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Original Research

Effect of pressure on the denaturation of whey antibacterial proteins

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ABSTRACT:

Lactoferrin, IgG and lactoperoxidase provide health benefits. The antimicrobial properties of these proteins permit the use of them as supplements for nutraceutical products. These properties are vulnerable during processing. High-Pressure (HP) treatment is an appealing option in contrast to the customary heat processing of food. In this research, the impact of HP treatment on the denaturation of proteins present in skim milk and whey, and in buffer, were analyzed over a pressure range of 450 to 700 MPa at 20°C. The process of denaturation was analyzed by kinetic analysis. Denaturation of lactoferrin and IgG were estimated utilizing a sandwich ELISA and radial immunodifussion, individually. Denaturation of lactoperoxidase was recorded by estimating the loss of enzymatic activity. Results obtained indicated that the effect of HP depend widely on the protein. At the point when milk was treated at 500 MPa, higher values of D-value, of 74×10^2, and 123×10^2 sec, were recorded for lactoferrin and IgG, respectively. For lactoperoxidase, no loss of activity was seen after 30 min. Lactoferrine and IgG are denatured more slowly in buffer and in milk than in whey. The stability of lactoferrin, IgG and lactoperoxidase is to be considered to use them as bioactive components in food.

Keywords:

High-pressure treatment, Denaturation, Bovine whey proteins, Antibacterial activity, Food, Kinetic analysis.

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INTRODUCTION

Whey proteins are widely used in the food industry for their technological and biological properties. Lactoferrin, IgG and lactoperoxidase are whey proteins with biological properties that may provide health benefits to consumers. The antimicrobial properties of these proteins give them the potential to be used as supplements for special foods or nutraceutical products. It is therefore important to know that technological treatments have on their structure and functionality. The usual methodology to preserve milk is the application of thermal treatments. However, these treatments also have undesirable effects such as the loss of some nutritional components and the modification of sensorial properties. Likewise, heat treatment induces the denaturation of whey proteins, which could cause changes in their functional properties. In order to avoid these adverse effects, alternative systems are being developed for food preservation that prioritize replacing heat treatments. These processing technologies called "non-thermal preservation methods" include, among others, high hydrostatic pressure treatment.

High pressure treatment is known to induce protein denaturation by altering the equilibrium between the interactions that stabilize the folded conformation of native proteins (Considine *et al*., 2007). Thus, high pressure treatment of whey protein products has shown to induce changes in proteins that modify their functional properties for different applications in the dairy industry. A better understanding of the effects of high pressure on whey proteins is still required to open new perspectives in the control of their technological and biological properties, as well as to optimize treatment conditions.

The aim of this study was to determine the effect of high pressure treatment on denaturation of minor whey proteins with antibacterial activity, lactoperoxidase, lactoferrin and IgG. The present work includes the treatment of these proteins present in skim milk and whey and as isolated proteins in buffer.

MATERIALS AND METHODS

Materials

Bovine lactoferrin and lactoperoxidase were gotten from Fina Research (Seneffe, Belgium). Lactoperoxidase substrate and bovine IgG were obtained from Sigma (Poole, UK). Fresh raw bovine milk samples were provided by Quesos Villacorona (El Burgo de Ebro, Zaragoza, Spain). Recombinant chymosin was given by Chr. Hansen (Horsholm, Denmark) (Mazri *et al*., 2012b).

Pressure treatment

Samples of the skimmed milk, whey acquired by enzymatic coagulation, and pure proteins in 150 mm NaCl, 10 mm potassium phosphate cushion, pH 7.4 (PBS) were treated from 450 to 700 MPa at 20°C in an intermittent isostatic system from Stansted Fluid Power FPG 11500 B (Stansted, Essex, UK). Post pressure treatment, samples were put away at 4°C overnight before investigation. Samples from two autonomous experiments were analyzed in triplicates for affirmation of results (Trujillo *et al*., 2007).

