Baric Oligomerization in α-Lactalbumin/β-Lactoglobulin Mixtures

Marianne Jegouic,[†] Valerij Ya. Grinberg,[‡] André Guingant,[§] and Thomas Haertlé*,[†]

Institut National de la Recherche Agronomique, B.P. 1627, Rue de la Géraudiére, 44316 Nantes, Cedex 03, France, Institute of Biochemical Physics, Russian Academy of Sciences, Vavilov Str. 28, 117813 Moscow GSP-1, Russia, and Faculté des Sciences, Laboratoire de Synthesé Organique, Rue de la Houssiniére 2, 44072 Nantes, Cedex 03, France

The influence of high pressure on α-lactalbumin(ALA)/β-lactoglobulin (BLG) mixtures of various compositions was studied at pH 8.5 (50 mM Tris) by gel-permeation chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without 2-mercaptoethanol. High-molecular-weight disulfide-linked protein oligomers formed at a pressure of 10 kbar (1000 MPa) if the weight fraction of BLG (W_{BLG}^0) in the protein mixture exceeded 0.2. The maximum yield of these oligomers, of the order 80−85%, was observed at $W_{BLG}^0 \geq 0.4$. Conversion of both proteins in the oligomers was roughly the same. The estimates of the oligomerization yield obtained by gel-permeation chromatography and SDS-PAGE agreed well. SDS-PAGE results indicated that the formation of intermolecular disulfide bonds is necessary for the oligomerization. Thus, the oligomerization of pressure-denatured ALA and BLG is driven by thiol ↔ disulfide exchange in which the free thiol group of BLG acts as an initiator.

Keywords: *High pressure; α-lactalbumin; β-lactoglobulin; protein aggregation; gel-permeation chromatography; SDS-PAGE; intermolecular disulfide bonds; thiol ↔ disulfide exchange*

INTRODUCTION

It was shown recently (Jegouic et al., 1996) that α-lactalbumin (ALA) forms high-molecular-weight disulfide oligomers at high pressure (10 kbar, 1000 MPa) in the presence of low-molecular-weight thiol reducers such as cysteine, mercaptoethanol, and dithiothreitol. These compounds act as initiators of the thiol↔disulfide exchange reaction between unfolded molecules of this protein. It is essential to clarify whether high-molecular-weight entities, such as proteins containing free thiol groups [e.g., β-lactoglobulin (BLG)], are able to perform the same function.

Several aspects of the protein denaturation by high pressure may be of interest for food technology in connection with the use of food proteins as functional ingredients of many foods. These aspects were developed in the series of studies by Dumay et al. (1994), Friedman (1994), Hines et al. (1993), and Monahan et al. (1993). Previous studies by Huggins et al. (1951), Dickinson and Matsumura (1991), Monahan et al. (1993), and Tanaka et al. (1996) suggest that proteins containing free thiol groups may be able to perform the same functions.

Studying the gelation of proteins denatured by concentrated solutions of urea, Huggins et al. (1951) observed that the gelation threshold of γ-globulin with a very low free thiol content (0.07 mol/mol) was diminished by 50% by the addition of small amounts of a protein possessing more free thiols, such as bovine serum albumin (BSA). This study showed that a protein with free thiol groups could trigger thiol ↔ disulfide exchange between molecules of another disulfide-bond-rich protein provided both proteins were unfolded.

Similar results have been obtained in studies of the interactions between unfolded molecules of ALA and BLG at the oil/water interface (Dickinson and Matsumura, 1991; Monahan et al., 1993) in neutral pH. The results showed that the free thiol group of BLG induces the formation of disulfide oligomers of ALA at the interface. ALA has four disulfide bonds and no free thiol group (Kronman, 1989). It should be noted, however, that in the absence of free thiols, the intermolecular recombination of disulfide bonds at the interface proceeds slowly.

Additional evidence for strong interactions between unfolded molecules of ALA and BLG was obtained by Hines and Foegeding (1993) by analysis of the kinetics of thermal aggregation of these proteins at pH 7.0. They observed that the aggregation rate of ALA at 80 °C increased an order of magnitude in the presence of BLG.

