Iron powder was bought from Acros Organics (Geel, Belgium). All of the chemicals for electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA, USA). Double-distilled water was used to prepare all aqueous solutions.

Preparation of Emulsion Samples. *Sample Composition and Heat Treatment Combinations.* One percent (w/v) of β-lactoglobulin in 0.05 M sodium chloride and 0.05 M disodium phosphate solution was prepared at room temperature, during which the mixing time for ensuring complete solvation of the protein was at least 3 h and the pH was eventually adjusted to 7.0. The protein solution was used to prepare 4% (v/v) fish oil-in-water emulsions via HPH. The emulsion preparation process involved homogenization at various temperatures, with or without preheating the protein solution beforehand. Preheating was carried out by filling the protein solution into Kimax 15 mL disposable glass tubes (17 mm o.d. × 126 mm length) with screw thread tops and then immersing the tubes into a water bath. The tubes were cooled in room temperature water immediately after preheating. Table 1 shows the heat treatment combinations studied.

Table 1. Heat Treatment Combinations Used To Prepare Fish Oil Emulsions

preheating of protein solution to 65 °C in a water bath	homogenization temp (°C)
none (native)	8
none (native)	28
none (native)	48
none (native)	58
none (native)	68
none (native)	77
1 min	28
1 min	48
1 min	58
2 min	28
2 min	48
2 min	58

Emulsification via High-Pressure Homogenization. A 4% (v/v) fish oil-in-water coarse emulsion was made using a VirtiShear homogenizer (Virtis, Gardiner, NY, USA) at 30000 rpm for 10 s, immediately followed by HPH using a Nano DeBEE 45 high-pressure homogenizer (BEE International, South Easton, MA, USA) for three passes at 20 kpsi (137.9 MPa) and the corresponding treatment temperature. For temperature control during HPH, a countercurrent tubular heat exchanger that was connected to a temperature-regulated water bath was positioned downstream of the emulsifying cell. With the sample at the inlet kept within 2–3 °C of the designated temperature, the homogenization time was typically 2 min, except when homogenization was carried out at 8 and 28 °C, during which the homogenization time was ≤13 min. Each emulsion sample was prepared in triplicates.

Ferric Thiocyananate Peroxide Value Assay. Emulsion samples were pipetted in 1 mL volumes into 1.775 mL screw-capped Eppendorf tubes and incubated in an environmental shaker in the dark at 55 °C. Triplicates were removed daily over a period of 7 days for the determination of oxidative stability, based on the spectrophotometric measurement of the ability of peroxides to oxidize ferrous ions to ferric ions.[21] Emulsion sample (0.3 mL) was added to 1.5 mL of isooctane/2-propanol (3:1 v/v) extraction solvent. The mixture was vortexed three times for 10 s each and centrifuged in an Eppendorf microcentrifuge (5415D, Hauppauge, NY, USA) for 8 min at 720*g* rcf. Then 50 μL of the separated organic phase was added to 2.95 mL of methanol/butanol (2:1 v/v) mixture. This was followed by the addition of 15 μL of a 3.94 M ammonium thiocyanate aqueous solution and 15 μL of a 0.072 M ferrous iron acid solution. The 3.03 mL aliquot was vortexed, and absorbance at 510 nm was measured after 20 min of incubation at room temperature, against a ferric ion standard curve using a UV–vis spectrophotometer (DU 800 Beckman Coulter, Schaumburg, IL, USA). All absorbance measurements were adjusted by an average of three blank measurements, in which the 50 μL volume of the separated organic phase was replaced by 50 μL of methanol/butanol (2:1 v/v) mixture.

A ferric ion standard curve was created on the basis of the measurement of ferric ion dilutions of a 10 μg/mL stock solution. The procedures for the preparation of both the ferric ion stock solution and the ferrous iron acid solution were described in ref 21.

Emulsion Drop Size Measurement. The same triplicate samples prepared for the ferric thiocyananate peroxide value assay were diluted 100 times by deionized water, and each triplicate was measured twice for particle size (*Z* average) at 25 °C by dynamic light scattering (DLS), using a Zetasizer Nano ZS90 with optical arrangement at 90° (Malvern Instruments Ltd., Malvern, UK). During size measurement, the refractive indices of water and oil were set at 1.33 and 1.46, respectively, whereas the dispersant viscosity was that of water at 25 °C (i.e., 0.89 cP). Each measurement comprised 15 trial runs each 10 s long.

According to Thomas,[22] the average value of the *Z* average (Z_{av}) was converted to the average sauter mean diameter (d_{32}) as

$$\text{av } d_{32} = Z_{av} \times (1 + Q)^2 \quad (1)$$

where *Q* was the average experimentally determined polydispersity obtained during particle size measurement.

Calculations of the Intrinsic Rate of Oxygen Depletion. The total rate of oxygen depletion during oxidation of oil was proportional to the rate of peroxide formation

$$\frac{\partial(\text{Abs}_t - \text{Abs}_0)}{\partial t}$$

which was in turn proportional to $(6/d_{32})$ × intrinsic rate of oxygen depletion per unit of interfacial area of the emulsion drops, where Abs_0 was the average absorbance reading of a sample emulsion corrected by that of the blank at time = 0, Abs_t was that at time = *t*, and $(6/d_{32})$ represented the ratio of the total surface area of the emulsion drops to total volume of the drops.

Hence, the relative intrinsic rate of oxygen depletion per unit of interfacial area of the emulsion drops was calculated as

$$\frac{\partial(\text{Abs}_t - \text{Abs}_0)}{\partial t} \times \frac{d_{32}}{6} \quad (2)$$

Circular Dichroism (CD). CD spectra were collected using a Jasco J-810 spectrometer (Jasco Inc., Easton, MD, USA). A quartz cuvette with a path length of 2 mm was used. Ellipticity (mdeg) data were collected in continuous scanning mode, with the bandwidth set at 2 nm and the data pitch set at 0.2 nm. During the investigation of protein secondary structure in the wavelength range of 190–260 nm, the concentration of β-lactoglobulin in deionized water used was 0.005% (w/v) to avoid oversaturation of signal, whereas when tertiary protein structure was investigated in the wavelength range of 250–320

nm, the protein concentration was at 1% (w/v) to obtain a satisfactory signal/noise ratio.

Protein Structure at Different Temperatures. To record the secondary and tertiary structures of β-lactoglobulin at different temperatures, CD spectra were collected within 2−8 min after the solutions were equilibrated to 8, 28, 48, 58, 68, and 77 °C in the sample chamber. The average spectrum at each temperature was plotted using the Spectra Manager software from Jasco, based on six scans from duplicate samples performed during that time frame. The response time was set at 4 s, and the scanning speed was set at 50 nm/min.

Protein Structural Changes during Heating and Subsequent Cooling. The protein solution was heated to and held at 65 °C in the sample chamber within 1 or 2 min and then cooled immediately to 25 °C. CD spectra were recorded just before cooling and also in the next 3−8 min when the solution had cooled to 25 °C. The response time was set at 2 s and the scanning speed at 100 nm/min. The average spectrum at each scanning time was plotted using the Spectra Manager software, based on two scans from duplicate samples.

Differential Scanning Calorimetry (DSC). Modulated DSC was performed using a Q2000 differential scanning calorimeter (TA Instruments, New Castle, DE, USA) to study the enthalpy of denaturation of 5% (w/v) β-lactoglobulin samples. About 20 μL of sample was sealed in a Tzero aluminum hermetic pan for each run. Modulation was controlled at ±1 °C every 60 s, and scanning was done at 3 °C/min from 25 to 100 °C. In the simulation of preheating to 65 °C within 1 min, the sample was heated at 40 °C/min from 25 to 65 °C within the sample chamber and promptly cooled at 30 °C/1.5 min to 25 °C before a second thermal scanning at 3 °C/min from 25 to 100 °C was made. In the simulation of preheating to 65 °C within 2 min, the sample was heated at 40 °C/1.5 min from 25 to 65 °C within the sample chamber and isothermally held for another 0.5 min before being promptly cooled at 30 °C/1.5 min to 25 °C followed by the second thermal scanning. Data analysis was done using the TA analysis software.

ANS Binding Fluorescence Assay of Protein Solutions. The protein solutions studied were those mentioned in Table 2, but with the heat treatment during HPH simulated by the immersion of the solutions in 28, 48, 58, 68, and 77 °C water baths (8 °C was omitted) for 3 min. The assay involved the mixing of 200 μL of 1% (w/v) protein solution with 10 μL of 4.8×10^{-4} M ANS in ethanol in the dark at room temperature. The assay of the entire mixture volume was performed in 96-well black plates with flat bottoms (Costar) using a FlexStation 2 fluorescence spectrophotometer (Molecular Devices, Inc., Sunnyvale, CA, USA) The excitation wavelength was 360 nm, and emission fluorescence spectra in the wavelength range of 450−550 nm were collected. All data were collected at 25 °C.

Table 2. ANS Assay Results of β-Lactoglobulin Subjected to Different Heat Treatments[a]

preheating in a 65 °C water bath	heating temp in water bath (°C)	av fluorescence intensity (arbitrary units)
none (native)	28	69500 ± 2600aA
none (native)	48	76700 ± 600abA
none (native)	58	88500 ± 1000cA
none (native)	68	86400 ± 200bc
none (native)	77	112500 ± 1400d
1 min	28	85900 ± 1400A
1 min	48	84100 ± 500A
1 min	58	83800 ± 2400A
2 min	28	82900 ± 2200A
2 min	48	71600 ± 1600A
2 min	58	75100 ± 3900A

[a]Each reported average intensity value was based on the readings of duplicate samples taken at 25 °C. The emission wavelength was 462 nm. The error is in standard deviation. Values with different letters indicate statistically significant differences ($p < 0.05$).

