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Study of ethanol-induced conformational changes of holo and apo α-lactalbumin by spectroscopy and limited proteolysis

Zeina Wehbi[1], María-Dolores Pérez[1], Michèle Dalgalarrondo[2], Lourdes Sánchez[1], Miguel Calvo[1], Jean-Marc Chobert[2] and Thomas Haertlé[2]

[1]Tecnología y Bioquímica de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet, Zaragoza, Spain
[2]BIA-Fonctions et Interactions des Protéines Laitières, Institut National de la Recherche Agronomique, Nantes, France

This study was performed to contribute to the analysis of α-lactalbumin ``molten globule" state by using spectral and proteolysis techniques. Samples of holo and apo α-lactalbumin in the presence of different concentrations of ethanol were analyzed. Results of fluorescence spectroscopy of both forms showed that as ethanol concentration increased, the tryptophanyl residues became more accessible to the solvent. Near circular dichroism spectra of holo α-lactalbumin indicated that its tertiary structure was maintained in 20% ethanol whereas it was altered in 30 and 40% ethanol. For apo α-lactalbumin, spectra were similar in all samples studied. Holo α-lactalbumin was resistant to trypsinolysis in 0% ethanol, whereas it was easily hydrolyzed in 20 and 30% ethanol. In the case of the apo form and in the absence of ethanol, 70% of the protein was degraded after 1 h. However, in the presence of 20 and 30% ethanol, the overall reaction rate was lowered. Peptides obtained after tryptic hydrolysis were identified by reversed-phase high-performance liquid chromatography coupled to mass spectrometry. Differences in population of produced peptides indicate the changes of folding intermediates present in the studied α-lactalbumin solutions. This study demonstrated that proteolytic enzymes are suitable tools to determine protein structure complementing physico-chemical studies.

Keywords: Bovine α-lactalbumin / Conformation / Ethanol / Proteolysis / Spectroscopy

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1 Introduction

α-Lactalbumin is the second most abundant protein of bovine whey, after β-lactoglobulin, its concentration reaching about 1.2 mg/mL [1]. It is an acidic, low molecular mass globular protein (14.2 kDa) produced in the mammary gland during lactogenesis [2]. Bovine α-lactalbumin possesses a single strong Ca^{2+}-binding site [3]. The removal of calcium bound to the protein causes pronounced changes in structure and function, mostly in tertiary, but not in secondary structure, which is easily observed in fluorescence [4, 5] and circular dichroism data [6]. Cation binding to the strong calcium-binding site increases considerably the stability of the protein under the action of heating and other denaturing agents such as urea and guanidine hypochloride [3]. As demonstrated by differential scanning calorimetric data, the binding of calcium shifts the heat transition to higher temperatures by about 30°C [7].

An interesting property of α-lactalbumin is its ability to interact with a variety of hydrophobic compounds including fatty acids [8 – 10] and hydrophobic peptides such as melittin [11]. It has been shown that α-lactalbumin binds 5-doxylstearic, stearic, palmitic and oleic acids and the binding affinity depends upon the protein state [8 – 10]. Studies performed using fluorescence spectroscopy and partition equilibrium techniques indicated that the apo form of the bovine α-lactalbumin displays one binding site for oleic acid with an association constant of $4.6 \times 10^6\ M^{-1}$ [8]. However, holo α-lactalbumin is unable to bind fatty acids [8, 10].

The main function of α-lactalbumin is to modulate the substrate specificity of the lactose-synthetase complex from N-acetyl-glucosamine to glucose, allowing the formation of lactose in the lactating udder [2]. Recently, folding variants of human and bovine α-lactalbumin were shown to induce

Correspondence: Dr. María-Dolores Pérez, Tecnología y Bioquímica de los Alimentos, Facultad de Veterinaria, Universidad de Zaragoza, Miguel Servet 177, 50013-Zaragoza, Spain
E-mail: dperez@unizar.es
Fax: + 34-976-761-612

Abbreviations: CD, circular dichroism; **TFE**, trifluoroethanol

apoptosis in tumor cells and immature cells but to spare healthy differentiated cells. The conversion of α-lactalbumin to the active apoptotic form required the removal of calcium by treatment with EDTA and the passage of the apo protein throughout an ion-exchange column that had been conditioned previously with oleic acid [12, 13].

On the other hand, several studies have shown that the "molten globule state" is a relevant species in the pathway of protein folding. Therefore, it is of considerable interest to generate and characterize partially folded protein forms, occurring at equilibrium [14] and as their surface activity is increased in molten globule state, to study their functional, including biological properties. In α-lactalbumin, these forms can be generated upon exposing the protein to acid solutions (pH 2), to mild denaturant agents such as organic solvents or by removing calcium bound to the protein using chelating agents [3]. The available data show that with the increase of alcohol concentrations, α-lactalbumin undergoes conformational transitions followed by changes in their spectral properties [14 – 16] and thermal stability [15]. Likewise, the use of proteolytic enzymes has been already used to study protein conformation and to characterize alcohol-induced changes in α-lactalbumin and other proteins [14, 16 – 18].

The present work was performed to study conformational properties of the partly unfolded forms of α-lactalbumin induced by ethanol using a combination of spectral and proteolysis techniques. The structural changes and proteolytic susceptibilities of holo and apo α-lactalbumin, the latter with or without oleic acid bound, were analyzed at pH 8.0 and in the presence of different concentrations of ethanol.

