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Article *in* European Food Research and Technology · May 2012

DOI: 10.1007/s00217-012-1695-x

Eur Food Res Technol (2012) 234:813–819
DOI 10.1007/s00217-012-1695-x

ORIGINAL PAPER

Effect of high-pressure treatment on denaturation of bovine β-lactoglobulin and α-lactalbumin

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Received: 31 October 2011 / Revised: 27 January 2012 / Accepted: 3 February 2012 / Published online: 22 February 2012

Abstract The effect of high-pressure treatment on denaturation of β-lactoglobulin and α-lactalbumin in skimmed milk, whey, and phosphate buffer was studied over a pressure range of 450–700 MPa at 20 °C. The degree of protein denaturation was measured by the loss of reactivity with their specific antibodies using radial immunodiffusion. The denaturation of β-lactoglobulin increased with the increase of pressure and holding time. Denaturation rate constants of β-lactoglobulin were higher when the protein was treated in skimmed milk than in whey, and in both media higher than in buffer, indicating that the stability of the protein depends on the treatment media. α-Lactalbumin is much more baroresistant than β-lactoglobulin as a low level of denaturation was obtained at all treatments assayed. Denaturation of β-lactoglobulin in the three media was found to follow a reaction order of $n = 1.5$. A linear relationship was obtained between the logarithm of the rate constants and pressure over the pressure range studied. Activation volumes obtained for the protein treated in milk, whey, and buffer were -17.7 ± 0.5, -24.8 ± 0.4, and -18.9 ± 0.8 mL/mol, respectively, which indicate that under pressure, reactions of volume decrease of β-lactoglobulin are favoured. Kinetic parameters obtained in this work allow calculating the pressure-induced denaturation of β-lactoglobulin on the basis of pressure and holding times applied.

Keywords β-lactoglobulin · α-lactalbumin · Whey proteins · High hydrostatic pressure · Denaturation kinetics

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Introduction

Thermal treatment of milk is commonly used to inactivate pathogenic and spoilage microorganisms and also enzymes, in order to make it safe for human consumption and to extend its shelf life. Although thermal processing is effective, economical, and readily available, in many cases, it has undesirable effect on the nutritive value and the sensorial properties of milk, depending on their intensity [1].

The increasing demand for fresh-like and nutritious products has led to the development of milder preservation technologies to replace existing thermal treatments [2]. Among these technologies, high hydrostatic pressure treatment is being investigated as an alternative to heat treatment of milk because it has the capacity of inactivating vegetative microorganisms at lower temperatures than those used in conventional heat treatments [3]. Thus, it has been reported that to achieve a shelf life of 10 days at a storage temperature of 10 °C, a pressure treatment of 400 MPa for 15 min or at 600 MPa for 3 min at 20 °C is necessary [4].

Pressure treatment of milk has considerable effects on milk proteins. High-pressure treatment induces disruption and reformation of casein micelles [5]. For the whey proteins, high pressure produces changes in conformation, followed by aggregation, mainly via sulphydryl-disulfide interchange reactions, either with other whey proteins or in the case of β-lactoglobulin, as well with κ-casein and

potentially α_{s2}-casein [6]. The majority of denatured β-lactoglobulin is apparently associated with casein micelles [7, 8]. The interaction of casein micelles and whey proteins in milk may have significant implications for properties of products made from high-pressure-treated milk like cheese and yoghurts [9–11].

On the other hand, whey protein products such as concentrates, isolates, or fractions enriched in β-lactoglobulin or α-lactalbumin have been used as food ingredients because of their functional properties like emulsifying, foaming, and gelling properties. High-pressure treatment of these products induced changes in proteins that were shown to modify those functional properties for different applications in the dairy industry [12, 13].

