High-Pressure Unfolding and Aggregation of β-Lactoglobulin and the Baroprotective Effects of Sucrose

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The effects of processing at 450 MPa and 25 °C for 15 min on the unfolding and aggregation of an industrial β-lactoglobulin protein isolate (βLG) have been studied at pH 7.0 (at 0.1 MPa) and two protein concentrations, at 0–5% sucrose. After 2.5 or 5% protein solutions (no sucrose) were pressure processed, βLG remained soluble, but the residual enthalpy of denaturation (ΔH, as determined by DSC 20–27 h after pressurization) was decreased by 44 or 54%, respectively, indicating significant unfolding. Solubility in 2 M ammonium sulfate was similarly decreased, evidencing pressure-induced protein aggregation. Some soluble aggregates (36–10^3 kDa) were observed by gel permeation chromatography. Pressure-induced unfolding or aggregation (at 2.5% protein) was found to be partially reversible with storage time after pressurization (up to 26 or 33%, respectively, of the initial changes, after 7 days at 4 °C). The presence of 5% sucrose during pressurization at 2.5% protein reduced βLG unfolding (ΔH was decreased by 27% instead of 44%) and slightly increased the rate of recovery of protein solubility in 2 M ammonium sulfate.

Keywords: *High pressure; β-lactoglobulin; denaturation; unfolding; protein aggregation; calorimetry; polyols; sucrose; baroprotectant*

INTRODUCTION

β-Lactoglobulin is the major constituent of whey protein isolates prepared industrially from bovine skimmed milk by ion-exchange chromatography. The biochemical and physicochemical properties of β-lactoglobulin have been extensively studied, and its primary, secondary, and tertiary structures are known (MacKenzie, 1971; Green et al., 1979; Papiz et al., 1986; Monaco et al., 1987). It is a highly water-soluble protein and constitutes an adequate model to investigate the denaturing effects of high pressure on globular proteins and the possible baroprotective effects of polyols.

The effects of high pressure upon proteins have been reviewed by Heremans (1982), Weber and Drickamer (1983), and Balny et al. (1989). Pressure-induced denaturation of proteins is a complex phenomenon that depends on the protein structure, pressure, temperature, pH, ionic strength, and solvent composition (Masson, 1992). At low protein concentrations (0.05–0.2%), pressure-induced denaturation is often partly or completely reversible (Suzuki and Miyosawa, 1965; Brandts et al., 1970; Zipp and Kauzmann, 1973; Chryssomallis et al., 1981; Silva et al., 1989). In the case of oligomeric proteins, dissociation generally precedes unfolding (Silva et al., 1989). At high protein concentration (10%), intermolecular interactions and irreversible aggregation are favored (Wong and Heremans, 1988). Only a few studies are available concerning the effects of pressure on bovine β-lactoglobulin. The adiabatic compressibility of β-lactoglobulin has been determined by Gekko (1991), using sound velocity and density measurements in aqueous solution, and found to be $\approx 8.5 \times 10^{-12}$ cm^2 dyn^{-1}. This high value, as compared to that of other proteins, may be indicative of relatively high stability against temperature or pressure denaturation (Gekko, 1991). The specific proteolysis of β-lactoglobulin (in whey protein concentrate) by thermolysin is enhanced under pressure (200 MPa), probably as a result of changes in the structure of β-lactoglobulin (Hayashi et al., 1987; Okamoto et al., 1991; Dufour et al., 1992). Nakamura et al. (1993) found that treatment of whey at 200–600 MPa induced extensive aggregation of β-lactoglobulin and enhanced its proteolysis at atmospheric pressure.

Polyols, including some mono- or disaccharides, partly stabilize the native structure of proteins against heat or freeze denaturation (Itoh et al., 1976; Di Paola and Belleau, 1978; Back et al., 1979; De Wit, 1981; De Wit and Klarenbeek, 1981; Arakawa and Timasheff, 1982). Some of these authors found an increase of 3–17 °C in the denaturation temperature of globular proteins in the presence of 20–50% sucrose or sorbitol near neutral pH. With lower polyol contents (near 5% glucose, lactose, or sorbitol), De Wit and Klarenbeek (1981) found an increase of 1–2 °C in the T_m (temperature of the maximum of the endothermal peak) of β-lactoglobulin (10% protein solutions at pH 6.7). The protection mechanisms appear to be related to changes at the protein–water interface, resulting in enhanced hydrophobic interactions and in a more compact protein structure. Dreyfus et al. (1988) have shown that mitochondrial ATP synthase was inhibited at 210 MPa and 25 °C but that enzymatic activity was completely recovered after pressure release provided pressure processing was carried out in the presence of 30% glycerol.

Polyols may also stabilize proteins against pressure-induced denaturation. Numerous studies indicate that high concentrations of sucrose or glycerol protect microorganisms against pressure inactivation (Cheftel, 1992; Knorr et al., 1992).

The aim of the present study was to differentiate the effects of high hydrostatic pressure (450 MPa) on two different phenomena, namely the unfolding and aggregation of an oligomeric protein, β-lactoglobulin. Experiments were carried out at pH 7.0 (without buffer) and at two protein concentrations (2.5 and 5%), in the presence or the absence of a potential baroprotectant, sucrose.

