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Influence of high hydrostatic pressure on the proteolysis of β-lactoglobulin A by trypsin

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This work describes the effect of the hydrolysis time and pressure (0·1–400 MPa) on the proteolysis of β-lactoglobulin A (β-lg A) with trypsin, either conducting hydrolysis of β-lg under pressure or hydrolysing β-lg that was previously pressure treated. Pressurisation, before or during enzyme treatments, enhanced tryptic hydrolysis of β-lg. Trypsin degraded pressure-modified β-lg and pressure-induced β-lg aggregates, favouring proteolysis to the intermediate degradation products: (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}). These were further cleaved at the later stages of proteolysis to yield: (Val_{15}-Tyr_{20}), (Ser_{21}-Arg_{40}), (Val_{41}-Tyr_{60}), (Trp_{61}-Lys_{69})S-S(Leu_{149}-Ile_{162}) and (Trp_{61}-Lys_{70})S-S(Leu_{149}-Ile_{162}). Particularly, in the tryptic hydrolysates of pre-pressurized β-lg, two other fragments linked by disulphide bonds: (Lys_{101}-Arg_{124})S-S(Leu_{149}-Ile_{162}) and (Tyr_{102}-Arg_{124})S-S(Leu_{149}-Ile_{162}), were found. These corresponded to rearrangement products induced by SH/SS exchange between the free thiol group of Cys_{121} and Cys_{160}, that normally forms the disulphide bond Cys_{66}-Cys_{160}. In the light of these results, structural modifications of β-lg under high pressure are discussed.

Keywords: β-lactoglobulin, high pressure, proteolysis, trypsin.

The serum milk protein β-lactoglobulin (β-lg) has been reported to exhibit an improved proteolytic digestibility when hydrolysed under high hydrostatic pressure, and this has been applied to its selective removal from whey concentrates to be used as hypoallergenic additives to modified milk for infants (Hayashi et al. 1987; Okamoto et al. 1991). Such enhancement of proteolysis under pressure might benefit, not only from conformational changes of β-lg, that make it more accessible to proteolysis, but also from pressure effects on the enzyme or a direct effect on the β-lg–enzyme interaction (Stapelfeldt et al. 1996). Protease activity under high pressure is determined by the enzyme stability, with many monomeric enzymes retaining their native structures up to pressures of 400–800 MPa, as well as by changes in the activation free volume of the reaction ($\Delta V^{\neq}$) (Kudryashova et al. 1998). It should also be noted that the activity of some enzymes, such as chymotrypsin, is greatly increased at 360 MPa (Mozhaev et al. 1996).

Tryptic hydrolysis of β-lg at pH 7·7–8·0 is accelerated under pressure, with an optimum at 300 MPa (Van Willige & Fitzgerald, 1995; Maynard et al. 1998). Steady-state kinetic experiments showed that such enhanced digestibility results from an increase in the catalytic constant up to 100 MPa, presumably promoted by pressure-induced exposure of normally hindered cleavage sites, and from a continuous decrease in Michaelis constant, which indicates changes in the protein conformation that facilitate binding of the substrate to the enzyme (Olsen et al. 2003). Bonomi et al. (2000, 2003) postulated that, under conditions of sub-denaturing intensity, transient protein conformations are formed with ample regions of the hydrophobic core unfolded and this exposes new cleavage sites to enzymatic hydrolysis. However, according to Maynard et al. (1998), the nature of the tryptic end-products of β-lg produced at 300 MPa was the same as those obtained at 0·1 MPa.

In addition, pressure-induced denaturation of β-lg also enhances its susceptibility to subsequent proteolysis at atmospheric pressure (Stapelfeldt et al. 1996; Otte et al. 1997; Maynard et al. 1998; Knudsen et al. 2002; Bonomi et al. 2003). In this case, differences between the peptide profiles resulting from hydrolysis of pressure-treated and native β-lg have been reported (Knudsen et al. 2002).

The aim of this paper was to elucidate whether the use of high pressure on β-lg A changes the proteolytic pattern and/or promotes cleavage of sites that are not accessible in the native protein. For this purpose we examined the effect of trypsin, under different hydrolysis times and

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pressure levels, either conducting hydrolysis of β-lg under pressure or hydrolysing β-lg that was previously pressure treated.

