

Hydrolysis under High Hydrostatic Pressure as a Means To Reduce the Binding of β -Lactoglobulin to Immunoglobulin E from Human Sera

R. CHICÓN,¹ J. BELLOQUE,¹ E. ALONSO,² P. J. MARTÍN-ÁLVAREZ,¹ AND R. LÓPEZ-FANDIÑO^{1*}

¹*Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain; and*

²*Hospital General Universitario Gregorio Marañón, Doctor Esquerdo 46, 28007 Madrid, Spain.*

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ABSTRACT

Cows' milk allergy is the most frequent food allergy in children, and β -lactoglobulin (β -Lg) is a major allergen. Milk-based hypoallergenic ingredients are manufactured by enzymatic hydrolysis, a process that could be improved by the application of high-pressure treatments. This study showed that the treatment of β -Lg dissolved in buffer with chymotrypsin and trypsin under high pressure for relatively short times accelerated proteolysis by leading to a rapid removal of the intact protein. The rapid proteolysis of the β -Lg substrate under pressure led to the production, in 20 min, of hydrolysates with lower immunoglobulin (Ig) G binding than those produced in 8 h (chymotrypsin) or 48 h (trypsin) at atmospheric pressure. However, those hydrolysates retained some residual IgE-binding properties that could be traced to the preferential release, during the initial stages of proteolysis, of peptides containing IgE epitopes, such as (Val-41–Lys-60), (Leu-149–Ile-162), and (Ser-21–Arg-40). The formation of these fragments was favored when proteolysis was conducted under high pressure due to the preferential hydrolysis of Arg-40 and Arg-148 by trypsin, and Tyr-42 and Leu-149 by chymotrypsin, all located at the dimer interface of β -Lg or very close to it. Although our results do not support that trypsin and chymotrypsin under high pressure selectively address the allergenic regions of β -Lg, it is possible to select the conditions that quickly produce hydrolysates with reduced potential allergenicity that could be used in hypoallergenic foods.

β -Lactoglobulin (β -Lg), the major milk whey protein, is considered to be one of the most important milk allergens (25). β -Lg is absent in human milk, resistant to gastric acid and to proteolytic attack, and able to cross intact the intestinal mucosa. Although different processing practices have been tested to develop hypoallergenic foods, enzymatic hydrolysis of milk proteins for infant formula is the only efficient strategy that has led to marketed hypoallergenic products (11). The optimal extent of hydrolysis with respect to immunological properties and nutritive value is unknown. In fact, when using hydrolysis, the main challenge is to eliminate the allergenic epitopes while maintaining the palatability, nutritional, and functional properties of the original protein (23).

A novel approach is to use physical treatments of denaturing intensity to provide better substrates for the action of proteinases (5). Thus, under certain temperature and high-pressure conditions, β -Lg forms transient conformers, with ample regions of the hydrophobic core unfolded and new target bonds exposed to enzymatic hydrolysis, as compared with the compact native protein or the aggregated products formed upon extensive denaturation (4, 12). In fact, high-pressure treatment of β -Lg either before or, particularly during, enzyme treatments, considerably enhances its hydrolysis (4, 9, 14, 15, 17, 22). An important reduction in the immunochemical reactivity was reported in whey

protein hydrolysates obtained under high pressure with different enzymes (19, 20).

We have previously reported that pressures up to 400 MPa enhanced tryptic and chymotryptic hydrolysis of β -Lg A dissolved in buffer (6, 7). Our studies revealed quantitative differences in the hydrolysis pattern when high pressures were applied during the enzyme action, because high pressure promoted a quick attack toward β -Lg, which led to the temporary accumulation of intermediate peptide products. The specificity of these two enzymes is very different: Trypsin is specific toward positively charged amino acids, most of them located on the surface of the β -Lg molecule, and chymotrypsin is specific for hydrophobic/aromatic residues, buried in the hydrophobic core of the native protein. However, the extent of proteolysis achieved with both enzymes was very similar. This supports the hypothesis that the conformational state of the protein under high pressure determines the similar behavior of both proteinases (4). In fact, hydrolysis of β -Lg by pepsin and chymotrypsin could be related to pressure-induced changes in the structure of the protein, which showed an increased flexibility at pressures around and above 200 MPa, with the extent of unfolding increasing with the pressure level (1, 2).

Therefore, chymotryptic or tryptic hydrolysis of β -Lg under high pressure for relatively short times (5 to 20 min) can accelerate proteolysis and produce hydrolysates with high contents of high-molecular-weight peptides. These hy-

* Author for correspondence. Tel. + 34 91 5622900; Fax. + 34 91 5644853; E-mail: rosina@ifi.csic.es.

drolysates may combine reduced allergenicity with good functional properties, which makes them good candidates to be used as ingredients in hypoallergenic foods (4). In this work, we have studied the immunoglobulin (Ig) G- and IgE-binding activities of these hydrolysates and attempted to correlate these properties with the presence and relative abundance of the different peptide fragments. Our aim was to assess the potential of hydrolysis under high pressure to produce hypoallergenic foods by checking whether this process could selectively address the allergenic regions of food proteins.

