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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of Binding of Sodium Dodecyl Sulfate, All-*trans*-retinol, and 8-Anilino-1-naphthalenesulfonate on the High-Pressure-Induced Unfolding and Aggregation of *β*-Lactoglobulin B

THÉRÈSE CONSIDINE,[†,‡] HARJINDER SINGH,[†] HASMUKH A. PATEL,[†,‡,§] AND LAWRENCE K. CREAMER*[,‡]

Riddet Centre, Massey University, Private Bag 11 222, Palmerston North, New Zealand, Fonterra Research Centre, Private Bag 11 029, Palmerston North, New Zealand, and Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11 222, Palmerston North, New Zealand

Bovine *β*-lactoglobulin B (*β*-LG) is susceptible to pressure treatment, which unfolds it, allowing thiol-catalyzed disulfide bond interchange to occur, facilitating intermolecular bonding (both noncovalent and disulfide). In the present study, *β*-LG was mixed with sodium dodecyl sulfate (SDS), all-*trans*-retinol (retinol), or 8-anilino-1-naphthalenesulfonate (ANS) on a 1:1.1 molar basis, and aliquots were held at pressures between 50 and 800 MPa for 30 min at pH 7.2 and 20 °C. Polyacrylamide gel electrophoresis (PAGE) showed that *β*-LG alone (control) was converted into a non-native monomer and a series of dimers, trimers, etc., at pressures beyond 100 MPa; SDS inhibited the formation of non-native species up to 200 MPa, and neither retinol nor ANS inhibited the formation of the non-native species as effectively as SDS. At pressures beyond 350 MPa, SDS ceased to have any inhibitory effect, but both ANS and retinol showed significant inhibition. The near- and far-UV CD patterns and the ANS fluorescent data were consistent with the PAGE data, but the retinol fluorescent data did not show sufficient change to interpret. The results suggested that there were three discernible structural stages. In Stage I (0.1−150 MPa), the native structure is stable; in Stage II (200−450 MPa), the native monomer is reversibly interchanging with non-native monomers and disulfide-bonded dimers; and in Stage III (>500 MPa), the free CysH in non-native monomer and dimer interacts with −S−S− bonds to produce high molecular weight aggregates of *β*-LG. SDS inhibited the Stage I to Stage II transition at 200 MPa, and ANS and retinol inhibited the Stage II to Stage III transition at 600 MPa.

KEYWORDS: Sodium dodecyl sulfate; 8-anilino-1-naphthalenesulfonate; retinol; *β*-lactoglobulin B; pressure-induced aggregation; binding site

INTRODUCTION

The structure of bovine *β*-lactoglobulin (*β*-LG) has been studied intensively for many years, and it is now known (*1*) that it has a molecular mass of about 18 300 Da, two sheets, and a three-turn helix, and normally exists as the native dimer between pH 5 and pH 7.5. It has two disulfide bonds and a cysteine (CysH) residue (CysH121) that is hidden in the native protein (shown in **Figure 1** in ref *2*). When *β*-LG is in an unfolding environment, that is, in a chaotropic solvent, at high temperature, at high pH, or at high pressure, CysH121 interacts reversibly with a physically close disulfide bond to create a non-native species with Cys121 bonded to another Cys to form a new disulfide bond and a new CysH residue. It has been shown recently, for a high temperature, neutral pH environment, that Cys160 is likely to become bonded to another Cys in another *β*-LG molecule or different protein such as α-lactalbumin, forming an inter-protein disulfide bond (e.g., refs *3*−*6*).

The structure of the protein at high pressure has been determined in a number of studies (*7*−*12*). Typically, the *β*-LG sample is pressurized in a special cell and examined at a range of high pressures, using spectral methods to follow the pressure-induced changes. Subirade et al. (*13*), using Fourier transform infrared (FTIR) spectroscopy, monitored the effect of pressure on the conformation of *β*-LG. They confirmed that the *β*-sheet is the major structural element of *β*-LG and that its secondary structure is not affected by pressure in the range 0−1400 bar (0−140 MPa). However, Panick et al. (*9*) reported that, at 130

* Author to whom correspondence should be addressed [telephone +64-6-350-4649; fax +64-6-356-1476; e-mail lawrie.creamer@fonterra.com].
† Riddet Centre, Massey University.
‡ Fonterra Research Centre.
§ Institute of Food, Nutrition and Human Health, Massey University.

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MPa, the amount of β-sheet and α-helical structures decreased with a concomitant increase in the content of disordered structure. No differences in pressure sensitivity between β-LG A and β-LG B were noted. In contrast to this result, Botelho et al. (*14*) and Belloque et al. (*10*) showed that β-LG B had greater pressure sensitivity than β-LG A and suggested that it was related to the existence of a cavity in β-LG at the site of replacement of Val119 by Ala, which does not affect the rearrangement of the surrounding protein structure at low pressure.

Tanaka and Kunugi (*7*) analyzed a mixture of β-LG and D_2O at a pD of 7.0, using proton nuclear magnetic resonance spectroscopy (NMR) at 300 MHz and FTIR spectroscopy. They concluded that pressures of 100 MPa for 3 h allowed a high degree of H/D exchange of the peptide bond amide protons. Belloque et al. (*10*), using a similar strategy, pressurized β-LG in D_2O at neutral pH for 5 min and then analyzed the mixture at normal pressure at low pH by two-dimensional NMR to determine the accessibility of individual amide protons. They reported that there was some unfolding of β-LG at 100 MPa but that the core was still structured. At pressures between 100 and 200 MPa, changes had occurred to some parts of the β-LG structure, and, at pressures between 300 and 400 MPa, β-LG became very flexible. Kuwata et al. (*11*) used an ^{15}N-labeled sample of β-LG and measured two-dimensional NMR spectra at 36 °C and pH 2.0 and at pressures between 0.1 and 200 MPa. They found that there was an increasing flexibility of the protein on the NMR time-scale with increasing pressure and that the protein had very little tertiary structure at 200 MPa.