In this work, we describe the results of a study of the effect of application of 10 bar pressure at pH 8.5 on oligomerization of ALA and BLG in solutions at different ratios. Observed oligomerization of both proteins proceeds mainly by the mechanism of thiol ↔ disulfide exchange of intensively unfolded proteins, which is made possible by the presence of BLG.

MATERIALS AND METHODS

The procedure of Maillart and Ribadeau Dumas (1988) was used to isolate variant B of BLG and a crude ALA fraction from homozygous cow's milk. ALA was obtained from the crude fraction after chromatography on DEAE Sephacel [50 mM Tris (pH 8.0), 5 mM $CaCl_2$] and eluted with the linear NaCl gradient (0.0−0.1 M). The homogeneity of the protein preparations was checked with high-performance gel-permeation chromatography (GPC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). As a rule, the purity of the protein components used in the preparations was >97%. All other chemicals (buffer salts, SDS) used were of analytical grade.

For all high-pressure experiments, ALA/BLG mixed solutions with different weight fractions of BLG ($W_{BLG}^0 \approx 0.0-1.0$) were prepared in 50 mM Tris (pH 8.5) at a constant total

* Author to whom correspondence should be addressed [telephone (33) 40 67 50 91; fax (33) 40 67 50 84].

[†] Institut National de la Recherche Agronomique.

[‡] Institute of Biochemical Physics.

[§] Faculté des Sciences.

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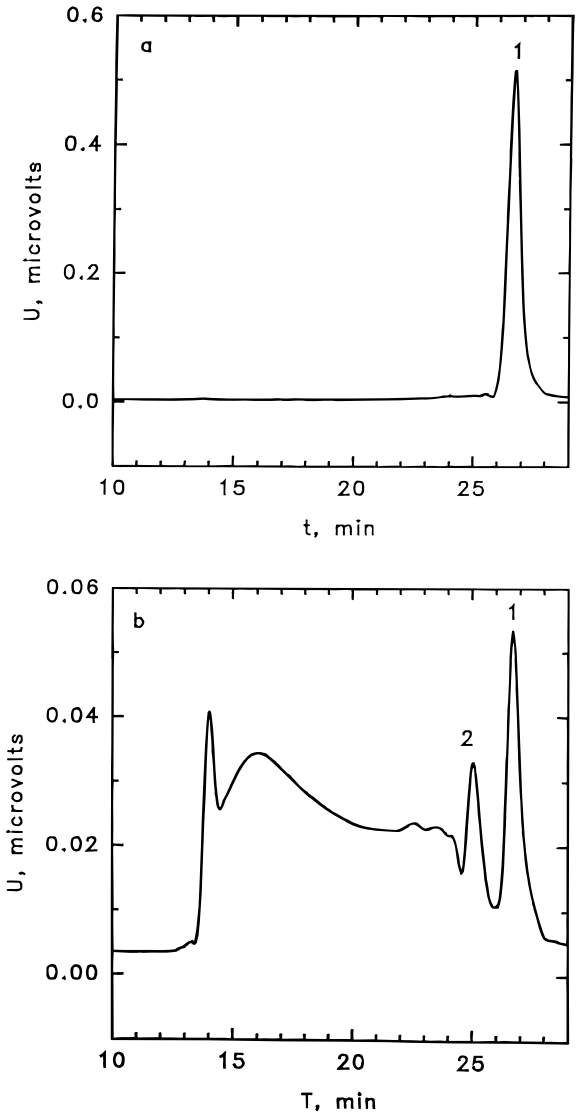

Figure 1. Gel-permeation chromatography of BLG (a) before and (b) after high-pressure treatment: (1) BLG monomer; (2) BLG dimer.

