Proteolytic Pattern, Antigenicity, and Serum Immunoglobulin E Binding of β -Lactoglobulin Hydrolysates Obtained by Pepsin and High-Pressure Treatments

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ABSTRACT

This study evaluates the use of high pressure to enhance pepsin hydrolysis of β -lactoglobulin (β -LG). The protein was subjected to high pressure before and during the proteolytic process. Analysis of remnant β -LG, identification of the peptides produced, and evaluation of antigenicity (binding to commercial antibodies) and binding to IgE of allergic patients' sera were conducted in the hydrolysates. The results showed that the application of high pressure before the enzyme treatment slightly improved the proteolytic process but did not reduce the antigenicity or IgE binding of the hydrolysates. The application of high pressure during the enzymatic treatment enhanced the production of large intermediate fragments that were further proteolysed to smaller fragments as proteolysis proceeded for longer periods. At 400 MPa, all the intact protein was removed in minutes, simultaneously decreasing its antigenicity and serum IgE binding properties. However, for considerable reduction of the antigenicity and IgE binding of β -LG, extending the incubation time with the enzyme was needed to reduce the amount of potentially allergenic intermediate peptides. Changes of β -LG under pressure at acidic pH are discussed.

Key words: milk whey protein, allergen, high pressure, proteolysis

INTRODUCTION

Cow's milk allergy is the most common allergy in early childhood. In most cases, it is an IgE-mediated reaction against cow's milk proteins, with β -LG being one of the major sensitizing agents (Wal, 2001). To prevent allergic symptoms, hypoallergenic formulas are widely used. These formulas are basically protein hydrolysates, frequently manufactured with milk whey

proteins, which contain a high amount of β -LG. To efficiently remove the allergenic epitopes, extensive hydrolysis of the protein and ultrafiltration is used. Improving the proteolysis process is of interest to the manufacturing industry to achieve an optimal extent of hydrolysis with respect to allergenic properties, palatability, and nutritional and functional properties (Høst and Halcken, 2004).

β -Lactoglobulin is very resistant to enzyme digestion, particularly to pepsin, a phenomenon attributed to its unique structural stability at acid pH (Reddy et al., 1988; Dalgalarondo et al., 1995). At pH values around 2, optimum for pepsin activity, β -LG is dissociated into monomers (Townend et al., 1960), but its folding is similar to neutral pH (Uhrinova et al., 2000; Ragona et al., 2003). The structure of β -LG is basically a β -barrel where most hydrophobic amino acids, targets for pepsin, point inward and are not easily accessible to the enzyme. Different means have been used to improve β -LG digestibility by pepsin, such as chemical modification of the protein (Chobert et al., 1995), addition of denaturing agents to the proteolysis medium (Dalgalarondo et al., 1995; Guo et al., 1995) and physical treatments, such as heating (Guo et al., 1995; Iametti et al., 2002) or high pressure (Dufour et al., 1995; Stapelfeldt et al., 1996; Peñas et al., 2006).

High-pressure-treated β -LG is more susceptible to enzyme hydrolysis than the native protein (Maynard et al., 1998; Knudsen et al., 2002). In particular, if the enzyme reaction is performed under high pressure, a significant increase in the proteolysis rate is achieved with several enzymes (Dufour et al., 1995; Stapelfeldt et al., 1996; Maynard et al., 1998; Bonomi et al., 2003; Chicón et al., 2006a,b). This has been attributed to the effect of high pressure on the protein conformation, which favors unfolding, giving rise to regions more exposed to the enzyme action (Dufour et al., 1995; Bonomi et al., 2003; Belloque et al., 2007). However, under high pressure, β -LG is proteolysed by pepsin at acid pH with more difficulty than by other proteases at neutral pH (Dufour et al., 1995; Stapelfeldt et al., 1996; Peñas et

Received August 31, 2007.

Accepted November 8, 2007.

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al., 2006). This is due to the nature of β -LG, which structure becomes more flexible as the pressure increases but requires higher pressures to unfold at pH 2.5 than at pH 6.8 and, upon decompression, refolds faster at acid than at neutral pH (Belloque et al., 2007). The hydrolysates obtained by enzymatic treatment of β -LG under high pressure may exhibit reduced antigenicity, IgE binding, or both (Bonomi et al., 2003; Fritsché, 2003; Peñas et al., 2006).

Previous studies in our laboratory have investigated the peptides released by the application of high pressure and proteolysis with trypsin and chymotrypsin on β -LG (Chicón et al., 2006a,b). In contrast with these enzymes, the use of pepsin allows the evaluation of not only a different enzyme but also an acidic environment that causes structural changes on β -LG, becoming a highly stable monomer, and reduces cross-linking due to SH/SS exchange. The objective of this research was to identify the peptides released by pepsin on β -LG, during or after the application of high pressure, and to evaluate their antigenicity and IgE binding. The aim was to obtain a better insight into the behavior of β -LG under pressure and to estimate the usefulness of this process to produce hypoallergenic hydrolysates.

