

Multiple forms of equine α -lactalbumin: evidence for *N*-glycosylated and deamidated forms

J.-M. Girardet*, M.-A. N'negue, A.S. Egito¹, S. Campagna, A. Lagrange, J.-L. Gaillard

Laboratoire des BioSciences de l'Aliment, Faculté des Sciences et Techniques, Université Henri Poincaré-Nancy 1, USC, INRA no. 885, BP 239, Vandœuvre-lès-Nancy Cedex 54506, France

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Abstract

Equine raw milk whey proteins were separated by anion-exchange fast protein liquid chromatography and characterised by alkaline polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate PAGE, and bi-dimensional PAGE. Approximately, 1% of α -lactalbumin (α -LA) was *N*-glycosylated. A minor *N*-glycosylated form of lysozyme was also found in equine milk. On the other hand, two non-glycosylated α -LA isoforms with similar molecular masses ($14,215 \pm 4$ Da) were shown to be present. Their respective apparent isoelectric points were 5.25 and 4.94. These isoforms did not correspond to different genetic variants and were not the result of α -LA modulation by calcium ions. They corresponded rather to a non-enzymatic deamidation process of a single asparagine side-chain, the most acidic isoform being spontaneously generated from the less acidic isoform by simple incubation of α -LA at 37°C. The initial rate of this chemical degradation was 4.5 μ M ammonia liberated per hour, in 150 mM sodium phosphate buffer pH 7.4 at 37°C. Deamidation induced a slight variation in secondary structure content, but no significant change in the tertiary structure of the equine α -LA was studied by circular dichroism in the near- and far-UV regions.

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1. Introduction

The physical, chemical and functional properties of α -lactalbumin (α -LA) and lysozyme (LZ) have been extensively compared. The two proteins have striking structural homology in spite of marked differences in their functions (Acharya, Stuart, Phillips, McKenzie, & Teahan, 1994; Qasba & Kumar, 1997). α -LA is a part of the lactose synthetase complex that promotes the synthesis of lactose in the lactating mammary gland in modifying the substrate specificity of the UDP-galactose-4-epimerase (Kuhn, Carrick, & Wilde, 1980). Human α -LA has a strong Ca^{2+} binding site and a weaker secondary binding site (Chandra, Brew, & Acharya, 1998). Calcium binding strongly influences the molecu-

lar stability of α -LA and is required for refolding and disulphide bond formation in the reduced, denatured protein. α -LA can also bind different metal ions in addition to calcium (Permyakov & Berliner, 2000). LZ in equine milk has the essential features of a chicken-type (*c*-type) LZ (McKenzie & Shaw, 1985) that catalyses the hydrolysis of glycosidic bonds of mucopolysaccharides in bacterial cell walls. Equine milk LZ is a calcium binding protein (Lyster, 1992), although bovine and hen egg white LZs have no calcium. Therefore, on the basis of metal binding properties, the equine LZ is more similar to bovine α -LA than to bovine and hen LZs (Sugai, Ikeguchi, & Shimizu, 1999).

The refolding of bovine α -LA (e.g., Dolgikh et al., 1981) and equine LZ (Sugai et al., 1999; Morozova-Roche, Jones, Noppe, & Dobson, 1999) has been extensively studied in order to elucidate the mechanisms by which proteins fold. The two proteins show an intermediate, partially unfolded state termed “molten globule” state that does not bind calcium any more.

In the mare, three genetic variants of α -LA have been identified (Kaminogawa, McKenzie, & Shaw, 1984;

*Corresponding author. Tel.: +33-383-68-42-66; fax: +33-383-68-42-74.

E-mail address: jean-michel.girardet@scbiol.uhp-nancy.fr (J.-M. Girardet).

¹Present address: EMBRAPA-CAPRINOS, Cx. Postal D10, CEP 62011-970, Sobral, Ceara, Brazil.

Godovac-Zimmermann, Shaw, Conti, & McKenzie, 1987). Donkey's α -LA presents two isoforms A and B which show an isoelectric point (IEP) difference of 0.23 pH units (Giuffrida, Cantisani, Napolitano, Conti, & Godovac-Zimmermann, 1992). Their aminoacid sequences reveal, however, no differences. This apparent heterogeneity of α -LA would be due to binding of Ca^{2+} (Giuffrida et al., 1992).

Among the different species investigated, α -LA has been reported to be glycosylated either totally (Prasad, Hudson, Butkowsky, Hamilton, & Ebner, 1979; Cantisani, Napolitano, Giuffrida, & Conti, 1990), or partially (Brew, Castellino, Vanaman, & Hill, 1970; MacGillivray, Brew, & Barnes, 1979; Hopp & Woods, 1979; Halliday, Bell, McKenzie, & Shaw, 1990; Giuffrida et al., 1997). No glycosylation has been reported up to now for horse's and donkey's α -LAs. Moreover, no glycoform of LZ has been found, except the hen egg white LZ, for which only 0.3% of the total protein is glycosylated (Trudel & Asselin, 1995).