Ligands, such as palmitic acid and all-*trans*-retinol (retinol), have been added to β-LG and have been shown to stabilize the native structure in unfolding environments such as urea solution (e.g., ref *15*), in the presence of an enzyme, trypsin (*16*), and at elevated temperatures (*2*) by binding in the β-LG calyx (*17*, *18*). For example, Dufour et al. (*19*) measured the Trp fluorescence of β-LG, and the extrinsic fluorescence of *cis*-parinaric acid (PnA) and retinol in β-LG solutions at pressures between 0.1 and 400 MPa at pH 3.0 and pH 7.0. At pH 3.0, β-LG changed conformation reversibly, but, at pH 7.0, an irreversible change occurred at about 150 MPa; however, the binding of these probes is hydrophobic and the association with β-LG at high pressure may not be comparable, even if the protein structure is unchanged, with binding at atmospheric pressure. Stapelfeldt and Skibsted (*20*) pressurized β-LG in the presence of PnA. They reported a decrease in the fluorescence quantum yield of PnA with increasing pressure. The largest effect was seen for moderate pressure, corresponding to the pressure region in which they suggested that pressure melting of β-LG preceded pressure unfolding. However, they did highlight that the binding of PnA may affect the pressure stability of β-LG.

Yang et al. (*21*−*23*) explored the structure of β-LG after pressure treatment with ligand probing using retinol, ANS, and PnA. They suggested that the resultant formation of non-native disulfide bonds caused conformational changes within the calyx.

Considine et al. (*2*) studied the effect of four ligands on the heat-induced aggregation of β-LG B and found that the two ligands that were strongly bound in the β-LG calyx, SDS and palmitate, retarded the primary thiol-catalyzed disulfide bond interchange that results in the formation of early intermediates. They also found that retinol and ANS, a so-called fluorescent hydrophobic probe, did not retard the early denaturation step but that ANS appeared to take part in the β-LG aggregation reaction, confirming a suggestion of Kamen and Woody (*24*). Thus, the aim of this study was to expand the work of Considine et al. (*2*) to investigate the effect of SDS, retinol, and ANS on the pressure-induced changes to the native structure and aggregation of β-LG.

MATERIALS AND METHODS

β-LG was prepared as described by Manderson et al. (*25*). Retinol, butylated hydroxytoluene (BHT), and ANS were obtained from Sigma Chemical Co., St. Louis, MO. Sodium dodecyl sulfate (SDS; special grade; catalog number 44215) and all other chemicals were AnalaR grade and were from BDH Laboratory Supplies, Poole, England; Coomassie Blue R250 and the polyacrylamide gel electrophoresis (PAGE) chemicals were obtained from BioRad Laboratories, Hercules, CA; Amido black 10B was obtained from Merck, Darmstadt, Germany. The water was from an artesian bore and was purified by reverse osmosis followed by ion exchange and carbon treatment using a Milli-Q system (Millipore Corp., Bedford, MA). The conductivity of the water was checked routinely.

β-LG (1.5 mg/mL) was mixed with 26 mM sodium phosphate buffer, pH 7.2, containing 68 mM sodium chloride. It is important to note that various pressure-sensitive and pressure-resistant buffers can be used during pressure treatment and that the buffer type and the buffer molarity influence the type of aggregates produced (*26*).

Retinol, ANS, and SDS (1 mg/mL) solutions were prepared as outlined by Considine et al. (*2*). Aliquots of ANS, SDS, and retinol solutions were added to β-LG (1.5 mg/mL) solutions at a molar ratio of 1:1.1 protein:ligand. Aliquots (approximately 6 mL) of the β-LG mixtures were transferred into Beckman Polyallomer Quick-Seal centrifuge tubes (13 mm internal diameter, 15 mm high, Beckman Instruments, Inc., Palo Alto, CA), which were heat sealed and transferred to the pressure chamber of a high-pressure unit ("Food-Lab" food processor, model S-FL-085-9-W, Stansted Fluid Power Ltd, Essex, UK). Samples were individually treated at pressures from 50 to 800 MPa for 30 min at 20 °C. A homogenized emulsion, consisting of a mixture of 10% vegetable-oil-in-water with surfactant and preservative, was used as the pressurizing fluid. The dimensions of the chamber of the high-pressure rig were 17 mm × 132 mm. The pressurization and depressurization rates were 5 and 14.5 MPa per second, respectively. The average adiabatic heating during compression was 1.4 °C per 100 MPa, and the cooling during decompression was 1.3 °C per 100 MPa. After the pressurizing treatment, each tube was immediately placed in an ice/water mixture for 5 min and then allowed to stand for 2 h at room temperature. Possible loss of sample, or change of concentration, was checked by weighing the tubes before and after treatment. PAGE, circular dichroism (CD), and fluorescence data acquisition and analysis were carried out as described by Considine et al. (*2*).

RESULTS

PAGE. A SDS-PAGE (nonreduced) gel pattern of the proteins in the pressure-treated β-LG samples is shown in **Figure 1A**. The patterns of the pressure-treated β-LG solutions (e.g., 700 MPa) showed monomer, dimer, a region of closely overlapping lower mobility bands, and material that could not enter the stacking gel. Similar electrophoretic patterns were shown by Considine et al. (*2*) for heat-treated β-LG.

The measured quantities of monomer β-LG in the SDS-PAGE gels were plotted as a function of pressure (**Figure 2A**). Samples pressurized up to 150 MPa showed very little change in the quantities of monomeric β-LG (**Figures 1A** and **2A**). Between 150 and 800 MPa, a decrease in the intensity of the monomer band was evident (**Figures 1A** and **2A**), with a concomitant increase in the intensity of the dimer band up to 500 MPa (**Figures 1A** and **2C**). Subsequent increases in pressure resulted in a slight decrease in the dimer concentration. This decrease in dimer concentration was not observed by Considine et al. (*2*) when β-LG was heat-treated.