MATERIALS AND METHODS

Treatments with Pepsin at Atmospheric and High Pressure

β -Lactoglobulin A was isolated from the pH 4.6 soluble fraction of a milk sample obtained from a homozygote cow, as described by Ebeler et al. (1990). Proteolysis was conducted with pepsin (EC 3.4.23.1) from porcine stomach mucosa (570 units/mg; Sigma Chemicals Co., St. Louis, MO). A 2.0-mL solution containing native β -LG A (5.0 mg) and pepsin (0.25 mg) in 50 mM citrate buffer, pH 2.5, was immediately incubated at 37°C, at atmospheric pressure in a water bath for up to 48 h, or under high pressure (i.e., 100, 200, 300, and 400 MPa) for up to 120 min, using a 900-HP apparatus (Eurotherm Automation, Lyon, France). For the hydrolysis experiments conducted at atmospheric pressure on the prepressurized protein, β -LG A was dissolved in 50 mM Tris-HCl buffer, pH 6.8, and treated at 100, 200, 300, or 400 MPa and 22 to 25°C for 20 min. Immediately after depressurization, the pH was adjusted to 2.5 with 1 N HCl and pepsin (1:20) was added to the pressurized substrate, incubating in a water bath at 37°C for 5 to 60 min. The enzyme reaction was stopped in all cases by raising the pH to 6.5–7 by the addition of 2 N NaOH. Samples were immediately freeze-dried and kept at refrigeration temperatures until used. Duplicate samples were prepared and analyzed.

SDS-PAGE

Lyophilized hydrolysates were reconstituted in 10 mM Tris-HCl, pH 8.0, containing 2.5% SDS, 10 mM EDTA, and 5.0% β -mercaptoethanol and immediately heated at 100°C for 10 min. The SDS-PAGE was done on a PhastSystem Electrophoresis apparatus, using precast high-density gels and PhastGel SDS buffer strips (Amersham Biosciences, Uppsala, Sweden), following the electrophoretic and silver staining conditions of the manufacturer.

HPLC-MS/MS

The reversed-phase (RP)-HPLC with UV detection, online with electrospray ionization and quadrupole ion trap instrument (ESI-MS/MS) analyses, were performed as previously described by Chicón et al. (2006a), using an Agilent 1100 Series HPLC equipment (Agilent Technologies, Waldbronn, Germany) with a Hi-Pore Reversed Phase RP-318 Column (250 $\times$ 4 mm i.d., BioRad Laboratories, Hercules, CA) and an Esquire 3000 mass spectrometer (Bruker Daltonik, Bremen, Germany). Using Data Analyze (version 3.0, Bruker Daltonik), the m/z spectral data were processed and transformed to spectra representing mass values. Biotools (version 2.1, Bruker Daltonik) was used to process the MS(n) spectra and to perform peptide sequencing. To aid the identification of disulfide linked fragments the hydrolysates were also analyzed by RP-HPLC-MS/MS after a reducing step using dithiothreitol, at a final concentration of 70 mM and pH 7.0, for 1 h at 37°C.

To compare the peptide content in different eluting fractions of the chromatographic separation, a quantitative estimation of the area under defined limits of the HPLC chromatograms was done by manually setting the integration limits and automatically integrating the areas using Agilent Chemstation software (revision A.05.01, Agilent Technologies). The calculated chromatogram areas in the different fractions corresponded to F1 (12 to 23 min), F2 (23 to 30 min), F3 (30 to 36 min), F4 (36 to 50 min), and the β -LG peak. The total peptide fraction was calculated as $PF = F1 + F2 + F3 + F4$, and the total content of protein and peptides was $Tot = PF + \beta\text{-LG} = F1 + F2 + F3 + F4 + \beta\text{-LG}$.

Antigenicity and IgE Binding

Residual antigenicity (i.e., IgG binding) of the samples was evaluated by indirect ELISA. High binding polystyrene microtiter plates (Corning, Cambridge, MA) 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 PBS solution, pH 7.4, and incubated overnight at 6°C. Plates were washed with

PBS containing 0.05% Tween 20. Plates were blocked with PBS containing 2.5% Tween 20 for 1 h at room temperature and incubated with 50 μ L/well of horseradish peroxidase-conjugated rabbit anti-bovine β -LG IgG (Bethyl Laboratories, Montgomery, TX) diluted 1:20,000 in PBS. A solution of *o*-phenylene-diamine dihydrochloride (DakoCytomation) containing H_2O_2 was added following the instructions of the manufacturer. Plates were incubated for 30 min at room temperature in the dark, and the reaction was stopped by adding 50 μ L/well of 0.5 M H_2SO_4 . Optical densities were read at 492 nm on an automated ELISA plate reader Multiskan Ascent (Labsystems, Helsinki, Finland).

The IgE binding was evaluated by indirect ELISA, using patients' sera. Individual serum samples from 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, particularly β -LG, as determined by the FEIA-CAP System (Pharmacia Diagnostics, Uppsala, Sweden). The pooled serum of 5 healthy nonallergic people was used as negative control. The assay was performed similarly as above but incubating with 50 μ L/well of serum diluted 1:20 in PBS containing 0.05% Tween 20 overnight at 6°C. After blocking, 50 μ L/well horseradish peroxidase-conjugated rabbit anti-human IgE (DakoCytomation, Glostrup, Denmark) was added at a 1:1,000 dilution in PBS-Tween, and plates were incubated for 1 h at room temperature. A signal amplification system (ELAST ELISA amplification system, PerkinElmer Life Sciences, Waltham, MA) was used following the instructions of the manufacturer. The reaction was revealed with *o*-phenylene-diamine dihydrochloride as described above.

The ELISA determinations were carried out in triplicate and measurements averaged. A blank without antigen (PBS) and a positive control containing intact β -LG were included in each plate.