MATERIALS AND METHODS

Materials. β -Lg, genetic variant A, was isolated from the pH 4.6-soluble fraction of a milk sample obtained from a homozygotic cow. The protein content of the purified preparation, as determined by the Kjeldhal method, was 82% (6). Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.21.4, 11,000 *N*-benzoyl-L-tyrosine ethyl ester [BTEE] U/mg [1 BTEE unit corresponds to ΔA_{256} of 0.001/min, when 0.55 mM BTEE is used as substrate, in 3 ml of 50 mM Tris-HCl, pH 7.8, at 25°C]), TLCK-treated chymotrypsin (EC 3.4.21.1, 55 *N*-acetyl-L-tyrosine ethyl ester [ATEE] U/mg [1 ATEE unit corresponds to ΔA_{237} of 0.001/min with 0.97 mM ATEE in 3.1 ml at pH 7.0 at 25°C]) were purchased from Sigma (St. Louis, Mo.).

Enzyme treatments at high and atmospheric pressure. For the proteinase treatments under high pressure, β -Lg (2.5 mg/ml) was dissolved in 50 mM Tris-HCl buffer (pH 6.8). The substrate was mixed with trypsin or chymotrypsin (enzyme-to-substrate ratio of 1:20) and immediately poured into Eppendorf vials (1,700 μ l), avoiding headspace, and treated at 100, 200, 300, or 400 MPa, using a 900 HP apparatus (Eurotherm Automation, Lyon, France). The pressure was raised at a rate of 2.5 MPa/s, maintained for 10 and 20 min, and released at the same rate. The temperature of the hydrostatic fluid medium was controlled by water circulating through a jacket surrounding the pressure vessel and, before pressure processing, it was set to 37°C. The temperature increase as a result of the pressure treatments was approximately 2°C/100 MPa. An increase in the temperature from 37 to 45°C does not substantially increase proteolysis, neither at atmospheric pressure nor at pressures up to 600 MPa (4). Immediately after removal from the high-pressure unit, the enzyme reactions were stopped by the addition of 0.5 M HCl to pH 3.0.

Controls were obtained by conducting the hydrolysis at atmospheric pressure (0.1 MPa) on native β -Lg at 37°C. Samples were immediately freeze-dried and reconstituted as needed for subsequent analyses. All reactions were performed in triplicate.

Preparation of peptide fractions. Semipreparative reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters Series 600 HPLC apparatus, equipped with Millennium 3.2 Software for data acquisition (Waters Corp., Milford, Mass.). A Prep Nova Pak HR C₁₈ column (inside diameter, 300 by 7.8 mm; 6- μ m particle size; 60-Å pore size) (Waters) was used. Solvent A was a mixture of water-trifluoroacetic acid (1,000:1) and solvent B a mixture of acetonitrile-trifluoroacetic acid (1,000:0.8). Operating conditions were column temperature, 30°C; flow rate, 4 ml/min; and injection volume, 300 μ l (protein concentration of approximately 2.5 mg/ml). The elution was performed with a linear gradient of solvent B in A from 0 to 50% B in 60 min, followed by washing at 70% B and conditioning of the column. Absorbance was recorded at 214 nm. Three fractions (F) were collected using a Waters Fraction Collector II: F1 (re-

tention time from 20 to 29 min), F2 (retention time from 29 to 34 min), and F3 (retention time from 34 to 44 min). The fractions were lyophilized, dissolved in water, and their protein concentrations was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.), using bovine serum albumin as standard. Then, each fraction was further diluted with water to a protein concentration of 2.5 mg/ml and 2.5 μ g/ml for RP-HPLC-tandem mass spectrometry (MS/MS) and enzyme-linked immunosorbent assay (ELISA) analyses, respectively.

RP-HPLC-MS/MS. RP-HPLC with UV detection, online with electrospray ionization (ESI) quadrupole ion trap-MS/MS instrument (ESI-MS/MS) analyses were performed as previously described by Chicón et al. (6), using an Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany) with a Hi-Pore Reversed Phase RP-318 Column (inside diameter, 250 by 4 mm) (Bio-Rad Laboratories, Hercules, Calif.) and an Esquire 3000 mass spectrometer (Bruker Daltonik, Bremen, Germany).

Indirect ELISA. Individual serum samples from 13 children with proven allergy to bovine milk proteins were collected with written consent at the hospital Gregorio Marañón (Madrid, Spain), fulfilling ethical requirements. All patients showed specific IgE antibodies toward milk proteins, determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). Individual sera were always used for IgE-binding experiments, while the pooled sera of 5 healthy, nonallergic people were used as negative control.