Measurement of protein concentration and enzymatic activity

Antisera against bovine lactoferrin and IgG were acquired in rabbits. Specific antibodies to lactoferrin were purified by immunoadsorption and conjugated with horseradish peroxidase utilizing the periodate technique (Mazri *et al*., 2012a). The concentration of IgG was estimated by radial immunodiffusion. Lactoferrin was quantified utilizing a sandwich ELISA and enzymatic activity of lactoperoxidase was measured by a spectrophotometric method using 2,2'-azino-bis (3-ethylbenzothiazoline-sulfonic acid) (ABTS) as substrate according to Mazri *et al*. (2012a and 2012b).

Calculation of D and Z values

The time required for 90% protein denaturation

at constant pressure (D value) was calculated for each treatment by regression analysis of the lines obtained by plotting the logarithm of immunoreactive lactoferrin, IgG and enzymatic activity of lactoperoxidase expressed as the percentage of the initial protein concentration against time. The D values correspond to the reciprocal of the slope of those lines. The effect of pressure on the D value was also studied: the pressure (MPa) necessary to reduce the D value by 1 logarithmic cycle (Z value) was calculated by regression analysis of the line obtained by plotting the logarithm of D values against the corresponding pressures. Z value corresponds to the reciprocal of the slope of that line (Wehbi *et al*., 2005).

Kinetic data analysis

Determination of D and Z values, the reaction order and the denaturation rate constants were performed as reported by Wehbi *et al*. (2005). The calculation of activation volumes and frequency factors were performed according to Anema *et al*. (2005).

RESULTS AND DISCUSSION

We studied the effect of different high pressure treatments on the denaturation of bovine IgG, lactoferrin and lactoperoxidase. In the case of IgG by measuring the immunoreactive concentration of the protein at different pressures and for various holding times, the degree of denaturation of lactoferrin subjected to pressure treatment was estimated by measuring the loss of reactivity with its specific antibodies using a sandwich ELISA. Conventional immunoassays relied on labeling the interaction of a protein and antibodies and,

