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Effects of High-Pressure Processing at Low Temperature on the Molecular Structure and Surface Properties of β-Lactoglobulin

MARCIA K. WALKER,† DANIEL F. FARKAS,† SONIA R. ANDERSON,§ AND LISBETH MEUNIER-GODDIK*,†

Department of Food Science and Technology and Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

High-pressure processing (HPP) was utilized to induce unfolding of β-lactoglobulin (β-LG). β-Lactoglobulin solutions at concentrations of 0.5 mg/mL, in pH 7.5 phosphate buffer, were pressure treated at 510 MPa for 10 min at either 8 or 24 °C. The secondary structure, as determined by circular dichroism (CD), of β-LG processed at 8 °C appeared to be unchanged, whereas β-LG processed at 24 °C lost α-helix structure. Tertiary structures for β-LG, as determined by near-UV CD, intrinsic protein fluorescence spectroscopy, hydrophobic fluorescent probe binding, and thiol group reactivity, were changed following processing at either temperature. The largest changes to tertiary structure were observed for the samples processed at 24 °C. Model solutions containing the pressure-treated β-LG showed significant decreases in surface tension at liquid−air interfaces with values of 54.00 and 51.69 mN/m for the samples treated at 24 and 8 °C, respectively. In comparison, the surface tension for model solutions containing the untreated control was 60.60 mN/m. Changes in protein structure during frozen and freeze-dried storage were also monitored, and some renaturation was observed for both storage conditions. Significantly, the sample pressure-treated at 8 °C continued to display the lowest surface tension.

KEYWORDS: High-pressure processing; β-lactoglobulin; molecular structure; surface tension; low temperature

INTRODUCTION

Bovine β-lactoglobulin (β-LG) is a 162 amino acid residue whey protein that has been the subject of extensive research (*1*−*3*). On the basis of results obtained by circular dichroism (CD), the secondary structure of β-LG appears to contain 10−15% α-helix, 50% β-sheet, and 15−20% β-turns (*3*). The relationship between β-LG's molecular structure and functionality has been investigated with special emphasis on its sensitivity to thermal denaturation (*4*, *5*). As the temperature is increased, β-LG undergoes structural modification, which exposes its hydrophobic interior (*6*) and results in enhanced foaming properties (*7*).

Unfolding of β-LG by high-pressure processing (HPP) at 50−1000 MPa and ≥25 °C has been previously investigated using methods such as fluorescence (*8*), differential scanning calorimetry (DSC) (*9*), CD (*6*, *10*, *11*), Fourier transform infrared (FTIR) spectroscopy, and nuclear magnetic resonance (NMR) (*12*). Pittia et al. (*10*) used high pressure (300−900 MPa) in conjunction with a fixed temperature (25 °C) and holding time to investigate the effect of HPP on the functionality and structure of β-LG. Because of an increased potential for the formation of undesirable aggregates, the pressure-modified β-LG displayed both reduced emulsifying capacity and foam stability when compared to native β-LG. Other studies also suggest that HPP treatments at room temperature using pressures of 150−450 MPa favor extensive protein aggregation (*9*, *13*).

Kolakowski et al. (*14*) suggested that lowering the temperature during HPP enhances the exposure of the hydrophobic regions of β-LG to water. Using relatively low pressures (300 MPa), they reported that low-temperature (4 °C) pressurization minimizes the loss of native structure and decreases undesirable aggregation. Their research strongly suggests that low-temperature pressurization may be useful to minimize protein aggregation and subsequent losses in protein functionality. Valente-Mesquita et al. (*15*) also investigated the effects of hydrostatic pressure on the structure of β-LG at low temperature. Application of pressures up to 350 MPa at 3 °C caused an increase in intrinsic fluorescence emission. They concluded that the β-LG dimer unfolds during compression and that upon decompression partial but incomplete refolding occurs.

With the current commercial trend toward using higher pressures for shorter times, there is a need for information on

* Corresponding author [telephone (541) 737-8322; fax (541) 737-1877; e-mail Lisbeth.goddik@oregonstate.edu].
† Department of Food Science and Technology.
§ Department of Biochemistry and Biophysics.

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the effects of higher pressures on β-LG and other food proteins. The objective of this study was to induce a targeted unfolding of β-LG that would enhance the protein's ability to lower surface tension at air−water interfaces, yet minimize undesirable aggregation. Additionally, conformational changes of β-LG after frozen and freeze-dried storage were monitored. Surface tension at air−water interfaces was selected as an indicator of β-LG's functional properties because of the importance of this property in dispersed food systems.