Of particular interest for milk and dairy products is the study of high-pressure-induced denaturation of the major whey proteins, β-lactoglobulin, and α-lactalbumin because they are largely responsible for the functional properties of whey [12]. Denaturation of β-lactoglobulin in milk occurs above about 150 MPa and application of higher pressures results in considerable denaturation of this protein [5, 12]. Using fluorescence spectroscopy, important changes in fluorescence intensity and the emission maximum of β-lactoglobulin occur between 100 and 200 MPa [14, 15]. Using circular dichroism, Tedford et al. [16] have shown significant changes in secondary and tertiary structures of β-lactoglobulin whereas Iametti et al. [17] found no detectable changes in the secondary structure and only a 10% loss of tertiary structure even at 900 MPa. It has been also reported that reversible modifications of the structure of β-lactoglobulin by pressure treatment lead to dissociation of dimers, mainly by destabilization of hydrophobic interactions. However, a treatment above certain threshold intensity induces monomers to unfold and to interact to form aggregates that are stabilized by covalent and non-covalent interactions [5, 12]. The formation of inter- and intra-molecular interactions of the sulphydryl group is suggested to be the main cause for the pressure-induced irreversible denaturation of β-lactoglobulin [15]. Compared to β-lactoglobulin, α-lactalbumin is stable to pressures up to about 400–500 MPa in milk at 20 °C [12, 18].

The kinetics of denaturation of bovine β-lactoglobulin and α-lactalbumin subjected to high-pressure treatment has been previously studied. Denaturation of individual whey proteins in these works has been determined using electrophoresis [19, 20] and reversed-phase high performance liquid chromatography (RP-HPLC) [21, 22] after subjected pressure-treated milk or whey protein isolate samples to acid precipitation at pH 4.6, to remove precipitated casein and aggregated whey proteins. The order of reaction determined for the pressure-induced denaturation of β-lactoglobulin was found to range between 2 and 3 [19–22] and of α-lactalbumin between 2 and 2.5 [20–22].

The aim of this work was to study the effect of high-pressure treatment on the denaturation of bovine β-lactoglobulin and α-lactalbumin in milk and whey and as isolated proteins in buffer. The degree of denaturation was determined by measuring the loss of reactivity with their specific antibodies using radial immunodiffusion. This technique does not require any manipulation of the sample after pressure treatment. We calculated the kinetic parameters for the denaturation process of β-lactoglobulin that allow the prediction of the denaturation degree of this protein on the basis of pressure and holding time applied.

Materials and methods

Materials

Fresh raw bovine milk samples were kindly supplied by Quesos Villacorona (El Burgo de Ebro, Zaragoza, Spain). Recombinant chymosin (EC 3.4.23.4) was provided by Chr.Hansen (Horsholm, Denmark). Bovine β-lactoglobulin and α-lactalbumin were purchased from Sigma (Poole, UK).

Antisera

Antisera against purified bovine β-lactoglobulin and α-lactalbúmin were obtained in rabbits according to Wehbi et al. [19]. All procedures were performed under Project Licence PI48/10 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed following the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes. Immunoelectrophoresis was used to check the specificity of the antisera, which showed to be specific for each protein.

Milk and whey preparation

Milk was skimmed by centrifugation at 2,000×g at 4 °C for 30 min, followed by filtration of the subnatant through glass wool to remove fat particles. Fat concentration in skimmed milk, as determined by the Gerber method, was found to be less than 0.18% (w/w). Recombinant chymosin (1 mg/mL) was added to skimmed milk (30 μL/mL), and the milk was coagulated at 35 °C for 45 min. Finally, clotted milk was centrifuged at 2,000×g at 4 °C for 15 min to obtain whey.

Pressure treatment

Skimmed milk, whey samples, and β-lactoglobulin and α-lactalbumin solutions in 150 mM NaCl, 10 mM potassium

phosphate buffer, pH 7.4 were introduced into eppendorf tubes without headspace and treated in a Stansted Fluid Power FPG 11500B high-pressure food processor (Stansted, Essex, UK). The temperatures of the samples, the chamber, and the pressurization fluid were pre-equilibrated to 20 °C for at least 1 h before treatment. Temperature and pressure in the chamber were monitored throughout the run using data logging equipment. Rate of pressure increase was maintained at 240 MPa per min, and depressurization time was less than 30 s. The increase and decrease of temperature during pressurization or depressurization was about 2 °C per 100 MPa as a result of adiabatic heating or cooling. Samples were treated from 450 to 700 MPa for different times at 20 °C. Samples from two independent experiments were analyzed by duplicate. After pressure treatment, the samples were stored at 4 °C overnight before analysis.

