JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Changes in Chymotrypsin Hydrolysis of *β*-Lactoglobulin A Induced by High Hydrostatic Pressure

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β-Lactoglobulin (*β*-Lg) was subjected to high pressures up to 400 MPa and proteolysis with chymotrypsin. The hydrolysates were analyzed by SDS-PAGE and RP-HPLC, and the fragments obtained were identified by ESI-MS/MS. The results obtained showed that *β*-Lg was hydrolyzed by chymotrypsin in a "progressive proteolysis" manner at either atmospheric or high pressure. Proteolysis during or after high-pressure treatment showed longer and more hydrophobic peptides than proteolysis at atmospheric pressure. Chymotrypsin showed a behavior similar to that of trypsin, with some differences, probably related to the orientation of the target residues specific for each enzyme. The similarities between proteolytic fragments produced by the two enzymes support that proteolysis enhancement under high pressure depends on the substrate changes rather than the enzyme. High pressure seemed to accelerate the first steps of proteolysis, probably through dimer dissociation, while leaving portions of the molecule more resistant to the enzyme.

KEYWORDS: *β*-**Lactoglobulin; high hydrostatic pressure; proteolysis; chymotrypsin; milk whey proteins**

INTRODUCTION

High-pressure treatments of *β*-lactoglobulin (*β*-Lg), before or during enzyme treatments, have been reported to enhance its enzymatic hydrolysis. This effect can derive from the exposure of normally hindered cleavage sites on the protein and from changes in the protein conformation that facilitate binding to the enzyme (*1*). According to Bonomi et al. (*2*), under high-pressure conditions, *β*-Lg forms transient conformers with ample regions of the hydrophobic core unfolded and new bonds exposed to enzymatic hydrolysis.

Some controversy remains regarding the existence of pressure-induced cleavage specificity changes. Thus, whereas hydrolysis of *β*-Lg by thermolysin under pressure at pH 7.0 may be favored by partial unfolding of the substrate and pressure-induced thermolysin activation, enhanced hydrolysis by pepsin at its optimum pH (2.5−3.0) is likely to be driven by negative reaction volume changes, as *β*-Lg is more difficult to unfold under pressure at acidic pH (*3*). Furthermore, according to Maynard et al. (*4*) tryptic hydrolysis of *β*-Lg at pH 7.7 was accelerated under pressure, with an optimum at 300 MPa, but the nature of the tryptic end-products was the same as that of those obtained at 0.1 MPa.

The literature regarding the effect of high pressure on the proteolytic process of *β*-Lg with chymotrypsin is very limited (*2, 5, 6*), particularly the identification of peptides derived from it. However, the use of chymotrypsin offers some advantages because its major target residues are hydrophobic and most of them lie in the inner part of the protein, whereas trypsin, which

is more commonly used, acts mainly on Lys and Arg, which lie at the outer part of the protein, facing the solvent. This makes chymotrypsin a good tool, complementary to trypsin, to understand the unfolding behavior and hydrolysis of the protein under high pressure.

In a previous work we studied the effect of the hydrolysis time and pressure level (0.1−400 MPa) on the proteolysis of *β*-Lg with trypsin, either conducting hydrolysis of *β*-Lg under pressure or hydrolyzing *β*-Lg that was previously pressure treated (*7*). As compared to the proteolysis at atmospheric pressure, proteolysis under or following pressure treatments led to quantitative differences in the hydrolysis pattern, mainly at the shortest hydrolysis times. High pressure promoted a quick attack toward *β*-Lg that led to the initial formation of disulfide-linked peptide products of medium size that were further cleaved at the later stages of proteolysis. The nature of the main intermediate hydrolysis products suggested that structural modifications of *β*-Lg under pressure lead to dimer dissociation, in a way similar to what occurs under elevated temperatures. Our work also supported previous studies that have suggested that pressure-induced unfolding of *β*-Lg may increase surface hydrophobicity (*8, 9*) and provided evidence for the formation on pressurization of a new disulfide bond involving Cys_{121} and Cys_{160} (*10*).

The aim of this paper was to provide elements for a better understanding of the effect of high pressures on *β*-Lg and its proteolysis process, using chymotrypsin, a proteinase with a specificity different from that of trypsin. We have examined the influence of the pressure level and hydrolysis time, either conducting simultaneous hydrolysis and pressurization or hydrolyzing *β*-Lg that was previously pressure treated.

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MATERIALS AND METHODS

Proteinase Treatments at High and Atmospheric Pressures. β-Lg, genetic variant A, was isolated from the pH 4.6 soluble fraction of a milk sample obtained from a homozygotic cow (*11*). Hydrolyses with TLCK-treated chymotrypsin (EC 3.4.21.1, 55 units/mg, from bovine pancreas, Sigma Chemical Co., St. Louis, MO) were conducted as described previously (*7*). Briefly, β-Lg A (5.0 mg) and chymotrypsin (0.25 mg) were dissolved in 2.0 mL of 50 mM Tris-HCl buffer, pH 6.8, and immediately pressurized at 100, 200, 300, or 400 MPa, for 5, 10, and 20 min at 37 °C, using a 900 HP apparatus (Eurotherm Automation, Lyon, France). For the hydrolysis experiments conducted at atmospheric pressure on the prepressurized protein, β-Lg A, 4.75 mg/mL, in 50 mM Tris-HCl buffer, pH 6.8, was treated at 100, 200, 300, or 400 MPa and 22−25 °C for 20 min. Immediately after depressurization, chymotrypsin was added to the pressurized substrate, so that the same enzyme-to-substrate ratio explained above was maintained (1:20), and the mixture was incubated in a water bath at 37 °C for 5, 30, and 60 min. Controls were obtained by conducting the hydrolyses at atmospheric pressure on native β-Lg A at 37 °C for different periods (5 min−48 h). The enzyme reaction was stopped by the addition of 0.5 M HCl to pH 3.0. Samples were immediately freeze-dried and reconstituted as appropriate for subsequent analyses. All reactions were performed at least in duplicate.

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

RP-HPLC-MS. RP-HPLC with UV detection, on-line with electrospray ionization and quadrupole ion trap instrument (ESI-MS/MS), analyses were performed on Agilent 1100 series HPLC equipment (Agilent Technologies, Waldbronn, Germany) and an Esquire 3000 mass spectrometer (Bruker Daltonik, Bremen, Germany). Absorbance was recorded at 214 nm with an Agilent 1100 series variable-wavelength detector. Chromatographic separations were performed with a Hi-Pore reversed phase RP-318 column (250 × 4 mm i.d.) (Bio-Rad Laboratories, Hercules, CA). Operating conditions were as follows: column at ambient temperature; flow rate, 0.8 mL/min; injection volume, 50 μL; solvent A, 0.37 mL/L TFA in Milli-Q water; solvent B, 0.27 mL/L TFA in HPLC-grade acetonitrile (Scharlau Chemie, Barcelona, Spain). The elution was performed with a linear gradient of solvent B in A going from 0 to 50% in 60 min. The flow was split post UV detector by placing a T-piece (Valco, Houston, TX) connected with a 75 μm i.d. peek outlet tube of an adjusted length to give ~20 μL min^{-1} of flow directed into the mass spectrometer via the electrospray interface. Ion source parameters were as follows: nebulizer pressure, 60 psi; dry gas, 12 L/min; dry temperature, 350 °C. The capillary was held at 4 kV. The m/z range scanned was 200−2500. About 10 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10000, and the precursor ions were isolated within a range of 4.0 m/z and fragmented with a voltage ramp going from 0.30 to 2 V. Using Data Analyse (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 (DTT), at a final concentration of 70 mM and pH 7.0, for 1 h at 37 °C. Quantitative estimation of the area under defined limits of the HPLC chromatograms was done by setting manually the integration limits and automatically integrating the areas using Agilent Chemstation software (revision A.05.01, Agilent Technologies).

