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Jian Yang, Washington State University
A. Keith Dunker, Washington State University
Joseph R. Powers, Washington State University
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Barry G. Swanson, Washington State University

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β-Lactoglobulin Molten Globule Induced by High Pressure

Jian Yang,1 A. Keith Dunker,1 Joseph R. Powers,1 Stephanie Clark,1 and Barry G. Swanson*1†

Department of Food Science and Human Nutrition and Department of Biochemistry and Biophysics,
Washington State University, Pullman, Washington 99164-6376

β-Lactoglobulin (β-LG) was treated with high hydrostatic pressure (HHP) at 600 MPa and 50 °C for selected times as long as 64 min. The intrinsic tryptophan fluorescence of β-LG indicated that HHP treatment conditions induced a conformational change. HHP treatment conditions also promote a 3-fold increase in the extrinsic fluorescence of 1-anilinonaphthalene-8-sulfonate and a 2.6-fold decrease for cis-paranemic acid, suggesting an increase in accessible aromatic hydrophobicity and a decrease in aliphatic hydrophobicity. Far-ultraviolet circular dichroism (CD) spectra reveal that the secondary structure of β-LG converts from native β-sheets to non-native α-helices following HHP treatment, whereas near-ultraviolet CD spectra reveal that the native tertiary structure of β-LG essentially disappears. Urea titrations reveal that native β-LG unfolds cooperatively, but the pressure-treated molecule unfolds noncooperatively. The noncooperative state is stable for 3 months at 5 °C. The nonaccessible free thiol group of cysteine in native β-LG became reactive to Ellman’s reagent after adequate HHP treatment. Gel electrophoresis with and without β-mercaptoethanol provided evidence that the exposed thiol group was lost concomitant with the formation of S-S-linked β-LG dimers. Overall, these results suggest that HHP treatments induce β-LG into hydrophobic molten globule structures that remain stable for at least 3 months.

Keywords: β-Lactoglobulin; high pressure; molten globule; fluorescence; hydrophobic probes

INTRODUCTION

Protein structural properties contribute to functional properties such as solubility, emulsification, foaming, gelation, coagulation, viscosity, water binding, fat, and flavor binding (1, 2). A major factor affecting protein functionality is the hydrophilicity or hydrophobicity of protein resulting from the environments of the polar and nonpolar amino acids. Polar and charged amino acids at the surface of globular proteins provide protein solubility and water-binding properties (3). Nonpolar and uncharged amino acids on the surface of globular proteins provide protein hydrophobicity favorable to protein gelation and affinity for nonpolar ligands (3). Amphiphilic properties of protein contribute foaming and emulsion functionality due to protein migration to the oil-water or air-water interfaces. The formation of viscoelastic films through intermolecular hydrophobic interactions at the interfaces plays an important role in stabilizing foams and emulsions (4). Nonpolar amino acid residues in the interior of the protein structure provide hydrophobic pockets contributing to protein ligand binding (5).

Proteins may exist in three structurally distinct, thermodynamically stable states (6), the ordered state, the molten globular state, and the fully unfolded state. We are replacing the term native state used by Ptitsyn and many others with the term ordered state. This change in terminology recognizes that there exist numerous proteins that are incompletely folded in their native states, perhaps including native molten globules. Incompletely folded yet native proteins were called natively unfolded by Weinreb et al. (7) or intrinsically unstructured by Wright and Dyson (8).

The molten globule state is defined as a protein structural state intermediate between the ordered and fully unfolded states, existing either at equilibrium or transiently during refolding (9, 10). Protein in the molten globule state usually retains the secondary structure of the ordered state and exhibits a compact tertiary structure, but with increased mobility and looser packing of the protein chain (6). The molten globule also preserves many native intermolecular hydration sites and possesses surface hydration like the ordered state (11). Disulfide bonds and especially disulfide rearrangement favor the formation and stabilization of the molten globule (12, 13).