MATERIALS AND METHODS

Proteins and Chemicals. Chromatographically purified, lyophilized β-LG (no. L3908), ANS fluorescent probe (no. A-1028), and Ellman's reagent (D-8130) [5,5′-dithiobis(2-nitrobenzoic acid), DTNB] were all of reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). Distilled water was utilized.

Samples. Freshly prepared pH 7.5 sodium phosphate buffer (10 mM) was used to prepare β-LG solutions at 0.5 mg/mL. The final protein concentrations were spectrophotometrically confirmed using a Shimadzu Bio Spec 1601 (Kyoto, Japan) at 280 nm ($\epsilon_{280} = 17600\ M^{-1}\ cm^{-1}$). The pH of the solutions was verified using a Fisher Scientific Accumet AR25 pH-meter (Pittsburgh, PA). Samples, containing 60 mL of solution, were vacuum packaged into polyethylene pouches (TRS-95250-KAPAK, Minneapolis, MN), heat-sealed, and equilibrated at either 4 or 24 °C for 2 h prior to processing.

HPP Treatment Conditions. Sample pouches were run at 510 MPa for 10 min using a 7-in. diameter by 36-in. long Engineered Pressure Systems (Haverhill, MA) 22-L isostatic press. The time it took for pressure to come up was ∼6−7 min, and decompression time was <1 min. Samples were run at ambient temperature (22−24 °C) and at low temperature (8 °C). For low-temperature processing, the vessel was precooled by filling the unit with ice water and allowing it to equilibrate for 2 h while samples were equilibrated at 4 °C and packed in 4 °C ice water for loading. Control samples were treated similarly to HPP samples except that no pressure was applied. The temperatures of the samples were measured before and after processing. Triplicate runs were performed for each treatment.

Adiabatic Compression Heating. Adiabatic compression heating was monitored by inserting thermocouples into protein samples and pressure transmitting fluid inside an Engineered Pressure Systems 2-L 100 000 psi isostatic press. Sample pouches, containing 50 mL, were run at 510 MPa, for 10 min. Pressure come-up time was ∼4 min, and pressure release was <1 min. The chamber was packed with ice and allowed to cool for 1 h. Samples were prepared and equilibrated in a water bath at 8 °C. Thermocouples were inserted into the sample and pressure transmitting fluid. The sample was put into an outer bag filled with ice water and heat sealed. The increase in temperature following HPP was <2 °C.

Surface Tension Measurements. Immediately following HPP, samples were analyzed for surface tension (γ) changes using a Du Nouy ring tensiometer (model 70535, CSC Scientific Co., Inc., Fairfax, VA). Samples of 20 mL at room temperature were poured into a 60 mm × 15 mm tissue culture dish (no. 3002, Becton Dickson Labware, Lincoln Park, NJ). A 6-cm platinum−iridium ring (70537, CSC Scientific Co.) was used for measurements, and apparent surface tension (ρ) was recorded (*16*). Samples were monitored over a period of time up to 3 h, although surface tension results were recorded after equilibration for 1 h, after which time no further changes in tension occurred.

Native Polyacrylamide Gel Electrophoresis. Equipment, sample buffers, molecular weight standards (161-0362) 10−250 kDa, running buffer, 10−20% Tris HCl-30 μL load size ready gels, and staining and destaining solutions were obtained from Bio-Rad (Bio-Rad Labs, Hercules, CA). β-LG samples were diluted 1:2 in Bio-Rad native sample buffer (161-0738), and 25-μL samples were loaded into wells within 4 h of HPP.

Protein Structure Characterization. *Circular Dichroism (CD).* Far-UV CD spectra were measured from 184 to 260 nm, and near-UV was measured from 260 to 320 nm using a Jasco spectropolarimeter (J720, Japan Spectroscopic Co., Inc. Tokyo, Japan) and a 0.1 cm square quartz cell. Near-UV measurements were performed using a 1-cm cylindrical quartz cell at ambient temperature. The spectral data were collected at 1-nm intervals using a scan rate of 20 nm/min. Triplicate spectra were run on all samples and averaged by the instrument software. Baseline samples of buffer were run, and all samples were baseline corrected prior to calculation. Instrument software was used to smooth all spectra. The instrument was calibrated using an aqueous solution of (+)-10-camphorsulfonic acid (CSA) at a concentration of 1 mg/mL in a 1-mm cell (*17*). Samples were diluted 3:1, with buffer, to ensure signal within linear range, and final concentration was calculated using Beer's law. Measurements on fresh samples were performed within 1 h of HPP.

