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Unfolding and Refolding of β-Lactoglobulin Subjected to High Hydrostatic Pressure at Different pH Values and Temperatures and Its Influence on Proteolysis

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The unfolding of β-lactoglobulin during high-pressure treatment and its refolding after decompression were studied by ^{1}H NMR and ^{2}H/^{1}H exchange at pH 6.8 and 2.5 and at 37 and 25 °C. The extent of unfolding increased with the pressure level. The structure of β-lactoglobulin required higher pressures to unfold at pH 2.5 than at pH 6.8. More flexibility was achieved at 37 °C than at 25 °C. Results indicated that the structural region formed by strands F, G, and H was more resistant to unfold under acidic and neutral conditions. The exposure of Trp^{19} at an earlier time, as compared to other protein regions, supports the formation of a swollen structural state at pH 2.5. Refolding was achieved faster when β-lactoglobulin was subjected to 200 MPa than to 400 MPa, to 37 °C than to 25 °C, and to acidic than to neutral pH. After treatment at 400 MPa for 20 min at neutral pH, the protein native structure was not recovered. All samples at acidic pH showed that the protein quickly regained its structure. Hydrolysis of β-lactoglobulin by pepsin and chymotrypsin could be related to pressure-induced changes in the structure of the protein. Compared to the behavior of the protein at atmospheric pressure, no increased proteolysis was found in samples with no increased flexibility (100 MPa, 37 °C, pH 2.5). Slightly flexible structures were associated with significantly increased proteolysis (100 MPa, 37 °C, pH 6.8; 200 MPa, 37 °C, pH 2.5). Highly flexible structures were associated with very fast proteolysis (≥200 MPa, 37 °C, pH 6.8; ≥300 MPa, 37 °C, pH 2.5). Proteolysis of prepressurized samples improved only when the protein was significantly changed after the pressure treatment (400 MPa, 25 °C, 20 min, pH 6.8).

KEYWORDS: β-Lactoglobulin; structure; nuclear magnetic resonance; high pressure; chymotrypsin; pepsin

INTRODUCTION

β-Lactoglobulin constitutes about half of the protein content in milk whey and has been extensively studied (*1*). It is a small protein (∼18 kDa) whose structure is known from X-ray and NMR studies (*2*−*4*). It consists basically of an antiparallel β-calyx formed by eight strands (A−H) that leave an internal cavity able to bind hydrophobic ligands. An α-helix and a ninth strand (I) are found at the external face, near the closure of the barrel. At neutral pH, β-lactoglobulin forms dimers that dissociate under acidic conditions (*5*). At acidic pH, the protein is very stable (*6*) and maintains a structure similar to that at neutral pH, although some changes regarding the A−B loop, involved in the dimer formation, and the E−F loop, which could modulate the ligand access to the open end of the calyx, have been reported (*7*, *8*).

In general, the susceptibility of β-lactoglobulin to proteolysis is relatively low, being particularly resistant to pepsin, a fact that has been attributed to its high stability at low pH (*9*, *10*). Subjecting β-lactoglobulin to high pressure has been shown to favor its hydrolysis by chymotrypsin, trypsin, pepsin, and other proteinases (*11*−*18*), a phenomenon that could be attributed to structural changes that enhance its susceptibility to proteolysis.

The structural changes induced in β-lactoglobulin by high pressure include dissociation of the dimer, unfolding, and irreversible aggregation (*19*−*35*). NMR is a structure-sensitive technique that combined with H^2/H^1 exchange demonstrates transiently unfolded states of proteins, allowing the study of structural changes of β-lactoglobulin under heat (*36*, *37*) and high pressure (*20*, *25*, *27*).

The aim of this work was to evaluate the unfolding and refolding of β-lactoglobulin subjected to high pressures at neutral and acidic pH at two different temperatures and to relate these changes to the susceptibility to proteolysis. The enzymes chosen, chymotrypsin and pepsin, are specific for hydrophobic residues, most facing the inner face of the protein, thus not easily accessible in the structured protein.

MATERIALS AND METHODS

β-Lactoglobulin, TLCK-treated chymotrypsin (EC 3.4.21.1), and pepsin (EC 3.4.23.1) were obtained from Sigma (St. Louis, MO).

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Sample Preparation and Pressure Treatments for NMR Study. Two types of H^2/H^1 exchange studies were performed: the unfolding of the protein during the high-pressure treatments and the refolding of the protein after decompression.

For the unfolding studies, acidic and neutral samples of β-lactoglobulin (1%, w/v) were prepared in ^2H_2O, taken to pH 6.8 or 2.5 with NaO^2H or ^2HCl, and distributed into Eppendorf tubes. One of the aliquots was kept as the control sample (0.1 MPa), while the remaining tubes were sealed and pressurized at 100, 200, 300, and 400 MPa in a 900 HP instrument (Eurotherm Automation, Lyon, France). The samples were compressed at a rate of 2.5 MPa/s, the final pressure was maintained for 20 min, and decompression was at the same rate as compression. The temperature was set to 25 or 37 °C, although compression led to a temporary increase of temperature of about 2 °C/100 MPa. It has to be noted that this temporary temperature increase could lead to an additional effect on the protein structure. Afterward, the neutral samples were immediately acidified with ^2HCl to pH 2.5 to minimize further $^2H/^1H$ exchange. All samples were kept at room temperature until they were analyzed.