The rigid packing of ordered proteins excludes small hydrophobic molecules from their densely packed interiors, whereas the nonrigid packing of the molten globule state leads to substantial affinity for hydrophobic ligands, conferring an almost detergent-like character to molten globules. For example, Semisotinov et al. (14) reported that proteins in the molten globule state (bovine α-lactalbumin, bovine carbonic anhydrase and Staphylococcus aureus β-lactamase) exhibit high affinity for the hydrophobic probe 1-anilinonaphthalene-8-sulfonate (ANS). The ANS fluorescence intensity of proteins in the molten globule is 10–50 times the intensity of native proteins (14).

The molten globular state of proteins can be induced using chemical methods. At acid conditions in the pH 2–3 range and at appropriate ionic strengths, selected native proteins transform to the molten globule state (15, 16). Dilute molar concentrations of chemical denaturants such as urea or guanidine hydrochloride...
also induce selected proteins to form the molten globule state (16).

High pressure can depolymerize protein oligomers and unfold protein monomers (17–19). Although the detailed mechanism underlying pressure-induced protein unfolding is in some dispute (20, 21), high pressure nevertheless provides an alternative for driving proteins into the molten globule state (22–25). At high pressures, denaturation of proteins results in the incorporation of water into the protein, whereas heat denaturation results in the transfer of nonpolar groups into water (21, 26). Proteins such as cholinesterase, trypsin, and carboxypeptidase are induced into the molten globule state in the range of pressures from 50 to 150, 650, and 150–550 MPa, respectively, and retain a molten globular form after pressure release (22–24).

The functionalities of β-lactoglobulin (β-LG), a major whey protein, are diverse, contributing to solubility, gelation, foaming, emulsification, and flavor binding (27). β-LG consists of 162 amino acid residues and contains two disulfide bridges and one free cysteine. β-LG is predominantly β-sheet with nine antiparallel β-strands and one short α-helix (5, 28). Given its eight strands of antiparallel β-sheet that form a binding pocket for small hydrophobic ligands, β-LG is categorized as a member of the lipocalin superfamily of proteins, similar to plasma retinol-binding proteins (29). Thus, β-LG is capable of binding hydrophobic ligands including retinal, fatty acids, alkanes, aliphatic ketones, and aromatic compounds (30).

During refolding experiments, β-LG exhibits an intermediate structure that closely resembles a molten globule structure induced by guanidine hydrochloride (31) and urea (32). Incubation at temperatures slightly above 70 °C apparently induces β-LG into the molten globule state (33). Finally, methanol (40%) and ethanol (30%) also transform β-LG molecules into molten globular forms (34).

Hirose (35) proposed that proteins in the molten globule state will provide improved protein functionality with regard to food science applications, although to our knowledge this proposal has not been tested. Given the information presented above, a molten globule derived from β-LG would be a good candidate for testing the practical utility of such protein forms. Here we report the formation and structural characterization of highly stable molten globular forms of β-LG induced by high pressure, which we believe to have significant hydrophobic functional advantages compared to other native proteins in formulated foods. The stability and reproducibility of high hydrostatic pressure (HHP) treated β-LG provides the basis for experiments to explore possible food science applications of the highly interesting molten globular protein form.

MATERIALS AND METHODS

Protein and Chemicals. β-LG (no. L-6879) and ANS fluorescent probe (no. A-1028) were purchased from Sigma Chemical Co. (St. Louis, MO). αs-Parinaric acid (CPA) (no. 46164) was obtained from Pierce (Rockford, IL). Urea and sodium phosphate (monobasic, dibasic) were purchased from J. T. Baker (Phillipsburg, NJ). Chemicals for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA).

HHP Treatment. Sodium phosphate buffer (0.01 M, pH 7.0) was used to prepare β-LG at 0.5 mg/mL. The final concentration of β-LG was spectrophotometrically determined using a molar extinction coefficient at 280 nm, ε280 = 17600 M⁻¹ cm⁻¹ (36). β-LG was treated with HHP of 600 MPa at 50 °C for holding times of 0, 4, 8, 32, or 64 min. The zero holding time is the compression required to reach a pressure of 600 MPa. After exposure to high pressure, the β-LG was immediately cooled or stored at 5 °C.

