High Pressure Promotes β-Lactoglobulin Aggregation through SH/S−S Interchange Reactions

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Solutions of β-lactoglobulin (β-Lg) isolate (23 g of protein/kg, pH 7.0, in 50 mM Bis-Tris buffer) were either flushed with N_2 or O_2 or brought to given concentrations of *N*-ethylmaleimide (NEM), β-mercaptoethanol (MSH), cysteine (CYS), or glutathione (GSH) and then pressurized at 450 MPa and 25 °C for 5, 15, or 30 min. Sulfhydryl groups (SH), half-cystine residues, and S−S bonds were not influenced by pressure (0−30 min), with or without prior flushing with N_2, thus revealing no significant oxidation of SH groups. Polyacrylamide gel electrophoresis (PAGE) and PAGE carried out in the presence of sodium dodecyl sulfate (SDS−PAGE) revealed a progressive decrease in β-Lg from 5 to 30 min, with a corresponding formation of oligomers and high molecular weight aggregates, whether pressure was applied in N_2, air, or O_2. SDS−PAGE with or without MSH demonstrated the progressive increase in S−S-bonded oligomers and aggregates from 5 to 30 min. High concentrations of NEM (30× the SH group content, on a molar basis) or of MSH (50×) prevented the pressure-induced formation of all aggregates, or only of S−S-bonded aggregates, respectively. High (30×) concentrations of CYS or GSH prevented the formation of S−S-bonded aggregates, probably through interchange reactions between CYS or GSH and the intramolecular S−S bonds of β-Lg. These data confirm that most pressure-induced S−S bonds resulted from SH/S−S interchange reactions rather than from oxidation of SH groups.

Keywords: *Hydrostatic pressure; β-lactoglobulin; aggregation; disulfide bonds; sulfhydryl groups; SH/S−S interchange reactions; intermolecular S−S*

INTRODUCTION

The thermal aggregation and gelation of β-lactoglobulin (β-Lg) or whey protein concentrates have been extensively investigated. The role of intermolecular disulfide bonds in these phenomena is clearly established, especially at neutral or alkaline pH (Sawyer, 1967; Watanabe and Klostermeyer, 1976; Shimada and Cheftel, 1988, 1989; Matsudomi *et al*., 1991; Monahan *et al*., 1995), and it is likely that the free and reactive -SH group of Cys 121 of β-Lg promotes SH/S−S interchange reactions with the 119-106 and possibly the 66-160 disulfide bond of other β-Lg molecules. These S−S bonds are responsible both for polymer formation and for the elasticity characteristics of thermal gels. Site-directed mutagenesis to introduce one or two supplementary -SH groups in β-Lg enhanced its ability to form high molecular weight (M_w) aggregates and gels upon heating, while the absence of Cys 121 or its conversion into an intramolecular S−S bond prevented thermal aggregation or increased β-Lg thermostability (Batt *et al*., 1994). The participation of hydrophobic interactions to the thermal aggregation and/or gelation of β-Lg is also confirmed, but less is known concerning the degree of unfolding of the β-barrel structure (based on eight antiparallel β-sheets accounting for 55% of the molecule), the extent of formation of intermolecular β-sheets (dependent on pH, ionic strength, temperature and protein concentration, Matsuura and Manning, 1994), and the sequence of these events.

The pressure-induced unfolding of β-Lg has been recently investigated. Dumay *et al*. (1994) subjected a solution of β-Lg isolate containing 25 or 50 g/kg protein to 450 MPa at 25 °C for 15 min. A 50% reduction in the enthalpy of thermal denaturation was measured by differential scanning calorimetry after pressure release, demonstrating partial unfolding of the secondary/tertiary structure by pressure processing. Enthalpy reduction was partly reversible when the 25 (but not the 50) g/kg protein solution was kept at 4 °C for 24 h after pressure release. Some protection against unfolding was apparent when pressurization was carried out in the presence of 5−50 g/kg sucrose.

Dufour *et al*. (1994) carried out measurements under pressure (intrinsic fluorescence of the tryptophan residues of β-Lg; fluorescence of added retinol in the β-Lg−retinol complex), using 2.2 g/L solutions of β-Lg in pH 7 Tris buffer or in pH 3 acetate buffer. Results indicated that β-Lg unfolds in the 150−300 MPa range. Unfolding was extensive and irreversible at pH 7, but much smaller and reversible at pH 3 (β-Lg is also more resistant to heat and to proteolysis at acid than at neutral pH). Dissociation by pressure of the β-Lg−retinol complex was reversible at acid but not neutral pH. It is likely that pressure unfolding at neutral pH enhances the reactivity of the SH group of β-Lg, and that intermolecular S−S bonds contribute to the irreversibility of unfolding.

The proteolysis of β-Lg under pressure was studied with thermolysin (pH 7), pepsin (pH 4), trypsin, or chymotrypsin (pH 8), at low concentrations (0.5−20 g/L) of pure β-Lg or higher concentrations (20−100 g/kg) of whey protein concentrate (Hayashi *et al*., 1987; Okamoto *et al*., 1991; Dufour *et al*., 1995; Van Willige and Fitzgerald, 1995). Proteolysis was markedly enhanced up to 200, 250, or 300 MPa, depending on the protease. This enhancement was attributed to pressure-induced unfolding of β-Lg, especially at pH 7 or 8. Protease inactivation occurred at higher pressures.

Previous studies from this laboratory concerned the pressure-induced aggregation of β-Lg isolate in water, at pH 7 and protein concentrations of 25 or 50 g/kg (Dumay *et al*., 1994). β-Lg remained largely soluble

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after processing at 450 MPa and 25 °C for 15 min. However, solubility in 2 M ammonium sulfate was decreased and some soluble aggregates were observed by gel permeation chromatography. The molecular mass of these aggregates (36×10^3 kDa) markedly increased with protein concentration. Pressurized solutions of the same β-Lg isolate (25 g/kg protein) at pH 7.0 in water, phosphate buffer, or pressure-resistant buffers (Tris, Bis-Tris, or Bis-Tris-propane) were analyzed by electrophoresis in the presence or absence of sodium dodecyl sulfate and β-mercaptoethanol, 24 h after processing at 150−450 MPa and 25 °C for 15 min (Funtenberger *et al*., 1995). Electrophoresis as well as the nitrogen solubility of the protein constituents at pH 4.7 indicated that β-Lg aggregation at pH 7.0 was more extensive in pressure-resistant buffers than in phosphate buffer or in water. Electrophoresis also revealed the progressive formation of dimers to hexamers and of higher polymers of β-Lg as a function of pressure level and of buffer type and molarity. All high molecular weight aggregates and most oligomers disappeared when pressurized solutions were treated with β-mercaptoethanol before electrophoresis. Thus pressure induced the formation of intermolecular S−S bonds, especially when the pH was kept close to 7.

Pressure processing of β-Lg isolate solutions at higher protein concentrations (80−160 g/kg) and pH 7.0 in water or various buffers induced β-Lg gelation at low temperature (25 °C) (Cheftel *et al*., 1995; Zasypkin *et al*., 1996). All solutions were almost fully gelled immediately after processing and pressure release, but pressure-induced gels had a sponge-like texture and a porous microstructure and underwent progressive syneresis and exudation with storage time after pressure release. In contrast, heat-induced gels prepared from the same β-Lg isolate solutions (87 °C for 40−45 min) displayed a finely stranded network and high water retention. Pressure induced lower gel rigidity and elasticity than heating (Zasypkin *et al*., 1996), suggesting weaker intermolecular or interparticular forces. Van Camp and Huyghebaert (1995a,b) also obtained weaker gels by pressurization (400 MPa, 20−30 °C, 30 min) of solutions of whey protein concentrates than by heating (80 °C, 30 min). The decreasing solubility (in various dissociating media) of the protein constituents of pressure-induced gels as a function of storage time after pressure release suggests that aggregation and gelation resulted from hydrophobic interactions and also S−S bonds and that a progressive build up of these interactions and bonds took place after pressure release (Cheftel *et al*., 1995).