For the refolding studies, a 10% (w/v) solution of β-lactoglobulin was prepared in H_2O at pH 6.8 and 2.5. One aliquot of the solution was kept as a control, and the remaining samples were pressurized at 200 and 400 MPa for 20 min at 25 and 37 °C. After decompression, they were allowed to refold at room temperature for 5 min and 6 h. After this time, the samples were diluted (1/10, v/v) in ^2H_2O, and the pH was adjusted to 2.5. The samples were kept at room temperature until they were analyzed.

For comparison with the proteolysis susceptibility, it was verified that the addition of buffer salts to the protein solution (Tris, pH 6.8, and citrate, pH 2.5) did not appreciably modify the flexibility of β-lactoglobulin on pressurization as compared with the samples dissolved in ^2H_2O.

NMR Spectra. ^1H NMR spectra were recorded at 35 °C on a 400 MHz Varian ^{UNITY}INOVA spectrometer (Varian NMR Instruments, Palo Alto, CA 94304), with a 3.7 s acquisition time and 5 s delay time, and water was suppressed using presaturation for 1−2.5 s. Spectra for unfolding studies were done using 256 transients, while those for structure recovery needed 4096 transients due to the dilution of the sample. Raw data were processed using Varian software. Resonances were assigned following a previous study (*36*).

Proteinase Treatments. β-Lactoglobulin was treated with chymotrypsin and pepsin, conducting proteolysis either under high pressure or under atmospheric pressure on the prepressurized protein.

For the proteinase treatments conducted under high pressure, β-lactoglobulin (2.5 mg/mL) and chymotrypsin or pepsin (E/S = 1/20) were dissolved in 50 mM Tris−HCl buffer, pH 6.8 (for chymotrypsin), or in 50 mM citrate buffer, pH 2.5 (for pepsin). Samples were immediately pressurized at 100, 200, 300, and 400 MPa for 5, 10, and 20 min at 37 °C.

For the hydrolysis treatments conducted at atmospheric pressure on the prepressurized protein, β-lactoglobulin (2.5 mg/mL) dissolved in 50 mM Tris−HCl buffer, pH 6.8 (for chymotrypsin experiments), or in 50 mM citrate buffer, pH 2.5 (for pepsin experiments), was treated at 200 and 400 MPa at 25 and 37 °C for 20 min. Immediately after decompression, the enzyme (chymotrypsin or pepsin) was added to the pressurized substrate so that the E/S ratio was the same as above (1/20). Then the substrate and enzyme were incubated in a water bath at 37 °C for 5, 30, and 60 min.

Controls were obtained by conducting the hydrolyses at atmospheric pressure (0.1 MPa) on native β-lactoglobulin at 37 °C. The enzyme reaction with chymotrypsin was stopped by lowering the pH to 3 with HCl. Pepsin was inactivated by raising the pH to 6.5 with NaOH. Samples were immediately freeze-dried and reconstituted as needed for HPLC analyses. All reactions were performed at least in duplicate.

Analysis of Remnant β-Lactoglobulin. The amount of intact β-lactoglobulin left in the hydrolysates was determined by RP-HPLC with UV detection on Agilent 1100 series HPLC equipment (Agilent Technologies, Waldbronn, Germany). The absorbance was recorded at 214 nm with an Agilent 1100 series variable-wavelength detector. Chromatographic separations were performed with a 250 × 4 mm i.d. Hi-Pore reversed-phase RP-318 column (Bio-Rad Laboratories, California). The operating conditions were as follows: column at ambient temperature; flow rate, 0.8 mL/min; injection volume, 50 μL; solvent A, 0.37 mL/L TFA in Milli-Q water; solvent B, 0.27 mL/L TFA in HPLC-grade acetonitrile (Scharlau, Barcelona, Spain). The elution was performed with a linear gradient of solvent B in solvent A from 0% to 50% in 60 min. Characteristic chromatographic patterns of β-lactoglobulin hydrolysates are described in previous papers (*17*, *18*). The concentration of residual protein present in the hydrolysates was determined by interpolation of the area of the β-lactoglobulin peak in a standard curve of the protein (peak area vs concentration).

RESULTS AND DISCUSSION

Unfolding during Pressurization. The evolution of the unfolding of β-lactoglobulin was followed by $^2H/^1H$ exchange. Basically, H atoms belonging to the amide groups are observable in the spectra while they are not exposed to the solvent (^2H_2O) due to the protective effect of the structure and H-bonding. As the structure becomes unfolded or increasingly flexible, new amide groups are exposed to the solvent, allowing for $^2H/^1H$ exchange. This decreases the resonance intensity in the amide region of the spectra.

The pressure level, pH, and temperature influenced the flexibility of the protein as determined by the $^2H/^1H$ exchange (**Figure 1**). As expected, the effect of pressure on flexibility was considerable. No or little $^2H/^1H$ exchange was detected when the samples were treated at 100 MPa, but higher pressures greatly increased $^2H/^1H$ exchange. It has to be noted that the slight $^2H/^1H$ exchange found at 100 MPa, neutral pH, and 25 °C had not been noticed in earlier experiments (*25*) due to the shorter time used for pressurization (5 min). This time dependency is consistent with fast transient structural changes that allow amide groups to be exposed to the solvent only for a short time.