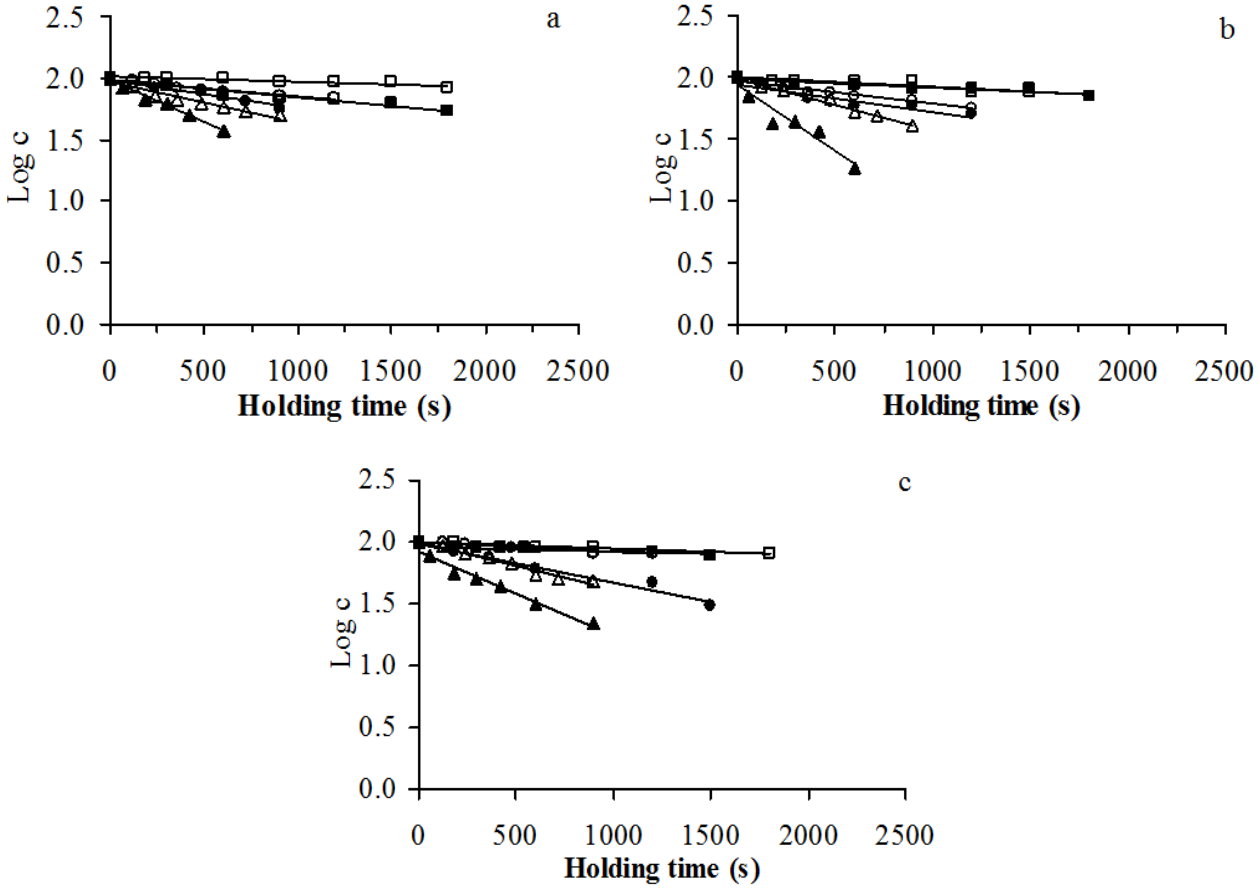

Figure 1. Effect of pressure treatment on the denaturation of IgG

(a): Milk; (b): Whey and (c): Buffer at different pressures (-·- 450, -■- 500, -▲- 550, -o- 600, -□- 650, -D- 700 MPa); c is the concentration of immunoreactive protein at each holding time expressed as percentage of the initial concentration.

in general, are particularly useful for detecting conformational changes in proteins with concomitant loss of epitopes as a result of denaturation (Tremblay *et al*., 2003). Denaturation of lactoperoxidase was evaluated by determining the residual enzymatic activity. This activity was measured by a spectrophotometric method using ABTS as the substrate. These data were evaluated using similar processes to those used in studying the thermal denaturation (Anema *et al*., 2005) to determine kinetic parameters for pressure-induced denaturation of IgG, lactoferrin and lactoperoxydase.

The decrease of proteins concentration, expressed as log of residual IgG and lactoferrin concentration, versus the holding time, are shown in Figure 1 and 2. The graphs showed results of individual experiments although mean values from two different experiments were used to calculate D values. At all pressures, immunoreactive IgG and lactoferrin decreased with time of treatment in the three media. D values decreased with the increase of pressure (Figure 1 and 2) giving Z values shown in the graphs of Figure 3.

Results obtained for the decrease of the concentration of immunoreactive IgG in pressure-treated milk indicated a loss of 45% of IgG after treatment at 500 MPa for 30 min. This reduction is in agreement with that obtained by Trujillo *et al*. (2007) for caprine IgG and by Indyk *et al*. (2008) for bovine IgG, both treated in colostrum at the same conditions. Likewise, Felipe *et al*. (1997) determined the denaturation of immunoglobulins in caprine milk and they did not observe differences in the level of