The aim of the present work was to study the effects of time under pressure on β-Lg aggregation phenomena, and to investigate some of the mechanisms of pressure-induced aggregation, especially the formation of intermolecular S−S bonds. Experiments were carried out in a 50 mM bis-Tris buffer, pH 7.0, at 23 g/kg protein.

MATERIALS AND METHODS

β-Lactoglobulin Isolate. The β-lactoglobulin isolate (β-Lg isolate; batch no. 650) prepared from sweet whey by Besnier-Bridel (Rétiers, France) was the same as in a previous study (Funtenberger *et al*., 1995). It contained 58 g/kg moisture, and on a dry basis, 5.6 g/kg non-protein nitrogen (NPN), 859 ± 4 g/kg protein [(total N − NPN) × 6.38)], 788 ± 29 g/kg protein (as measured with the Folin reagent according to Bensadoun and Weinstein, 1976), 50 g/kg ash, 0.4 g/kg calcium, <10 g/kg fat, and ca. 40 g/kg lactose. The difference between the two methods of protein determination corresponds to the glycomacropeptide (from κ-casein), free of tyrosine and phenylalanine, that is not measured with the Folin reagent. The β-Lg isolate contained 89 g of native β-lactoglobulin (β-Lg) and 2 g of native α-lactalbumin (α-La) per 100 g of soluble protein [(total N − NPN) × 6.38)], as determined by gel permeation chromatography at pH 6.0. The β-Lg isolate was highly soluble (nitrogen solubility of 99.9% at pH 7.0 and 92.0% at pH 5.3) and in a highly native state (denaturation enthalpy of 14.1 J per g of protein). In the following sections, the grams of protein are determined with the Folin reagent and therefore correspond to β-Lg (∼98% w/w) and α-La (∼2%).

Reagents. All chemicals were of analytical grade. Tris-[hydroxymethyl]aminomethane (Tris) and [bis[hydroxyethyl]-imino]tris[hydroxymethyl]methane (Bis-Tris), cysteine (CYS), glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), sodium dodecyl sulfate (SDS), *N*-ethylmaleimide (NEM), and noncrystallized β-Lg (L-2506) were from Sigma (St. Louis, MO). Acrylamide, *N*,*N*′-methylenebisacrylamide (both twice crystallized), and ammonium persulfate were from Serva (Heidelberg, Germany). Dithiothreitol (DTT), β-mercaptoethanol (MSH), and Temed (tetramethylethylenediamine) were from Merck (Darmstadt, Germany).

Preparation of β-Lg Isolate Solutions before High-Pressure Processing. β-Lg isolate was solubilized in 50 mM bis-Tris-HCl buffer (pH 7.0) at a protein concentration of 23 g/kg of buffer (= 23.1 g/L), measured according to Bensadoun and Weinstein (1976). After moderate magnetic stirring for 45 min at room temperature, the pH was adjusted to 7.0 with 0.05 M NaOH. Bis-Tris-HCl buffer was selected in view of its pressure-resistant pH characteristics (Neuman *et al*., 1973; Kunugi, 1993) and of previous results (Funtenberger *et al*., 1995).

In some cases, β-Lg isolate solutions (23 g protein/kg) were incubated for 1 or 2 h at room temperature with slow bubbling of oxygen (O_2 > 99.5%, Aligal 3, L'Air Liquide, Vedène, France) or nitrogen (type U, N_2 > 99.995%, 5 mg/kg O_2, 5 mg/kg H_2O) before high-pressure processing (HPP). In other cases, NEM or MSH was added to β-Lg isolate solutions (23 g of protein/kg) at concentrations of 4.1 and 41 mM (NEM) or 13.7 and 137 mM (MSH), respectively. The highest concentrations correspond to ∼30 or 100 times the β-Lg molarity. The pH was adjusted to 7.0 with 0.05 M NaOH or HCl. The solutions were then incubated under moderate magnetic stirring for 1 h at room temperature, before HPP.

In another series of experiments, CYS or GSH was added to β-Lg isolate solutions (23 g of protein/kg) at various concentrations: 2.7 or 41 mM CYS or 2.6 or 41 mM GSH (∼2 or 30 times the β-Lg molarity). The pH was adjusted to 7.0 with 0.05 M NaOH. Solutions were then incubated for 1 h under mild stirring at room temperature for the lowest CYS or GSH concentrations or for 1 h under mild stirring at room temperature plus 1 night at 4 °C for the highest CYS and GSH concentrations.

High-Pressure Processing (HPP). The β-Lg isolate solutions were poured into polyvinylidene chloride tubing (35 mm in diameter, Krehalon, Eygalières, France) and processed in water at 450 ± 1 MPa and 25 ± 1 °C for 15 min, using a 1 L vessel (ACB, Nantes, France) as previously described (Funtenberger *et al*., 1995). For some experiments (influence of the duration of pressure application) pressure was maintained at 450 ± 1 MPa (and 25 ± 1 °C) for 5, 15, or 30 min. The rate of pressure increase or decrease was ∼120 or 270 MPa/min, respectively. After pressure release, pressure-processed samples were kept at 4 °C for 20−24 h in the sealed tubing before analysis, for practical reasons.

Determination of SH Group and Half-Cystine Contents. β-Lg isolate solutions were either left as such (presence of air) or flushed with N_2 prior to pressurization. 4 g of the pressurized solution (and nonpressurized controls) was diluted with 6 g of one of the two following pH 8.0 buffers: (1) 0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, 8 M urea, and 17.3 mM (= 5 g/L) SDS; (2) same as buffer 1 plus 10 mM DTT. The resulting protein concentration was 9.2 g/kg, corresponding to 9.85 g/L. The resulting concentrations in urea, SDS, or DTT were 4.58 M, 9.90 mM, or 5.72 mM, respectively. The diluted

solutions were centrifuged at 17400*g* for 15 min. 1 mL of supernatant fraction was diluted again with 9 mL of buffer 1, or with 5.66 mL of buffer 2, to a protein concentration of 0.98 or 1.48 g/L, respectively, and analyzed for soluble protein (both buffers), SH groups (buffer 1), and half-cystine contents (buffer 2, after removal of DTT as indicated below). Protein solubility (buffer 1) (expressed as 100 × protein content of the diluted supernatant/protein content of a solution diluted at the same level without centrifugation) was found to vary from 90 to 100%, except for the 30 min pressurization where it fell to 75−80%.

For each set of experimental conditions (pressurization time, air or N_2, nonpressurized controls), it was checked that decreasing the ratio of initial solution/first diluting buffer, with resulting concentrations lower in protein (1.25 g/kg), and higher in urea, SDS, or DTT (7.7 M, 16.6 mM, or 9.4 mM, respectively) before the centrifugation step, did not change significantly the protein, SH, or half-cystine contents. At these concentrations of protein and dissociating agents, protein solubility (buffer 1) varied from 85 to 100% for all samples.

Protein content was determined according to the Lowry method as modified by Bensadoun and Weinstein (1976). SH groups were determined with DTNB (Ellman, 1959), as indicated by Shimada and Cheftel (1988) with a reaction time of 30 min, and expressed as μmol of SH group per g of protein solubilized with buffer 1. Half-cystine contents were determined with DTNB after reduction of S−S bonds into SH groups by DTT (see Shimada and Cheftel, 1988): β-Lg isolate solutions diluted with buffer 2 were incubated for 6−8 h at 25 °C after the second dilution step. DTT was then removed on a Sephadex G-25 column (2 cm in diameter, 7.4 cm long), using buffer 1 for elution. The half-cystine content was expressed as μmol of half-cystine residues per g of protein solubilized with buffer 1. The disulfide bond content was calculated as the difference between half-cystine content and SH groups and was expressed as μmol of S−S bond/g of protein.

