

Reduction of immunoreactivity of bovine β -lactoglobulin upon combined physical and proteolytic treatment

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Received 3 July 2001 and accepted for publication 4 February 2002

Bovine β -lactoglobulin was hydrolyzed with trypsin or chymotrypsin before, during and after treatment at 600 MPa and pH 6.8 for 10 min at 30, 37 and 44 °C. The extent of β -lactoglobulin hydrolysis under pressure was noticeably higher than at atmospheric pressure, particularly when chymotrypsin was used. Addition of proteases at ambient pressure to previously pressure-treated β -lactoglobulin gave only a modest increase in proteolysis with respect to the untreated protein. Products of enzyme hydrolysis under pressure were separated by reverse-phase HPLC, and were found to be different from those obtained at atmospheric pressure when chymotrypsin was used. The residual immunochemical reactivity of the products of combined pressure-enzyme treatment was assessed on the unresolved hydrolysates by ELISA tests using polyclonal and monoclonal antibodies, and on individual hydrolytic fractions by Western Blotting using sera of paediatric patients allergic to whey proteins in cow milk. The immunoreactivity of the whole hydrolysates was related to their content of residual intact β -lactoglobulin, and no immunochemical reactivity was found for all the products of chymotrypsin hydrolysis under pressure. The results indicate that chymotrypsin effectively hydrolysed hydrophobic regions of β -lactoglobulin that were transiently exposed during the pressure treatments and that were not accessible in the native protein or in the protein that had been previously pressure treated.

Keywords: β -lactoglobulin, high-pressure, proteolytic enzymes, protein immunoreactivity.

Bovine β -lactoglobulin (β -lg) is one of the proteins responsible for intolerance and/or allergenic response to cows' milk in humans. Although intolerance or allergy to cows' milk is usually limited to infancy and disappears spontaneously in most children at age 2–3, it represents a major challenge to paediatricians, nutritionists, and food technologists. Cows' milk formulae have been successfully used for many newborns who cannot be breastfed, but suitable formulae are necessary for those infants who develop allergy to bovine milk proteins. Cows' milk contains more than 30 proteins and many of them can induce IgE-mediated responses in allergic children. Soya and protein hydrolysed formulae are the most widely used alternatives for allergic subjects.

Hydrolysed formulae have been developed to lower or to eliminate the antigenicity of milk proteins. However, several studies have reported residual antigenic activities in both partially and extensively hydrolysed formulae (Plebani et al. 1990; Sampson et al. 1992; Ragno et al. 1993; Restani et al. 1995, 1996). Moreover, several clinical studies have shown that anaphylactic reactions can occur in severely allergic children fed extensive hydrolysates (Businco et al. 1989; Saylor & Bahna, 1991). For these reasons, it is to be hoped that new technological treatments will be developed in order to produce novel, improved-tolerance formulae.

Residual immunoreactivity in these preparations probably stems from the inability of the hydrolytic reaction to address all the sequential epitopes even in the denatured protein. High-temperature heat denaturation is commonly used in the preparation of extensive hydrolysates, because

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denaturation by itself leads to the removal of conformational epitopes (Davis & Williams, 1998), and because the concomitant thermal precipitation of denatured β -lg minimizes the amount of residual intact protein in the preparation. In spite of the apparent simplicity of this approach, formation of aggregated forms of β -lg may hide putative sites of attack from the action of proteases, therefore leaving intact some regions of the protein that may be relevant to its allergenic properties.

Denaturation of β -lg at neutral pH by physical means is a complex phenomenon, that occurs through a series of intermediate steps, the kinetics and equilibrium of which are dependent on treatment conditions, on protein concentration and interaction with other components in complex systems such as milk and whey. Most of the steps occurring below a given threshold intensity for physical treatment (temperatures below 60–65 °C or pressures below 600 MPa) and resulting in spectroscopically or analytically detectable modifications of β -lg are known to be reversible in solutions of pure β -lg at neutral pH (Cairolì et al. 1994; Iametti et al. 1997). Transient β -lg conformers are formed by both the physical treatments mentioned above, and the properties of these conformers have been investigated in some detail (Iametti et al. 1996, 1997; Roefs & De Kruif, 1994).

The β -lg conformers transiently formed during a physical treatment of sub-denaturing intensity may represent good substrates for the action of proteases, since ample regions of the hydrophobic protein core are unfolded and solvent-exposed (Cairolì et al. 1994), thus making even the innermost parts of the protein accessible – at least in principle – to enzymatic hydrolysis. In more advanced steps of physical denaturation at neutral pH (for instance, at temperatures above 65 °C) collapse of the hydrophobic portion of the structure may occur (Cairolì et al. 1994), possibly making the same enzyme attack sites inaccessible again.