2 Materials and methods

2.1 Materials

Bovine holo and apo α-lactalbumin, Sephadex G-50, oleic acid, and TPCK-trypsin were from Sigma (Poole, UK). Nucleosil C_{18} column (4.6 mm id × 25 cm) was from SFCC (Gagny, France) and Pepmap C_{18} column (75 μm id × 15 cm length) was from LC Packings, Dionex (Amsterdam, The Netherlands). The polyacrylamide gels and gel buffers were purchased from Pharmacia (Uppsala, Sweden). Other reagents and chemicals were of analytical grade.

2.2 Preparation of α-lactalbumin samples

Holo and apo α-lactalbumin were dissolved in 10 mM potassium phosphate, 0.15 M NaCl buffer, pH 7.4, and applied to a Sephadex G-50 column (75 × 3 cm) equilibrated with the same buffer. The fractions with the highest absorbance were collected, dialyzed against double distilled water and lyophilized. α-Lactalbumin purity was assessed by SDS-PAGE, using gels with a gradient of acrylamide from 8 to 25%, and densitometric analysis. Purity was found to be higher than 97%.

Binding of oleic acid to apo α-lactalbumin was performed by incubation of the protein (280 μM) dissolved in 25 mM Tris-HCl buffer, pH 8.0, with the fatty acid dissolved in ethanol at 37°C for 1 h. The concentration of oleic acid added was twice the molar concentration of the protein and the final concentration of ethanol in the protein solution was 3%.

Stock solutions of holo and apo α-lactalbumin were prepared in 25 mM Tris-HCl buffer, pH 8.0. Protein concentration in the stock solutions was determined spectrophotometrically using the extinction coefficient at 280 nm $E^{1\%}$ = 20.9. Protein solutions in different ethanol percentages (20, 30 and 40% v/v) were obtained by dilution of the stock solutions with ethanol and water to obtain the same protein concentration in each sample.

2.3 Fluorescence spectroscopy

Fluorescence spectra of holo and apo α-lactalbumin solutions were recorded in the ratio mode at 25°C with an Aminco SLM 4800C spectrofluorimeter using a quartz cuvette with 1-cm excitation path length. Intrinsic tryptophan fluorescence was recorded between 300 and 400 nm (excitation 290 nm). The excitation and emission bandwidths were set at 5 nm. Protein concentration was of 10 μM in 25 mM Tris-HCl buffer, pH 8.0.

2.4 Circular dichroism spectroscopy

Circular dichroism (CD) spectra of holo and apo α-lactalbumin solutions were measured using a Jobin Yvon Mark III dichrograph. Spectra were the averages of three accumulated scans with subtraction of the baseline. The cylindrical cells used had a path length of 1 cm in the near UV (250 – 350 nm) spectral region. The wavelength step was 1 nm, the response time was 4 s and the scan rate was 10 nm/min. All the spectra were taken at 37°C and at a protein concentration of 140 μM in 25 mM Tris-HCl buffer, pH 8.0. The results are expressed in terms of molar ellipticity ($deg \times cm^2 \times dmol^{-1}$).

2.5 Tryptic hydrolysis

Solutions of holo and apo α-lactalbumin (140 μM) in 25 mM Tris-HCl buffer, pH 8.0 were hydrolyzed with

TPCK-trypsin (initial concentration 1 mg/mL in 10^{-3} N HCl) with an E/S ratio of 1%. Proteolysis was performed in stabilized temperature of 37°C. Aliquots were removed after 1, 2, 3, 6, and 24 h. Hydrolysis was stopped by lowering the pH to 2.0 with the addition of 0.1 M glycine-HCl, pH 2.0 at a given reaction time. In a certain number of experiments, 0.1 M DTT was added to the aliquots to a final concentration of 10 mM and incubated at 37°C for 30 min before acidification. All aliquots were kept frozen until analysis by RP-HPLC.

2.6 Separation of tryptic peptides by RP-HPLC

Tryptic peptides were analyzed by RP-HPLC on a Nucleosil C_{18} column equilibrated with solvent A (0.11% TFA in H_2O) and eluted with a linear gradient from 35% solvent B (70% ACN, 30% H_2O, 0.09% TFA) to 65% solvent B in 30 min. The flow rate was 0.8 mL/min and the absorbance was recorded at 220 nm. A non-hydrolyzed sample was added to each set of chromatographic analysis as a control.

2.7 MS analysis

2.7.1 LC-MS analysis of α-lactalbumin

Capillary LC-ESI/MS analysis of α-lactalbumin was performed with a Waters-616 pump and a Waters-600 controller system (Waters, Milford, MA, USA), coupled to an IT mass spectrometer (LCQ Advantage, Thermo-Finnigan, San Jose, USA). The IT operated in the positive ionization mode. Mass data were acquired using the X-Calibur version 1.3 software (Thermo-Finnigan) on the *m/z* range 400 – 2000.

Chromatographic separation was performed by RP-HPLC on a Nucleosil C_{18} column at a flow rate of 300 µL/min. The flow was split 25-fold post column, to decrease the flow rate into the mass spectrometer to approximately 12 µL/min. The gradient used for chromatographic separation was as previously described (Section 2.6).

2.7.2 LC-MS/MS analysis of hydrolyzed α-lactalbumin

Nanoscale capillary LC-MS/MS analyses of the digested α-lactalbumin were performed using a Switchos-Ultimate II capillary LC system (LC Packings) coupled to a hybrid quadrupole orthogonal acceleration TOF mass spectrometer (Q-TOF Global, Micromass/Waters, Manchester, UK). Chromatographic separation was conducted on a Pepmap C_{18} column at a flow rate of 200 nL/min. The gradient consisted of a linear increase from 2 to 40% of ACN in 40 min, followed by an increase to 50% of ACN within 10 min.