Intrinsic and Extrinsic Fluorescence. Conformational changes of β-LG were monitored by intrinsic tryptophan fluorescence spectra using an excitation wavelength of 295 nm and observing an emission wavelength of 350 nm. The hydrophobicity of β-LG was assayed as extrinsic ANS fluorescence using an excitation wavelength of 390 nm and observing emission at a wavelength of 470 nm or as extrinsic CPA fluorescence using an excitation wavelength of 325 nm and observing emission at a wavelength of 420 nm. For these assays, 15 μL of ANS (8.0 mM in 0.1 phosphate buffer, pH 7.0) or 10 μL of CPA (3.6 mM in absolute ethanol containing equimolar butylated hydroxytoluene) solution was added to 2 mL of native or pressure-treated β-LG solution (0.1 mg/mL). Intrinsic and extrinsic fluorescence spectra were collected with an Aminco-Bowman J-8961 spectrophotofluorometer (Aminco Division of Travenol Laboratories, Inc., Silver Spring, MD).

Protein Structure Characterization. Circular dichroism (CD) spectra of β-LG were collected using an Aviv SF-202 CD spectrophotometer (Aviv Instruments, Lakewood, NJ). Cuvettes with path lengths of 0.2 and 1.0 cm were used for collecting data in the far-ultraviolet (UV) (190–270 nm) CD spectra and the near-UV (260–320 nm) CD spectra, respectively. The molar ellipticity ([θ]λ) at a wavelength λ is quoted in units of degree-square centimeters per decimole (deg·cm²·dmol⁻¹) and is given by

\[ [\theta]_\lambda = \frac{(MRW)\theta_0/\text{d}c}{c} \]

where MRW (mean residue weight) is the molecular weight mass divided by the number of repeating units, θ0 is the measured ellipticity, d is the cuvette path length (cm), and c is the protein concentration (g/mL). The molar ellipticity [θ]λ was plotted versus the wavelength in the 190–320 nm region.

To compare stabilities, protein unfolding as a function of increasing urea concentrations was carried out on both the native and HHP-treated β-LG. For these experiments, β-LG solutions (0.5 mg/mL) were mixed with various proportions of 8 M urea and 0.01 M phosphate buffer at pH 7.0 to obtain final urea concentrations from 0 to 6 M and final protein concentrations of 0.1 mg/mL. Next, 15 μL aliquots of 8.0 mM ANS in 0.1 phosphate buffer, pH 7.0, were added to each mixture. The ANS fluorescence was normalized as F/F₀ and plotted against urea concentration, where F represents ANS fluorescence in the presence of β-LG at the different urea concentrations and F₀ is the ANS fluorescence in the presence of β-LG without urea.

Polyacrylamide Gel Electrophoresis (PAGE). Polyacrylamide gel electrophoresis without SDS (native PAGE) was used to assay β-LG. Acrylamide gels at 12% (w/v) with a 4% stacking gel were prepared according to the instruction manual of the Mini-Protean II Ready Gels (catalog no. 161-0900). Native and HHP-treated β-LG solutions (0.5 mg/mL) were diluted with the native sodium phosphate buffer (1:1 ratio) prepared according to the instruction manual of the Mini-Protean II Ready Gels (catalog no. 161-0900). Aliquots of 50 μL of the diluted mixtures were loaded onto the gels. Electrophoresis was carried out at ambient temperature for 55 min at 200 V.

PAGE (12%) in the presence of sodium dodecyl sulfate (SDS-PAGE) with or without β-mercaptoethanol was performed according to the instruction manual of the Mini-Protean II Ready Gels (catalog no. 161-0900). Native and HHP-treated β-LG solutions (0.5 mg/mL) were diluted with the sodium phosphate buffer (1:1 ratio) and heated at 100 °C for 4 min. Aliquots of 50 μL of the diluted mixtures were loaded onto the gels. Electrophoresis was run at ambient temperature for 36 min at 200 V. Prestained SDS-PAGE standards (catalog no. 161-0318, Bio-Rad) were used to calibrate the gels. The protein standards included aprotinin (6.9 kDa), lyszyme (20.7 kDa),...
soybean trypsin inhibitor (29.1 kDa), carbonic anhydrase (34.9 kDa), ovalbumin (52.4 kDa), bovine serum albumin (70.0 kDa), β-galactosidase (121.1 kDa), and myosin (205.0 kDa).