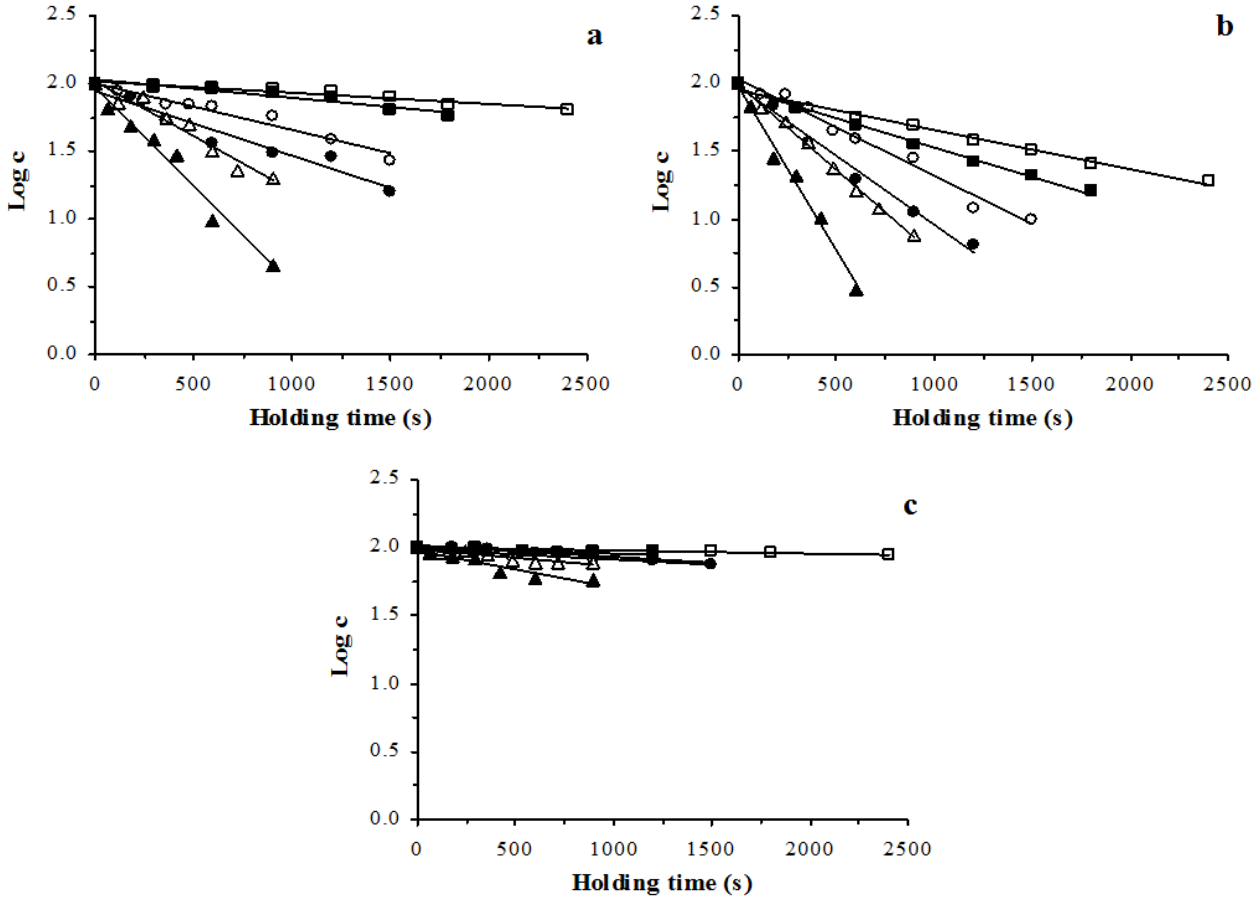

Figure 2. Effect of pressure treatment on the denaturation of lactoferrin

(a): Milk; (b): Whey and (c): Buffer at different pressures (-·- 450, -■- 500, -▲- 550, -o- 600, -□- 650, -D- 700 MPa); c is the concentration of immunoreactive protein at each holding time expressed as percentage of the initial concentration.

immunoglobulins with pressure treatments up to 300 MPa and only a small amount of denaturation at 400-500 MPa for 10 min at 25ºC.

D values obtained in this work were similar for IgG treated in milk and phosphate buffer. Furthermore, at all pressures, D values were higher when IgG was treated in milk or PBS than in rennet whey, differences among D values being lower when the pressure increased. The higher barosensitivity of IgG in rennet whey could be attributed to the presence of β-lactoglobulin which unfolds by pressure. The similar baroresistance of IgG in milk and buffer observed in this work is in disagreement with the behaviour observed by Indyk *et al*. (2008) who treated IgG in colostrum and in 150 mm NaCl, 10 mm HEPES buffer, pH 7.4. These authors indicated that the loss of IgG in bovine colostrum was less extensive than in buffer, except for the most extreme conditions, suggesting a protective role of milk or colostral components on IgG denaturation.