Measurement of protein concentration

The concentration of β-lactoglobulin and α-lactalbumin was determined by radial immunodiffusion as previously described [23]. Agarose at 1% in 25 mM sodium 5,5′-diethyl barbiturate buffer, pH 8.2 was prepared containing an appropriate amount of specific antiserum and spread onto 9 × 12 cm glass plates. Samples and appropriate protein standards (8 µL) were applied to the wells, and the plates were incubated in moist chambers at room temperature for 48 h. After immunodiffusion, the plates were washed in 0.25 mM NaCl, 10 mM potassium phosphate buffer, pH 7.4, for 24 h with frequent changes. Finally, the plates were washed once with distilled water and allowed to air-dry and stained with Coomassie Blue.

Kinetic data analysis and reaction order

The denaturation process of a protein can be described by the general equation:

$$-\mathrm{d}c/\mathrm{d}t = kc^n \quad (1)$$

where dc/dt represents the rate of protein denaturation, k the rate constant, c the residual protein concentration at each pressure, and n the reaction order.

The integration of Eq. 1 for the order $n = 1$, results in:

$$\ln(c_t/c_0) = -kt \quad (2)$$

where c_0 is the initial protein concentration (for time 0) and c_t the concentration of undenatured protein at each holding time.

For an order $n \neq 1$, when the general Eq. 1 is integrated results in:

$$(c_t/c_0)^{1-n} = \left[1 + (n-1)\,k(c_0)^{n-1}t\right]^{1/1-n} \quad (3)$$

To determine k values, the least-squares criterion by the Solver function of the Excel 5.0 package (Microsoft, Seattle, WA) was used. Correlation coefficients (r^2) and root mean square errors (RMSE) between predicted and observed data were used to test the fitness of the model [24].

Calculation of activation volumes and frequency factors

The activation volumes and frequency factors were calculated as indicated by Anema et al. [19] according to the following equation:

$$\ln k = \ln k_0 - pV_a/RT \quad (4)$$

where k is the rate constant, k_0 the frequency factor, R the universal gas constant, T the absolute temperature, p the pressure, and V_a the activation volume.

Results and discussion

In this study, the degree of denaturation of β-lactoglobulin and α-lactalbumin subjected to high-pressure treatments was determined by measuring the loss of reactivity of each protein with specific antibodies using radial immunodiffusion. Conventional immunochemical techniques are particularly useful for detecting conformational changes in proteins with concomitant loss of epitopes as a result of denaturation [25]. This technique has been widely employed to study the denaturation of whey proteins subjected to technological treatments such as heat treatment [23], high-intensity pulse electric fields processing [26], or high-pressure treatment [27]. High correlation coefficients ($r^2 > 0.98$) were obtained when representing the square of the diameter of precipitating rings corresponding to protein standards versus their concentration, being the relationship linear within the range from 10 to 250 µg/mL for both proteins. The concentration of β-lactoglobulin in skimmed milk and whey samples ranged from 3.8 to 4.2 mg/mL and the concentration of α-lactalbumin from 1.3 to 1.5 mg/mL. These values are in the range of concentrations found for both proteins in bovine milk [28].

In this work, immunoreactive protein determined includes native protein and protein that has been refolded to the original native state, as well as unfolded and aggregated proteins that still could react with antibodies because they maintain at least in part their conformational structure. However, when whey proteins are determined in the pH 4.6-soluble fraction of pressure-treated milk, protein determination could be underestimated. It has been shown by electrophoresis that acid precipitates from pressurized milk at 500 MPa for 10 min at 25 °C contain appreciable amounts of whey proteins, being most of the β-lactoglobulin precipitated at that pH, whereas α-lactalbumin, albumin, immunoglobulins, and lactoferrin also

