Roumanian Biotechnological Letters *Vol. 11, No. 2, 2006, pp. 2675-2680*

ORIGINAL PAPER

The effects of high pressure and low temperature on betalactoglobulin structure revealed by spectrophotometry online

Received for publication, 10 March, 2006
Accepted, 10 April, 2006

C. POHRIB[1], FLORENTINA MATEI -RADOI[1], STEFANA JURCOANE[1], CAMILLE LOUPIAC[2], JEAN-MARIE PERRIER-CORNET[2]
[1] Faculty of Biotechnology, U.S.A.M.V.Bucuresti, Romania
[2] G P A B - Laboratoire de Génie des Procédés Alimentaires et Biotechnologiques, ENSBANA, France

Abstract

A solution of betalactoglobulin (b-LG) isolate (10% protein, w/w, in 0,5M TRIS buffer, pH 7 and phosphate buffer 0,5M, pH 7) was processed for about 10 min. at 1, 500, 1000, 1500, 1750 barr at 25°C, in water (25°C) as pressure transmitting medium (PTM). Sample temperature was monitored with T thermocouples located in the sample cylinder. The same b-LG isolate solution was also processed at different pressure and low temperature and the effects were studied with two methods: the spectrophotometry and differential scanning calorimetry. The results measured by spectrophotometry after pressure treatments at 25°C indicated vary small shift, about 1nm, these results indicating that the effects of pressure are not very significant. By measuring the effects of high pressure on b-LG with spectrophotometry online, the results indicated a very large shift, about 10nm at 1750 bar and 25°C, this meaning that the structure of protein is changed under high pressure and tryptophan residues became more exposed to an aqueous microenvironment. The application of 2000 bar at different temperatures (25°C and 0°C) revealed that the changes of bLG structures are more significant at low temperature.

Keywords: Cold temperature denaturation; High pressure; b-lactoglobulin

Introduction

β-lactoglobulin (b-LG) is the major whey protein secreted in the milk of ruminants like the cow or sheep, and monogastrics like pigs, horse, dog or cat. It is absent in the milk of humans or rodents. Bovine b-LG can be readily isolated and, since its isolation from milk by Palmer in 1934, it has been extensively studied by every essentially technique available. Despite this, however, its biological function remains uncertain.

b-LG is very pressure sensitive in proportion to its small size and globular structure. Upon pressure treatment, conformational changes occur resulting in solvent exposure of tryptophan residues and increased reactivity of the free thiol group on Cys121 [9, 15]. Solution studies of native b-lactoglobulin have indicated that pressure treatment has a notable effect on the protein's conformational and aggregation properties, which are more extensive at higher concentrations.

Its pressure-induced denaturation at ambient and higher temperatures has been reported in terms of unfolding and aggregation. b-LG unfolding and loss of native structure through processing at 50-450 MPa and 25°C was investigated during or after pressure processing using methods such as differential scanning calorimetry (DSC) [2] fluorescence of the b-LG molecule [1, 13, 14] or of the retinol/b-LG complex [16], circular dichroism (CD) [8], Fourier transform infra-red (FTIR) spectroscopy, nuclear magnetic resonance (NMR) [6,

17] or reactivity of the free thiol group to Ellman's reagent [9, 17]. Unfolding and aggregation of b-LG appear to be both pressure and time dependent [7, 13, 14, 17] and to be more extended at pH 7.0 than at pH 3.0 [1]. Structural changes were observed at 50 MPa, as shown by hydrogen/deuterium exchange and NMR at pD 7.0 [16] or by spectrofluorimetric methods [13]. The enthalpy of thermal denaturation (DHtd) of 2-5% b-LG solutions, pH 7.0, was reduced by half after pressurization at 450 MPa and 25°C for 15 min, indicating extensive but subtotal unfolding of the protein structure [2]. Partial refolding was observed with time after pressure release [2, 9, 13]. It was suggested that partial unfolding induced at 150-450 MPa and 25°C favors the aggregation [2]. Pressure-induced aggregation of b-LG (2.5% protein, pH 7.0) was due to the formation of intermolecular S-S bonds through thiol/disulphide interchange reactions (rather than through SH oxidation) [4, 5], followed by the formation of b-LG polymers, as already observed for heat-induced aggregation [11, 12]. However, the microstructure and mechanical properties of pressure-induced b-LG gels (10-14% protein, pH 7.0), as well as the FTIR spectra of 10% b-LG solutions, suggest different mechanisms for temperature or pressure-induced gelation of b-LG [3, 6].