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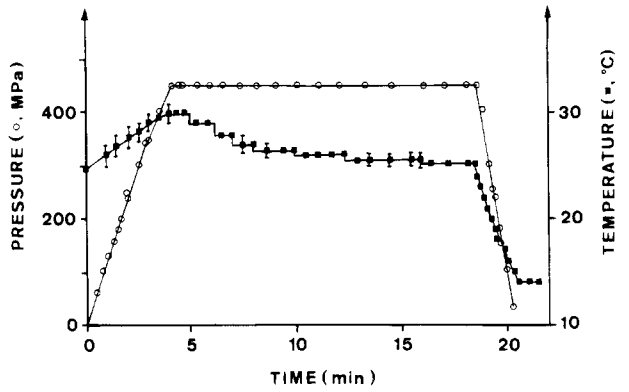

Figure 1. Pressure and temperature of the pressure-transmitting water as a function of time during processing of solutions of β-lactoglobulin isolate at 450 MPa and 25 °C for 15 min.

MATERIALS AND METHODS

β-Lactoglobulin Protein Isolate. A single batch of β-lactoglobulin protein isolate (βLG) from Besnier-Bridel (Rétiers, France) was used. This isolate was prepared from sweet or mixed whey by α-lactalbumin precipitation at acid pH, ion-exchange chromatography of the residual whey, ultrafiltration, and spray drying (Rialland and Barbier, 1988). It contained 5.8% moisture and, on a dry basis, 0.56% non-protein nitrogen (NPN), 86% protein [(total N − NPN) × 6.38], 5% ash, 0.04% calcium, <1% fat, and ca. 4% lactose. It also contained 89% native β-lactoglobulin and 2% native α-lactalbumin (αLA) per 100 g of soluble protein, as determined by gel permeation chromatography at pH 6.0. These two proteins only (without any aggregates) were detected by chromatography or by SDS–polyacrylamide gel electrophoresis, but some glycomacropeptide (from κ-casein) was present, according to the manufacturer. The nitrogen solubility of βLG was 100% at pH 7.0 and 95% at pH 6.0, 5.3, or 4.7. The nitrogen solubility in 1, 2, or 2.5 M ammonium sulfate was 100, 97, or 93%, respectively. The denaturation enthalpy measured by DSC for a 2.5% (w/w) protein solution at pH 7.0 was 14.1 J g^{-1}, a value close to that of native β-lactoglobulin (De Wit and Klarenbeek, 1981).

βLG solutions (2.5 or 5% protein, w/w) were prepared in deionized water. Sucrose (analytical grade) was added at 0.5–5% (w/w). The spontaneous pH of the solutions (6.8) was adjusted to 7.0 by addition of 0.1 N NaOH. No buffer was used, and possible pH changes under pressure were not monitored or controlled. Sucrose and protein concentrations are given in percent w/total w of solution.

High-Pressure Processing. Pressure processing was carried out in water using a 1-L unit manufactured by ACB (Nantes, France). The liquid samples were poured into poly(vinylidene chloride) tubing (35 mm in diameter, Krehalon, Eygalières, France), sealed with double knots at both ends. The pressure was raised to 450 MPa in 3 min 45 s, maintained at 450 MPa (±1 MPa) for 15 min, and then released in 1 min 20 s. The temperature of the sample before high-pressure processing and during most of the holding period was 25 ± 1 °C (Figure 1).

βLG solutions were analyzed within 2 h after pressurization (20 min in the case of ammonium sulfate precipitation) or after storage at 4 °C for various periods of time in the sealed tubing.

Differential Scanning Calorimetry (DSC). A Setaram (Caluire, France) microcalorimeter was used with C276 hastelloy hermetic cells. Samples (containing 2.5 or 5% protein, 850 mg) were heated from 25 to 99 °C, at 1 °C min^{-1}, using water as a reference. After cooling, the sample and reference were heated again under the same conditions. No peaks were observed in the second run. The enthalpy of denaturation (ΔH, J g^{-1}) and the temperature of the maximum of the endothermal peak (T_m, °C) were calculated after subtraction of the second run from the first. Results are the mean of three or more measurements.

Precipitation by Ammonium Sulfate. Tubes containing increasing amounts of ammonium sulfate in a 50 mM Tris-HCl buffer at pH 7.5 were used so that, on addition of the βLG solution, the final ammonium sulfate concentration varied between 0 and 3 M and the protein concentration was always 0.12% (w/w). After standing overnight at 20 °C, the contents were centrifuged (12000g × 20 min, Sorvall RC2B, rotor SS34) to remove protein precipitates. The absorbance at 280 nm of the supernatant in 2 M ammonium sulfate was measured using a PU 8740 Philips spectrometer and taken as a denaturation index.

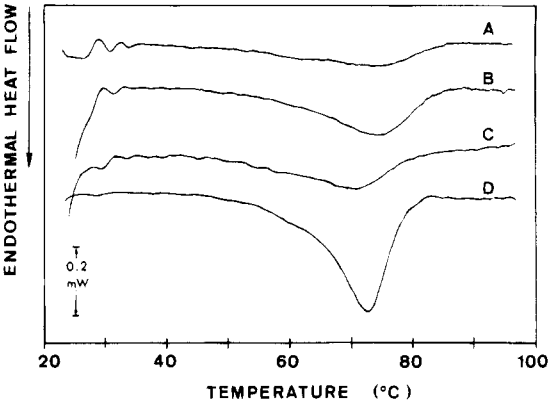

Figure 2. Pressure-induced unfolding of β-lactoglobulin isolate (βLG) as influenced by protein concentration: DSC endotherms of βLG solutions (pH 7.0) containing (w/w) 2.5% (A, B) or 5% protein (C, D); control (unpressurized) βLG solution (B, D); βLG solutions processed at 450 MPa and 25 °C for 15 min and then stored unopened at 4 °C for 20 h before DSC (A, C). Heating rate was 1 °C min^{-1}.