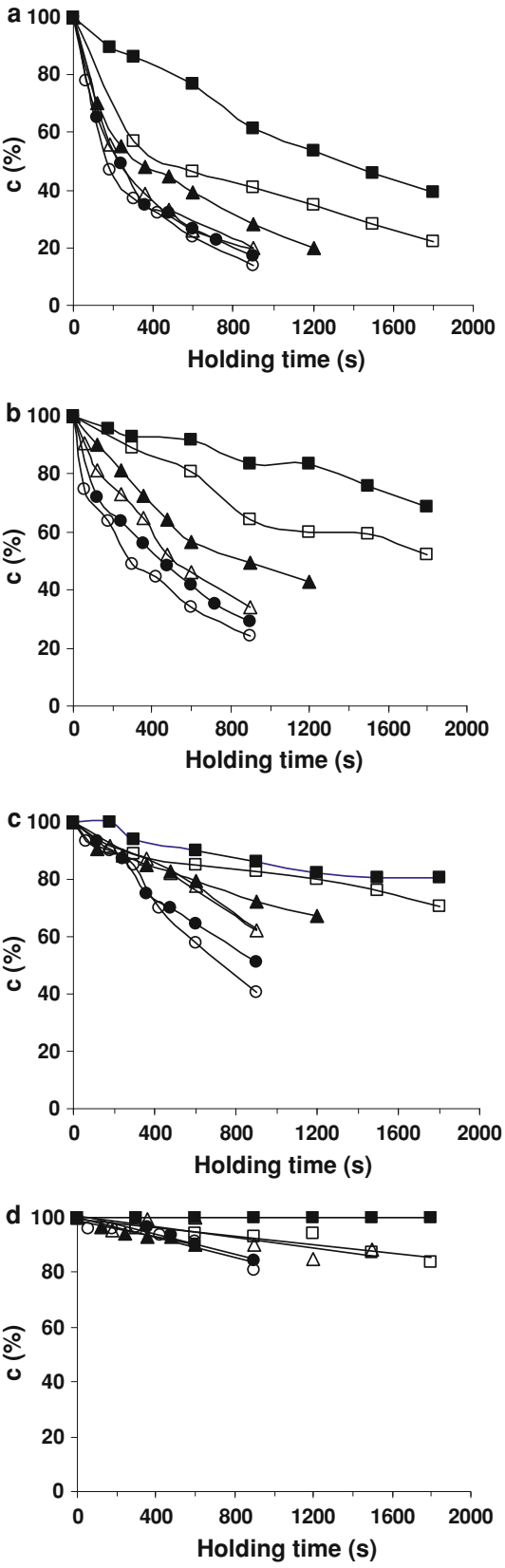

Fig. 1 Effect of pressure treatment on the denaturation of ▶ β-lactoglobulin in skimmed milk (**a**), whey (**b**), and phosphate buffer (**c**) and of α-lactalbumin in skim milk (**d**) at different pressures (*filled square* 450; *empty square* 500; *filled triangle* 550; *empty triangle* 600; *filled circle* 650; *empty circle*, 700 MPa). *c* is the concentration of immunoreactive protein at each holding time expressed as percentage of the protein concentration of untreated sample

precipitated but in a much lower extent. Then, aggregated whey proteins or whey proteins that have interacted with caseins, and which may maintain part of their structure, would not be accounted when samples are adjusted to pH 4.6.

The process of denaturation was studied by analyzing the concentration of individual proteins at each holding time after pressure treatment. These data can be evaluated using similar processes to those used in studying the thermal denaturation [19, 29].

Previously, preliminary experiments were performed to determine an appropriate range of pressures and times of treatment. We observed that β-lactoglobulin in phosphate buffer and α-lactalbumin in milk, whey, and buffer denatured slowly at 400 MPa. Therefore, a range from 450 to 700 MPa was chosen to reach a degree of protein denaturation along time that allows the prediction of kinetic parameters.

The loss of concentration of immunoreactive β-lactoglobulin and α-lactalbumin increased with pressure and time of treatment (Fig. 1a, b, c, d). Furthermore, it was observed that baroresistance of β-lactoglobulin varied depending on the medium of treatment. Thus, at all pressures, the degree of denaturation was higher for the protein treated in milk than in whey ant in both media than in buffer.