For the estimation of the IgE binding, high-binding polystyrene microtiter plates (Corning, Cambridge, Mass.) were used as solid support. Single wells were coated with 50 μ l of antigen (2.5 μ g/ml of β -Lg or β -Lg hydrolysates) in 0.01 M phosphate-buffered saline solution (PBS; pH 7.4), and incubated overnight at 6°C. Plates were washed three times with PBS containing 0.05% Tween 20, using a Microplate Washer (Nunc, Roskilde, Denmark). This washing system was used after each incubation step. Residual free binding sites were blocked with PBS containing 2.5% Tween 20 for 1 h at room temperature. Then, plates were incubated with 50 μ l per well of serum diluted 1:20 in PBS containing 0.05% Tween 20 overnight at 6°C. The same dilution of negative control sera was used as negative control. Rabbit anti-human IgE conjugated with horseradish peroxidase (HRP; DakoCytomation, Glostrup, Denmark) was added (50 μ l per well, diluted 1:1,000 in PBS containing 0.05% Tween 20) and incubated for 1 h at room temperature. Before the addition of the enzyme substrate, a signal amplification system based on the subsequent addition of biotin-tyramide and streptavidin-HRP was used, following the instructions of the manufacturer (ELAST ELISA amplification system, PerkinElmer, Waltham, Mass.). A solution of freshly prepared *o*-phenylenediamine dihydrochloride (OPD; from Dako-Cytomation) containing H₂O₂ was added. Plates were incubated for 30 min at room temperature in the dark, and the reaction was stopped by adding 50 μ l per well of 0.5 M H₂SO₄. Optical densities were read at 492 nm on an automated ELISA plate reader (Multiskan Ascent, LabSystems, Helsinki, Finland). ELISA determinations were carried out in triplicate and measurements averaged. A blank without antigen (PBS), the negative control, and positive controls (different concentrations of β -Lg) were included in each plate.

IgG binding of the samples was evaluated against commercial anti-bovine β -Lg IgG, following the ELISA described above, with slight modifications. Briefly, wells were coated with 50 μ l of antigen as described above. After the blocking step, plates were incubated for 1 h at room temperature with 50 μ l per well of HRP conjugated rabbit anti-bovine β -Lg (Bethyl Laboratories, Mont-

TABLE 1. IgG binding of β -Lg hydrolyzed with trypsin and chymotrypsin at atmospheric pressure^a

Time	Trypsin	Chymotrypsin
0 min	3.51 $\pm$ 0.027 E ^b	3.43 $\pm$ 0.009 E
60 min (1 h)	3.42 $\pm$ 0.013 D	2.95 $\pm$ 0.001 D
480 min (8 h)	3.15 $\pm$ 0.020 C	0.35 $\pm$ 0.004 C
1,440 min (24 h)	3.02 $\pm$ 0.032 B	0.06 $\pm$ 0.005 B
2,880 min (48 h)	2.77 $\pm$ 0.002 A	0.02 $\pm$ 0.002 A

^a IgG binding, absorbance at 492 nm in the ELISA using anti-bovine β -Lg HRP conjugate. Values are means $\pm$ standard deviations ($n = 3$).

^b Mean values in the same column with different letters indicate significant differences ($P < 0.05$).

gomery, Tex.) diluted 1:20,000 in PBS. Finally, 50 μ l per well of the OPD solution were added and incubated for 30 min.

Statistical analysis. Triplicate analyses were conducted on each sample. The IgG- and IgE-binding results were analyzed by one-way analysis of variance (ANOVA), the least-significant difference test and the two-sample Student's *t* test to compare different groups of data. A two-way ANOVA was also used to assess the influence of the pressure level and hydrolysis time. Differences were considered statistically significant when $P < 0.05$. The SPSS program (version 14.0, SPSS, Inc., Chicago, Ill.) was used for data processing.

RESULTS AND DISCUSSION

Effect of the hydrolysis under high pressure with trypsin and chymotrypsin on the IgG- and IgE-binding properties of β -Lg. When hydrolysis of β -Lg was performed at atmospheric pressure, the one-way ANOVA showed that IgG binding significantly decreased with time, both with trypsin and chymotrypsin ($P < 0.05$; Table 1). The two-way ANOVA of the data revealed the contribution of the pressure and time factors, as well as their interaction, to statistical differences in IgG binding ($P < 0.05$). Table 2 shows the mean values, with their corresponding standard deviations, for the treatments studied and the results obtained after mean comparisons.

In agreement with previous results (6, 7), simultaneous application of high-pressure and enzymatic treatments considerably accelerated β -Lg A hydrolysis. Proteolysis with chymotrypsin was faster than that with trypsin, both at at-

mospheric and high pressure, leading to a lower residual IgG binding (Tables 1 and 2). RP-HPLC analyses (results not shown) revealed that, with both enzymes, proteolysis was enhanced at pressures ≥ 100 MPa, although intact β -Lg was left after treatments at 100 MPa for 20 min. At 200 to 400 MPa, a hydrolysis time of 10 min was enough to remove all intact protein. The more extensive proteolysis at 200 MPa, as compared with 100 MPa, was reflected in the lower IgG binding of the hydrolysates ($P < 0.05$; Table 2). However, the IgG binding of the hydrolysates obtained with trypsin at 300 and 400 MPa was higher ($P < 0.05$) than that of those obtained at 200 MPa and equivalent incubation periods. This is probably due to the hindrance to further proteolysis brought about by the pressure-induced inactivation of trypsin, which has been reported to start at around 300 MPa (6, 15, 24). On the contrary, no chymotrypsin inactivation occurs in the pressure interval considered (7, 16). In fact, the hydrolysates of β -Lg A produced with chymotrypsin at 300 and 400 MPa showed an IgG-binding activity similar than those obtained at 200 MPa. An increase in the incubation time with trypsin and chymotrypsin from 10 to 20 min reduced the IgG-binding response of the hydrolysates at all pressure levels, although the differences were not statistically significant at 300 MPa (Table 2).