Materials and Methods

Materials

β-Lg genetic variant A was isolated, following the method of Ebeler et al. (1990), from the pH 4·6 soluble fraction of a milk sample obtained from a homozygotic cow, previously genotyped for β-lg AA by isoelectric focusing. The protein content of the purified preparation, as determined by the Kjeldhal method, was 82 %. Analyses by SDS-PAGE, HPLC-MS (see below), and CE (Recio & Olieman, 1996) revealed that it was virtually free from other milk proteins. Comparison of the ^{1}H-NMR spectra with previously recorded spectra for pure β-lg A confirmed its purity and native conformation. TPCK-treated trypsin (EC 3.4.21.4) was purchased from Sigma Chemicals Co (St. Louis, MI, USA).

Trypsin treatments at high and atmospheric pressure

β-Lg A (5·0 mg) and trypsin (0·25 mg) were dissolved together in 2·0 ml 50 mM-Tris-HCl buffer, pH 6·8. For pressure experiments, substrate and enzyme mixtures were directly poured into Eppendorf vials (1700 µl) avoiding headspace and treated at 100, 200, 300 or 400 MPa, using a 900 HP equipment (Eurotherm Automation, Lyon, France). The pressure was raised at a rate of 2·5 MPa/s, maintained for 5, 10 or 20 min, and released at the same rate. The temperature of the hydrostatic fluid medium was controlled by circulating water 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 deg C/100 MPa. Immediately after removal from the high pressure unit, the reactions were stopped by the addition of 0·5 M-HCl to pH 3·0. Samples were freeze-dried and reconstituted as appropriate for subsequent analyses.

Proteolysis with trypsin was also conducted at atmospheric pressure on pressurized β-lg. For this purpose, β-lg A, dissolved in 50 mM-Tris-HCl buffer, pH 6·8, at a concentration of 3·2 mg/ml, was treated at 100, 200, 300 or 400 MPa and 20 °C for 20 min. Immediately after depressurisation, 400 µl of a trypsin solution were added to 1500 µl of the pressurized protein solution, so that the enzyme to substrate ratio (1 : 20) was the same as above, and 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 at 37 °C on native β-lg A in a water bath for 5, 10, 20, 30, 60 min, 8, 24 and 48 h. Reactions were stopped and processed as already indicated.

All reactions were performed in duplicate and the analytical determinations were performed at least in duplicate.

SDS-PAGE analyses

Lyophilized hydrolysates were dissolved either in 10 mM-Tris-HCl buffer, pH 8·0, 25 g SDS/l and 10 mM-EDTA (non-reducing conditions) or in the same buffer containing 50 g β-mercaptoethanol/l (β-ME) (reducing conditions), and heated at 100 °C for 10 min. Analysis by SDS-PAGE used the PhastSystem Electrophoresis apparatus, precast High Density Gels and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). Electrophoretic conditions and silver staining followed the procedures of the manufacturer.

RP-HPLC-MS/MS

RP-HPLC with UV detection, on-line electrospray ionisation and quadrupole ion trap instrument (ESI-MS/MS) analyses were performed on an 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 × 4·6 mm i.d.) (Bio-Rad Laboratories, CA, USA). Operating conditions were: column at ambient temperature; flow rate, 0·8 ml/min; injection volume, 50 µl;. solvent A, 0·37 ml TFA/l double distilled water (milli-Q); solvent B, 0·27 ml TFA/l 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. A standard curve of β-lg was used to determine the concentration of residual protein present in the hydrolysates. The flow was split post UV-detector by placing a T-piece (Valco, Houston, TX, USA) connected with a 75 µm ID peek outlet tube of an adjusted length to give approximately 20 µl/min of flow directed into the mass spectrometer via the electrospray interface. Ion source parameters were: nebulizer pressure, 60 psi; dry gas, 12 l/min and 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 10 000 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 Analyses™ (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. In addition, the known specificity of trypsin (Arg-X and Lys-X) was used in peptide labelling. To aid the identification of disulphide linked

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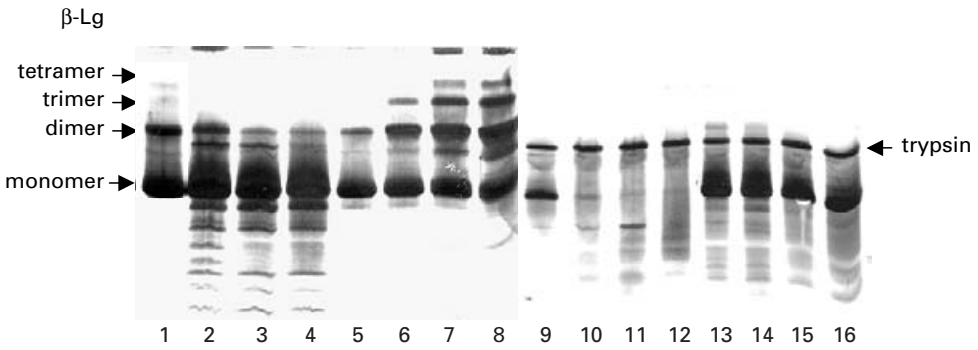