The aims of this study were (i) to evidence the existence of minor glycosylated forms of equine α -LA and (ii) to show that the existence of two major forms of equine α -LA was not the result of a modulation by Ca^{2+} as suggested in the case of the donkey's α -LAs, but was the consequence of a protein degradation process identified as the deamidation of one amidated side-chain. This degradation process was identified by *in vitro* study as non-enzymatic deamidation and its effects on the Ca^{2+} binding and on the secondary and tertiary structures of the equine α -LA were discussed.

2. Materials and methods

2.1. Preparation of equine whey soluble proteins

Milk samples were collected from a herd of Haflinger mares and immediately stored at -20°C until used. The milk was skimmed by centrifugation ($2100 \times g$ at 32°C for 30 min) and the whole casein was precipitated at pH 4.2 with 1 M HCl (the minimal solubility of the equine caseins was reached for pH 4.2 instead of pH 4.6 for bovine caseins). Milk whey was neutralised with 1 M NaOH, dialysed against distilled water in the presence of thymol at 4°C for 72 h, and the whey soluble proteins were lyophilised.

2.2. Anion-exchange FPLC

The whey soluble proteins were fractionated by anion-exchange FPLC (ÅKTA-FPLC model; Amersham Pharmacia, Uppsala, Sweden) on an analytical Mono Q HR 5/5 column ($50 \times 5 \text{ mm}^2$ internal diameter; $10 \mu\text{m}$ particle size; Amersham Pharmacia). Volumes of $200 \mu\text{L}$ of samples (10 mg mL^{-1}) were loaded onto the

column equilibrated in 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris/HCl) buffer, pH 7.4, containing 0.02% (w v^{-1}) sodium azide. The flow rate was 1 mL min^{-1} and detection was monitored at 280 nm. A linear gradient from 0 to 0.2 M NaCl was applied from 5 to 35 min. Four fractions (noted F1–F4) were collected, dialysed, and lyophilised.

2.3. Affinity chromatography on immobilised Con A

The F3 fraction (20 mg mL^{-1}) was separated onto a Con A-Sepharose column ($15 \times 1.5 \text{ cm}^2$, Amersham Pharmacia). The flow rate was 10 mL h^{-1} and detection was monitored at 280 nm. Con A-unbound fraction was eluted in 5 mM sodium acetate buffer, pH 6.8, containing 1 mM CaCl_2 , 1 mM MnCl_2 , 1 mM MgCl_2 , 1 M NaCl, and 0.02% (w v^{-1}) sodium azide. Con A-bound fraction was eluted in the same buffer containing 500 mM methyl- α -D-glucopyranoside (Krusius, Finne, & Ravaula, 1976). The Con A-unbound and Con A-bound fractions of F3 were dialysed and lyophilised.

2.4. Electrophoresis

SDS-PAGE was performed with a 4.9% (w v^{-1}) polyacrylamide concentration gel in 0.125 M Tris/HCl buffer, pH 6.8, containing 0.1% (w v^{-1}) SDS, and with a 15.4% (w v^{-1}) polyacrylamide separation gel in 0.38 M Tris/HCl buffer, pH 8.8, containing 0.1% (w v^{-1}) SDS (Laemmli & Favre, 1973). Samples were dissolved at 2 mg mL^{-1} in 0.125 M Tris/HCl buffer, pH 6.8, in the presence of 0.1% (w v^{-1}) SDS, 5% (v v^{-1}) 2-mercaptoethanol, 10% (v v^{-1}) glycerol, and 0.01% (w v^{-1}) bromophenol blue. After heating at 100°C for 3 min, $20 \mu\text{L}$ of sample was loaded in the gel. The molecular mass standards (Bio-Rad, Hercules, CA, USA) were myosin (200.0 kDa), β -galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), LZ (apparent molecular mass of 15.5 kDa), and aprotinin (6.5 kDa). Proteins were fixed with 12% (w v^{-1}) trichloroacetic acid for 30 min and then stained for 60 min by 0.5% (w v^{-1}) R-250 Coomassie blue dissolved in mixture of 50% (v v^{-1}) ethanol and 12% (w v^{-1}) trichloroacetic acid, followed by an overnight destaining in solution of 30% (v v^{-1}) ethanol, 7.5% (v v^{-1}) acetic acid, and 5% (w v^{-1}) trichloroacetic acid. Staining of glycoproteins fixed in 12% (w v^{-1}) trichloroacetic acid was carried out with Schiff's reagent after 1% (w v^{-1}) periodic acid oxidation (Kapitany & Zebrowski, 1973). Relative proportions of the electrophoretically separated proteins was performed by densitometry at 633 nm (Ultrosan XL densitometer; Amersham Pharmacia) after Coomassie blue staining. For microsequencing analysis, proteins of interest were electrotransferred onto polyvinylidene

difluoride membrane after SDS-PAGE and stained by 0.2% (w v⁻¹) Ponceau S red in 2% (v v⁻¹) acetic acid. Destaining was performed with ultra-pure H₂O.

Alkaline PAGE was carried out with a 9.25% polyacrylamide gel in 0.38 M Tris/HCl buffer, pH 8.9 (Pâquet, Nejjar, & Linden, 1988). Samples (2 mg mL⁻¹) were solubilised in 0.38 M Tris/HCl, pH 8.9, containing 10% (v v⁻¹) glycerol and 0.01% (w v⁻¹) bromophenol blue. Volumes of 20 µL were loaded in the gel. After electrophoresis, proteins were stained by Coomassie blue as described above.