Native Polyacrylamide Gel Electrophoresis (PAGE). The protein solution samples used in the ANS assay were diluted to 0.04% (w/v) and analyzed by native PAGE. A 15% resolving gel with a 4.5% stacking gel was hand cast, following closely instructions available from Bio-Rad.[23] During native PAGE, the native sample buffer (Bio-Rad, Richmond, CA, USA) was added to each diluted sample at a 1:1 volume ratio and vortexed. Thirty microliters of the mixture was loaded onto the gel (6 μg protein/well). Electrophoresis of the samples was run with a Mini Protean II (Bio-Rad) gel electrophoresis chamber, with a 10 times diluted Tris/glycine (Bio-Rad) running buffer. After electrophoresis, the gels were stained with Coomassie staining solution (50% methanol, 10% glacial acetic acid, 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad)) for 1 h and subsequently destained (with 10% glacial acetic acid, 5% methanol) overnight. The image of the gel was captured, and band densities were measured, using the Chemi-Doc XRS system (Bio-Rad).

Size Exclusion Chromatography−Multiangle Light Scattering (SEC-MALS). Three solution samples of 1% (w/v) of β-lactoglobulin in 0.05 M sodium chloride and 0.05 M disodium phosphate (pH 7) were studied: (i) native protein at room temperature; (ii) solution that was heated to 65 °C within 1 min followed immediately by cooling to room temperature; and (iii) solution that was heated at 77 °C for 3 min followed immediately by cooling to room temperature. The samples were analyzed in duplicates by SEC-MALS performed on a Dawn Heleos 2 system (Wyatt Technology Corp., Santa Barbara, CA, USA). The sample was injected onto an S500HR separation column and run at a flow rate of 1.3 mL/min under a pressure of 1.3 MPa. The mobile phase was deionized water, with 0.02% (w/v) sodium azide added. The total elution per run was 2 h. The obtained SEC profile from the refractive index (RI) signal was subsequently processed with the program ASTRA 5.3.4.14. from Wyatt Technology, using the Zimm fit and a refractive index increment (dn/dc) of 0.185.[24]

Atomic Force Microscopy. *Instrumentation.* The AFM studies were performed using an MFP-3D atomic force microscope from Asylum Research (Santa Barbara, CA, USA). IgorPro software (WaveMetrics Inc., Portland, OR, USA) was used to control the instrument and for data acquisition. The software WSxM v5.0 Develop 3.2 (Nanotec Electrónica, Madrid, Spain)[25] and Microsoft Excel 2007 were used for data analysis. The cantilevers used were from MikroMasch (Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria), 200 μm long, and had a nominal spring constant of 0.3 N/m. The probes were made of silicon and were cone-shaped, with a tip radius of 10 nm and a half angle of 36°.

Sample Preparation and Imaging. Three solution samples of 0.1% (w/v) of β-lactoglobulin in 0.05 M sodium chloride and 0.05 M disodium phosphate (pH 7) were prepared: (i) native protein solution at room temperature; (ii) solution that was heated to 65 °C within 1 min followed immediately by cooling to room temperature; and (iii) solution that was heated at 77 °C for 3 min followed immediately by cooling to room temperature. First grade HOPG (SPI Supplies, West Chester, PA, USA) was used as the surface substrate. A freshly cleaved HOPG surface was covered with plenty of sample solution overnight to allow protein adsorption. The next day, the HOPG was drained of the sample solution and fixed on top of a magnetic piece on a microscopic glass before it was transferred onto the magnetic stage of the atomic force microscope. A 0.05 M sodium chloride and 0.05 M disodium phosphate (pH 7) buffer was pipetted on top of the HOPG to simulate a fluid environment for the adsorbed protein. Images were acquired in the alternative current (ac) tapping mode.

Force Measurement. Force curves were acquired in the direct current (dc) tapping mode, with single penetration measurements made on different locations on the surface of zoomed-in topological features. The image area was scanned again after the force measurements to ensure that the analyzed features had not drifted away. Force measurements were also performed on bare hard mica substrate under the same buffer to get the inverse optical lever

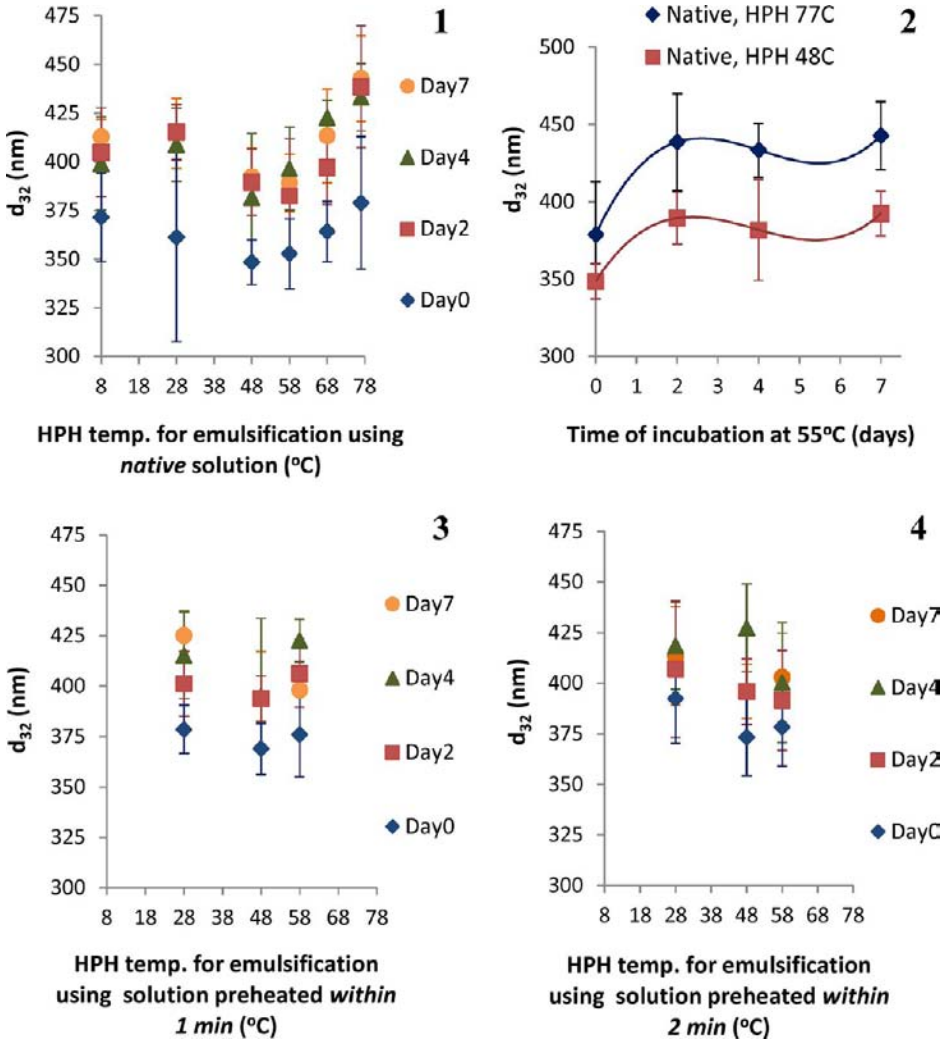


Figure 1. Plots showing the effect of HPH temperature on the size of emulsion droplets (d_{32}) using solutions of β-lactoglobulin (1, 2) in native state, (3) preheated within 1 min, and (4) preheated within 2 min. The emulsion samples were stored at 55 °C over a period of 7 days for analysis. Error bars indicate the standard deviation from the mean of d_{32} values derived from six measurements.

sensitivity (INVOLS) of the probe, so that the raw deflection data of the protein samples could be converted to force data.

Young's Modulus Calculation. The indentation depth δ on the surface of the protein was defined by the difference in the cantilever position z between an infinitely hard surface (e.g., mica) and the sample surface at every force level. The force measurement results were fitted to the Hertz model by plotting the graph of force versus $\delta^{3/2}$, because the Hertz model is expressed as

$$F = \frac{4}{3}E^*\sqrt{R}\,\delta^{3/2} = K\delta^{3/2} \tag{3}$$

where F is the force level, E^* is the reduced elastic modulus when two spheres are forced together by a force load, R is the radius of curvature of the tip under the assumption that the probed protein surface is infinitely planar compared to the tip, and K is the slope of the graph.

E^* is related to Young's modulus of the adsorbed protein surface, E_s, and that of the stiff tip, E_t, by

$$\frac{1}{E^*} = \left(\frac{1-\nu_s^2}{E_s}\right) + \left(\frac{1-\nu_t^2}{E_t}\right) \tag{4}$$

where ν_s and ν_t are the values of Poisson's ratio for the adsorbed protein surface and the tip, respectively. The value of ν_s is assumed to be 0.5 for soft biological samples,[26] such as protein in this case, whereas the value of ν_t is assumed to be near 1 due to the stiffness of the tip. Furthermore, because $E_t \gg E_s$ the second term in the right-hand side of eq 4 becomes negligible and may be simplified[26] to

$$\frac{1}{E^*} = \frac{1-0.5^2}{E_s} \tag{5}$$

The Young's modulus of the adsorbed protein could thus be calculated as

$$E_s = E^*(1-0.5^2) = \frac{3K}{4\sqrt{R}}(1-0.5^2) \tag{6}$$

Statistical Analysis. All experiments were performed in triplicate and measurements performed at least in triplicate. Data were analyzed using a general linear model or one-way ANOVA, and significance was established at $\alpha = 0.05$. Pairwise comparisons were done using Tukey's method.