In the case of lactoferrin, at all pressures, D values were lower when lactoferrin was treated in whey than in milk, and were much lower in both than when lactoferrin was treated in phosphate buffer (Figure 2). These results indicated that lactoferrin is denatured more slowly when it is treated in buffer than in milk, and more slowly in milk than in whey. Furthermore, D values changed as a function of pressure (Figure 3), giving Z values of 200.1, 283.5, and 243.1 MPa in milk, whey, and phosphate buffer, respectively. These findings suggested a greater pressure dependence of the denaturation process for lactoferrin when it is treated in milk or buffer than in whey. Results obtained in this work are in accordance with those reported for heat-treated lactoferrin, which was found to be more heat-sensitive when treated in milk than in phosphate buffer (pH 7.4). This fact had been attributed to changes in the calcium phosphate bound to caseins, which changes to a more amorphous state with increasing temperature. Thus, interactions of lactoferrin with caseins would be enhanced and, consequently, lactoferrin heat sensitivity increased (Sánchez *et al*., 1992). The greater decrease of milk pH compared with that of phosphate buffer could also contribute to the low thermoresistance of lactoferrin when treated in milk.

Results obtained in the pressure treatment of lactoperoxidase from 450 to 700 MPa indicated that the enzyme is highly resistant to pressure as it was not affected by any pressure treatment applied to milk, whey, or protein solutions, even after a treatment at 700

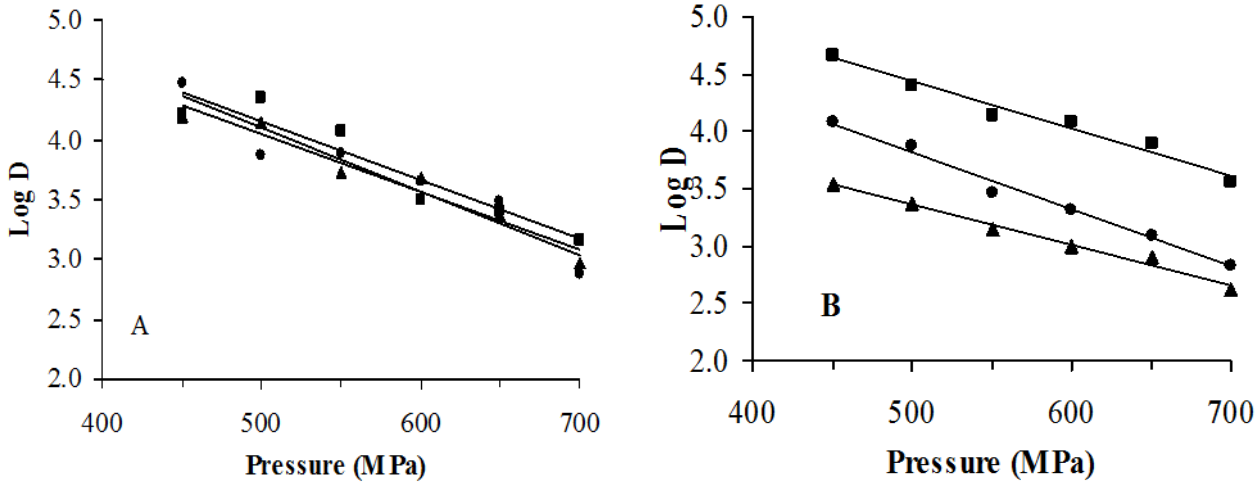

Figure 3. Effect of pressure on D-values for the loss of immunoreactivity of IgG (A) and lactoferrin (B)

milk (-·-), whey (-▲-), buffer (-■-). D value: time in seconds required for 90% protein denaturation at constant pressure; Z-value: pressure necessary to reduce D value in one logarithmic cycle.