As shown in **Figure 1**, H^2/H^1 exchange was lower at acidic than neutral pH. Regardless of the temperature used, the structure of β-lactoglobulin at pH 2.5 required a higher pressure to acquire similar flexibility than at pH 6.8, confirming the robustness of the protein structure at acidic pH (*6*). Neutral samples showed a considerable increase in flexibility at 200 MPa and above, in agreement with the decrease of α- and β-structure that has been found by FT-IR between 100 and 200 MPa (*23*). Processing at acidic pH led to low $^2H/^1H$ exchange at pressures up to 200 MPa, but at pressures of 300 MPa and higher, increased flexibility was observed.

It is worth noting that, at acidic pH, the indole proton resonance of Trp^{19} was visually lower at 200 MPa (**Figure 1**), while this pressure did not lead to an appreciable $^2H/^1H$ exchange on other amide resonances. Fluorescence studies have shown that Trp residues become part of a more hydrophilic environment under high-pressure treatment (*24*). In native β-lactoglobulin, Trp^{19} is located at the bottom of the calyx, in a hydrophobic and strained environment (*3*), thus some structure flexibility being necessary to make it accessible to the solvent. However, our results indicate that the side chain of Trp^{19} was exposed earlier to the solvent than the backbone amide groups at 200 MPa. This can be explained by taking into account that the hydrogen exchange rate in unstructured polypeptides is faster for Trp indole than for backbone amide protons and this difference is greater at acidic than at neutral pH (*38*). When transiently exposed for a very short time, the Trp indole proton can exchange more easily than the backbone amide protons. Therefore, the early exchange of Trp^{19} seems to be the first detectable sign of initial and fast transient structural changes. It has been suggested that pressure-denatured proteins retain a compact structure with water molecules penetrating their core

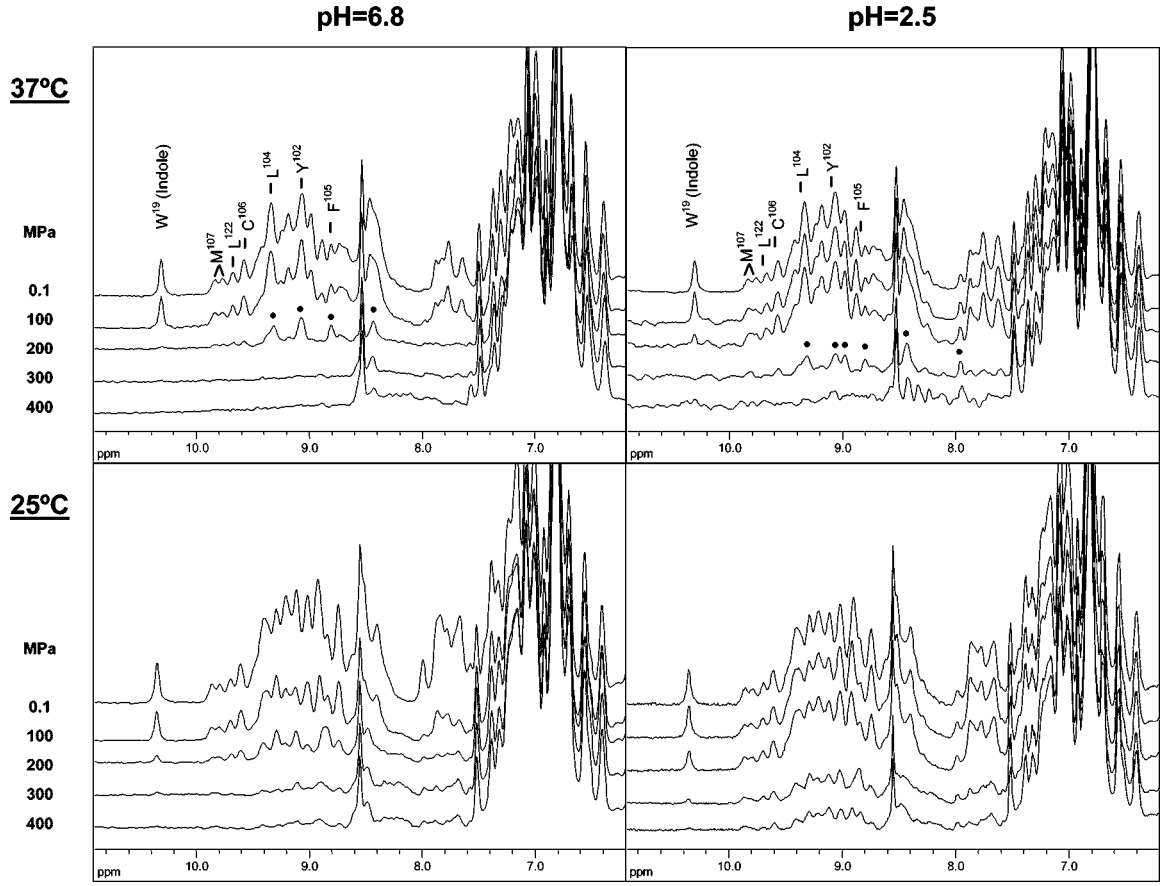

Figure 1. Unfolding of β-lactoglobulin during pressurization at different pH values and temperatures, as followed by NMR and $^2H/^1H$ exchange. Labeled resonances belong to some identified residues (letter and residue number). Residues more resistant to exchange at 37 °C are marked with dots. Spectra were taken at 35 °C on the acidified sample (pH 2.5).