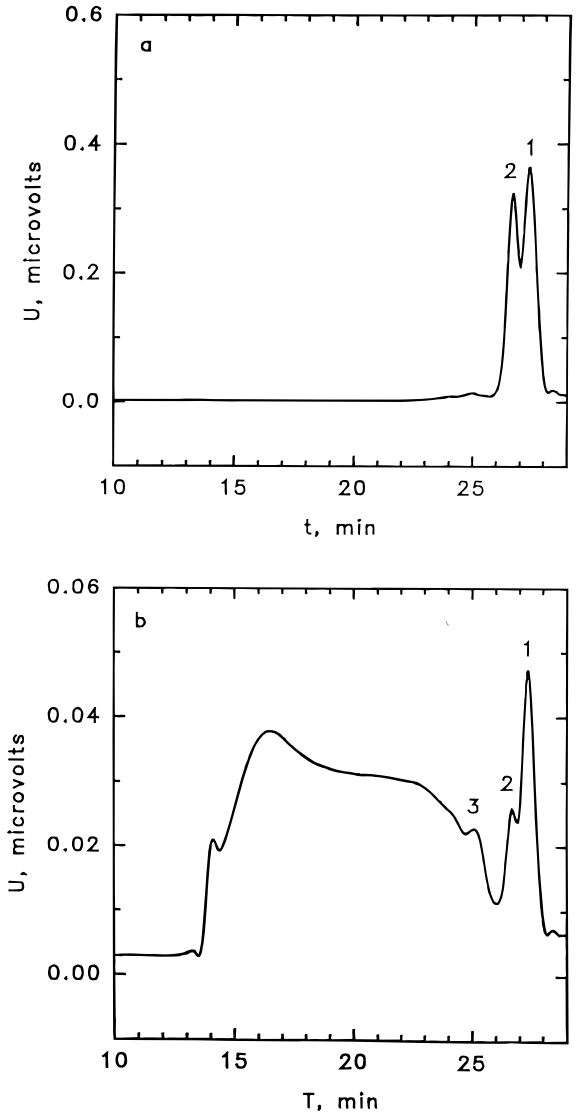

Figure 2. Gel-permeation chromatography of the ALA/BLG mixture at $W_{BLG}^0 = 0.6$ (a) before and (b) after the high-pressure treatment: (1) ALA monomer; (2) BLG monomer; (3) BLG or ALA/BLG dimers.

protein concentration of 50 mg/mL. The mixtures were prepared from stock solutions of the proteins. Protein concentrations in the stock solutions were estimated spectrophotometrically using the extinction coefficients $E^{1\%}_{280} = 20.9$ for ALA (Weltlaufer, 1961) and $E^{1\%}_{280} = 9.77$ for BLG.

The high-pressure experiments were carried out at 10 kbar (1000 MPa) for 30 min as described elsewhere (Jegouic et al., 1996). The changes of the populations of high-molecular-weight oligomers in protein mixtures before and after the high-pressure treatment were monitored by ultrafiltration, high-performance GPC, and SDS-PAGE in the presence and the absence of 2-mercaptoethanol (ME) as described by Jegouic et al., (1996). A TSK-G4000SWXL column (Tosohaas) with a protein exclusion limit of ~7 MDa was used for the GPC analysis. The column was calibrated with eight protein standards covering the molecular weight range from 12.4 to 669 kDa. In addition, α-lactalbumin was used as an external standard in the each experiment.

Processed protein was diluted with 50 mM Tris buffer (pH 7.5) to the concentration of ~2 mg/mL, filtered through a Chromafil Type P-45/25 ultrafilter (Macherey-Nagel) with 0.45-μm pore size, and loaded on the column. Total peak area, relative contents, and retention times of main components were calculated for each chromatogram. In some cases, the compressed samples contained particles retained by the 0.45-μm filter. These particles were considered to be formed by protein oligomers with an apparent molecular size or diameter (R) exceeding 450 nm.

RESULTS AND DISCUSSION

Baric oligomerization of ALA alone is not observed in the absence of low-molecular-weight reducing thiols, even at high pressures applied for extended periods of time (Jegouic et al., 1996). This resistance to oligomerization is due to the fact that ALA has no free thiol groups capable of inducing thiol ↔ disulfide exchange after unfolding of the protein by high pressure (Rao et al., 1989).