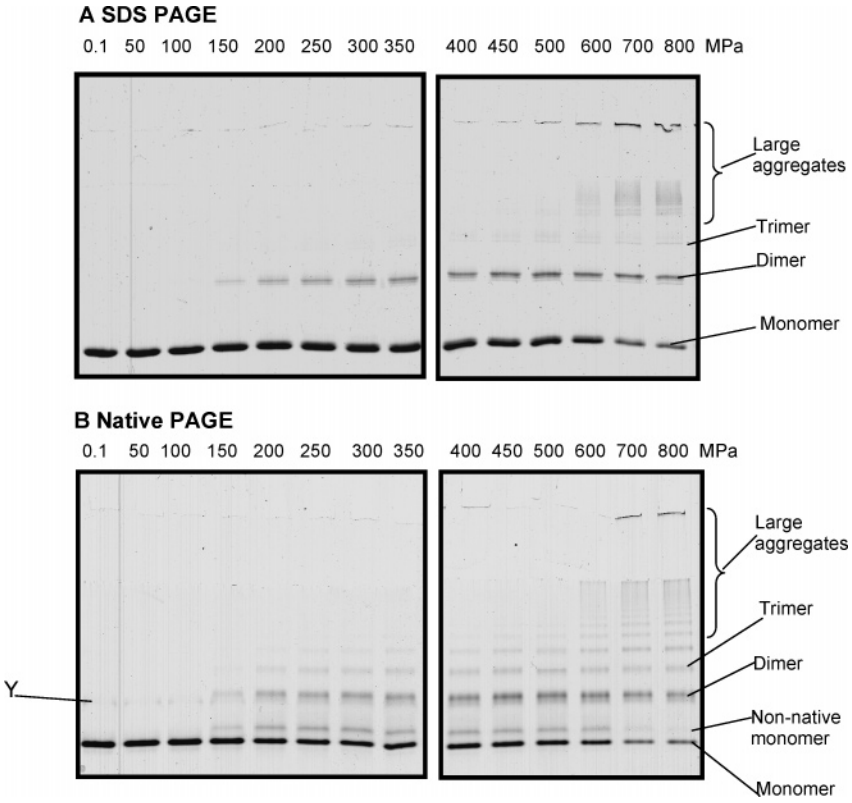

Figure 1. (**A**) SDS-PAGE and (**B**) native-PAGE patterns of samples of β-LG B after pressurization between 50 and 800 MPa. β-LG unpressurized (0.1 MPa), β-LG pressurized at 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, and 800 MPa. See text for experimental details.

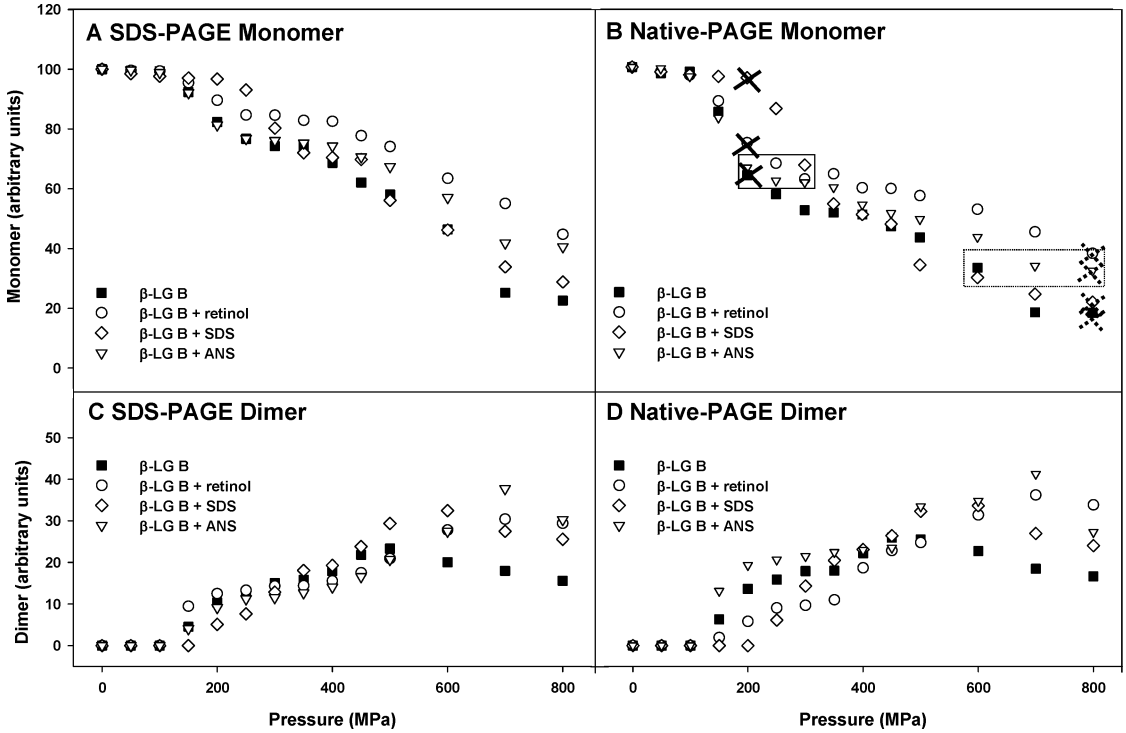

Figure 2. Plots of band intensities of SDS (**A**, **C**) and native (**B**, **D**) monomer (**A**, **B**) and dimer (**C**, **D**) of β-LG B with and without ligands as a function of pressure between 50 and 800 MPa. See **Figure 3** for explanations of X and the rectangular boxes.

The alkaline- (native-) PAGE pattern of the same samples is shown in **Figure 1B**. The major band present up to 100 MPa was native monomeric β-LG, but the pressure-treated samples showed the appearance of the dimer (**Figures 1B** and **2D**) at higher pressures. A similar trend of dimer formation and decrease to that in the SDS-PAGE pattern was evident in the native-PAGE pattern. The non-native monomer band was present in all samples pressurized from 150 to 800 MPa, and the intensity of the band was at a maximum in the samples pressurized between 400 and 500 MPa. Further pressurization resulted in a decrease in this non-native monomer band (**Figure 1B**).