RESULTS

The raw z-average data of the emulsion samples obtained from the dynamic light scattering studies suggested that droplet size in each of the emulsion samples studied was increasing from day 0 to day 2 before stabilizing. However, Figure 1 shows that from day 0 to day 2, there was no significant increase in the calculated d_{32} ($p > 0.05$), which was converted from z-average[22] considering the polydispersity index obtained during droplet

dx.doi.org/10.1021/jf304834n | J. Agric. Food Chem. 2013, 61, 1954−1967

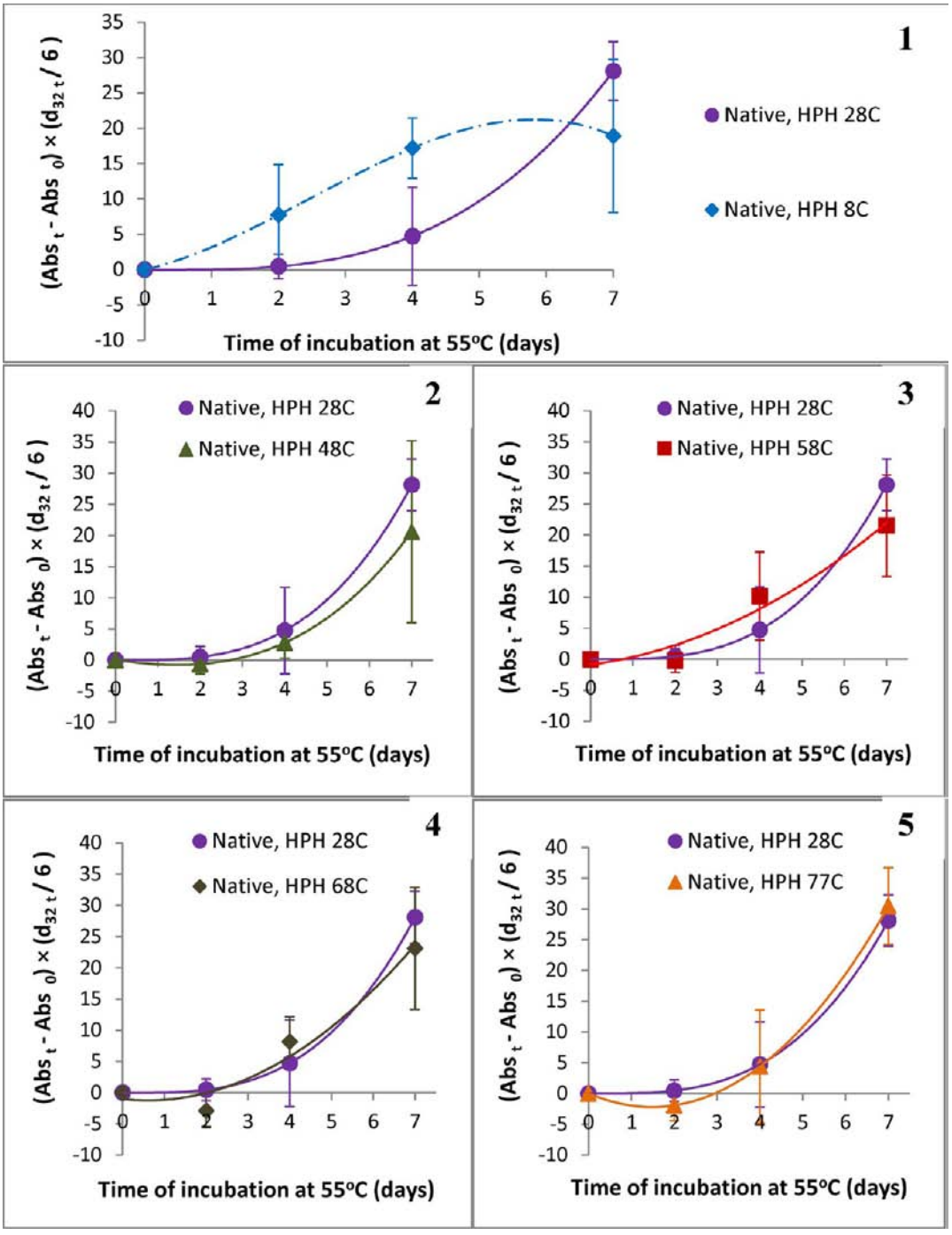


Figure 2. Plots of relative intrinsic rate of oxygen depletion in fish oil per unit interfacial area versus time, as influenced by the HPH temperature at (1) 8 °C, (2) 28 °C, (3) 48 °C, (4) 58 °C, and (5) 68 °C, for emulsion samples created with solutions of 1% (w/v) β-lactoglobulin in native state. Error bars indicate the standard deviation from the mean of oxygen depletion. In all panels, the data are compared with corresponding data for HPH 28 °C.

size measurement. This is evidenced by the overlapping of the error bars of standard deviation of the displayed data points. It could be inferred that all of the emulsions studied were physically stable. Notably, the emulsion created at 77 °C with native β-lactoglobulin solution had the largest droplet size, as can be seen from Figure 1.2; however, the difference became significant ($p < 0.05$) only after incubation at 55 °C for 7 days.

From the intrinsic rates of oil oxidation in the emulsions depicted in Figures 2, 3, and 4, it was evident that the ability of β-lactoglobulin to protect the encapsulated oil against oxidation was poorest when the HPH temperature was at a low temperature of 8 °C (see Figure 2.1), using the oxidative stability of the emulsion formed with native β-lactoglobulin during HPH at 28 °C as a benchmark for comparison. Interestingly, however, very brief preheating of β-lactoglobulin to 65 °C within 1 min and subsequent emulsification at a moderate temperature of 28 °C led to the best oxidative stability attained (see Figure 3.1). Without such a thermal history of β-lactoglobulin, emulsification at a temperature below 65 °C (i.e., 28, 48, and 58 °C) did not lead to significantly

dx.doi.org/10.1021/jf304834n | J. Agric. Food Chem. 2013, 61, 1954−1967

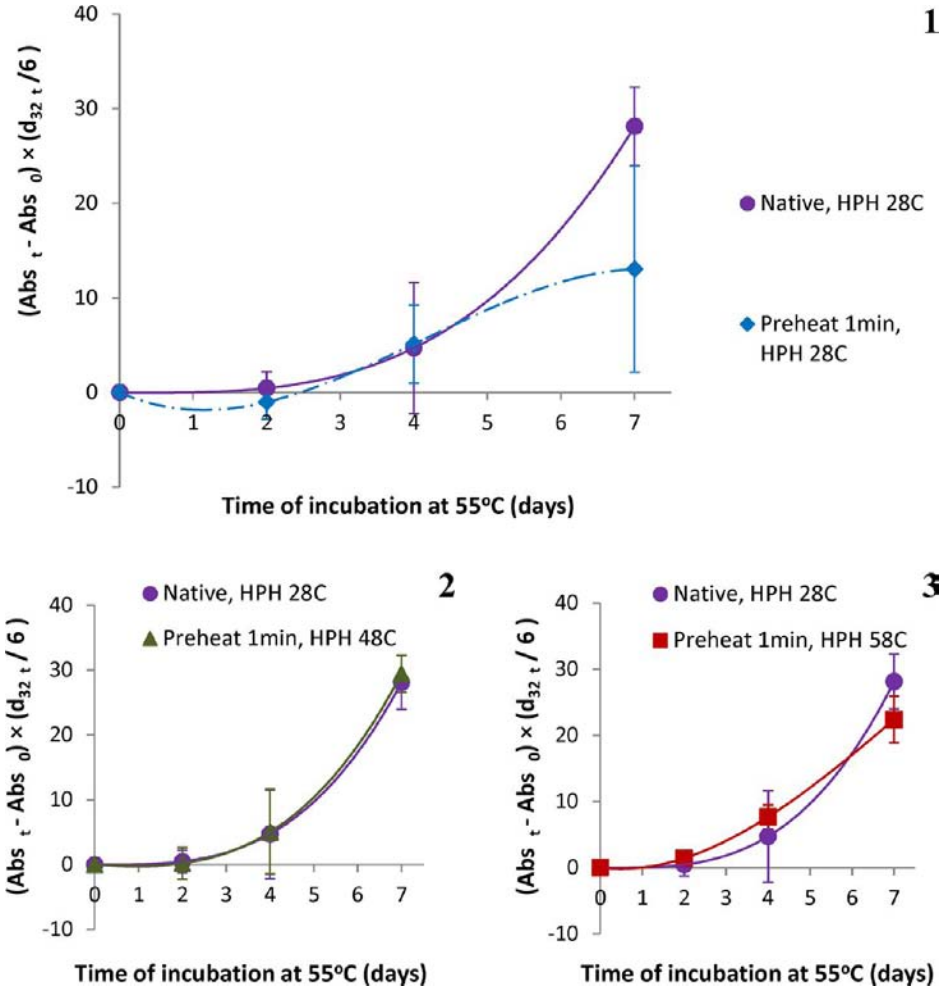


Figure 3. Plots of relative intrinsic rate of oxygen depletion in fish oil per unit interfacial area versus time, as influenced by the HPH temperature at (1) 28 °C, (2) 48 °C, and (3) 58 °C, for emulsion samples created with solutions of 1% (w/v) β-lactoglobulin preheated to 65 °C within 1 min. Error bars indicate the standard deviation from the mean of oxygen depletion. Except for panel 1, the data are compared with corresponding data for HPH 28 °C without preheating.

improved oxidative stability as compared with the benchmark. Yet, more intense thermal combinations of preheating and temperature setting during HPH, that is, preheating to 65 °C within 1 min plus HPH at 48 or 58 °C and preheating to 65 °C within 2 min plus HPH at 28, 48, and 58 °C, did not lead to an improvement in oxidative stability as can be seen from Figure 3.2 and 3.3 and Figure 4.1, 4.2, and 4.3, respectively. Emulsification at 68 and 77 °C using native β-lactoglobulin—deemed to be the most intense heat treatments studied here—resulted in oxidative stability similar to that of the benchmark as well.

CD studies in Figure 5 reveal that protein secondary structure was conserved until 77 °C, indicating the set-in of denaturation at 77 °C. Figure 6 shows that protein tertiary structure was the most rigid at 8 °C and that it was gradually lost with increase in temperature, as indicated by the peak areas between 280 and 288.8 nm and between 288.8 and 300 nm.

