

Purification and physicochemical characterization of ovine β -lactoglobulin and α -lactalbumin

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Ovine whey proteins were fractionated and studied by using different analytical techniques. Anion-exchange chromatography and reversed-phase high-performance liquid chromatography (HPLC) showed the presence of two fractions of β -lactoglobulin but only one of α -lactalbumin. Gel permeation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis allowed the calculation of the apparent molecular mass of each component, while HPLC coupled to electrospray ionisation-mass spectrometry (ESI-MS) technique, giving the exact molecular

masses, demonstrated the presence of two variants A and B of ovine β -lactoglobulin. Amino acid compositions of the two variants of β -lactoglobulin differed only in their His and Tyr contents. Circular dichroism spectroscopy profiles showed pH conformation changes of each component. The thermograms of the different whey protein components showed a higher heat resistance of β -lactoglobulin A compared to β -lactoglobulin B at pH 2, and indicated high instability of ovine α -lactalbumin at this pH.

1 Introduction

Milk proteins are a heterogeneous mixture with two dominant groups, caseins and whey proteins, each of which contains different components. This heterogeneity is influenced by genetic or nongenetic factors such as phase of lactation and feeding [1]. Recently, electrospray ionisation-mass spectrometry (ESI-MS) has become a powerful alternative tool for the determination of the relative molecular mass (M_r) of bovine milk proteins [2]. It may detect changes in proteins caused by insertion, deletion, mutation or modification of amino acids. It may identify genetic variants and post-translational modifications such as phosphorylation and glycosylation [3].

Bovine β -lactoglobulin was one of the first proteins whose amino acid composition and sequence were established. Its polypeptide chain is composed of 162 amino acid residues including two disulfide bonds (Cys 66-Cys 160 and Cys 106-Cys 119) and one free cysteine 121 [4]. The molecule is constituted by nine anti-parallel β -strands and one α -helix [5, 6] and its molecular mass is 18400 Da. Bovine β -lactoglobulin shows a remarkable stability at low pH, resisting to denaturation even at pH 2 [7] but denatures easily in alkaline pH and aggregates at intermediate pH values undergoing multiple conformational changes [8]. Bovine α -lactalbumin is a small (M_r 14200 Da), acidic (pI 4–5), Ca^{2+} -binding milk whey protein, which consists of 123 amino acid residues, and contains two domains: a large α -helical domain and a small β -sheet domain, connected by a calcium-binding loop. The α -helical domain is composed of three major α -helices (residues 5–11, 23–24, and

86–98) and two short 3_{10} helices (residues 18–20 and 115–118). Bovine α -lactalbumin forms high-molecular mass-disulfide oligomers at high pressure (10 kbar, 1000 Mpa) in the presence of low-molecular-mass thiol reducers such as cysteine, mercaptoethanol, and dithiothreitol [9]. These compounds act as initiators of the thiol $\rightleftharpoons$ disulfide exchange reaction between unfolded molecules of this protein.

Ovine β -lactoglobulin and α -lactalbumin were isolated from milk whey of Portuguese native Lacaune breeds by preparative gel filtration and further purified by ion-exchange chromatography. Genetic variants were characterized by isoelectric focusing. Separation of β -lactoglobulin and α -lactalbumin by mass exclusion chromatography from whey was efficient. β -Lactoglobulin eluted as a single peak during ion-exchange chromatography by linear gradient of NaCl. Isoelectric focusing showed that α -lactalbumin appears as a single variant, whereas β -lactoglobulin migrates as two bands [10].

ESI-MS was used to determine the molecular mass of major ovine milk proteins and to study the great complexity of these proteins by coupling high-performance liquid chromatography (HPLC) and ESI-MS for on-line separation. Whey proteins were further separated by HPLC coupled to ESI-MS, after purification by fast protein liquid chromatography (FPLC) and analysis by ESI-MS using flow injection analysis (FIA) [2, 3]. Amigo *et al.* [11] described three genetic variants A, B, and C of β -lactoglobulin and two of α -lactalbumin of ovine milk on basis of capillary electrophoresis, amino acid composition, and sequence studies. Compared with bovine whey proteins there is only a limited amount of information on physicochemical properties of ovine whey proteins, therefore there is a need for better characterization of structures of both ewe β -lactoglobulin and α -lactalbumin. The techniques used in the present study included gel permeation chromatography, polyacrylamide gel electrophoresis, HPLC-coupled ESI-MS, FPLC-coupled ESI-MS, amino acid composition analysis, circular dichroism, and calorimetry.