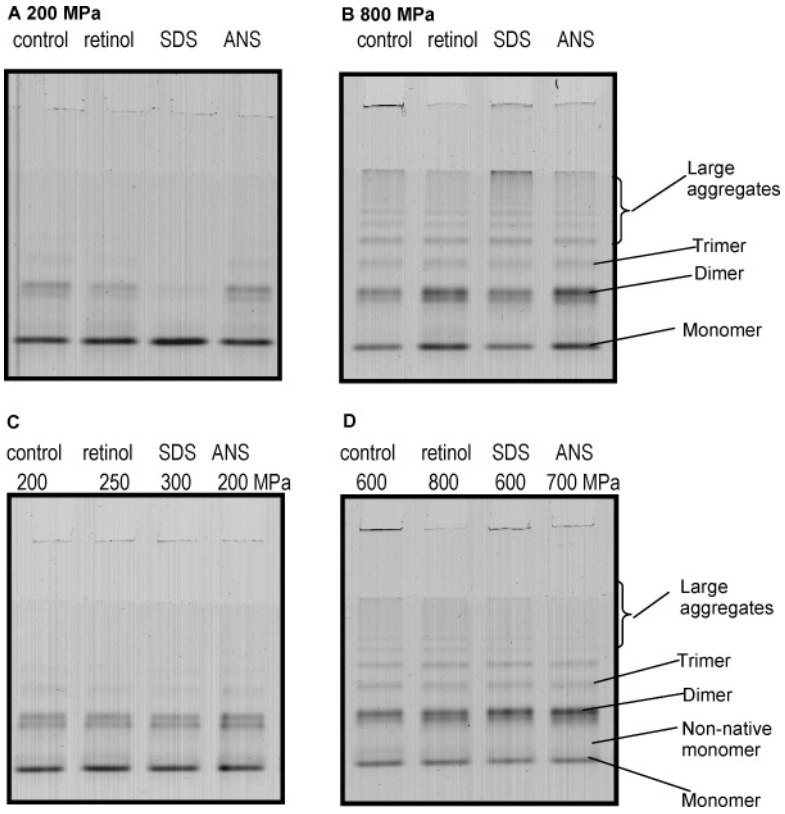

Figure 3. Comparison of the effect of ligands on the native-PAGE patterns. Native-PAGE of β-LG B pressurized in the presence of ligands at (**A**) 200 MPa (denoted by X in **Figure 2**) and (**B**) 800 MPa (denoted by dashed X in **Figure 2**) for 30 min: control; retinol; SDS; ANS. Native-PAGE of β-LG B pressurized in the presence of ligands at various pressures that gave approximately (**C**) 30% (denoted by rectangular box in **Figure 2**) and (**D**) 60% (denoted by dashed rectangular box in **Figure 2**) β-LG denaturation: with no ligand added, pressurized at 200 MPa (**C**) or 600 MPa (**D**); with added retinol, pressurized at 250 MPa (**C**) or 800 MPa (**D**); with added SDS, pressurized at 300 MPa (**C**) or 600 MPa (**D**); with added ANS, pressurized at 200 MPa (**C**) or 700 MPa (**D**).

Effect of Ligands. SDS-PAGE analysis of the pressure-treated solutions of β-LG plus ligand showed that there were monomers, dimers, and ranges of less mobile bands with patterns similar to those present in the control solution (**Figure 1A**). However, the β-LG solutions containing SDS or retinol and pressurized between 200 and 450 MPa had more monomer protein than the control samples (**Figure 2A**). Interestingly, the amount of monomer remaining at 800 MPa was higher in β-LG solutions containing retinol or ANS than in those containing SDS, which offered the most protection to β-LG at lower pressure treatments. These results are in contrast to those of Considine et al. (*2*), who carried out a similar experiment but using heat treatment instead of pressure treatment. They found that, with heating to 93 °C, all samples had similar levels of monomer, with the exception of the β-LG ANS mixture, which had much higher levels of monomer. Dimer (**Figure 2C**) trends comparable to the control were evident when ligands were present, with SDS being the most effective at slowing dimer formation at treatments $\leq$250 MPa.

The addition of all ligands caused higher levels of dimer in the pressure-treated mixtures after pressure treatments of $\geq$500 MPa. Below this pressure, the levels of dimer in the SDS-PAGE patterns were relatively similar (**Figure 2C**), but the native-PAGE patterns (**Figure 2D**) showed that mixtures of β-LG and ANS had higher levels of dimer than the control. Conversely, mixtures of β-LG and either SDS or retinol had lower levels of dimer than the control at pressures between 150 and 300 MPa. The subsequent slight decrease in dimer formation for β-LG mixtures containing ligands occurred at higher pressures (700−800 MPa) than for the control (500 MPa). Similar results were obtained using native-PAGE (**Figure 2D**). Moreover, the type and the quantity of the various products in the samples pressure treated at 200 and 800 MPa (**Figure 3A** and **B**, respectively) or after about 30% and 60% β-LG denaturation (**Figures 3C** and **D**) were different. The stabilizing power of the ligands appeared to follow the order SDS > retinol > ANS $\approx$ control between 150 and 300 MPa, but followed the order retinol > ANS > SDS $\approx$ control at pressures greater than 300 MPa (**Figure 2A**,**B** and **3D**).

Noncovalently Bonded Protein Polymers. The difference between native-PAGE and SDS-PAGE represents the species linked by hydrophobic bonds, as SDS dissociates all of the hydrophobically linked trimers, tetramers, etc., into disulfide-linked dimers, trimers, etc., and/or SDS monomers. With respect to β-LG, the native-PAGE monomer consists only of native monomer, whereas the SDS-PAGE monomer contains both native monomer and non-native monomers. Thus, the difference between SDS-PAGE monomer and native-PAGE monomer gives an estimate of the non-native monomers in treated β-LG samples (**Figure 4**). Non-native β-LG was present in control samples pressurized at pressures as low as 50 MPa. The proportion of non-native β-LG increased up to 350 MPa but decreased thereafter. A similar trend was evident when retinol or ANS was mixed with β-LG prior to treatment. However, when SDS was added to β-LG, very little non-native monomer was evident below 200 MPa, and there were moderate levels in pressurized samples up to 500 MPa. Above 500 MPa, the levels of non-native monomer decreased.