Whereas Figure 7 suggests there was apparently not much change in the secondary structure because the CD patterns were still closely similar, Figure 8 suggests that preheating of β-lactoglobulin to 65 °C within 1 or 2 min led to a transient change in tertiary structure as indicated by smaller peak areas between 280 and 288.8 nm and between 288.8 and 300 nm. This indicates that the protein was in a molten globule state at 65 °C. However, Figure 9 hinted at a slight irreversible change in tertiary structure due to rapid preheating, as signaled by a decrease in the enthalpy of denaturation with increasing preheating time. The results in Figure 9 are also in general agreement with the results in Table 2, which depicts that heating β-lactoglobulin at 48−68 °C, with or without preheating, led to permanent gradual increase in surface hydrophobicity as compared to the native protein at 28 °C. Consistent with the results of CD, heating the protein at 77 °C caused a drastic increase in surface hydrophobicity.

Figure 10 shows the appearance of darker bands of higher molecular weight for β-lactoglobulin heated at 77 °C, thus clearly indicating irreversible protein aggregation. Figure 11 shows that the onset of elution of β-lactoglobulin heated in a 77 °C-water bath was near 35 min, much earlier than the onset of elution of native β-lactoglobulin (heated only in a 28 °C water bath) at around 49 min as a result of aggregation at 77 °C. HPH at 28 °C shifted the onset of elution to around 46 min, probably as a result of aggregation due to heating during homogenization. Interestingly, HPH at 77 °C delayed the onset of elution from near 35 to 42 min, possibly as a result of breakup of aggregates during HPH due to shear. Noteworthily, the elution profiles of β-lactoglobulin that was preheated in a water bath to 65 °C within 1 min with and without HPH were

dx.doi.org/10.1021/jf304834n | J. Agric. Food Chem. 2013, 61, 1954−1967

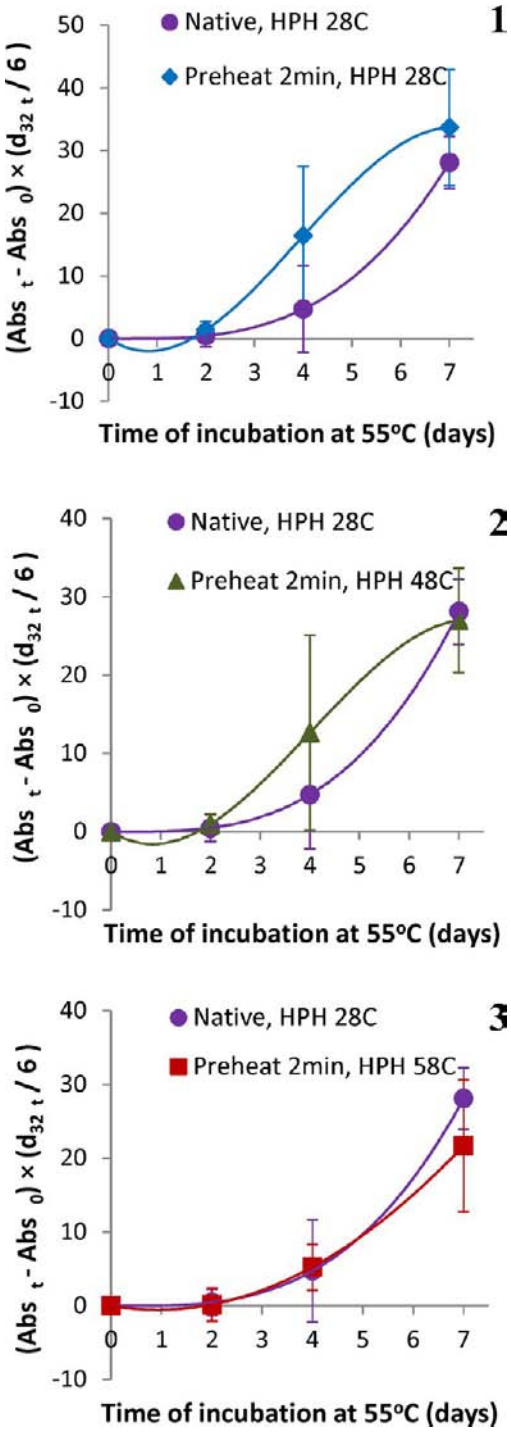


Figure 4. Plots of relative intrinsic rate of oxygen depletion in fish oil per unit interfacial area versus time, as influenced by the HPH temperature at (1) 28 °C, (2) 48 °C, and (3) 58 °C, for emulsion samples created with solutions of 1% (w/v) β-lactoglobulin preheated to 65 °C within 2 min. Error bars indicate the standard deviation from the mean of oxygen depletion.

similar to that of the native protein. Similarly, the pattern of band distributions during native PAGE, as shown in Figure 10, was similar, even though the average measured density of the band analyzed was higher when there was preheating to 65 °C within 1 min (i.e., 2079) compared to being in the native state at 28 °C (i.e., 1903).

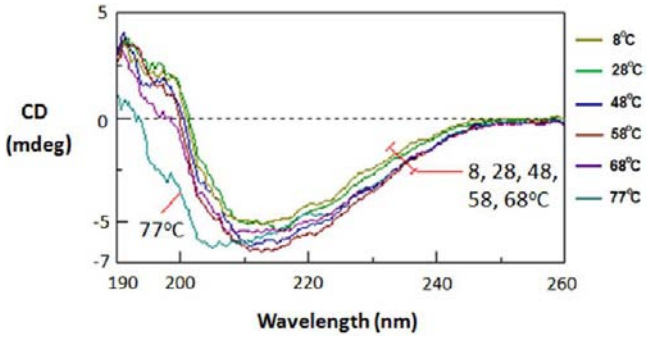


Figure 5. CD spectra of 0.005% (w/v) β-lactoglobulin solutions collected in the wavelength range of 190−260 nm, within 2−8 min after the solutions were equilibrated to 8, 28, 48, 58, 68, and 77 °C in the sample chamber. Each spectrum was an average of six scans from duplicate samples.

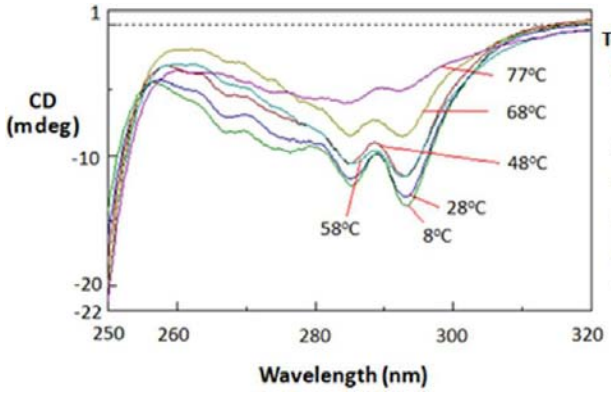
Figure 12.1 shows that there was hardly any protein globule found on the hydrophobic graphite substrate in the native β-lactoglobulin, whereas preheating at 65 °C for 1 min led to globule formation on the graphite (see Figure 12.2). With further increase in the intensity of thermal treatment prior to AFM imaging, as in the case of β-lactoglobulin heated at 77 °C, there was an increase in globules in terms of globule size and number (see Figure 12.3). Strands were observed in the sample that was preheated at 65 °C for 1 min (see Figure 13.1.1), as noted during scanning of different areas, but they were rather rare. Strands were even rarer in the native sample (not shown). For the preheated sample, the strand and globule species were within 20−30 nm in height, as depicted in Figure 13.1.2. On the other hand, for the sample that was heated at 77 °C, the typical height of a globule was around 160 nm.

The phase contrast images of the features portrayed in Figure 13 are shown in Figure 14. Panels 1.1 and 1.2 of Figure 14 show negligible phase contrast between the surface of the features and the background, suggesting the same material composition. On the contrary, panels 2.1 and 2.2 of Figure 14 show that the feature was composed of a material different from that of the background, with a greater phase contrast as much as 16°.

From Figure 15, it could be inferred that β-lactoglobulin was similar in stiffness when in the native state or when it had been preheated at 65 °C for 1 min, but became more adhesive and softer upon denaturation at 77 °C. The stiffness of the strand and globule species under these three conditions was quantitatively analyzed, via the fitting of data to the Hertz model, as delineated in Figure 16. Table 3 shows the stiffness expressed in the Young's modulus of elasticity. The adhesive globules of the denatured β-lactoglobulin typically had a Young's modulus of 0.45 GPa, whereas the globules and strands of the native protein and the protein heated at 65 °C had Young's moduli larger by >2-fold (1.13−1.52 GPa). The stiffness of the HOPG background surface not covered by any strand or globule also included in Table 3. The similar order of magnitude but a large standard deviation in the stiffness was found for the background surface, compared the strands and globules.

DISCUSSION

Although most of the oxidative stability plots in Figures 2−4 (except Figures 2.1 and 3.1) are very similar to that of the native sample homogenized at 28 °C, the CD spectra show that the heat treatments had clearly induced differences in protein



Temp. (°C)	Peak areas 280-288.8nm	288.8-300nm
8	-11.3	-32.0
28	-9.0	-28.0
48	-8.6	-24.8
58	-7.3	-20.9
68	-6.6	-14.6
77	-3.2	-4.1

Figure 6. (Left) CD spectra of 1% (w/v) β-lactoglobulin solutions collected in the wavelength range of 250−320 nm, within 2−8 min after the solutions were equilibrated to 8, 28, 48, 58, 68, and 77 °C in the sample chamber. Each spectrum was an average of six scans from duplicate samples. (Right) Tabulation of the peak areas (in arbitrary units) in the ranges of 280−288.8 and 288.8−300 nm for each temperature treatment.