Table 1. Kinetic parameters for pressure denaturation of IgG assuming a reaction order

S. No	MPa	Milk			Whey			Buffer		
		k	b	r^2	*k*	b	r^2	*k*	b	r^2
1	450 MPa	0.34	0.988	0.893	1.02	1.000	0.940	0.49	0.999	0.860
2	500 MPa	1.02	1.026	0.942	1.39	1.019	0.949	0.96	0.999	0.970
3	550 MPa	1.77	1.014	0.977	2.50	1.006	0.967	1.71	1.003	0.976
4	600 MPa	3.21	1.028	0.977	4.54	1.066	0.988	3.92	1.000	0.997
5	650 MPa	5.24	1.022	0.989	8.40	1.000	0.970	5.88	0.983	0.982
6	700 MPa	10.82	1.013	0.990	17.48	1.047	0.990	12.08	1.035	0.996
	Va		**- 31.7**			**- 28.1**			**- 30.9**	

n: 1.5, at different pressures; K: rate constant ($10^4 \times sec^{-1}$); b: ordinate intercept; r^2: square correlation, Va: activation volume (ml/ mol).

MPa for 15 min. Our results agreed with those previously reported, which indicated that lactoperoxidase shows extreme resistance to high-pressure treatment. Ludikhuyze *et al*. (2001) observed that no substantial inactivation, or only minor inactivation, occurred after treatment of lactoperoxidase in milk and diluted whey, respectively, at 700 MPa for 140 min at temperatures between 20ºC and 65ºC. In fact, a very pronounced antagonistic effect between high temperature and pressure was observed by those authors; that is, at 73ºC, a temperature where thermal inactivation of lactoperoxidase at atmospheric pressure occured rapidly; application of pressure up to 700 MPa completely inhibits enzyme inactivation. The fact that pressure greatly slows the rate of inactivation during the early stage of thermal treatment and indicated that a considerable increase in volume of the molecules occurs on moving from the native to the activated state during pressure treatment (Johnson and Campbell, 1945). Likewise, Rademacher and Kessler (1997) reported that even after treatment of lactoperoxidase for 4 h at 800 MPa and 40 to 60ºC, residual activities >50% were measured. They attributed the high-pressure stability of lactoperoxidase to its monomeric structure, which is stabilized by eight disulfide bonds. Seyderhelm *et al*. (1996) found that the pressure treatment of raw bovine milk at 600 MPa and 25 to 40ºC for 30 min resulted in about 20% reduction of lactoperoxidase activity. He also noted that about 70% of activity was lost when the enzyme was treated in tris buffer, pH 7, indicating that lactoperoxidase is more stable in milk than in buffer. However, we did not observe any reduction in lactoperoxidase activity in phosphate buffer after treatment at 600 MPa at 20ºC for 25 min. It was reported that the combination of high-pressure treatment and the lactoperoxidase system cause a strongly

Table 2. Kinetic parameters for pressure denaturation of lactoferrin assuming a reaction order

S. No	MPa	Milk			Whey			Buffer		
		k	b	r^2	*k*	b	r^2	*k*	b	r^2
1	450 MPa	1.06	0.979	0.943	5.26	1.017	0.992	0.25	0.998	0.877
2	500 MPa	1.77	0.963	0.921	8.17	0.972	0.997	0.46	1.002	0.950
3	550 MPa	5.54	0.967	0.921	15.26	0.810	0.954	0.91	1.020	0.951
4	600 MPa	8.97	1.007	0.945	24.79	0.838	0.990	1.02	0.984	0.940
5	650 MPa	14.59	0.901	0.935	28.94	0.812	0.972	1.61	1.016	0.896
6	700 MPa	40.14	0.805	0.934	73.87	0.701	0.906	3.75	1.021	0.910
	Va		**- 35.8**			**- 22.4**			**- 15.5**	

n: 1.5, at different pressures; K: rate constant ($10^4 \times sec^{-1}$); b: ordinate intercept; r^2: square correlation, Va: activation volume (ml/ mol).

synergistic inactivation of a wide range of gram-negative and gram-positive bacteria. Therefore, lactoperoxidase might be an interesting additional technology to improve the safety of high-pressure food preservation (García-Graells *et al*., 2003).