RESULTS

Hydrolysis of β-Lg with Chymotrypsin at Atmospheric Pressure. The RP-HPLC chromatograms of the hydrolysate of β-Lg with chymotrypsin obtained at atmospheric pressure for

1 and 24 h are shown in **Figures 1a** and **2a**. The peak labels refer to the peptide sequences identified by ESI-MS/MS (**Table 1**). Disulfide-linked oligopeptides were identified with the aid of reduction with DTT (**Figures 1b** and **2b**). Treatment with DTT effectively reduced the disulfide bonds and gave rise to new peaks that corresponded to peptides labeled with an asterisk in **Table 1** (numbers 36−46).
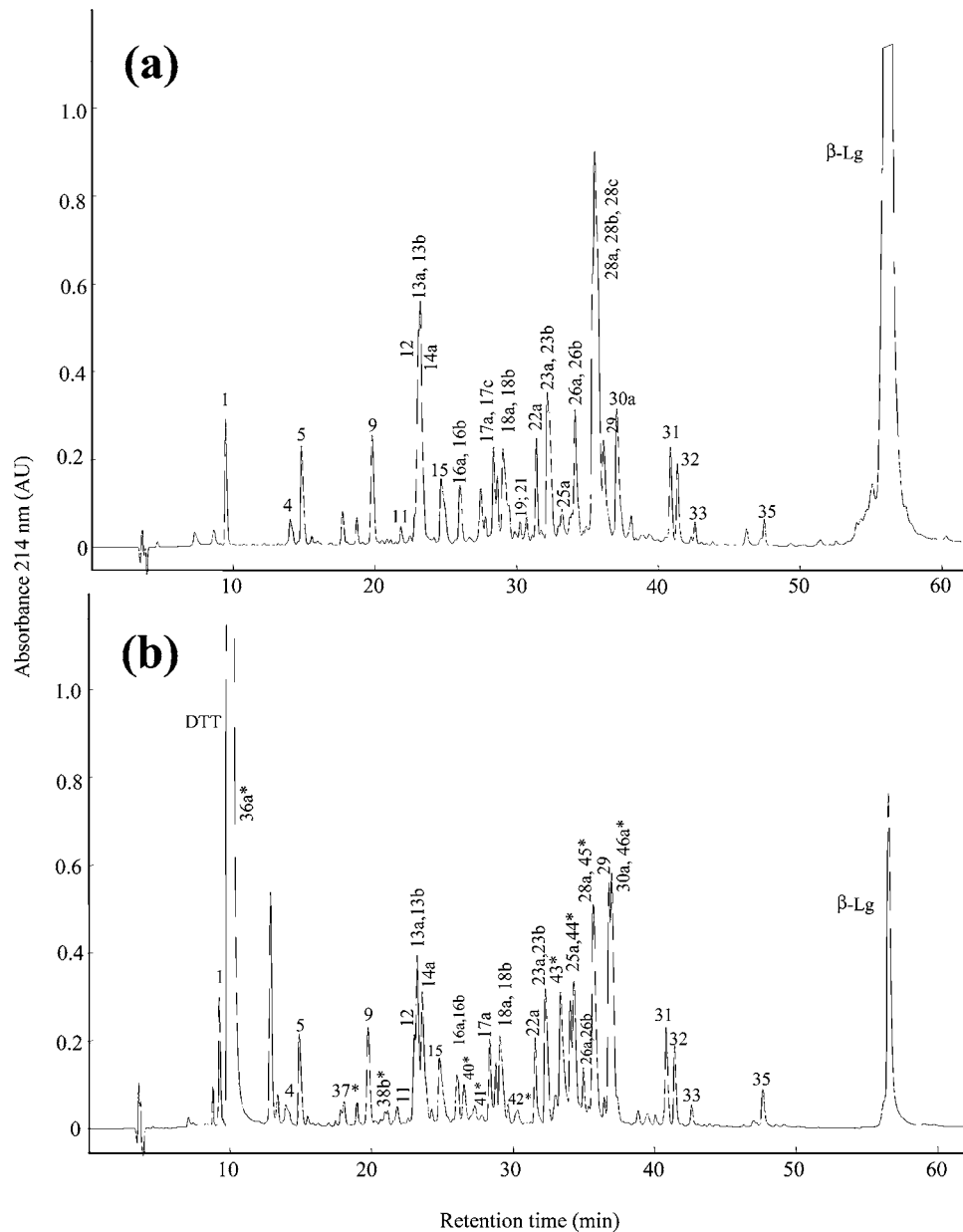
Chymotrypsin hydrolyzed β-Lg after Tyr, Trp, Phe, Met, and Leu, which is consistent with its specificity. Peptides corresponding to the cleavage of bonds with Glu, Gln, and Lys at the N-terminal side were also found. These fragments were more abundant after exhaustive proteolysis but, in some cases, such as Gln$_{59}$, fragments showed up at short proteolysis times (see peak 32, **Figure 1a**). Gln can be cleaved by chymotrypsin, particularly when followed in the sequence by Lys, Arg, Ser, or Thr. Glu is a less specific target, but its cleavage is favored by the presence of Lys or Thr. Lys is also a weak target but, when followed by Lys, Arg, or Gly, its cleavage is favored (*12*, *13*). This agrees with most of the Gln, Glu, and Lys residues cleaved. In addition, other nonspecific residues cleaved, such as Glu$_{55}$, Lys$_{70}$, and Ile$_{71}$, were followed by Ile.

The RP-HPLC chromatograms showed that, after 1 h, at the initial stages of proteolysis, the peak corresponding to β-Lg was very important (**Figure 1a**), whereas at 24 h it was completely absent (**Figure 2a**). Increased proteolysis at longer incubation times was also shown by estimating the relative area covered by the peptide fraction within the total area of peaks in the chromatogram (PF/total), a value related to the amount of peptides produced from the protein cleavage (**Table 2**). This relative area increased from 0.1 to 1 (complete hydrolysis) in samples incubated for 5 min−24 h. **Table 2** also shows the relative areas of four different peptide fractions (F1−F4) within the area of the total peptide fraction and their evolution with the hydrolysis time. The most relevant peaks in the hydrolysates obtained at 1 h were peptide fragments included in fraction F4, with long retention times and corresponding to large/hydrophobic peptides. Most of these had disappeared after 24 h of hydrolysis, becoming as prominent as those in F2 that eluted earlier and correspond to smaller peptides.