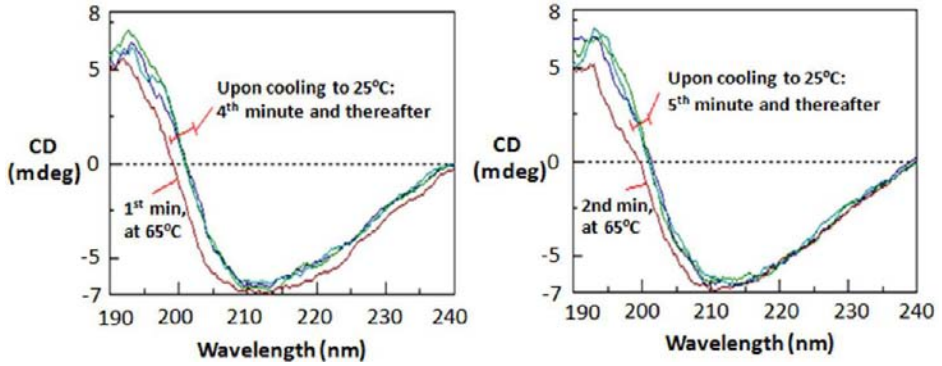


Figure 7. CD spectra of 0.005% (w/v) β-lactoglobulin solutions collected in the wavelength range of 190−260 nm. (Left) Solution was heated to and held at 65 °C in the sample chamber within 1 min and then cooled immediately to 25 °C. (Right) Solution was heated to and held at 65 °C in the sample chamber within 2 min and then cooled immediately to 25 °C. Each spectrum was an average of two scans from duplicate samples.

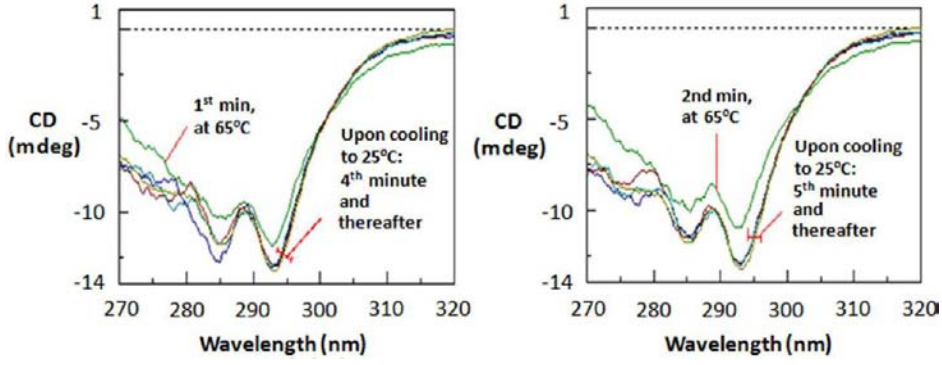


Figure 8. CD spectra of 1% (w/v) β-lactoglobulin solutions collected in the wavelength range of 270−320 nm. (Left) Solution was heated to and held at 65 °C in the sample chamber within 1 min and then cooled immediately to 25 °C. (Right) Solution was heated to and held at 65 °C in the sample chamber within 2 min and then cooled immediately to 25 °C. Each spectrum was an average of two scans from duplicate samples.

structure, especially at the tertiary level. The highest initial rate of oil oxidation in the 8 °C emulsion might be related to the rigidity of the protein. β-Lactoglobulin encompasses an eight-stranded antiparallel β-barrel enclosing a highly hydrophobic ligand-binding cavity[27,28] with an amphiphilic α-helix on the exterior. Although it is likely that this native α-helix might have played the role of an oil-binding site,[29] the energy barrier required to be overcome for the opening and unfolding of the β-barrel at the oil−water interface might have been greater than it would have been at higher homogenization temperatures. Consequently, this translated into poorer unfolding of the protein molecules during the first pass of HPH, before they are surrounded by other protein molecules in the subsequent passes of HPH.

The results in Figure 2 imply that any associated conformational change below 60 °C during processing, such as the dissociation of dimers to monomeric species at pH 7.0 in solution, had no effect on the oxidative stability of the emulsion. This was whether or not the order of species had any influence on molecular mobility, steric hindrance, and adsorption at the oil−water interface. The results in Figures 2−4 also suggest that there was an optimal heat treatment,

dx.doi.org/10.1021/jf304834n | J. Agric. Food Chem. 2013, 61, 1954−1967

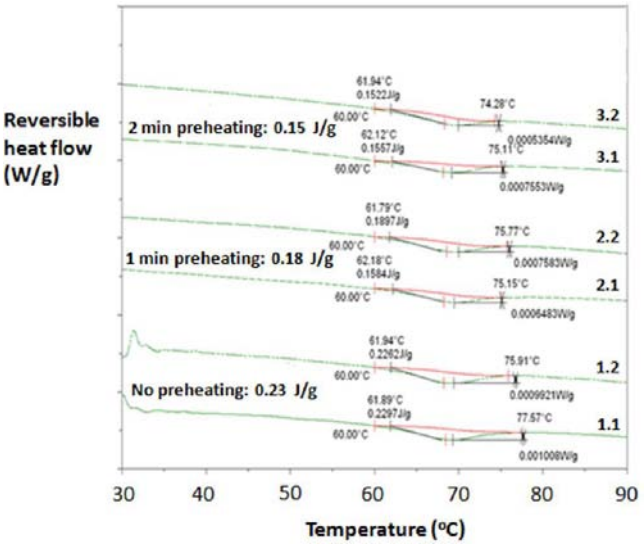


Figure 9. Plots of reversible heat flow versus temperature from modulated DSC studies of 5% (w/v) solutions of (1) native β-lactoglobulin, (2) β-lactoglobulin with a 1 min preheating history at 65 °C in the sample holder; and (3) β-lactoglobulin with a 2 min preheating history at 65 °C in the sample holder. The average enthalpies of denaturation from duplicate samples are included in the figure.

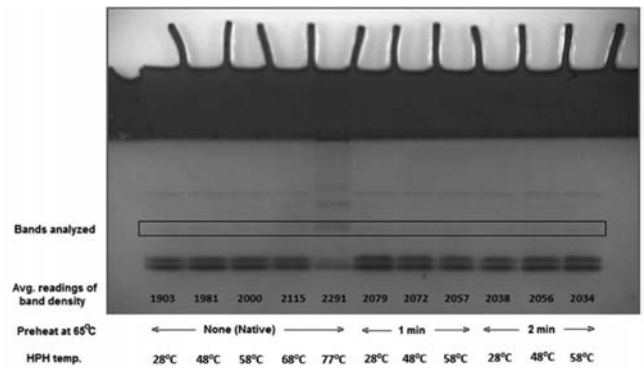


Figure 10. Results of native PAGE of β-lactoglobulin subjected to different combinations of heat treatment in water baths. The density of each band analyzed within the boxed region framed in black was an average of three readings.

beyond which the oxidative stability was comparable to that at the 28 °C benchmark, despite the expectation that more intense heat treatment would have increased protein adsorption to the oil. There are grounds for such an expe−tation. First, Kiokias et al.[30] had reported how heat denaturation resulted in whey protein emulsions with less production of conjugated diene hydroperoxides from primary oxidation. They speculated that this was due to the development of a thick, viscoelastic protein film at the interface. Second, as evident in Table 2, the surface hydrophobicity increased with heat treatment and was highest for the sample that was treated at the highest temperature and became denatured. It was conceivable that this unfolding enhanced the solvent accessibility to other buried amino acids with antioxidant activity, such as cysteine, tryptophan, and methionine,[6,8] as well as improved protein functionality in terms of emulsifying properties such as the emulsifying activity index (EAI).[31] However, the relationship between heat denaturation and EAI did not seem that

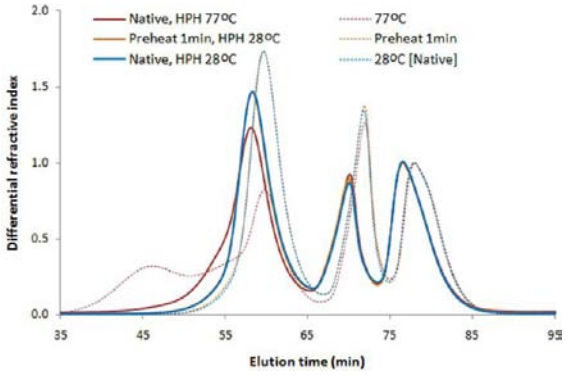


Figure 11. SEC-MALS elution profiles of selected 1% (w/v) β-lactoglobulin solutions, each normalized to the last peak. The samples were subjected either to different heat treatments in water baths to simulate the temperature conditions during HPH (as represented by dotted lines) or to actual HPH, with or without preheating history (as represented by continuous lines). Each elution profile was an average based on duplicate samples.

straightforward for β-lactoglobulin: Voutsinas et al.[32] reported that the EAI of heat-denatured β-lactoglobulin, at pH 7.4, was adversely affected. Emulsion stability was found to be maximum for intermediate heat treatment that resulted in partial unfolding of the molecule.[19] From here, the discussion shall be focused on the following three systems: (i) native β-lactoglobulin at 28 °C; (ii) β-lactoglobulin that was preheated to 65 °C within 1 min; and (iii) β-lactoglobulin exposed to 77 °C for 3 min (i.e., denatured). An alternative nanorheological perspective from the AFM studies shall be offered.