Fig. 1. SDS-PAGE patterns under non-reducing conditions of: β-lg treated with trypsin at an enzyme to substrate ratio 1 : 20 and 37 °C, at 0·1 MPa for 0 (1), 1 (2), 8 (3) and 24 h (4); β-lg pressurized for 20 min at 20 °C at 100 (5), 200 (6), 300 (7), and 400 (8) MPa; β-lg treated with trypsin at an enzyme to substrate ratio 1 : 20 and 37 °C, for 20 min at 100 (9), 200 (10), 300 (11) and 400 (12) MPa and β-lg pre-pressurized at 100 (13), 200 (14), 300 (15), and 400 (16) MPa at 20 °C for 20 min, and subsequently treated with trypsin at an enzyme to substrate ratio 1 : 20, 37 °C and 0·1 MPa for 60 min.

fragments the hydrolysates were also analysed 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.

Results

Effect of high pressure on the susceptibility of β-lg A to the proteolytic attack by trypsin

The concentration of residual β-lg A was determined by RP-HPLC, after treatment with trypsin under high pressure and after hydrolysis at atmospheric pressure of the native and pre-pressurized proteins. In addition, the hydrolysates were analysed by SDS-PAGE, as shown in Fig. 1. At atmospheric pressure (0·1 MPa), hydrolysis of the native protein with trypsin was very slow and approximately 40% of the original substrate remained after 48 h at 37 °C, which corroborated that β-lg A was very resistant to the action of this enzyme (Iametti et al. 2002). Simultaneous application of high-pressure and enzymatic treatment considerably accelerated β-lg A hydrolysis. Treatment with trypsin at 100 MPa for 20 min was not enough to remove all intact β-lg, but at 200 MPa for 5 min and above, all β-lg was proteolysed.

RP-HPLC and SDS-PAGE analyses showed that when the enzyme treatment was conducted at atmospheric pressure, pre-pressurized β-lg A was also hydrolysed more rapidly than native β-lg and the proteolysis level increased with the intensity of the pressure treatment applied. However, after 60 min of hydrolysis of β-lg pre-pressurised at 400 MPa for 20 min, there was a considerable amount of intact β-lg, showing that previous pressurisation was less efficient than simultaneous pressurisation for the enzymatic removal of the intact protein. It should be noted that a slightly enhanced hydrolytic efficiency was found even when the hydrolysis was conducted at atmospheric pressure on the substrate pre-pressurised at 100 MPa. This does not agree with the observation made by Knudsen et al. (2002), who indicated that pre-treatment of β-lg A at 150 MPa hardly affected subsequent hydrolysis reactions due to protein renaturation. A feasible explanation would be that, in the present work, proteolysis was performed immediately after pressurisation, while in the previous paper there was a lapse of 30 min between pressurisation and hydrolysis, that would allow for better structural recovery of the protein.

The electrophoretic pattern of native β-lg, showed, in addition to the monomer band, a band corresponding to the β-lg dimer (Fig. 1, lane 1). Pressurisation of β-lg gave rise to bands corresponding to dimers, trimers and tetramers, whose intensity increased with the pressure level (Fig. 1, lanes 5–8). Dimers and higher order aggregates were effectively reduced in the presence of β-ME (results not shown), although a small amount of dimers remained always visible, probably due to incomplete SS reduction (Morgan et al. 1999). This confirmed that SS bonds were the major intermolecular force involved in pressure induced aggregates (Funtenberger et al. 1995).

When β-lg was treated with trypsin under high pressure, no dimers or higher aggregates were found (Fig. 1, lanes 9–12). This indicated that either pressure-induced polymers were efficiently hydrolysed by the enzyme or that a previous attack on β-lg by the proteinase prevented them from being formed. When pre-pressurized β-lg was hydrolysed by trypsin at atmospheric pressure the aggregates that had been formed upon pressurisation were also efficiently hydrolysed (Fig. 1, lanes 13–16).