The initial stages of hydrolysis with chymotrypsin showed some major hydrolysis products, such as (Lys$_{60}$−Phe$_{82}$) S−S (Ser$_{150}$−Ile$_{162}$) and (Glu$_{62}$−Phe$_{82}$) S−S (Ser$_{150}$−Ile$_{162}$) (peaks 28c and 28b, **Figure 1a**), which were further cleaved to (Glu$_{62}$−Lys$_{70}$) S−S (Ser$_{150}$−Ile$_{162}$), (Glu$_{62}$−Ile$_{71}$) S−S (Ser$_{150}$−Ile$_{162}$), and (Glu$_{62}$−Glu$_{74}$) S−S (Ser$_{150}$−Ile$_{162}$) (peaks 17c, 19, and 21, **Figure 2a**). Other fragments derived from cleavage of Phe$_{151}$ and Leu$_{156}$, particularly Asn$_{152}$−Ile$_{162}$ and Glu$_{157}$−Ile$_{162}$, were also released at a late stage of proteolysis, as detected in DTT-treated samples (peaks 42 and 38a, **Figures 1b** and **2b**).

The longer peptides Val$_{43}$−Gln$_{59}$, Val$_{43}$−Leu$_{58}$, and Val$_{43}$−Trp$_{61}$ (peaks 32, 33, and 35, **Figure 1a**) were hydrolyzed to Val$_{43}$−Glu$_{55}$, Leu$_{58}$−Trp$_{61}$, and Val$_{43}$−Leu$_{57}$ (peaks 16a, 14a, and 30a, **Figure 2a**), respectively, as proteolysis proceeded. Also, Ser$_{21}$−Leu$_{32}$ (peak 31) was hydrolyzed to Ala$_{23}$−Leu$_{32}$ and Ala$_{25}$−Leu$_{32}$ (peaks 30b and 25b, respectively) through the cleavage of Leu$_{22}$ and Met$_{24}$, respectively. Lys$_{8}$−Tyr$_{20}$, Asp$_{11}$−Tyr$_{20}$, and Lys$_{8}$−Trp$_{19}$ (peaks 28a, 22a, and 26b, respectively) were also hydrolyzed to Asp$_{11}$−Trp$_{19}$ and Lys$_{14}$−Trp$_{19}$ (peaks 18a and 14b). In contrast, some short peptides, such as Lys$_{100}$−Tyr$_{102}$, Arg$_{40}$−Tyr$_{42}$, Asp$_{33}$−Leu$_{39}$, and His$_{146}$−Leu$_{149}$ (peaks 1, 5, 9, 15, respectively, **Figure 1a**), released at the initial stages, were not further cleaved.

From the information gathered above, it can be inferred that Met$_7$, Leu$_{10}$, Tyr$_{20}$, Leu$_{32}$, Tyr$_{42}$, Leu$_{58}$, Gln$_{59}$, Trp$_{61}$, and Leu$_{149}$

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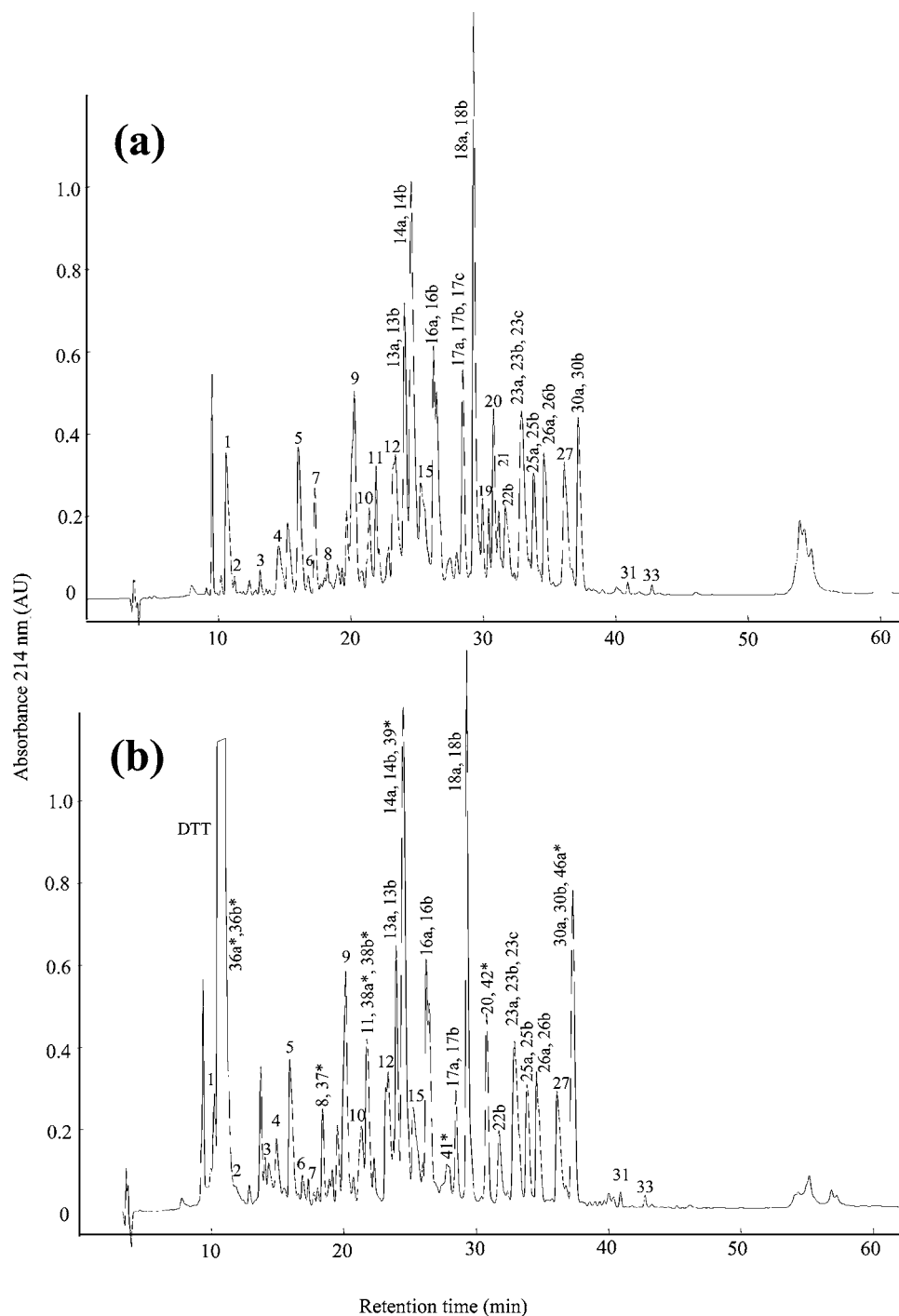
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Figure 1. RP-HPLC pattern of native β-Lg A treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 0.1 MPa for 1 h, before (**a**) and after (**b**) reduction with DTT. Peak numbers refer to the peptide sequences indicated in **Table 1**.

were easy targets for chymotrypsin, whereas Gln_{13}, Leu_{22}, Met_{24}, Leu_{57}, Phe_{151}, and Leu_{156}, together with less specific targets, such as Lys_{70}, Ile_{71}, and Glu_{55}, were more difficult to cleave.