2 Materials and methods

2.1 Preparation of ewe's whey and isolated proteins

Fresh ewe's whey was prepared from skimmed milk (Lacaune breed, dairy farm of INRA, Rennes, France) by precipitation of caseins at pH 4.6 with 1 N HCl. Caseins were

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Abbreviations: CD, circular dichroism; ESI-MS, electrospray ionization mass spectrometry; FIA, flow injection analysis; FPLC, fast protein liquid chromatography; GP, gel permeation; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-performance liquid chromatography

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removed by centrifugation at $10000 \times g$ for 30 min at 4°C . The supernatant containing whey proteins was dialyzed for 48 h against distilled water then frozen and lyophilized.

2.2 Anion-exchange chromatography

β -Lactoglobulin and α -lactalbumin were isolated from fresh whey using the anion-exchange chromatography on DEAE-Sephacrose Fast Flow column (300×50 mm). Elution was made in 25 mM Tris, pH 8.0, containing 5 mM CaCl_2 by using an 0–1 M NaCl linear gradient. In another series of experiments, elution was made in 20 mM MES, pH 6.4, by using an 0–1 M NaCl linear gradient. Homogeneity of the protein preparation was checked by high-performance gel-permeation (GP) chromatography and SDS-Tricine polyacrylamide gel electrophoresis (PAGE) according to Schagger and von Jagow [12]. α -Lactalbumin and β -lactoglobulin were over 90% pure.

2.3 Gel-permeation chromatography

GP chromatography was performed on a TSK-G3000 SWXL column (300×7.8 mm; Tosohaas, Montgomeryville, PA, USA). The elution was carried out at a flow rate of 0.8 mL/min with 30 mM sodium phosphate, pH 6.7, or 30 mM glycine, pH 2.5. The column was calibrated with protein standards covering a molecular mass range from 6.5 to 2000 kDa (Dextran Blue, 2000; alcohol dehydrogenase, 150; bovine serum albumin, 66.7; ovalbumin, 43; carbonic anhydrase, 29; bovine β -lactoglobulin, 18.4; cytochrome *c*, 16; ribonuclease A, 13.7, aprotinin, 6.5 kDa).

2.4 SDS-Tricine PAGE

SDS-Tricine PAGE was performed on 10% acrylamide plates in denaturing conditions according to Schagger and von Jagow [12]. All the samples containing 5 μg protein were prepared in denaturing buffer with β -mercaptoethanol and heated at 100°C for 3 min before migration. Buffers used for migration were 0.2 M Tris-HCl, pH 8.9, at the anode and 0.1 M Tris, 0.1 M Tricine containing 0.1% SDS at the cathode. Electrophoresis was performed at 10 and 20 mA in the stacking and the running gels, respectively. Gels were stained with Coomassie Brilliant Blue R-250. The relative molecular mass of the purified proteins was determined using proteins standards of known molecular mass (lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase *b*) in the range 14.2–99.0 kDa.

2.5 Reversed-phase HPLC (RP-HPLC)

A modification of the method of Visser *et al.* [13] was used to separate and identify the major ewe's whey proteins. Chromatographies were carried out on a Waters 2695 separation module equipped with a Waters 996 photodiode array detector using the Millenium software, all from Millipore (Concord, MA, USA). RP-HPLC was run on a Nucleosil C_{18} column (250×4 mm; Macherey Nagel, France), equilibrated with solvent A (0.11% trifluoroacetic acid (TFA) in H_2O). Elution was performed by using a gradient from 10% solvent B (80% acetonitrile, 19.91% H_2O , 0.09 % TFA) to 100% solvent B in 23 min. The temperature of the column was maintained at 30°C , the flow rate was 0.6 mL/min, and the absorbance was recorded at 220 nm.

2.6 Amino acid composition

Amino acid composition of whey proteins was determined after acid hydrolysis under vacuum (6 N HCl, 24 h, 110°C) in a Pico-Tag station (Waters Associates, Milford, MA, USA). Amino acids were derivatized with phenylisothiocyanate according to Bidlingmeyer *et al.* [14], and quantified by RP-HPLC on a Pico-Tag C_{18} column (150×3.9 mm, Waters).

2.7 HPLC–ESI-MS

In order to study the molecular mass of whey proteins which were not resolved directly by HPLC–ESI-MS, ovine β -lactoglobulin and α -lactalbumin were purified from whey by GP-FPLC. Each isolated whey milk protein fraction was dissolved in an acetonitrile/ H_2O solution (1/1 v/v) acidified with 0.5% v/v formic acid, and then introduced in the fluidic way *via* FIA. ESI-MS experiments were performed on a Thermo Finnigan LCQ Advantage (San Jose, CA, USA) by infusion at 2.5 $\mu\text{L}/\text{min}$. Samples were dissolved at 5 pmol/ μL in acetonitrile/ H_2O (1/1 v/v) solution acidified with 0.5% v/v formic acid.