RESULTS AND DISCUSSION

Effect of High Pressure on the Susceptibility of β -LG to Pepsin Hydrolysis

The extent of hydrolysis of β -LG by pepsin under the different pressure conditions was estimated by SDS-PAGE as shown in Figure 1. In addition, the RP-HPLC-UV chromatograms, illustrated in Figure 2, allowed for the evaluation of the evolution of the peptide pattern. No major qualitative differences were found in the hydrolysates, but the amounts of the different peptides varied depending on the conditions of enzyme treatment (Figure 2 a, c, and e). The total peptide content was calculated as the area of the peptide fraction (PF)

Figure 1. The SDS-PAGE of hydrolysates of β -LG obtained with pepsin under different pressure conditions. Lane 1: control native β -LG; lanes 2 and 3: hydrolysates obtained at atmospheric pressure on native β -LG for 8 and 24 h, respectively. Lanes 4, 5, 6, and 7: hydrolysates obtained at atmospheric pressure for 1 h on prepressurized β -LG (100, 200, 300, and 400 MPa, respectively, for 20 min); lanes 8, 9, 10, and 11: hydrolysates obtained under high pressure, at 100, 200, 300, and 400 MPa, respectively, for 20 min on native β -LG.

relative to the area of all peaks (**Tot**) that included, when present, the peak of intact β -LG (PF/Tot in Table 1). In addition, we quantified the peak areas of 4 peptide groups corresponding to different regions of the chromatogram (F1, F2, F3, and F4) relative to the total peptide area (PF) that included all peptide groups (Table 1).

The results obtained from SDS-PAGE (Figure 1, lanes 2 to 3) and the total peptide content estimated by HPLC-UV (Figure 2a, Table 1) showed that native β -LG was weakly susceptible to pepsin at atmospheric pressure, in agreement with previous reports (Reddy et al., 1988; Dalgalarondo et al., 1995; Otte et al., 1997). The peptide fraction slowly increased with time, but complete hydrolysis was not achieved even after 48 h of incubation with the enzyme. Proteolysis time caused a progressive decrease in the higher retention time fraction, F4, whereas F1 and F2 increased, indicating that F4 contained mainly intermediate fragments that were proteolysed further to smaller peptides.

Treatment of β -LG at neutral pH and pressures ≥ 200 MPa promoted its subsequent proteolysis by pepsin at atmospheric pressure, although a considerable amount of the protein was still present in its full length after 60 min of enzymatic treatment (Figure 1, lanes 4 to 7, Figure 2c, Table 1). Proteolysis was more efficient because the pressure used was higher. This supports previous findings showing that pressure-induced struc-

Figure 2. HPLC-UV chromatograms of β -LG hydrolysates obtained with pepsin in the absence (a, c, e) and presence of dithiothreitol (DTT; b, d, f). Native β -LG hydrolyzed at atmospheric pressure for 48 h (a, b); prepressurized β -LG (400 MPa, 20 min) hydrolyzed at atmospheric pressure for 60 min (c, d); native β -LG hydrolyzed under 400 MPa for 5 min (e, f). Peak numbers correspond to the peptides identified by MS/MS (Table 2). F1, F2, F3, and F4 correspond to the fractions in Table 1.

tural changes on β -LG facilitate pepsin action (Dufour et al., 1995; Stapelfeldt et al., 1996; Belloque et al., 2007). The total peptide fraction in the hydrolysates obtained at atmospheric pressure on β -LG increased with prepressurization from 200 to 400 MPa (see PF/Tot in Table 1), but extending the hydrolysis time from 5 to 60 min did not make much difference in the overall amount of peptides released. This indicates that little proteolysis of the intact protein occurred after the first 5 min, probably due to the capacity of the protein to recover its structure after decompression (Ikeuchi et al., 2001; Belloque et al., 2007). However, the incubation time of the prepressurized substrate with pepsin changed the relative proportion of the different peptide fractions, with F4 decreasing markedly from 5 to 60 min of incubation with the enzyme. This suggests that

the intermediate peptides contained in F4 were better substrates for the enzyme action than the whole protein.

Under high pressure, the protein was hydrolyzed very efficiently. As compared with atmospheric pressure, enzyme treatments at 200 to 400 MPa dramatically increased the proteolysis rate of β -LG. The peptide fraction (PF/Tot) increased with pressure and with incubation time, and no intact β -LG was found after 5 min at 400 MPa (Table 1). Previous studies performed under similar conditions of pressure and time but with different enzymes working at neutral pH, such as trypsin and chymotrypsin, showed that proteolysis was enhanced at 100 MPa (Chicón et al., 2006a,b). The observation that proteolysis was not promoted at 100 MPa under the present conditions can be attributed to the

Table 1. Relative area of peptide fractions (from HPLC chromatograms) and antigenicity (from ELISA with a commercial anti- β -LG antibody), obtained from pepsin hydrolysis of β -LG A subjected to different treatments with high pressure¹