In this study, we used high pressure as the physical denaturant, since the intensity threshold of pressure treatment appears less critical than that of thermal processing with respect to the occurrence of irreversible changes in the aggregation state of β -lg, and to the sensitivity of the aggregation process to protein concentration (Iametti et al. 1995).

The literature reports the increased sensitivity of pressure-treated β -lg to proteases added either after or during the pressure treatment itself. Most of the available studies were limited to pressures around 300–400 MPa, and were characterized by the use of very long incubation times, typically leading to extensive protein hydrolysis and to enzyme inactivation (Dufour et al. 1995; Van Willige & Fitzgerald, 1995; Stapelfeldt et al. 1996; Maynard et al. 1998). Some of the earliest studies in this field have already addressed the possibility of combining pressure and enzyme hydrolysis to reduce the allergen content of whey (Okamoto et al. 1991). However, only preliminary studies of a comparative nature were carried out on short proteolytic/pressure treatment of food proteins, including β -lg, in conditions where the effects of pressure on the protein structure were known (Bonomi et al. 1999, 2000).

Following those preliminary guidelines, in this work we tested different enzymes for the combined proteolytic/pressure treatment of β -lg at relatively high enzyme/ β -lg ratios (1/20 w/w), and at the highest pressure/time combinations compatible with at least partial retention of enzyme activity. A possible synergy between pressure and temperature was also investigated. Our goal was to maximize hydrolysis during the physical treatment, while producing hydrolytic fragments fulfilling at least the minimum requirement for increased tolerability, that is, loss of detectability by suitable sera or antibodies in immunochemical tests *in vitro*.

Materials and Methods

Chemicals and proteins

Chemicals were reagent or HPLC grade. Unless otherwise specified, buffer was 50 mM-sodium phosphate, pH 6.8. β -lg was from Sigma (Sigma Chemical Co., St. Louis, MO 63187, USA). Each protein batch was tested, as received for content of multimeric forms and disulphide-linked dimers, by HPLC gel-permeation and SDS-PAGE under non-reducing conditions. Bovine pancreatic trypsin (TPCK treated, type XIII), chymotrypsin (TLCK treated, type VII), bromelain, thermolysin (protease, type X), and the synthetic substrates for assaying proteolytic activities were also from Sigma. The activity of bromelain and trypsin was measured on benzoyl-L-arginine-*p*-nitroanilide, and the activity of chymotrypsin and thermolysin on N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Monoclonal antibodies were prepared according to Bonde et al. 1991.

Pressure treatment, proteolytic procedures, and sample handling

High pressure treatments were performed on 5 ml of a 2.5-mg/ml β -lg solution in buffer, sealed in a plastic bag before pressure treatment. An aliquot of each sample was kept as a non-pressurized control at the same temperature reached by the sample in the pressure vessel. Pressure treatment was carried out in a vessel thermostatted by external circulation of water at different temperatures (25, 30 and 37 °C) in an isostatic high-pressure generator ABB Model QFP-6 (Flow Pressure Systems, Västerås 72166, Sweden), set at 600 MPa. Under our conditions, temperature increase was in the range of 2 deg C/100 MPa, so that upon reaching 600 MPa the sample temperatures were ~30, 37 and 44 °C. Pressurization took between 60 and 90 s depending on the final pressure achieved. During the holding time (which is referred to as the actual treatment time), and the following instantaneous decompression, the temperature of samples decreased. The temperature and pressure profile in the course of a high-pressure treatment in the conditions used in the present study is shown in Fig. 1, for a treatment carried out at an average sample temperature of 44 °C (exterior circulating water at 37 °C).

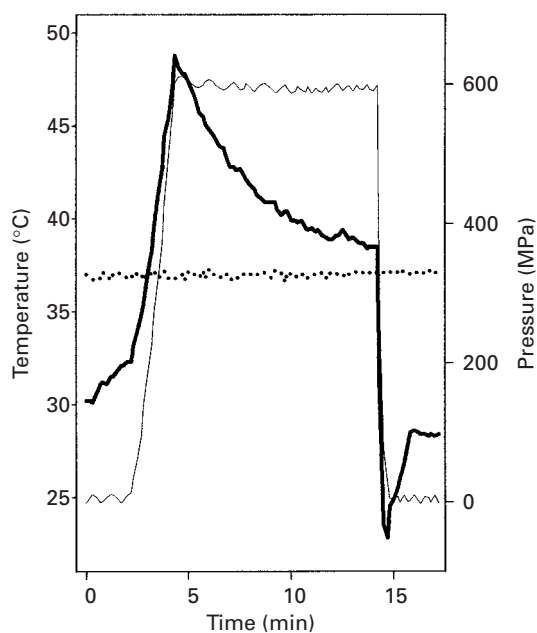


Fig. 1. Changes in pressure and sample temperature during a high-pressure treatment at 600 MPa for 10 min at an average sample temperature of 44 °C. Thin line, pressure. Thick line, sample temperature. Dotted line, temperature in the circulating exterior water, set at 37 °C.