**Estimation of Total Free and Exposed Sulphydryl Groups.** Total free SH groups were determined using Ellman’s reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB),52 Sodium phosphate buffer (0.01 M, pH 7.0), 8.0 M urea, and 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA) were used for preparation of DTNB solution (2.5 mM). A 0.5 mL aliquot of a β-LG solution was mixed with 1.95 mL of sodium phosphate buffer (0.01 M, pH 7.0), 8.0 M urea, and 1 mM EDTA, and 0.05 mL of the DTNB solution was added. Fifteen minutes after mixing, the absorbance at 412 nm was recorded. The final concentration of urea was 6.4 M. A molar extinction coefficient of 1.36 × 10^4 M^{-1} cm^{-1} was used to estimate the number of moles of SH per mole of β-LG.

The exposed (accessible) free SH groups in β-LG were determined with DTNB (2.5 mM) in a 0.01 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (pH 7.0), without urea to induce protein unfolding. A 0.5 mL aliquot of β-LG solution was mixed with 1.95 mL of sodium phosphate buffer (0.01 M, pH 7.0), and 0.05 mL of DTNB solution was added. Fifteen minutes after mixing, the absorbance at 412 nm was recorded. The results were used to estimate the number of moles of SH per mole of β-LG.

To estimate the number of new S–S bonds formed per mole of β-LG, the total moles of SH per mole of β-LG at each time point was subtracted from 1, the theoretical number of SH groups per mole of β-LG, to give the number of oxidized SH groups. This number was then divided by 2 to estimate the number of new S–S bonds per β-LG that formed during the treatment. A second estimate of S–S bond formation was calculated from the area ratios of bands on SDS-PAGE, assuming one disulfide per every two molecules in the dimer band and two disulfides per three molecules in the trimer band.

**RESULTS AND DISCUSSION**

**Conformational Change.** Following a series of exploratory experiments, 600 MPa at 50 °C for 4, 8, 16, 32, or 64 min was selected for HHP treatment of β-LG. The selected pressure, temperature, and times were readily produced with accessible equipment. Higher temperatures begin to induce denaturation in the absence of pressure, and significantly lower temperatures required inconveniently long times to induce change at the selected pressure.

The tryptophan fluorescence spectra of β-LG treated at 600 MPa and 50 °C for selected times and of β-LG in 8 M urea are presented in Figure 1. Most proteins exhibit a decrease in intrinsic fluorescence and a red shift upon unfolding. The large increase in fluorescence following incubation in 8 M urea demonstrates that β-LG is one of several proteins exhibiting increases in intrinsic tryptophan fluorescence and a red shift upon unfolding. A fluorescence increase and red shift typically indicate movement from a less polar (less quenching) environment to a more polar (more quenching) environment (37).

The fluorescence changes of tryptophan residues, Trp39 and Trp51, upon denaturation of β-LG were considered previously. Manderson et al. (38) suggested that both Trp39 and Trp51 become separated from specific, strong fluorescence quenchers upon denaturation, with the former moving away from the adjacent Arg89 and the latter from the adjacent Cys66–Cys160 disulfide bond observed in the crystal structure. Although previous studies selected a heat treatment and we selected high pressure, the similar increases in the intensity and red shift of the intrinsic tryptophan fluorescence following either treatment suggest that both methods result in tryptophan movement away from quenchers located in the adjacent three-dimensional structure.

The fluorescence intensity increases and the red shifts show much smaller changes per unit of time as the total time of treatment increases, essentially reaching plateaus at a 1.7-fold increase in intrinsic fluorescence and at a 4 nm red shift after 64 min of treatment (Figure 1). These values are significantly less than the 1.9-fold intensity increase and 13 nm red shift found for 8 M urea. A reasonable explanation, which is consistent with pressure studies on other proteins (25), is that unfolding is more or less complete in 8 M urea but that partially folded, perhaps molten globular, forms remain after the pressure treatment.