2.8 Circular dichroism (CD) spectroscopy

CD spectra were recorded using a Jobin Yvon CD 6 dichrograph equipped with a thermostated cell holder. The cylindrical cells used had a path length of 0.01 cm in the far-UV region (185–260 nm) and of 1 cm in the near-UV region (250–320 nm). An average of five and three scans in the far- and near-UV region, respectively, was used for final spectral analysis. All spectra were obtained at 25°C using a protein concentration of 2 mg/mL in a 10 mM glycine-HCl buffer at pH 2.5 or in a 30 mM sodium phosphate-NaOH buffer at pH 7.0. The baseline spectrum of buffer was subtracted from each spectrum and the resultant values were expressed in molar ellipticity ($[\Theta]$) according to Provencher and Glöckner [15].

2.9 Calorimetry studies

Calorimetric measurements were carried out with the high sensitivity differential scanning microcalorimeters MSC System and VP-DSC (Micro cal, USA) within the temperature range of 8– 110°C at a heating rate of 1 K/min and an excess pressure of 2 atm. Data processing was carried out using Origin software (Micro cal). Absolute molar heat capacity of β -lactoglobulin was calculated assuming a molecular mass of 18.4 kDa and partial specific volume of $0.751 \text{ cm}^3/\text{g}$ [4, 16]. Molar heat capacity of α -lactalbumin was calculated assuming a molecular mass of 14.26 kDa and partial specific volume of $0.709 \text{ cm}^3/\text{g}$ according to Privalov and Potekhin [16]. Molar heat capacity of the protein *vs.* temperature were fitted with the Sigma Plot 3.2 software. Concentration of protein solutions for CD and calorimetric measurements was determined spectrophotometrically, with $\epsilon_{278} = 15654 \text{ M}^{-1} \text{ cm}^{-1}$ in the case of β -lactoglobulin, and $\epsilon_{278} = 28508 \text{ M}^{-1} \text{ cm}^{-1}$ in the case of α -lactalbumin.

3 Results and discussion

3.1 Anion-exchange chromatography, RP-HPLC, and SDS-PAGE

Anion-exchange chromatography profiles on DEAE-Sephacrose (25 mM Tris, 5 mM CaCl_2 , pH 8, eluted with an 0–1 M NaCl linear gradient) of ovine whey proteins (Fig. 1A) showed

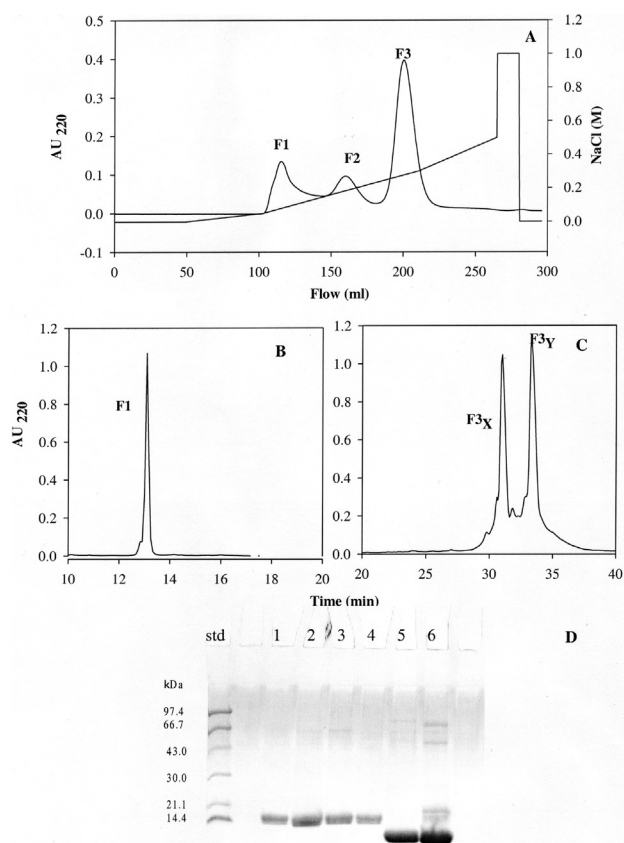


Figure 1. (A) Anion-exchange chromatography on DEAE-Sepharose (25 mM Tris, pH 8, with an 0–1 M NaCl linear gradient) of ovine whey proteins; (B), (C) RP-HPLC on C_{18} column of fractions F1 and F3 obtained by anion-exchange chromatography; (D) SDS-Tricine PAGE of bovine β -lactoglobulin, ewe's β -lactoglobulin (F3), ewe's β -lactoglobulin (F3X), ewe's β -lactoglobulin (F3Y), bovine α -lactalbumin, and ewe's α -lactalbumin (F1) on lanes 1–6, respectively. Std is a mixture of proteins with known molecular masses.