The present study deals with the effects of low temperature and high pressure under b-LG and revealing the effects of structure changes online, during pressure treatments by fluorecence spectophotometry and DSC after pressure treatments.

Materials and methods

Materials

b-LG derived from bovine milk, approximately 90% chromatographically purified and lyophilized (Sigma Chemical), was dissolved in either 0,5 M phosphate buffer pH 7.0 or 0,5 M TRIS buffer, pH 7 to provide concentration of 10% protein, w/w for differential scanning calorimetry DSC and spectrofluoresence. The concentration of protein solutions were determined by spectrophotometry with an extinction coefficient of 0.96 for a 10 mg ml^{-1} solution at 278 nm in a 10 cm path length.

Methods

High pressure processing

Prepared samples of the protein were placed in 1.5 ml plastic microcapsules (Life Sciences, UK) and loaded into a 316 stainless steel pressure vessel with a 3 ml working capacity. The remaining volume was filled with water used as the pressurizing medium. The temperature of the vessel was regulated by immersion in a thermostated water bath Julabo F81, Germany. Hydraulic pressurization was achieved using a hand pump (Novaswiss, Suisse). A series of test conditions used different combinations of pressures and temperatures was achieved. For the study of structure changing of b-LG on line under high pressure was used a special optic cell ((Institute of High Pressure Physics, Poland), with three windows.

Spectrofluorometry

Intrinsic fluorescence emission spectroscopy, used to study the tertiary structure, dynamics and interactions of pressure-temperature treated b-LG in solution, was measured using Fluorolog III (Jobin Yvon, Horiba group, USA) fluorometer. Emission spectra were obtained using an excitation wavelength (λ_{ex}) of 293 nm to excite selectively tryptophan (W19 and W61) in the protein's structure.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis, used to examine the energetic of the pressure-temperature processed b-LG, was performed using a DSC III Setaram microcalorimeter. Samples were heated from 20°C to 110°C at 1°C/min using the buffer solution as a reference. The enthalpy of denaturation (ΔH) and the temperature of the maximum of the endothermal peak (T_{max}) were determined from baseline-corrected thermograms with the concentration data normalized for comparative purposes. A maximum variation in T_{max} of ±0.5°C was found for replicated runs.

Results and discussions

Spectrofluorometry

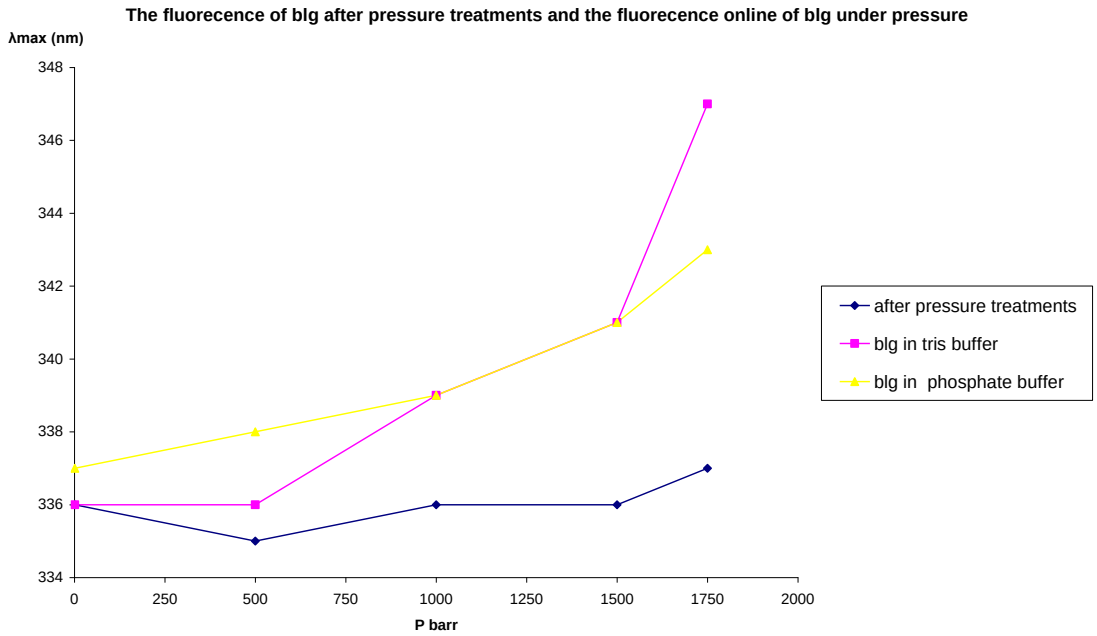

Figure 1. The fluorescence of b-LG after pressure treatment and the fluorescence online of b-LG under pressure