Results obtained for the denaturation of β-lactoglobulin in milk treated at 500 MPa are similar to those reported by Anema et al. [19] using native electrophoresis of whey proteins after removing caseins and aggregated whey proteins by precipitation at pH 4.6. These authors found that denatured β-lactoglobulin treated at 500 MPa and at 20 °C for 15 and 30 min was about 45 and 25% compared to the untreated milk sample, as found in our work. However, we obtained a higher level of denaturation than that estimated by Anema et al. [16] when treated milk at 600 MPa. By contrast, Hupertz et al. [30] when measuring β-lactoglobulin in the pH 4.6 soluble fraction of milk by RP-HPLC found a denaturation of 95% after treatment of milk at 600 MPa for 30 min at 20 °C, which is higher than that estimated in this work. These differences could be attributed to the different conditions of the pressure treatment and to the different techniques used to measure the residual protein concentration.

Baroresistance of α-lactalbumin was much higher than that obtained for β-lactoglobulin This fact is shown by the low level of denaturation obtained after treatment of milk, which was less than 20% at the highest pressure and time applied (700 MPa for 15 min). The higher stability of α-lactalbumin to pressure-induced denaturation in milk, compared with β-lactoglobulin, is consistent with previous observations. Huppertz et al. [30] determined that about 36% of the protein was denatured after treatment of milk at 800 MPa at 20 °C for 30 min. Hinrichs and Rademacher [20] reported that about 10% of α-lactalbumin was denatured after treatment of milk at 600 MPa for 30 min at 10 °C.

The higher stability of α-lactalbumin, to pressure-induced denaturation, compared to β-lactoglobulin has been attributed to the higher number of intramolecular disulfide bonds (4 in α-lactalbumin and 2 in β-lactoglobulin) and to the presence of a free sulphydryl group in β-lactoglobulin [12, 30]. Furthermore, the binding of calcium to α-lactalbumin has been shown to stabilize the protein against pressure and heat treatments [23, 31].

On the other hand, results obtained in our work indicated that β-lactoglobulin and α-lactalbumin were more sensitive to pressure when the proteins were treated in milk than in whey. This fact is reflected by the higher level of denaturation obtained for β-lactoglobulin treated in milk compared to whey. Likewise, in the case of α-lactalbumin, the degree of denaturation was lower in whey and buffer than in milk. Thus, the concentration of immunoreactive protein treated in whey and buffer decreased less than 8% after treatments up to at 700 MPa for 15 min (results not shown). These findings are in accordance with those reported by Huppertz et al. [30] who estimated that only about 16.6% of α-lactalbumin was denatured after treatment of whey at 800 MPa for 30 min at 20 °C.

Our results are also in agreement with those obtained by Huppertz et al. [30] as they observed that the level of pressure denaturation of β-lactoglobulin and α-lactalbumin increased with increasing level of milk in mixtures of whey and milk, as it has been also indicated for the heat denaturation of both proteins [30, 32].

This fact has been attributed to the unfolding of β-lactoglobulin by pressure, which results in the exposure of its free sulphydryl group. Unfolded β-lactoglobulin can interact with other proteins containing disulfide bonds such as αs_2 caseins, k-casein, α-lactalbumin, and β-lactoglobulin, through sulphydryl-disulfide interchange reactions. On high-pressure treatment of milk, most denatured β-lactoglobulin interacts with casein micelles and the level of β-lactoglobulin associated with them is proportional to the level of denatured β-lactoglobulin [30]. In fact, immunolabeling studies have revealed the presence of β-lactoglobulin dots very close to micelle fragments in high-pressure-treated milk [11]. Therefore, the lower extent of pressure-induced denaturation of β-lactoglobulin in whey than in milk may be explained by the lower number of molecules available for interaction in whey, mainly due to the absence of casein micelles [30].

Pressure-induced denaturation of β-lactoglobulin was considerably faster when it was treated in whey than in buffer. This fact could be due in part to the absence of calcium in the medium. It has been observed that denaturation of β-lactoglobulin and α-lactalbumin is more extensive in milk than in colloidal calcium phosphate-free milk, suggesting that a higher level of calcium enhances denaturation of these proteins as it was also observed for heat denaturation of the same proteins in milk [23, 30].