The main factors, pressure, time, and their interaction, also contributed to statistical differences in IgE binding ($P < 0.05$), as shown by two-way ANOVA, except for serum (S) 3 in the ELISA against trypsin hydrolysates and S2 in the ELISA against chymotrypsin hydrolysates. In fact, individual serum from patients allergic to β -Lg were shown to vary in their specificity toward the fractions (Table 3). In any case, the pressure level influenced the reactivity toward IgE of the samples in a fashion slightly different from the IgG binding, as β -Lg allergenic epitopes only partially overlap antigenic determinants (3). The proteolysis products of β -Lg A produced by trypsin under pressure exhibited a lower IgE-binding activity than those produced in 48 h at atmospheric pressure ($P < 0.05$, by the least-significant difference test). However, it should be noted that, even if no residual β -Lg was left after enzymatic treatments at 200 MPa for 10 min, the hydrolysates retained a noticeable reactivity against IgE. The lowest IgE binding corresponded to the hydrolysates produced with trypsin at 200 MPa for 20 min ($P < 0.05$). An increase in the pressure level from

TABLE 2. IgG binding of β -Lg hydrolyzed with trypsin and chymotrypsin under different high-pressure conditions^a

Pressure (MPa)	Trypsin		Chymotrypsin	
	10 min	20 min	10 min	20 min
100	1.33 $\pm$ 0.111 C b ^b	0.87 $\pm$ 0.004 B a	0.62 $\pm$ 0.059 B b	0.30 $\pm$ 0.007 C a
200	0.84 $\pm$ 0.160 A b	0.45 $\pm$ 0.003 A a	0.25 $\pm$ 0.002 A b	0.20 $\pm$ 0.001 B a
300	1.02 $\pm$ 0.029 B a	0.98 $\pm$ 0.008 C a	0.21 $\pm$ 0.001 A a	0.20 $\pm$ 0.001 B a
400	1.13 $\pm$ 0.002 C b	1.03 $\pm$ 0.016 D a	0.24 $\pm$ 0.031 A b	0.15 $\pm$ 0.003 A b

^a IgG binding, absorbance at 492 nm in the ELISA using anti-bovine β -Lg HRP conjugate. Values are means $\pm$ standard deviations ($n = 3$).

^b Mean values in the same column with different capital letters indicate significant differences ($P < 0.05$). Mean values in the same row with different lowercase letters, and for each enzyme, indicate significant differences ($P < 0.05$).

TABLE 3. IgE binding of β -Lg hydrolyzed with trypsin and chymotrypsin under different high-pressure conditions and at atmospheric pressure^a

Serum ^b	High pressure				Atmospheric pressure ^c	
	Time (min)	200 MPa	300 MPa	400 MPa	Time (min)	0.1 MPa
Trypsin						
S1	10	1.07 ± 0.123 A b ^d	0.98 ± 0.145 A a	0.98 ± 0.166 A a	2,880 (48 h)	2.97 ± 0.02
	20	0.55 ± 0.090 A a	0.69 ± 0.178 A a	1.26 ± 0.182 B a		
S2	10	2.49 ± 0.061 C b	2.00 ± 0.122 A a	2.19 ± 0.005 B b	2,880 (48 h)	3.27 ± 0.19
	20	1.61 ± 0.025 A a	1.98 ± 0.211 B a	2.06 ± 0.037 B a		
S3	10	0.26 ± 0.069 A a	0.20 ± 0.108 A a	0.27 ± 0.032 A a	2,880 (48 h)	1.32 ± 0.01
	20	0.16 ± 0.002 A a	0.20 ± 0.138 A a	0.23 ± 0.036 A a		
S4	10	1.59 ± 0.073 B b	1.44 ± 0.055 A b	1.65 ± 0.080 B a	2,880 (48 h)	2.39 ± 0.11
	20	0.92 ± 0.067 A a	0.95 ± 0.089 A a	1.82 ± 0.061 B b		
Chymotrypsin						
S2	10	1.29 ± 0.014 A a	1.02 ± 0.222 A a	1.23 ± 0.140 A a	480 (8 h)	0.74 ± 0.25
	20	0.98 ± 0.387 A a	1.24 ± 0.071 A a	1.00 ± 0.008 A a	2,880 (48 h)	0.24 ± 0.06
S5	10	2.00 ± 0.055 A a	2.46 ± 0.044 B b	2.35 ± 0.137 B b	480 (8 h)	2.23 ± 0.07
	20	1.94 ± 0.043 A a	1.78 ± 0.157 A a	1.74 ± 0.134 A a	2,880 (48 h)	0.91 ± 0.22
S6	10	1.69 ± 0.029 A a	2.19 ± 0.176 B b	2.11 ± 0.108 B b	480 (8 h)	1.36 ± 0.25
	20	1.36 ± 0.265 B a	1.87 ± 0.006 C a	0.68 ± 0.072 A a	2,880 (48 h)	0.20 ± 0.11
S7	10	0.61 ± 0.178 A a	0.90 ± 0.072 B b	0.85 ± 0.058 B a	480 (8 h)	0.43 ± 0.13
	20	0.66 ± 0.056 A a	0.64 ± 0.139 A a	0.83 ± 0.115 A a	2,880 (48 h)	—