On closer analysis of the results from heat treatment involving preheating to 65 °C for 1 min followed by HPH at 28 °C, with specific reference to Figures 7−9 and Table 2, it could be inferred that β-lactoglobulin remained in a molten globule state and that renaturation was not 100%. These findings were in general agreement with similar work reported by other authors. For instance, Subramaniam et al.[33] reported that when β-lactoglobulin was refolded after being denatured by the reducing agent guanidine hydrochloride, the tryptophan phosphorescence lifetime—a measure of the amino acid residue local environment—was significantly shorter than that of a native β-lactoglobulin. In the works of Bhattacharjee and Das[34] and Bhattacharjee et al.,[35] it was revealed from extrinsic fluorescence study and FT-IR and CD that β-lactoglobulin that was refolded from thermal denaturation had less tertiary structure compared to the native protein. Despite the physical changes observed in Figures 7−9 and Table 2, the temporary heating to 65 °C within 1 min did not seem sufficient to induce aggregation, as suggested in Figures 10 and 11. Even if there was any aggregate formed during the temporary heating, it might be metastable such that it broke up under the operational pressure of 1.3 MPa during column separation in the SEC-MALS analysis. As such, the aggregates spotted in Figure 12.2 were probably not a result of deposition of existing aggregates from the aqueous phase, but rather due to protein molecules that managed to gradually pile up at sporadic locations upon a seemingly absent but pre-existing protein monolayer. The entire area excluding the aggregates in Figure 12.2 was believed to be covered by a protein monolayer because, first, this area and the aggregates seemed to be of the same material with negligible phase contrast (see Figure 14.1.1 and 14.1.2). Second, this area could not have been bare HOPG because

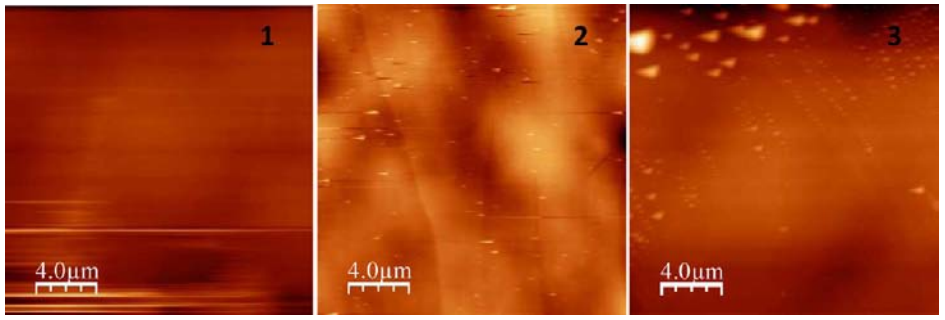


Figure 12. Representative AFM images, obtained in contact mode, of β-lactoglobulin adsorbed overnight at room temperature onto hydrophobic graphite substrate in buffer: (1) native β-lactoglobulin; (2) β-lactoglobulin preheated at 65 °C for 1 min and immediately cooled; (3) β-lactoglobulin previously heated at 77 °C for 3 min (denatured) and immediately cooled. The scan size was 20 μm × 20 μm.

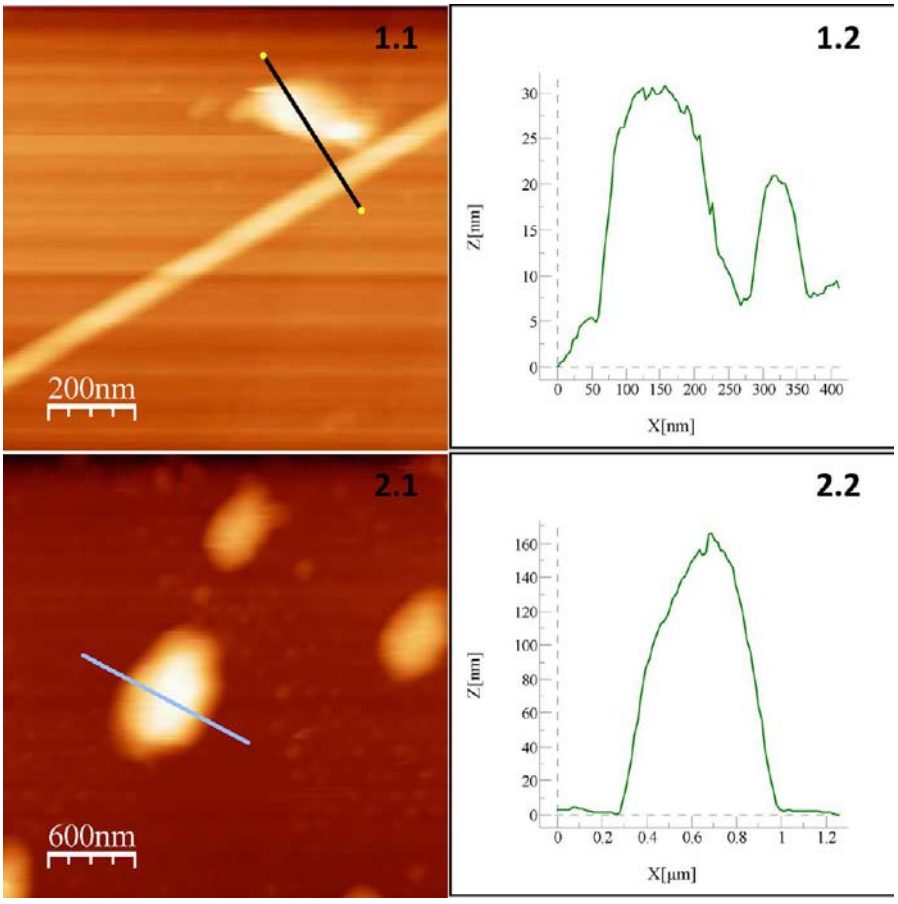


Figure 13. (Left) Close-up scans, in contact mode, of topological species of β-lactoglobulin adsorbed overnight at room temperature onto hydrophobic graphite substrate in buffer. (Right) Height profiles at the cross sections of the topological features marked: (1) β-lactoglobulin preheated at 65 °C for 1 min and immediately cooled; (2) β-lactoglobulin previously heated at 77 °C for 3 min (denatured) and immediately cooled. The strand observed in panel 1.1 was scarce, as noted during scanning of different areas.

freshly cleaved HOPG had a reported Young's modulus of 18 GPa.[36] Third, Helstad et al.[26] did a similar study on the nanorheological properties of casein globules on graphite in buffer, and they showed that with the use of 0.02% (w/v) casein—at a concentration lower than the 0.1% (w/v) protein used here—the force data obtained from the space between the globules was well fitted by the scaling theory of Alexander and de Gennes,[37,38] implying the presence of a protein monolayer.

The consequence of the incomplete renaturation of β-lactoglobulin could be a greater ease of unfolding again in the presence of a new denaturing agent, such as heat or, more relevant in this context, a hydrophobic surface such as oil. Tavel et al.[39] reported that the heat-induced molten globule of β-lactoglobulin was able to bind to hydrophobic ligands better than the native protein, due to a looser structure, especially within the β-barrel cavity. Relkin[40] reported that when solutions of β-lactoglobulin had been heated in the sample chamber of DSC at 70 °C for 1 min followed by quenching to 20 °C, the thermal transition past the denaturation temperature was only ~50% reversible when the protein was thermally scanned again. This agreed with the results depicted in Figure 9. Therefore, it is proposed that during the first pass of HPH, the molten globules that resulted from preheat treatment at 65 °C not only unfolded at the oil surface more easily and to a

dx.doi.org/10.1021/jf304834n | J. Agric. Food Chem. 2013, 61, 1954−1967

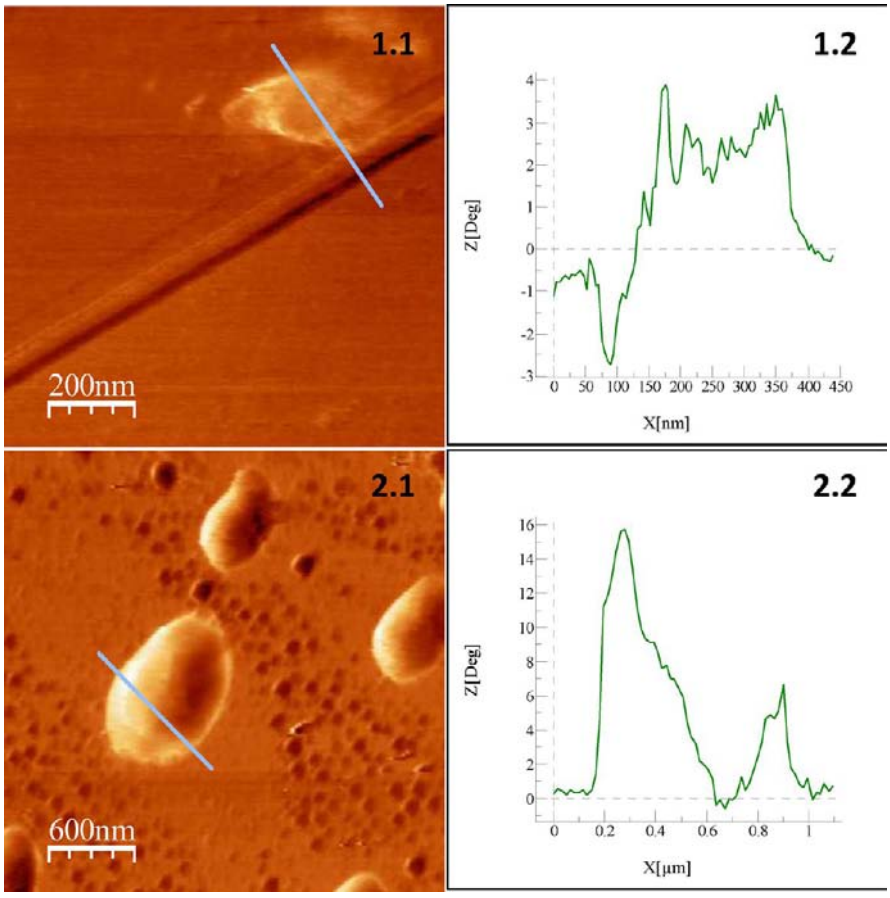


Figure 14. (Left) Phase contrast images of the close-up scans from Figure 13. (Right) Phase contrast profiles at the cross sections of the topological features marked: (1) β-lactoglobulin preheated at 65 °C for 1 min and immediately cooled; (2) β-lactoglobulin previously heated at 77 °C for 3 min (denatured) and immediately cooled. The strand observed in panel 1.1 was scarce, as noted during scanning of different areas.