Hydrophobic Interaction Chromatography. The supernatants from ammonium sulfate precipitation were analyzed by hydrophobic interaction chromatography using a fast protein liquid chromatograph (Pharmacia, Uppsala, Sweden) as previously described by Dumay and Cheftel (1989). For this, they were adjusted to 1.27 M ammonium sulfate, filtered through a Durapore membrane (0.22 μm, Millipore, Bedford, MA), and injected onto a Phenyl-Superose column (HR 5/5, Pharmacia). The protein constituents were separated at room temperature by means of a linear gradient, where buffer A (50 mM Tris-HCl, 1.27 M ammonium sufate, pH 7.5) is progressively replaced by buffer B (50 mM Tris-HCl, 35% acetonitrile, pH 7.5) during 20 min at a flow rate of 0.5 mL min^{-1}. Reference proteins were from Sigma (St. Louis, MO): noncrystallized β-lactoglobulin (L-2506); β-lactoglobulin variant A (L-7880); β-lactoglobulin variant B (L-8005); α-lactalbumin (L-6010). Results are the mean of three chromatography runs.

Gel Permeation Chromatography. Gel permeation chromatography was carried out at room temperature with the same fast liquid chromatograph. Elution was done on a TSK SW3000 column (7.5 × 300 mm, Toyo Soda, Tokyo, Japan), at a flow rate of 0.5 mL min^{-1}, using a 0.06 M potassium phosphate buffer, pH 6.0, containing 0.15 M Na_2SO_4 (Laligant et al., 1991). It is known that β-lactoglobulin at pH 6.0 is present as a dimer (molecular mass = 36 kDa) (MacKenzie and Sawyer, 1967). Results are the mean of three chromatography runs. Molecular mass evaluation was based on the following standard proteins from Pharmacia or Sigma: thyroglobulin (669 kDa); apoferritin (443 kDa); aldolase (158 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); β-lactoglobulin dimer (36 kDa); α-lactalbumin (14.4 kDa); ribonuclease (13.7 kDa). The coefficient of regression of the molecular weight calibration curve was 0.994.

RESULTS AND DISCUSSION

Pressure Unfolding of β-Lactoglobulin As Determined by Differential Scanning Calorimetry. *Effects of Protein Concentration.* DSC thermograms of βLG solutions (2.5 and 5% protein, w/w), before and after pressure processing, are shown in Figure 2.

Table 1. Pressure-Induced Unfolding of β-Lactoglobulin Isolate (βLG) As Influenced by Protein and Sucrose Concentrations and by Storage Time after Pressurization: Residual Enthalpy of Denaturation (ΔH) and Temperature of Endothermal Peak (T_m) of βLG Solutions Processed at 450 MPa and 25 °C for 15 min in the Presence or Absence of Sucrose and Then Stored at 4 °C for 2 h to 7 Days before Analysis[a]

composition of solutions (%, w/w)	control (unpressurized) βLG solutions		pressure-processed βLG solutions					
			ΔH (J g^{-1})			T_m (°C)		
	ΔH (J g^{-1})	T_m (°C)	2-3-h storage[b]	20-27-h storage[b]	5-7-day storage[b]	2-3-h storage[b]	20-27-h storage[b]	5-7-day storage[b]
5% protein + 0% sucrose	11.8 (0.1)	72.9 (0.1)	5.7 (0.2)***	5.4 (0.1)***	5.6 (0.4)***	71.7 (0.5)*	71.3 (0.4)**	71.5 (0.4)**
2.5 % protein + 0% sucrose	14.1 (0.3)	74.9 (0.3)	6.9 (0.2)***	7.9 (0.4)***	8.8 (0.4)***	75.3 (0.5)	74.9 (0.1)	75.1 (0.5)
+ 0.5% sucrose	14.7 (0.4)	75.4 (0.3)	ND	10.1 (0.1)***•••	9.9 (0.4)***•	ND	75.1 (0.7)	75.6 (0.2)
+ 1% sucrose	14.7 (0.3)	75.2 (0.3)	ND	10.3 (0.9)**•	10.2 (0.8)***	ND	75.1 (0.4)	75.2 (0.2)
+ 2% sucrose	14.2 (0.2)	74.9 (0.2)	ND	9.3 (0.6)***•	9.7 (0.7)***	ND	75.3 (0.5)	75.4 (0.4)
+ 5% sucrose	14.2 (0.6)	75.8 (0.2)••	10.1 (0.6)***•••	10.3 (0.7)***••	10.5 (0.6)***•	75.7 (0.4)	75.8 (0.2)••	76.2 (0.3)•

[a] Differential scanning calorimetry at 1 °C min^{-1}. Means of three to five measurements (with standard deviation). Significant differences relative to control solution (same sucrose content), for $p = 0.05$ (*), $p = 0.01$ (**), or $p = 0.001$ (***). Significant differences relative to the corresponding solution at 2.5% protein without sucrose, for $p = 0.05$ (•), $p = 0.01$ (••), or $p = 0.001$ (•••). ND, nondetermined. [b] Samples were stored in sealed tubing at 4 °C between pressure processing and analysis.