2D-PAGE coupling isoelectrofocalisation (IEF) and SDS-PAGE was adapted from the method of Herbert et al. (1998). For the first dimension (IEF), the proteins (100 µg) were dissolved in 200 µL of a solution containing 8 M urea, 4% (w v⁻¹) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate, 2 mM tributyl phosphine, 40 mM Tris, 0.2% (w v⁻¹) ampholytes 3–10 (Bio-Lytes 3–10; Bio-Rad), and 0.001% (w v⁻¹) bromophenol blue. A pre-cast immobilised pH gradient gel strip (IPG-strip) at 4% (w v⁻¹) polyacrylamide with a linear pH gradient range of 4–7 (IPG ReadyStrip pH 4–7, length 11 cm, Bio-Rad) was placed in the Protean IEF Cell (Bio-Rad) and was rehydrated with the protein solution for 15 h at 20°C under active conditions (constant voltage of 50 V). The focusing was performed at 20°C in three steps. First, a low voltage (250 V for 15 min) was required to remove excess salts. Secondly, a voltage ramp from 250 to 8000 V for 150 min was applied with a current limit of 50 µA per IPG-strip. Thirdly, the voltage was kept at a constant value of 8000 V for 5 h. The IPG-strip was then equilibrated for 15 min in 0.125 M Tris/HCl buffer, pH 6.8, containing 6 M urea, 2% (w v⁻¹) SDS, 20% (v v⁻¹) glycerol, and 5 mM tributyl phosphine. The IPG-strip was embedded on top of an SDS-PAGE concentration gel using molten 1% (w v⁻¹) agarose in electrode buffer containing 384 mM glycine and 0.1% (w v⁻¹) SDS and adjusted to pH 8.3 using Tris. The second dimension (SDS-PAGE) was performed as described above. The IEPs of sample proteins were determined with the help of 2D SDS-PAGE Standards (purchased from Bio-Rad) composed of a mixture of trypsin inhibitor, actin, albumin, carbonic anhydrase, conalbumin, myoglobin, and glyceraldehyde-3-phosphate dehydrogenase. With a pH gradient range of 4–7, only trypsin inhibitor (IEP=4.5), actin (5.1), albumin (5.5), carbonic anhydrase (6.2), and conalbumin (6.3) were visible in 2D-PAGE gel.

2.5. Electrophoretic shift of equine α -LA isoforms

The whey soluble proteins, the F2 and F3 fractions (8 mg mL⁻¹ corresponding to approximately 0.5 mM α -LA in the case of F2 and F3) were solubilised in 20 mM Tris/HCl buffer, pH 8.0, containing 0.02% (w v⁻¹)

sodium azide, in the absence or in the presence of 5 mM ethylenediaminetetra-acetate (EDTA) disodium salt or 5 mM calcium chloride. The protein solutions were incubated at 37°C for 16 h. Controls were performed in the absence of EDTA and calcium chloride with protein solutions stored at –20°C instead of 37°C. For alkaline PAGE analysis, 180 µL of 0.38 M Tris/HCl buffer, pH 8.9, containing 50% (v v⁻¹) glycerol and 0.01% (w v⁻¹) bromophenol blue were added to 20 µL of protein solutions. Volumes of 40 µL (whey soluble proteins) or 20 µL (F2 and F3 fractions) were loaded in the gel.

2.6. *In vitro* chemical degradation of α -LA

The whey soluble proteins (15 mg mL⁻¹) were solubilised in 20 mM Tris/HCl, in 150 mM Tris/HCl, and in 150 mM sodium phosphate buffer, all buffers at pH 7.4 and containing 0.02% (w v⁻¹) sodium azide. The protein solution was then filtered at 0.45 µm and incubated at 37°C. In the case of the 150 mM buffers, volumes of 100 µL were taken from the protein solution as a function of time and were diluted four times with the FPLC buffer prior to injection of 100 µL onto the Mono Q column. In the case of the 20 mM Tris/HCl buffer, no dilution was performed. Separation by anion-exchange FPLC was carried out as previously described. The relative proportions of the α -LA A1 and A2 were determined by peak area measurements of the F2 and F3 fractions, respectively.

2.7. Determination of ammonia

The whey soluble proteins (15 mg mL⁻¹) were solubilised in 150 mM sodium phosphate buffer, pH 7.4, containing 0.02% (w v⁻¹) sodium azide and filtered. The total ammonia released after incubation at 37°C as a function of time was measured by an NADPH-glutamate dehydrogenase method with an ammonia determination kit according to the manufacturer's instruction (Sigma Chemical Co., St. Louis, MO, USA). A volume of 100 µL of protein solution was added to 1 mL of ammonia assay solution. The decrease in absorbance due to the oxidation of NADPH was determined at 340 nm.