**Hydrophobic Probe Binding.** High-pressure treatment results in an increase in the extrinsic fluorescence intensity of ANS associated with β-LG (Figure 2). By 64 min of treatment at 600 MPa and 50 °C, there is a 3-fold increase in the ANS fluorescence intensity. This increase in ANS fluorescence intensity is less than the 10–50-fold increase typically associated with the transitions from the ordered state to the molten globule state (14). However, the native ordered state of β-LG contains an intrinsic pocket that binds ANS. Studies on S. aureus β-lactamase, another protein having a pre-existing pocket that binds ANS, exhibited a <2-fold ANS fluorescence increase following transition from the ordered to molten globular state (14). Thus, the observed increase in ANS fluorescence is consistent with pressure-induced molten globule formation.
In addition to the fluorescence increase, a 12 nm blue shift was observed for the ANS emission spectra in the presence of β-LG following 600 MPa pressure treatment. The blue shift indicates that pressure-treated β-LG binds ANS in a less polar environment compared to the environment surrounding native β-LG. Given that the ligand free pocket of native β-LG is filled with water (39), such a polarity decrease is reasonable and consistent with pressure-induced molten globule formation. In contrast to the results with the ANS probe, high-pressure treatment of β-LG resulted in a decrease in fluorescence intensity of CPA, a second extrinsic fluorescence probe (Figure 2). An overall decrease in fluorescence intensity of 65% followed 64 min of high-pressure treatment.

Two independent hydrophobic binding sites were identified on β-LG (40). The primary hydrophobic binding site is located within the calyx formed by eight strands of antiparallel β-sheets, and a second hydrophobic binding site lies in a cleft between the helix and an edge of the β-barrel. An accepted function of the primary hydrophobic binding site of β-LG is to bind fatty acids and retinol (5, 40). The importance of the second hydrophobic binding site is unclear. Comparison of ANS and CPA fluorescence upon binding to proteins having different proportions of aromatic and aliphatic hydrophobic groups led Hayakawa and Nakai (41) to suggest that ANS is more sensitive to aromatic hydrophobicity, whereas CPA is more sensitive to aliphatic hydrophobicity. This explanation at first might seem unlikely, but it should be kept in mind that aromatic/aromatic interactions exhibit a large polar component due to edge-to-face interactions (42), whereas aliphatic/aliphatic interactions arise primarily from van der Waals interactions. Also, one of the cardinal features of the native to molten globular transition is a loss of near-UV CD intensity from the aromatic side chains, suggesting that the aromatic groups in particular become more mobile during this transition (43). Increased mobility of the aromatic groups could lead to specific enhancement of their accessibility for binding with ANS.

Besides differences in interaction energies between aromatic groups as compared to those between aliphatic groups, structural factors may also be important. Because the side-chain packing of molten globules tends to be nonrigid, the clusters of hydrophobic side chains may be expected to approximate a spherical form. Depending on molecular sizes, the spherical clusters may accommodate a somewhat less isometric structure such as ANS while not easily accommodating a long, lean molecule such as CPA.

As a further test, we compared ANS and CPA affinities after conversion of α-lactalbumin to its molten globular form. As reported previously, ANS fluorescence exhibited a marked increase concomitant with conversion of α-lactalbumin to the molten globule (14). In contrast, CPA exhibited little or no measurable increase in fluorescence upon conversion of α-lactalbumin to the molten globule state. Additional experiments are necessary to assess the extrinsic fluorescence of CPA associated with conversion of proteins into the molten globular state.

Evidence for the Molten Globule State. Proteins converted to the molten globule state typically exhibit little or no change in the far-UV CD spectrum, indicating little change in secondary structure. Proteins in the molten globule state exhibit large decreases in the magnitude of peaks in the near-UV CD spectrum, indicating loss of aromatic dichroism from the motional averaging that arises from nonrigid side-chain packing (6, 43). Thus, determining the changes in the near- and far-UV CD spectra provides a direct test of whether pressure treatment induces conversion of β-LG into the molten globule state. The changes in the far-UV CD spectra of β-LG treated at 600 MPa and 50 °C for selected times are presented in Figure 3. The magnitude of the mean residue ellipticity (deg cm² dmol⁻¹) increases markedly and the spectral minimum shifts from 215 to 208 nm following high-pressure treatment.