Effect of high pressure on the peptide patterns of β-lg A treated with trypsin

Fig. 2 shows the RP-HPLC chromatograms of the hydrolysate of β-lg A treated with trypsin at atmospheric pressure for 48 h and those obtained upon hydrolysis at 200 and 400 MPa for 20 min. Reduction with DTT was

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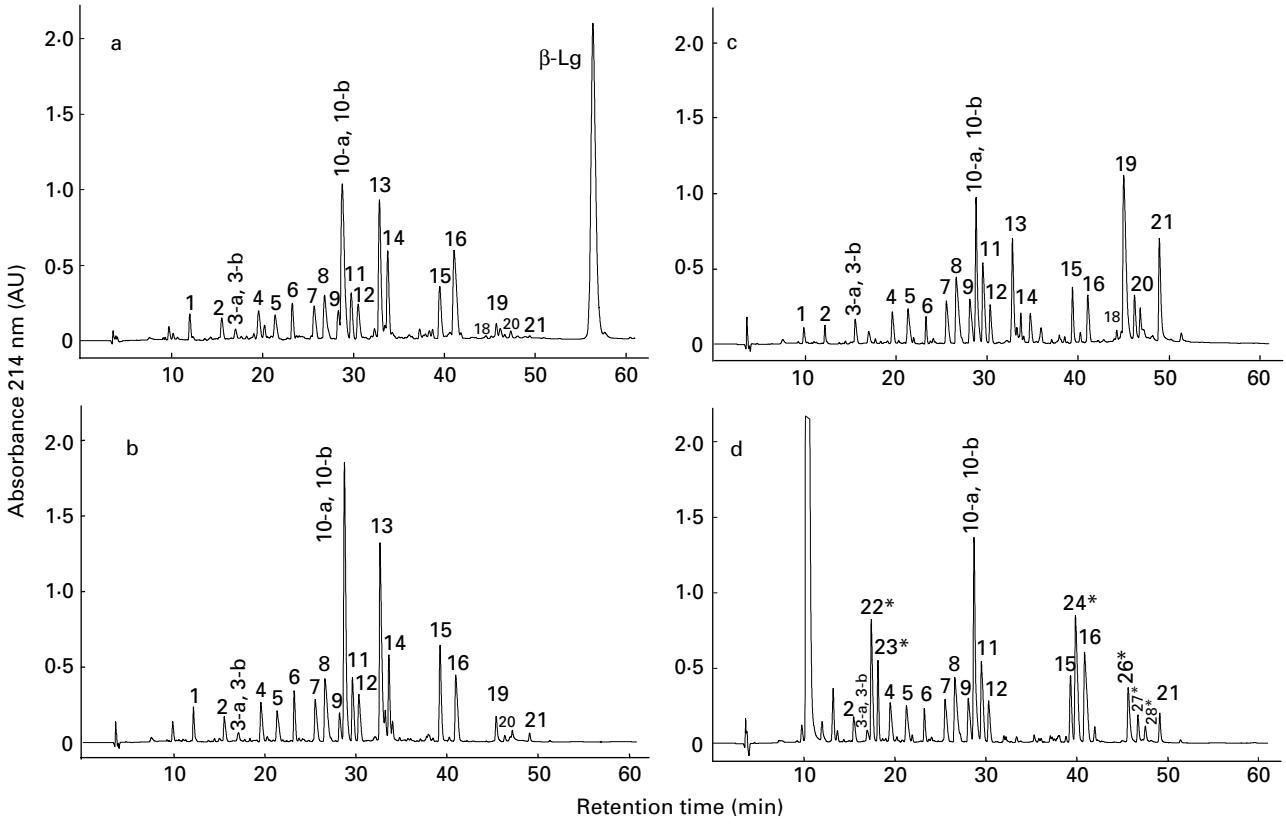

Fig. 2. RP-HPLC patterns of β-lg treated with trypsin at an enzyme to substrate ratio 1 : 20 and 37 °C, at 0·1 MPa for 48 h (a), at 200 MPa for 20 min (b) and at 400 MPa for 20 min, before (c) and after (d) reduction with DTT. Peak numbers refer to the peptide sequences indicated in Table 1.