Native b-LG prepared at pH 7.0 and exhibited emission maximum wavelengths, λ_{em}, of 335 nm and 336 nm, respectively. No major changes was found for the pressure – temperature treated protein prepared at pH 7.0 suggesting that the pressure treatment was unable to affect the protein's tryptophan environment. By measuring the effects of high pressure on b-LG with spectrophotometry online, the results indicated a very large shift, about 10nm at 1750 bar and 25°C (Figure 1), this meaning that the structure of protein is changed under high pressure and tryptophan residues became more exposed to an aqueous microenvironment. Because after pressure treatments the λ_{em} of b-LG is same as native b-LG values, the conclusion is that the changes of protein are reversible.

Lowering the temperature under pressure also enhanced the exposure of the hydrophobic zones of b-LG to water considering the results of fluorescence online at optic cell (Figure 2). So, decreasing the temperature of the b-LG solution under high pressure reveals that tyrosine and tryptophan residues became significantly more exposed to an

aqueous microenvironment at low temperatures. Also, after these treatments the b-LG structure changes are reversible.

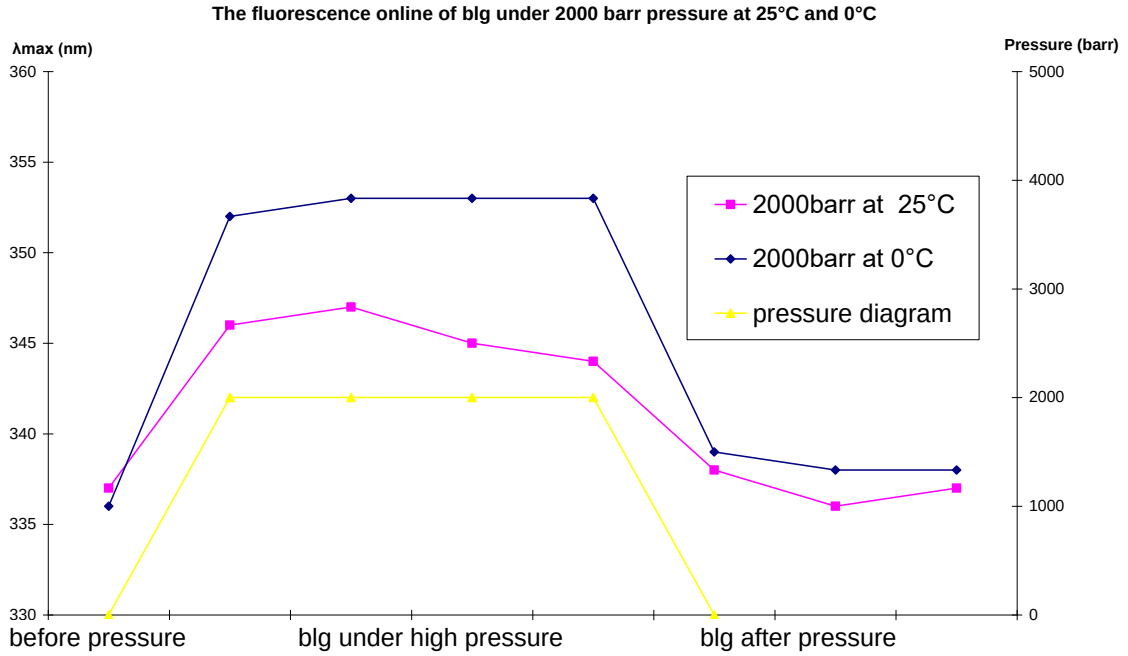

Figure 2. The fluorescence online of b-LG under 200 bar pressure at 25ºC and 0ºC
Differential scanning calorimetry

The residual enthalpies of denaturation (ΔH) and temperature of the maximum endothermal peak (T_{max}) are shown in Table 1. The endothermal effects on the DSC thermograms are the result of disruption of intramolecular hydrogen bonds. The reduction in ΔH, therefore, indicates a partial loss of protein structure after both (combined) pressure and temperature treatment.