When appropriate, proteolytic enzymes were added at 0.1 mg/ml (a 1/20 mass ratio to β -lg, corresponding to a molar ratio β -lg monomer/enzyme of about 30). When proteolysis was carried out during the pressure treatment, a β -lg/protease mixture was prepared immediately before the pressure treatment and kept on ice until placed in the pressure chamber. Immediately after the pressure treatment, treated solutions were placed on ice, and split into three aliquots. One aliquot was diluted ten-fold into trifluoroacetic acid (1 g/l) for chromatographic separations and ELISA measurements, another was diluted with an equal volume of 200 g/l trichloroacetic acid and centrifuged to precipitate proteins, and a third was frozen in liquid nitrogen. All these manipulations were carried out within 2 mins from the end of pressure treatment.

Analytical determinations

Enzyme activities were determined spectrophotometrically with the synthetic substrates listed above. In the case of pressure treated enzymes, residual activity was assessed after thawing the frozen protein samples.

Reversed-phase (RP) HPLC separations were performed directly on the sample aliquots diluted into 1 g/l trifluoroacetic acid. A Deltapak-C18 column (3.9 × 150 mm; Waters Corporation, Milford, MA 01757, USA), fitted to a Waters 625 HPLC equipped with a Waters 490E dual wavelength detector was used. Elution of hydrolytic products and residual non-hydrolyzed protein was performed by applying a linear gradient from 200 to 600 ml acetonitrile/l

trifluoroacetic acid (1 g/l) in 30 min. Detection was at 220 nm. The amount of residual non-hydrolyzed protein was quantified by on-line integration, using untreated β -lg as a standard.

Non-reducing SDS-PAGE was performed on the precipitates obtained by addition of trichloroacetic acid to the treated material. The precipitates were washed with trichloroacetic acid (1 g/l), carefully drained, and suspended in sample buffer (0.25 M-Tris-HCl buffer, 225 g glycerol/l, 40 g SDS/l, pH 6.8,) at a final protein concentration of 2–5 mg/ml. Separation was performed by using a gradient running gel (9–19% polyacrylamide) prepared according to Restani et al. (1995). After the electrophoresis, gels were stained with Coomassie Brilliant Blue G-250 according to Neuhoff et al. (1988). All materials and instruments were from Bio-Rad (Bio-Rad Laboratories, Hercules, CA 94547, USA). Image analysis was performed after high-resolution scanning of the stained gels.

Immunochemistry

After SDS-PAGE, immunoblotting was performed as described previously (Restani et al. 1995, 1996). After performing protein transfer and appropriate blocking procedures, the blocked membranes were immersed in 10 ml 2.5 g gelatine/l solution containing 0.3 ml of a patient's serum. Detection of bound antibody was performed by using a phosphatase-conjugated goat anti-human IgE and a developing solution containing alkaline phosphatase substrates.

ELISA tests were performed as competitive capture ELISA by using β -lg-specific monoclonal or polyclonal antibodies as capture antibodies (Rosendal & Barkholt, 2000). Polyclonal rabbit antibodies were diluted 4000–10 000 times in carbonate buffer (15 mM- Na_2CO_3 –35 mM- NaHCO_3 , pH 9.6) and coated directly to the wells of microtiter plates, 0.1 ml/well. When using monoclonal antibodies, polyclonal anti-mouse antibodies (Z109, 1 : 1000, 0.1 ml/well, DAKO A/S, 2600 Glostrup, Denmark) were used for coating followed by incubation of 0.1 ml/well of monoclonal antibody diluted to approximately 250 ng/ml in PBS buffer (PBS-T; 1.5 mM- KH_2PO_4 –6.5 mM- Na_2HPO_4 , 0.5 M- NaCl , 2.7 mM- KCl , 1 ml Triton X-100/l). These steps were carried out at 4 °C, and all subsequent steps at room temperature. Plates were washed four times with PBS-T buffer diluted 1 : 10, and the various samples diluted in PBS-T were applied to the wells in serial two-fold dilutions and incubated for 1 h. After four washes, the plates were incubated with a fixed concentration of biotinylated β -lg (10–200 ng/ml, depending on the antibody). After incubation and another four washes, plates were incubated with horse radish peroxidase labelled streptavidin (HRP-streptavidin, DAKO, diluted 1 : 5000 in PBS-T), and washed four times. Bound HRP activity was measured by using a substrate-containing buffer (0.2 M-potassium citrate, 3 mM- H_2O_2 , 0.6 mM-3,3',5,5'-tetramethylbenzidine; Sigma). The reaction was terminated by addition of 2 M- H_3PO_4 , 0.1 ml/well. Absorbance at 450 nm was determined