^a IgE binding, absorbance at 492 nm in the ELISA. Values are means $\pm$ standard deviations ($n = 3$).

^b S, serum. The ELISA used sera from patients allergic to cow's milk. Specific IgE antibody titers to β -Lg in the sera used were S1 = 18.9 kU/liter, S2 = 31.9 kU/liter, S3 = 23.7 kU/liter, S4 = 20.1 kU/liter, S5 = 31.9 kU/liter, S6 = 68.6 kU/liter, S7 = 55.7 kU/liter, and S8 = 62.7 kU/liter. All sera were diluted 1:20 except for S7, which was diluted 1:50.

^c Data corresponding to the hydrolysis at atmospheric pressure are not included in the comparison.

^d Mean values in the same row with different capital letters, and for each serum, indicate significant differences ($P < 0.05$). Mean values in the same column with different lowercase letters, and for each serum, indicate significant differences ($P < 0.05$).

200 to 300 MPa led to hydrolysates with lower IgE binding only after 10 min of hydrolysis, while trypsin treatment at 400 MPa did not further reduce the reactivity toward IgE over 300 MPa. On the other hand, even if proteolysis of β -Lg with chymotrypsin was enhanced at 300 and 400 MPa over 200 MPa, in general terms, this did not result in a decreased reactivity toward human sera from allergic patients, probably because of the release of specific immunoreactive fragments that contributed to the IgE response to different extents. However, Bonomi et al. (4) reported that there was a complete lack of recognition of the large-sized hydrolytic products produced by chymotrypsin on β -Lg (under 600 MPa of pressure and at the same enzyme to substrate ratio that we used in our work) in Western blots with β -Lg-specific antibodies or with sera from allergic patients.

In an attempt to explain the residual IgG and IgE binding of the hydrolysates obtained with trypsin and chymotrypsin under pressure, the peptide pattern was studied by RP-HPLC-MS/MS.

IgE binding of the peptides released by trypsin on β -Lg under pressure. As an example, in Figure 1a and 1b the RP-HPLC chromatograms of β -Lg hydrolyzed by trypsin at 200 for 10 and 20 min are compared. The main difference observed was the disappearance, with the hydrolysis time, of three hydrophobic, late-eluting peptides that were identified as (Val-41-Lys-70) S-S (Leu-149-Ile-162),

(Val-41-Lys-69) S-S (Leu-149-Ile-162), and (Val-15-Arg-40), as well as the decrease in (Ser-21-Arg-40) and (Val-41-Lys-60). These peptides had been previously found in β -Lg hydrolysates produced by trypsin at atmospheric and high pressure (6, 8, 14, 15, 18). In addition, the comparison among the hydrolysates obtained at pressures of 200, 300, and 400 for 10 min (Fig. 1a, 1c, and 1d) shows that the late-eluting peptides were also very abundant at higher pressures. However, in agreement with previous results, these were not further proteolyses as hydrolysis proceeded from 10 to 20 min at 300 and 400 MPa, due to enzyme inactivation (6).

It could, therefore, be hypothesized that the presence of the fragments (Ser-21-Arg-40), (Val-41-Lys-60), (Val-41-Lys-70) S-S (Leu-149-Ile-162), (Val-41-Lys-69) S-S (Leu-149-Ile-162), and (Val-15-Arg-40) in the hydrolysates obtained under high pressure for 10 min could be responsible for their IgE reactivity, even once all intact β -Lg had disappeared. In fact, these are related to the tryptic β -Lg fragments (Val-41-Lys-60) and (Leu-149-Ile-162), previously identified by Sélo et al. (21) as major IgE-binding epitopes, and to (Ser-21-Arg-40), identified as intermediate binding epitope. In particular, the sequence (Leu-149-Ile-162) is known to comprise two distinct linear IgE-binding epitopes, which makes it capable to trigger IgE-sensitized mast cell degranulation (10). Following the same interpretation, residual IgE reactivity could also be