Table 1. Values of the calorimetry parameters

Sample	Endothermal peak (T_{max})	ΔH (kcal/mol)
Native	71,64	4,75
1500b, -20°C	71,6	3,1
2000b, -20°C	71,68	4,2
4000b, -20°C	71,56	3,35

Considering the results of DSC at various pressures and negative temperatures, it is likely that low temperatures minimize the loss of native structure induced by pressurization (DSC data), and reduce subsequent aggregation reactions under high pressure. This may be due to a more hydrated state of the protein under pressure at low or sub-zero temperatures than at ambient temperature.

The application of high pressure (1000-4000 bar) and very low temperatures (-20°C) at pressure cell and measuring the florescence after these treatments, showed that the structure changes of b-LG are reversible, but measuring ΔH by DSC revealed that the structure changes of b-LG are not completely reversible (Figure 3).

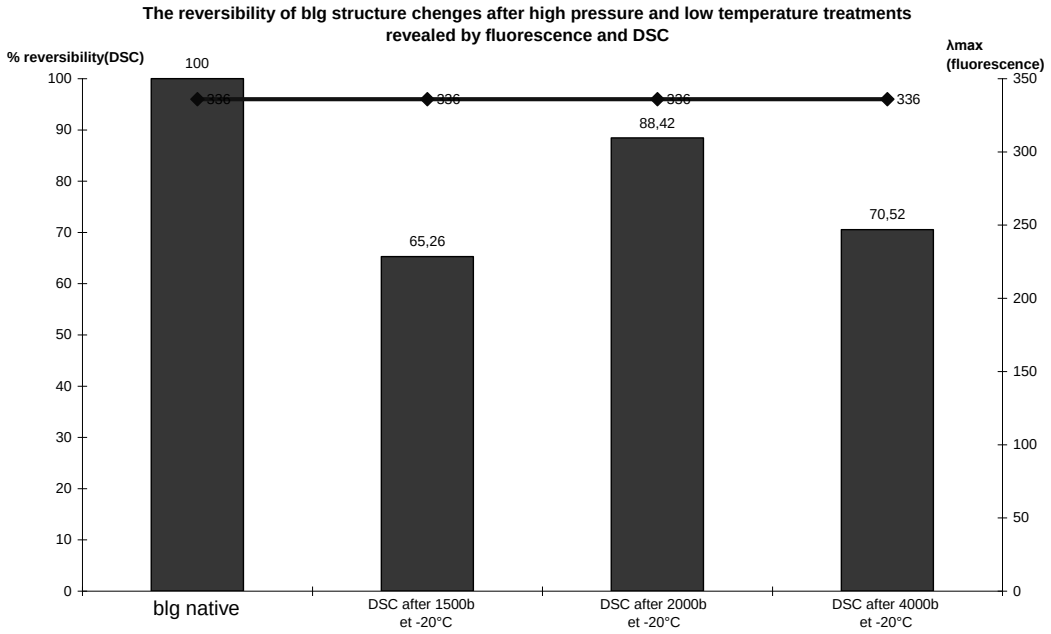

Figure 3. The reversibility of b-LG structure changes after high pressure and low temperature treatments revealed by fluorescence and DSC

Conclusions

The application of high pressure at very low temperatures on b-LG protein causes changes in the protein structure revealed by spectrophotometry of fluorescence online. It can say that the tryptophan residues became more exposed to an aqueous microenvironment at low temperature in rapport with high pressure and 25°C.

After high pressure and 25°C and high pressure and low temperature treatments of b-LG, it appears that the changes in structure are reversible if they are studied by fluorescence, but not if they are studied by calorimetry.

Acknowledgments

The author has been financially supported by Leonardo Da Vinci E.U. Programme (RO/2004/PL93055/S). Special thanks to the laboratories members of G P A B - Laboratoire de Génie des Procédés Alimentaires et Biotechnologiques especially to Dr Jean-Marie PERRIER-CORNET and to the members of IMSAPS "Ingénierie Moléculaire et Sensorielle de l'Aliment et des Produits de Santé" , especially to Dr Camille LOUPIAC.