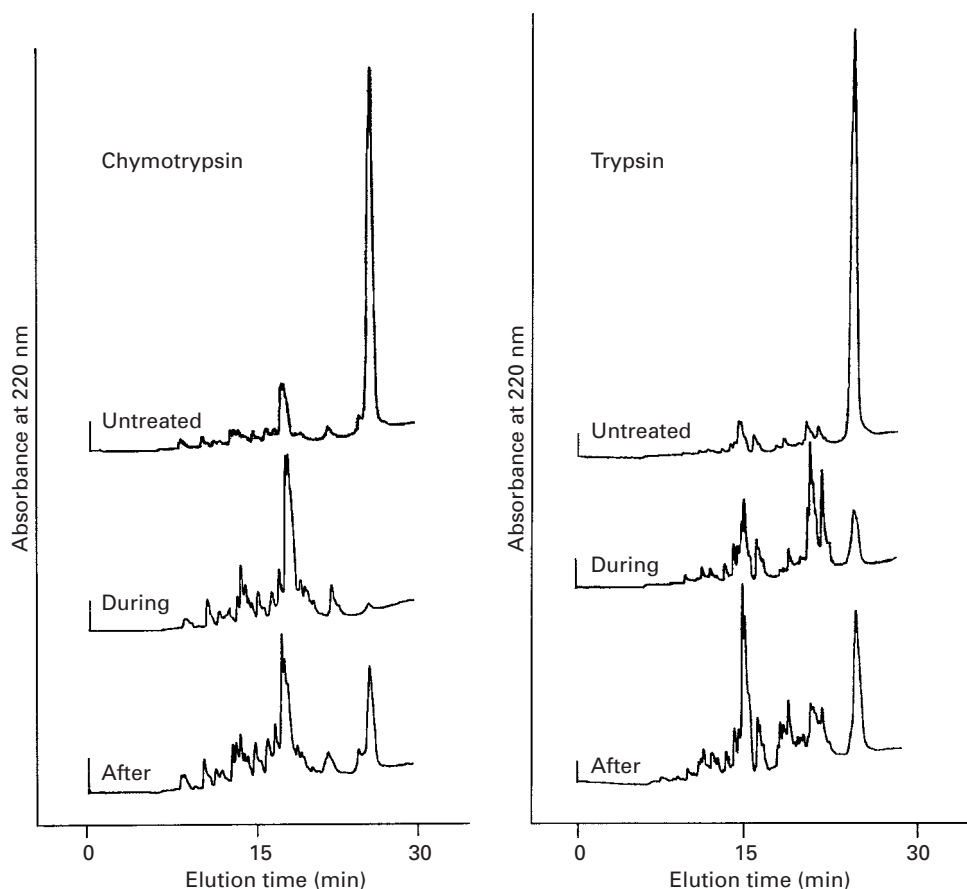


Fig. 2. RP-HPLC separation of the products of β -lactoglobulin hydrolysis at 37 °C before, during and after a pressure treatment at 600 MPa for 10 min. Concentrations: 2.5 mg β -lg/ml buffer and 0.125 mg protease/ml. Chromatographs are offset for clarity.

on a microtiter plate reader (EL312el, Bio-Tek Instruments, Inc., Winooski, VT 05404, USA).

Results and Discussion

Proteolysis under pressure

In the absence of a denaturation step, β -lg appeared to be virtually resistant to hydrolysis under the conditions used in this study, since less than 10% of the native protein was degraded at atmospheric pressure by trypsin, chymotrypsin, bromelain, and thermolysin, each added at the same weight ratio to the substrate protein.

When treatment with proteases was performed concurrently with high-pressure treatment at 600 MPa, bromelain was completely ineffective in β -lg hydrolysis, and the low-specificity thermolysin left 86% of the β -lg intact. Trypsin and chymotrypsin gave much higher hydrolysis figures (see below). The barostability of the four enzymes was also very different, based on residual activity using synthetic substrates at the end of a pressure treatment in the absence of substrate protein. We found bromelain to be relatively barostable at neutral pH (88% residual activity after 10 min at 600 MPa), whereas thermolysin – a remarkably

thermostable protease – lost ~90% activity under the same conditions, confirming previous reports on the isolated protein (Kunugi et al. 1997). Residual activity for the two pancreatic enzymes was 53% (trypsin) and 16% (chymotrypsin) under the same conditions. In view of these results, and of their higher specificity, we focused on trypsin and chymotrypsin.