three major peaks (F1, F2, and F3), which correspond to α -lactalbumin, ovine serum albumin and β -lactoglobulin, respectively, as shown by SDS-PAGE (Fig. 1D). Peaks F1 and F3 were chromatographed by RP-HPLC (Fig. 1B, C). Only one peak was obtained in the case of α -lactalbumin and two different peaks (F3X and F3Y) in the case of β -lactoglobulin. As shown by SDS-PAGE, F3, F3X and F3Y have the same molecular mass (Fig. 1D, lanes 2–4). Consequently, two different proteins with a similar mass identical to that of bovine β -lactoglobulin could be purified by RP-HPLC. On the other hand, the SDS-PAGE of the sole peak of α -lactalbumin obtained by RP-HPLC (Fig. 1B) showed a main band with a molecular mass comparable to that of bovine α -lactalbumin (Fig. 1D; lanes 5 and 6).

Fraction F3 obtained by anion-exchange chromatography was submitted to further anion-exchange chromatography under less alkaline conditions (20 mM MES, pH 6.4, with a linear 0–1 M NaCl gradient). Fraction F3 was separated into two fractions (F3X and F3Y, Fig. 2A) similar to those obtained by RP-HPLC (Fig. 1C). Hence, it is possible to purify two β -lactoglobulin fractions either by RP-HPLC or by anion-exchange chromatography under well-defined conditions. A further separation of each peak by RP-HPLC did not lead to any additional separation confirming that only two variants were present in the ovine β -lactoglobulin studied. In a recent work [17],

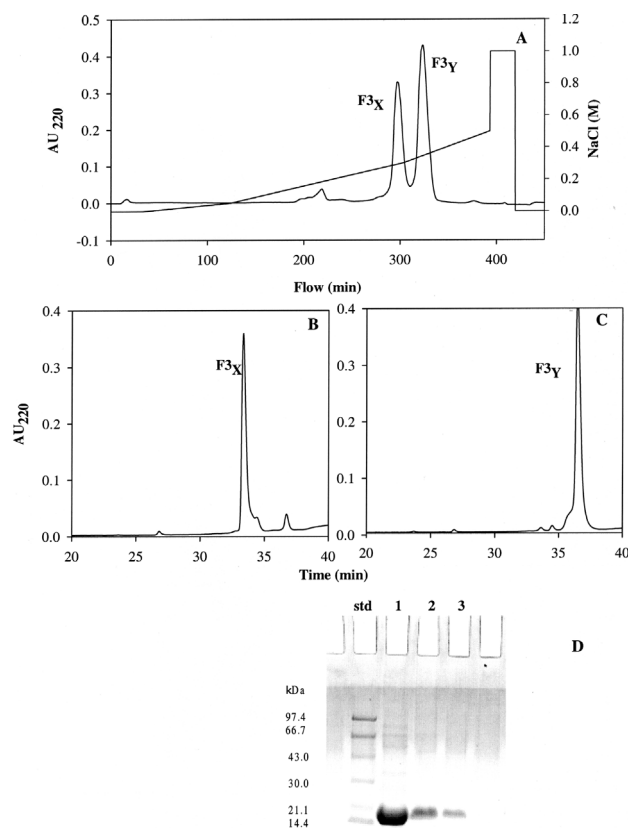


Figure 2. (A) Anion-exchange chromatography on DEAE-Sepharose (20 mM MES, pH 6.4, with a linear 0–1 M NaCl gradient) of fraction F3 issued from anion-exchange chromatography in 25 mM Tris, pH 8.0 (see Fig. 1); (B), (C) RP-HPLC on C_{18} column of fractions F3X and F3Y obtained by anion-exchange chromatography in 20 mM MES, pH 6.4, buffer; (D) SDS-Tricine PAGE of a mixture of fractions F3X and F3Y obtained by anion-exchange chromatography, fraction F3X and fraction F3Y obtained by RP-HPLC, on lanes 1–3, respectively. Std is a mixture of proteins with known molecular masses.

bovine isoforms A and B of β -lactoglobulin could not be separated by DEAE-cellulose chromatography, probably because of the different ionic strength and buffer composition used. SDS-PAGE of the last two RP-HPLC fractions (Fig. 2D) indicated that F3X (Fig. 2D, lane 2) and F3Y (Fig. 2D, lane 3) have identical molecular masses similar to that of whole ovine β -lactoglobulin. These fractions isolated either by RP-HPLC or anion-exchange chromatography may represent two genetic variants of ovine β -lactoglobulin, which could be assigned to the main β -lactoglobulin variants (A and B) predominant in sheep breeds [11]. The absence of the third variant (β -lactoglobulin C) may be due to its scarcity [18]. The presence of a unique peak in the case of α -lactalbumin on RP-HPLC and anion-exchange chromatograms demonstrates the presence of one variant which may probably be α -lactalbumin A since the variant B is limited to very specific sheep breeds [11].