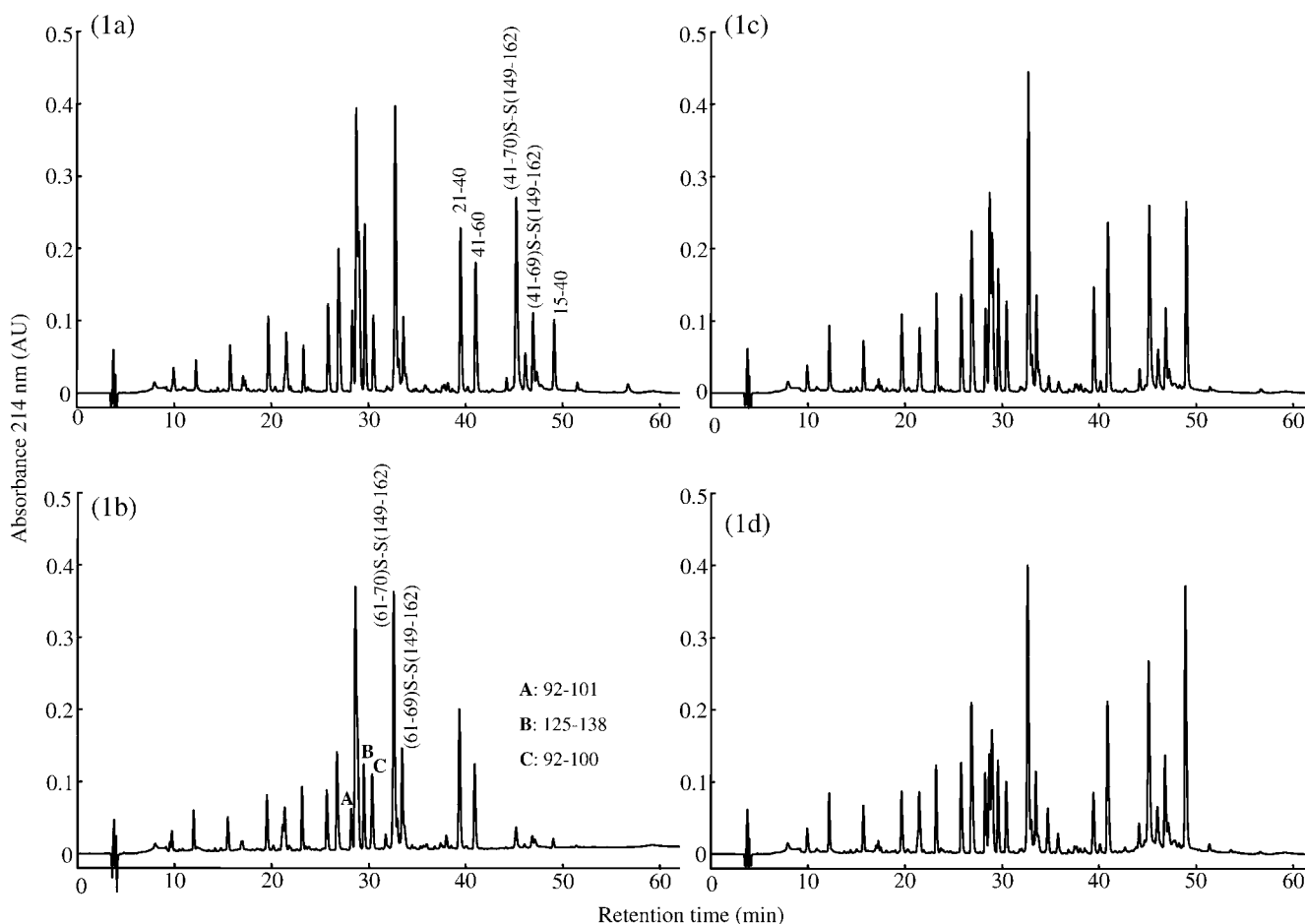


FIGURE 1. RP-HPLC patterns and relevant peptides identified by RP-HPLC-MS/MS of β -Lg hydrolyzed with trypsin at an enzyme-to-substrate ratio of 1:20 and 37°C, at 200 MPa for 10 min (a), 200 MPa for 20 min (b), 300 MPa for 10 min (c), and 400 MPa for 10 min (d).

attributed to the appearance of the hydrolysis products (Trp-61-Lys-69) S-S (Leu-149-Ile-162) and (Trp-61-Lys-70) S-S (Leu-149-Ile-162), which were major peptides in the hydrolysate produced at 200 MPa for 20 min (Fig. 1b).

IgE binding of the peptides released by chymotrypsin on β -Lg under pressure. In agreement with previous reports (7), and as it was the case with trypsin, the main differences among the peptides produced by chymotrypsin when acting under high pressure, as compared with atmospheric pressure, were also found in the RP-HPLC late-eluting peaks. These (marked with an asterisk in Fig. 2a) formed preferentially during the first 10 min of incubation with the enzyme under high pressure (in the order of 400 MPa > 300 MPa > 200 MPa) and decreased at longer incubation times. With the aim to investigate the peptides responsible for the IgE-binding activity of the chymotryptic hydrolysates of β -Lg, and in view of the fact that no previous reports were found on the IgE-binding activity of β -Lg chymotryptic peptides, the hydrolysate obtained at 400 MPa for 10 min was separated by RP-HPLC at a semi-preparative scale into three different fractions (Fig. 2). These fractions were collected after several separation rounds to obtain enough material for analyses of protein concentration, IgG- and IgE-binding activities, and RP-HPLC-MS/MS.