2.8. Mass spectrometry

The F2 fraction (16 mg mL⁻¹) was solubilised in 20 mM Tris/HCl buffer, pH 7.4, containing 0.02% (w v⁻¹) sodium azide, and incubated at 37°C for 16 h. One volume of a mixture of 20% (v v⁻¹) acetonitrile and 0.2% (v v⁻¹) trifluoroacetic acid was added to one volume of the protein solution prior to filtration at 0.45 µm. The protein solution was then fractionated by reversed-phase high performance liquid chromatography

(RP-HPLC). A volume of 300 μL (corresponding to 2.4 mg protein) was loaded onto a Delta-Pak C4 column ($300 \times 7.8 \text{ mm}^2$ inner diameter, 5 μm particle size, 30 nm porosity) connected to an HPLC model Alliance 2690 (Waters, Milford, MA, USA). A linear gradient from 30% to 50% (v v^{-1}) acetonitrile in water in the presence of 0.1% (v v^{-1}) trifluoroacetic acid was applied for 15 min, followed by an isocratic step at 50% (v v^{-1}) acetonitrile for 10 min. The flow rate was 1 mL min^{-1} and detection was monitored at 280 nm. α -LA isoforms free from salt were collected and lyophilised. The molecular mass determination of the equine α -LA isoforms was performed by electrospray ionisation-mass spectrometry analysis as previously described (Léonil et al., 1995).

2.9. Microsequencing

Amino-terminal microsequencing was performed on a Model 476A microsequencer with on line identification of the phenylthiohydantoin derivatives (Applied Biosystems, Foster city, CA, USA).

2.10. Circular dichroism (CD)

Measurements were carried out on a CD6 dichrograph (Jobin Yvon, Longjumeau, France) at room temperature. The instrument was routinely calibrated with an aqueous solution of d-10-camphorsulphonic acid. CD spectra in the far-UV region and in the near-UV region were collected using cells with path length of 0.1 and 1 cm, respectively. Five to eight scans were averaged and corrected for baseline. Ellipticity is reported as $[\theta]$, the mean residue molar ellipticity (in $\text{deg cm}^2 \text{ dmol}^{-1}$ unit).

Protein solutions were made in 10 mM sodium phosphate buffer, pH 7.1, at a protein concentration of $1.6 \times 10^{-5} \text{ M}$ as determined by absorbance at 280 nm using an extinction coefficient of $27,880 \text{ cm}^{-1} \text{ M}^{-1}$. The pH of the mixture was systematically controlled after addition of the protein. The estimation of secondary structure content from CD data was made using K2d, a neural net retrieval program (Andrade, Chacon, Merelo, & Moran, 1993).

3. Results and discussion

3.1. Chromatography of equine whey proteins

The whey soluble proteins were separated in four fractions by anion-exchange FPLC (Fig. 1). Each of the four fractions was checked for purity by anion-exchange FPLC and was submitted to amino-terminal microsequencing (Fig. 1). LZ was not retained on the Mono Q column and was entirely recovered in F1. Its amino-

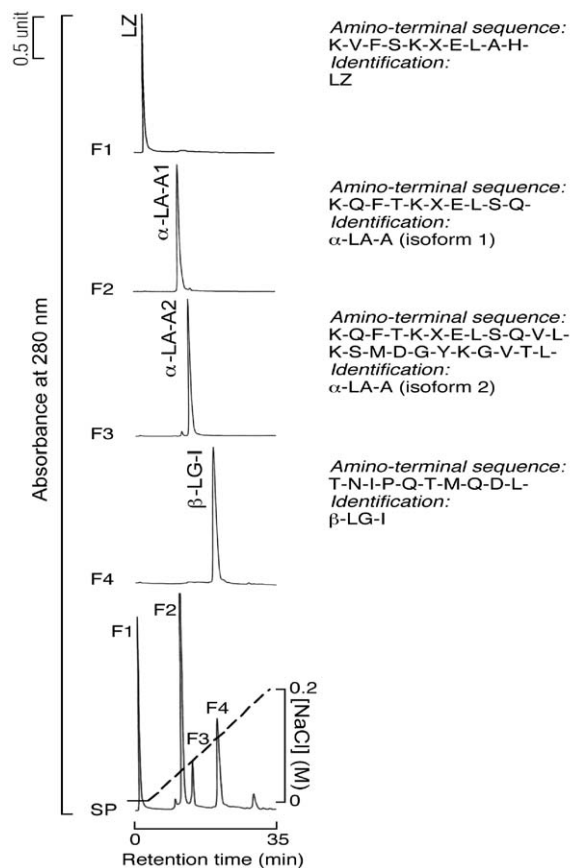


Fig. 1. Anion-exchange FPLC on a Mono Q column of equine whey soluble proteins (SP) and of the F1–F4 fractions collected. The major proteins were identified by amino-terminal microsequencing. LZ: lysozyme; α -LA-A: α -lactalbumin variant A; β -LG-I: β -lactoglobulin variant I.

terminal region was identical to that reported for equine LZ (accession number P11376; McKenzie & Shaw, 1985). α -LA displayed two isoforms recovered in F2 and F3, respectively. The two isoforms, which had a Glu residue at position 7 of the polypeptide chain, corresponded to the same genetic variant A of α -LA (accession number P08334; Kaminogawa et al., 1984), although they were eluted in two different fractions. Residue at position 7 corresponds to Glu in variant A, the most commonly found in the different mare's breeds, and to Gln in variants B and C (accession number P08896; Godovac-Zimmermann et al., 1987) that have been identified in colostrum of a Persian Arab mare (Godovac-Zimmermann et al., 1987). The minor isoform α -LA A2 recovered in F3 had stronger adsorption on the Mono Q column than the major isoform α -LA A1 recovered in F2 ($A1/A1 + A2 = 73\%$). β -LG was entirely recovered in F4 and corresponded to variant I (accession number P02758; Godovac-Zimmermann & Braunitzer, 1987). Among the numerous differences between the β -LG variants I and II, Asn was found at position 2 for the variant I instead of Asp for the variant II.