3.2 Gel-permeation chromatography

β -Lactoglobulin and α -lactalbumin obtained by anion-exchange chromatography were submitted to GP chromatography at two pH values (Fig. 3). At pH 2.5, β -lactoglobulin was eluted at 10.16 min in one single peak corresponding to a molecular mass of 17943 Da since it exists in a monomeric form at acid pH and low ionic strength. At pH 6.7, β -lactoglo-

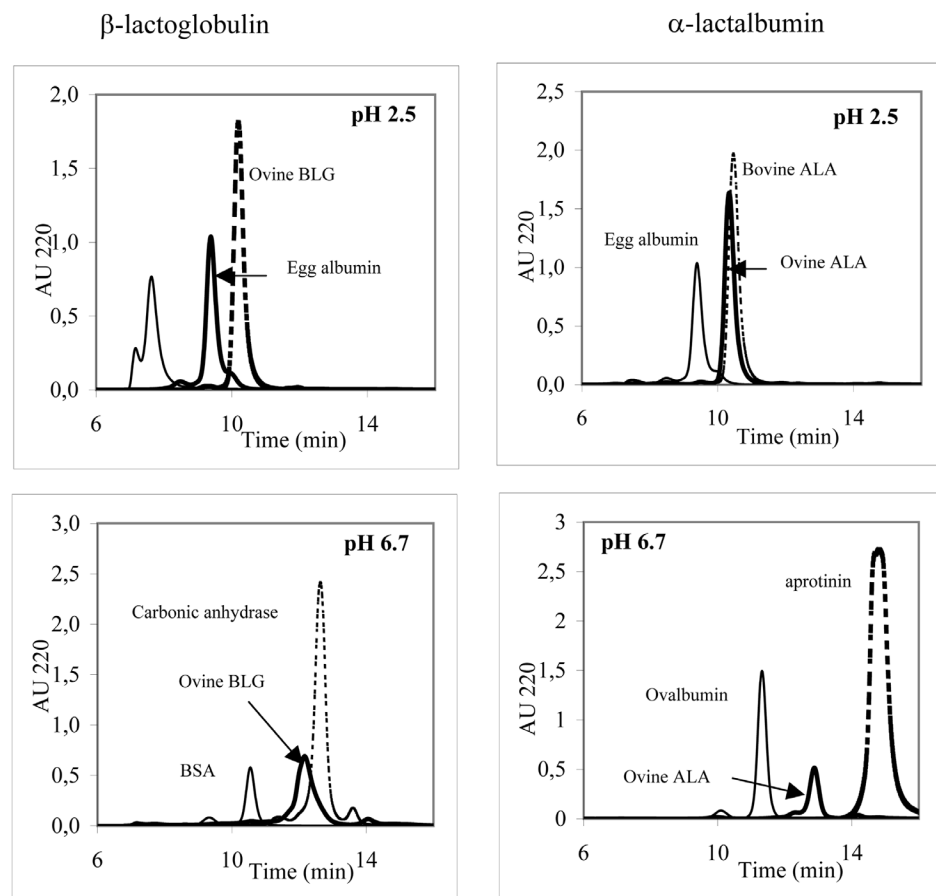


Figure 3. Gel-permeation chromatography on a TSK-G3000 SWXL column of ewe's α -lactalbumin (ALA) and β -lactoglobulin (BLG). The elution was carried out at a flow rate of 0.8 mL/min with 30 mM sodium phosphate, pH 6.7, or 30 mM glycine, pH 2.5.

bulin elution was shifted to longer retention time (12.165 min) corresponding to a molecular mass of 34111 Da, demonstrating that ovine β -lactoglobulin, as its bovine counterpart, exists in dimeric form at neutral pH. α -Lactalbumin eluted at 10.34 and 12.87 min at pH 2.5 and 7.0, respectively, corresponding to molecular masses of 14927 and 20445 Da, respectively. The last component with a relatively high molecular mass may result from the ability of α -lactalbumin to bind organic compounds or peptides as previously described [19, 20].