Each high-pressure experiment was repeated on three different days, starting from a freshly made solution of β-Lg isolate. Each resulting sample was analyzed three times for protein, SH, or half-cystine contents.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) without dissociating or denaturing agent was carried out at pH 7.0 in 50 mM sodium phosphate buffer, using a vertical slab apparatus (Pharmacia, Uppsala, Sweden). Minigels were prepared using glass plates of 80 × 80 mm. The separating gel at 105 g/L in acrylamide (acrylamide/bisacrylamide ratio, a/ba = 22.5) was prepared in the above mentioned buffer. The stacking gel (3 mm high) at 35 g/L acrylamide (a/ba = 22.5) was prepared in pH 7.0, 25 mM sodium phosphate buffer. Ammonium persulfate and Temed were added at 1.8 and 2.8 mM, respectively. Protein samples were diluted in 25 mM sodium phosphate buffer (pH 7.0) in the presence of glycerol (150 mL/L). 2 μL (3.8 mg of protein/mL) of these solutions were pipetted on the plates. Electrophoresis was carried out at a constant power of 35 W and 18 ± 1 °C for 3.5−4 h.

PAGE in the presence of SDS (SDS−PAGE) with or without MSH was performed according to Laemmli (1970) and as previously described (Funtenberger *et al.*, 1995), using a stacking gel (67 g/L in acrylamide, pH 6.8) and a separating gel with a linear gradient (80−200 g/L in acrylamide, pH 8.7; a/ba = 22.5). 10 μL samples (3.8 mg/mL protein) in pH 6.8 Tris-HCl buffer (0.1 M final) with SDS (20 g/L), glycerol (150 mL/L), and with or without MSH (210 mM) were pipetted on the plates. Electrophoresis was carried out in 25 mM Tris-glycine buffer, pH 8.3, containing 1 g/L SDS, at a constant power of 35 W and 18 ± 1 °C for 5 h.

After the gel was stained with R-250 Coomassie blue and washing (Funtenberger *et al.*, 1995), the relative intensity of stained bands was determined by scanning at 590 nm (GS-300 densitometer, Hoefer Scientific Instruments, San Francisco, CA). It was checked with pure β-Lg that the relative intensity of stained bands was proportional to the amounts of protein deposits, below the saturation. Protein markers from Sigma were used for protein identification and molecular weight determination: α-La (L-6010, 14.4 kDa); noncrystallized β-Lg (L-2506, 18.4 kDa for monomers), β-Lg variant A (L-7880), β-Lg variant B (L-8056), SDS 6H kit containing carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa). The following equations were found between the molecular weight (M_w) of protein markers and their electrophoretic mobility (d) in the separating gel: (1) $\log(M_w) = 2.104 - 0.0153d$ ($R^2 = 0.997$) in the case of SDS−PAGE and (2) $\log(M_w) = 2.070 - 0.0139d$ ($R^2 = 0.995$) in the case of SDS−PAGE plus MSH.

Table 1. Influence of the Duration of Pressurization on SH Groups, Half-Cystine Residues, and S−S Bonds of β-Lg Solutions,[a] with or without Prior N_2 Flushing

duration of pressurization (min)	SH groups (μmol)[b]	half-cystine residues (μmol)[b]	S−S bonds (μmol)[b]
	Nonpressurized β-Lg without N_2 Flushing[c]		
0	42.7 (6.3)	257 (17)	107 (11)
	Nonpressurized β-Lg Flushed with N_2[d]		
0	37.3 (0.8)	265 (25)	114 (15)
	Pressurized β-Lg without N_2 Flushing[e]		
5	39.6 (3.3)	263 (20)	112 (11)
15	40.3 (9.3)	257 (6)	109 (8)
30	37.0 (6.7)	264 (26)	114 (13)
	Pressurized β-Lg Flushed N_2[e]		
5	38.4 (2.9)	268 (15)	115 (7)
15	44.1 (6.6)	273 (20)	114 (7)
30	38.1 (7.0)	284 (7)	123 (4)

[a] Solutions of β-lactoglobulin isolate (23 g/kg protein) in 50 mM Bis-Tris-HCl buffer, pH 7.0, with or without prior flushing with nitrogen, pressurized (or not) at 450 MPa and 25 °C for 5, 15, or 30 min. Determinations carried out 24 h (4 °C) after pressure release (mean values ± standard deviation). [b] μmol per g of protein (three determinations per solution). [c] Mean values from six different solutions. [d] Mean values from two different solutions. [e] Mean values from three different solutions pressurized independently. The analysis of variance shows that the $F_{18/7}$ ratios are = 1.69, 1.32, and 1.21 for SH, half-cystine and S−S bonds, respectively, and nonsignificant for $p = 0.05$.

RESULTS AND DISCUSSION

Influence of High-Pressure on the SH Groups of β-Lactoglobulin Isolate. The sulfhydryl groups and half-cystine residues of control and pressurized solutions of β-Lg isolate (23 g of protein/kg of solution in Bis-Tris-HCl buffer, pH 7.0), determined after pressure release and storage for 20−24 h at 4 °C, are given in Table 1. In pure β-Lg from Sigma, the SH group and half-cystine contents were found to be 53.7 ($\sigma = 2.9$) and 281 ($\sigma = 10$) μmol/g of β-Lg, respectively, corresponding to 0.99 ($\sigma = 0.05$) and 5.2 ($\sigma = 0.18$) mol/mol of β-Lg. In β-Lg isolate (nonpressurized) these contents were found to be 42.7 ($\sigma = 6.3$) and 257 ($\sigma = 17$) μmol/g of protein, corresponding to 0.80 ($\sigma = 0.12$) and 4.61 ($\sigma = 0.31$) mol/mol of β-Lg, respectively (assuming 89 g of β-Lg, 2 g of α-lactalbumin, and 9 g of glycomacropeptide per 100 g of [(total N − NPN) × 6.38)] in the β-Lg isolate). The β-Lg molecule is known to contain one SH group, two S−S bonds, and therefore five half-cystines. The relatively low SH group content found for the β-Lg isolate may reflect a prior oxidation of some SH groups into S−S bonds (with the formation of covalent dimers of β-Lg). α-Lactalbumin has four S−S bonds, and the glycomacropeptide contains no cysteine nor cystine.

The contents in SH groups and in half-cystine (Table 1) did not change significantly ($p = 0.05$) as a result of processing at 450 MPa and 25 °C for 5, 15, or 30 min, whether this treatment was carried out under air (nondegassed) or under N_2 (with prior N_2 flushing of the β-Lg solution so as to eliminate O_2). The apparent content in S−S bonds, calculated as the difference between half-