Mass data acquisitions were piloted by the Masslynx software (Micromass/Waters) using the so-called "survey" scanning mode: MS data were recorded for 1 s on the *m/z* range 400 – 1500, after which the two most intense ions (doubly or triply charged ions only) were selected and fragmented in the collision cell (MS/MS measurements). MS/MS data were recorded on each of these ions for 10 s on the *m/z* range 50 – 1500. The energy in the collision cell was optimized as a function of the *m/z* and charge state of the precursor ions.

2.7.3 Peptide assignment: Databank searching

Mass data collected during LC-MS/MS analysis were processed with the Protein Lynx Global Server software version 2.1 (Micromass/Waters). α-Lactalbumin (entry P00711) was unambiguously identified by searching the peptide masses and MS/MS sequence stretches against the Swiss-Prot databank (release February 1, 2005). Several peptides displaying disulfide bridges could be assigned to the sequence of α-lactalbumin after a *de novo* interpretation of the MS/MS spectra. Mass tolerance for peptide identification was set to 120 ppm.

3 Results and discussion

3.1 Influence of ethanol on the structural changes of α-lactalbumin

Results of spectral measurements of ethanol-induced conformational changes of holo and apo α-lactalbumin are shown in Figs. 1 and 2. In the case of holo α-lactalbumin, the maximum of tryptophan emission was observed at 330 nm. As ethanol concentration increased, this maximum shifted to longer wavelengths and its magnitude increased, being most intense in the presence of 40% ethanol (Fig. 1 a). Red shift of the emission maximum implies that under the influence of ethanol, the tryptophanyl residues, which in aqueous solution are sheltered in the hydrophobic core of the protein molecule, become more exposed to a polar environment [19]. These results are in agreement with those of Grinberg *et al*. [15]. For the apo form, fluorescence intensity reached its maximum at 350 nm. No changes in the maximum wavelength were observed whereas the intensity magnitude increased with increasing ethanol concentrations (Fig. 1 b). This increase in fluorescence intensity corresponds to an increase in solvent polarity and may be due to a decrease of the quenching of some tryptophanyl residues.

Near-UV CD spectra of holo and apo α-lactalbumin are shown in Fig. 2. The spectrum of the holo form has two minima at 270 and 298 nm arising from spectral contribution of aromatic chromophores (tryptophan, tyrosine and phenylalanine) as previously reported [8, 15]. In the presence of 20% ethanol, the spectrum is similar to that of the holo protein in the absence of ethanol (Fig. 2 a). In contrast,

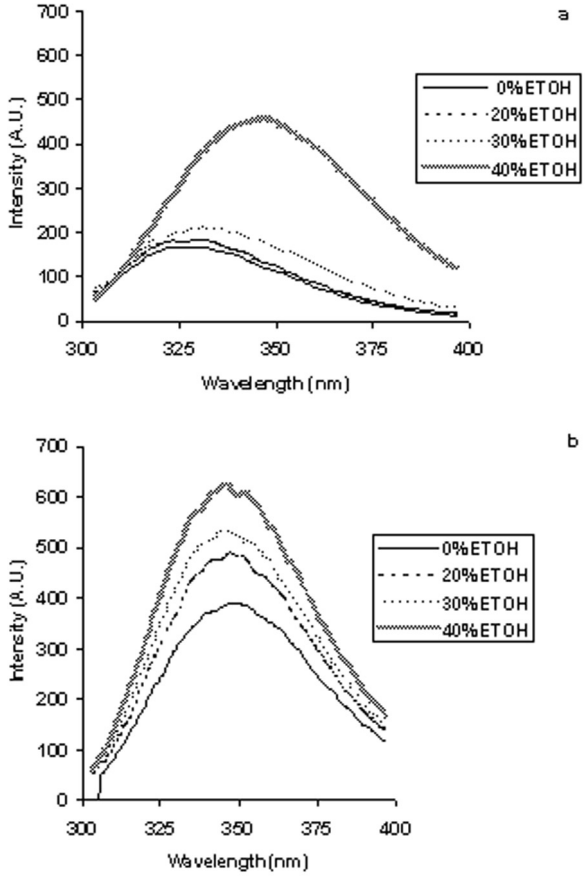

Figure 1. Fluorescence spectra of holo (a) and apo (b) α-lactalbumin in the presence of increasing concentrations of ethanol. Protein concentration was 10 μM in 25 mM Tris-HCl buffer, pH 8.0. The spectra were recorded at 25°C (A.U., arbitrary units).

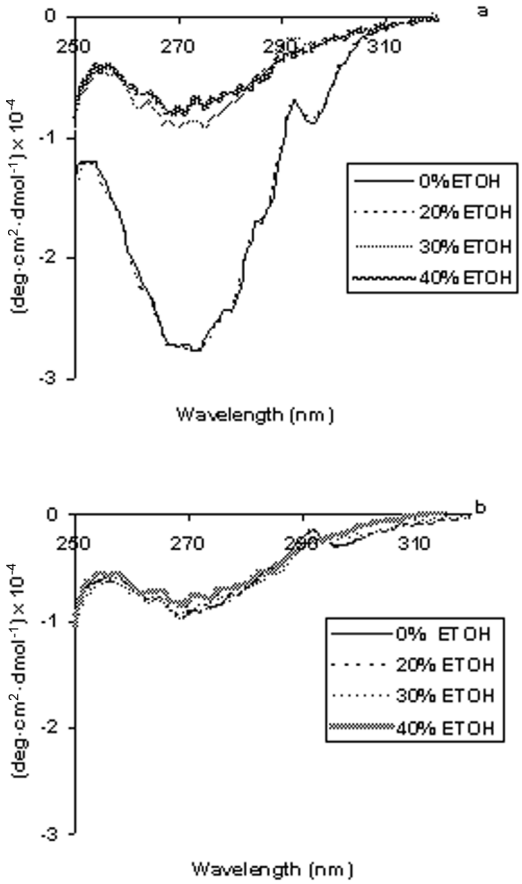

Figure 2. Near-UV CD spectra of holo (a) and apo (b) α-lactalbumin in the presence of increasing concentrations of ethanol. Protein concentration was 140 μM in 25 mM Tris-HCl buffer, pH 8.0. The spectra were recorded at 37°C.