3.3 CD spectroscopy

Near UV-CD spectrum of α -lactalbumin at pH 2.0 (Fig. 4) did not show any interpretable signal, which is mainly due to a total loss of tertiary structure or to the occurrence of a classical molten globule state, which consists of a compact state with fluctuating tertiary structure [21]. However, at neutral pH, α -lactalbumin gave a characteristic near UV-CD profile with two specific signals at 253 and 272 nm. This pH dependence of near UV-CD profile of α -lactalbumin is due to its sensitivity to acid conditions, which dramatically affect its Ca^{++} binding properties [22]. Far UV-CD profiles of α -lactalbumin at pH 6.7 (Fig. 4) showed a characteristic profile with three specific signals at 189, 207, and 223 nm. Although these specific signals were less visible when shifting to acid conditions (pH 2) showing a change towards a random coiled conformation, the overall shape of the profile was still obtained indicating the low influence of this change. Consequently, acid conditions affect greatly the tertiary structure but only slightly the secondary structure of ewe's α -lactalbumin.

The CD spectrum of β -lactoglobulin in the aromatic region (near-UV) at pH 2.0 (Fig. 5) showed a profile with a minimum

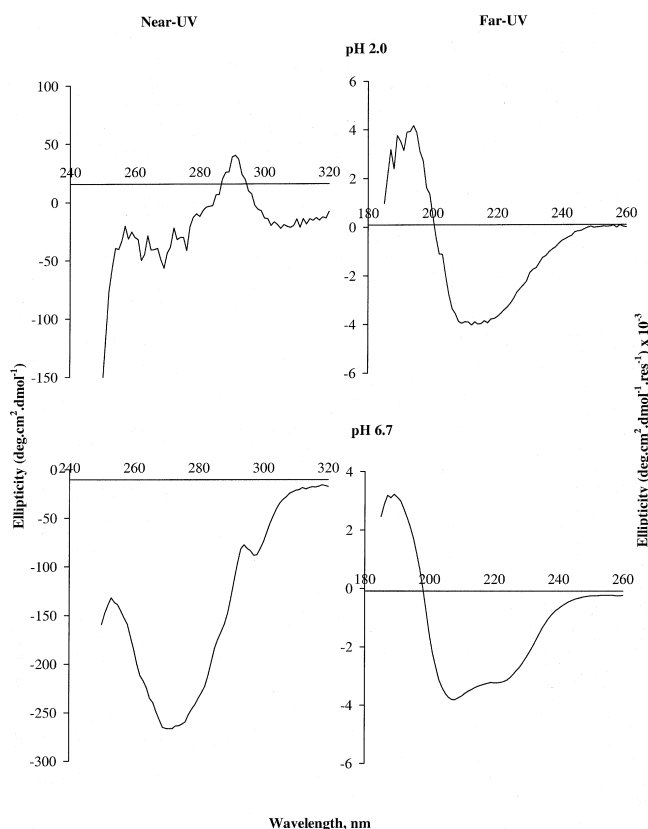


Figure 4. Near- and far-UV CD spectroscopy profiles of α -lactalbumin at pH 2.0 and 6.7.

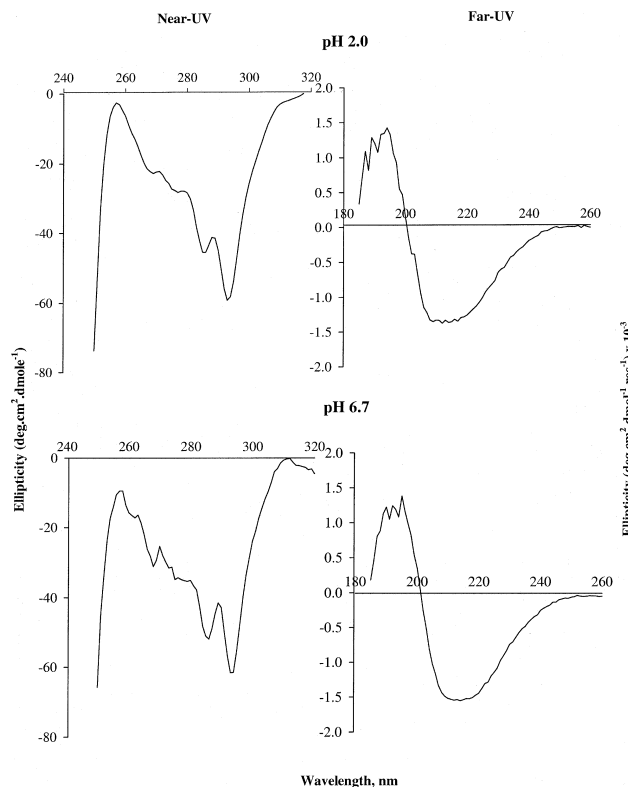


Figure 5. Near- and far-UV CD spectroscopy profiles of β -lactoglobulin at pH 2.0 and 6.7.