It should be noted that no peptides corresponding to the fragment Cys_{106}–Leu_{122} of β-Lg were detected in the RP-HPLC chromatograms before DTT reduction (**Figures 1a** and **2a**; **Table 1**). That region contains the amino acids Cys_{106} and Cys_{119} that form, in native β-Lg, a disulfide bond, in addition to the one that links Cys_{66} and Cys_{160}, and also the free thiol group of Cys_{121}. Peptides corresponding to the fragments Cys_{106}–Leu_{117} and Cys_{106}–Val_{118} were detected in the RP-HPLC chromatograms of β-Lg hydrolyzed with chymotrypsin for 1 and 24 h upon reduction of disulfide bonds with DTT (peaks 39 and 41, **Figures 1b** and **2b**).

Hydrolysis of β-Lg with Chymotrypsin under High-Pressure Conditions. The SDS-PAGE analysis showed that pressurization of β-Lg gave rise to covalently linked aggregates, which became more evident as the pressure increased (**Figure 3**, lanes 5–8) and were linked through disulfide bonds, because they disappeared in the presence of β-mercaptoethanol (results not shown). Bands attributed to low-order aggregates (i.e., dimers and trimers) showed up at lower pressures and were more intense than those corresponding to higher order aggregates. Some peptides with low molecular weight were present in pressurized samples (**Figure 3**, lanes 5–8), suggesting that some peptide fragments were released only by applying high pressure. When β-Lg was treated with chymotrypsin under high pressure, no dimers, trimers, or higher aggregates were found (**Figure 3**, lanes 9–12). Compared to the SDS-PAGE pattern of native β-Lg completely hydrolyzed at atmospheric pressure (**Figure 3**, lane 4), hydrolysis under high pressure led to the disappearance of the initial substrate with a higher accumulation of intermediate degradation forms (**Figure 3**, lanes 9–12). This suggests that the application of pressure, during chymotrypsin treatment, promoted a very rapid disappearance of the β-Lg

Figure 2. RP-HPLC pattern of native β-Lg A treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 0.1 MPa for 24 h, before (**a**) and after (**b**) reduction with DTT. Peak numbers refer to the peptide sequences indicated in **Table 1**.

monomers and pressure-induced aggregation forms, without extensive hydrolysis.

RP-HPLC analyses revealed that β-Lg was completely proteolyzed after treatment with chymotrypsin at ≥ 200 MPa for 5 min, as compared with >8 h required at atmospheric pressure (**Table 2**). However, it has to be noted that complete proteolysis was actually achieved at 200 MPa for 10 min or at ≥ 300 MPa for 5 min, because SDS-PAGE, with silver staining, showed β-Lg traces when processed at 200 MPa for 5 min. The main differences among the peptides produced by chymotrypsin when acting on β-Lg at atmospheric and high pressure were found in the hydrophobic or largest peptide portion of the RP-HPLC chromatograms (**Figure 4a**). The peaks in fraction F4

(**Table 2**) were very important in the chymotryptic hydrolysates obtained at high pressure, as compared with those obtained at atmospheric pressure and a similar degree of disappearance of β-Lg. Those peptides formed preferentially during the first 5−10 min of incubation with the enzyme under high pressure, and they decreased at longer incubation times. This peptide evolution was observed even at 400 MPa, suggesting that no chymotrypsin inactivation occurred in the pressure interval considered.

Peptides that were released to a greater extent when hydrolysis with chymotrypsin was conducted under high pressure were $Asp_{11}-Tyr_{20}$, Lys_8-Tyr_{20}, and Lys_8-Trp_{19} (peaks 22a, 28a, and 26b, respectively); $Ala_{23}-Leu_{32}$ and $Ser_{21}-Leu_{32}$ (peaks 30b and 31, respectively); $Val_{43}-Leu_{57}$, $Val_{43}-Gln_{59}$, and $Val_{43}-$

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Table 1. Peptide Sequences Arising from the Hydrolysis of β-Lg A with Chymotrypsin under Different Pressure Conditions (0.1–400 MPa) and of Prepressurized β-Lg A, As Determined by RP-HPLC-MS (Peaks with Equal Number but Different Letters Are RP-HPLC Coeluting Peptides)