RP-HPLC chromatograms (Fig. 2) show that the products obtained when proteases were present during pressure treatment may be different in nature (composition) or in relative abundance from the hydrolysis products obtained at atmospheric pressure from native or pressure-treated β -lg. Compositional differences were particularly evident in the case of trypsin, suggesting different cleavage sites were available to this enzyme under pressure (compared with native or pressure-treated protein). Fig. 2 also shows that treatment conditions affect the amount of residual intact protein.

Amounts of intact protein remaining after enzymatic hydrolysis before, during and after treatment at 600 MPa are presented in Table 1.

The sensitivity of β -lg to proteolytic action at ambient pressure was increased ten-fold (trypsin) and four-fold (chymotrypsin) after 10 min treatment at 600 MPa. Our

Table 1. Hydrolysis of β -lactoglobulin during pressure treatment at different temperatures. β -lg concentration, 2.5 mg/ml buffer. Proteolysis was carried out with 0.125 mg/ml enzyme during pressure treatment at 600 MPa or at atmospheric pressure (0.1 MPa) for 10 min at the given temperature. Residual intact β -lg was determined by RP-HPLC

Values are means $\pm$ SD for $n=$ three separate experiments

	Residual β -lg, % of initial					
	Trypsin			Chymotrypsin		
	30 °C	37 °C	44 °C	30 °C	37 °C	44 °C
At 0.1 MPa	3 $\pm$ 1	13 $\pm$ 3	14 $\pm$ 4	10 $\pm$ 4	24 $\pm$ 4	26 $\pm$ 6
During treatment at 600 MPa	69 $\pm$ 3	89 $\pm$ 4	91 $\pm$ 3	63 $\pm$ 5	97 $\pm$ 2	98 $\pm$ 1
At 0.1 MPa, after treatment of β -lg at 600 MPa	29 $\pm$ 4	n.a.	n.a.	38 $\pm$ 5	n.a.	n.a.

n.a., not available

findings confirm previous reports on increased accessibility to proteases of pressure-treated β -lg (Staplefeldt et al. 1996; Maynard et al. 1998). After pressure treatment of pure β -lg under the same conditions used here, very modest irreversible changes in the protein structure were detected by spectroscopic, analytical, and separation techniques (Iametti et al. 1997). The increased sensitivity to proteolysis of pressure-treated protein could indicate that some of the residues specifically recognized by each protease were left exposed in the treated protein even in the absence of spectroscopically-detectable structural modifications. Chymotrypsin gave an extent of proteolysis similar to that of trypsin after pressure treatment of β -lg. This is somewhat surprising, since both enzymes were added in almost identical molar ratio to β -lg, but the specific activity of trypsin (on synthetic substrates) was $\sim$ 200-fold higher than that of chymotrypsin. We had no evidence for a transient change in catalytic activity under pressure for either enzyme when pressure-treated in the presence of synthetic substrates (not shown). All these observations confirm earlier reports on the hydrolysis of pressure-treated proteins (Heremans & Heremans, 1989; Ruan et al. 1997; Iametti et al. 1998a, 1999), and provide yet more evidence that the accessibility of cleavage sites in the protein substrate – rather than the intrinsic catalytic ability of a given protease – is what limits the effectiveness of the enzyme action on actual protein substrates.

When proteases were present during pressure treatment, hydrolysis of β -lg was extensive, in spite of significant irreversible pressure inactivation of the enzymes and the pressure-induced pH decrease in phosphate buffer (Kuntz & Kauzmann, 1974).

Pressure treatment was shown to promote transient modifications of β -lg structure at neutral pH, such as transient dimer dissociation with concomitant exposure of hydrophobic sites and charged residues at the dimer interface (Iametti et al. 1997; Moller et al. 1998). These transiently modified intermediates are probably responsible of the irreversible interaction of β -lg with other milk proteins when whole milk or milk fortified with whey protein concentrate are treated at high pressure (Needs et al. 2000a, b). Our data

show that the transient conformers originating in the course of pressure treatment expose novel access sites for either enzyme. Another observation is that the extent of hydrolysis in these conditions was similar with both enzymes, in spite of chymotrypsin being much less pressure-resistant than trypsin. A protective effect of β -lg against pressure inactivation of trypsin was reported (Maynard et al. 1998), although in those experiments the concentration of β -lg was 50 mg/ml.