at 268 nm originating from its phenylalanyl residues and two characteristic deep minima at 287 and 297 nm arising from tryptophanyl residues, especially Trp19. At pH 6.7, the intensity of the two minima, slightly shifted at 286 and 294 nm, increased; a more significant increase of the intensity at 268 nm was observed, which agrees well with the results of Manderson *et al.* [23]. The main β -lactoglobulin fluorescent residue is Trp 19 and is responsible for 80% of the protein fluorescence emission [24]. Thus, fluorescence spectroscopy measurements on β -lactoglobulin mainly concern this Trp emission spectrum. In the far-UV region, a slight decrease of the minimum at about 217 nm and a minor shift of the positive band at 194 nm occurred at pH 2. At pH 6.7 a shift of the minimum at 215 nm and of the maximum at 196 nm was observed. However, far-UV CD spectra of β -lactoglobulin showed close similarity in secondary structure in native and acid conditions.

3.4 Calorimetry measurements

Thermograms of β -lactoglobulin and α -lactalbumin at different pH values are shown in Fig. 6. A single peak of heat capacity corresponding to the thermal unfolding transition was generally observed for the studied proteins at pH 6.7. No transition could be observed on thermograms of α -lactalbumin at pH 2.0 in 30 mM glycine-HCl buffer (data not shown), demonstrating the extensive denaturation of this sample as previously observed by CD experiments (Fig. 4). At low pH, α -lactalbumin loses its ability to bind Ca^{2+} due to the protonation of three abnormally titrating carboxyl groups responsible for the specific binding of Ca^{2+} [25]. At pH 8, a slight transition from 69.5 to 67.8 °C was observed indicating changes in the tertiary structure of the protein arising mainly from the changes in Ca binding to the molecule.

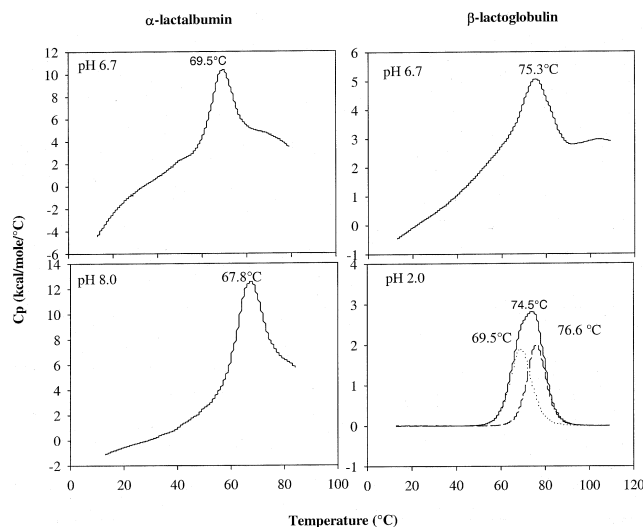


Figure 6. Partial heat capacity curves of α -lactalbumin and β -lactoglobulin at different pH values. At pH 2.0: (—) whole β -lactoglobulin, (---) β -lactoglobulin variant A, (···) β -lactoglobulin variant B.

In the presence of 1 mM EDTA, the thermograms of α -lactalbumin showed a single irreversible transition at 45 °C (data not shown). The disappearance of the transition at 69 °C upon addition of EDTA to α -lactalbumin can be attributed to the disappearance of the contribution of Ca^{2+} ions. Two variants of ewe α -lactalbumin (A and B) have been described [26]. Variant B occurs in very specific breeds only. In the present study only one genetic variant was observed.

The thermogram of β -lactoglobulin at pH 6.7 showed the effect of temperature on the main transition, substantially smaller than that observed at pH 2.0. At neutral pH, the slope of the heat capacity curve of β -lactoglobulin at temperatures between 40 and 70 °C was higher than that observed at acid pH. This indicates endothermic heat effects within a wide temperature range, suggesting low cooperativity of thermal transition of β -lactoglobulin under the applied conditions. At temperatures above denaturation, the heat capacity of β -lactoglobulin was higher than the value which can be expected for completely unfolded β -lactoglobulin, suggesting that some residual structure in native β -lactoglobulin melts at temperature higher than 110 °C [27].