Experimental data obtained in this work were analyzed using Eqs. 2 and 3 to determine both the rate (k) and the reaction order (n) of denaturation in the three treatment media at all pressures. When using that prediction, an n value between 1.4 and 1.6 was obtained (results not shown). However, as the comparison of the rate constants is not permissible for reactions with different values of the reaction order, the mean value was calculated from results obtained in the three media at all pressures, which was of $n = 1.5$. Then, the experimental data were evaluated again considering that fixed reaction order (Table 1). The values

Table 1 Rate constants of pressure denaturation of β-lactoglobulin in skimmed milk, whey, and phosphate buffer assuming a fixed reaction order of $n = 1.5$

Pressure	Milk			Whey			Buffer		
	k	r^2	RMSE	k	r^2	RMSE	k	r^2	RMSE
450 MPa	6.06	0.990	0.023	1.98	0.957	0.021	1.52	0.941	0.019
500 MPa	13.94	0.964	0.054	4.42	0.971	0.028	2.14	0.955	0.024
550 MPa	23.03	0.979	0.042	9.71	0.991	0.017	4.04	0.977	0.023
600 MPa	32.84	0.995	0.024	15.33	0.993	0.018	4.96	0.959	0.026
650 MPa	34.45	0.994	0.023	19.50	0.985	0.031	8.19	0.988	0.021
700 MPa	41.68	0.990	0.033	26.40	0.978	0.047	9.80	0.953	0.039

Parameters shown are rate constant (k, $s^{-1} \times 10^4$), correlation coefficient (r^2), and root mean square error (RMSE)

of the coefficients of correlation (r^2) were higher than 0.941, and the values of the root mean square errors (RMSE) were lower than 0.054, which indicates that the measured data were well described by the kinetic parameters determined with Eq. 3 for $n = 1.5$. The correlation between concentrations experimentally determined and those calculated for experiments performed with the three treatment media is shown in Fig. 2. In the case α-lactalbumin, it was not possible to estimate the reaction order due to its low level of denaturation obtained at all pressures studied.

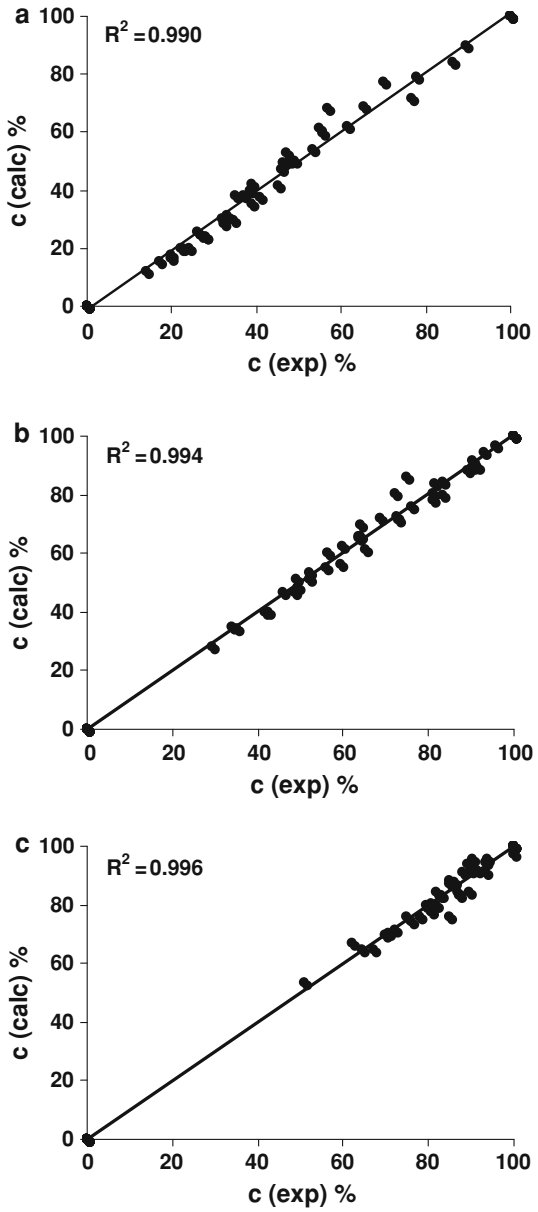

Fig. 2 Correlation between values experimentally determined (exp) after pressure treatment of milk (**a**), whey (**b**), and phosphate buffer (**c**) and those calculated (calc) by means of kinetic parameters for β-lactoglobulin assuming a reaction order of $n = 1.5$. c is the concentration of immunoreactive protein at each holding time expressed as percentage of the protein concentration of untreated sample. R^2, correlation coefficient