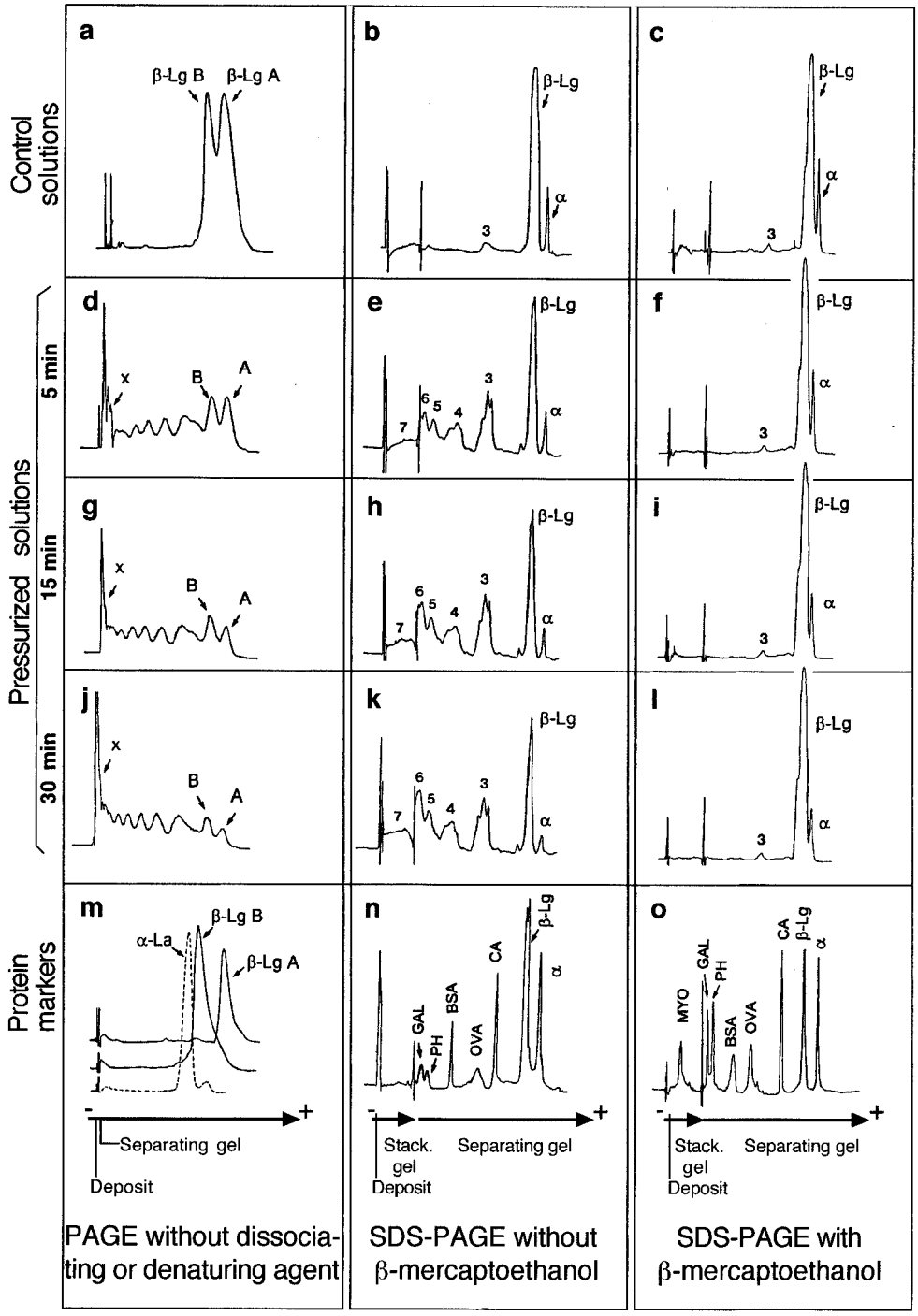

Figure 1. Influence of the duration of pressurization at 450 MPa and 25 °C on β-lactoglobulin aggregation. Polyacrylamide gel electrophoretic (PAGE) patterns of control (a−c) or pressurized β-Lg isolate solutions for 5 (d−f), 15 (g−i), or 30 (j−l) min. Protein markers (m−o). PAGE without dissociating or denaturing agent (a,d,g,j,m); SDS−PAGE in the presence of sodium dodecyl sulfate (SDS) (b,e,h,k,n); SDS−PAGE in the presence of additional β-mercaptoethanol (c,f,i,l,o). A = β-Lg variant A; B = β-Lg variant B; x = high molecular weight aggregates present at the top of PAGE gel; α = α-lactalbumin (14.4 kDa); β-Lg = β-lactoglobulin monomers (18.4 kDa); peak 3 = β-Lg di-trimers (37−47 kDa); peak 4 = β-Lg tetramers (62−78 kDa); peak 5 = β-Lg pentamers (96−100 kDa); peak 6 = β-Lg hexamers (≥120 kDa); 7 = protein aggregates present in the stacking gel; CA = carbonic anhydrase (29 kDa); OVA = ovalbumin (45 kDa); BSA = bovine serum albumin (66 kDa); PH = phosphorylase *b* (97.4 kDa); GAL = β-galactosidase (116 kDa); MYO = myosin (205 kDa). Solutions of β-Lg isolate (23 g/kg protein) in 50 mM Bis-Tris-HCl buffer at pH 7.0. Conditions of electrophoresis described in materials and methods. Analysis carried out 20−24 h (4 °C) after pressure release.

cystine and SH group contents, remained almost constant (Table 1). These results appear to indicate that there was no significant oxidation of SH groups into S−S bonds during the pressure-processing of β-Lg isolate. It is not excluded, however, that a decrease in slow reacting (with DTNB) SH groups from cysteine 121 (Shimada and Cheftel, 1989) due to oxidation could be compensated for by a lesser increase in fast reacting SH groups from other cysteines due to SH/S−S interchange reactions.

Influence of the Time of Pressurization on β-Lactoglobulin Isolate Aggregation. Electrophoresis of β-Lg isolate solutions was carried out 20−24 h (at 4 °C) after processing at 450 MPa and 25 °C for 0

(controls, Figure 1a−c), 5 (Figure 1d−f), 15 (Figure 1g−i), or 30 min (Figure 1j−l). Protein markers are shown in Figure 1m−o. Results were obtained from both PAGE at pH 7.0 without dissociating or denaturing agent, and SDS−PAGE with or without MSH. PAGE patterns without dissociating or denaturing agent may reflect indiscriminately changes in protein molecular weight and/or in surface electric charge. This allows the separation of the two β-Lg variants A and B in dimer and/or monomer forms and may also permit the detection of protein aggregates linked through noncovalent bonds (labile to SDS). It is known that there is an equilibrium between β-Lg monomers and dimers at pH 7.0 (McKenzie and Sawyer, 1967) and that variant A contains one additional negative charge (aspartyl instead of glycyl residue in position 64) as compared to variant B (Eigel *et al.*, 1984).

PAGE patterns of control (nonpressurized) β-Lg isolate solutions (Figure 1a) revealed variants A and B of B-Lg as 2 peaks, and the monomer−dimer equilibrium was probably reflected by the asymmetry in the peaks. Purified commercial β-Lg variants A and B were used to confirm their positions (Figure 1m). α-La was not visible on PAGE patterns, probably because it was present in amounts too small to be differentiated from the B variant of β-Lg. On SDS−PAGE patterns of nonpressurized β-Lg isolate, α-La was visible as one narrow peak and β-Lg was visible as one major peak containing both variants as monomers (dimers are dissociated by SDS) (Figure 1b). With SDS−PAGE in the presence of MSH (Figure 1c), nonpressurized β-Lg isolate displayed a major peak corresponding to β-Lg monomers. α-La was also visible.