(*39*) and that a swollen state of β-lactoglobulin at 150 MPa could initiate the unfolding process (*32*). The early exchange of Trp^{19} at 200 MPa and acidic pH supports the hypothesis of a swollen state of the protein as a step preceding the unfolding of the overall structure.

The temperature also influenced unfolding as, in general terms, more $^2H/^1H$ exchange was attained at 37 °C than at 25 °C, indicating an increased flexibility at the highest temperature, probably due to increased thermal motions. Circular dichroism studies have shown that combining the high-pressure process with heating results in a less rigid structure for β-lactoglobulin as the temperature is raised (*28*). In addition, the resonance intensity decreased in a different manner at the two temperatures. Whereas at 25 °C the intensity of many resonances decreased in parallel, at 37 °C certain resonances disappeared while leaving some signals particularly prominent (**Figure 1**). This indicates that some regions of the protein easily became flexible while other parts were more robust. The difference among resonances in the $^2H/^1H$ exchange rates was clearer at 37 °C than at 25 °C, probably as a consequence of the enhancement of the hydrophobic effect at the highest temperature, which may protect particular regions of the protein. As shown in **Figure 1**, the amide groups of Tyr^{102}, Leu^{104}, and Phe^{105} were found more resistant than others to H^2/H^1 exchange at 37 °C and either neutral (200 MPa) or acidic (300 MPa) pH. These three residues lie on the G strand and participate in the H-bonding between the FGH strands, a region with a considerable number of interacting hydrophobic residues and an SS bond, which has been identified as a very stable region of the protein upon heating (*36*, *37*) and high-pressure treatments (*25*). Kuwata and colleagues (*27*) studied the unfolding of β-lactoglobulin at pH 2 and 36 °C by variable-pressure NMR up to 200 MPa. These authors found local conformational fluctuations in the hydrophobic core at low pressure, the formation of two different intermediates at higher pressures involving either the hydrophobic core (strands F−H) or the opposite side (strands B−E) and a totally unfolded structure at 200 MPa. Our data were not consistent with a completely unfolded structure, since many of the amide protons were quite resistant to exchange at 200 MPa during the 20 min process, but these differences can be related to the different pressurization procedures. However, these authors also found that Tyr^{102} was more resistant to exchange than Cys^{106}, which agrees with our findings. On the other hand, at the same pressure level, the $^2H/^1H$ exchange of the indole group of Trp^{19} was greater at 37 °C than at 25 °C (**Figure 1**).

Refolding after Pressurization. In these experiments, the protein was dissolved in H_2O and subjected to high pressure, and thus, no $^2H/^1H$ exchange was allowed during pressurization. It was only after decompression that dilution with ^2H_2O led to $^2H/^1H$ exchange. The capture of two different intermediate refolded states was achieved by diluting the pressurized protein with ^2H_2O either 5 min or 6 h after pressurization. The spectrum of each refolded protein was compared with that of the native protein dissolved under the same conditions (**Figure 2**). If the spectra were superimposable, the protein was considered to recover its original structure.

The results showed that the structural recovery of β-lactoglobulin was more rapid when lower pressures were used during the treatment (**Figure 2**). At neutral pH, refolding of β-lactoglobulin was achieved faster following pressurization at 200 MPa than at 400 MPa. Upon treatment at 200 MPa and 25 °C,