A small quantity of a second band, which had slightly greater mobility than the dimer band, denoted by a "Y", was observed

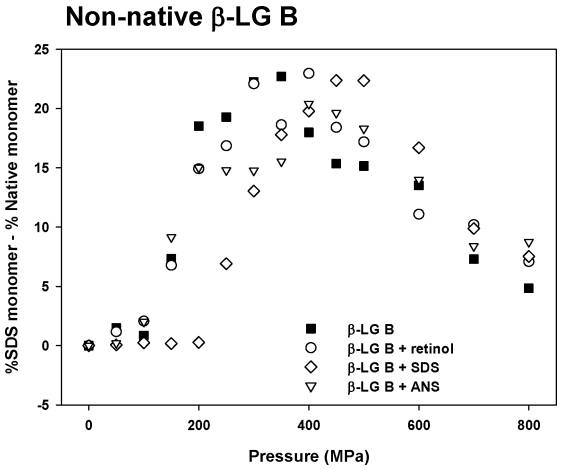

Figure 4. Effect of pressure treatment at between 50 and 800 MPa for 30 min on the concentration of β-LG B non-native monomer (difference between SDS-monomer and native monomer, shown in **Figure 2**) with and without ligands.

after storing (4 °C) samples of β-LG mixed 1:1.1 with SDS prior to native-PAGE analysis (**Figure 1B**) regardless of treatment. Similar findings were reported by Considine et al. (*2*) when mixtures of palmitate and β-LG were stored before heat treatment.

Near-UV CD. The near-ultraviolet (near-UV) CD spectra of unpressurized β-LG and a selection of pressurized β-LG solutions with no added ligand and with added SDS are shown in **Figure 5A** and **B**, respectively. The native protein spectrum had two sharp troughs at 285 and 293 nm, which have been ascribed to Trp19 alone (*27*) and indicate that Trp19 is in a chiral environment. The loss of these bands as a consequence of the pressure treatment of β-LG suggests that Trp19 had moved to a less chiral environment and that the tertiary structure had been altered. However, even after pressurizing β-LG at 800 MPa for 30 min, some of the protein appeared to retain an ordered tertiary structure. Comparable results were shown by Considine et al. (*2*) when β-LG was heat-treated in the presence of a range of ligands. This conclusion is also consistent with the SDS-PAGE and native-PAGE results (**Figure 1A** and **B**). These CD results are in general agreement with those obtained in previous pressure treatment (*8*, *28*) and heat treatment (*2*, *27*, *29*) studies.

The effect of pressure on the intensity of the 293 nm trough of β-LG is shown in **Figure 5C**. The 293 nm signal of native β-LG decreased with increasing pressure. The 293 nm signals of the β-LG samples with added ligands also decreased with increasing pressure (**Figure 5C**). The pressure at which the decrease began was higher for samples with SDS or retinol than for the control (**Figure 5C**). Retinol itself displays a CD signal, which results in a plot that is ~2 mdeg lower than the rest of the results. This CD result is in agreement with the findings of Considine et al. (*2*) for heat-treated β-LG solutions and is consistent with the loss of native structure as shown by the native-PAGE results (**Figures 1B** and **2B**).