the tertiary structure in the presence of 30 and 40% ethanol is largely disorganized as shown by the strong reduction in the CD signal in the 250–300 nm region. These results are in agreement with previous measurements performed for α-lactalbumin in the presence of increasing concentrations of ethanol [15] or trifluoroethanol (TFE) [14]. In the case of apo α-lactalbumin, the near CD spectra showed the characteristic absence of signal at 270 nm of this form, as compared with that previously reported [6, 8]. These results indicate an increase of the rotation freedom of aromatic chromophores (loss of the rigid tertiary structure of the protein). Furthermore, the near-UV CD spectra of the apo form in the presence of increasing concentrations of ethanol were hardly distinguishable from that of the apo form without ethanol (Fig. 2b). Similar results were obtained by Polverino de Laureto *et al.* [16] for apo α-lactalbumin at 37°C in the presence of increasing concentrations of TFE. Far-UV CD studies performed with holo α-lactalbumin indicated that increased concentrations of ethanol increased the amplitudes of spectral bands at 208 and 232 nm, which are characteristic of increased α-helix content [15].

The conformation of apo α-lactalbumin, previously incubated with oleic acid, in the presence of increasing ethanol concentrations was also studied by near-UV CD spectroscopy. No changes were observed when comparing with the CD spectra of apo α-lactalbumin under the same solvent conditions (results not shown). However, Polverino de Laureto *et al.* [16] observed a reduction of the CD signal at 270 nm when adding oleic acid to apo α-lactalbumin dissolved in aqueous solutions at pH 8.3. This different behavior of the apo form could be attributed to the different temperatures at which measurement was performed, or to a decreased signal due to micellization of oleic acid.

3.2 Proteolysis of α-lactalbumin as a function of ethanol concentration

Holo and apo forms of α-lactalbumin were subjected to trypsinolysis in the presence of increasing ethanol concentrations and tryptic peptides were separated by RP-HPLC. This protease has the advantage of being stable and active

at neutral pH in the presence of aqueous organic solvents [20]. Furthermore, due to its substrate specificity the tryptic cleavage is expected to depend mainly on the stereochemistry and the dynamics of the α-lactalbumin substrate and not on the specificity of the enzyme.

The rate of disappearance of holo and apo α-lactalbumin peak is shown in Fig. 3. In the absence of ethanol, holo α-lactalbumin was resistant to hydrolysis by trypsin (more than 95% of the protein remained undigested after 24 h of incubation) as expected for a fully folded and rigid structure of the calcium-loaded protein. These results are in agreement with those previously reported under similar experimental conditions [21 – 23].

In the presence of 20% ethanol, holo α-lactalbumin was susceptible to trypsinolysis, about 57% of the protein remaining non-hydrolyzed after 6 h of hydrolysis. Hydrolysis rate increased substantially in the presence of 30% ethanol, the proportion of non-hydrolyzed α-lactalbumin decreased from 50 after 1 h to 10% after 6 h of hydrolysis (Fig. 3 a). Structural changes induced by ethanol make the protein more susceptible to hydrolysis mainly because of exposure of its hidden tryptic cleavage sites as it has been also found with other globular proteins in alcohols [17]. Similarly, it was observed that after its thermal denaturation, α-lactalbumin was susceptible to tryptic hydrolysis due to a looser conformation that facilitated the accessibility of the enzyme [22].

It is remarkable here that in the presence of 20% ethanol when spectral measurements did not show any significant secondary or tertiary changes [15] there might be transitory conformational changes, indicated by enhanced proteolysis, such as an increase in amino acid side chain rotation. Therefore, limited proteolysis might be a more sensible tool to determine conformational changes of α-lactalbumin than spectroscopic techniques as it has been also reported in the case of β-lactoglobulin modified by site-directed mutagenesis [24].

In the presence of 40% ethanol, only a small peak eluting just before α-lactalbumin at all incubation times was observed (data not shown). This peak disappeared after reduction with DTT, indicating that the protein aggregates formed would hinder the sites of cleavage. The analysis of the results of limited proteolysis experiments performed on globular proteins of known structure reveals that helical segments are not the site of proteolytic attack [25]. Therefore, the high helical conformation state acquired by α-lactalbumin dissolved in aqueous buffer containing 40% ethanol [15] might be the origin of this inhibitory effect as it was also reported in the case of α-lactalbumin in the presence of high concentration of TFE [14] or in the case of other globular proteins in high ethanol concentration [15, 17, 18].