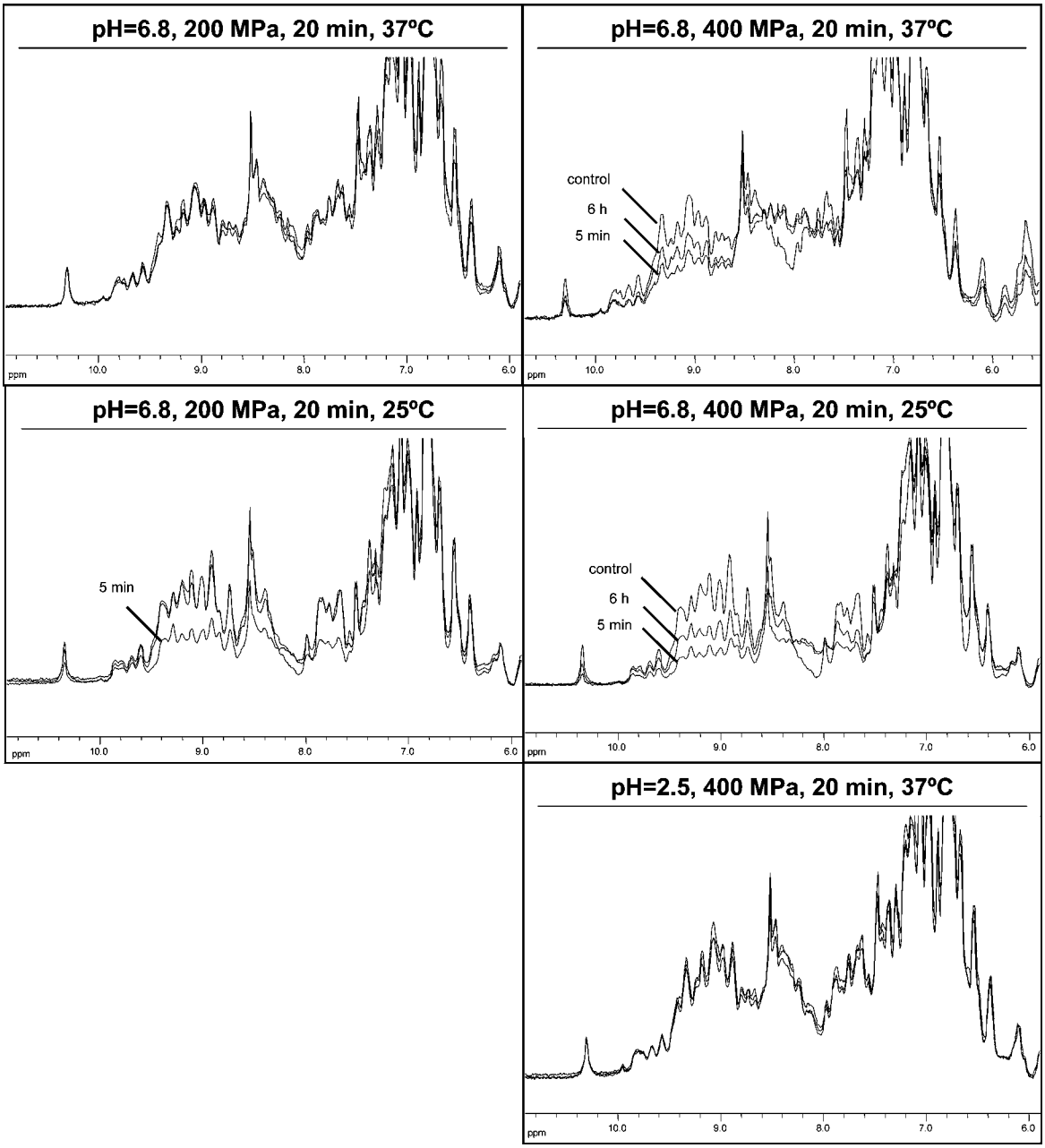

Figure 2. Structure recovery of β-lactoglobulin subjected to high-pressure treatment under different conditions of pressure, pH, and temperature and allowed to $^2H/^1H$ exchange after 5 min and 6 h of decompression. Each of the figures shows the overlapping of three spectra: β-lactoglobulin treated with high pressure and allowed to refold for 5 min and for 6 h and native β-lactoglobulin (control). Spectra not labeled showed good overlapping with the spectrum of native β-lactoglobulin. Spectra were taken at 35 °C on the acidified sample (pH 2.5).

β-lactoglobulin did not refold after 5 min but recovered its structure after 6 h. After treatment at 400 MPa, it was shown that the refolding also evolved with time, but even after 6 h, the spectra of these samples did not resemble that of the native protein, indicating that no structural recovery was detected. As the result of increased pressure treatment native monomers of β-lactoglobulin evolve toward the formation of non-native monomers, dimers, trimers, and polymers (*35*). The correct refolding can be hampered by the formation of non-native disulfide bonds that result from SH/SS exchange reaction between the free thiol group of Cys^{121} and a native SS bond (*24*, *40*). The rearrangement of $Cys^{66}-Cys^{160}$ into new non-native SS bonds in β-lactoglobulin treated with high pressures has been demonstrated (*17*). On the other hand, all acidic samples refolded faster than neutral samples, showing full structural recovery 5 min after pressurization. This is consistent with previous research showing that, upon decompression, β-lactoglobulin does not revert to the original structure at neutral pH while it does at acidic pH (*24*, *26*).

Proteins refolded faster after treatment at 37 °C than at 25 °C. Neutral solutions treated at 200 MPa and 37 °C were fully recovered within 5 min, while it took longer when they were treated at 25 °C, a likely consequence of the different unfolding process, which can lead to structural species that act as different starting points for refolding. No differences were found among acidic samples due to their rapid refolding.

Relationship between β-Lactoglobulin Structure and Proteolysis. Treatment of β-lactoglobulin with chymotrypsin at neutral pH, 37 °C, and pressures of 0.1−400 MPa (**Figure 3A**) showed that proteolysis was enhanced at pressures ≥100 MPa and, at 200−400 MPa, a hydrolysis time of 5 min was enough