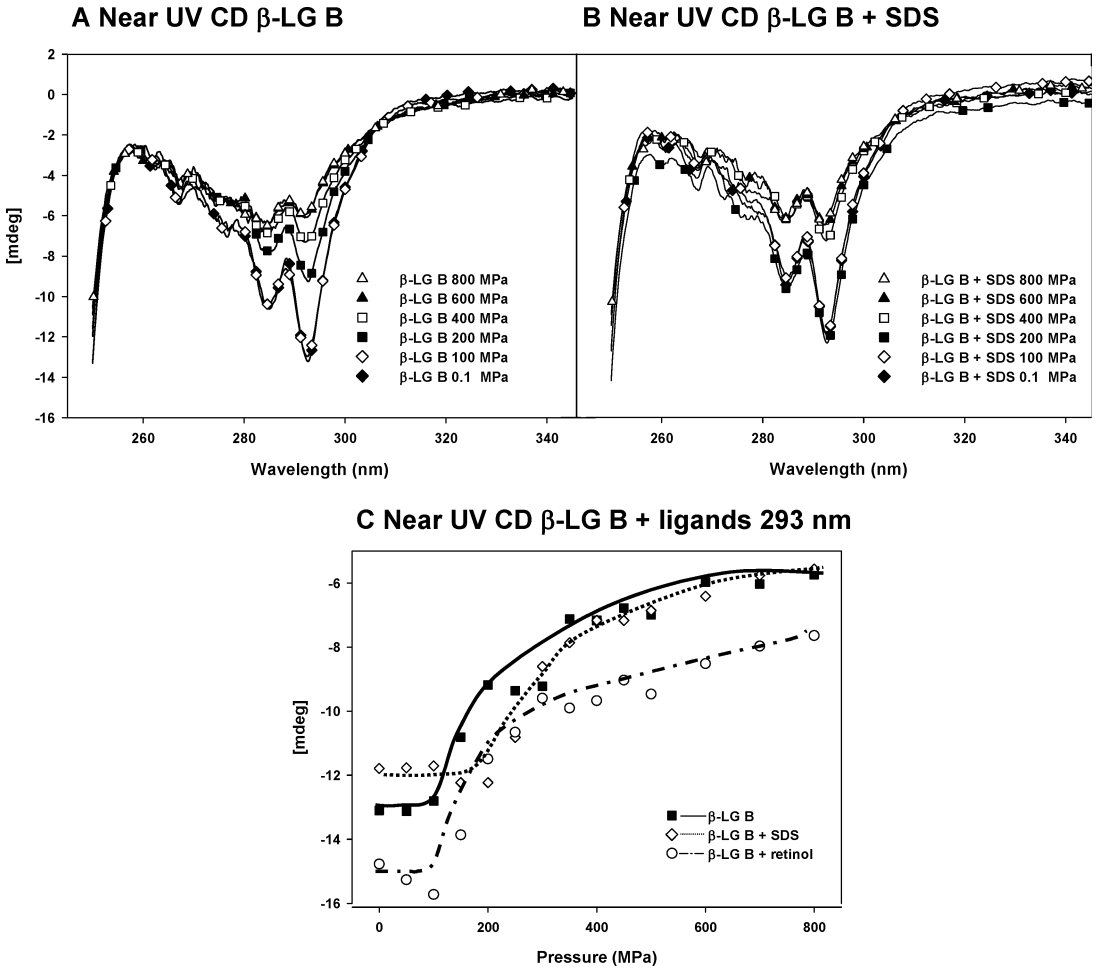

Figure 5. Near-UV CD spectra between 245 and 345 nm of a pressure-treated solutions of (**A**) β-LG with no added ligand and (**B**) β-LG with added SDS, and (**C**) a plot of the CD intensity at 293 nm versus pressure for β-LG with and without ligands.

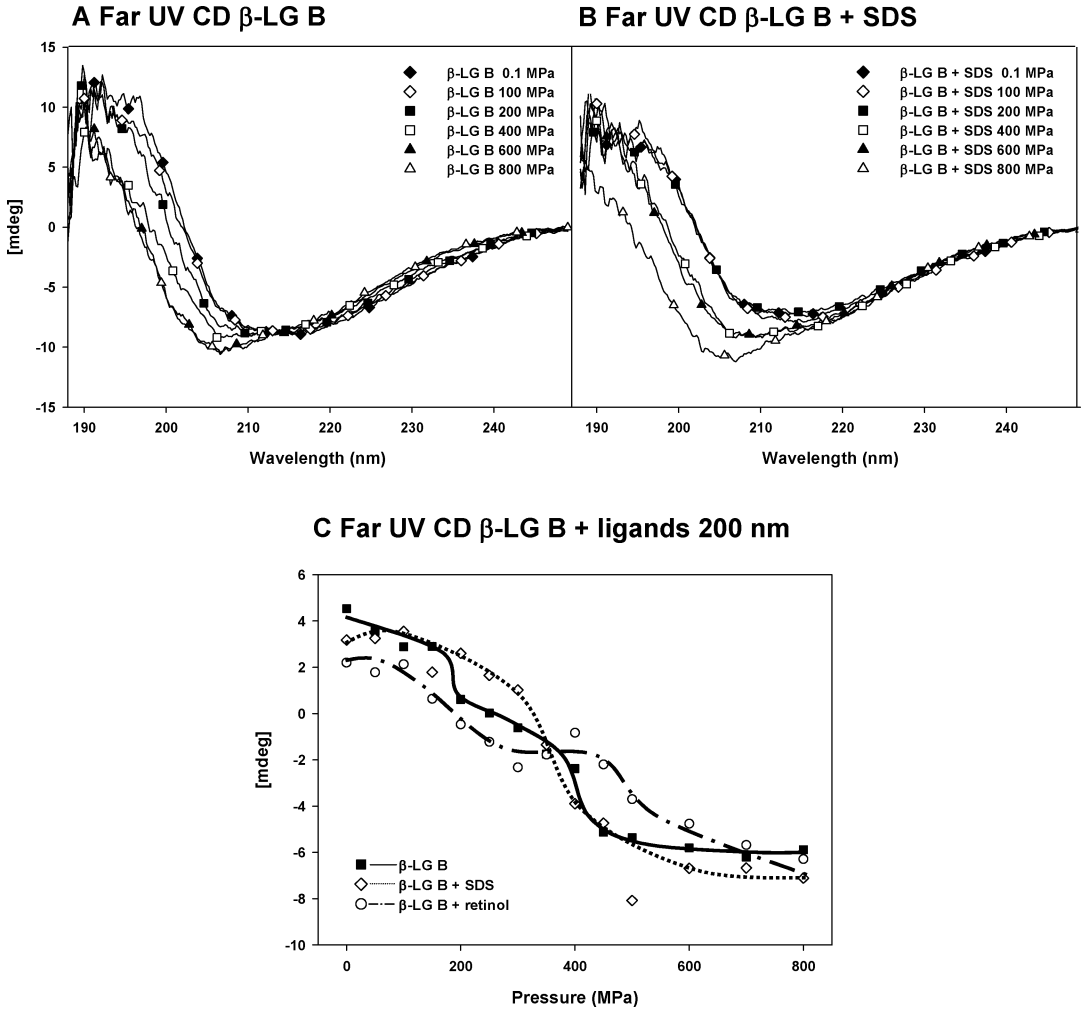

Figure 6. Far-UV CD spectra between 188 and 249 nm of pressure-treated solutions of (**A**) β-LG B with no added ligand and (**B**) β-LG B with added SDS, and (**C**) a plot of the CD intensity at 200 nm versus pressure for β-LG B with and without ligands.

Far-UV CD. The far-UV CD spectra of native β-LG and β-LG with added SDS are shown in **Figure 6A** and **B**, respectively. As the pressure was increased, the trough at 216 nm gradually broadened and deepened and the trough minimum shifted to a lower wavelength (approximately 207 nm) (**Figure 6A**). These results are consistent with those reported by Yang et al. (*23*), who pressurized β-LG at 600 MPa and 50 °C for various holding times, and Ikeuchi et al. (*28*) who pressurized β-LG between 0.1 and 400 MPa. Our results indicate that between 200 and 400 MPa, there was a broadening of the spectrum and, at higher pressures, the trough both deepened and broadened further (**Figure 6A**). On addition of a ligand, for example, SDS (**Figure 6B**), the broadening of the spectrum occurred at higher pressure, 400−600 MPa. The observed spectral shift may be regarded as reflecting the shift from β-sheet to α-helix (*30*), which is similar to molten globule formation (*31*, *32*). Similar trends were seen for heated samples, and this spectral shift was observed only after treatment temperatures >70 °C (*2*, *33*) for β-LG and >75 °C for β-LG with added ligand (e.g., SDS, palmitic acid; ref *2*).