Solutions were stored at 4 °C in the sealed tubing, for 20 h to 7 days, before analysis. The mean residual enthalpies of denaturation and temperatures of the maximum of the endothermal peak (T_m) are listed in Table 1. Since endothermal effects on DSC thermograms result mainly from the disruption of intramolecular hydrogen bonds, a reduction in residual enthalpy indicates a partial loss of βLG structure after high-pressure processing. The enthalpy reduction measured 2−3 h after pressure processing was 51−52% at both 2.5 and 5% protein, while 20−27 h after pressure processing, the enthalpy reduction was 44 (2.5% protein) or 54% (5% protein). At 5% protein, but not at 2.5%, there was also a slight decrease in the T_m after pressure processing (Table 1). The effect of pressure on protein unfolding is apparently enhanced at a higher protein concentration, but this may be due to a lesser reversibility at higher protein concentration (see next section).

At the relatively low protein concentration of 2.5%, thermal aggregation of β-lactoglobulin during DSC measurements was probably limited. Aggregation phenomena are known to be moderately exothermal and should not interfere extensively with the evaluation of (endothermal) unfolding. As a control experiment for this statement, the enthalpy of denaturation of unpressurized βLG was measured at 2.5 and 5% protein, using 21.25 mg of protein in the calorimeter cell in both cases. The enthalpies of denaturation were 14.1 (Table 1) and 12.1 J g^{-1} of protein, respectively. The T_m values were 74.9 and 72.9, respectively.

The βLG solutions used in the present study were not buffered except by the protein itself. It is thus possible that pH changes taking place under pressure contribute to the significant molecular unfolding observed here (the pH after pressure release was close to 6.9). An investigation of unfolding induced by pressure at different pH values (with or without pressure-resistant buffers such as Tris or Bis Tris propane) would therefore be of interest. Other methods of analysis, such as circular dichroism, could also reveal the proportions of the eight β strands and of the single α helix of β-lactoglobulin remaining after pressure treatment.

Effects of Storage Time after Pressure Processing. Data given in Table 1 were obtained 22 h to 7 days (at 4 °C) after pressurization, as it was found that the high-pressure unfolding of β-lactoglobulin was partly reversible and that changes occurred particularly rapidly in the first few hours after pressure treatment. It was possible to keep samples several days at 4 °C, due to the pasteurizing effect of pressure processing. At a protein concentration of 2.5%, 14% of the enthalpy reduction due to pressure processing was recovered after 20−27 h and 26% after 5−7 days. This indicates that a significant proportion of the unfolding occurring on pressure processing is reversible. The high compressibility and flexibility of β-lactoglobulin may be responsible for this phenomenon. At 5% protein however, no reversibility was observed, perhaps because of interfering aggregation phenomena

Effects of Sucrose. The effects of 0.5−5% (w/total w) sucrose addition to βLG solutions were studied at a protein concentration of 2.5%. The slight increase in the T_m (Table 1) of the control (unpressurized) βLG in the presence of sucrose at pH 7.0 becomes significant at 5% sucrose and suggests some protection against thermal denaturation [see also De Wit and Klarenbeek (1981)]. It should be noted that there is no notable change in the ΔH, with or without sucrose (Table 1).

Pressure processing in the presence of sucrose (0.5−5%) significantly decreased the residual enthalpy of denaturation of βLG (as compared to that of unpressurized samples with the same sucrose content). However, the residual enthalpy after pressure processing in the presence of sucrose was significantly larger than after processing in the absence of sucrose (Table 1; Figure 3).

The presence of sucrose during pressure processing also canceled the time dependence (after pressure release) of the residual enthalpy of denaturation (Table 1). The results clearly indicate that sucrose exerts some baroprotective effects on βLG. The mechanisms of these effects, especially at such low sucrose concentrations (e.g., 12 mol of sucrose/mol of β-lactoglobulin), are not easily explained. Sucrose may act at the protein−water interface, perhaps at a specific position on the β-lactoglobulin molecule, or may stabilize the protein through a preferential exclusion mechanism that affects the surface tension of water, as proposed by Lee and Timasheff (1981).

Pressure Aggregation of β-Lactoglobulin As Determined by Ammonium Sulfate Precipitation and by Gel Permeation Chromatography. βLG remained largely soluble after pressure processing of solutions containing 2.5 or 5% protein. Indeed, the

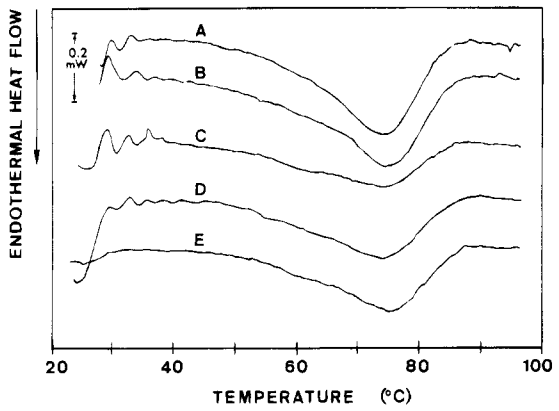

Figure 3. Pressure-induced unfolding of β-lactoglobulin isolate (βLG) as influenced by sucrose concentration: DSC endotherms of control (unpressurized) βLG solutions (pH 7.0) containing (w/w) 2.5% protein and 0 (A) or 5% (B) sucrose; DSC endotherms of βLG solutions containing (w/w) 2.5% protein and 0 (C), 0.5% (D), or 5% (E) sucrose, processed at 450 MPa and 25 °C for 15 min and then stored unopened at 4 °C for 20 h before DSC. Heating rate was 1 °C min^{-1}.