After HPP, samples were slightly opalescent, in contrast to the translucid β-Lg isolate control. PAGE patterns of β-Lg isolate solutions processed at 450 MPa and 25 °C for 5, 15, or 30 min (Figure 1d,g,j) indicate that the peaks of native β-Lg decreased markedly already after 5 min of pressure processing (as determined after 24 h at 4 °C). An evaluation of the remaining β-Lg (A + B variants) on PAGE patterns after 5, 15, or 30 min of HPP calculated from areas of β-Lg peaks on three different minigels indicated 29 (±5), 14.9 (±1.7), or 10.5 (±3.3)% of the initial β-Lg (A + B variants), respectively (Figure 1a,d,g,j). These β-Lg peaks were followed by a succession of well differentiated bands corresponding probably to β-Lg aggregates (Figure 1d,g,j). Some of these aggregates could result from enhanced hydrophobic interactions subsequent to the pressure-unfolding of β-Lg. SDS−PAGE patterns reveal a progressive decrease in β-Lg monomers and an increase in SDS-resistant polymers (peaks 3−6; Figure 1b,e,h,k). The M_w of these polymers [calculated according to eq 1 found within the M_w of protein markers and their electrophoretic mobility] were 37, 40, 43.5, and 47 kDa for the four main bands in peak 3; 62, 68, and 78 kDa for the three bands in peak 4; 96−100 kDa for peak 5; and ≥120 kDa for peak 6. These M_w determinations are in agreement with our previous results (Funtenberger *et al.*, 1995) and could be attributed to di- and trimers (peak 3), tetramers (peak 4), pentamers (peak 5), and hexamers (peak 6). The peak of α-La clearly decreased, suggesting some aggregation between β-Lg and α-La via SH/S−S interchange reactions (Figure 1b,e,h,k). The β-Lg aggregation phenomenon was further illustrated by increasing amounts of aggregates in the stacking gel (Figure 1e,h,k, band 7) and at the beginning of the separating gel (Figure 1d,g,j, band x; and 1e,h,k, band 6). β-Lg aggregation clearly increased with the duration of pressurization. An evaluation of the remaining β-Lg monomers calculated from areas of β-Lg peaks on two to three SDS−PAGE patterns after 5, 15, or 30 min of HPP indicated 63 (±1), 52 (±2.6), or 40 (±2.5)% of the initial β-Lg monomers, respectively (Figure 1b,e,h,k). When samples were treated with MSH before SDS−PAGE (Figure 1c,f,i,l), almost all the high M_w aggregates and most oligomers observed by PAGE or SDS−PAGE without MSH disappeared, whereas the β-Lg and the α-La peaks totally recovered their initial size. These results are in agreement with those previously reported (Funtenberger *et al.*, 1995) and indicate that intermolecular S−S bonds were formed during pressure-induced aggregation. β-Lg aggregation increased with the duration of pressure processing, indicating that the formation of intermolecular disulfide bonds under pressure is a time-dependent process. The decrease in β-Lg on SDS−PAGE reflects the formation of S−S-bonded aggregates, while that on PAGE reflects the sum of noncovalent (hydrophobic?) and covalent aggregates (and possibly of unfolded β-Lg molecules with a modified surface charge). Comparing the values of remaining β-Lg obtained on PAGE and SDS−PAGE, it appears that the ratio of S−S-bonded aggregates (SDS−PAGE)/total denatured forms of β-Lg (PAGE) was ∼0.52, 0.56, or 0.68 after 5, 15, or 30 min of HPP, respectively. This indicates that covalent aggregates (as observed after 24 h of storage at 4 °C) represented more than half of the total aggregates induced by pressure and increased with pressurization time from 5 to 30 min. The fact that S−S linked oligomers are formed indicates that SH/S−S interchange reactions take place, since the sole oxidation of SH groups into S−S bonds could only lead to covalent dimers.

Influence of Various Gas Atmospheres on the Pressure-Induced Aggregation of β-Lactoglobulin Isolate. β-Lg isolate solutions were first incubated for 1−2 h at room temperature in the presence of O_2 (Figure 2a−f) or N_2 (Figure 2g−l), then subjected (or not) to HPP, and finally analyzed by PAGE and SDS−PAGE with or without MSH.

Nonpressurized samples flushed with O_2 or N_2 displayed identical electrophoretic patterns (Figure 2a−c,g−i), indicating that there was no spontaneous SH oxidation and that O_2 or N_2 bubbling did not induce β-Lg denaturation (by comparison to Figure 1a−c). Samples pressurized in the presence of air (nondegassed) (Figure 1g,h) or O_2 (Figure 2d,e) were identical, while the one pressurized in the presence of N_2 appeared to be only slightly less aggregated (Figure 2j,k). The evaluations of the remaining β-Lg after 15 min of HPP in the presence of air, N_2, or O_2 indicated, respectively, 14.9 (±1.7), 16.1 (±5.9), or 12.2 (±1.9)% of the corresponding β-Lg controls, as calculated from areas of β-Lg peaks (A + B variants) on three to four different PAGE patterns; and 52 (±2.8), 49 (±3.1), or 50 (±2.9)% of the corresponding β-Lg controls, as calculated from areas of β-Lg peaks on two to three different SDS−PAGE patterns. These results indicated that there was no significant difference for $p = 0.05$ within the values of remaining β-Lg. Since S−S-bonded oligomers and polymers are formed in all pressurized samples, including under N_2 (as demonstrated by SDS−PAGE patterns with and without MSH, Figure 2k,l), it is clear that most of these intermolecular S−S bonds result from SH/S−S interchange reactions rather than from SH oxidation.