In contrast to ALA, baric oligomerization of BLG occurs readily without additional initiators. This oligomerization is clearly demonstrated by GPC data presented in Figure 1. BLG denatured by high pressure gives a large population of monomer, dimers, and high-molecular-weight oligomers. Pressure-induced oligomerization of BLG is due to the presence of a free thiol in this protein. The thiol group is capable of inducing the oligomerization of BLG by the mechanism of thiol ↔ disulfide exchange. As seen previously by Tanaka et al. (1996), BLG can form lower size oligomers (dimers, tetramers) after its unfolding by high pressure. Thanks to the presence of this reducing group (the cysteine), BLG can also initiate the oligomerization of ALA as

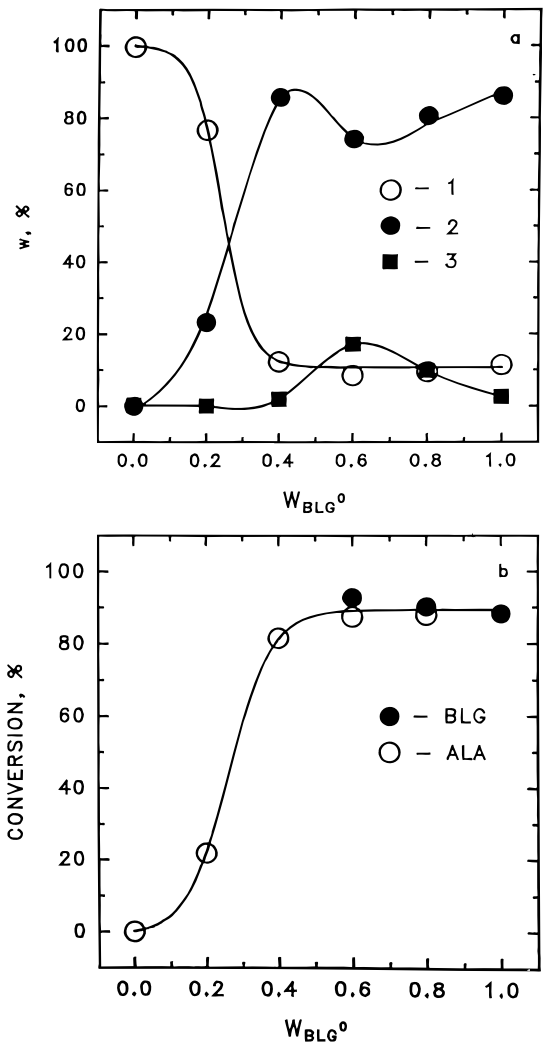

Figure 3. (a) Products of the baric oligomerization of ALA/BLG mixtures according to the analysis of obtained GPC data: (1) monomers; (2) oligomers with (diameter) R < 450 nm; (3) oligomers with R > 450 nm; (W_{BLG}^0) weight fraction of BLG in the mixture. (b) Conversion of the proteins during baric oligomerization of ALA/BLG mixtures as a function of weight fraction of BLG in the solution.

demonstrated with GPC data for the ALA/BLG mixture at $W_{BLG}^0 \geq 0.6$ (Figure 2). In this case, mixing and denaturation of BLG with ALA results in formation of a large heterogeneous population of oligomers in which BLG or ALA/BLG dimers can be still detected. The upper limit of molecular weights of these oligomers can be estimated (according to maximal resolution capacity of the column and previously obtained calibration curve with applied standards) as ~7 MDa. These oligomers are presumably composed of several hundreds of BLG and ALA molecules. It should be highlighted that according to previous work of Jegouic et al. (1996) use of low-molecular-weight reducers during high-pressure unfolding of ALA (in the otherwise identical ionic and baric conditions) results in different molecular size aggregate distrubution, yielding smaller aggregates with the predominant mass of 500 kDa. Comparison of these results indicates that the use of BLG as a reducer contributes to increase of size, very likely producing heterooligomer or heteropolymers.