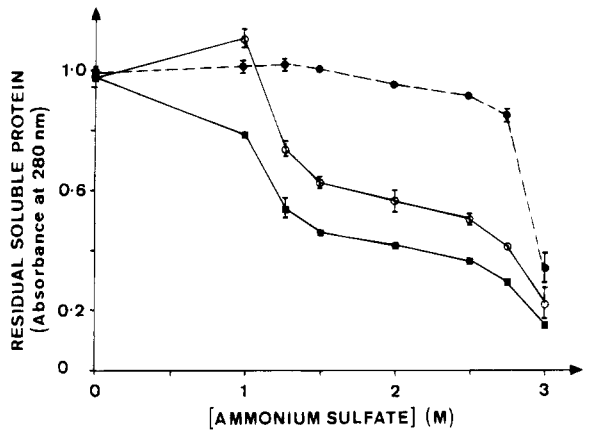

Figure 4. Pressure-induced aggregation of β-lactoglobulin isolate (βLG) as influenced by protein concentration: residual soluble protein after precipitation by ammonium sulfate of βLG solutions (pH 7.0); control (unpressurized) βLG solution (●); βLG solutions containing (w/w) 2.5% (○) or 5% (■) protein, processed at 450 MPa and 25 °C for 15 min and then stored unopened at 4 °C for 20–27 h between pressure processing and precipitation. Values are means of three independent experiments ± standard deviation. Protein content of solutions on precipitation was 0.12% (w/w).

absorbance of the diluted βLG solutions (0.12% protein in Tris-HCl buffer, pH 7.5) remained identical before and after pressure processing (Figure 4), even after storage in sealed tubing at 4 °C for up to 7 days.

Effect of Protein Concentration. The residual soluble protein (absorbance at 280 nm) remaining after precipitation of βLG solutions (2.5 or 5% protein) by increasing concentrations of ammonium sulfate is plotted in Figure 4. The control βLG remained largely soluble up to 2.75 M ammonium sulfate, precipitation starting at a concentration near 3 M, in agreement with previous results (Dumay and Cheftel, 1989). With βLG solutions processed at 450 MPa and 25 °C for 15 min, precipitation started at 1.27 M ammonium sulfate and was, as expected, greater in the case of βLG solutions pressurized at 5% than at 2.5% protein. Forty and 56% reductions in soluble protein (absorbance at 280 nm) were observed at 2 M ammonium sulfate for the 2.5 and 5% protein solutions, respectively, as analyzed 20 h after pressurization (Figure 4). Control (unpressurized) βLG only showed a 3–4% reduction in soluble protein at 2 M ammonium sulfate relative to the soluble protein in the absence of ammonium sulfate (Figure 4).

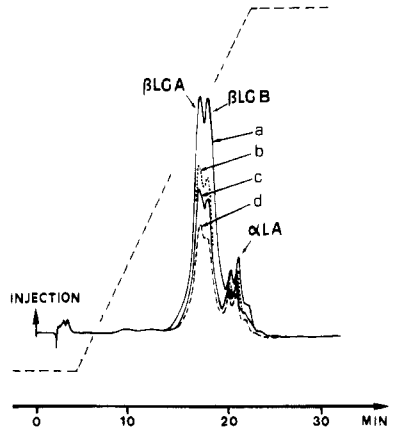

Figure 5. Hydrophobic interaction chromatography (Phenyl-Superose column) of soluble protein constituents from solutions of β-lactoglobulin isolate (βLG) (pH 7.0) processed at 450 MPa and 25 °C for 15 min and then precipitated by 2 M ammonium sulfate: control (unpressurized) βLG solution (a); pressure-processed βLG solutions (2.5% protein, w/w), with (b, d) or without (c, d) 5% sucrose (w/w), stored unopened for 20 min (d) or for 5 days at 4 °C (b, c), before precipitation with ammonium sulfate. Injection was 100 μL of a solution containing 0.1% protein (w/w). Absorbance was measured at 280 nm. The dashed line represents the elution gradient.

Hydrophobic interaction chromatography of the residual soluble protein constituents at 2 M ammonium sulfate indicates that they correspond principally to β-lactoglobulin (variants A and B), with minor components consisting mainly of α-lactalbumin (Figure 5) [see also Dumay and Cheftel (1989)]. The greater decrease in absorbance observed in the case of βLG pressure processed at 5% corresponds to an extensive precipitation of β-lactoglobulin (variants A and B) by ammonium sulfate (not shown in Figure 5). Hydrophobic interaction chromatography is used here to assess the proportion of remaining native β-lactoglobulin after the precipitation of denatured–aggregated forms by ammonium sulfate (2 M). It is assumed that a large increase in surface hydrophobicity takes place, due to the unfolding of β-lactoglobulin, and is immediately followed by the formation of soluble aggregates. The latter are then precipitated at high concentrations of ammonium sulfate.