carried out to detect disulphide linked peptides. The peak numbers refer to the peptide sequences identified by ESI-MS/MS (Table 1). No qualitative differences were observed in the peptide pattern when β-lg was hydrolyzed with trypsin under pressure as compared with atmospheric pressure, but quantitative differences were noticeable. At the highest pressure level assayed (400 MPa), hydrophobic, late eluting peptides were predominant in the chromatograms (peaks 19, 20, 21, Fig. 2c). These corresponded to two peptides linked by a disulphide bond between residues Cys_{66} and Cys_{160}: (Val_{41}-Lys_{69})S-S(Leu_{149}-Ile_{162}) and (Val_{41}-Lys_{70})S-S(Leu_{149}-Ile_{162}), as well as to the sequence (Val_{15}-Arg_{40}). As illustrated in Fig. 2d, the two former fragments were effectively split using reduction with DTT into (Val_{41}-Lys_{69}), (Val_{41}-Lys_{70}) and (Leu_{149}-Ile_{162}) (peaks 27*, 26* and 24*). Both had been previously identified in β-lg hydrolysates produced by trypsin at atmospheric pressure (Dalgalarrondo et al. 1990; Turgeon et al. 1992; Chen et al. 1993; Otte et al. 1997). Knudsen et al. (2002) also reported their presence after tryptic hydrolysis of β-lg pre-pressurized at 300 MPa for 15 min. The residue (Val_{15}-Arg_{40}) had also been described as an intermediate product formed in the early stages of tryptic hydrolysis at atmospheric pressure of native and pressurized β-lg (Dalgalarrondo et al. 1990; Maynard et al. 1998; Knudsen et al. 2002).

The fragments (Val_{41}-Lys_{69})S-S(Leu_{149}-Ile_{162}), (Val_{41}-Lys_{70})S-S(Leu_{149}-Ile_{162}) and (Val_{15}-Arg_{40}) (peaks 19, 20 and 21) were not so abundant in the hydrolysates obtained at lower pressures during an equivalent time period (i.e. 200 MPa, 20 min, Fig. 2b), but there was a higher level of their hydrolysis products: (Val_{41}-Tyr_{60}), (Trp_{61}-Lys_{69})S-S(Leu_{149}-Ile_{162}), (Trp_{61}-Lys_{70})S-S(Leu_{149}-Ile_{162}), (Val_{15}-Tyr_{20}), (Ser_{21}-Arg_{40}) (peaks 16, 14, 13, 10-b and 15 in Fig. 2b, respectively). In fact, at pressures from 100 to 300, the hydrophobic peptides 19–21 were initially released at the beginning of the hydrolysis, and progressively disappeared after 10 to 20 min, as they gave rise to their degradation products. The relative abundance of these long hydrophobic intermediates in β-lg hydrolysed under 400 MPa for 20 min (Fig. 2c) could be explained by the impairment of further proteolysis due to the pressure-induced inactivation of trypsin, which has been reported to occur at this pressure level (Van Willige & Fitzgerald, 1995; Maynard et al. 1998).

In the hydrolysates produced with trypsin on pre-pressurized β-lg A, hydrophobic and disulphide linked peptides were also very prominent, particularly at the

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Table 1. Peptide sequences arising from the hydrolysis of β-lg with trypsin under different pressure conditions (0·1–400 MPa) and of pre-pressurized β-lg, as determined by RP-HPLC-MS/MS. Peaks 22–28 (marked with an asterisk) arose from reduction of the hydrolysates with 70 mM-DTT

Peak	Observed mass	Calculated mass[a]	Molecular ion (m/z) selected for MS/MS (charge)	Protein fragment
1	408·2	408·201	409·2 (2)	$Phe1_{36}$-Lys_{138}
2	572·5	572·353	573·5 (1)	Ile_{71}-Lys_{75}
3-a	700·6	700·448	701·6 (1)	Lys_{70}-Lys_{75}
3-b	856·5	856·440	857·5 (1)	Asp_{33}-Arg_{40}
4	915·5	915·466	916·5 (1)	Ile_{84}-Lys_{91}
5	672·5	672·381	673·5 (1)	Gly_{9}-Lys_{14}
6	1244·5	1244·577	1245·5 (1)	Thr_{125}-Lys_{135}
7	932·6	932·536	933·6 (1)	Leu_{1}-Lys_{8}
8	836·6	836·469	837·6 (1)	Ala_{142}-Arg_{148}
9	1192·6	1192·678	1193·6 (1)	Val_{92}-Lys_{101}
10-a	902·6	902·559	903·6 (1)	Thr_{76}-Lys_{83}
10-b	695·4	695·328	696·4 (1)	Val_{15}-Tyr_{20}
11	1634·5	1634·768	1635·5 (1)	Thr_{125}-Lys_{138}
12	1064·6	1064·575	1065·6 (1)	Val_{92}-Lys_{100}
13	2906·0	2905·317	1454·0 (2)	(Trp_{61}-Lys_{70})S-S(Leu_{149}-Ile_{162})
14	2777·0	2777·222	1389·5 (2)	(Trp_{61}-Lys_{69})S-S(Leu_{149}-Ile_{162})
15	2029·7	2029·051	2030·7 (1)	Ser_{21}-Arg_{40}
16	2313·0	2312·252	1157·5 (2)	Val_{41}-Lys_{60}
17	4458·3	4458·098	1487·1 (3)	(Lys_{101}-Arg_{124})S-S(Leu_{149}-Ile_{162})
18	4330·5	4329·997	1444·5 (3)	(Tyr_{102}-Arg_{124})S-S(Leu_{149}-Ile_{162})
19	5199·6	5199·557	1734·6 (3); 1300·9 (4)	(Val_{41}-Lys_{70})S-S(Leu_{149}-Ile_{162})
20	5072·7	5071·467	1691·9 (3); 1269·2 (4)	(Val_{41}-Lys_{69})S-S(Leu_{149}-Ile_{162})
21	2707·0	2706·369	1354·5 (2)	Val_{15}-Arg_{40}
22*	1249·5	1249·540	1250·4 (1)	Trp_{61}-Lys_{70}
23*	1121·4	1121·445	1122·4 (1)	Trp_{61}-$Lys_{6}9$
24*	1657·5	1657·777	1658·5 (1)	Leu_{149}-Ile_{162}
25*	2802·2	2802·321	1402·1 (2)	Lys_{101}-Arg_{124}
26*	3544·4	3543·781	1773·2 (2)	Val_{41}-Lys_{70}
27*	3415·4	3415·686	1708·7 (2)	Val_{41}-Lys_{69}
28*	2674·0	2674·226	1338·0 (2)	Tyr_{102}-Arg_{124}