Large spectral changes in the far-UV CD spectra are atypical for proteins that undergo transitions to the molten globule state. However, similar spectral changes were reported previously for β-LG (31, 34, 44, 45), although the conditions for inducing the conversion were different from conditions in our experiments.

The observed spectral shifts are consistent with a substantial shift from β-sheet to α-helix that occurs concomitant with molten globule formation (31, 34). This secondary structural change was previously explained in the following way. Secondary structure depends not only on the local sequence but also on interactions between regions of the chain that are separated in the sequence. Reduction of the interaction energies occurs upon conversion to the molten globule state. Reducing the interaction energies will allow each region of sequence to assume a locally preferred secondary structure. Supporting this conjecture is the observation that secondary structure predictors indicate local preferences for α-helix in many parts of the molecule that are observed as β-sheet in the folded structure (31).

The near-UV CD spectra of β-LG following selected times of treatment at high pressure are presented in Figure 4. Unlike the unusual changes observed in the far-UV CD, the changes in the near-UV CD exhibit the typical loss in intensity that accompanies conversion to the molten globule state. The signal associated with the rigid tertiary structure of native β-LG decreased with time of high-pressure treatment, exhibiting substantial reduction after 4 min and nearly complete disappearance after 8 min (Figure 4).

The near- and far-UV CD spectra compare favorably with previous studies on the conversion of β-LG into the molten globule state. Thus, there is little doubt that...
high-pressure treatment at 600 MPa and 50 °C induces β-LG into the molten globule state.

**Cooperativity of Protein Unfolding.** Conversion of an ordered protein into the molten globular state is accompanied by a marked decrease in the cooperativity of protein unfolding (6). Although the unfolding of molten globules is generally noncooperative, some molten globules such as the pH 4 form of apomyoglobin exhibit a slight degree of cooperativity upon unfolding. However, even with apomyoglobin at pH 4, the overall free energy stabilizing the molten globular form is very small, so the particles are quite fragile and are disrupted at low concentrations of denaturant. Thus, the urea unfolding profile provides a reliable means to distinguish molten globular from ordered forms. However, the cooperativity of the ordered state and the extent to which the cooperativity is lost during conversion to the molten globule state vary for different proteins (10, 46). To study changes in cooperativity of unfolding for β-LG, urea titrations were conducted in the presence of ANS (Figure 5). The native β-LG exhibited a characteristic sigmoidal curve due to cooperative unfolding, whereas the pressure-treated protein displayed a hyperbolic curve due to noncooperative unfolding.

With regard to the possible practical use of molten globular β-LG, a major concern is the possibility of refolding to the ordered state during storage. Urea titrations to induce unfolding provide a simple measure to estimate the amount of refolding during storage. The molten globular preparations of β-LG treated for 32 and 64 min were maintained at 5 °C for various periods of time and subjected to urea titrations to determine the degree of cooperativity. The short times of storage resulted in essentially no change, so only the longest time examined, 3 months, is presented (Figure 6). Even after a 3 month storage period, only a modest amount of refolding occurred.

The lack of change following 3 months of storage suggests that refolding involves a substantial energy barrier. The explanation of the stability of the molten globular form of β-LG is uncertain. Two prior observations suggest features that could contribute to long-term stability. The β-LG protein is one of several observed to undergo cold denaturation (47). Because the molten globular forms were stored in a refrigerator, nominally at 5 °C, the tendency of β-LG to cold denature may contribute to the long-term stability. A second important factor for long-term stability may be disulfide bond scrambling. The β-LG molecule contains one disulfide bond and one free sulfhydryl group. As indicated in Figure 8, formation of the molten globule first involves exposure of the free sulfhydryl, followed by concomitant formation of protein dimers and loss of free sulfhydryl groups. Three months of storage is associated with stabilization of the molten globule state, not refolding to the ordered state, so it is very likely that non-native disulfide bonds form during the pressure treatment. In the absence of free sulfhydryls at pH 7 used to store the protein, the non-native, scrambled disulfides are stable indefinitely. Indeed, such disulfide scrambling is reported to provide stability to the α-lactalbumin molten globule (48).