β -LG pretreatment	Enzyme hydrolysis		Fraction relative area					Antigenicity
Pressure (MPa)	Pressure (MPa)	Time (min)	PF/Tot	F1/PF	F2/PF	F3/PF	F4/PF	IgG binding
None	0.1	0	—	—	—	—	—	3.55
None	0.1	5	0.10	0.14	0.17	0.11	0.58	3.34
None	0.1	60	0.17	0.14	0.34	0.20	0.32	3.74
None	0.1	480	0.33	0.17	0.47	0.17	0.20	3.07
None	0.1	1,440	0.51	0.21	0.56	0.18	0.05	2.68
None	0.1	2,880	0.76	0.24	0.57	0.12	0.07	2.27
100 ²	0.1	5	0.05	0.16	0.11	0.17	0.55	4.02
100 ²	0.1	30	0.14	0.11	0.29	0.13	0.47	3.96
100 ²	0.1	60	0.13	0.13	0.36	0.17	0.33	3.97
200 ²	0.1	5	0.28	0.16	0.37	0.14	0.33	3.76
200 ²	0.1	30	0.27	0.17	0.43	0.16	0.24	3.96
200 ²	0.1	60	0.27	0.17	0.45	0.16	0.22	3.79
300 ²	0.1	5	0.47	0.07	0.32	0.12	0.50	3.81
300 ²	0.1	30	0.47	0.11	0.45	0.19	0.25	3.35
300 ²	0.1	60	0.49	0.17	0.48	0.18	0.17	3.34
400 ²	0.1	5	0.57	0.06	0.41	0.14	0.39	3.26
400 ²	0.1	30	0.62	0.11	0.46	0.18	0.26	3.60
400 ²	0.1	60	0.62	0.14	0.49	0.19	0.19	3.60
None	100	5	0.22	0.09	0.30	0.18	0.42	3.53
None	100	10	0.24	0.10	0.35	0.18	0.37	3.98
None	100	20	0.34	0.10	0.38	0.15	0.37	4.17
None	200	5	0.76	0.05	0.32	0.14	0.48	3.13
None	200	10	0.87	0.06	0.34	0.13	0.47	2.26
None	200	20	0.94	0.08	0.39	0.16	0.38	1.11
None	300	5	0.97	0.07	0.34	0.19	0.40	1.10
None	300	10	0.98	0.08	0.35	0.16	0.41	0.82
None	300	20	0.99	0.08	0.38	0.19	0.34	0.43
None	400	5	1.00 ³	0.06	0.30	0.17	0.47	0.42
None	400	10	1.00 ³	0.08	0.35	0.19	0.38	0.32
None	400	20	1.00 ³	0.09	0.39	0.24	0.28	0.10
None	400	60	1.00 ³	0.14	0.47	0.26	0.13	0.06
None	400	120	1.00 ³	0.17	0.51	0.22	0.10	0.02

¹The areas of fractions correspond to F1 (12 to 23 min), F2 (23 to 30 min), F3 (30 to 36 min), and F4 (36 to 50 min), PF (F1+F2+F3+F4), Tot (F1+F2+F3+F4+ β -LG). See Figure 2 for visualization of the fractions. Antigenicity values in absorbance (492 nm) units.

²Prepressurization at given pressure for 20 min at 22 to 25°C.

³Complete hydrolysis.

structural stability of the protein at acid pH (Reddy et al., 1988; Dufour et al., 1995; Stapelfeldt et al., 1996; Belloque et al., 2007). Between 200 and 400 MPa there were no clear differences in the relative proportions of the peptide fractions, but at each pressure level, F4 decreased and F1/F2 increased with the incubation time.

The comparison between the hydrolysates obtained under atmospheric pressure and under high pressure with a similar level of β -LG degradation (PF/Tot) revealed that proteolysis under high pressure led to higher peptide content in fraction F4, whereas the peptide contents in F1 and F2 were considerably lower. We have previously proposed that proteolysis of β -LG under high pressure at neutral pH follows a progressive mechanism that involves the rapid disappearance of the pro-

tein to yield large peptides, which are then hydrolyzed more slowly to release smaller peptides (Chicón et al., 2006a,b). This is supported by the present findings at acid pH: intact β -LG was hydrolyzed rapidly under high pressure, leading to the accumulation of intermediate peptides during the early stages of proteolysis that were further degraded on extending incubation with the enzyme. In contrast, at atmospheric pressure, the persistent presence of the intact protein after 48 h of hydrolysis and the low amount of intermediate peptides indicate that the proteolysis on the protein was difficult and slow, but once the resulting intermediate peptides were released, they were further hydrolyzed to smaller fragments with more ease.

It has to be mentioned that no SS-bonded aggregates of β -LG were found when the protein was subjected to

high pressure in the absence of enzyme (results not shown), in agreement with the low reactivity of the thiol group at acid pH.

Peptides Released by Pepsin on β -LG as Influenced by Previous or Simultaneous High-Pressure Treatments

Table 2 shows the sequences of the peptides identified by RP-HPLC-MS/MS in the hydrolysates of β -LG. Most fragments derived from the cleavage of the Phe, Tyr, Trp, and Leu residues, in agreement with the specificity of the enzyme. Other cleaved bonds involved Ile, Glu, Asp, and Gln residues.