CD Data Analysis of Secondary Structure. Far-UV CD spectra were analyzed using the methods of Hennessey et al. (*18*) and Manavalan and Johnson (*19*). Data are reported as percentages of α-helix, 3_{10}-helix, β-sheet, β-turn, polyproline-like 3/1-helix, and "other" structure.

Intrinsic Fluorescence. Measurements were taken using a Perkin-Elmer LS 50 luminescence spectrometer, connected to a circulating constant-temperature bath set at 25 °C. To minimize the contribution of tyrosyl residues to the emission spectra, the excitation wavelength was set at 290 nm. Emission spectra were scanned from 290 to 450 nm. The samples were freeze-dried and stored at 4 °C prior to analysis.

Surface Hydrophobicity—Extrinsic Fluorescence. Determination of surface hydrophobicity was carried out using 1,8-anilinonaphthalene-sulfonate (ANS) as a fluorescent probe according to the modified method of Yang et al. (*8*). The fluorescence intensity was measured at room temperature using the Perkin-Elmer LS50 luminescence spectrometer. ANS fluorescence was monitored using a fixed excitation wavelength of 390 nm and scanning emission wavelengths of 400−600 nm. Each sample was analyzed in duplicate. The samples were freeze-dried and stored at 4 °C prior to analysis.

Reactivity of Exposed Thiol Groups. The modified method of Shimada et al. (*20*) based on Ellman's reagent was employed using a Shimadzu Bio Spec 1601 (Kyoto, Japan) spectrophotometer for absorbance measurements at 412 nm. An extinction coefficient of 13 600 $M^{-1}\ cm^{-1}$ was used to calculate the concentration of free sulfhydryl groups (*21*). The samples were freeze-dried and stored at 4 °C prior to analysis. Each sample was analyzed in duplicate.

Freeze-Drying. Samples were slab frozen in a blast freezer at −40 °C for 24 h and then freeze-dried with the product temperature remaining <25 °C during the drying cycle. The final moisture content ranged from 4.38 to 5.6%. Freeze-dried samples were transferred into double-lined polypropylene bags and stored at 4 °C until analysis.

Statistical Treatment. The statistical significance of differences between treatments was determined by Student's *t* test. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Selection of HPP Conditions. A number of processing variables were initially tested to select conditions that would decrease the surface tension of β-LG model solutions compared to the control. β-LG concentrations were tested from 0.1 to 1.5 mg/mL. Concentrations >0.5 mg/mL led to increased surface tension, presumably due to protein aggregation. Buffer pH values of 5.2, 6.0, 7.0, and 7.5 were tested, and the buffer pH of 7.5 was selected because this sample exhibited the largest decrease in surface tension; in addition, visible precipitation was observed within 24 h of storage at 4 °C for the pH 5.2 and 6.0 solutions. Pressures during processing were tested at 450, 510, and at 600 MPa, which is the maximum pressure for the vessel. No effects were observed on surface tension for the model solutions treated at 450 MPa, whereas the highest pressure (600 MPa) led to only a minimal decrease in surface tension when compared with the control. The lowest surface tension was obtained at 510 MPa with a β-LG concentration of 0.5 mg/mL, pH 7.5, at 8 °C (**Table 1**). Thus, the structures of β-LG treated at these conditions as well as the corresponding treatment at room temperature were investigated. Native PAGE, utilized to monitor the formation of protein aggregates following HPP,

Table 1. Surface Tension of Model β-Lactoglobulin Solutions[a] Containing Control (Untreated) and High Pressure Processing[b] Treated β-Lactoglobulin

sample	control	8 °C[d]	24 °C[d]
surface pressure[c] ± SD (mN/m)	60.60* ± 0.11	51.69* ± 0.17	54.00* ± 0.11

[a] pH 7.5, β-lactoglobulin concentration of 0.5 mg/mL phosphate buffer. [b] HPP treatment conditions: 10 min, at 510 MPa, pH 7.5. [c] Means of triplicate runs. *, means within row are significantly different ($P < 0.05$). [d] Initial high-pressure processing temperature.