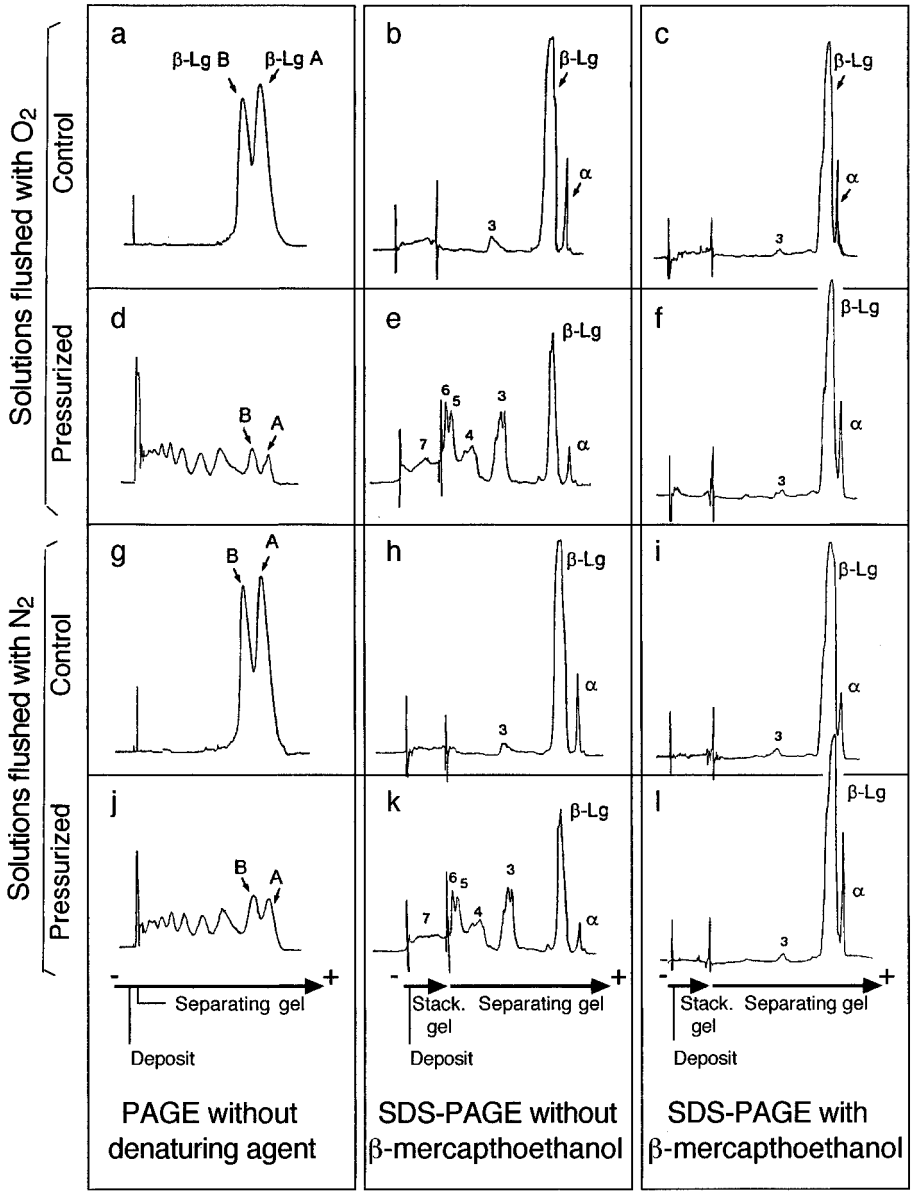

Figure 2. Influence of O_2 (a−f) or N_2 (g−l) flushing on the pressure-induced aggregation of β-lactoglobulin. Polyacrylamide gel electrophoretic (PAGE) patterns of control (a−c, g−i) or pressurized (d−f, j−l) β-Lg isolate solutions (23 g/kg protein) in 50 mM Bis-Tris-HCl buffer, pH 7.0. PAGE without dissociating or denaturing agent (a,d,g,j); SDS−PAGE in the presence of sodium dodecyl sulfate (b,e,h,k); SDS−PAGE in the presence of additional β-mercaptoethanol (c,f,i,l). β-Lg isolate solutions were flushed with O_2 (a−f) or N_2 (g−l) prior to pressurization, pressurized or not (controls) at 450 MPa and 25 °C for 15 min, then stored at 4 °C for 20−24 h in impermeable tubings. Conditions of electrophoresis, peak numbers and protein markers as for Figure 1.

This is supported by the constant content in SH groups (Table 1). It is of interest to speculate that if oxidation of SH groups into S−S bonds were the predominant reaction, the degree of polymerisation of β-Lg would be much lower, since there would be fewer SH groups to catalyze SH/S−S interchanges.

Effects of *N*-Ethylmaleimide and of Reducing Agents on the Pressure-Induced Aggregation of β-Lactogobulin Isolate. After 1 h of incubation at room temperature in the presence of *N*-ethylmaleimide (NEM) or β-mercaptoethanol (MSH), β-Lg isolate solutions were pressurized or not and then analyzed by PAGE (Figure 3a−d) or SDS−PAGE (Figure 3e).

NEM, an SH-blocking agent, was added to β-Lg isolate solutions at a concentration of 4.1 (Figure 3a) or 41 mM (Figure 3b,e), corresponding to about 3 or 30 times the SH group content, respectively. The PAGE pattern of the nonpressurized β-Lg isolate indicates that NEM had some denaturing effect on the protein, since it increased the mobility (and probably the negative charge) of some β-Lg molecules. This effect increased with the NEM concentration: the addition of 4.1 mM NEM (Figure $3a_{1,2}$) led to a third β-Lg population with a higher mobility than that of the other two β-Lg variants initially present, and the addition of 41 mM NEM (Figure $3b_{1,2}$) led to a broad peak with shoulders probably corresponding to three or four differently charged β-Lg populations. Pressurization increased the proportion of modified β-Lg molecules so that the lower NEM concentration now lead to two high-mobility β-Lg populations (Figure $3a_3$), and the highest NEM concentration to one main high-mobility β-Lg population (Figure $3b_3$). Pressurization thus appeared to enhance the NEM-induced effects. Apart from the traces of di-trimers already present before pressure processing (Figure $3e_{1,2}$), SDS−PAGE patterns revealed no further aggregation of β-Lg (Figure $3e_3$). This indicates that the blockage of SH groups by NEM prevented the

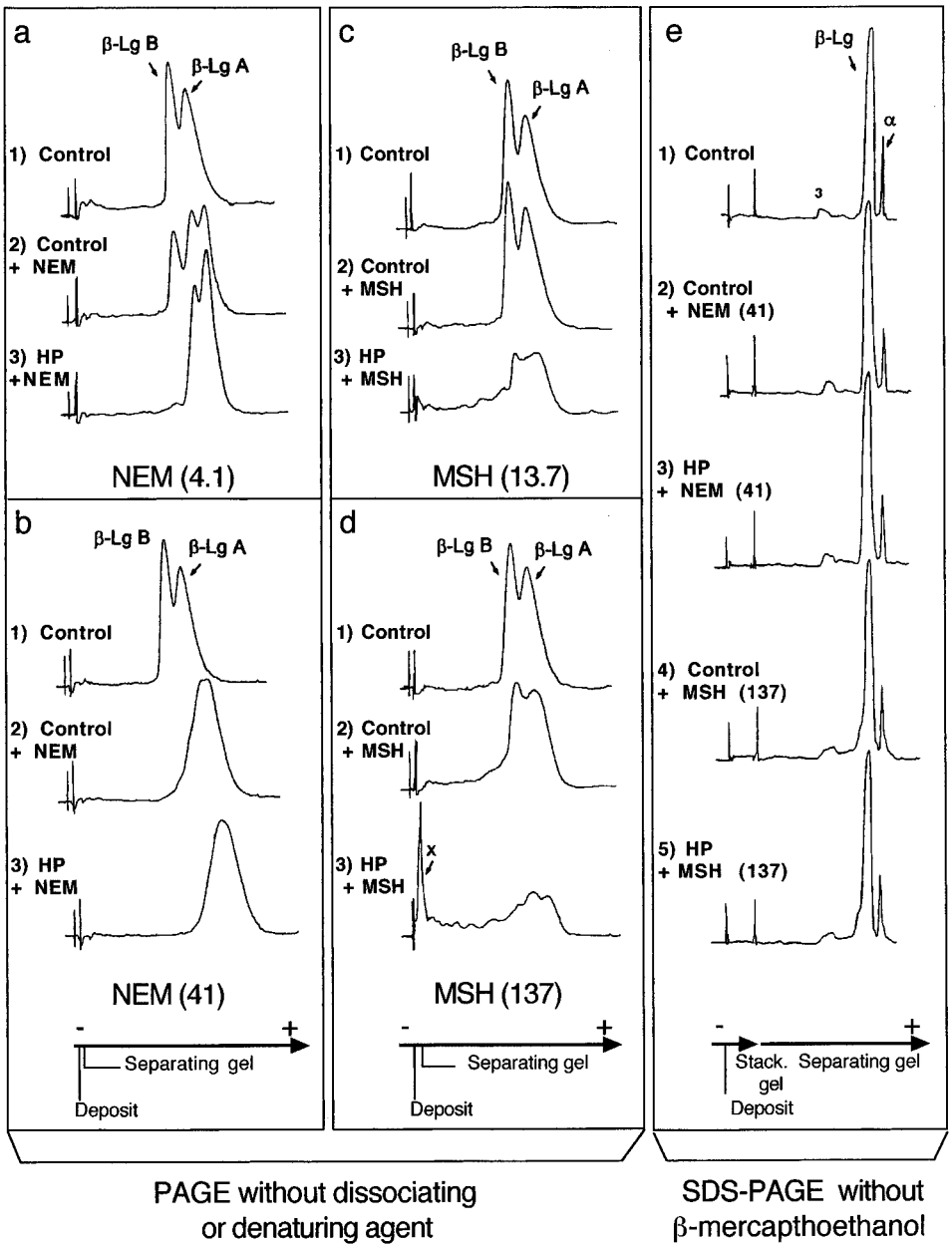

Figure 3. Influence of *N*-ethylmaleimide (NEM) and β-mercaptoethanol (MSH) on the pressure-induced aggregation of β-lactoglobulin. Polyacrylamide gel electrophoretic (PAGE) patterns without dissociating or denaturing agent (a−d); SDS−PAGE in the presence of sodium dodecyl sulfate (e). Solutions of β-Lg isolate containing 23 g/kg protein in 50 mM Bis-Tris-HCl buffer at pH 7.0. Control β-Lg solutions (a_1,b_1,c_1,d_1,e_1). Nonpressurized β-Lg isolate solutions containing 4.1 mM NEM (a_2), 41 mM NEM (b_2, e_2), 13.7 mM MSH (c_2), 137 mM MSH (d_2, e_4). Pressurized β-Lg solutions (HP) containing 4.1 mM NEM (a_3), 41 mM NEM (b_3, e_3), 13.7 mM MSH (c_3), or 137 mM MSH (d_3, e_5). Conditions of pressurization as for Figure 2. Other conditions and symbols as for Figure 1.

formation of intermolecular disulfide bonds and subsequent polymerization normally observed as a result of pressure processing of β-Lg isolate.