The comparable data for ALA/BLG mixtures at different ratios of the proteins were obtained by SDS-PAGE and GPC. These results in combination with data on protein losses deduced from the spectroscopic

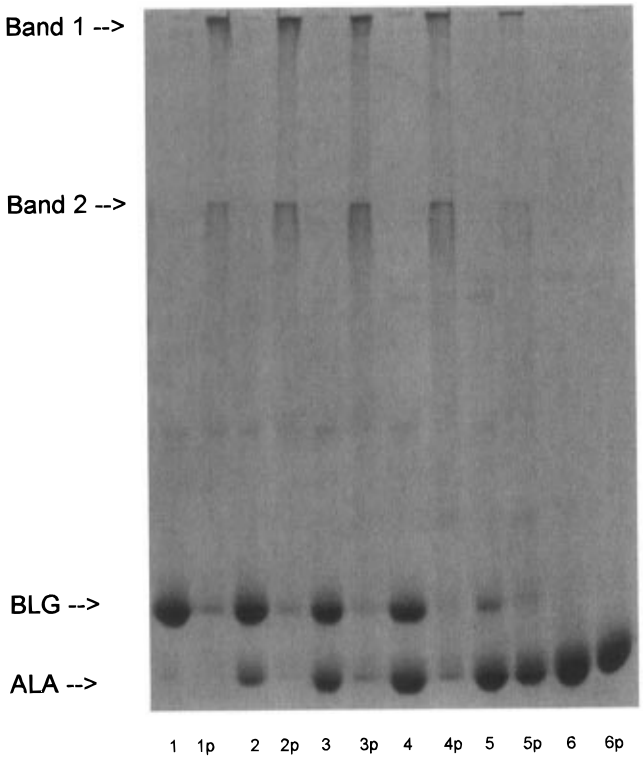

Figure 4. SDS-PAGE (without mercaptoethanol) of ALA/BLG mixtures at W_{BLG}^0 = 1.0 (1,1p), 0.8 (2,2p), 0.6 (3,3p), 0.4 (4, 4p), 0.2 (5,5p), and 0.0 (6,6p) before (1−6) and after (1p-6p) high-pressure treatment. Bands 1 and 2 correspond to high-molecular-weight oligomers unable to penetrate the spacer gel boundaries. Bands ALA and BLG represent their monomeric forms.

measurements of protein content in the controls and presurized samples, observed during the ultrafiltration of the mixture after their high-pressure denaturation, give the information on the size distribution of oligomers as a function of an initial composition of the mixture (Figure 3a). As seen in this Figure, ~90% of monomers were converted to oligomers at $W_{BLG}^0 \geq 0.4$. The maximal yield of the biggest oligomers with diameter R > 450 nm (oligomers retained on the filters) is observed, however, at $W_{BLG}^0 \geq 0.6$. Independently of the mixture composition, the conversion of ALA and BLG to oligomers is practically the same (~ 90%) when $W_{BLG}^0 \geq 0.6$ (Figure 3b). Unfortunately, at $W_{BLG}^0 < 0.6$, the data on the BLG conversion could not be obtained because the monomeric BLG was insufficiently resolved on the GPC column used.

To show that the thiol ↔ disulfide exchange is involved in the baric oligomerization of ALA/BLG mixtures, products of the oligomerization were analyzed by SDS-PAGE in the presence and absence of ME. In the presence of ME, only the monomers of both proteins are present regardless of the composition of the initial pressurized mixture. In contrast, SDS-PAGE without the reducing agent (ME) shows the presence of high-molecular-weight oligomers formed by disulfide bonding (Figure 4). The oligomers are separated into two bands. The first band is located at the beginning of the stacking gel (band 1). The second (band 2) stops on a boundary between the stacking and separating gel. Rough quantitative data on the oligomerization products could be obtained by comparing the intensities of the two bands by scanning the SDS gels (Figure 5). The composition of the studied mixtures before the high-pressure treatment was applied is illustrated in Figure 5a. As