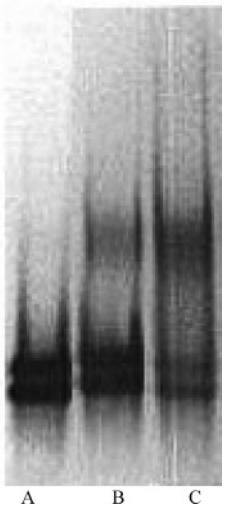

Figure 1. Native PAGE of β-LG samples: sample A, untreated control; sample B, HPP-treated at 510 MPa and 8 °C for 10 min; sample C, HPP-treated at 510 MPa and 24 °C for 10 min.

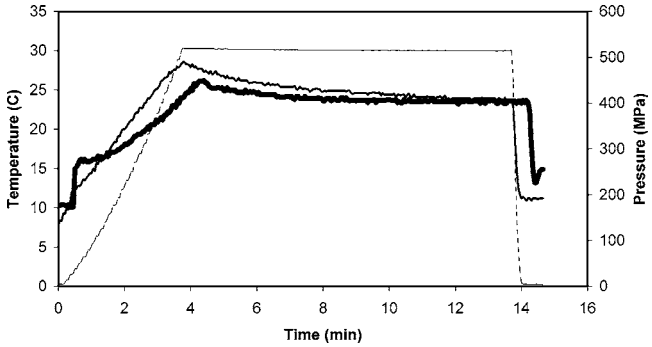

Figure 2. Adiabatic compression heating during HPP treatment of β-LG solutions at 510 MPa, for 10 min: (—) product temperature; (—) pressure transmitting fluid temperature; (- - -) pressure.

indicated that samples treated at room temperature may have more high molecular weight aggregates than samples treated at 8 °C (**Figure 1**). These results are consistent with those of Kolakowski et al. (*14*), who concluded, from the results of gel permeation chromatography, that β-LG aggregates are formed when pressurized at 25 °C but not at lower temperatures.

Adiabatic Compression Heating. Temperature changes during HPP at low temperature are shown in **Figure 2**. A thermocouple in the center of the sample package and a thermocouple placed near the top of the pressure unit in the pressure transmitting fluid reported close readings, indicating good temperature distribution within the chamber. However, it is important to note that even though samples entered the cycle at 8 °C, they reached a temperature of 28.6 °C before cooling to 11.8 °C upon decompression. Although this temperature increase is not sufficient to lead to protein unfolding at low-temperature processing, it could be significant when starting at or above room temperature. The extent of compression heating is comparable with the results presented by Rasanayagam et al. (*22*), who reported at 15 °C temperature increase when HPP water with an initial temperature of 25 °C at 600 MPa.

Changes in β-LG Secondary Structure Following HPP. Comparisons between the secondary structure of native and HPP-treated samples, determined immediately following pressure treatment and after being held at 4.4 °C for 24 h, are presented in **Table 2**. The native control was stable in solution for the 24-h period. HPP treatment at 8 °C resulted in a decrease in α-helix, from 16 to 11%, and an increase in the apparent β-sheet component. After 24 h of storage, the sample treated at 8 °C had a similar proportion of α-helix but a smaller proportion of β-sheet than the native sample. In contrast, samples treated at 24 °C showed significant decreases in α-helix content both immediately following HPP and following the 24-h storage period. These results suggest that secondary structure of samples treated at 8 °C recovered relatively quickly, whereas samples treated at 24 °C exhibited permanent changes in secondary structure.

Changes in β-Lactoglobulin Tertiary Structure Following HPP. Tertiary structure was investigated by using four different techniques: near-UV CD, intrinsic protein fluorescence spectroscopy, hydrophobic fluorescent probe binding, and determination of the reactivity of exposed thiol groups. These methods do not quantify changes in tertiary structure in the same sense that far-UV CD quantifies secondary structure, but rather indicate differences in the response of the protein to the environment.

Near-UV CD. CD bands in the near-UV region (250−320 nm) of folded protein result from the presence of aromatic amino acids. The bands observed at 285−295 nm and at 265−277 nm are attributed to tryptophan and tyrosine, respectively. Decreases in the tryptophan bands indicate a loss of local asymmetric structure and hence a loss of tertiary structure (*23*). The near-UV CD spectra of HPP samples, immediately following pressure treatment, were compared with the spectrum of the native control (**Figure 3**). The changes in the spectra are consistent with previously reported results showing that the tertiary structure of β-LG decreases with increasing severity of the conditions of HPP. Tedford et al. (*11*) observed that increased temperature (up to 79 °C) is the processing variable resulting in the most significant changes in tertiary structure (as indicated by a flattened near-UV CD spectrum). Yang et al. (*8*) reported complete disappearance of tertiary structure following 8 min of HPP at 600 MPa and 50 °C.