[a] Monoisotopic mass for the neutral molecule, calculated from amino acid sequence

shortest hydrolysis times and at the highest pressures. As illustrated in Fig. 3a, tryptic hydrolysis of β-lg treated at 200, 300 or 400 MPa, for only 5 min, released peptides that eluted from 38 to 53 min. These were further cleaved at later stages of proteolysis, but remained to a greater extent when pre-pressurisation was conducted at 400 MPa compared to 300 and 200 MPa.

In addition to the peptides previously described, two other fragments linked by disulphide bonds: (Lys_{101}-Arg_{124})S-S(Leu_{149}-Ile_{162}) and (Tyr_{102}-Arg_{124})S-S(Leu_{149}-Ile_{162}) (peaks 17 and 18), were found by ESI-MS/MS and confirmed with DTT reduction (peaks 25*, 28* and 24*, Fig. 3b). These corresponded to rearrangement products induced by SH/SS exchange between the free thiol group of Cys_{121} and Cys_{160}, that normally takes part of the disulphide Cys $_{66}$-Cys_{160} bond:

$$HS\text{-}Cys_{121} + Cys_{66}\text{-}S\text{-}S\text{-}Cys_{160} \rightarrow Cys_{121}\text{-}S\text{-}S\text{-}Cys_{160} + HS\text{-}Cys_{66} \quad (1)$$

These fragments were present in the highest amounts when proteolysis was performed at atmospheric pressure on pre-pressurised β-lg (Fig. 3a). However, traces of (Tyr_{102}-Arg_{124})S-S(Leu_{149}-Ile_{162}) were also found in the hydrolysate of β-lg with trypsin at atmospheric pressure and at 400 MPa (peak 18, Fig. 3a & c). The possibility of SH/SS interactions between β-lg peptides produced at atmospheric pressure was reported, particularly at high pH, due to an increased reactivity of the SH groups (Turgeon et al. 1992; Maynard et al. 1998; Caessens et al. 1999).

The existence of rearrangement products was further substantiated by the presence of fragments (Trp_{61}-Lys_{70}), (Trp_{61}-Lys_{69}), and (Leu_{149}-Ile_{162}) (peaks 22*, 23* and 24*), in the hydrolysates that were not reduced with DTT (Fig. 3a). These peptides contain either Cys_{66} or Cys_{160} in a SH form. Since these residues form a SS bond in the native β-lg, their presence might indicate that a SH/SS exchange reaction has occurred, not only following