The change in CD intensity at 200 nm with pressure is shown in **Figure 6C**. All samples showed an increase in intensity with higher pressures. Pressure-treated β-LG containing retinol was similar to the control. However, when SDS was added to β-LG, the increase in intensity at 200 nm occurred at higher pressure than for the control.

ANS Fluorescence. The effect of the addition of ANS to β-LG before pressurization was analyzed using ANS fluorescence (**Figure 7A**). The emission spectrum of the native β-LG: ANS mixture contained a broad peak at 480 nm. The sample pressurized at 800 MPa for 30 min had an ANS emission maximum at 468.5 nm. The I_{ANS} (emission intensity at λ_{max}) increased 6-fold with increasing pressure and decreased the λ_{max} from 480 to 468 nm. This finding is in agreement with Stapefeldt and Skibsted (*20*) and Stapelfeldt et al. (*34*), who reported an overall exposure of hydrophobic residues to the solvent upon pressurization (600 MPa), which increased the binding of ANS to β-LG, resulting in a decreasing emission maximum and an increasing fluorescence quantum yield.

A plot of the 468.5 nm emission intensity versus the pressure (**Figure 7B**) suggested that the change in the ANS environment could not be treated as a simple two-stage system, as previously found for the heat treatment of β-LG (*2*) and solvent unfolding (*15*), but could perhaps be treated as a three-stage system. Collini et al. (*35*) showed that ANS has a low affinity for β-LG and that two binding sites, internal and external, are available. As the pressure treatment increases, β-LG begins to unfold, making more hydrophobic sites available, thus promoting ANS binding (*36*). Similar findings were reported by Considine et al. (*2*) for the heat treatment of β-LG and ANS, but with a clear two-stage system being evident, with ANS being in one environment at up 55 °C and in a different environment after heat treatments

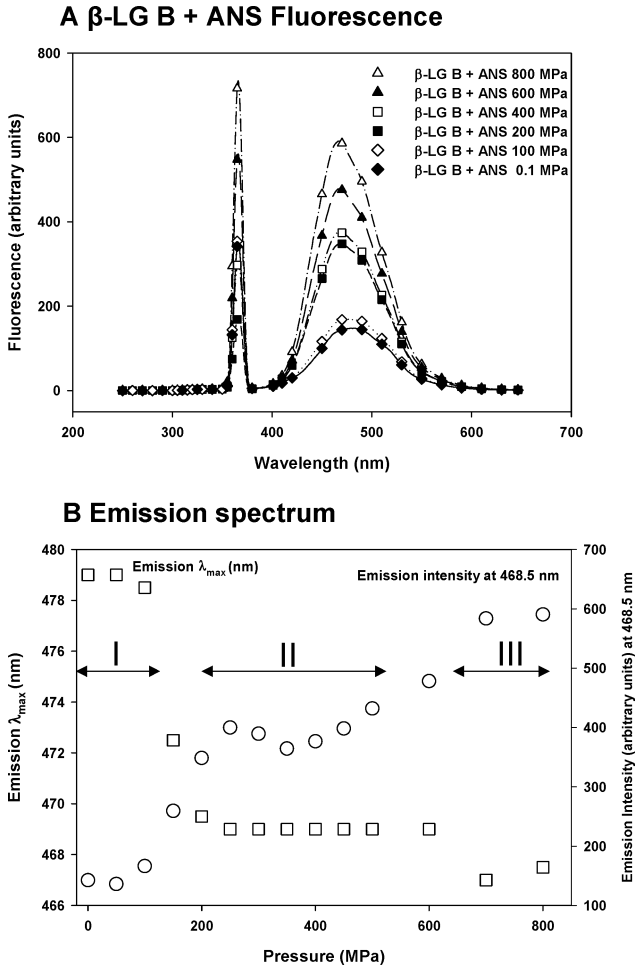

Figure 7. Effect of pressure on (**A**) the ANS fluorescence emission spectrum of β-LG B and (**B**) I_{ANS} (○) and λ_{max} (□), which have been excited at 370 nm. The horizontal lines show the approximate regions or pressure ranges in which the three protein structural stages might be stable.

of β-LG greater than 84 °C. Between 55 and 84 °C, ANS was in one or the other environment.

DISCUSSION

An NMR study by Kuwata et al. (*11*), in which disulfide bond interchange was inhibited because of the low pH (~2), clearly demonstrated that β-LG unfolded increasingly as the pressure was increased. They interpreted their results in terms of the observed structures of globular proteins in solution as being the average of a number of similar conformations; consequently, increasing or decreasing the pressure can rapidly change the average structure as the proportions of the various conformations alter. This approach has been used to determine the refolding of proteins after they have been almost completely unfolded in a chaotropic solvent. However, when the pH is near neutral, a thiol can catalyze the interchange of disulfide bonds (*37*−*39*). Thus, pressure treatment of β-LG solutions at neutral pH results in irreversible changes because of the consequential disulfide bond interchange and not the pressure effect per se.