Intrinsic Fluorescence Spectra. β-LG exhibits an increase in intrinsic tryptophan fluorescence and a red shift upon unfolding. The tryptophan fluorescence spectra for the treatments selected are shown in **Figure 4**. An increase in fluorescence was observed, indicating that unfolding had occurred in both HPP treatments, which exposed tryptophan residues, thus potentially leading to higher surface hydrophobicity of the protein. These results are consistent with the work done by Yang et al. (*8*) and Valente Mesquita et al. (*15*), who studied HPP at 50 °C, 600 MPa, and at 3 °C, 350 MPa, respectively.

Hydrophobic Probe Binding/Extrinsic Fluorescence. The native structure of β-LG contains an internal hydrophobic binding site located within the β-barrel and an external hydrophobic site located between the β-barrel and the α-helix (*24*, *25*). Usually the hydrophobic core of β-LG is protected from solvent by the rigid tertiary structure, giving it a low

Table 2. Secondary Structure of Control (Untreated) and High Pressure Processing[a] (HPP) Treated β-Lactoglobulin Solutions[b] As Determined by Circular Dichroism

sample	temp of HPP (°C)	recovery time after HPP (h)	α-helix	3_{10}-helix	β-sheet	β-turn	polyproline-like 3/1-helix	other
control		0	0.16	0.04	0.24	0.14	0.06	0.37
control		24	0.17	0.04	0.24	0.13	0.07	0.36
510 MPa	8	0	0.11	0.04	0.26	0.14	0.07	0.38
510 MPa	8	24	0.18	0.04	0.20	0.14	0.07	0.37
510 MPa	24	0	0.08	0.05	0.26	0.13	0.07	0.41
510 MPa	24	24	0.07	0.05	0.24	0.14	0.07	0.42

[a] Samples were analyzed within 1 h of HPP treatment, and all treatments were repeated in triplicate. HPP conditions: 510 MPa, 10 min. [b] Model solution: 0.5 mg/mL β-lactoglobulin phosphate buffer, pH 7.5.

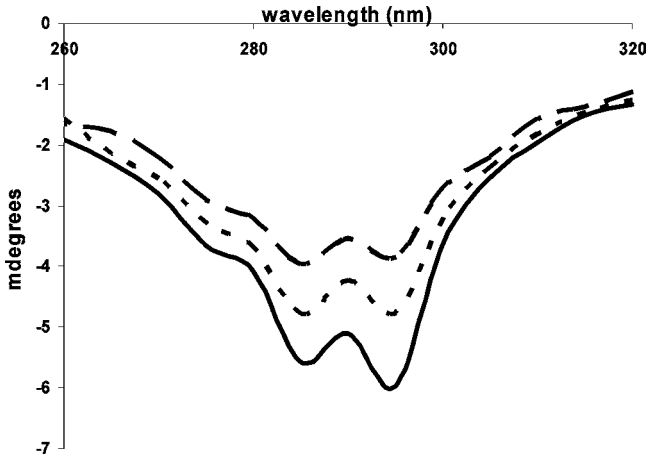

Figure 3. Near-UV CD spectra of β-LG immediately following HPP treatment at 510 MPa, pH 7.5, 0.5 mg/mL, for 10 min. Treatments: (—) control (untreated); (– – –) 24 °C initial processing temperature; (- - -) 8 °C initial processing temperature.

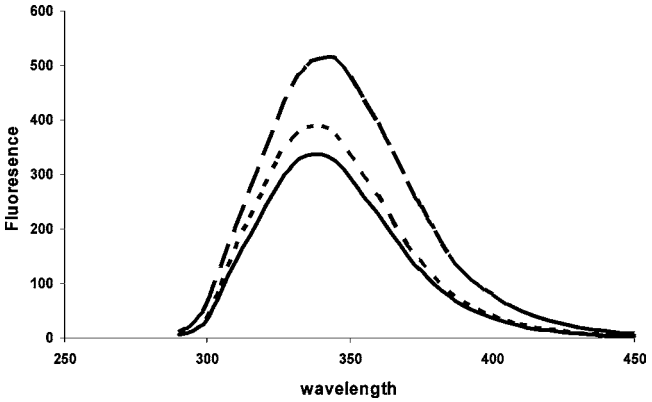

Figure 4. Intrinsic fluorescence spectra of β-LG immediately following HPP treatment at 510 MPa, pH 7.5, 0.5 mg/mL, for 10 min. Treatments: (—) control (untreated); (– – –) 24 °C initial processing temperature; (- - -) 8 °C initial processing temperature.