Bibliography

1. Dufour, E., Hui Bon Hoa, G., & Haertle, T. (1994). High-pressure effects on b-lactoglobulin interactions with ligands studied by fluorescence. Biochimica and Biophysica Acta, **1206**, 166-172
2. Dumay, E. M., Kalichevsky, M. T., & Cheftel, J. -C. (1994). High-pressure unfolding and aggregation of b-lactoglobulin and the baroprotective effects of sucrose. Journal of Agricultural and Food Chemistry, **42**, 1861-1868.

3. Dumay, E., Kalichevsky, M. T., & Cheftel, J. -C. (1998). Characteristics of pressure-induced gels of b-lactoglobulin at various times after pressure release. Lebensmittel-Wissenschaft und-Technologie, **31**, 10-19
4. Funtenberger, S., Dumay, E., & Cheftel, J. -C. (1995). Pressure-induced aggregation of b-lactoglobulin in pH 7.0 buffers. Lebensmittel-Wissenschaft und-Technologie, **28**, 410-418.
5. Funtenberger, S., Dumay, E., & Cheftel, J. -C. (1997). High pressure promotes b-lactoglobulin aggregation through SH/S±S interchange reactions. Journal of Agricultural and Food Chemistry, **45**, 912-921.
6. Heremans, K., Van Camp, J., & Huyghebaert, A. (1997). High-pressure effects on proteins. In S. Damodaran & A. Paraf, Food proteins and their applications (pp. 473-502). New York: Marcel Dekker.
7. Hinrichs, J., Rademacher, B., & Kessler, H. G. (1996). Reaction kinetics of pressure-induced denaturation of whey proteins. Milchwissenschaft, **51**, 504-509
8. Iametti, S., Transidico, P., Bonomi, F., Vecchio, G., Pittia, P., Rovere, P., & Dall'Aglio, G. (1997). Molecular modi®cations of b-lactoglobulin upon exposure to high pressure. Journal of Agricultural and Food Chemistry, **45**, 23-29
9. Mùller, R. E., Stapelfeldt, H., & Skibsted, L. H. (1998). Thiol reactivity ureunfolded b-lactoglobulin. Antioxidative properties and thermal refolding. Journal of Agricultural and Food Chemistry, **46**, 425-430
10. Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E. & Kraulis, P. J. 1986. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Letters to Nature* , **342**, 383-385.
11. Shimada, K., & Cheftel, J. -C. (1988). Texture characteristics, protein solubility, and sulfhydryl group/disul®de bond contents of heat-induced gels of whey protein isolate. Journal of Agricultural and Food Chemistry, **36**, 1018±-025.
12. Shimada, K., & Cheftel, J. -C. (1989). Sulfhydryl group/disul®de bond interchange reactions during heat-induced gelation of whey protein isolate. Journal of Agricultural and Food Chemistry, **37**, 161-168.
13. Stapelfeldt, H., Petersen, P. H., Kristiansen, K. R., Qvist, K. B., & Skibsted, L. H. (1996). Effect of high hydrostatic pressure on the enzymic hydrolysis of b-lactoglobulin B by trypsin, thermolysin and pepsin. Journal of Dairy Research, **63**, 111-118
14. Stapelfeldt, H. and Skibsted, L.H., Pressure denaturation and aggregation of β-lactoglobulin studied by intrinsic fluorescence depolarization, Rayleigh scattering, radiationless energy transfer and hydrophobic fluoroprobing. *Journal of Dairy Research* **66** (1999), pp. 545–558.
15. Stapelfeldt, H., & Skibsted, L. H. (1999). Pressure denaturation and aggregation of b-lactoglobulin studied by intrinsec fuorescence depolarization. Rayleigh scattering, radiationless energy transfer and hydrophobic fluoroprobing. Journal of Dairy Research, **66**, 545-558.
16. Tanaka, N., Tsurui, Y., Kobayashi, I., & Kunugi, S. (1996). Modifcation of the single unpaired sulfhydryl group of b-lactoglobulin under high pressure and the role of intermolecular S-S exchange in the pressure denaturation [Single SH of b-lactoglobulin and pressure denaturation]. International Journal of Biological Macromolecules, **19**, 63-68.
17. Tanaka, N., & Kunugi, S. (1996). Effect of pressure on the deuterium exchange reaction of a-lactalbumin and of b-lactoglobulin. International Journal of Biological Macromolecules, **18**, 33-39.