The results shown in **Figure 1B** indicate that treatment of β-LG at increasing pressure at pH 7.2 results in an increased proportion of disulfide-bonded aggregates with non-native monomer predominating at low pressures and the largest aggregates predominating at the highest pressures. The quantity of native protein remaining in the control (i.e., no ligands) after

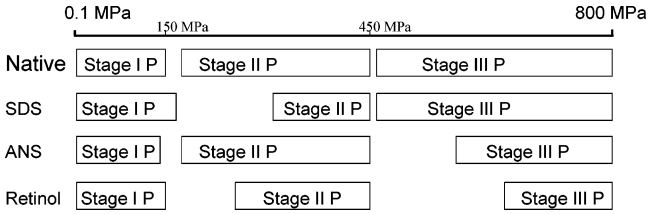

Figure 8. Proposed three-stage model of the pressure denaturation of β-LG B, and β-LG B with added ANS, retinol, or SDS.

pressure treatment (**Figure 2B**) decreased markedly between 100 and 250 MPa. Based on our knowledge of the effects of temperature or heat on β-LG solutions, it seems likely that, at pressures below about 150 MPa, the critical CysH121 is not exposed and a potential disulfide bond target is not available for reaction and is called Stage I P (**Figure 8**). In this stage, the native structure is the major component after pressure treatment as shown by PAGE and CD (**Figures 1**, **5**, and **6**). Examination of β-LG patterns (SDS-PAGE, **Figure 1A**) indicates that pressure treatment between 200 and 450 MPa (Stage II P) forms disulfide-bonded dimers. The same samples examined using native-PAGE show the presence of non-native monomer, trimer, and tetramer, and disulfide-bonded dimers (**Figure 1B**) indicated that noncovalent interactions had been involved. Manderson et al. (*25*) demonstrated that heat-induced trimers observed in native-PAGE could be formed from three disulfide-bonded β-LG as well as a noncovalently associated non-native monomer and a disulfide-bonded dimer. Similarly they showed that tetramers could be composed of four disulfide-bonded monomers or of two noncovalently disulfide-bonded dimers. In summary, the results of the present study suggest that pressure treatments between 150 and 450 MPa for 30 min produced only non-native monomers and disulfide bonded dimers, which could then associate noncovalently to form stable trimer and tetramer adducts, but not trimers, tetramers, etc., via disulfide bond interchange.

From this present study, we propose that a three-stage model (**Figure 8**) can be used to describe the denaturation and aggregation of pressure treated β-LG. In Stage I P (0.1−150 MPa), the native structure is the stable structure after pressure treatment, as shown by PAGE, CD, and fluorescence (**Figures 1**, **2**, **5**, **6**, and **7**). In Stage II P (200−450 MPa), the native monomers are reversibly interchanging with a non-native monomer and a disulfide-bonded dimer. Finally, in Stage III P (beyond about 500 MPa), the stable state after pressure treatment is unfolded β-LG stabilized by chains of disulfide-bonded β-LG molecules.

Stepwise unfolding of β-LG as a result of pressure treatment has been reported previously (*11*, *20*). Stapelfeldt and Skibsted (*20*) also proposed a three-stage pressure denaturation of β-LG based on studies at high pressure. In their first stage, moderate pressures (up to 50 MPa) induced changes in β-LG that could be described as pressure melting with an increased thiol reactivity, and induced collapse of the inner calyx but did not increase the hydrodynamic volume of β-LG. In their second stage, at pressures up to 200 MPa, a pressure unfolding occurred that was partly reversible after pressure release in a slow renaturation process. In stage three, high pressures (300 MPa) showed irreversible changes becoming evident with aggregation and gel formation. Our results on the consequences of high pressure as opposed to the structures present at high pressures support and extend their earlier studies.

Effect of Ligands. The addition of hydrophobic ligands (e.g., SDS) stabilizes the native form at about 250 MPa (**Figures 2B**

and **5C**), thus shifting the transition between Stage I P and Stage II P (**Figure 8**). Similarly addition of low levels of retinol and/or ANS to β-LG inhibited transition from Stage II P to Stage III P at 450 MPa (to ~600 MPa) (**Figures 2B**,**D**, **4**, **5C**, and **6C**), indicating that these ligands stabilize the non-native monomers and dimers.

High Affinity Anionic Ligands. In an earlier study on the effect of temperature on β-LG denaturation and aggregation (*2*), in which both SDS and palmitate were used as high affinity ligands, very similar results were obtained. Because SDS is water soluble and palmitate is less soluble, SDS was the chosen ligand for the present study and SDS has the ability to inhibit the transition from Stage I P to Stage II P, but not the transition to Stage III P, and it is likely that palmitate would act in a similar way. Two other fatty acids, myristic acid and conjugated linoleic acid (CLA), were recently shown to protect β-LG from both early pressure denaturation (*40*), shifting the transition between Stage I P and Stage II P from 150 to 200 MPa and also CLA had the ability to shift the transition from Stage II P to III P, from 450 to 600 MPa. It may be that these ligands not only bind strongly in the native protein calyx, but their anionic region could bind to Lys60 and Lys69, thus stabilizing the native state more effectively.

Low Affinity Ligands. Retinol binds strongly in the calyx of β-LG at higher pHs (pH 8 being optimum) but not as strongly at the pH of this study. The PAGE data indicate that retinol does not affect the transition between Stage I P and Stage II P, but, beyond this pressure (150 MPa), it inhibits the loss of native β-LG (**Figure 2B**) and the loss of β-LG in SDS-PAGE (**Figure 2A**). ANS does not inhibit the loss of native β-LG up to 400 MPa (SDS-PAGE, **Figure 2A**) or 300 MPa (native-PAGE, **Figure 2B**). In addition, both of these ligands modify the pattern of β-LG polymer distribution (**Figure 3B** and **D**). In both the control sample and the SDS sample, whether taken after pressure treatment at 800 MPa (**Figure 3B**) or after pressure treatments that give comparable monomer concentrations (**Figure 3D**), the quantity of high molecular weight material caught in the sample slot, or on the top of the stacking gel, was significantly greater than that in the samples with added ANS or retinol. Thus, the mode of action of these two ligands (ANS and retinol) is qualitatively as well as quantitatively different, with ANS and retinol inhibiting the Stage II P to Stage III P transition. SDS stabilizes Stage I P and inhibits conversion to Stage II P, but does not affect further conversions to higher stages. Thus, these ligands stabilize the various stages differently, probably because the binding sites of β-LG in each stage are different.

ABBREVIATIONS USED

ANS, 8-anilino-1-naphthalenesulfonate; PnA, *cis*-parinaric acid; β-LG, bovine β-lactoglobulin B; BHT, butylated hydroxytoluene; CD, circular dichroism; Cys, CysH, cysteine; FTIR, Fourier transform infrared; I_{ANS}, emission intensity at λ_{max}; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; retinol, all-*trans*-retinol; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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