The factors affecting protein thermostability, the different natures of the samples, and the experimental conditions used to follow the denaturation of this whey protein by differential scanning calorimetry make it difficult to compare the heat stability of ovine and bovine β -lactoglobulins. However, data reported here support the fact that native ovine β -lactoglobulin is more heat sensitive than its bovine homologue, as it has been yet reported by Law [28]. De Wit and Swinkels [29] obtained an identical T_d of 75 °C for samples of unknown variant composition at pH 6.5, in 0.03 M phosphate buffer and under a very rapid rate of heating of 10 °C/min. These authors attributed the different thermostability of both ovine variants to the structural changes consecutive to the substitution of a His residue in variant B for a Tyr residue in variant A. The transition peak of β -lactoglobulin B is shifted to lower temperatures and is characterized by a lower C_p as compared with variant A. Reversibility of thermal unfolding was checked by scanning protein solutions after a preliminary heating to 90 °C and a subsequent cooling.

Ovine β -lactoglobulin A showed a higher temperature of denaturation ($76.61^\circ\text{C} \pm 0.096$) than β -lactoglobulin B ($69.51^\circ\text{C} \pm 0.145$) when heated from 10 to 100°C at pH 2.0. This phenomenon can be attributed to the main role of hydrophobic interactions in the stability of proteins and particularly of tyrosyl residues of β -lactoglobulin A in such interactions. Pintado and Malcata [10] have found a good correlation between the pI of whey proteins and the temperature of denaturation (78 – 97°C) for pH values in the range 3.5–7.0, in the presence of NaCl. Therefore, ovine β -lactoglobulin B shows a higher temperature of denaturation than ovine β -lactoglobulin A. Different thermograms were obtained for bovine β -lactoglobulin variants [30]. It was also found that heat capacity at pH 2 is lower than at neutral pH for the whole β -lactoglobulin since the unfolded state (prevailing at neutral pH) has a higher heat capacity than the compact denatured state observed at acid pH [31]. The difference observed between the two variants in their temperature of denaturation is dependent on the conditions used in the experiments (pH and ionic strength), which affect the thermal transition of the two β -lactoglobulin variants [10]. This difference in T_d at pH 2 shows that β -lactoglobulin A is more heat resistant than the variant B, what agrees with the results of Calavia and Burgos [26] working at a different pH (6.6) but is in disagreement with those reported in the case of the bovine counterpart [32].

3.5 ESI-MS and amino acid composition

Ovine α -lactalbumin and β -lactoglobulin purified by RP-HPLC were analyzed by HPLC coupled to ESI-MS. Both β -lactoglobulin fractions (F3X and F3Y), as well as the whole β -lactoglobulin showed well-resolved mass spectra (data not shown). Fraction F3Y showed a main component with an M_r of 18171 ± 2.07 Da, suggesting that it represents variant A of β -lactoglobulin. The other β -lactoglobulin fraction (F3X) gave a main component with an M_r of 18148.7 ± 2.87 Da, confirming the previous conclusion that it represents variant B of β -lactoglobulin. These mass values for the two β -lactoglobulin variants are in complete agreement with those reported by Trujillo *et al.* [33].

Table 1. Amino acid composition (g amino acid/100 g protein) of ovine whey proteins

Amino acid	BLG total	BLG (A)	BLG (B)	ALA
Asx	7.70	8.64	8.92	15.98
Glx	15.28	15.05	14.99	11.70
Ser	4.10	3.15	3.17	4.70
Gly	3.43	3.05	2.99	4.85
His	1.55	1.08	1.62	2.14
Arg	2.10	1.93	1.94	1.55
Thr	4.74	4.69	4.71	4.77
Ala	9.30	9.81	9.78	6.12
Pro	6.05	5.04	4.98	2.35
Tyr	1.97	2.24	1.85	2.91
Val	6.51	6.33	6.30	4.72
Met	2.57	2.69	2.69	0.30
Cys	1.60	1.84	1.98	3.41
Ile	5.23	5.40	5.55	6.30
Leu	13.69	14.36	14.08	11.56
Phe	3.11	2.82	2.83	3.81
Lys	11.00	11.88	11.58	12.83

ALA, α -lactalbumin
BLG, β -lactoglobulin

The amino acid compositions of the two β -lactoglobulin fractions are similar except in the contents of His and Tyr residues (Table 1). This demonstrates that fraction F3X and F3Y represent β -lactoglobulins B and A, respectively, since the following substitutions have been demonstrated [34] for ovine β -lactoglobulin variants A, B, and C: His₂₀ (B) $\rightarrow$ Tyr (A and C) and Arg₁₄₈ (A and B) $\rightarrow$ Gln (C). The ESI mass spectrum of purified α -lactalbumin showed a main component with an M_r of 14152.4 ± 1.03 Da (data not shown). This value corresponds to that of the main variant A of α -lactalbumin. This mass value is very close to that obtained by Trujillo *et al.* [33]. The amino acid composition obtained for F1 did not differ to that yet described for α -lactalbumin, confirming the identity of this fraction.

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