peak	obsd mass	calcd mass[a]	m/z^b	protein fragment
1	437.3	437.3	438.3 (1)	$Lys_{100}-Tyr_{102}$
2	502.4	502.3	503.4 (1)	$Asp_{11}-Lys_{14}$
3	536.4	536.3	537.4 (1)	$Lys_{135}-Lys_{138}$
4	445.3	445.2	446.3 (1)	$Asp_{137}-Leu_{140}$
5	436.3	436.2	437.3 (1)	$Arg_{40}-Tyr_{42}$
6	828.6	828.5	829.6 (1)	$Lys_{69}-Lys_{75}$
7	929.6	929.6	930.6 (1)	$Lys_{69}-Thr_{76}$
8	801.6	801.5	802.6 (1)	$Lys70-Thr76$
9	700.5	700.3	701.5 (1)	$Asp_{33}-Leu_{39}$
10	672.5	672.4	673.5 (1)	$Lys_{83}-Asn_{88}$
11	572.4	572.4	573.4 (1)	Leu_1-Gln_5
12	558.4	558.3	559.4 (1)	$Lys_{141}-Met_{145}$
13a	724.4	724.3	725.4 (1)	$Val_{94}-Tyr_{99}$
13b	720.5	720.4	721.5 (1)	$Lys_{135}-Leu_{140}$
13c	1143.5	1143.6	1144.5 (1)	$Val_{94}-Tyr_{102}$
14a	573.4	573.3	574.4 (1)	$Leu_{58}-Trp_{61}$
14b	660.4	660.4	661.4 (1)	$Lys_{14}-Trp_{19}$
15	537.4	537.3	538.4 (1)	$His_{146}-Leu_{149}$
16a	1454.5	1454.7	1455.5 (1)	$Val_{43}-Glu_{55}$
16b	1371.5	1371.7	1372.5 (1)	$Val_{123}-Glu_{134}$
17a	804.5	804.4	805.5 (1)	Leu_1-Met_7
17b	1242.5	1242.6	1243.5 (1)	$Val_{123}-Leu_{133}$
17c	2606.0	2606.2	1304.0 (2)	$(Glu_{62}-Lys_{70})$ S–S $(Ser_{150}-Ile_{162})$
18a	1016.5	1016.5	1017.5 (1)	$Asp_{11}-Trp_{19}$
18b	1255.6	1255.7	1256.6 (1)	$Lys_{83}-Leu_{93}$
18c	985.6	985.6	986.6 (1)	$Asp1_{37}-Met_{145}$
19	2718.8	2719.2	1360.4 (2)	$(Glu_{62}-Ile_{71})$ S–S $(Ser_{150}-Ile_{162})$
20	805.4	805.4	806.4 (1)	$Ser_{150}-Leu_{156}$
21	3032.0	3032.4	1517.0 (2)	$(Glu_{62}-Glu_{74})$ S–S $(Ser_{150}-Ile_{162})$
22a	1179.5	1179.6	1180.5 (1)	$Asp_{11}-Tyr_{20}$
22b	902.6	902.6	903.6 (1)	$Lys_{75}-Phe_{82}$
23a	1646.6	1646.8	1647.6 (1)	$Val_{123}-Phe_{136}$
23b	1215.6	1215.7	1216.6 (1)	$ILe_{72}-Phe_{82}$
23c	1584.8	1585.0	1585.8 (1)	$Lys_{69}-Phe_{82}$
24	1102.6	1102.6	1103.6 (1)	Leu_1-Leu_{10}
25a	1456.7	1456.9	1457.7 (1)	$Lys_{70}-Phe_{82}$
25b	788.4	788.4	789.4 (1)	$Ala_{25}-Leu_{32}$
26a	1328.7	1328.8	1329.7 (1)	$Ile_{71}-Phe_{82}$
26b	1314.6	1314.7	1315.6 (1)	Lys_8-Trp_{19}
27	1186.7	1186.6	1187.7 (1)	Gly_9-Trp_{19}
28a	1477.6	1477.8	1478.6 (1)	Lys_8-Tyr_{20}
28b	3917.8	3916.9	1959.9 (2)	$(Glu_{62}-Phe_{82})$ S–S $(Ser_{150}-Ile_{162})$
28c	4230.6	4231.1	1411.2 (3)	$(Lys_{60}-Phe_{82})$ S–S $(Ser_{150}-Ile_{162})$
28d	1546.4	1546.8	1547.4 (1)	Thr_6-Trp_{19}
29	2074.4	2074.1	1038.2 (2)	$Val_{123}-Leu_{140}$
30a	1680.6	1680.9	1681.6 (1)	$Val_{43}-Leu_{57}$
30b	990.5	990.5	991.5 (1)	$Ala_{23}-Leu_{32}$
30c	1710.7	1710.9	1711.7 (1)	Thr_6-Tyr_{20}
31	1190.7	1190.6	1191.7 (1)	$Ser_{21}-Leu_{32}$
32	1921.9	1922.0	1922.9 (1)	$Val_{43}-Gln_{59}$
33	1793.8	1794.0	1794.8 (1)	$Val_{43}-Leu_{58}$
34	1353.4	1353.7	1354.4 (1)	$Tyr_{20}-Leu_{32}$
35	2237.0	2236.2	1119.5 (2)	$Val_{43}-Trp_{61}$
36a*[c]	1063.5	1063.5	1064.5 (1)	$Glu_{62}-Lys_{70}$
36b*	935.4	935.4	936.4 (1)	$Glu_{62}-Lys_{69}$
37*	1176.5	1176.5	1177.5 (1)	$Glu_{62}-Ile_{71}$
38a*	757.4	757.3	758.4 (1)	$Glu_{157}-Ile_{162}$
38b*	1489.5	1489.7	1490.5 (1)	$Glu_{62}-Glu_{74}$
39*	1336.4	1336.5	1337.4 (1)	$Cys_{106}-Leu_{117}$
40*	1666.0	1666.7	1667.0(1)	$Cys_{106}-Gln_{120}$
41*	1435.4	1435.6	1436.4 (1)	$Cys_{106}-Val_{118}$
42*	1310.5	1310.6	1311.5 (1)	$Asn_{152}-Ile_{162}$
43*	2374.2	2374.3	1188.1 (2)	$Glu_{62}-Phe_{82}$
44*	2688.4	2688.4	1345.2 (2)	$Lys_{60}-Phe_{82}$
45*	2929.4	2929.6	1465.7 (2)	$Leu_{58}-Phe_{82}$
46a*	1544.5	1544.7	1545.5 (1)	$Ser_{150}-Ile_{162}$
46b*	1882.5	1882.8	1883.5 (1)	$Cys_{106}-Leu_{122}$

[a] Monoisotopic mass for the neutral molecule, calculated from amino acid sequence. [b] Molecular ion (m/z) selected for MS/MS charge. [c] The asterisk indicates peptide fragments obtained in DTT-treated samples.

Table 2. Relative Area of Peptide Fractions Obtained from Chymotrypsin Proteolysis of β-Lg A Subjected to Different Treatments with High Pressure, As Calculated from RP-HPLC Chromatograms [Areas of Fractions Correspond to F1 (Peaks 1–11), F2 (Peaks 12–18), F3 (Peaks 19–26), F4 (Peaks 27–35), PF (F1+F2+F3+F4), Total (F1+F2+F3+F4+β-Lg)]

β-Lg pretreatment prepressurization for 20 min (MPa)	enzyme treatment		relative area				
	pressure (MPa)	time (min)	PF/total	F1/PF	F2/PF	F3/PF	F4/PF
none	0.1	5	0.11	0.16	0.24	0.18	0.43
none	0.1	60	0.50	0.11	0.27	0.20	0.41
none	0.1	480 (8 h)	0.97	0.19	0.38	0.23	0.21
none	0.1	1440 (24 h)	1.00[a]	0.22	0.52	0.18	0.08
none	100	5	0.93	0.15	0.24	0.19	0.42
none	100	10	0.97	0.13	0.23	0.17	0.47
none	100	20	1.00[a]	0.15	0.27	0.22	0.36
none	200	5	1.00[a]	0.12	0.20	0.19	0.49
none	200	10	1.00[a]	0.13	0.24	0.22	0.41
none	200	20	1.00[a]	0.15	0.26	0.24	0.36
none	300	5	1.00[a]	0.15	0.21	0.19	0.46
none	300	10	1.00[a]	0.12	0.22	0.23	0.43
none	300	20	1.00[a]	0.14	0.25	0.28	0.33
none	400	5	1.00[a]	0.15	0.23	0.22	0.39
none	400	10	1.00[a]	0.12	0.24	0.28	0.37
none	400	20	1.00[a]	0.12	0.28	0.34	0.26
100	0.1	5	0.29	0.15	0.25	0.18	0.42
100	0.1	60	0.83	0.12	0.27	0.24	0.37
200	0.1	5	0.54	0.10	0.23	0.17	0.50
200	0.1	60	0.85	0.12	0.26	0.23	0.39
300	0.1	5	0.62	0.10	0.19	0.14	0.58
300	0.1	60	0.86	0.11	0.26	0.24	0.39
400	0.1	5	0.77	0.10	0.17	0.12	0.61
400	0.1	60	0.93	0.12	0.27	0.24	0.36

[a] Complete hydrolysis.

Leu_{58} (peaks 30a, 32, and 33, respetively); and $Ile_{72}-Phe_{82}$, $Lys_{70}-Phe_{82}$, and $Ile_{71}-Phe_{82}$ (peaks 23b, 25a, and 26a, respectively). In addition, the two disulfide-linked peptides, $(Lys_{60}-Phe_{82})$ S–S $(Ser_{150}-Ile_{162})$ and $(Glu_{62}-Phe_{82})$ S–S $(Ser_{150}-Ile_{162})$ (peaks 28b and 28c, respectively), were predominant in the chromatograms (**Figure 4a**). As reported above, treatment with DTT reduced disulfide bonds between residues Cys_{66} and Cys_{160} and between residues Cys_{106} and Cys_{119}. In fact, the peptides $Cys_{106}-Val_{118}$ and $Cys_{106}-Gln_{120}$ were detected in the RP-HPLC chromatograms only following reduction with DTT (peaks 40 and 41, respectively, **Figure 4b**).