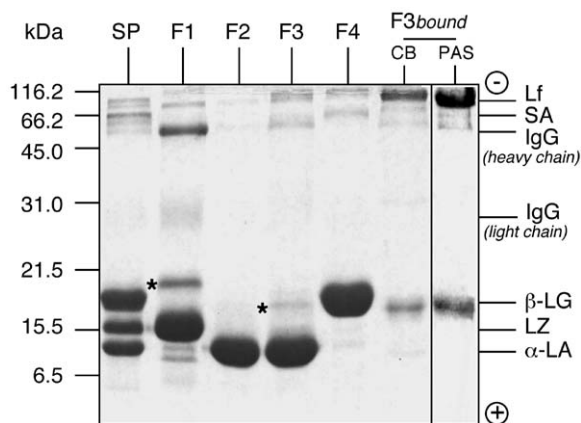


Fig. 2. SDS-PAGE of the equine whey soluble proteins (SP), of the F1–F4 chromatographic fractions, and of the subfraction of F3 bound onto immobilised concanavalin A (F3bound). Lanes SP, F1–F4, and CB of F3bound: proteins were revealed by Coomassie blue; Lane PAS of F3bound: glycoproteins were revealed by Schiff's reagent after periodic acid oxidation; Lf: lactoferrin; SA: serumalbumin; β -LG: β -lactoglobulin; LZ: lysozyme; α -LA: α -lactalbumin; stars pointed out glycosylated forms of lysozyme (lane F1) and α -lactalbumin (lane F3).

The whey soluble proteins fractionated by FPLC were characterised by SDS-PAGE (Fig. 2). The principal proteins β -LG, LZ, and α -LA displayed apparent molecular masses of 18.8, 15.5, and 14.4 kDa, respectively. Although the molecular masses of both LZ and α -LA are about 14 kDa, LZ (a very basic protein) migrates as a 15.5-kDa protein (Alston-Mills & Thompson, 1996). It was noted that the two α -LA isoforms of the F2 and F3 fractions displayed the same apparent molecular masses (14.4 kDa; Fig. 2).

3.2. Glycosylated equine α -LA and lysozyme

Large amounts of each chromatographic fraction have been loaded in the SDS-PAGE gel for a better visualisation of the minor components in the different samples (Fig. 2). In addition to the well-known serum proteins present in whey, i.e. lactoferrin, serumalbumin, and immunoglobulins, two other minor bands with apparent molecular masses of 19 and 17 kDa were found in F1 and F3, respectively. These two bands, as well as lactoferrin, were stained by Schiff's reagent after periodic acid oxidation. After electrotransfer onto a polyvinylidene difluoride membrane of the F1 and F3 fractions, the two bands of interest were excised and directly microsequenced. The 19-kDa protein was identified as a mixture of approximately 60% of glycosylated LZ and 40% of an equine β -casein carboxy-terminal peptide. The 17-kDa protein was identified as glycosylated α -LA A. By densitometry measurements, the glycosylated form of α -LA represented approximately 1% of the whole α -LA (A1 + A2). The F3 fraction was separated by Con A affinity

chromatography into two Con A-unbound and Con A-bound fractions. The 17-kDa glycoprotein was recovered in the Con A-bound fraction together with lactoferrin (Fig. 2). As it displayed affinity toward Con A, the 17-kDa glycoprotein corresponded to N -glycosylated α -LA A.

Several α -LAs of both ruminant and non-ruminant species are known to be N -glycosylated either totally or partially. Most mammals possess the usual N -glycosylation site for α -LA, which is Asn⁴⁵ in the triplet ⁴⁵Asn-Xaa-Ser⁴⁷. This N -glycosylation consensus site is not found in equine α -LA, because an unusual Lys takes the place of the usual Ser at position 47 (Fig. 3; Godovac-Zimmermann et al., 1987). Approximately 1% of the human α -LA is glycosylated; the site of carbohydrate attachment is located at position 71 in the triplet ⁷¹Asn-Ile-Cys⁷³, although the usual triplet ⁴⁵Asn-Glu-Ser⁴⁷ is present in the primary structure (Giuffrida et al., 1997). The same unusual triplet is also found in the region 71–73 of the equine α -LA and seemed to be the only possibility of carbohydrate attachment, as no usual N -glycosylation site is present in the equine protein (Fig. 3). Moreover, the apparent molecular mass difference observed between the non-glycosylated and glycosylated α -LAs (difference of 2600 Da) indicated that only one carbohydrate chain was attached on the polypeptide chain (the molecular mass of a biantennary N -acetylglucosamine-type carbohydrate chain is approximately 2000 Da).