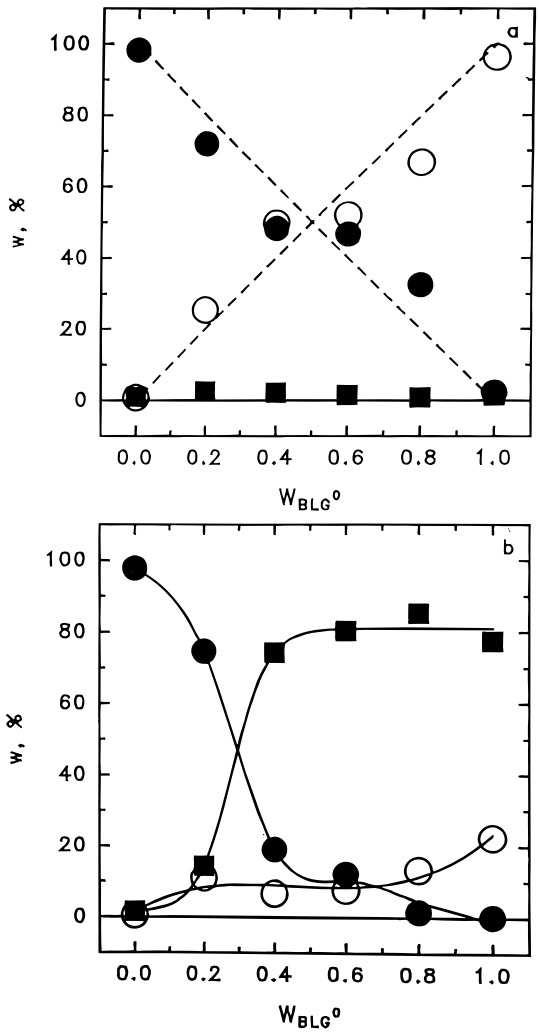

Figure 5. Compositions of ALA/BLG mixtures (a) before and (b) after high-pressure treatment calculated from SDS-PAGE data (without mercaptoethanol): (○) BLG monomer; (●) ALA monomer; (■) oligomers; (W_{BLG}^0) weight fraction of BLG in the mixture.

expected, no oligomerization is detected at any W_{BLG}^0. After the high-pressure treatment, the content of the high-molecular-weight forms increases sharply with increasing W_{BLG}^0 and approaches a constant level (~80%) at $W_{BLG}^0 \geq 0.4$ (Figure 5b). According to the SDS-PAGE data, the conversion of the both proteins in the same composition range of the mixture is more or less the same reading of 80%. Thus, the results of SDS-PAGE agree well with those obtained by GPC and indicate that disulfide bonds are of major importance for the stabilization of oligomers produced by high pressure in the ALA/BLG mixtures.

CONCLUSIONS

The free thiol group of BLG is able to induce the baric oligomerization of ALA by triggering thiol ↔ disulfide exchange reactions. The results obtained in this study indicate that the baric oligomerization of ALA/BLG 5% mixture, at ratios that are found in whey, can be achieved and the yields of oligomers exceed 80%. Both proteins, mixed at whey concentrations og 0.7% and ratios, display roughly the same reactivity at this composition of the mixture. It is assumed that besides the homo-oligomers of ALA and BLG, mixed ALA/BLG oligomers may be formed during this reaction. Higher-molecular-weight reducing agents, such as BLG, can act as initiators of the thiol ↔ disulfide exchange, so it may be postulated that any protein or peptide containing free cysteines can also initiate the oligomerization, gelation, or coagulation of any soluble disulfide-linked protein that has been denatured (no matter its character). The high pressure use for food processing is getting gradually more widespread, so the results of this work can indicate the way to induce nutritionally acceptable chemical changes during food processing by trying to induce desired functional changes (Haertlé and Grinberg, 1996).

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