Some peptides present in the hydrolysates of β-Lg obtained at atmospheric pressure were not found when proteolysis was conducted under high-pressure conditions (peaks 2, 3, 6–8, 10, 13b, 16a, 17b, 17c, 20, 22b, 23c, 25a, and 27). Residues that were cleaved under atmospheric pressure but not under high pressure were, in most cases, nonspecific targets, and their absence was probably due to the short hydrolysis times.

Hydrolysis of Prepressurized β-Lg with Chymotrypsin at Atmospheric Pressure. Prepressurized β-Lg was also hydrolyzed more rapidly than native β-Lg at atmospheric pressure, although a considerable amount of the intact protein was still present under the conditions assayed (**Figure 3**, lanes 13–16). The SDS-PAGE band patterns were qualitatively very similar to those of β-Lg hydrolyzed under high pressure (**Figure 3**, lanes 9–12). The aggregates formed on the pressurization step (**Figure 3**, lanes 5–8) disappeared when the prepressurized β-Lg was hydrolyzed by chymotrypsin at atmospheric pressure.

The RP-HPLC analysis showed that the relative area of the total peptide fraction increased with higher pressure (**Table 2**). The hydrophobic peptide portion was very important in the

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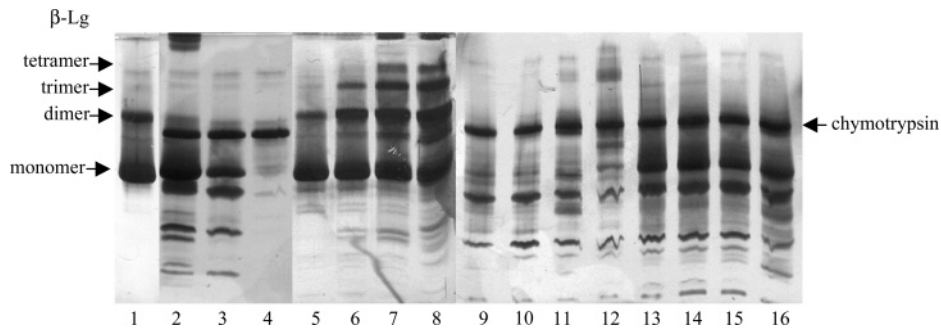
Figure 3. SDS-PAGE pattern under nonreducing conditions of native β-Lg A (lane 1); β-Lg A treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 0.1 MPa for 1 (lane 2), 8 (lane 3), and 24 h (lane 4); β-Lg A pressurized for 20 min at 20 °C at 100 (lane 5), 200 (lane 6), 300 (lane 7), and 400 MPa (lane 8); β-Lg A treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20 and a temperature of 37 °C for 20 min at 100 (lane 9), 200 (lane 10), 300 (lane 11), and 400 MPa (lane 12); and β-Lg A prepressurized at 100 (lane 13), 200 (lane 14), 300 (lane 15), and 400 MPa (lane 16) at 20 °C for 20 min and subsequently treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 0.1 MPa for 60 min.
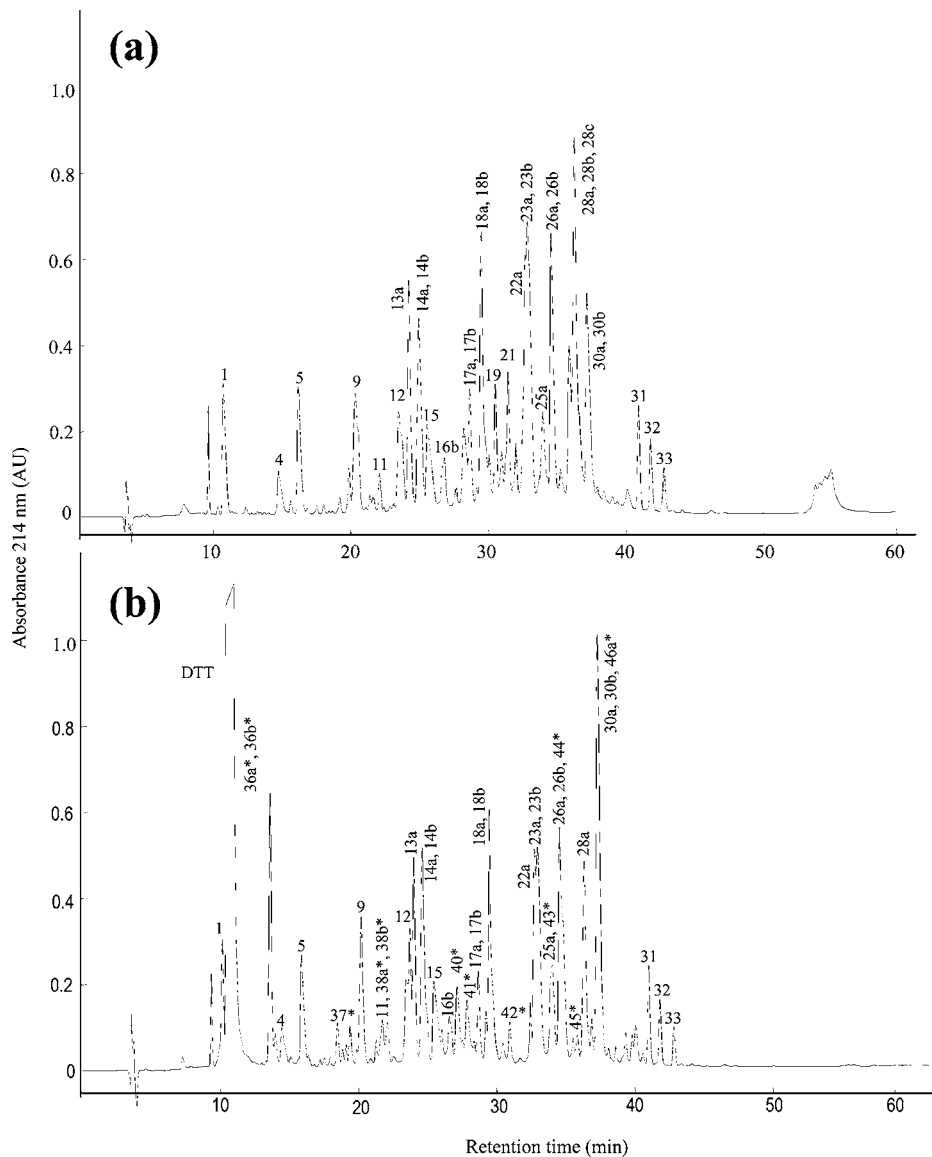
Figure 4. RP-HPLC pattern of native β-Lg A treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 400 MPa for 20 min, before (**a**) and after (**b**) reduction with DTT. Peak numbers refer to the peptide sequences indicated in **Table 1**.