**Aggregation of β-LG.** Funtenberger et al. (49) reported that treatment of β-LG at 450 MPa and 25 °C results in aggregation, and selected gel electrophoresis was used to observe stable β-LG dimers through hexamers. Further analysis indicated that the aggregates were stabilized by disulfide bonds arising mostly from disulfide interchange rather than from oxidation. We also used electrophoresis to assess β-LG aggregation after various times of pressure treatment (Figure 7). The native gels and the SDS gels without β-mercaptoethanol demonstrate that high-pressure treatment brings about dimer formation with a small fraction of trimers (Figure 7a,b). Following addition of β-mercaptoethanol, only monomers were observed (Figure 7c),
suggesting that intermolecular disulfide bonds are formed during high-pressure treatment.

The thiol group of native \( \beta \)-LG is buried and not accessible to Ellman’s reagent unless the protein is first unfolded by urea \((32)\). Although high-pressure treatment causes the buried SH groups to become accessible, the total number of SH groups assayed in the presence of urea decreases with time of treatment (Figure 8), indicating that thiol oxidation occurs during or after high-pressure treatment of \( \beta \)-LG.

The native and SDS gels exhibit equivalent formation of dimers and trimers (Figure 7a,b). Furthermore, the number of disulfide bonds estimated by the decrease in free SH concentration and the number of disulfide bonds estimated from the assumption that every dimer and trimer are disulfide-linked agree very well (Figure 9). Thus, \( \beta \)-LG forms stable aggregates in the presence of disulfide bonds.

Only dimers and trimers of \( \beta \)-LG were observed in our studies, whereas dimers through hexamers were reported previously. Total SH decreased concomitantly with formation of aggregates in our studies, whereas the total SH remained approximately constant in the previous studies \((49)\). These differences may relate to buffer systems and protein concentrations: bis-Tris-HCl buffer instead of sodium phosphate and 23 mg/mL instead of 0.5 mg/mL were used by Funtenberger et al. \((49)\) and herein, respectively.

The pH of the phosphate buffer decreases reversibly by \(0.2-0.3\) pH unit per 100 MPa of HHP treatment \((50)\). Reducing the pH decreases the rate of disulfide interchange. Thus, the estimated decrease of 1.2–1.8 pH units at 600 MPa in this research and the significantly reduced protein concentration together will very likely lead to a substantial reduction in the intermolecular disulfide interchange, reducing the fraction of higher molecular weight aggregates.

Apparent refolding of \( \beta \)-LG in the molten globule state back to the native state (Figure 4) is dependent on disulfide shuffling during storage. If so, one possible way to increase the shelf life of molten globular \( \beta \)-LG will be to promote complete oxidation of SH groups to disulfides. In the absence of SH to catalyze interchange, the molten globular \( \beta \)-LG molecules containing non-native disulfide bonds will be stable indefinitely.

**Comparing the Time Courses for the Observed Structural Changes.** In this study, \( \beta \)-LG was subjected to pressure treatment, and six measures of structure were carried out: 1, intrinsic tryptophan fluorescence; 2, extrinsic fluorescence with associated ANS; 3, extrinsic fluorescence with associated CPA; 4, far-UV CD; 5, near-UV CD; and 6, disulfide bond formation (e.g., protein aggregation). To compare the time courses for the selected measures of structural change, each was converted to the fraction of the observed change and then plotted versus time (Figure 10).

The near-UV CD peak at 295 nm exhibited the shortest time course for its change, suggesting that side-
Due to the unique hydrophobic structure of the molten globule, the highly stable, pressure-induced molten globular form of β-LG may exhibit hydrophobic functional properties substantially different from those of either native or typically denatured proteins. Because of the stability of the resulting molten globular preparations, we are in a position to evaluate whether the pressure-induced molten globule of β-LG displays enhanced functionality for use as a food ingredient; if so, this would enable improved food quality and increased utilization of whey protein concentrates and isolates.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; ANS, 1-anilinonaphthalene-8-sulfonate; CPA, cis-parinaric acid; CD, circular dichroism; UV, ultraviolet; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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