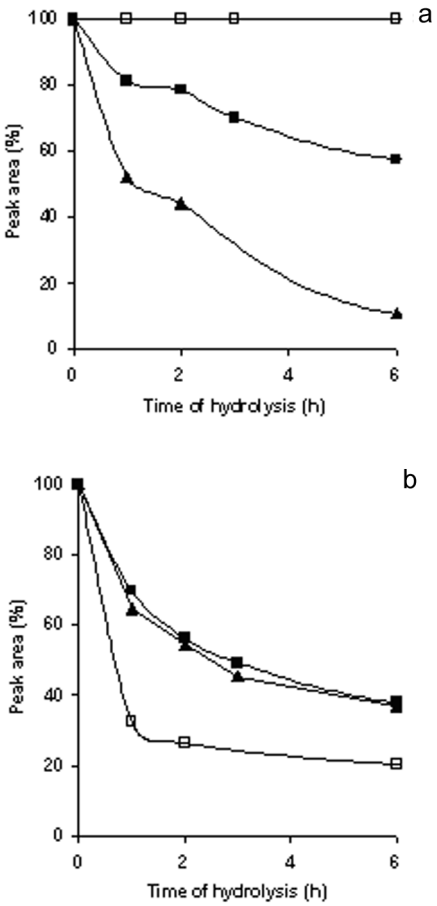

Figure 3. Disappearance with time of bovine holo (a) and apo (b) α-lactalbumin peak after proteolysis with trypsin as a function of ethanol concentration: 0 (□), 20 (■) and 30% (▲). Peak area corresponds to residual α-lactalbumin (%).

Fig. 3 b shows the disappearance of apo α-lactalbumin. In the absence of ethanol, the protein is degraded quickly, leaving only 30% of the intact protein after 1 h of trypsinolysis. These results are in agreement with those reported by Hirai *et al.* [23] who determined the lactose synthase activity of α-lactalbumin after hydrolysis by trypsin. The half-times of the tryptic digestion of calcium loaded protein was 150 times higher than that of apo α-lactalbumin. These authors concluded that calcium afforded α-lactalbumin a very strong resistance to tryptic attack while apo α-lactalbumin was highly susceptible to digestion [23]. Likewise, the partially folded states of α-lactalbumin exposed to acid solutions at pH 2.0 or at neutral pH upon EDTA-mediated removal of the single protein-bound Ca^{2+} ion were easily digested by chymotrypsin and proteinase K whereas the native form was resistant to proteolysis [26]. In contrast, when adding 20 and 30% ethanol to the apo form, an inhibitory effect of proteolysis can be observed and the overall reaction rates slightly lowered. The proportion of unde-

graded apo α-lactalbumin decreased from 64 to 36% after 1 and 6 h of hydrolysis, respectively, at both ethanol concentrations. Similar results were observed for the apo form of α-lactalbumin in the presence of increasing concentrations of TFE [14]. These authors found that the addition of TFE up to 20% greatly accelerates the proteolysis by thermolysin though at higher concentrations the proportion of degraded protein decreased considerably. In the presence of 40% ethanol, no hydrolysis of the apo α-lactalbumin was observed in our work after 24 h of incubation as it was observed in the case of the holo form of the protein.

When apo α-lactalbumin was previously incubated with oleic acid to form a complex, the rate of disappearance of the protein was similar to that obtained in the case of the apo form without fatty acid bound (results not shown). In contrast, it was reported that the binding of other low molecular mass apolar substances, such as 4,4′-bis-[1-(phenylamino)-8-naphtalene sulfonate, to apo α-lactalbumin increased its resistance to tryptic digestion [23]. Our results indicate that the binding of oleic acid to α-lactalbumin is not sufficient to increase its conformational stability in order to resist tryptic degradation, which is the contrary to what it has been observed in the case of other fatty acid-binding proteins such as β-lactoglobulin [27].

3.3 Chromatographic analysis of tryptic peptides

Chromatographic profiles of holo and apo α-lactalbumin obtained on a Nucleosil C_{18} column after 6 h of tryptic hydrolysis are presented in Fig. 4. In the case of holo α-lactalbumin, most of the protein was found to be resistant to trypsinolysis since few hydrophilic peptides were eluted from the column. In the presence of 20% ethanol, a large population of peptides much earlier eluted than intact protein, including hydrophilic and hydrophobic peptides was observed. In the presence of 30% ethanol, the population of peptides showed a similar pattern to that obtained in the presence of 20% ethanol. However, after 1 h of hydrolysis two long hydrophobic peptides were observed at both sides of the α-lactalbumin peak (results not shown). Along incubation time with trypsin, the intensity of the peak eluted earlier than α-lactalbumin decreased while that of the peak eluted later increased.

In the case of the apo form of α-lactalbumin, with or without oleic acid bound, the tryptic peptides profiles were quite similar in the presence of 20 and 30% ethanol. Long hydrophobic peptides eluted just before α-lactalbumin peak were produced first, as they were observed after 1 h of proteolysis. Their concentration decreased with time leaving only one peak, which corresponded, to non-hydrolyzed protein after 6 h in 0% ethanol and after 24 h hydrolysis in the presence of 20 and 30% ethanol. Likewise, several hydrophilic and hydrophobic peptides earlier eluted than the whole protein appeared and their concentration increased with time.

3.4 Peptide identification

Peptides obtained by tryptic hydrolysis were identified by RP-HPLC coupled to IT-MS. Samples analyzed were the holo and apo forms hydrolyzed in 0% ethanol and the holo form hydrolyzed in the presence of 20 and 30% ethanol after 24 h of incubation. For some peptides, identification was completed by LC MS/MS spectroscopy. Results are shown in Fig. 5 and Table 1. The recovery of sequence was complete except for tryptic tri-peptides Glu11-Lys13 and Asp14-Lys16. Peak 1, containing residues 1 to 5 was present in all samples studied. This pentapeptide has shown to have bactericidal activity against Gram-positive bacteria [28]. Some peptides of high molecular mass remained unidentified in spite of some attempts of identification after reduction of disulfide bonds.