In general terms, most peaks were found in all the samples, but in very different amounts (Figure 2a, c, and e). Similarly, Dufour et al. (1995) reported that hydrolysis of β -LG by pepsin shows only pressure-induced rate increases that are not followed by any pressure-induced cleavage specificity. In the hydrolysates obtained at atmospheric pressure on the prepressurized protein and, especially in those obtained under high pressure, particularly at 400 MPa, the late eluting HPLC peptides were prominent (Figure 2 a, c, and e). Some fragments appeared to be the consequence of primary cuts on the protein, whereas others were the result of further proteolysis of the primary fragments. All primary fragments were formed more easily when the protein was treated under high pressure and, thus, they accumulated at short hydrolysis times. When proteolysis was performed at 400 MPa and long times (e.g., 120 min), proteolysis evolved so that the late-eluting peptides, such as Val¹²³-His¹⁴⁶ (58), Val¹²³-Leu¹⁴⁹ (61), Phe⁸²-Leu⁹⁵ (57), Lys⁸³-Leu⁹⁵ (51), and Ile²⁹-Val⁴¹ (54) disappeared, giving rise to smaller peptides with shorter retention times (results not shown).

In the hydrolysates obtained under high pressure, the peptide Val¹²³-Leu¹⁴⁹ (61) was present in high amounts, suggesting that pressurization promoted a primary cleavage at Leu¹²²-Val¹²³ and Leu¹⁴⁹-Ser¹⁵⁰ bonds. The shorter sequence Val¹²³-His¹⁴⁶ (58) was also prominent, indicating that the longer fragment, once released, was easily proteolyzed to the smaller one. Some peptides resulting from proteolysis of both fragments, such as (52, 59, 60, 43, 8, and 28), were evidenced. The SS bonded peptide Leu⁵⁸-Val⁸¹(SS)Ser¹⁵⁰-Ile¹⁶² (44) was very important, as well as its separate peptides, Leu⁵⁸-Val⁸¹ (69) and Ser¹⁵⁰-Ile¹⁶² (71), and other related ones [i.e., Leu⁵⁸-Phe⁸² (70)]. This indicated that, in addition to Leu¹⁴⁹-Ser¹⁵⁰, the bonds Leu⁵⁷-Leu⁵⁸ and Val⁸¹-Phe⁸²-Lys⁸³ were very susceptible to proteolysis under high pressure. In addition, the simultaneous presence of Ser¹⁵⁰-Ile¹⁶² (71) and the products resulting from its cleavage through the bond Leu¹⁵⁶-Glu¹⁵⁷ (35, 63, and

46) indicated that the latter is rapidly cleaved once the longer fragment is released of the protein. Other important fragments were Lys⁸³-Leu⁹⁵ (51) and Phe⁸²-Leu⁹⁵ (57), and shorter fragments within these sequences such as (38, 38, 15, and 13), but their signals were not as high. This confirmed the higher susceptibility of Val⁸¹-Phe⁸²-Lys⁸³ bonds and indicated that Leu⁹⁵-Glu⁹⁶ cleavage was also favored by high pressure. It was found that Leu⁹⁵-Leu¹⁰⁴ (45) was only present in high pressure treated samples and Asp⁹⁶-Phe¹⁰⁵ (56) was considerably high, indicating that Val⁹⁴-Leu⁹⁵-Asp⁹⁶ and Leu¹⁰⁴-Phe¹⁰⁵-Cys¹⁰⁶ cleavages were favored by high pressure. The peptide Ile²⁹-Val⁴¹ (54) was also very high in the pressure-treated sample, as compared with the samples hydrolyzed at atmospheric pressure. This indicated that the bonds Val⁴¹-Tyr⁴²-Val⁴³ and Asp²⁸-Ile²⁹ were particularly susceptible to cleavage under high pressure. Its proteolysis products (21 and 30) were slightly enhanced. Other fragments that showed the favored cleavage of the bond Val⁴¹-Tyr⁴² were Tyr⁴²-Leu⁵⁴ (31) and Tyr⁴²-Glu⁵⁵ (29). Their presence, besides, indicated that bonds Leu⁵⁴-Glu⁵⁵-Ile⁵⁶ were favorably broken under high pressure. Fragments coming from these 2 latter peptides (24 and 11) were increased in the samples hydrolyzed under high pressure. It has to be noted that no SS-bonded peptides resulting from SH/SS exchange reactions were found, in agreement with the absence of pressure-induced SS-bonded protein aggregates (see above).

It can be inferred from the rapid formation of all primary fragments, which sequences were distributed throughout the whole protein (Figure 3), that Asp²⁸/Ile²⁹, Tyr⁴², Leu⁵⁴, Leu⁵⁷/Leu⁵⁸, Phe⁸², Leu⁹⁵, Leu¹⁰⁴/Phe¹⁰⁵, Leu¹²², Leu¹⁴⁹ were target residues that became more available to pepsin by the application of high pressure. Some residues (Leu⁵⁴, Phe⁸², Leu⁹⁵, Phe¹⁰⁵, and Leu¹²²) were not accessible to the enzyme in the native protein. The residues Leu⁵⁴ and Phe⁸² are closely located within the protein, as well as Leu⁹⁵, Phe¹⁰⁵, and Leu¹²², a fact that is probably related to their similar behavior. Residues Leu⁹⁵, Phe¹⁰⁵, and Leu¹²² lie in the FGH strands, a robust region of the protein (Belloque et al., 2000). The exposure to the solvent of Leu¹⁰⁴ and Phe¹⁰⁵ is difficult at acid pH, but they become increasingly exposed as the pressure increases from 200 to 400 MPa (Belloque et al., 2007). The residues Arg⁴⁰ and Arg¹⁴⁸, as well as Tyr⁴² and Leu¹⁴⁹ are favorably cleaved by trypsin and chymotrypsin, respectively, under high pressure, a fact that has been mainly attributed to the dissociation of the dimer (Chicón et al., 2006a,b). However, at acid pH, β -LG is already a monomer at atmospheric pressure, and thus, the proteolysis enhancement of these residues must be due to other conformational changes. Because Leu¹⁴⁹ is located in the I strand,