3.3. Electrophoretic properties of α -LA isoforms

Alkaline PAGE separated the soluble proteins of equine whey in three major bands (lane 1, Fig. 4). LZ did not migrate toward the anode, as its net charge is positive at pH 8.9. The band with the highest migration rate was identified as β -LG from the PAGE profile of F4. The two other principal bands corresponded to the isoforms of α -LA A. The major isoform A1 found in F2 had slower migration rate than the minor isoform A2 found in F3 (lanes 5 and 9, Fig. 4). This was correlated to the elution order from anion-exchange FPLC. In the donkey, two isoelectric forms of α -LA with identical aminoacid sequences (accession number P28546) have been evidenced by preparative IEF (Giuffrida et al., 1992). The existence of two α -LA isoforms in the donkey's milk has been explained by the absence or the presence of Ca²⁺ bound to α -LA. In the present study, α -LA A2 could be generated by incubation of α -LA A1 (present in the F2 fraction) at 37°C for 16 h (compare lane 1 with lane 2, and lane 5 with lane 6, Fig. 4). The reverse was, however, not observed (compare lane 9 with lane 10, Fig. 4). As the irreversible "A1 toward A2" modification might be related to Ca²⁺ displacement, EDTA was added in the protein solutions prior to incubation at 37°C for 16 h. It was expected that one

		1		50
Human	α -LA A	KQFTKCELSQ	LLKDIDGYGG	IALPELICTM FHTSGYDTQA IVENNESTEY
Bovine	α -LA B	EQLTKCEVFR	ELKDLKGYGG	VSLPEWVCTT FHTSGYDTQA IVQNNSTEY
Asinine	α -LA AB	KQFTKCELSQ	VLKSMDGYKG	VTLPEWICTI FHSSGYDTQT IVKNNGKTEY
Equine	α -LA A	KQFTKCELSQ	VLKSMDGYKG	VTLPEWICTI <u>FHSSGYDTQT</u> IVKNNGKTEY
Equine	α -LA B	KQFTKCQLSQ	VLKSMDGYKG	VTLPEWICTI <u>FHNSGYDTQT</u> IVKNNGKTEY
Equine	α -LA C	KQFTKCQLSQ	VLKSMDGYKG	VTLPEWICTI <u>FHNSGYDTQT</u> IVKNNGKTEY
		51		100
Human	α -LA A	GLFQISNKLW	CKSSQVPQSR	NICDISCDKF <u>LDDDLTDDIM</u> CAKKILDIKG
Bovine	α -LA B	GLFQINNKIW	CKDDQNPSS	NICNISCDKF <u>LDDDLTDDIM</u> CVKKILDKVG
Asinine	α -LA AB	GLFQINNKMW	CRDNQILPSR	NICGISCNKF <u>LDDDLTDDVM</u> CAKKILDSEG
Equine	α -LA A	GLFQINNKMW	CRDNQILPSR	<u>NICGISCNKF</u> <u>LDDDLTDDVM</u> CAKKILDSEG
Equine	α -LA B	GLFEINNKMW	CRDNQILPSR	<u>NICGISCNKF</u> <u>LDDDLTDDVM</u> CAKKILDSEG
Equine	α -LA C	GLFEINNKMW	CRDNQILPSR	<u>NICGISCNKF</u> <u>LDDDLTDDVM</u> CAKKILDSEG
		101	123	
Human	α -LA A	IDYWLAKHAL	CTEKLEQWLC	EKL <i>P00709</i>
Bovine	α -LA B	INYWLAKHAL	CSEKLDQWLC	EKL <i>P08037</i>
Asinine	α -LA AB	IDYWLAKHPL	CSEKLEQWLC	EEL <i>P28546</i>
Equine	α -LA A	IDYWLAKHPL	CSEKLEQWLC	EEL <i>P08334</i>
Equine	α -LA B	IDYWLAKHPL	CSEKLEQWLC	EEL <i>P08896</i>
Equine	α -LA C	IDYWLAKHPL	CSEKLEQWLC	EEL <i>P08896</i>

Fig. 3. Comparison of the aminoacid sequences of α -lactalbumin genetic variants of different species (human, cow, ass, and mare). Sequences were obtained from Swissprot (accession numbers indicated in italic characters). The putative sites of deamidation and *N*-glycosylation of the equine α -lactalbumin A are underlined by one and two lines, respectively. For the four species, the calcium-binding site was boxed.