Some differences appeared when comparing hydrolysis of apo and holo forms of α-lactalbumin. Peaks 5 (Leu115-Lys122 -SS- Cys6-Arg10) and 9 (Ala109-Lys114 -SS-Gly17-Lys58) were present in higher amounts in the apo form of the protein hydrolyzed in the absence of ethanol. Peaks 7 (Leu115-Leu123 -SS- Cys6-Arg10) and 10 (Ala109-Leu123 -SS- Cys6-Arg10 + Gly17-Lys58) having a molecular mass of 1798 and 7082, respectively, are characteristic of the holo form.

When trypsinolysis of the holo form is performed in the presence of ethanol, peptide bonds Lys114-Leu115 and Lys122-Leu123 are resistant to the protease. This indicates a better accessibility of the enzyme to the C-terminal region in the apo form.

Another difference could be observed in peak 8 (Ile59-Lys93) present in higher amount in the apo form. This peptide presents two putative tryptic cleavage sites (Lys62-Asn63 and Lys79-Phe80) maintained by two disulfide bridges (Cys61-Cys77 and Cys72-Cys91). These three forms were found in the chromatogram. The form without cleavage has a mass of 4041 (peak 15), that with one cleavage has a mass of 4059 (peak 8) and that with 2 cleavages has a mass of 4077 (peak 7). During the trypsinolysis of the holo form two different masses could be found in peak 8: 4059 and 4187. The mass 4187 corresponds to peptide Ile59-Lys94 with one cleavage indicating that in the holo form Lys93-Lys94 bond is less sensitive to tryptic hydrolysis.

A very small peak (peak 4) was observed only in the apo form of α-lactalbumin. Two peptides of mass 1300 and 1681 were found in this peak; analysis by LC MS/MS showed that they correspond to peptide Ala109-Lys114 di-

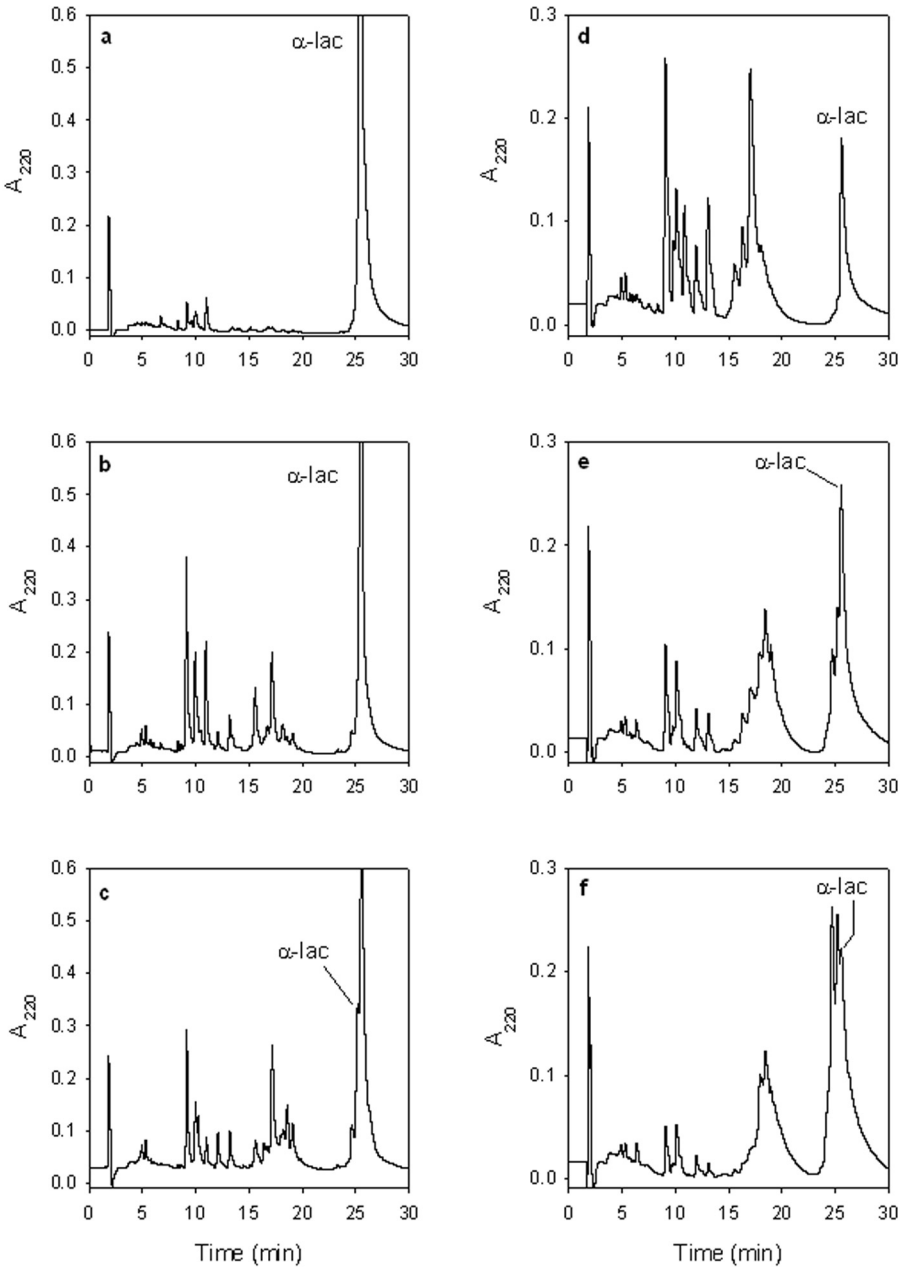

Figure 4. RP-HPLC of holo and apo α-lactalbumin tryptic peptides after 6 h of hydrolysis in the presence of increasing concentrations of ethanol: holo (a, b, c) and apo (d, e, f) α-lactalbumin in 0 (a, d), 20 (b, e) and 30% ethanol (c, f).

sulfide linked to peptides Cys6-Arg10 and Leu115-Lys122, respectively. These peptides were in very low quantities; however, if some disulfide interchange could occur in the apo form, this suggests a flexibility of N-and C-terminal parts of α-lactalbumin.