hydrolysates (see, for instance, peptide peaks 28−30 in **Figure 5a** and the high relative area of fraction F4 in **Table 2**). In addition, new peaks with high retention times appeared after hydrolysis of β-Lg prepressurized at 300 and 400 MPa. Among

these, components 28d, 30c, 34, and 35 were identified, respectively, as the fragments Thr$_6$−Trp$_{19}$, Thr$_6$−Tyr$_{20}$, Tyr$_{20}$−Leu$_{32}$, and Val$_{43}$−Trp$_{61}$, whereas for other peaks, eluting at 38−48 min (**Figure 5a**), ionization was not strong enough to allow

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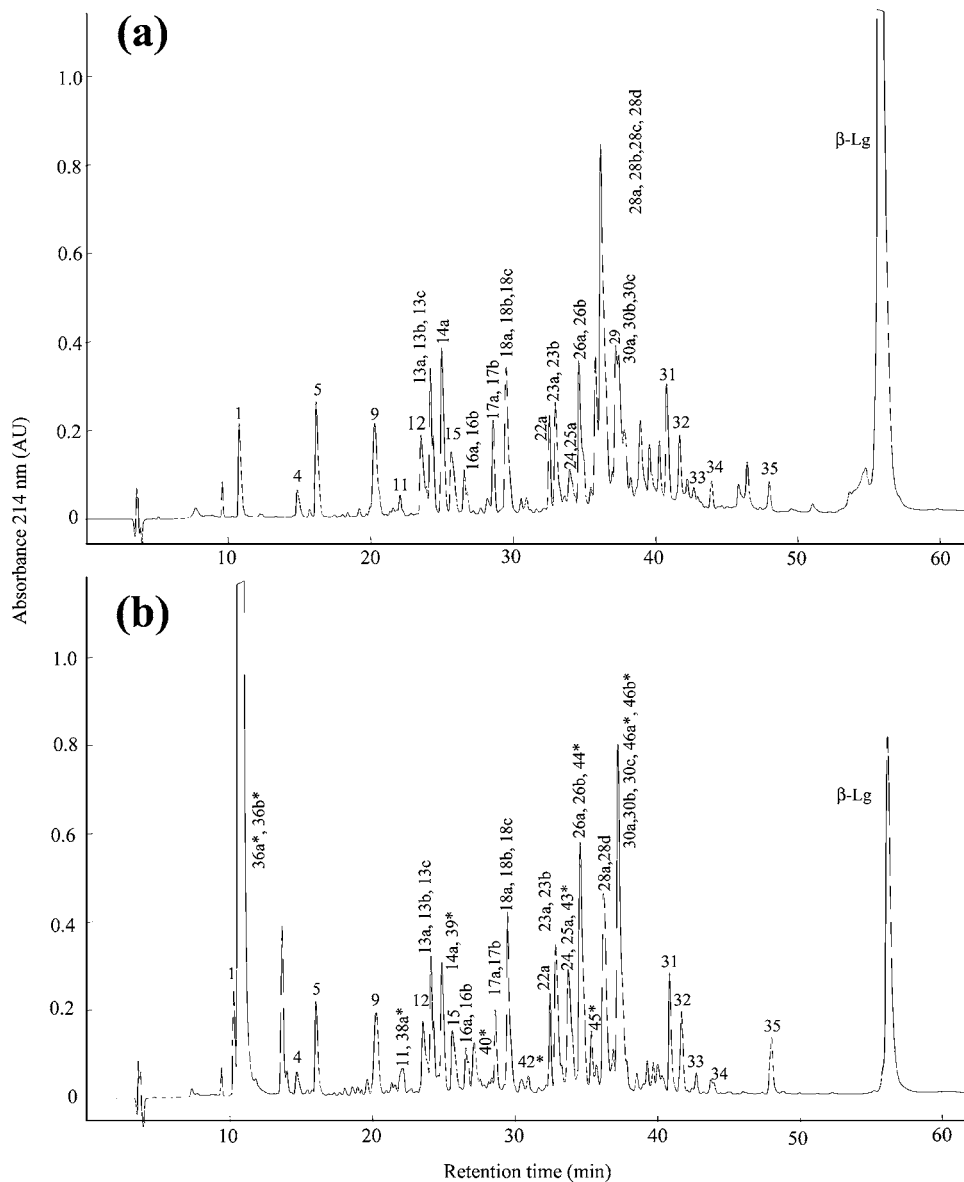
Figure 5. RP-HPLC pattern of prepressurized β-Lg A (300 MPa, 20 min), treated with chymotrypsin at an enzyme-to-substrate ratio of 1:20, a temperature of 37 °C, and a pressure of 0.1 MPa for 5 min, before (**a**) and after (**b**) reduction with DTT. Peak numbers refer to the peptide sequences indicated in **Table 1**.

for sequence elucidation. These components were absent from the hydrolysates of β-Lg prepressurized at 100 MPa, and they were very small in those of β-Lg prepressurized at 200 MPa (results not shown), suggesting that they might be related to the pressure level, and tended to disappear at longer hydrolysis times. It should be mentioned that, after reduction with DTT, a peptide corresponding to the fragment $Cys_{106}-Leu_{122}$ was found (**Figure 5b**), which contains Cys_{106}, Cys_{119}, and Cys_{121}.

Similar to the results obtained for proteolysis conducted under high pressure, many fragments found in the hydrolysates obtained under atmospheric pressure from native β-Lg were absent in the prepressurized samples. Most of these peptides were the result of cleavage at nonspecific targets and thus likely not to be favorably cleaved due to the short time used for hydrolysis of prepressurized β-Lg.

DISCUSSION

Simultaneous or previous pressurization of β-Lg increased its susceptibility to hydrolysis by chymotrypsin. These results are in agreement with previous reports showing that pressuriza-tion during or before enzyme treatment enhances hydrolysis of β-Lg by different enzymes (*2*, *4*, *6*, *7*). When the enzyme treatment was conducted at atmospheric pressure on prepres-surized β-Lg, this was hydrolyzed more rapidly than native β-Lg. Proteolysis with chymotrypsin was slightly faster than with trypsin (*7*) and, for both enzymes, more efficient as the protein was pretreated with increasing pressure. However, the presence of intact protein in these hydrolysates shows that proteolysis conducted after the pressure treatment does not very efficiently remove the protein. Simultaneous application of high pressure and enzymatic treatment considerably accelerated the hydrolysis. β-Lg was completely proteolyzed after treatment with chymotrypsin at 200 MPa for 10 min or at 300 MPa for 5 min. Complete hydrolysis had been achieved with trypsin at 200 MPa for 5 min (*7*).

The specificity of these two enzymes is different: trypsin is specific toward positively charged amino acids, mostly located on the surface of the β-Lg molecule, and chymotrypsin is specific for aromatic, Leu and Met residues, many of them buried in the hydrophobic core of the native protein. The number

and rate of formation of peptides released by chymotrypsin are thus expected to increase as a result of partial unfolding of the core of β-Lg (*6*). On the other hand, pressure-induced inactivation of trypsin has been reported to occur at 400 MPa (*4, 5*), whereas the activity of chymotrypsin is greatly enhanced at 360 MPa (*14*). However, despite their different specificities and pressure sensitivities and, in agreement with previous reports (*2*), the extents of proteolysis achieved with both enzymes were very similar. This supports the hypothesis that the accessibility of cleavage sites on the substrate is what limits the effectiveness of the enzyme action (*2*).