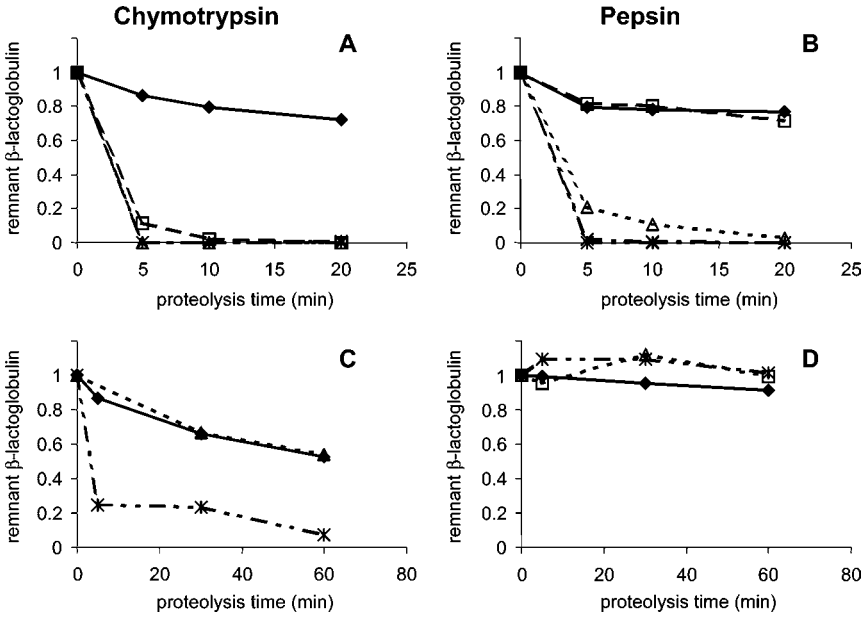

Figure 3. Effect of high pressure on the proteolysis of β-lactoglobulin, as assessed by the concentration of remnant β-lactoglobulin present in the hydrolysates, analyzed by RP-HPLC: (**A**) proteolysis under high pressure (chymotrypsin, pH 6.8, 37 °C) of native β-lactoglobulin; (**B**) proteolysis under high pressure (pepsin, pH 2.5, 37 °C) of native β-lactoglobulin; (**C**) proteolysis at atmospheric pressure (chymotrypsin, pH 6.8, 37 °C) of prepressurized β-lactoglobulin (pH 6.8, 25 °C); (**D**) proteolysis at atmospheric pressure (pepsin, pH 2.5, 37 °C) of prepressurized β-lactoglobulin (in citrate buffer, pH 2.5, 25 °C). Pressure treatments: 0.1 MPa (◆), 100 MPa (□), 200 MPa (△), 400 MPa (∗).

to remove all intact protein. Proteolysis with chymotrypsin under high-pressure conditions increased in parallel with the flexibility of the protein at neutral pH and 37 °C (**Figure 1**). For treatments at 100 MPa proteolysis was enhanced even though the structure of β-lactoglobulin did not show much flexibility. However, there are conformational changes that cannot be detected under the experimental conditions used, such as the dissociation of β-lactoglobulin induced by high pressure (*41*), which has been suggested to be a major cause for proteolysis enhancement of β-lactoglobulin under high pressure (*17*, *18*) and heat (*42*).

Proteolysis with pepsin (**Figure 3B**) followed the same trend as the structural flexibility under acidic pH and 37 °C (**Figure 1**). It required higher pressures to attain similar results than at neutral pH, thus supporting that the structural stability of β-lactoglobulin at acidic pH is responsible for its low hydrolysis by pepsin (*9*) even under high pressure (*11*, *13*). No structural flexibility nor increased proteolysis was found at 100 MPa as compared with atmospheric pressure. However, when the protein was treated at 200 MPa, the slightly increased structural flexibility was associated with a significant activation of proteolysis, similar to that found at neutral pH and 100 MPa. Since at this pH the protein is a monomer, the increased proteolysis could not be attributed to dissociation. However, the finding of Trp^{19} exposure to the solvent at 200 MPa, attributed above to a possible swollen state of the protein, could indicate easier accessibility of pepsin to buried targets and, as a consequence, improved proteolysis. At pressures of 300−400 MPa, complete proteolysis of β-lactoglobulin was quickly achieved, which is consistent with the increased flexibility found at these pressures.

Prepressurization of β-lactoglobulin enhanced proteolysis to a lesser extent, in agreement with previous work (*15*, *16*). Treatment of prepressurized β-lactoglobulin with chymotrypsin showed no differences between samples pretreated at 0.1 and 200 MPa, but proteolysis was notably enhanced in samples pretreated at 400 MPa (**Figure 3C**), but still lower than that of the protein hydrolyzed under high pressure. This agrees with previous results that have shown that hydrolysis of chymotrypsin on prepressurized β-lactoglobulin increases as the pressure used for treatment is higher (*15*, *18*). When pepsin acted on prepressurized β-lactoglobulin at acidic pH, no evidence was found for increased proteolysis (**Figure 3D**). These results can be correlated with the NMR data obtained from neutral and acidic samples treated at 200 and 400 MPa and 25 °C for 20 min and allowed to refold for 5 min (**Figure 2**). Hydrolysis with chymotrypsin was not promoted in samples treated at 200 MPa that showed a fully recovered structure after the pressure treatment. However, the misfolded protein produced by treatment at 400 MPa and neutral pH for 20 min, as revealed by NMR analysis, exhibited an increased proteolytic susceptibility to chymotrypsin. Similarly, the negligible proteolysis of β-lactoglobulin with pepsin following prepressurization at acidic pH up to 400 MPa is consistent with the full and fast (<5 min) refolding of the protein found for all acidic samples.

From our results, it can be concluded that the proteolysis enhancement of β-lactoglobulin subjected to high-pressure treatments is in good agreement with the structural flexibility of the protein, which supports the hypothesis that the accessibility of cleavage sites on the substrate is what limits the effectiveness of the enzyme action under high pressure. Proteolysis was enhanced when rapid transient structural changes occurred and was very fast when the unfolding of the protein was extensive. Proteolysis after pressurization was enhanced when the protein was not refolded to the native structure.