Another very small peak eluted just before peak 5 was found only in the hydrolysate of the holo form in the presence of 20% ethanol. This peak of molecular mass 1755 was identified by LC-MS/MS as peptide Asn44-Lys58 demonstrating an atypical tryptic cleavage (Gln-Asn).

Peak 6 (Val99-Lys108), of molecular mass 1200 is present in both hydrolysates.

Some amounts of non-hydrolyzed α-lactalbumin were present in the apo form only. However, during trypsinolysis of the holo form, a hydrophobic peptide (peak 12) with a molecular mass of 12 884 was present, which corresponds to α-lactalbumin deleted of N-terminal tryptic peptides Glu1-Lys5, Glu11-Lys13 and Asp14-Lys16.

3.5 Kinetics of trypsinolysis

MS identification of some peptides allowed us to follow the tryptic cleavage of some peptides.

Table 1. Identification by mass spectrometry of tryptic peptides of bovine holo and apo α-lactalbumin hydrolyzed in the presence of increasing concentrations of ethanol. Peak numbers as in Fig. 5

Peak number	Molecular mass	Identification	Sequence obtained by LC MS/MS
1	618.3	Glu1 – Lys5	
2	488.3	Ile95 – Lys98	
3	616.4	Lys94 – Lys98	
4	1300	Ala109 – Lys114 + Cys6 – Arg10	AL.....CEVFR
	1681	Leu115 – Lys122 + Ala109 – Lys114	LDQWL.....K
5	1684.6	Leu115 – Lys122 + Cys6 – Arg10	LDQWL.....VFR
6	1200.6	Val99 – Lys108	(K)KILDKVGINYWLAHK(A)
7	4077.2	Ile59 – Lys93 (two intramolecular tryptic cleavage)	
	4187	Ile59 – Lys94	
8	4059	Ile59 – Lys93 (one intramolecular tryptic cleavage)	
9	5303.1	Gly17 – Lys58 + Ala109 – Lys114	
10	7082	Ala109 – Leu123 + Cys6 – Arg10 + Gly17 – Lys58	
11	α-lactalbumin		
12	12886		
13	6739.4		
14	1798	Leu115 – Leu123 + Cys6 – Arg10	LDQWL.....R
15	4041	Ile59 – Lys93	
16	9100		

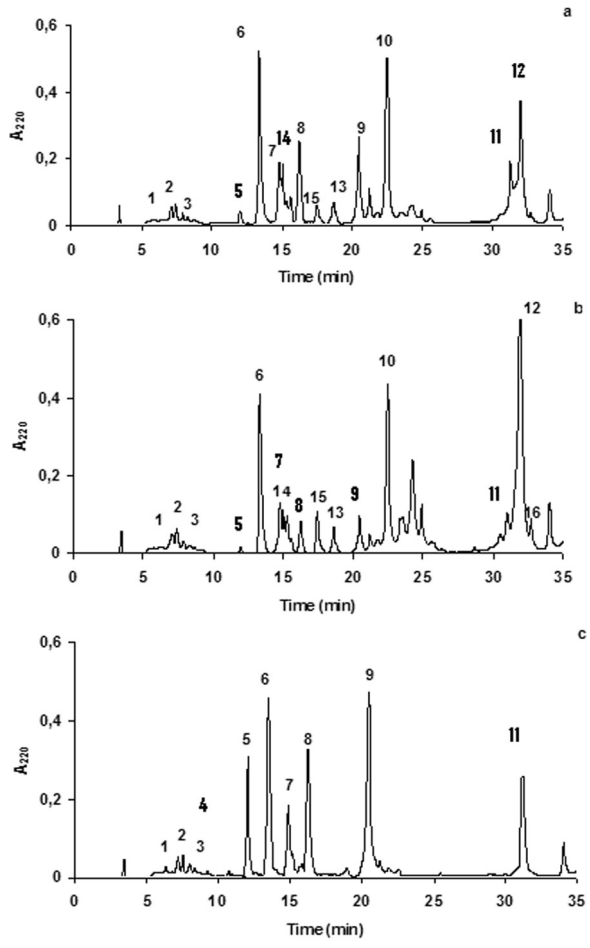

Figure 5. RP-HPLC of holo and apo α-lactalbumin tryptic peptides after 24 h hydrolysis in the presence of increasing concentrations of ethanol: holo α-lactalbumin in 20 (a) and 30% (b) ethanol; apo α-lactalbumin in 0% ethanol (c).