As shown in Table 4, the highest IgG binding corresponded to F3, followed by F2, while F1 presented a very low reactivity against IgG ($P < 0.05$). The IgE-binding properties of the fractions did not follow the same trend, because, in general terms, the highest IgE binding corresponded to F2. It should be noted that not all of the peptides might contribute equally to the IgE-binding signal found in the ELISA, as the small size of some of them may prevent their binding to the polystyrene plate. A similar behavior was found among patients less likely to outgrow their allergy (≥ 4 years old) as compared with younger patients (1 to 2 years old).

The peptides that were released to a greater extent when hydrolysis with chymotrypsin was conducted under high pressure were (Val-43-Leu-57), (Val-43-Gln-59), (Val-43-Leu-58) and two disulfide linked fragments, (Lys-60-Phe-82) S-S (Ser-150-Ile-162), and (Glu-62-Phe-82) S-S (Ser-150-Ile-162) (F3; Fig. 2d). These are related to the tryptic β -Lg fragments (Val-41-Lys-60) and (Leu-149-Ile-162) reported by Sélo et al. (21) as major IgE-binding epitopes. The two disulfide-linked fragments are coincident with the main peptides found in the early steps of chymotryptic hydrolysis of β -Lg at 55°C and atmospheric pressure by Iametti et al. (12); although, these authors did not find any residual immunoreactivity of these hydrolysis products

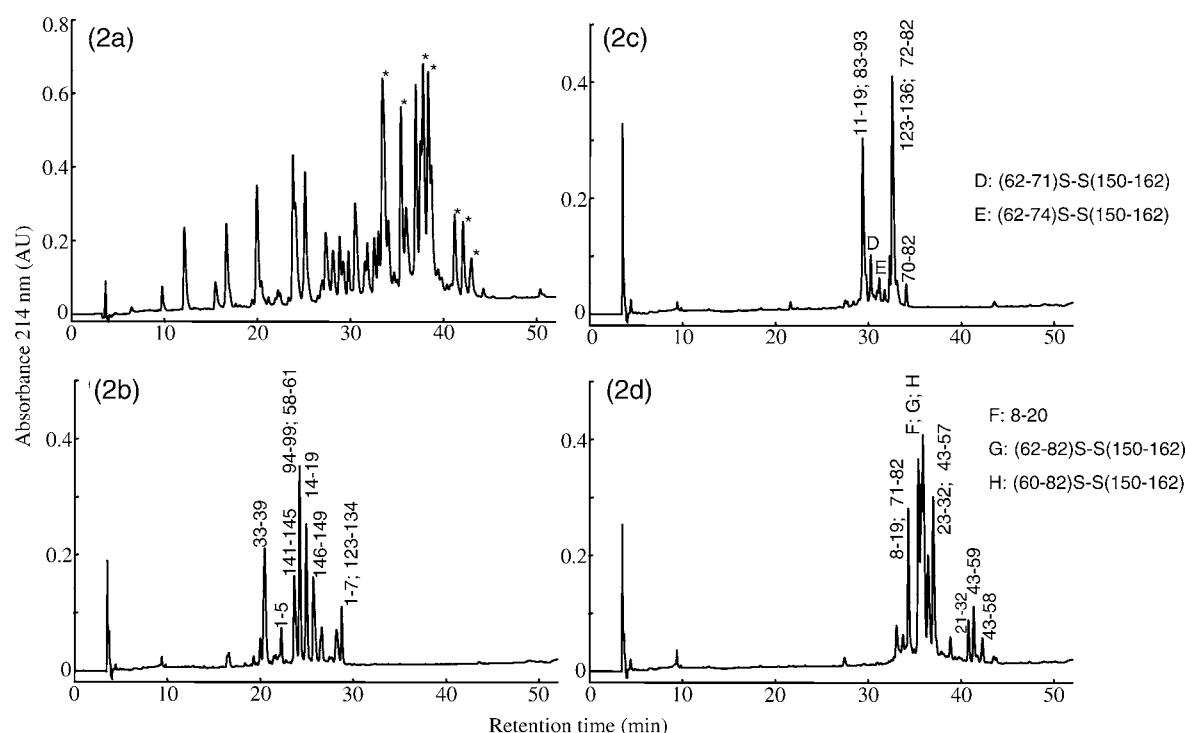


FIGURE 2. RP-HPLC patterns and relevant peptide sequences identified by RP-HPLC-MS/MS of β -Lg hydrolyzed with chymotrypsin at an enzyme-to-substrate ratio of 1:20 and 37°C, at 400 MPa for 10 min (a) and F1 (b), F2 (c), and F3 (d) isolated from the hydrolysate shown in (a) by semipreparative RP-HPLC.

against monoclonal antibodies or rabbit antiserum. In addition, F3 also contained the fragments (Ala-23–Leu-32) and (Ser-21–Leu-32), related to the intermediate reactive epitope (Ser-21–Arg-40) (21), that appeared as important transitional hydrolysis products enhanced by the pressure treatment.