During the pressure treatment, disulfide-linked aggregates were formed (**Figure 3**, lanes 5–8), but we did not find evidence of peptides resulting from disulfide bond rearrangements involving Cys_{121} and Cys_{66} or Cys_{160}, as was previously proven for high-pressure-induced (*7*) and heat-induced aggregates (*15*). In agreement with Knudsen et al. (*6*), an increase in disulfide-linked oligomers was observed as the pressure applied increased. Low-order oligomers were detectable at lower pressures than higher order aggregates, in agreement with the mechanism proposed for the early stages of β-Lg aggregation induced by heat (*16*) and high pressure (*17*), which include the dissociation of the dimer into monomers, the subsequent activation of monomers, and the formation of intermediate oligomers. The aggregates formed on the prepressurized protein (**Figure 3**, lanes 5–8) disappeared after being treated with chymotrypsin at atmospheric pressure (**Figure 3**, lanes 13–16), thus being efficiently hydrolyzed. On the other hand, when proteolysis was performed under high pressures, no aggregates were found (**Figure 3**, lanes 9–12). In this case, aggregation could be prevented by the proteolytic process (*2, 4*), although the formation and further proteolysis of a small amount of aggregates could also be involved (*7*).

The main difference encountered between hydrolysates obtained at high (**Figures 4a** and **5a**) and at atmospheric pressure (**Figure 2a**) with chymotrypsin was the accumulation of hydrophobic peptides, representing intermediate stages of hydrolysis. Proteolysis simultaneous or after pressurization favored the release of peptides contained in the sequences Lys_8–Tyr_{20}, Ser_{21}–Leu_{32}, and Lys_{70}–Phe_{82}. 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}), appeared as important intermediate hydrolysis products enhanced by the pressure treatment. These are coincident with the main peptides found in the early steps of chymotryptic hydrolysis of β-Lg at 55 °C and atmospheric pressure (*18*) and support previous findings which suggest that β-Lg behaves under pressure in a way similar to what occurs under elevated temperatures (*7, 17, 19*). Val_{43}–Leu_{57}, Val_{43}–Leu_{58}, and Val_{43}–Gln_{59} also increased in pressure-treated samples.

The accumulated fragments indicate that chymotrypsin attacked Leu_{149} and Tyr_{42} more easily in pressure-treated samples. Similarly, previous studies have shown that fragments Val_{15}–Arg_{40}, (Val_{41}–Lys_{69}) S–S (Leu_{149}–Ile_{162}), and (Val_{41}–Lys_{70}) S–S (Leu_{149}–Ile_{162}), which involve cleavage of Arg_{40} and Arg_{148}, are favorably released by trypsin under high pressures (*7*). This suggests that the conformational state of the protein under high pressure determines the similar behavior of both enzymes. It has been proposed that Arg_{148} and Arg_{40} can be preferred targets for trypsin due to their location at the dimer interface or very close to it (*7*). Similarly, Leu_{149} is located in the I strand, participating in the dimer bonding, and Tyr_{42} is close to it (**Figure 6**). This reinforces the hypothesis of dimer dissociation being the major cause for the rapid disappearance
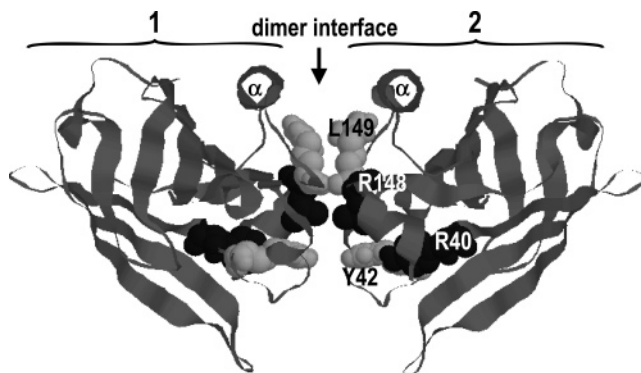
Figure 6. Three-dimensional model of the β-Lg A dimer, showing some chymotryptic (light gray) and tryptic (black) cleavage sites enhanced by high pressure. Monomers are labeled 1 and 2 and α-helix is labeled as α, for reference. Model built with RasWin Molecular Graphics, version 2.6, using the coordinates from the Protein Data Bank, PDB ID: 1BEB (*22*).

of β-Lg under proteinase action and high pressure (*7*). At atmospheric pressure, the equilibrium between the monomer and dimer forms would allow only a portion of the β-Lg molecules to have Leu_{149} and Tyr_{42} exposed for the proteinase action. However, under high pressure, induced dimer dissociation (*6*) would lead to the availability of these residues in all molecules.

Cleavage of other chymotrypsin-specific targets was difficult to achieve even under high pressure. For instance, Phe_{151}, Leu_{22}, and Met_{24} were cleaved slowly, as supported by the persistent presence of fragments Ser_{150}–Ile_{162} and Ser_{21}–Leu_{32} in all samples and the small proportion of their proteolysis products. High pressure promoted their cleavage only slightly. These residues lie in the protein region formed by the bound β-strands A–I. In the same region lies Leu_{149} that, in contrast, was a target promoted by high pressures. This suggests that high pressure may cause a partial disruption of this region, leaving some of its structure resistant to the action of high pressures and thus to the protease. On the other hand, no peptides resulting from cleavage after Leu_{46} and Leu_{54} were found even under high pressure, whereas the fragments containing them were present in considerable amounts. It is tempting to attribute it to their location, the bound β-strands B–C, that has shown to be very robust and difficult to unfold (*20, 21*).

It has to be pointed out that some sequential residues displayed different susceptibilities to cleavage by proteases. In this respect, Gln_{59} and Trp_{61} were easy targets for chymotrypsin. On the contrary, it has been previously shown that Lys_{60} is a difficult target for trypsin (*7*). Although the three residues form a tripeptide, Gln_{59} and Trp_{61} are more accessible to the enzyme, probably due to their orientation, opposite Lys_{60} in native β-Lg.

The results that show some portions of the molecule being more labile than others to proteolysis demonstrate that high-pressure promotes proteolysis of β-Lg but maintains a progressive proteolytic mechanism, because it led to a rapid removal of the intact protein, whereas intermediate fragments were proteolyzed at a slower rate. A similar behavior has been found when proteolysis has been conducted during a thermal treatment under sub-denaturing conditions (*18*). It seems that, under high pressure, the dissociation of the dimer could be a major event that leads to the rapid disappearance of β-Lg. However, high pressure does not seem to promote so efficiently other structural events that could favor complete proteolysis, even though an increased flexibility of the whole protein occurs at pressures around and above 200 MPa (*10*). The use of high-pressure chymotryptic or tryptic hydrolysis of β-Lg for relatively short

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times (5−20 min) can accelerate proteolysis and produce hydrolysates with high contents of high molecular weight peptides. It is likely that these types of hydrolysates show good functional properties.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; DTT, dithiothreitol; EDTA, ethylene-diaminetetraacetic acid; ESI-MS/MS, electrospray ionization mass spectrometry/mass spectrometry; RP-HPLC, reversed phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; UV, ultraviolet.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of February 18, 2006, contained an error in Table 1. This has been corrected in the posting on February 22, 2006.

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