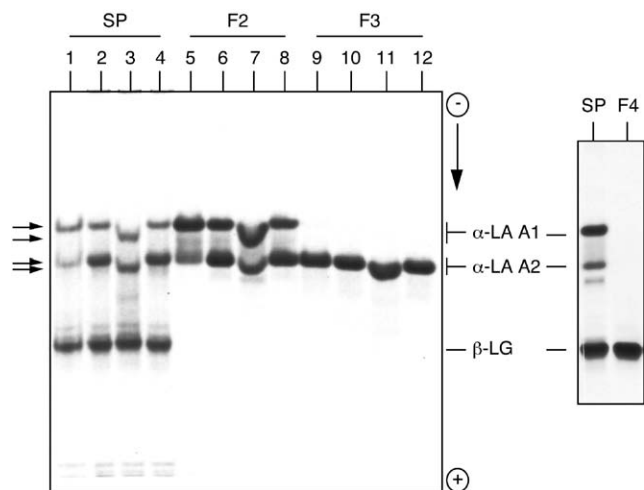


Fig. 4. Alkaline PAGE of equine whey soluble proteins (SP), of the F2 and F3 fractions after different treatments, and of the F4 fraction. The proteins of the SP, F2, and F3 fractions were first solubilised (8 mg mL^{-1}) in 20 mM Tris/HCl buffer, pH 8.3, containing 0.02% (w v^{-1}) sodium azide. Lanes 1, 5, and 9: storage at -20°C for 16 h. Lanes 2, 6, and 10: incubation at 37°C for 16 h without any additives. Lanes 3, 7, and 11: incubation at 37°C for 16 h in the presence of 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA). Lanes 4, 8, and 12: incubation at 37°C for 16 h in the presence of 5 mM calcium chloride. α -LA A1 and A2: isoforms 1 and 2 of α -lactalbumin variant A; β -LG: β -lactoglobulin. Arrows show the electrophoretic shifts of the α -lactalbumin isoforms treated by EDTA. The gel was stained with Coomassie blue.

single band corresponding to one of the two isoforms would be observed in the presence of EDTA. However, the two bands were still present (lanes 3 and 7, Fig. 4).

Moreover, the “A1 toward A2” modification still occurred even in the presence of chelating agent (compare lane 1 with lane 3, and lane 5 with lane 7, Fig. 4).

Electrophoretic shifts were observed for each of the two A1 and A2 bands, but not for the β -LG band, after EDTA treatment (arrows on Fig. 4). It was reported that Ca^{2+} -modulated proteins such as α -LAs undergo a Ca^{2+} -dependent electrophoretic shift by alkaline PAGE in the presence of Ca^{2+} chelating agent (Thompson et al., 1988). The electrophoretic shift is related to protein conformational changes upon binding of Ca^{2+} . The addition of calcium chloride instead of EDTA had no effect on the migration rate of the α -LA isoforms (lanes 4, 8, and 12, Fig. 4). It was thus concluded that the A2 form did not arise from the A1 form by the loss of Ca^{2+} upon EDTA action; the two isoforms corresponded to equine holo- α -LA.

The change “A1 toward A2” might rather be due to eventual chemical modification of the equine α -LA occurring at 37°C , spontaneously. After incubation at 37°C for 16 h in the absence of EDTA and calcium chloride, the F2 fraction was fractionated by RP-HPLC and the two isoforms α -LA A1 and A2 were submitted to mass spectrometry analysis. Identical molecular masses ($14,215 \pm 4 \text{ Da}$) were found for the two α -LA isoforms, close to the predicted molecular mass ($14,214 \text{ Da}$) of the equine α -LA A. Therefore, no important chemical modification such as proteolysis or oligomerisation was observed between the two α -LA

isoforms. However, the effective charges of the proteins A1 and A2 were different, as they displayed different electrophoretic and chromatographic behaviours. Changes in peptide and protein structure through the spontaneous non-enzymatic deamidation of Asn or Gln residues have been observed in many *in vitro* and *in vivo* experiments (Robinson, 2002). Such a modification of an amide group to an acidic group changes the molecular mass by only 1 Da. The experimental error limits of ± 4 Da did not allow one to detect small differences of molecular masses caused by deamidation of one or a few side-chains. Thus, the experimental observations supported the hypothesis of a non-enzymatic deamidation process occurring spontaneously.

3.4. Determination of IEPs

The three acidic proteins of equine whey, i.e. β -LG, α -LA A1 and A2, were characterised by 2D-PAGE with respect to their molecular mass and IEP (Fig. 5). A very good linearity of the pH gradient was obtained with standard proteins between pH 4.5 and 5.5. This allowed one to determine the apparent IEPs of the whey proteins with precision. The different proteins were identified on the electrophoretic profile by 2D-PAGE analysis of the F2–F4 fractions (data not shown). LZ (in F1) was not visible in the IEF gel (and then in the 2D-PAGE gel), as it migrated close to the cathode. β -LG displayed the most acidic character (apparent IEP of 4.74). The two α -LA A1 and A2 isoforms had respective apparent IEPs of 5.25 and 4.94 (Fig. 5). The IEP values were in accordance with the FPLC elution order and with the alkaline PAGE migration rates of the two α -LA isoforms. α -LA A2 (with the lowest IEP) was more strongly adsorbed onto the anion-exchange column, as

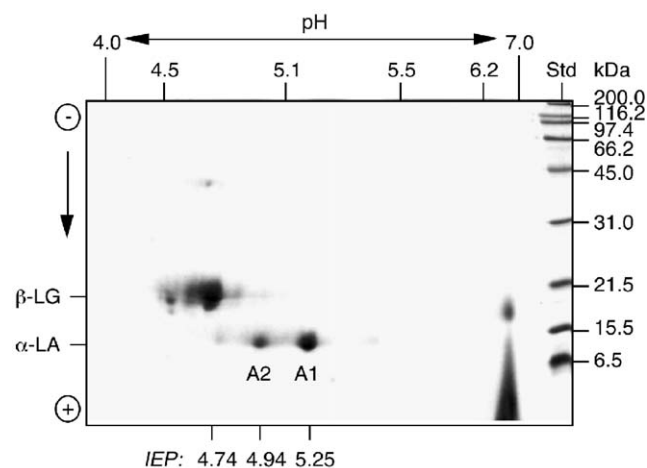


Fig. 5. 2D-PAGE of equine whey soluble proteins. The gel was stained with Coomassie blue. Std: molecular mass standards; β -LG: β -lactoglobulin; α -LA: α -lactalbumin; A1 and A2: isoforms 1 and 2 of α -lactalbumin variant A; IEP: isoelectric point.