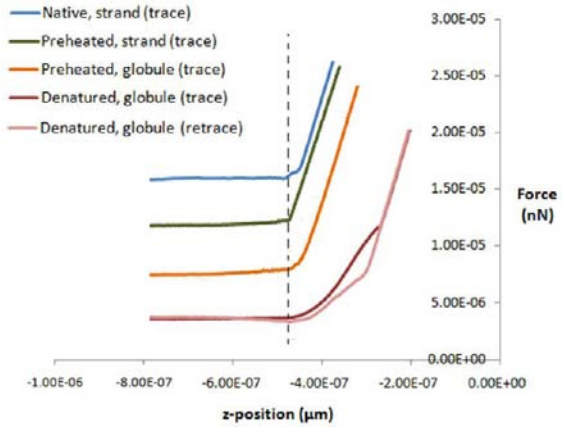


Figure 15. Representative force curves, as a function of the cantilever position z, of β-lactoglobulin adsorbed onto hydrophobic graphite substrate in buffer: (1) β-lactoglobulin preheated at 65 °C for 1 min and immediately cooled; (2) β-lactoglobulin previously heated at 77 °C for 3 min (denatured) and immediately cooled.

greater extent than the native protein but also provided a stronger anchorage pad for other protein molecules that were convectively transported to the interface during the second and third passes of HPH. Consequently, there was greater surface loading but without reduced stiffness of the interfacial region (see Table 3), in turn leading to better oxidative stability.

On the other hand, when heat treatment was carried to too great an extent, as in the case of denaturation, β-lactoglobulin unfolded even more and became even more flexible, as discussed by Kim et al.[19] on the changes in surface hydrophobicity and surface pressure−area isotherm of β-lactoglobulin with increased heating. The presence of aggregates in the solution that were identified in Figures 10 and 11 must have been a consequence of this greater extent of unfolding accompanying denaturation. Adsorption of a macromolecular emulsifier to the oil surface occurs during HPH, as described by Håkansson et al.[41]

$$\left(\frac{\partial \Gamma}{\partial t}\right)_{\text{turbulent}} = 0.272\pi\varepsilon^{1/3}(d_E + d)^{7/3}\frac{c_E(t)}{\pi d^2}\alpha_{ads},\ d \geq 2\lambda \quad (7)$$

where Γ is the surface load, t is time, ε is the local turbulent dissipation rate in the turbulent jet, d_E denotes the hydrodynamic diameter of the protein emulsifier, d denotes the hydrodynamic diameter of the oil droplet, $C_E(t)$ denotes the protein concentration in the continuous aqueous phase, α_{ads} is the efficiency or number of adsorptions per collision, and λ is the microscale of turbulence.

It was likely that during HPH the larger d_E and enhanced α_{ads} (the latter due to the adhesiveness of the aggregates—as indicated in Figure 15) promoted the adsorption of the aggregates onto the oil surface. Unlike the case when β-lactoglobulin was heated to 65 °C within 1 min, it was possible that the globules in Figure 12.3 represented the pre-existing

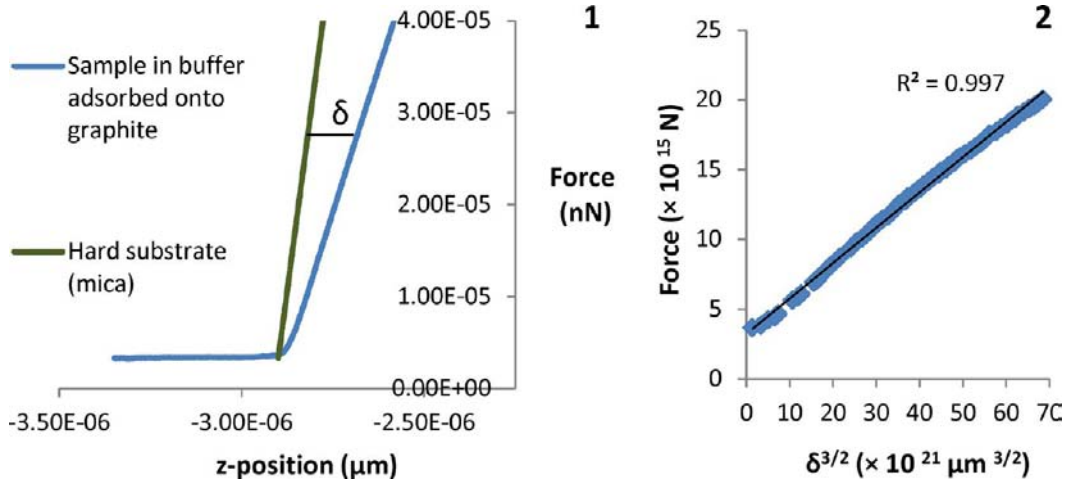


Figure 16. Typical plots used in the fitting of force measurement results to the Hertz model, during data analysis: (1) derivation of indentation depth δ, as defined by the difference in cantilever position z between an infinitely hard surface and the sample surface at every force data (only trace force curves were used; specifically, in the case of the globules from denaturation, only the nonlinear slope of the trace force curve was considered); (2) graph of force versus $\delta^{3/2}$.

Table 3. Calculated Young's Modulus E of β-Lactoglobulin, Which Was Adsorbed Overnight at Room Temperature onto Hydrophobic Graphite Substrate in Buffer[a]

β-lactoglobulin sample adsorbed onto graphite in buffer	calcd Young's modulus E (GPa)
surface not covered by features[b]	0.710 (±0.690)ab
preheated, strand	1.519 (±0.253)b
preheated, globule	1.169 (±0.276)b
denatured, globule	0.463 (±0.025)a

[a]Each reported E was based on two to five force curves performed at different spots on the surface of the species identified in Figure 13. The error is in standard deviation. Values with different letters indicate statistically significant differences ($p < 0.05$). [b]From Figure 13.1.1.

aggregates that were adsorbed onto the graphite. This probably explained the striking difference in the height profile shown in Figure 13.2.2, as compared to the height profile in Figure 13.1.2.

Uricanu et al.[42] reported that, in another AFM study on casein micelles, heating at a higher temperature caused an increase in the formation of intermolecular links, which led to greater stiffness of the micelles. In contrast, Table 3 reveals that the adsorbed globules from denaturation were significantly softer than native β-lactoglobulin, with their Young's modulus being less than half that of the native state and when heating was only to 65 °C within 1 min. Considering the larger size of the adsorbed globules from denaturation and their softness, it was conjectured that the these globules were composed of loose, porous flocs, implying greater oxygen permeability. An inverse relationship between the stiffness of whey protein gel and gel permeability had also been reported by Verheul and Roefs.[43] Perhaps the influence of the disparity in Young's moduli on the rate of oil oxidation is best exemplified by comparing the oxidation trends of the β-lactoglobulin emulsions reported in this study versus sodium caseinate emulsions reported in our previous work.[44] For both emulsion systems, the protein concentration in the solution was 1% (w/v), the buffer composition was the same, with the ionic strength at 0.15 M Na^+, the volumetric fraction of oil was equal to 4% (v/v), and the emulsions were formed via HPH under 20 kpsi (137.9 MPa). Casein had a Young's modulus of the magnitude of ~0.1 MPa under an ionic strength of 0.05 M Na^+,[26] which was far smaller than that of the values reported in Table 3 for β-lactoglobulin. Unlike the curves in Figures 2−4, where there was mostly a delay in the onset of oxidation until day 2, the curves for sodium caseinate were all convex, with a greater initial slope of $(Abs_t - Abs_0) \times (d_{32}/6)$ against time from day 0 to day 2. Sørensen et al.[45] had also found that at the oil−water interface, β-lactoglobulin had a greater positive effect on the oxidative stability of fish oil-enhanced milk emulsions than casein. On the basis of the aforementioned, a reduced Young's modulus might hold the key to why the oxidative stability for the denatured protein emulsion was not necessarily better than, but only comparable to, that of the native protein emulsion, despite the additional coverage of aggregates.

In conclusion, it was discovered that oil oxidation was more effectively impeded when β-lactoglobulin was in the molten globule state than in the native or denatured state, during adsorption at the oil−water interface. From AFM experiments conducted in a fluid environment, it was shown that when β-lactoglobulin was preheated to a molten globule state before being adsorbed onto HOPG, there was additional coverage of aggregates on HOPG that retained the original stiffness of the native protein (Young's modulus = 1.2 GPa). On the other hand, these aggregates were absent when native β-lactoglobulin was adsorbed onto HOPG. The aggregates imaged in the case of denatured β-lactoglobulin were larger, but they were also much softer (Young's modulus = 0.45 GPa), and it was postulated that the increased porosity due to extensive heating offset the advantage of increased layer coverage.

AUTHOR INFORMATION

Corresponding Author

*E-mail: msanmart@purdue.edu. Phone: +1 (765) 496-1140. Fax: +1 (765) 494-7953.

Present Address

[§]Sensient Flavors LLC, 5600 W. Raymond St., Indianapolis, IN 46241, USA.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Xin Xu of the Birck Nanotechnology Center at Discovery Park (Purdue University) for the acquisition of AFM images.