Pressure-induced denaturation of IgG and lactoferrin was analyzed assuming different orders of reaction. The best fit was found to be for the reaction order of n = 1.5, as indicated by the linearity obtained when representing (Ct/C0)×1-n as a function of holding time, for which the values of the correlation coefficients (r^2) were >0.86. Table 1 and 2 shows results of individual experiments corresponds to the denaturation rate constants (K) of proteins obtained in two different experiments. Different orders of reaction were published for explaining the denaturation process of IgG, subjected to heat treatment. First-order reactions were assumed by Li-Chan *et al*. (1995), whereas Law *et al*. (1994) and Mainer *et al*. (1997) considered an order of reaction of n=1.5 and Lucisano *et al*. (1994) estimated a second order reaction. The value of the reaction order n>1, for thermal denaturation of IgG could be attributed to a complex reaction involving many consecutive and/or concurrent steps (Anema and Mckena, 1996; Mainer *et al*., 1997). The same could be also assumed the for pressure-induced denaturation of IgG because under high pressure, the unfolding of protein occurs and it is followed by the formation of aggregates (Anema *et al*., 2005; Hinrichs and Rademacher, 2005). The reaction order recorder for the thermal denaturation of lactoferrin in skim milk and phosphate buffer has been accounted for to be n=1 (Sánchez *et al*., 1992). To clarify contrasts in the order of reaction of whey proteins exposed to thermal treatment, Dannenberg and Kessler (1988) proposed intermediate and consecutive reactions that would show up as a reaction of higher order. The same could be accepted for pressure initiated denaturation on the grounds that the folding of proteins happens under high-pressure conditions and is trailed by the formation of aggregates (Hinrichs *et al*., 1996; Anema *et al*., 2005). Thus, the presence of disulfide bonded aggregates consisting of lactoferrin and other whey proteins has been observed in whey protein concentrated solutions subjected to the high-pressure treatment (Patel *et al*., 2004). Differences in the reaction order of induced-denaturation of lactoferrin between thermal and pressure treatments suggested differences in the predominance of individual steps in the overall denaturation mechanism, reported for other whey proteins (Anema and McKenna, 1996, Anema *et al*., 2005).

When plotting the logarithm of the rate constants (ln k) against pressure, straight lines were obtained, for which the volume of activation (Va) and the frequency factors (ln k0) can be calculated (Table 1 and 2). The values of Va obtained for IgG and lactoferrin treated in skimmed milk, rennet whey and buffer were in the range reported for the denaturation of other whey proteins by high pressure (Anema *et al*., 2005; Hinrichs and Rademacher, 2005). In the three media, the negative Va values were obtained for denaturation of IgG and lactoferrin indicated that reactions of volume decrease were favoured by high pressure (Royer, 2002). Negative Va values also indicated that the rate of denaturation of IgG and lactoferrin increases with pressure, as it has been observed in this work.

CONCLUSION

Denaturation of IgG, lactoferrin and lactoperoxidase by pressure treatments depends on each protein, the pressure and holding time applied, as well as on the treatment medium. Baroresistance of IgG is similar when they are treated in milk and phosphate buffer, whereas a higher sensitivity is observed when treated in rennet whey.

Lactoferrin is denatured more slowly when it is treated in buffer than in milk, and more slowly in milk

than in rennet whey. Lactoperoxidase shows a high baroresistance whereas IgG and mainly lactoferrin are much more pressure sensitive proteins. The enzymatic activity of lactoperoxidase is stable when it is treated in milk, whey and buffer at all pressures and times assayed.

Denaturation of IgG and lactoferrin in the three media was found to follow a reaction order of n = 1.5. The negative values of the activation volume acquired for denaturation of lactoferrin and IgG demonstrated that reactions of volume decline are supported by high pressure. Kinetic parameters acquired in this work permitted expectation of the pressure induced denaturation of whey proteins based on pressure and holding times applied.

Results acquired ought to be considered in the plan of pressure treatments for conservation so as to keep up IgG or lactoferrin and lactoperoxydase integrity, and along these lines their biological function when they will be added to unique food or pharmaceutical items.

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