Gel permeation chromatography of control and pressure-processed βLG solutions is shown in Figure 6. Pressure processing was carried out at 2.5 or 5% protein, and most protein constituents remained soluble, as previously indicated. Gel permeation chromatography was carried out on samples diluted to 0.1% (w/w) protein in a phosphate buffer at pH 6.0. Under these conditions, native β-lactoglobulin was eluted in the dimer form. The proportion of dimer was considerably reduced after pressure processing, especially at a higher protein concentration. Results indicate a decrease in the β-lactoglobulin dimer by 20 or 40% after pressure processing at 2.5 or 5% protein, respectively. At the same time, soluble aggregates were eluted in the 36–500- (2.5% protein) or 36–10^3-kDa range (5% protein).

Electrophoretic studies are under way to further investigate these aggregation phenomena caused by pressure treatment, especially the patterns of formation of soluble aggregates.

Effects of Storage Time after Pressure Processing. The changes in protein precipitation by ammonium sulfate, as influenced by storage time at 4 °C after pressure

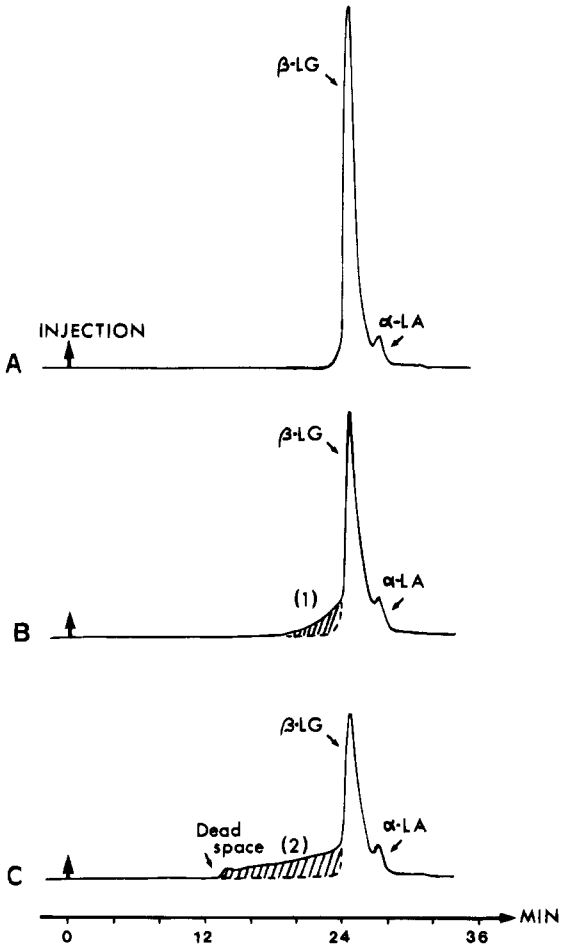

Figure 6. Pressure-induced aggregation of β-lactoglobulin isolate as influenced by protein concentration: gel permeation chromatography (TSK 3000 column) at pH 6.0 of soluble protein constituents of βLG solutions (pH 7.0); control (unpressurized) βLG solution (A); βLG solutions containing (w/w) 2.5% (B) or 5% (C) protein, processed at 450 MPa and 25 °C for 15 min and then stored unopened at 4 °C for 20–27 h before chromatography. Injection was 100 μL of a solution containing 0.1% protein (w/w). Absorbance was measured at 280 nm. Aggregate sizes were 36–400 kDa (1) and 36–1000 kDa (2).

processing of a βLG solution at 2.5% protein, are plotted in Figure 7. There is a significant increase in residual soluble protein after storage for 20–27 h, compared with that obtained less than 20 min after pressurization (a 28% recovery in the loss of absorbance at 280 nm after precipitation at 2 M ammonium sulfate, Table 2). After 27 h, changes proceed at a much slower rate, since the total recovery in the loss of soluble protein was close to 33% after storage for 5–7 days (Table 2). When βLG was pressure processed at 5% protein, a significant but smaller recovery was also observed with storage time (Table 2).

The greater recovery in residual soluble protein (observed after precipitation in ammonium sulfate), when compared to the recovery in enthalpy (Table 1), may be due to the fact that precipitation with ammonium sulfate was carried out 10–20 min and 20–27 h after pressure release, while calorimetric measurements were done 2–3 h and 20–27 h after pressure release. Much of the recovery in enthalpy may have taken place during the 2–3 h delay and escaped evaluation. This attempt to compare the extents of reversibility (after pressure release) in unfolding and in aggregation may be irrelevant since protein unfolding

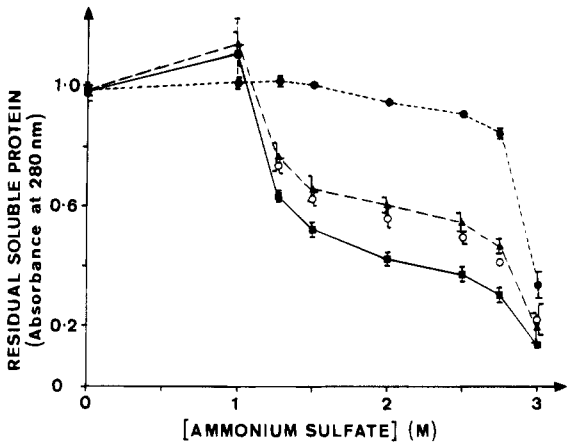

Figure 7. Pressure-induced aggregation of β-lactoglobulin isolate (βLG) as influenced by storage time after pressure processing: residual soluble protein after precipitation by ammonium sulfate of βLG solutions (2.5% protein, w/w, pH 7.0); control (unpressurized) βLG solution (●); βLG solutions processed at 450 MPa and 25 °C for 15 min and then precipitated within 20 min (■) or stored unopened at 4 °C for 20–27 h (○) or 5–7 days (▲) before precipitation. Means of three independent experiments ± standard deviation. Protein content of solutions on precipitation was 0.12% (w/w).