affinity to hydrophobic binding probes such as ANS. By disrupting the tertiary structure, the affinity of β-LG to ANS increases (*26*). As shown in **Figure 5**, increased ANS binding was observed as an increase in extrinsic fluorescence, when samples were HPP-treated at 24 °C compared to the untreated control or 8 °C HPP samples. The fluorescent intensity for the 24 °C sample was approximately doubled, whereas Yang et al. (*8*) reported a tripling in intensity following HPP at harsher conditions (600 MPa, 50 °C).

Reactivity of Exposed Thiol Groups. The internal thiol group plays an important role in surface characteristics when β-LG is

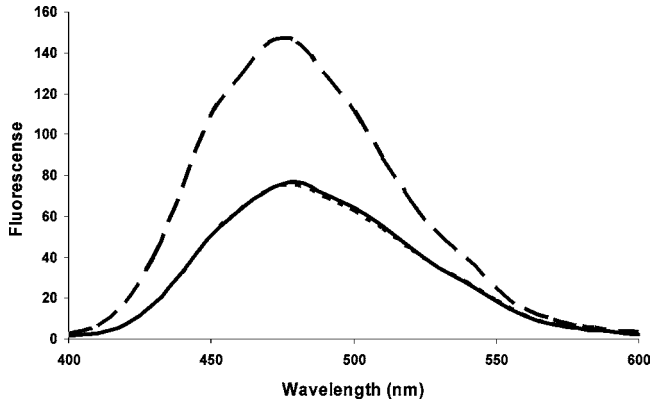

Figure 5. β-LG–ANS fluorescence spectrum immediately following HPP treatment at pH 7.5, 0.5 mg/mL, 510 MPa, 10 min. Treatments: (—) control (untreated); (– – –) 24 °C initial processing temperature; (- - -) 8 °C initial processing temperature. (Control and 8 °C sample plots are essentially superimposed.)

Table 3. Free Thiol Reactivity of β-Lactoglobulin Following High-Pressure Processing[a] with Initial Treatment Temperature at either 24 or 8 °C

	control	24 °C	8 °C
absorbance at 412 nm[b]	0.164* ± 0.008	0.195* ± 0.002	0.182* ± 0.003
free thiol concn (M)	1.2×10^{-6}	1.4×10^{-6}	1.34×10^{-6}
available free thiol groups[c] (%)	4.4	5.1	4.9

[a] High-pressure processing treatment conditions: 510 MPa, 10 min, pH 7.5, β-lactoglobulin concentration of 0.5 mg/mL. [b] Means within rows are significantly different ($P < 0.05$). [c] Calculation based on total theoretical amount of free thiol groups.

unfolded due to HPP. In native β-LG, of the five cysteines, four form intramolecular disulfide bonds and only Cys-121 is present as a free thiol group. This free thiol group is buried internally and remains less reactive. Partial unfolding by HPP increases the exposure of inner hydrophobic groups and the thiol group (*27*, *28*). Hydrophobic interactions and thiol−disulfide reactions that occur in this state can lead to aggregation (*29*, *30*). The increases in absorbance of DTNB at 412 nm (**Table 3**) show that the largely inaccessible SH group of β-LG becomes only slightly more reactive after HPP treatment at 8 and 24 °C.