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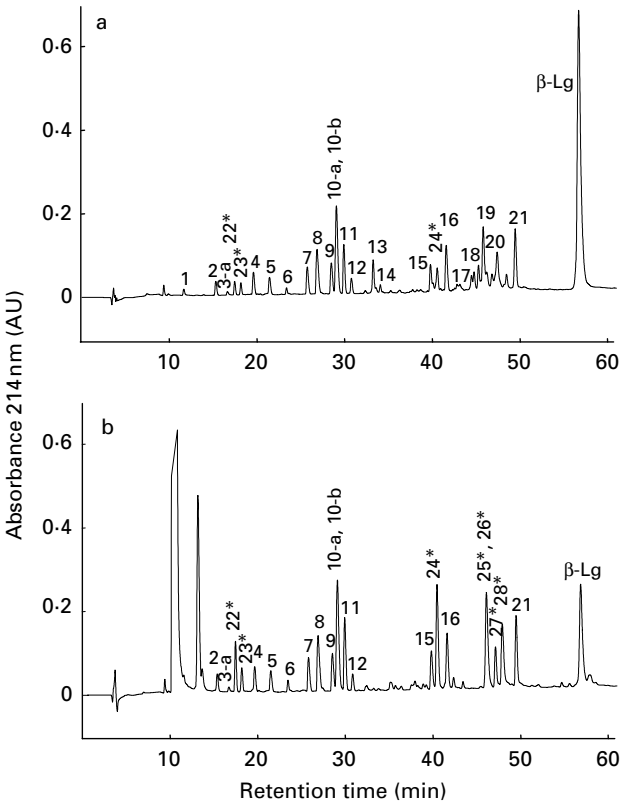

Fig. 3. RP-HPLC patterns of β-lg pre-pressurized at 300 MPa at 20 °C for 20 min and subsequently treated with trypsin at an enzyme to substrate ratio 1 : 20, 37 °C and 0·1 MPa for 5 min, before (a) and after (b) reduction with DTT. Peak numbers refer to the peptide sequences indicated in Table 1.

reaction (1), but also reaction (2):

$$HS\text{-}Cys_{121} + Cys_{66}\text{-}S\text{-}S\text{-}Cys_{160} \rightarrow Cys_{66}\text{-}S\text{-}S\text{-}Cys_{121} + HS\text{-}Cys_{160} \quad (2)$$

A proportion of the peptide (Leu_{149}-Ile_{162}) was also found in tryptic hydrolysates of heated β-lg (Creamer et al. 2004). It has to be pointed out that, in our study, these fragments were only observed in the pre-pressurised samples.

Discussion

In agreement with previous reports, our results show that pressurisation up to 400 MPa, before or during enzyme treatments, enhanced tryptic hydrolysis of β-lg (Maynard et al. 1998; Knudsen et al. 2002; Bonomi et al. 2003). As expected, β-lg A was more susceptible to proteolysis when the enzymatic treatments were carried out under pressure. Our study revealed quantitative differences in the hydrolysis pattern when high pressures were applied before or during trypsin action, mainly at the shortest hydrolysis times. Unlike the case of proteolysis at atmospheric pressure, that favoured the degradation of the β-lg substrate, as well as that of the intermediate hydrolysis products, high pressure promoted a quick attack towards β-lg A that led to the transient accumulation of intermediate peptide products.

Previous papers reported the presence of an increased level of hydrophobic peptides, representing intermediate stages of hydrolysis, in tryptic hydrolysates obtained under high temperatures (55 and 60 °C) at atmospheric pressure (Iametti et al. 2002), particularly the disulphide linked fragments (Val_{41}-Lys_{69})S-S(Leu_{149}-Ile_{162}) and (Val_{41}-Lys_{70})S-S(Leu_{149}-Ile_{162}), that also appeared as the main intermediate hydrolysis products in the present study. This strongly supports the existence of structural similarities between pressure and thermal denaturation of β-lg (Iametti et al. 1997; Panick et al. 1999) that may render similar portions of the molecule more accessible to the proteolytic action as compared to the native protein.

Furthermore, Iametti et al. (2002) suggested that the hydrolysis of β-lg under heat followed a sequential mechanism (or 'progressive proteolysis'), and our data bears out the existence of a similar process during hydrolysis under high pressure. A sequential mechanism would involve, initially, the release from the protein of large, intermediate, fragments, such as (Val_{41}-Lys_{69})S-S(Leu_{149}-Ile_{162}) and (Val_{41}-Lys_{70})S-S(Leu_{149}-Ile_{162}), susceptible to further hydrolysis. Under high pressure, these fragments tend to accumulate probably because they are formed at a rate faster than that of the subsequent proteolysis steps. Unlike our interpretation, Maynard et al. (1998) reported that, when tryptic hydrolysis of β-lg was conducted under high pressure, enhanced proteolysis was due to accelerated breakdown of large, intermediate products to final tryptic peptides. However, these authors conducted the enzymatic reactions for longer exposure times (100 min), which would allow for intermediate products to be extensively hydrolysed. As suggested by Bonomi et al. (2003), it is likely that under our conditions of high enzyme to substrate ratios and short incubation times, the attack towards the protein is maximized at the initial stages of the reaction without extensive degradation of the hydrolytic fragments. Therefore, assuming a sequential mechanism, significant amounts of intermediate fragments would be obtained if the pressure is high enough to provide good access to the first target and if the hydrolysis time is short enough to avoid further hydrolysis.