The order of reaction published for the pressure denaturation of β-lactoglobulin ranges between 2 and 3. Anema et al. [19] and Hinrichs and Rademacher [20] assumed a reaction order of $n = 2$ for β-lactoglobulin variants A and B treated in milk and in whey protein solutions, whereas a reaction order of $n = 2.5$ [21] and $n = 3$ [22] has been also reported for the denaturation of both protein variants by high-pressure treatment.

By comparison, thermal denaturation of β-lactoglobulin and α-lactalbumin in skim milk has been reported to follow a reaction order of $n = 1.5$ [23, 29, 33]. The unusual value of the reaction order estimated for the induced denaturation of both proteins subjected to thermal or pressure treatment has been attributed to a complex reaction mechanism that involves many consecutive and/or concurrent steps [19, 20]. This could be ascribed to the dissociation of β-lactoglobulin dimer into monomers and to unfolding of monomers followed by aggregation.

When the natural logarithms of the rate constants (ln k) obtained were plotted against the pressure, a linear relationship was found in the pressure range studied for β-lactoglobulin treated in the three media (Fig. 3). Some authors have suggested that unfolding is the rate-determining step in the reaction mechanism of β-lactoglobulin denaturation in the range of pressures between 300 and 600 MPa [19] whereas others have indicated that aggregation limits denaturation at pressures between 300 and 800 MPa [20]. The values of ln k_0 estimated in our work were of −10.3, −12.8, and −12.2, and the V_a values of were -17.7 ± 0.5, -24.8 ± 0.4, and -18.9 ± 0.8 mL/mol for the protein treated in milk, whey, and buffer, respectively. The values of V_a obtained are in the range reported for the pressure-induced denaturation of β-lactoglobulin [19–21]. In all cases, negative V_a values were

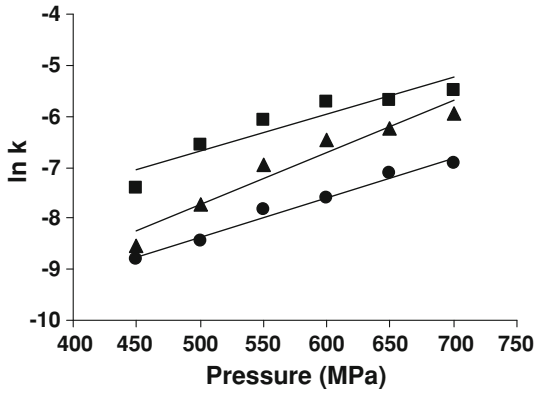

Fig. 3 Relationship between ln k and pressure for the denaturation of β-lactoglobulin in skimmed milk (*filled square*), whey (*filled triangle*), and phosphate buffer (*filled circle*)

obtained, which indicate that under pressure, reactions of volume decrease are favoured [21, 34].

High-pressure treatment of milk, whey, or milk protein fractions has the potential to produce dairy products with improved functional properties. These properties depend on great part on the denaturation of the predominant proteins. This study generated pressure and time dependent kinetic data that allow the calculation of the degree of denaturation of the major whey protein, β-lactoglobulin, on the basis of pressure and holding time applied.

Acknowledgments This work was supported by grant 3rd Plan Tecnológico de Navarra (Navarra Government) and by grant P1078/09 from Aragón Government and European Social Fund. Chafiaa Mazri from the Institut National de la Recherche Agronomique d'Algerie is recipient of a fellowship from AECID. We thank Quesos Villacorona for the generous collaboration in supplying fresh milk samples.