In the case of peak 10, corresponding to peptide Ala109-Leu123 disulfide linked to peptides Cys6-Arg10 and Gly17-Lys58 (Fig. 6) it can be observed that this peptide appears in the first time of hydrolysis then its quantity decreased after 3 h of hydrolysis of the apo-form and of the holo form in the presence of 20% ethanol. In the same time, an increasing amount of peptides Leu115-Leu123 + Cys6-Arg10 (peak 14) and Gly17-Lys58 + Ala109-Lys114 (peak 9) could be observed. The differences of kinetics of formation observed between these two peptides showed that in the case of peptide Leu115-Lys122 an intermediary Leu115-Leu123 was first produced; this intermediary was detected by MS but was too close to other peaks to be quantified. These results show that in the first time of hydrolysis bonds Lys5-Cys6, Arg10-Glu11, Lys16-Gly17, Lys58-Ile59, Lys108-Ala109 and Lys123-Leu124 are accessible to the protease. Bonds Lys114-Leu115 and Lys122-Leu123 are less accessible to the enzyme. When proteolysis was performed in the presence of 30% ethanol peptide Ala 109-Leu123 (peak 10) appeared in the first time of hydrolysis; however, its quantity did not decrease. The formation of peptide Ala 109-Lys122 (peak 4) was very slow and the quantity of peptide Leu115-Lys122 (peak 4) suggests that peptide bond Lys122-Leu123 is quite resistant to trypsinolysis.

Peptide Ile59-Lys93 (peak 15) appeared at the beginning of hydrolysis of apo form and of holo form in the presence of 20% ethanol. After 3 h of hydrolysis its amount decreased in parallel to the appearance of peptide Ile59-Lys93 (peak 6) with one intra-molecular cleavage. When hydrolysis was performed in the presence of 30% ethanol, the formation of this peptide was very low. The amount of the peak with two intra-molecular cleavages was very low and not easy to quantify.

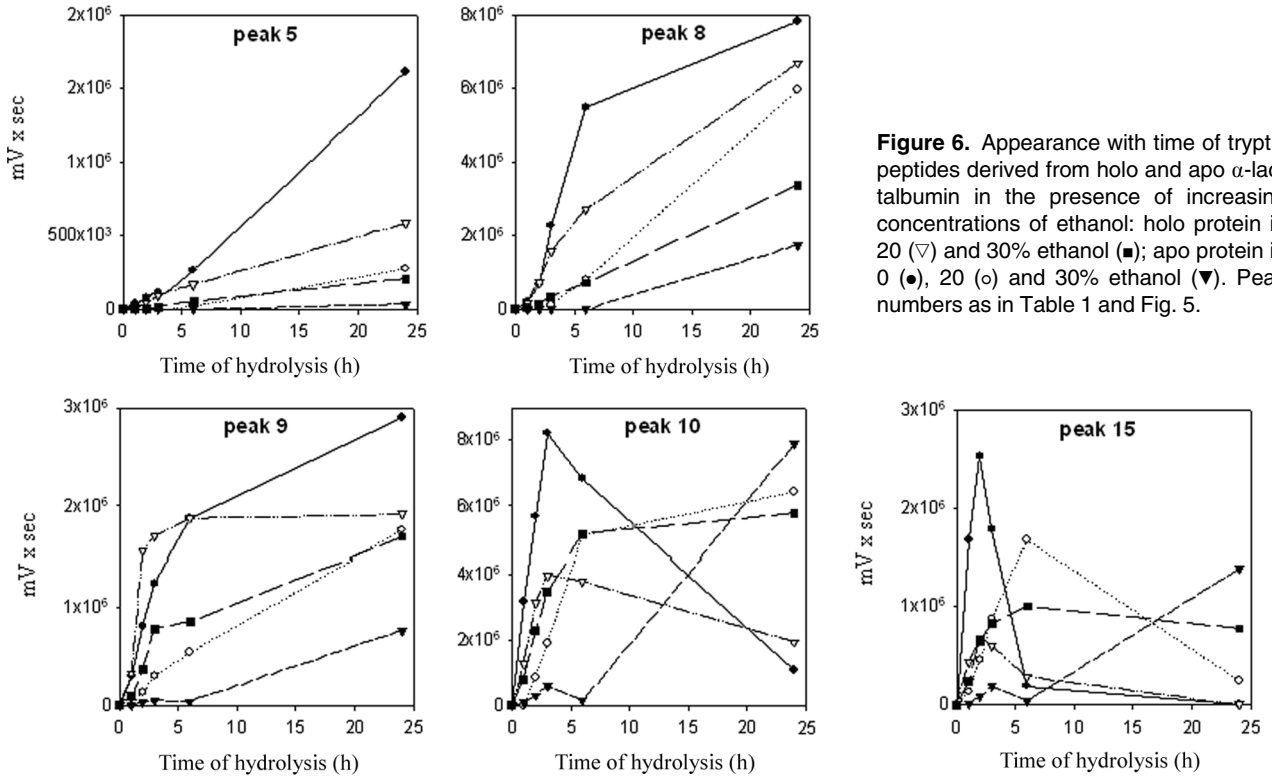

Figure 6. Appearance with time of tryptic peptides derived from holo and apo α-lactalbumin in the presence of increasing concentrations of ethanol: holo protein in 20 (▽) and 30% ethanol (■); apo protein in 0 (●), 20 (○) and 30% ethanol (▼). Peak numbers as in Table 1 and Fig. 5.

4 Concluding remarks

Considering the substrate specificity of trypsin and thus the numerous potential sites of proteolytic attack along the α-lactalbumin chain, these results indicate that this protein, in its partially folded apo or ethanol-induced states, is a more dynamic entity than in the holo state. This study and others [26] demonstrate that proteolytic enzymes can be used as suitable tools of determination of protein structure and dynamics, complementing other physico-chemical techniques.

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