Pressure is not the only physical treatment capable of generating transient conformers of β -lg. Reversible structural modification of high-order structures of β -lg has been shown to occur during treatment at temperatures below 65 °C (Cairolì et al. 1994; Iametti et al. 1995, 1996, 1997, 1998b). This prompted us to verify the effects on the proteolysis by trypsin and chymotrypsin during simultaneous application of temperature and pressure.

In these experiments, we aimed to keep the intensity of each physical treatment well below the known thresholds of irreversible denaturation in thermal or pressure treatments in the system and conditions under investigation, although no detailed information is available as for a possible synergism of these treatments in applications other than food sterilization.

As shown in Table 1, a temperature increase from 30 to 37 °C had significant effects on the extent of proteolysis by both enzymes at ambient pressure, whereas a further increase to 44 °C did not substantially increase proteolysis. An almost identical trend was observed at 600 MPa, with little improvement of proteolysis when the temperature was raised from 37 to 44 °C. Proteolysis by chymotrypsin was particularly sensitive to temperature: 10 min at 600 MPa and 37 °C were sufficient to achieve almost complete proteolysis of β -lg. This confirms that hydrophobic residues or regions are among the main target for conformational modifications induced by both pressure and temperature. It should be noted that only very modest and fully reversible structural modifications were detected at these temperatures by protein chemistry or by spectroscopy of pure β -lg solutions. The only remarkable molecular event observed in this temperature range was dimer dissociation (Iametti et al. 1996), that resulted in the exposure of hydrophobic residues

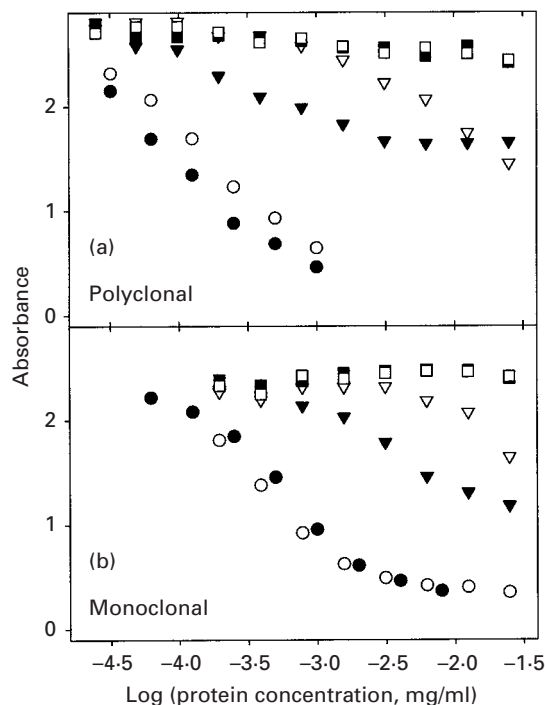


Fig. 3. Residual immunochemical reactivity of β -lactoglobulin following combined pressure/enzyme treatment. ELISA assay using (a) rabbit antiserum IV or (b) monoclonal antibody M 5G6. Hydrolysis with either ▼ ▽, trypsin or ■ □, chymotrypsin was performed at 37 °C (solid symbols) or 44 °C (open symbols). Controls were ●, untreated β -Ig and ○, pressure treated β -Ig with no enzymes added.

along the 'I' strand of the β -fold, and of positively charged residues on the edge of the large α -helix in each monomer (Brownlow et al. 1997). Very recently, preliminary evidence has been obtained that a heat-labile structural domain of the protein is modified irreversibly in this temperature range (Fessas et al. 2001), and that the same domain could be relevant to the stabilization of high-order associated forms of the native protein in solution (Iametti et al. 1998b).

Immunochemical properties of the proteolysis products

One of the goals of this work was to produce peptide fragments of sizable mass, but which are unrecognized by specific antibodies by standard immunochemical techniques. As shown in Fig. 3, pressure treatment alone resulted in a marginal decrease in the immunoreactivity of β -Ig, confirming previous observations on β -Ig and other proteins (Bonomi et al. 1999, 2000; Iametti et al. 1998a, 1999). However, a marked decrease in immunoreactivity (no less than ten-fold with respect to the native protein) was observed in the β -Ig samples treated under pressure in the presence of chymotrypsin or trypsin. A synergistic effect of temperature increase from 37 to 44 °C in reduction of immunoreactivity was observed only for trypsin. As reported in Table 2, the decrease in immunoreactivity after the combined treatment

was higher when chymotrypsin was used, and is consistent with the amounts of residual intact β -Ig reported in Table 2. Minor differences in the specificity of the various mono- and polyclonal antibodies made it evident that there were some differences in the immunoreactivity of the hydrolysis products obtained under the various treatment conditions.