References

1. Singh H (2004) Int J Dairy Tech 57:111–119
2. Butz P, Tauscher B (2002) Food Res Int 35:279–284
3. Rastogi NK, Raghavarao KSMS, Balasubramaniam VM, Niranjan K, Knorr D (2007) Crit Rev Food Sci Nutr 47:69–112
4. Rademacher B, Kessler HG (1997) In: Heremans K (ed) High pressure bio-science and biotechnology. Leuven University Press, Leuven, pp 291–293
5. Huppertz T, Kelly AL, Fox PF (2002) Int Dairy J 12:561–572
6. Huppertz T, de Kruif CG (2007) J Dairy Res 74:194–197
7. Felipe X, Capellas M, Law AJR (1997) J Agric Food Chem 45:627–631
8. Huppertz T, Fox PF, Kelly AL (2004) J Dairy Res 71:97–106
9. López-Fandiño R (2006) Int Dairy J 16:1131–1191
10. Trujillo AJ, Capellas M, Saldo J, Gervilla R, Guamis B (2002) Innov Food Sci Emerg Technol 3:295–307
11. Needs EC, Capellas M, Bland AP, Manoj P, MacDougal D, Paul G (2000) J Dairy Res 67:329–348
12. López-Fandiño R (2006) Crit Rev Food Sci Nutr 46:351–363
13. Rodiles-Lopez JO, Jaramillo-Flores ME, Gutierrez-Lopez GF, Hernandez-Arana A, Fosado-Quiroz RE, Barbosa-Canovas GV, Hernandez-Sanchez H (2008) J Food Eng 87:363–370
14. Dufour E, Hoa GHB, Haertlé T (1994) Biochim Biophys Acta 1206:166–172
15. Tanaka N, Tsurui Y, Kobayashi I, Kunugi S (1996) Int J Biol Macromol 19:63–68
16. Tedford LA, Smith D, Schaschke CJ (1999) Food Res Int 32:101–106
17. Iametti S, Transidico P, Bonomi F et al (1997) J Agric Food Chem 45:23–29
18. Rodiles-Lopez JO, Arroyo-Maya IJ, Jaramillo-Flores ME, Gutierrez-Lopez GF, Hernandez-Arana A, Barbosa-Canovas GV, Niranjan K, Hernandez-Sanchez H (2010) J Dairy Sci 93:1420–1428
19. Anema SG, Stockmann R, Lowe EK (2005) J Agric Food Chem 53:7783–7791
20. Hinrichs J, Rademacher B (2005) Int Dairy J 15:315–323
21. Hinrichs J, Rademacher B, Kessler HG (1996) Milchwissenschaft 51:504–508
22. Hinrichs J, Rademacher B (2004) J Dairy Res 71:480–488
23. Wehbi Z, Pérez MD, Sánchez L, Pocovi C, Barbana C, Calvo M (2005) J Agric Food Chem 53:9730–9736
24. Baranyi J, Pi C, Ross T (1999) Int J Food Microbiol 48:159–166
25. Tremblay L, Laporte MF, Leonil J, Dupont D, Raquin P (2003) In: Fox PF, McSweeney LH (eds) Advanced dairy chemistry. Vol. 1: proteins. P. F. Fox. Kluwer Academic, New York, pp 49–138
26. de Luis R, Arias O, Puértolas E, Benedé S, Sánchez L, Calvo M, Pérez MD (2009) Milchwissenschaft 64:422–426
27. Trujillo AJ, Castro N, Quevedo JM, Argüello A, Capote J, Guamis B (2007) J Dairy Sci 90:833–839
28. Caffin JP, Poutrel B, Rainard P (1985) J Dairy Sci 68:1087–1094
29. Anema SG, McKenna AB (1996) J Agric Food Chem 44:422–428
30. Huppertz T, Fox PF, Kelly AL (2004) J Dairy Res 71:489–495
31. Considine T, Patel HA, Anema SG, Singh H, Creamer LK (2007) Innov Food Sci Emerg Technol 8:1–23
32. Law AJR, Leaver J (1999) Int Dairy J 9:406–407
33. Dannenberg F, Kessler HG (1988) Milchwissenschaft 43:139–142
34. Royer CA (2002) Biochim Biophys Acta 1595:201–209