and protein aggregation are different phenomena. However, it is likely that partial unfolding of βLG precedes and causes the formation of βLG aggregates.

Hydrophobic interaction chromatography confirms the reversibility of βLG denaturation with storage time after pressure processing (Figure 5). Some reversibility was also observed by gel permeation chromatography (Figure 8). Immediately after pressure processing of βLG solutions at 2.5% protein, the peak corresponding to β-lactoglobulin dimers was greatly reduced in height, whereas the small peak corresponding to α-lactalbumin (14.4 kDa) was increased. This apparent increase in α-lactalbumin must be due to the elution of some β-lactoglobulin monomer (18.4 kDa) together with α-lactalbumin. A shoulder was also apparent between these two peaks. With increasing storage time after pressurization, the height of the second peak (α-lactalbumin plus β-lactoglobulin monomer) decreased and that of the first peak (β-lactoglobulin dimer) increased, while the shoulder became less apparent. Moreover, there was some increase in low molecular weight aggregates (eluted before the β-lactoglobulin dimer).

It is known that at pH 7.0 (the pH value selected here for pressure treatment of βLG) an equilibrium exists between the monomer and dimer forms of β-lactoglobulin (MacKenzie and Sawyer, 1967). This equilibrium is shifted toward the monomer at pH 7.5–8 and toward the dimer at pH 6.0.

The results of gel permeation chromatography reported above suggest that pressure processing partially dissociated the β-lactoglobulin dimer into modified monomers which were not able to re-form dimers when the pH was adjusted to 6.0 soon after pressure release.

It is also known that the native β-lactoglobulin dimer is stabilized through hydrophobic interactions between the strands I of two monomers (Green et al., 1979; Monaco et al., 1987). It is therefore likely that pressure affects the structure of β-lactoglobulin at or near strand I. The shoulder observed in Figure 8 could correspond to a modified β-lactoglobulin monomer with an increased apparent hydrodynamic volume or could perhaps result from rapid changes in the monomer/dimer equilibrium (during chromatography). βLG solutions stored at 4 °C

Table 2. Pressure-Induced Aggregation of β-Lactoglobulin Isolate (βLG) As Influenced by Sucrose Content and Storage Time after Pressure Processing: Residual Soluble Protein[a] after Precipitation by 2 M Ammonium Sulfate of βLG Solutions Processed at 450 MPa and 25 °C for 15 min in the Presence or Absence of Sucrose and Then Stored at 4 °C for 20 min to 7 Days before Precipitation

composition of solutions (%, w/w)	control (unpressurized) βLG solutions	pressure-processed βLG solutions		
		<20-min storage[b]	20-27-h storage[b]	5-7-day storage
5% protein				
+ 0% sucrose	0.97 (0.01)	0.32 (0.029)	0.43 (0.028)**	0.48 (0.036)**
2.5% protein				
+ 0% sucrose	0.97 (0.01)	0.43 (0.024)	0.58 (0.048)**	0.61 (0.036)**
+ 0.5% sucrose	0.96 (0.01)	0.42 (0.030)	0.58 (0.040)**	0.66 (0.031)***
+ 1% sucrose	0.96 (0.01)	0.41 (0.024)	0.60 (0.069)*	0.64 (0.023)***
+ 2% sucrose	0.97 (0.01)	0.41 (0.046)	0.59 (0.037)**	0.63 (0.039)**
+ 5% sucrose	0.97 (0.01)	0.41 (0.027)	0.62 (0.030)***	0.68 (0.016)•***

[a] Measured as absorbance (at 280 nm) after precipitation (in 2 M ammonium sulfate) and centrifugation and relative to absorbance in the absence of ammonium sulfate. Means of three or four independent experiments (with standard deviation). Significant differences relative to the corresponding solution (same sucrose content) analyzed within 20 min after pressurization, for $p = 0.05$ (*), $p = 0.01$ (**), or $p = 0.001$ (***). Significant differences relative to the corresponding solution at 2.5% protein and without sucrose, for $p = 0.05$ (•). [b] Samples were stored in sealed tubing at 4 °C between pressure processing and analysis.