Table 2. Peptide sequences arising from the hydrolysis of β -LG A with pepsin under different pressure conditions, as determined by reversed-phase HPLC-MS¹

Peak	m/z^2	Protein fragment	Sequence
1	494.2	24–28	(A)MAASD(I)
2	861.4	109–116	(E)NSAEPEQS(L)
3	561.3	85–89	(I)DALNE(V)
4	539.3	42–45	(V)YVEE(L)
5	645.5	137–142	(F)DKALK(A)
6	497.4	143–146	(A)LPMH(I)
7	877.6	2–9	(L)IVTQTMKG(L)
8	930.5	123–130	(L)VRTPEVDD(E)
9	1,059.5	123–131	(L)VRTPEVDDE(A)
10	974.4	109–117	(E)NSAEPEQSL(A)
11	856.5	47–54	(L)KPTPEGDL(E)
12	920.6	135–142	(E)KFDKALK(A)
13	802.6	83–89	(F)KIDALNE(N)
14	1,049.6	134–142	(L)EKFDKALK(A)
15	1,143.5	83–92	(F)KIDALNENKV
16	661.9	14–19	(Q)KVAGTW(Y)
17	809.6	140–146	(A)LKALPMH(I)
18	969.6	46–54	(E)LKPTPEGDL(E)
19	770.5	35–41	(A)QSAPLRV(Y)
20	990.5	1–9	LIVTQTMKG(L)
21	956.5	33–41	(L)DAQSAPLRV(Y)
22	1,105.5	2–11	(L)IVTQTMKGLD(I)
23	902.6	12–19	(D)IQKVAGTW(Y)
24	1,098.6	45–54	(E)ELKPTPEGDL(E)
25	1,233.6	132–142	(E)ALEKFDKALK(A)
26	1,337.0(2)	58–73(SS)157–162	
27	1,326.6	43–54	(Y)VEELKPTPEGDL(E)
28	1,243.5	123–133	(L)VRTPEVDDEAL(E)
29	1,618.6	42–55	(V)YVEELKPTPEGDL(E)
30	1,069.6	32–41	(L)LDAQSAPLRV(Y)
31	1,489.5	42–54	(V)YVEELKPTPEGDL(E)
32	1,232.6	42–42	(L)LDAQSAPLRV(Y)
33	1,527.6	134–146	(L)LPEKFDKALKALPMH(I)
34	827.6	99–104	(D)YKKYLL(F)
35	806.5	150–156	(L)SFNPTQL(E)
36	1,180.6(2)	58–81(SS)157–162	
37	1,290.6	82–92	(V)FKIDALNENKV(L)
38	1,355.7	83–94	(F)KIDALNENKVLV(L)
39	903.6	75–82	(E)KTKIPAVF(K)
40	1,158.6	96–104	(L)DLDYKKYLL(F)
41	1,032.6	74–82	(A)EKTIPAVF(K)
42	1,218.7	1–11	LIVTQTMKGLD(I)
43	1,711.8	132–146	(E)ALEKFDKALKALPMH(I)
44	1,443.4(3)	58–81(SS)150–162	
45	1,271.6	95–104	(V)LDTDYKKYLL(F)
46	1,844.1(2)	58–82(SS)157–162	
47	1,130.5	10–19	(G)LDIQKVAGTW(Y)
48	879.6	143–149	(A)LPMH(L)
49	1,370.6	94–104	(L)VLDTDYKKYLL(F)
50	1,840.7	131–146	(D)EALKFDKALKALPMH(I)
51	1,468.7	83–95	(F)KIDALNENKVLV(L)
52	1,909.9	134–149	(L)EKFDKALKALPMH(L)
53	1,393.3(2)	105–122(SS)157–162	
54	1,382.7	29–41	(D)ISLLDAQSAPLRV(Y)
55	1,483.7	93–104	(V)LVLDTDYKKYLL(F)
56	1,305.6	96–105	(L)DLDYKKYLL(F)
57	1,615.8	82–95	(V)FKIDALNENKVLV(L)
58	1,376.5(2)	123–146	(L)VRTPEVDDEALEKFDKALKALPMH(I)
59	1,112.1(2)	131–149	(D)EALKFDKALKALPMH(L)
60	1,169.5(2)	130–149	(D)DEALKFDKALKALPMH(L)
61	1,567.7(2)	123–149	(L)VRTPEVDDEALEKFDKALKALPMH(L)
62*	609.2	62–66	(W)ENDEC(A)
63*	758.5	157–162	(L)EEQCHI
64*	1,371.4	105–116	(L)FCMENSAEPEQS(L)
65*	871.5	156–162	(L)LEEQCHI
66*	1,164.5	58–66	(L)LQKWENDEC(A)

Continued

Table 2 (Continued). Peptide sequences arising from the hydrolysis of β -LG A with pepsin under different pressure conditions, as determined by RP-HPLC-MS¹

Peak	<i>m/z</i> ²	Protein fragment	Sequence
67*	565.3	118–122	(L)VCQCL(V)
68*	1,916.7	58–73	(L)LQKWENDECAQKKIIA(E)
69*	1,392.2(2)	58–81	(L)LQKWENDECAQKKIIAEKTKIPAV(F)
70*	1,465.7(2)	58–82	(L)LQKWENDECAQKKIIAEKTKIPAVF(K)
71*	1,545.5	150–162	(L)SFNPTQLEEQCHI
72*	1,578.9	56–82	(E)ILLQKWENDECAQKKIIAEKTKIPAVF(K)
73*	2,030.3	105–122	(L)FCMENSAEPEQSLVCQCL(V)

¹Peak numbers correspond to numbers in HPLC chromatograms (Figure 2). *Peptides marked with an asterisk were found in dithiothreitol-treated samples. Sequence amino acids between brackets represent flanking residues.