LITERATURE CITED

(1) Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry*, 3rd ed.; Fox, P. F., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publishers: New York, 2003; Vol. 1 (Proteins), Part B, pp 319−386.

(2) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C.; Findlay, J. B.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.
(3) Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8Å resolution - still an enigmatic lipocalin. *Structure* **1997**, *5*, 481−495.
(4) Uhrinova, S.; Uhrin, D.; Denton, H.; Smith, M.; Sawyer, L.; Barlow, P. N. Complete assignment of ^{1}H, ^{13}C and ^{15}N chemical shifts for bovine β-lactoglobulin: secondary structure and topology of the native state is retained in a partially unfolded form. *J. Biomol. NMR* **1998**, *12*, 89−107.
(5) Townend, R.; Weinberger, L.; Timasheff, S. N. Molecular interactions in β-lactoglobulin. IV. The dissociation of β-lactoglobulin below pH 3.5. *J. Am. Chem. Soc*. **1959**, *82*, 3168−3172.
(6) Kella, N. K.; Kinsella, J. E. Enhanced thermodynamic stability of β-lactogolbulin at low pH. A possible mechanism. *Biochem. J*. **1988**, *255*, 113−121.
(7) Uhrinova, S.; Smith, M. H.; Jameson, G. B.; Uhrin, D.; Sawyer, L.; Barlow, P. N. Structural changes accompanying pH-induced dissociation of the beta-lactoglobulin dimer. *Biochemistry* **2000**, *39*, 3565−3574.
(8) Ragona, L.; Fogolari, F.; Catalano, M.; Ugolini, R.; Zetta, L.; Molinari, H. EF loop conformational change triggers ligand binding in β-lactoglobulins. *J. Biol. Chem.* **2003**, *278*, 38840−38846.
(9) Reddy, I. M.; Kella, N. K.; Kinsella, J. E. Structural and conformational basis of the resistance of beta-lactoglobulin to peptic and chymotryptic digestion. *J. Agric. Food Chem.* **1988**, *36*, 737−741.
(10) Dalgalarrondo, M.; Dufour, E.; Chobert, J. M.; Bertrand-Harb, C.; Haertlé, T. Proteolysis of β-casein by pepsin in ethanolic media. *Int. Dairy J*. **1995**, *5*, 1−14.
(11) Dufour, E.; Hervé, G.; Haertlé, T. Hydrolysis of β-lactoglobulin by thermolysin and pepsin under high hydrostatic pressure. *Biopolymers* **1995**, *35*, 475−483.
(12) Van Willige, R. W. G.; Fitzgerald, R. J. Tryptic and chymotryptic hydrolysis of β-lactoglobulin A, B and AB at ambient and high pressure. *Milchwissenschaft* **1995**, *50*, 183−186.
(13) Stapelfeldt, H.; Petersen, P. H.; Kristiansen, K. R.; Qvist, K. B.; Skibsted, L. H. Effect of high hydrostatic pressure on the enzymatic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *J. Dairy Res.* **1996**, *63*, 111−118.
(14) Maynard, F.; Weingand, A.; Hau, J.; Jost, R. Effect of high pressure treatment on the typtic hydrolysis of bovine β-lactoglobulin AB. *Int. Dairy J*. **1998**, *8*, 125−133.
(15) Knudsen, J. C.; Otte, J.; Olsen, K.; Skibsted, L. H. Effect of high hydrostatic pressure on the conformation of β-lactoglobulin A as assessed by proteolytic peptide profiling. *Int. Dairy J*. **2002**, *12*, 791−803.
(16) Bonomi, F.; Fiocchi, A.; Frøkiær, H.; Gaiaschi, A.; Iametti, S.; Poiesi, C.; Rasmussen, P.; Restain, P.; Rovere, P. Reduction of immunoreactivity of bovine β-lactoglobulin upon combined physical and proteolytic treatment. *J. Dairy Res*. **2003**, *70*, 51−59.
(17) Chicón, R.; Belloque, J.; Recio, I.; López-Fandiño, R. Influence of high hydrostatic pressure on the proteolysis of β-lactoglobulin A with trypsin. *J. Dairy Res.* **2006**, *73*, 121−128.
(18) Chicón, R.; López-Fandiño, R.; Quirós, A.; Belloque, J. Changes in chymotrypsin hydrolysis of β-lactoglobulin A induced by high hydrostatic pressure. *J. Agric. Food Chem.* **2006**, *54*, 2333−2341.
(19) Hayakawa, I.; Linko, Y. Y.; Linko, P. Mechanism of high pressure denaturation of proteins. *Food Sci. Technol.* **1996**, *29*, 756−762.
(20) Tanaka, N.; Kunugi, S. Effect of pressure on the deuterium exchange reaction of alpha-lactalbumin and beta-lactoglobulin. *Int. J. Biol. Macromol.* **1996**, *18*, 33−39.
(21) Iametti, S.; Transidico, P.; Bonomi, F.; Vecchio, G.; Pittia, P.; Rovere, P.; Dall'Aglio, G. 1997 Molecular modifications of β-lactoglobulin upon exposure to high pressure. *J. Agric. Food Chem.* **1997**, *45*, 23−29.