The reason why some peptide bonds of the protein were hydrolysed preferentially to others should be found in the protein structure and the mechanism of action of trypsin. First, the target residues need to be accessible for the enzyme to bind. In addition, in order to cut the polypeptide chain after a Lys or Arg residue, this enzyme needs to bind the carboxyl group of this amino acid, which could be less available when forming H-bonds. The proteolytic attack on Arg_{40} was preferred over Lys_{60}. The lower accessibility of Lys_{60} could be influenced by its location, in strand C, where it participates in the H-bonding between strands C and D. The increased tryptic attack on

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Arg_{148} and Arg_{40} under pressure can be the consequence of the same structural change. Arg_{148}, located in the I strand, participates in dimer formation (Sawyer & Kontopidis, 2000). Better access to Arg_{148} could be provided if structural modifications of β-lg under high pressure include the dimer dissociation, in a way similar to that which occurs under elevated temperatures (Iametti et al. 2002; Creamer et al. 2004). Since Arg_{148} is involved in the H-bonding between strands A-I, it is very likely that loosening of the A-I strand interactions also occurs. These two structural changes would also make Arg_{40} more accessible, since, in the native form of β-lg, it is protected from exposure by the dimer interface and strand I. This is consistent with a generally accepted rule, that indicates that, under pressures lower than 150–200 MPa, dissociation of oligomers is induced (Lullien-Pellerin & Balny, 2002) and that pressure-induced unfolding of β-lg may increase surface hydrophobicity through the unmasking of buried hydrophobic groups (Pittia et al. 1996; Hosseini-nia et al. 1999).

In agreement with our results, Knudsen et al. (2002) reported that tryptic hydrolysis of pre-pressurized β-lg samples produced more hydrophobic or larger fragments than the native substrate. However, they attributed these differences to the formation of covalently linked oligomers, that rendered some parts of the pre-pressurized β-lg molecule, such as the dimer interface, less susceptible to the enzymatic attack. Our results on the hydrolysis of pre-pressurized β-lg A do not support this observation, because the peptide pattern also matched the hypothesis of dimer dissociation and exposure of the polypeptide backbone to the action of the trypsin explained above.

Knudsen et al. (2002) also found a decreased availability of parts of the β-lg molecule lying close to the Cys_{66}-Cys_{160} disulphide bond to enzymatic cleavage after pressure treatment. This supported previous reports on the establishment of intermolecular SS bonds by SH/SS exchange, with the participation of the free SH of Cys_{121} and the bond between Cys_{66}-Cys_{160} (Belloque et al. 2000). In the present paper we provide evidence for the formation on pressurisation of the peptides (Lys_{101}-Arg_{124}) S-S(Leu_{149}-Ile_{162}) and (Tyr_{102}-Arg_{124})S-S(Leu_{149}-Ile_{162}), as well as fragments with Cys_{66} and Cys_{160} in an SH form. All these fragments are consistent with the formation of a pressure-induced disulphide bond involving Cys_{121} and Cys_{66}/Cys_{160}, what confirms the low reactivity of the bond Cys_{106}-Cys_{119} to high pressure (Belloque et al. 2000). Tryptic hydrolysis of heated β-lg also indicated that the Cys_{66}-Cys_{160} disulphide bond had been broken during heating, leaving some Cys_{160} in the reduced form. This suggested that Cys_{160} was a major player in inter-protein disulphide bonding during heat induced β-lg cross-linking (Creamer et al. 2004).

SDS-PAGE analyses showed that when proteolysis was conducted under pressure, no β-lg aggregates were found. A possible explanation could be that proteolytic events preceded protein aggregation, as explained by Bonomi et al. (2003). This is supported by the study of Maynard et al. (1998), who found no rearrangement products after subjecting the hydrolysates to high pressures ('post-hydrolytic pressurisation'). However, the hypothesis of a high susceptibility of the pressure-induced aggregates towards proteolysis also seems likely, since they disappeared when pre-pressurized β-lg was hydrolysed by trypsin at atmospheric pressure. In addition, it has to be pointed out that the proteolytic products obtained from SS rearrangement were present not only in the hydrolysates made from pre-pressurised β-lg but, also in the samples hydrolysed under high pressure, although in lower amounts. These results suggest that both hypothesis stated above may be true when proteolysis is performed at high pressures, that is, less aggregates are formed due to previous proteolysis events and a small amount of protein is aggregated and further proteolysed.

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