The four methods utilized to examine changes in tertiary structure all agree on the observation that pressure clearly disrupts the tertiary structure of β-LG with the extent of disruption increasing with treatment temperature. However, the extrinsic fluorescence results indicate that 8 °C HPP leads to limited unfolding, which is not sufficient to expose additional ANS binding sites, and the unfolded protein responds to

Table 4. Effect of Storage Condition and Time on Secondary Structure and Surface Tension of Model β-Lactoglobulin Solutions[a] Containing Control (Untreated) and High Pressure Processing[b] (HPP) Treated β-Lactoglobulin

HPP temp (°C)	storage condition of sample	storage time (days)	α-helix	3_{10}-helix	β-sheet	β-turn	polyproline-like 3/1-helix	other	surface tension[c] (mN/m)
control	frozen[d]	1	0.18	0.04	0.21	0.13	0.07	0.37	65.08
control	frozen	12	0.18	0.02	0.26	0.10	0.08	0.35	66.6
control	freeze-dried	7	0.17	0.02	0.24	0.10	0.09	0.38	65.2
8	frozen	1	0.17	0.04	0.23	0.13	0.06	0.37	59.2
8	frozen	12	0.10	0.03	0.28	0.12	0.08	0.39	59.9
8	freeze-dried	7	0.18	0.03	0.22	0.12	0.09	0.36	59.7
24	frozen	1	0.10	0.05	0.23	0.13	0.08	0.40	62.2
24	frozen	12	0.17	0.05	0.18	0.13	0.08	0.39	63.4
24	freeze-dried	7	0.04	0.02	0.29	0.11	0.09	0.43	62.5

[a] 0.5 mg/mL β-lactoglobulin phosphate buffer, pH 7.5. [b] 510 MPa, 10 min, processed at either 25 or 8 °C. [c] Means of duplicate samples. [d] −40 °C.

environmental factors in a manner close to that of the untreated control. Near-UV CD and intrinsic fluorescence spectra yield a similar result, which is not surprising as both methods monitor tryptophan residues.

Structure−Function Relationship. Upon adsorption at the air−water interface, there is a change in protein conformation from that of the native state (*31*). The molecular rearrangements that occur as β-LG moves toward and into the water−air interface lead to the partial unfolding of the molecule and a loss of tertiary structure (*29*, *32*). Additionally, the conformation of the protein molecule in solution will greatly influence the rate and extent of the conformational change upon adsorption. Specifically, it appears that increased molecular flexibility facilitates the protein's rearrangement at interfaces (*31*, *33*). This research investigated increasing the surface activity of β-LG on the basis of these unfolding theories (*5*, *10*, *11*, *33*). The surface tension of β-LG model solutions was decreased after being subjected to HPP. Pressure treatment at room temperature caused significant and permanent changes in secondary and tertiary protein structure. However, the treatment failed to lead to optimum surface activity in model solutions, presumably due to protein aggregation. In contrast, pressure treatment at low temperature caused changes in tertiary structure, whereas secondary structure changes appeared to be minimal as α-helix content reverted to the original level during the initial 24 h. Despite the renaturation, the gain in surface activity of the model solutions was high. This was most likely due to the fact that protein aggregation remained minimal. It seems likely that β-LG in the partially unfolded state (510 MPa, 8 °C, and pH 7.5) has a more flexible, hydrophobic surface and therefore readily adsorbs at the interface, whereas the control has a more tightly folded conformation and therefore has a lower probability of collision at the interface. This suggests that the adsorbed protein at the interface has an average structure lying between the native folded and the completely unfolded state. These results indicate a favorable effect of unfolding on surface functionality when aggregation is avoided. They also illustrate that improved functional properties may be obtained when the changes in secondary structure are kept at a minimum as indicated by consistent levels of α-helix structure. Furthermore, small changes in tertiary structure appear to be preferable over larger changes or even loss of tertiary structure.

Effects of Freezing and Freeze-Drying of HPP Samples. It is important to understand molecular rearrangements occurring during subsequent processes and storage. Changes in the secondary structure of β-LG frozen or freeze-dried are listed in **Table 4**. Processing alone (freezing or freeze-drying) did not appear to affect the native secondary structure of β-LG (**Tables 2** and **4**). The α-helix component of samples that were HPP-treated at both 8 and 24 °C changed when the samples were held frozen for up to 12 days. However, no clear trend was observed because α-helix content increased during frozen storage for the sample processed at 24 °C and decreased for the sample processed at 8 °C. Dumay et al. (*9*) and Kolakowski et al. (*14*) report reversibility when processing at lower pressures (450 MPa) and 25 °C, whereas Yang et al. (*8*) report that the molten globule structure remains stable during frozen and 5 °C storage. Although we observed changes during storage, it is important to note that the improvement in functionality (ability to decrease surface tension) observed when HPP-treating β-LG at low temperature (8 °C) compared to room temperature was retained during both frozen and freeze-dried storage. Thus, HPP at low temperature can likely be utilized to develop protein ingredients with improved functional properties that remain stable during storage.

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