The amount of residual protein detected by SDS-PAGE (Fig. 4) in the proteolyzed samples was consistent with the figures reported in Table 2. The extensive proteolysis observed with chymotrypsin gave rise to appreciable amounts of proteolytic products capable of being retained by the gel. The estimated mass of the most abundant product of chymotryptic hydrolysis was ~6000 Da. Hydrolysis by trypsin under pressure resulted in the production of several peptides with a molecular mass ranging from 5000 to 16 000 Da.

Confirming previous reports on pressure treatments at lower temperatures (Funtenberger et al. 1997; Iametti et al. 1997), pressure-induced formation of soluble, covalently linked β -Ig aggregates (detected by SDS-PAGE) was modest. This finding is consistent with the hypothesized role of hydrophobic interactions in the early phases of β -Ig aggregation upon physical treatment (Cairolì et al. 1994; Roefs & De Kruif, 1994; Iametti et al. 1995, 1996). However, no formation of these covalently linked aggregates was observed in the enzyme-treated samples, indicating that proteolytic events took place more rapidly than protein aggregation. Apparently, pressure-induced unfolding also allowed the small fraction of covalently-linked β -Ig aggregates present in the native protein to be unfolded to the point where degradation by either enzyme occurred.

To verify the immunochemical reactivity of the large-sized fragments present in enzyme/pressure treated β -Ig and retained in SDS-PAGE gels, Western blots were performed with the unfractionated sera from paediatric patients allergic to whey proteins (Fig. 5). The most striking result was the complete lack of recognition of the large-sized hydrolytic product obtained with chymotrypsin. The apparently much higher recognition of covalently linked polymeric forms of β -Ig compared with native protein may be because the amount of β -Ig-bound antibody in immunoblots depends on the size of the protein being recognized, or because β -Ig is presented primarily in such polymeric forms to the immune system of the human gut. Incidentally, this peculiar feature of the immunoblotting approach confirms the small but appreciable formation of aggregates upon pressure treatment (Funtenberger et al. 1997; Iametti et al. 1997), and confirms the absence of any covalent β -Ig aggregates in the samples treated with chymotrypsin.

One of the two sera did barely recognize some intact β -Ig present in the hydrolysate obtained with trypsin under pressure. Since this particular serum also showed a decreased reactivity towards β -Ig that was pressure-treated in the absence of proteases, it is possible that this antiserum recognized conformational epitopes more specifically than sequential ones.

Use of relatively barostable enzymes during high-pressure treatment made it possible to attack hydrolytic sites

Table 2. Effects of various treatments on the residual immunoreactivity of β -lg as detected by different antibodies

Values are protein concentration in the original sample resulting in a 50% decrease in the absorbance in ELISA tests (I_{50} , $\mu\text{g/ml}$) and are estimated from plots similar to those presented in Fig. 3

Antibody	None	HP only 37 °C	HP+trypsin		HP+chymotrypsin	
			37 °C	44 °C	37 °C	44 °C
Rabbit antiserum I	0.04	0.10	0.32	7.08	50.1	25.1
Rabbit antiserum IV	0.14	0.26	19.8	46.4	n.d.	n.d.
Monoclonal M 5G6	0.43	0.54	5.91	32.7	n.d.	n.d.
Monoclonal M 9G10	1.08	0.87	>90	>120	n.d.	n.d.

n.d., not detectable

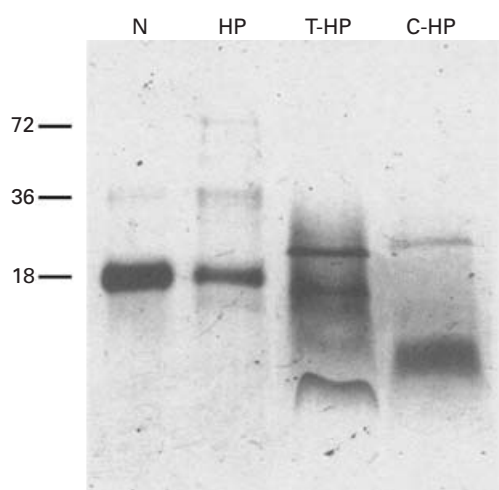


Fig. 4. SDS-PAGE under non-reducing conditions before and after treatment of β -lactoglobulin at 600 MPa for 10 min with or without protease. Concentrations: 2.5 mg β -lg/ml buffer and 0.125 mg protease/ml. Sample temperature was 37 °C. Samples and nominal protein loads, as expected from the protein content of the untreated samples, are as follows: N, native β -lg (12.5 μg); HP, pressure-treated β -lg (7.2 μg); T-HP, pressure treated with trypsin, β -lg (25 μg); C-HP, pressure treated with chymotrypsin, β -lg (12.5 μg). Molecular sizes of different aggregation states of β -lg are indicated.

that are not available in native β -lg or previously pressure-treated β -lg. When proteases specific for positively charged residues (i.e. trypsin) were used, the combined treatment generates products that are remarkably different from those produced by hydrolysis at atmospheric pressure of the native and of the pressure-treated protein (Maynard et al. 1998). With either protease, even the largest hydrolytic products were no longer recognized by β -lg-specific antibodies or by the sera of paediatric patients allergic to β -lg.