■ REFERENCES

(1) Gu, Y. S.; Decker, E. A.; McClements, D. J. Influence of pH and carrageenan type on properties of β-lactoglobulin stabilized oil-in-water emulsions. *Food Hydrocolloids* **2005**, *19*, 83−91.
(2) Gu, Y. S.; Regnier, L.; McClements, D. J. Influence of environmental stresses on stability of oil-in-water emulsions containing droplets stabilized by β-lactoglobulin−ι-carrageenan membranes. *J. Colloid Interface Sci.* **2005**, *286*, 551−558.
(3) Wooster, T. J.; Augustin, M. A. β-Lactoglobulin−dextran Maillard conjugates: their effect on interfacial thickness and emulsion stability. *J. Colloid Interface Sci.* **2006**, *303*, 564−572.
(4) Bouyer, E.; Mekhloufi, G.; Le Potier, I.; du Fou de Kerdaniel, T.; Grossiord, J. L.; Rosilio, V.; Agnely, F. Stabilization mechanism of oil-in-water emulsions by β-lactoglobulin and gum arabic. *J. Colloid Interface Sci.* **2011**, *354*, 467−477.
(5) Schmelz, T.; Lesmes, U.; Weiss, J.; McClements, D. J. Modulation of physicochemical properties of lipid droplets using β-lactoglobulin and/or lactoferrin interfacial coatings. *Food Hydrocolloids* **2011**, *25*, 1181−1189.
(6) Elias, R. J.; McClements, D. J.; Decker, E. A. Antioxidant activity of cysteine, tryptophan, and methionine residues in continuous phase β-lactoglobulin in oil-in-water emulsions. *J. Agric. Food Chem.* **2005**, *53*, 10248−10253.
(7) Kellerby, S. S.; McClements, D. J.; Decker, E. A. Role of proteins in oil-in-water emulsions on the stability of lipid hydroperoxides. *J. Agric. Food Chem.* **2006**, *54*, 7879−7884.
(8) Elias, R. J.; McClements, D. J.; Decker, E. A. Impact of thermal processing on the antioxidant mechanisms of continuous phase β-lactoglobulin in oil-in-water emulsions. *Food Chem.* **2007**, *104*, 1402−1409.
(9) Hu, M.; McClements, D. J.; Decker, E. A. Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *J. Agric. Food Chem.* **2003**, *51*, 1435−1439.
(10) Verheul, M.; Pedersen, J. S.; Roefs, S. P. F. M.; de Kruif, C. G. Association behavior of native β-lactoglobulin. *Biopolymers* **1999**, *49*, 11−20.
(11) Roefs, S. P. F. M.; de Kruif, C. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883−889.
(12) Ohgushi, M.; Wada, A. 'Molten-globule state': a compact form of globular proteins with mobile side-chains. *FEBS Lett.* **1983**, *164*, 21−24.
(13) Carrotta, R.; Bauer, R.; Waninge, R.; Rischel, C. Conformational characterization of oligomeric intermediates and aggregates in β-lactoglobulin heat aggregation. *Protein Sci.* **2001**, *10*, 1312−1318.
(14) Bauer, R.; Carrotta, R.; Rischel, C.; Øgendal, L. Characterization and isolation of intermediates in β-lactoglobulin heat aggregation. *Biophys. J.* **2000**, *79*, 1030−1038.
(15) De Wit, J. N. Thermal behaviour of bovine β-lactoglobulin at temperatures up to 150 °C. A review. *Trends Food Sci. Technol.* **2009**, *20*, 27−34.
(16) Zhai, J.; Miles, A. J.; Pattenden, L. K.; Lee, T. H.; Augustin, M. A.; Wallace, B. A.; Aguilar, M. I.; Wooster, T. J. Changes in β-lactoglobulin conformation at the oil/water interface of emulsions studied by synchrotron radiation circular dichroism spectroscopy. *Biomacromolecules* **2010**, *11*, 2136−2142.
(17) Fang, Y.; Dalgleish, D. G. Conformation of β-lactoglobulin studied by FTIR: effect of pH; temperature; and adsorption to the oil-water interface. *J. Colloid Interface Sci.* **1997**, *196*, 292−298.
(18) Lefèvre, T.; Subirade, M. Formation of intermolecular β-sheet structures: a phenomenon relevant to protein film structure at oil-water interfaces of emulsions. *J. Colloid Interface Sci.* **2003**, *263*, 59−67.
(19) Kim, D. A.; Cornec, M.; Narsimhan, G. Effect of thermal treatment on interfacial properties of β-lactoglobulin. *J. Colloid Interface Sci.* **2005**, *285*, 100−109.
(20) Meng, G.; Chan, J. C. K.; Rousseau, D.; Li-Chan, E. C. Y. Study of protein-lipid interactions at the bovine serum albumin/oil interface by raman microspectroscopy. *J. Agric. Food Chem.* **2005**, *53*, 845−852.
(21) Ogawa, S.; Decker, E. A.; McClements, D. J. Influence of environmental conditions on the stability of oil in water emulsions containing droplets stabilized by lecithin-chitosan membranes. *J. Agric. Food Chem.* **2003**, *51*, 5522−5527.
(22) Thomas, J. C. The determination of log normal particle size distributions by dynamic light scattering. *J. Colloid Interface Sci.* **1986**, *117*, 187−192.
(23) Criterion® Cassette Hand Casting Instructions. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110106B.pdf (accessed May 1, 2011), part of Bio-Rad Home Page (www.bio-rad.com/ (accessed May 1, 2011)).
(24) Huglin, M. B. Specific refractive index increments. In *Light Scattering from Polymer Solutions*; Huglin, M. B., Ed.; Academic Press: New York, 1972; pp 165−331.
(25) Horcas, I.; Fernandez, R.; Gomez-Rodriguez, J. M.; Colchero, J.; Gomez-Herrero, J.; Baro, A. M. *Rev. Sci. Instrum.* **2007**, *78*, 013705.
(26) Helstad, K. M.; Bream, A. D.; Trckova, J.; Paulsson, M.; Dejmek, P. Nanorheological properties of casein. In *Food Emulsions and Foams*; Dickinson, E., Ed.; Royal Society of Chemistry: London, UK, 2004; pp 218−229.
(27) Godovac-Zimmermann, J. The structural motif of β-lactoglobulin and retinol-binding protein: a basic framework for binding and transport of small hydrophobic molecules? *Trends Biochem. Sci.* **1988**, *13*, 64−66.
(28) Cowan, S. W.; Jones, T. A.; Newcomer, M. E. Crystallographic refinement of human serum retinol binding protein at 2Å resolution. *Proteins: Struct., Funct., Bioinf.* **1990**, *8*, 44−61.
(29) Shimizu, M.; Saito, M. Lipid-protein interaction at an emulsified oil surface: protein structures and their roles in lipid binding. In *Macromolecular Interactions in Food Technology*; Parris, N., Kato, A., Creamer, L. K., Pearce, J., Eds.; ACS Symposium Series 650; American Chemical Society: Washington, DC, 1996; pp 156−165.
(30) Kiokias, S.; Dimakou, C.; Oreopoulou, V. Effect of heat treatment and droplet size on the oxidative stability of whey protein emulsions. *Food Chem.* **2007**, *105*, 94−100.
(31) Morr, C. V. Conformation and functionality of milk proteins. In *Functionality and Protein Structure*; Pour-El, A., Ed.; ACS Symposium Series 92; American Chemical Society: Washington, DC, 1979; pp 65−79.
(32) Voutsinas, L. P.; Cheung, E.; Nakai, S. Relationships of hydrophobicity to emulsifying properties of heat denatured proteins. *J. Food Sci.* **1983**, *48*, 26−32.
(33) Subramaniam, V.; Steel, D. G.; Gafni, A. In vitro renaturation of bovine β-lactoglobulin A leads to a biologically active but incompletely refolded state. *Protein Sci.* **1996**, *5*, 2089−2094.
(34) Bhattacharjee, C.; Das, K. P. Thermal unfolding and refolding of β-lactoglobulin. *Eur. J. Biochem.* **2000**, *267*, 3957−3964.
(35) Bhattacharjee, C.; Saha, S.; Biswas, A.; Kundu, M.; Ghosh, L.; Das, K. P. Structural changes of β-lactoglobulin during thermal unfolding and refolding—an FT-IR and circular dichroism study. *Protein J.* **2005**, *24*, 27−35.
(36) HOPG-15M. http://www.brukerafmprobes.com/p-3742-hopg-15m.aspx (accessed June 1, 2011), part of Bruker AFM Probes home page (http://www.brukerafmprobes.com/ (accessed June 1, 2011)).
(37) Alexander, S. Adsorption of chain molecules with a polar head a scaling description. *J. Phys. (Paris)* **1977**, *38*, 983.
(38) De Gennes, P. G. Polymers at an interface; a simplified view. *Adv. Colloid Interface Sci.* **1987**, *27*, 189−209.
(39) Tavel, L.; Moreau, C.; Bouhallab, S.; Li-Chan, E. C. Y.; Guichard, E. Interactions between aroma compounds and β-lactoglobulin in the heat-induced molten globule state. *Food Chem.* **2010**, *119*, 1550−1556.

(40) Relkin, P. Reversibility of heat-induced conformational changes and surface exposed hydrophobic clusters of β-lactoglobulin: their role in heat-induced sol-gel state transition. *Int. J. Biol. Macromol.* **1998**, *22*, 59−66.
(41) Håkansson, A.; Trägårdh, C.; Bergenståhl, B. Dynamic simulation of emulsion formation in a high pressure homogenizer. *Chem. Eng. Sci.* **2009**, *64*, 2915−2925.
(42) Uricanu, V. I.; Duits, M. H. G.; Mellema, J. Hierarchical networks of casein proteins; an elasticity study on atomic force microscopy. *Langmuir* **2004**, *20*, 5079−5090.
(43) Verheul, M.; Roefs, S. P. F. M. Structure of whey protein gels; studied by permeability; scanning electron microscopy and rheology. *Food Hydrocolloids* **1998**, *12*, 17−24.
(44) Phoon, P. Y. *Proteins as Emulsifiers in Retarding Oil Oxidation in an Oil/Water Emulsion*. Ph.D. dissertation, Purdue University, West Lafayette, IN, USA, 2011.
(45) Sørensen, A.-D. M.; Baron, C. P.; Let, M. B.; Brüggemann, D. A.; Pedersen, L. R.; Jacobsen, C. Homogenization conditions affect the oxidative stability of fish oil enriched milk emulsions: oxidative linked to changes in protein composition at the oil-water interface. *J. Agric. Food Chem.* **2007**, *55*, 1781−1789.