(22) Møller, R. E.; Stapelfeldt, H.; Skibsted, L. H. Thiol reactivity in pressure-unfolded β-lactoglobulin. Antioxidative properties and thermal refolding. *J. Agric. Food Chem.* **1998**, *46*, 425−430.
(23) Panick, G.; Malessa, R.; Winter, R. Differences between the pressure- and temperature-induced denaturation and aggregation of beta-lactoglobulin A, B, and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry* **1999**, *38*, 6512−6519.
(24) Valente-Mesquita, V. L.; Botelho, M. M.; Ferreira, S. T. Pressure-induced subunit dissociation and unfolding of dimeric β-lactoglobulin. *Biophys. J.* **1998**, *75*, 471−476.
(25) Belloque, J.; López-Fandiño, R.; Smith, G. M. A ^{1}H-NMR study on the effect of high pressures on β-lactoglobulin. *J. Agric. Food Chem.* **2000**, *48*, 3906−3912.
(26) Ikeuchi, Y.; Nakagawa, K.; Endo, T.; Suzuki, A.; Hayashi, T.; Ito, T. Pressure-induced denaturation of monomer β-lactoglobulin is partially irreversible: comparison of monomer form (highly acidic pH) with dimer form (neutral pH). *J. Agric. Food Chem.* **2001**, *49*, 4052−4059.
(27) Kuwata, K.; Li, H.; Yamada, H.; Batt, C. A.; Goto, Y.; Akasada, K. High pressure NMR reveals a variety of fluctuating conformers in beta-lactoglobulin. *J. Mol. Biol.* **2001**, *305*, 1073−1083.
(28) Aouzelleg, A.; Bull, L. A.; Price, N. C.; Kelly, S. M. Molecular studies of pressure/temperature-induced structural changes in bovine β-lactoglobulin. *J. Sci. Food Agric*. **2004**, *84*, 398−404.
(29) Hinrichs, J.; Rademacher, B. High pressure thermal denaturation kinetics of whey proteins. *J. Dairy Res.* **2004**, *71*, 480−488.
(30) Ngarize, S.; Herman, H.; Adams, A.; Howell, N. Comparison of changes in secondary structure of unheated, heated and high-pressure-treated β-lactoglobulin and ovalbumin proteins using Fourier Transform Raman Spectroscopy and self-deconvolution. *J. Agric. Food Chem.* **2004**, *52*, 6470−6477.
(31) Nabhan, M. A.; Girardet, J. M.; Campagna, S.; Gaillard, J. L.; Le Roux, Y. Isolation and characterization of copolymers of β-lactoglobulin, α-lactalbumin, κ-casein and α_{s1}-casein generated by pressurization and thermal treatment of raw milk. *J. Dairy Sci.* **2004**, *87*, 3614−3622.
(32) Loupiac, C.; Bonetti, M.; Pin, S.; Calmettes, P. β-Lactoglobulin under high pressure studied by small-angle neutron scattering. *BBA−Proteins Proteomics* **2005**, *1764*, 211−216.
(33) Huppertz, T.; Fox, P. F.; Kruif, K. G. de; Kelly, A. L. High pressure-induced changes in bovine milk proteins: a review. *BBA−Proteins Proteomics* **2006**, *1764*, 593−598.
(34) López-Fandiño, R. Functional improvement of milk whey proteins induced by high hydrostatic pressure. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 351−363.
(35) Considine, T.; Patel, H. A.; Anema, S. G.; Singh, H.; Creamer, L. K. Interactions of milk proteins during heat and high hydrostatic pressure treatments. *Innovative Food Sci. Emerging Technol.* **2007**, *8*, 1−23.
(36) Belloque, J.; Smith, G. M. Thermal denaturation of β-lactoglobulin. A 1H-NMR study. *J. Agric. Food Chem*. **1998**, *46*, 1805−1813.
(37) Edwards, P. J. B.; Jameson, G. B.; Palmano, K. P.; Creamer, L. K. Heat-resistant structural features of bovine β-lactoglobulin. *Int. Dairy J*. **2002**, *12*, 331−344.
(38) Dempsey, C. E. Hydrogen exchange in peptides and proteins using NMR spectroscopy. *Prog. Nucl. Magn. Reson. Spectrosc.* **2001**, *39*, 135−170.

(39) Hummer, G.; Garde, S.; García, A. E.; Paulaitis, M. E.; Pratt, L. R. The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1552−1555.

(40) Funtenberger, S.; Dumay, E.; Cheftel, J. C. High pressure promotes β-lactoglobulin aggregation through SH/S-S interchange reactions. *J. Agric. Food Chem.* **1997**, *45*, 912−921.

(41) Hosseini-nia, T.; Ismail, A. A.; Kubow, S. Pressure-induced conformational changes of β-lactoglobulin by variable-pressure Fourier Transform Infrared Spectroscopy. *J. Agric. Food Chem.* **1999**, *47*, 4537−4542.

(42) Iametti, S.; Rasmussen, P.; Frøkiær, H.; Ferranti, P.; Addeo, F.; Bonomi, F. Proteolysis of bovine β-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur. J. Biochem.* **2002**, *269*, 1362−1372.

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