In other experiments, the reducing agent MSH was added to β-Lg isolate solutions at a concentration of 13.7 (Figure 3c) or 137 mM (Figure 3d,e), corresponding to about 5 or 50 times the disulfide bond concentration, respectively. After the addition of 13.7 mM MSH, the PAGE pattern of the nonpressurized sample was unchanged as compared to the non-MSH-treated sample (Figure $3c_{1,2}$). But, in the case of 137 mM MSH addition, PAGE patterns indicated an increase in the mobility of the two β-Lg variants, together with band broadening and height reduction (Figure $3d_{1,2}$). This clearly indicated a denaturing effect of MSH. Matsuura and Manning (1994) already observed that a 100-fold molar excess of MSH somewhat modified the circular dichroism spectrum of β-Lg. Samples pressurized after treatment with MSH became completely transparent, in contrast to the slightly opalescent sample of β-Lg isolate after pressurization without MSH. The PAGE pattern of the samples pressurized in the presence of MSH displayed an increase in β-Lg mobility plus a marked trail of charge-modified or/and aggregated β-Lg molecules following the peak of β-Lg. These changes were enhanced at the highest MSH concentration (Figure $3d_3$). Since these aggregates were labile to SDS (Figure $3e_5$), it is likely that they were formed through hydrophobic interactions subsequent to β-Lg unfolding induced by MSH and pressure. The SDS−PAGE pattern (Figure $3e_5$) clearly indicates that the presence of MSH inhibited the pressure-induced formation of intermolecular S−S bonds and SDS-resistant aggregates. This inhibition may be due to reduction of all S−S bonds

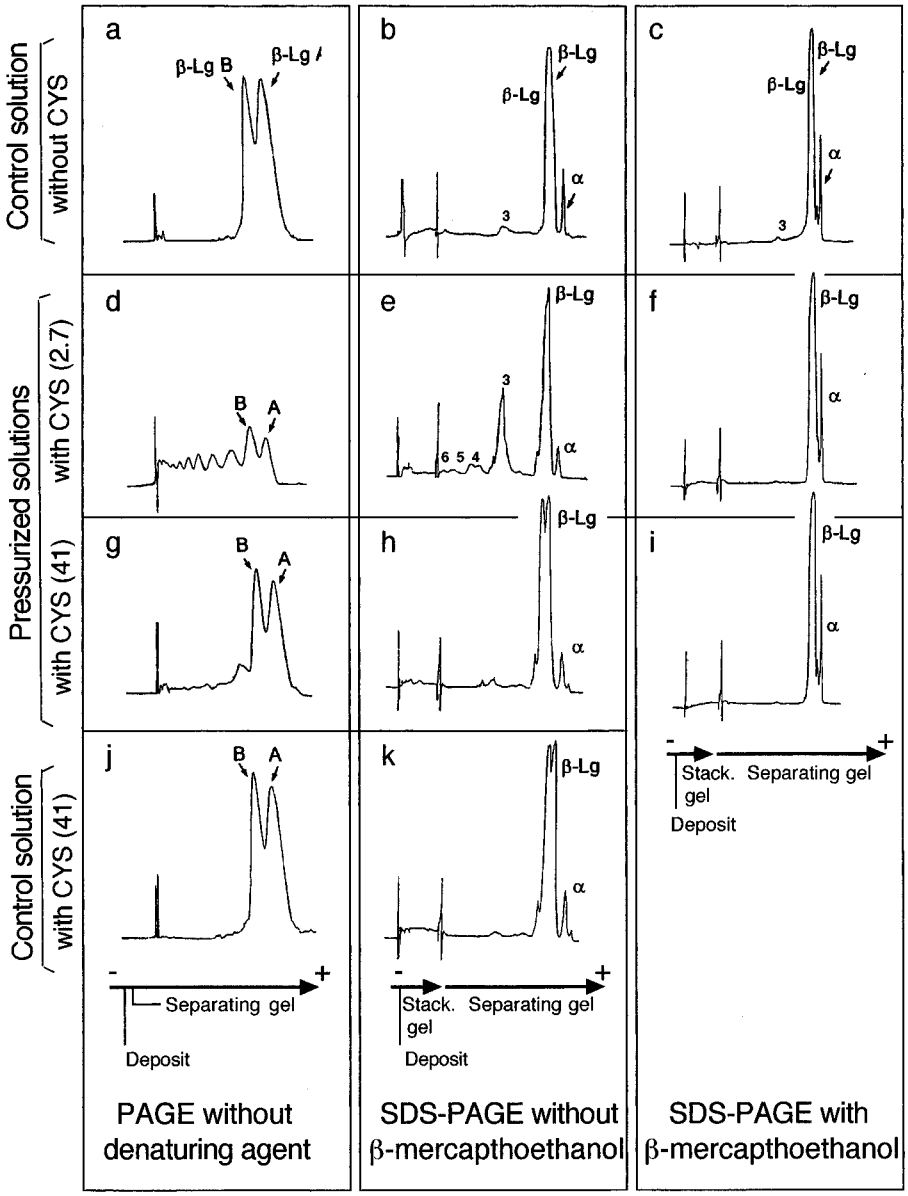

Figure 4. Influence of cysteine (CYS) on the pressure-induced aggregation of β-lactoglobulin. Polyacrylamide gel electrophoretic (PAGE) patterns of control β-Lg solutions (a−c), pressurized β-Lg solutions in the presence of 2.7 (d−f) or 41 (g−i) mM CYS, or nonpressurized β-Lg solutions in the presence of 41 mM CYS (j,k). PAGE patterns without dissociating or denaturing agent (a,d,g,j); SDS−PAGE in the presence of sodium dodecyl sulfate (b,e,h,k); SDS−PAGE in the presence of additional β-mercaptoethanol (c,f,i). Solutions of β-Lg isolate (23 g/kg protein) in 50 mM Bis-Tris-HCl buffer, pH 7. Conditions of pressurization as for Figure 2. Other conditions and symbols as for Figure 1.

and/or to SH/S−S interchange reactions between MSH and β-Lg before and/or during pressure treatment. Matsudomi *et al.* (1991) have shown that the hardness of thermal gels of β-Lg dramatically decreased when heating was done in the presence of an excess of dithiothreitol but increased at a very low concentration of this reducing agent. This increase could be due to the reduction of some intramolecular S−S of β-Lg: the resulting unfolding could enhance hydrophobic interactions, and the extra SH groups could enhance SH/S−S interchanges.

In further series of experiments, cysteine or reduced glutathione was added to β-Lg isolate solutions (Figures 4 and 5). Figure 4 shows the electrophoretic patterns of the control β-Lg isolate solution (Figure 4a−c), of the solutions pressurized in the presence of CYS at concentrations corresponding to about 2 or 30 times that of β-Lg (2.7 or 41 mM; Figure 4d−f or 4g−i) and of the nonpressurized β-Lg solution in the presence of 41 mM CYS (Figure 4j,k). Similarly, Figure 5 shows the electrophoretic patterns of the control β-Lg isolate solution (Figure 5a−c), of the solutions pressurized in the presence of 2.6 or 41 mM GSH (Figure 5d−f or 5g−i) and of the nonpressurized β-Lg solution in the presence of 41 mM GSH (Figure 5j,k). The PAGE patterns of nonpressurized β-Lg isolate with or without 41 mM CYS or GSH were similar (Figures 4a,j and 5a,j). However, the SDS−PAGE patterns differed (Figures 4b,k and 5b,k): the CYS-treated β-Lg isolate (Figure 4k) revealed two well-separated peaks. It is not excluded that cysteine interfered with SDS−protein binding. The SDS−PAGE pattern of nonpressurized β-Lg isolate containing 41 mM GSH displayed a single but large and asymmetrical β-Lg peak (Figure 5k).