From a molecular standpoint, we ascribe the unique effects of the combined enzyme/pressure treatment to generation of transient β -lg conformers, exposing otherwise inaccessible residues to enzyme action. The molecular nature and structural location of these target residues are currently being investigated.

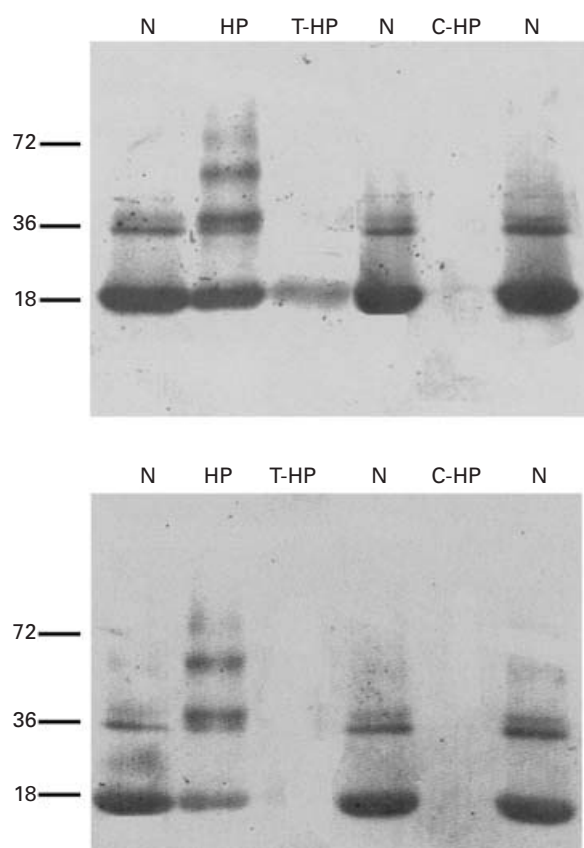


Fig. 5. Western blots for β -lactoglobulin before and after treatment at 600 MPa for 10 min with or without protease. Concentrations: 2.5 mg β -lg/ml buffer and 0.125 mg protease/ml. Sample temperature was 37 °C. Serum from each of two different paediatric patients allergic to β -lg was used to assess immunochemical reactivity in each blot. A sample volume corresponding to 12.5 μg β -lg was loaded in each lane. Treatment of samples is identified as follows: N, native β -lg; HP, β -lg pressure treated in the absence of proteases; T-HP, β -lg pressure-treated in the presence of trypsin; C-HP, β -lg pressure-treated in the presence of chymotrypsin.

From a practical standpoint, the major finding presented in this work is the absence of any residual immunochemical recognizability in the products of chymotrypsin hydrolysis under pressure. This could have relevant practical

application, because some of the hydrolysis products were of relatively high molecular size. However, other tests (e.g. skin prick tests, oral challenge to sensitive patients and *in vitro* assessment of the effects on cultured cells) will have to be performed to ensure that the products of the combined treatment are neither allergenic nor immunostimulating. On the other hand, the peptides derived from the combined treatment could possibly display novel interesting bioactivities, as observed for products of casein hydrolysis (Meisel & Bockelmann, 1999).

Combining pressure and temperature treatments to modulate the degree of hydrolysis could extend the possibilities for reducing or removing the immunoreactivity of β -lg, in conditions where no adduct formation with other milk components, such as lactose or caseins, has been reported. Pressure treatment does not promote any covalent protein modification other than disulphide exchange reactions (Rondanini et al. 2000).

Practical application will require refinement of treatment conditions, e.g. choice of enzymes and other parameters (length of hydrolysis, β -lg concentration, and enzyme/protein ratio). If extended to treatment of sweet whey or whole milk, interference by other proteins and non-protein components will have to be assessed.

This work was supported by grants from the Italian Ministry of Agricultural and Forestry Policies (MiPAF, Rome, Italy, to FB) and from the University of Milan (to SI). This is publication n. 2 of the project TOLLELAT.

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