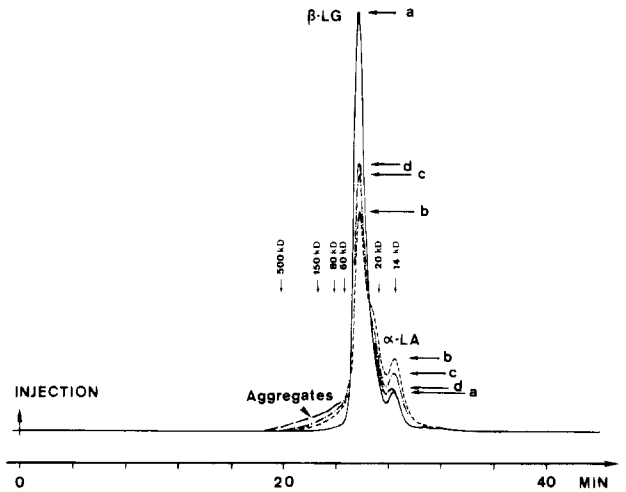

Figure 8. Pressure-induced aggregation of β-lactoglobulin isolate (βLG) as influenced by storage time after pressure processing: gel permeation chromatography (TSK 3000 column at pH 6.0) of soluble protein constituents from βLG solutions (2.5% protein, w/w, pH 7.0); control (unpressurized) βLG solution (a); βLG solution processed at 450 MPa and 25 °C for 15 min and analyzed within 40 min (b) or stored unopened at 4 °C for 20 h (c) or 5 days (d) before chromatography. Injection was 100 μL of a solution containing 0.1% protein, w/w. Absorbance was measured at 280 nm.

showed some reversibility after pressure release, as some monomers were then able to re-form dimers on adjustment to pH 6.0. Simultaneously, higher molecular weight aggregates were formed and increased with storage time (Figure 8).

Effects of Sucrose. Table 2 summarizes the data obtained by protein precipitation at 2 M ammonium sulfate (after pressure processing) as a function of the sucrose concentration present during pressure processing of βLG solutions at 2.5% protein. Less than 20 min after pressure processing, the residual soluble protein remaining after precipitation by ammonium sulfate was identical (ca. 42%) in the presence or absence of 0.5–5% sucrose (Table 2).

After 20–27 h of storage (4 °C), there appears to be no significant effect due to the presence of sucrose, except possibly a slight increase in reversibility at 5% sucrose. After 5–7 days at 4 °C, the increased recovery in residual soluble protein in the presence of sucrose became significant at 5% sucrose (Table 2).

The effect of 5% sucrose during pressure processing was also studied by gel permeation chromatography. Similar changes were observed as with βLG solutions pressure processed in the absence of sucrose (Figure 8), but in the presence of sucrose, the changes in chromatographic patterns (as a function of storage time at 4 °C after pressure treatment) were more rapid: the reversibility observed in the absence of sucrose after 20–27 h of storage at 4 °C corresponded approximately to what was observed 3–4 h after pressure processing in the presence of 5% sucrose. Furthermore, the extent of high molecular weight aggregates was reduced (36–400 kDa) after pressure processing in the presence of 5% sucrose.

These results, together with the DSC data of Table 1, do indicate that the presence of sucrose increased the rate and possibly the extent of βLG renaturation after pressure processing. It should be checked whether this effect is also observed if sucrose is added after pressure treatment.

DSC data at 2.5% protein clearly indicated less unfolding in the presence of sucrose. The effects of sucrose on pressure-induced aggregation would probably become more visible if studied at higher protein concentrations.

Conclusions. At the two protein concentrations studied (2.5 and 5%), β-lactoglobulin remained soluble after processing at 450 MPa and 25 °C for 15 min but had undergone significant unfolding and aggregation, as determined from the residual enthalpy of denaturation, solubility after addition of ammonium sulfate, and chromatographic behavior.

The exact mechanisms and extent of unfolding under pressure are not known. Pressure usually enhances hydrogen bonds and weakens hydrophobic interactions and salt bridges (Masson, 1992). This does not exclude, for example, an increase in hydrophobic interactions after pressure release. It would therefore be of interest to measure the remaining proportions of β strands and α helix of β-lactoglobulin and to determine the residual retinol binding ability of the molecule after pressure release. Some of the changes in protein structure could also be due to the effects of pressure on pH and dielectric constant.

Protein aggregation phenomena observed after pressure release are probably caused by an increased surface hydrophobicity of β-lactoglobulin due to pressure-induced unfolding. Electrophoresis under pressure would indicate whether aggregation takes place under pressure or only after pressure release.

The changes due to pressure treatment were partially reversible: 14% of ΔH and 28% of protein solubility in

2 M ammonium sulfate, lost soon after pressure treatment, were recovered after 20–27 h, and 26 and 33%, respectively, after 5–7 days. This does not necessarily imply that the original native structure was recovered.

Sucrose (5% w/w) was shown to reduce β-lactoglobulin unfolding (22% less structure lost in the presence of sucrose) and to slightly increase the rate of reversibility of aggregation (recovery of solubility at 2 M ammonium sulfate 5–7 days after pressure treatment increased from 33 to 48% of the initial loss). It is surprising that such relatively small concentrations (0.5–5%) of sucrose had a significant effect. A question that remains to be answered is whether the protective effect of sucrose occurred mainly during pressure treatment or during subsequent recovery (or both). Further experiments using other sugars and polyols (e.g., trehalose, glucose, lactose, sorbitol, or glycerol) may enable a greater understanding of the mechanism of stabilization. The influence of a wider range of protein concentration, pH, ionic strength, pressure, rate of pressurization or pressure release, and time of pressurization could also be important.

It would be especially interesting to use techniques that enable the protein to be studied under pressure, e.g., fluorescence and electrophoresis, as in the present study it has only been possible to investigate those modifications remaining minutes or hours after pressure processing.

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