²Molecular ion selected for MS/MS charge.

where it interacts in an antiparallel manner with the A strand, it is possible that it becomes more exposed through the dissociation of both strands under high pressure.

The restricted proteolysis of β -LG by pepsin at atmospheric pressure makes it difficult to identify the proteolysis products of this enzyme (Otte et al., 1997). Some authors have identified the peptides formed during pepsinolysis of native bovine (Chobert et al., 1995; Dalgarrondo et al., 1995) and ovine β -LG (El-Zahar et al., 2005), esterified β -LG (Chobert et al., 1995), or β -LG in ethanol solution (Dalgarrondo et al., 1995). Only one report has been found that describes 11 peptides released on proteolysis β -LG with pepsin under high pressure (Dufour et al., 1995). Comparing these fragments with those found in the present study, it can be inferred that Dufour and coworkers described mainly secondary products. It has to be pointed out that some secondary peptides are formed rapidly under high-pres-

sure conditions probably because the primary fragments are more readily available. Actually, many secondary peptides appeared simultaneously with primary peptides at short proteolysis times under high-pressure conditions. On the contrary, other secondary products, such as those derived from cleavage at Leu¹⁴³ and Glu⁷⁴, required longer times to appear, what indicated that these residues were more difficult to cleave even under high pressure. Finally, some sites (Leu³⁹, Leu⁸⁷, Leu¹⁰², and Leu¹⁰³) were evidenced as very tough targets for pepsin, as they were not attacked by the enzyme. The Leu³⁹ is preceded by a proline residue, which is known to inhibit pepsin action. The reason why Leu⁸⁷, Leu¹⁰², and Leu¹⁰³ were also difficult targets is less clear. However, it can be speculated that some of the primary fragments released, Phe⁸³–Leu⁹⁵ and Val⁹⁶–Leu¹⁰⁵, that have a hairpin conformation in the native structure (Figure 3) could maintain some secondary structure that would interfere with the enzyme activity.

Figure 3. View of the 3D structure of β -LG and some primary fragments favorably cleaved by proteolysis with pepsin under high pressure. Model built with RasWin Molecular Graphics, version 2.6, using the coordinates from the Protein Data Bank, PDB ID: 1BEB (Brownlow et al., 1997).

Table 3. Specific binding to IgE of allergic patients' sera of selected hydrolysates obtained by incubation of β -LG with pepsin at 37°C, different pressures and different times, in absorbance units, as measured by indirect ELISA¹

Sample	Pepsin hydrolysis		Serum IgE binding (ELISA response, Abs. 492 nm)						
	Pressure (MPa)	Time	S1	S2	S3	S4	S5	S6	S7
Control (native β -LG)	—	—	2.76	2.48	2.43	3.5	1.09	2.5	2.16
Hydrolysates at atmospheric pressure	0.1	24 h	2.85	2.67	—	—	—	—	—
(contain intact β -LG)	0.1	48 h	2.76	2.79	—	—	—	—	—
Hydrolysates at high pressure	400	5 min	1.92	1.17	1.78	—	—	—	—
(no intact β -LG present)	400	10 min	1.53	1.18	1.06	—	—	—	—
	400	20 min	0.82	0.70	1.02	0.30	<0.1	<0.1	<0.1
	400	60 min	0.24	0.55	0.50	0.25	<0.1	<0.1	<0.1
	400	120 min	0.22	0.20	<0.1	<0.1	<0.1	<0.1	<0.1
β -LG-specific serum titer (kU/L)			55.7	68.6	31.9	42.3	5.6	20.1	29.4

¹Individual sera labeled as S1 to S7. Absorbance values of <0.1 mean very low response. The protein/peptide content, corresponding to 2.5 μ g/mL, was the same in all samples.

Changes in Antigenicity and IgE Binding of β -LG Hydrolysates

The antigenic response of the different β -LG hydrolysates toward a commercial anti- β -LG antibody (Table 1) was high in all samples containing intact protein. These included all samples hydrolyzed at atmospheric pressure, either native or prepressurized. Only a slight reduction was observed in the native samples incubated with the enzyme for a long time (i.e., 24 and 48 h) at atmospheric pressure. This is consistent with the progressive removal of the intact protein revealed in the PF/Tot values. Some prepressurized samples (e.g., at 400 MPa) showed no significant reduction of IgG binding while containing a similar or higher peptide amount (PF/Tot) than the native samples hydrolyzed for 24 to 48 h. This could be explained by the accumulation of the peptides in the F4 fraction of the prepressurized hydrolysates, which probably carry a significant antigenic load. A clear decrease in antigenicity was evidenced when the protein was hydrolyzed under pressures ≥ 200 MPa, and the reduction was more intense as the incubation pressure and time increased. The extensive reduction of intact β -LG (PF/Tot ≥ 0.90) caused a considerable decrease in antigenicity. However, the complete removal of the protein (PF/Tot = 1.00) did not completely cancel the IgG response, which progressively decreased with time like the F4 fraction.