PAGE patterns of β-Lg isolate samples pressurized in the presence of the lowest concentration of CYS or of GSH were similar (Figure 4d or 5d). These patterns indicated a marked decrease in the peaks of β-Lg variants, similar to that observed in the sample pressurized without CYS or GSH (Figure 1g). Thus a low

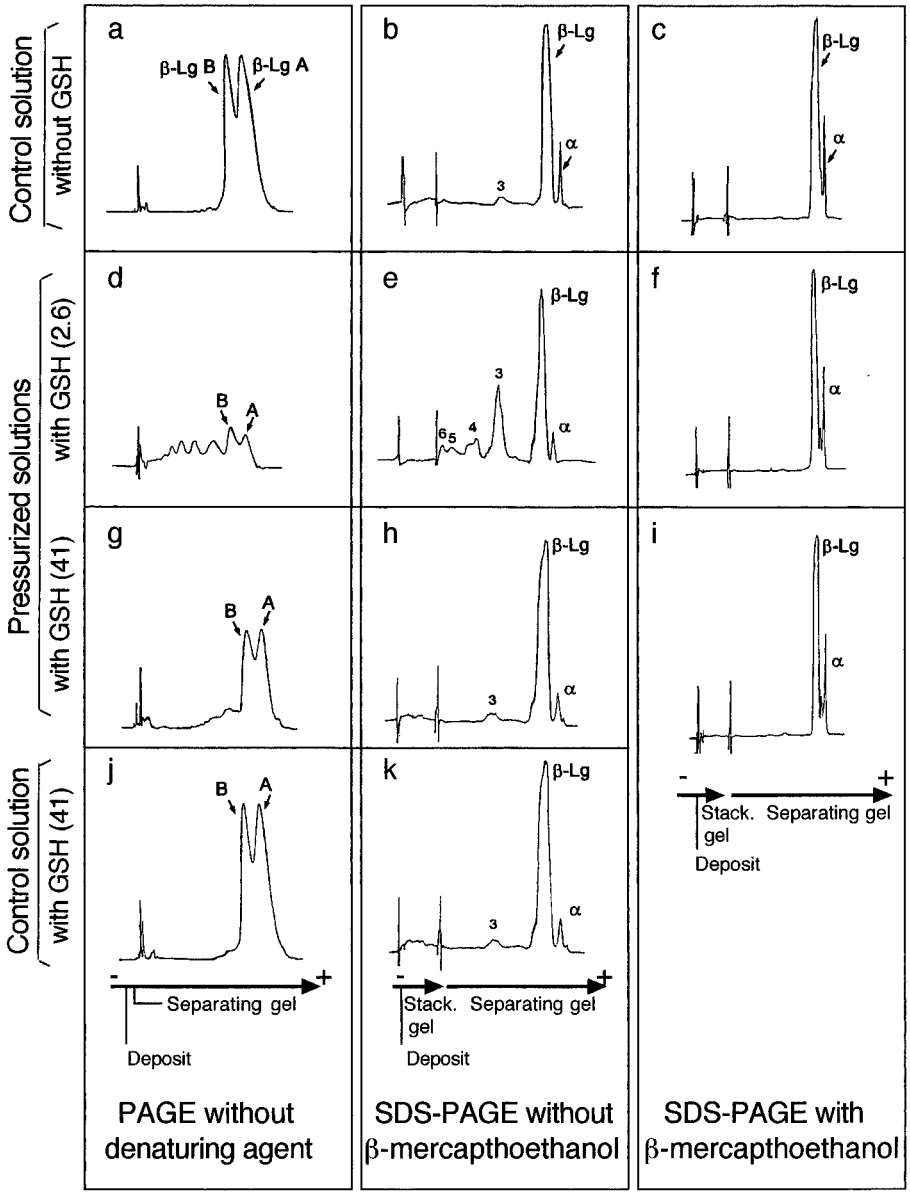

Figure 5. Influence of glutathione (GSH) on the pressure-induced aggregation of β-lactoglobulin. Polyacrylamide gel electrophoretic (PAGE) patterns of control β-Lg solutions (a−c), pressurized β-Lg solutions in the presence of 2.6 (d−f) or 41 (g−i) mM GSH, or nonpressurized β-Lg solutions in the presence of 41 mM GSH (j,k). PAGE patterns without dissociating or denaturing agent (a,d,g,j); SDS−PAGE in the presence of sodium dodecyl sulfate (b,e,h,k); SDS−PAGE in the presence of additional β-mercaptoethanol (c,f,i). Solutions of β-Lg isolate (23 g/kg protein) in 50 mM Bis-Tris-HCl buffer, pH 7. Conditions of pressurization as for Figure 2. Other conditions and symbols as for Figure 1.

concentration of CYS or GSH did not prevent the formation of β-Lg aggregates through pressure processing (except for the absence of band x, Figures 1g, 4d, and 5d). SDS−PAGE patterns, however, revealed fewer high M_w aggregates in β-Lg isolate pressurized with (peaks 4−7, Figure 4e or 5e) than without (Figure 1h) CYS or GSH, indicating that a low concentration of CYS or GSH partly prevented the pressure-induced aggregation of β-Lg through S−S bonds. CYS appeared to be slightly more effective than GSH. At 41 mM CYS or GSH, PAGE patterns of pressurized β-Lg isolate (Figure 4g or 5g) displayed higher peaks of β-Lg variants, and smaller trails of aggregates, than at 2.6−2.7 mM (Figure 4d or 5d). These aggregates appear to be fully dissociated by SDS, since they are not visible on SDS−PAGE patterns (Figure 4h or 5h). Cysteine and reduced glutathione both have an SH group. The presence of an excess of either reagent (about 30 times the β-Lg molar concentration, and therefore also 30 times the SH group concentration of β-Lg), clearly prevented the pressure-induced formation of β-Lg aggregates through intermolecular S−S bonds. It is likely that the effect of CYS or GSH rests on SH/S−S interchange reactions between the SH group of these reagents and the S−S bonds of β-Lg. Binding of CYS or GSH on β-Lg thus prevents S−S binding between β-Lg molecules. This hypothesis is consistent with the SDS−PAGE pattern observed for β-Lg isolate pressurized in the presence of CYS or GSH.

CONCLUSION

The results presented in this paper are in agreement with those of a previous study (Funtenberger *et al.*, 1995) and confirm that the pressure-induced aggregation of β-Lg into oligomers and high M_w polymers observed at a pH close to 7.0 largely depends on the

formation of intermolecular disulfide bonds through SH/S−S interchange reactions (via the nucleophilic attack of a disulfide bond by the ionized S^- form of an SH group). Such phenomena were also observed for the heat-induced aggregation of β-Lg at neutral or alkaline pH (Shimada and Cheftel, 1989). It would be of interest to repeat some of the reported experiments with pure β-Lg, so as to ascertain that the presence of small amounts of α-La, glycomacropeptide, and lactose in the industrial β-Lg isolate used does not change the mechanism of β-Lg aggregation by pressure processing.

It is likely that the formation of hydrophobic and S−S-bonded aggregates observed here after pressure release is enhanced by the prior unfolding of β-Lg under pressure (Dufour *et al.*, 1994; Dumay *et al.*, 1994). It is also likely that the pressure-induced gelation of β-Lg observed at higher protein concentrations (Cheftel *et al.*, 1995; Van Camp and Huyghebaert, 1995a,b; Zasypkin *et al.*, 1996) largely depends on the similar formation of hydrophobic interactions and disulfide bonds.

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