F2 contained a small proportion of the fragments (Glu-62–Ile-71) S-S (Ser-150–Ile-162), and (Glu-62–Glu-74) S-S (Ser-150–Ile-162), related to the tryptic β -Lg fragment

(Leu-149–Ile-162), and the peptides (Asp-11–Tyr-20), (Asp-11–Trp-19), and (Lys-83–Leu-93) related to tryptic β -Lg fragments, classified as minor epitopes (21). However, this fraction also included the coeluting peptides (Val-123–Phe-136) and (Ile-72–Phe-82), related to the β -Lg fragments (Asp-127–Hys-146) and (Lys-75–Ala-86), regarded as the most frequently IgE-bound epitopes by Järvinen et al. (13). Furthermore, according to these authors, the peptide (Lys-75–Ala-86) was only recognized by patients with

TABLE 4. IgG and IgE binding of the peptide fractions F1 through F3 (shown in Fig. 2), obtained by semipreparative RP-HPLC from the hydrolysate of β -Lg with chymotrypsin at 400 MPa for 10 min; antibody binding to nonhydrolyzed β -Lg is also shown^a

IgG/IgE binding	Serum ^b	F1	F2	F3	β -Lg
IgG	—	-0.05 ± 0.001 A ^c	0.19 ± 0.008 B	0.30 ± 0.008 C	2.60 ± 0.02
IgE group 1 (patients 1–2 yr old)	S1	0.00 ± 0.014 A	0.79 ± 0.018 C	0.33 ± 0.169 B	1.97 ± 0.10
	S8	0.21 ± 0.054 A	1.20 ± 0.084 C	0.65 ± 0.080 B	2.50 ± 0.03
	S9	0.27 ± 0.063 A	1.58 ± 0.045 C	0.84 ± 0.069 B	2.26 ± 0.04
	S10	0.00 ± 0.013 A	0.73 ± 0.041 C	0.60 ± 0.021 B	2.31 ± 0.05
IgE group 2 (patients ≥ 4 yr old)	S2	0.64 ± 0.169 A	0.99 ± 0.118 B	0.64 ± 0.102 A	2.28 ± 0.06
	S7	0.23 ± 0.024 A	1.16 ± 0.202 C	0.88 ± 0.094 B	2.07 ± 0.07
	S11	0.00 ± 0.076 A	1.49 ± 0.195 B	1.53 ± 0.390 B	1.81 ± 0.01
	S12	0.07 ± 0.009 A	0.65 ± 0.264 B	0.62 ± 0.006 B	2.08 ± 0.02
	S13	-0.05 ± 0.010 A	1.10 ± 0.076 C	0.32 ± 0.052 B	2.07 ± 0.05
	S14	0.11 ± 0.027 A	0.63 ± 0.043 C	0.17 ± 0.005 B	1.72 ± 0.03

^a IgG binding, absorbance at 492 nm in the ELISA using anti-bovine β -Lg HRP conjugate; IgE binding, absorbance at 492 nm in the ELISA using sera from patients allergic to cow's milk. Values are means $\pm$ standard deviations ($n = 3$).

^b Specific IgE antibody titers to β -Lg in the sera used (followed by the age in years of the children in parentheses) were S1 = 18.9 kU/liter (1), S2 = 31.9 kU/liter (10), S7 = 62.7 kU/liter (4), S8 = 21.3 kU/liter (2), S9 = 16.3 kU/liter (2), S10 = 13.0 kU/liter (2), S11 = 68.6 kU/liter (4), S12 = 7.8 kU/liter (8), S13 = 49.2 kU/liter (4), and S14 = 6.8 (6). All sera were diluted 1:20.

^c Mean values in the same row with different letters indicate significant differences ($P < 0.05$). β -Lg data were not included in the comparison.

persistent cow's milk allergy. The higher reactivity against IgE of F2, as compared to F3, with higher IgG antigenicity, could be attributed to these two sequences.

In conclusion, under the present conditions, the exposure of new peptide targets due to conformational changes of β -Lg under pressure only partially facilitated the removal of allergenic epitopes. The peptides released during simultaneous proteolysis and pressurization were the result of the preferential hydrolysis under high pressure of Arg-40 and Arg-148, by trypsin, and Tyr-42 and Leu-149 by chymotrypsin, all located at the dimer interface or very close to it. This promoted the release of β -Lg fragments that contain IgE-binding epitopes such as (Val-41–Lys-60), (Leu-149–Ile-162), and (Ser-21–Arg-40). A further reduction in the IgG- and IgE-binding properties of the hydrolysates would require the hydrolysis to proceed to yield shorter fragments, similar to those produced at atmospheric pressure. Our results do not support that trypsin and chymotrypsin under high pressure selectively address the allergenic regions of β -Lg. However, it is possible to select the appropriate enzyme, pressure, and hydrolysis time conditions that quickly produce hydrolysates with potential reduced allergenicity, as compared with those obtained by conventional enzymatic hydrolysis, showing that high-pressure processing represents a viable alternative to develop hypoallergenic foods.

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