Some hydrolysates were selected for testing their binding capacity to serum IgE from allergic patients (Table 3). No reduction in the response was found when β -LG was proteolyzed at atmospheric pressure, but the response decreased on incubation with the enzyme under high pressure. A considerably reduction in the IgE binding was already found in the hydrolysates obtained at 400 MPa for short periods, 5 to 10 min, and an increase in the incubation time with the enzyme led to a

progressive decrease of IgE binding. The serum titer greatly influenced the reactivity against IgE. Sera with lower titers (S5, S6, S7) showed no IgE binding for samples obtained at 400 MPa for 20 min, whereas sera with higher titers (S1 and S2) showed some reactivity against the hydrolysates obtained at incubation times as long as 120 min.

The observation that in the absence of protein there were still positive antigenic and IgE binding responses, particularly against the commercial antibody and the sera with higher titers, suggested again that the intermediate peptides, such as those in F4, preferentially formed at the initial stages of hydrolysis under high pressure, contained immunoreactive epitopes. As illustrated in Table 4, strong candidates for such reactivity are Leu⁹⁵–Leu¹⁰⁴ (45) and Asp⁹⁶–Phe¹⁰⁵ (56), which are similar to fragments 95–113 and 97–108 that have shown positive reactions with 100% of the sera from allergic patients (Ball et al., 1994; Heinzmann et al., 1999). In addition, Selo et al. (1999) found that a similar sequence, 92–100, still reacted with sera from 52% of the patients. The fragment Ser¹⁵⁰–Ile¹⁶² (71) would also be potentially allergenic because it is very similar to 149–162, which has reacted against sera belonging to 89% of the allergic patients (Selo et al., 1999). The Tyr⁴²–Leu⁵⁴ (31) is included in the sequence 41–60 that has been found to react with sera from 92% of the allergic patients (Selo et al., 1999). Selo et al. (1999) have also found a positive reaction in 72% of the allergic patients toward the fragment 25–40, similar to Ile²⁹–Val⁴¹ (54). Heinzmann et al. (1999) found the sequence 124–135 reactive toward IgE only for some patients. The peptides Val¹²³–His¹⁴⁶ (58) and Val¹²³–Leu¹⁴⁹ (61) contain it, as well as some shorter fragments (8 and 9). In addition, sequences similar to Phe⁸²–Leu⁹⁵ (57) or Lys⁸³–Leu⁹⁵ (51), particularly 84–91 and 85–96, have

Table 4. Primary peptides (released from the protein) and secondary peptides (released from primary peptides) found in β -LG hydrolysates, as deduced from their order of appearance as hydrolysis progressed, and related peptides described as allergenic in the literature¹

Primary peptides	Secondary peptides	Peptides described as allergenic ^{2,3,4}
29–41(54)	33–41 (21) 32–41 (30)	25–40 ²
42–54 (31)	42–45 (4)	41–60 ²
42–55 (29)	46–54 (18) 47–54 (11)	
58–81 (69)	58–66 (66)	
58–82 (70)	58–73 (68)	
58–82(SS)157–162 (46)	74–82 (41) 75–82 (39)	
82–95 (57)	82–92 (37)	84–91 ³
83–95 (51)	83–94 (38) 83–92 (15) 83–89 (13) 85–89 (3)	85–96 ²
95–104 (45)	ND ⁵	95–113 ³
96–105 (56)		97–108 ⁴ 92–100 ²
123–149 (61)	123–146 (58) 123–130 (8) 123–131 (9) 123–133 (28) 130–149 (60) 131–149 (59) 134–149 (52) 134–146 (33) 134–142 (14) 143–146 (6)	124–135 ³
150–162 (71)	150–156 (35) 157–162 (63) 58–82(SS)157–162 (46)	149–162 ²

¹Numbers in parentheses correspond to the peptide numbers in Figure 2 and Table 2.

²Selo et al. (1999).

³Ball et al. (1994).

⁴Heinzmann et al. (1999).

⁵Not detected.

reacted positively with sera from 40 and 44% of the allergic patients, respectively (Ball et al., 1994; Selo et al., 1999).

In conclusion, hydrolysis of β -LG with pepsin at acid pH was greatly accelerated by the application of high pressures during the enzymatic treatment. A progressive proteolysis mechanism was evidenced, and considering previous results, it is concluded that this mechanism occurs at atmospheric and high pressure as well as at neutral and acid pH. This mechanism allowed obtaining hydrolysates with different immunochemical properties by selecting the incubation time with the enzyme. At 400 MPa all the intact protein was removed by pepsin in minutes scale, reducing simultaneously the antigenicity and serum IgE binding properties. However, for obtaining hydrolysates with very low antibody binding capacity, it was necessary to remove the

intermediate peptides, which required hours of incubation with the enzyme under high pressure. This opens the possibility of obtaining hypoallergenic hydrolysates of β -LG, minimizing simultaneously the formation of new peptides cross-linked by disulphide bonds.

ACKNOWLEDGMENTS

This work has been supported by projects AGL-2004-03322 (Ministerio de Educación y Ciencia, Spain) and S-0505/AGR/0153 (Comunidad Autónoma de Madrid, Spain).

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