it might display more effective negative charges than α -LA A1. This also explained its greater electrophoretic migration rate toward the anode by alkaline PAGE. The IEP difference of 0.31 between A1 and A2 was in accordance with deamidation of only one Asn or Gln residue (Lutter et al., 2001).

The IEPs of the two isoforms of donkey's α -LA have been determined by preparative flat bed IEF with values of 5.48 and 5.25 (Conti, Napolitano, Lai, Pinna, & Godovac-Zimmermann, 1989). These values display differences of 0.23 and 0.31 pH unit with the IEPs of mare's α -LA A1 and α -LA A2, respectively. These differences could be either due to the different IEF methods used for the characterisation of the donkey's and mare's α -LAs, or to different α -LA aminoacid sequences between the two species. The mare's α -LA A possesses one additional negative charge comparatively to the donkey's protein, as the mare's and the donkey's sequences differ by one mutation (Asp⁷⁸ instead of Asn⁷⁸, respectively; Giuffrida et al., 1992).

3.5. Degradation of α -LA as a function of buffer ions

The modification rate of the equine α -LA A1 was studied *in vitro*, at pH 7.4 and 37°C, as a function of time and also as a function of the kind of buffer ions (Tris/HCl or sodium phosphate). The FPLC method (Fig. 6) was preferred to the alkaline PAGE to quantify the two isoforms A1 and A2, because a minimal delay was required in order to limit additional α -LA degradation during the analysis. In the three cases studied (20 mM Tris/HCl, 150 mM Tris/HCl, and 150 mM sodium phosphate), the relative proportions A1/A2 decreased according to a logarithmic function of time (Fig. 7). At

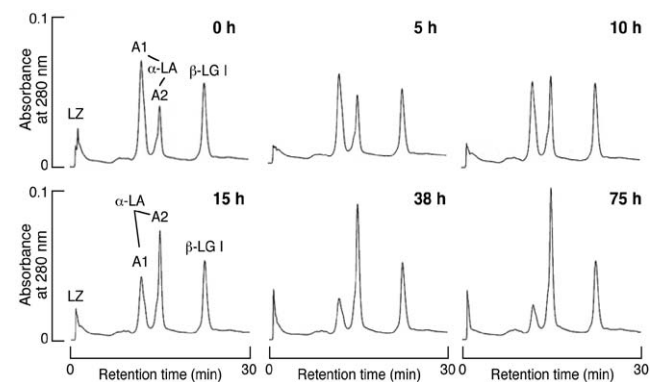


Fig. 6. Anion-exchange FPLC profiles on the time course of the degradation of α -lactalbumin. Equine whey soluble proteins (15 mg mL^{-1}) were incubated at 37°C in 150 mM sodium phosphate buffer, pH 7.4. Volumes of 100 μL were taken from the protein solution as a function of time (incubation time is indicated on each profile) and diluted four times prior to injection of 100 μL onto the Mono Q column. LZ: lysozyme; α -LA: α -lactalbumin; A1 and A2: isoforms 1 and 2 of α -lactalbumin variant A; β -LG I: β -lactoglobulin variant I.

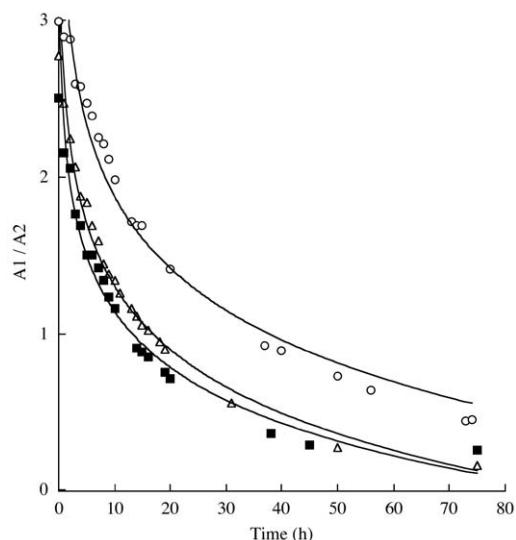


Fig. 7. Ratio of the peak areas of α -lactalbumin A1 over α -lactalbumin A2 (determined by FPLC) as a function of incubation time at 37°C in different buffers at pH 7.4. Equine whey soluble proteins (15 mg mL⁻¹) were solubilised in 20 mM Tris/HCl (Δ), 150 mM Tris/HCl ($\circ$), or 150 mM sodium phosphate buffer ($\blacksquare$). The mathematical functions of the different curves are: (Δ), $A1/A2 = -1.3 \log(\text{time}) + 2.6$, $r = 0.99$; ($\circ$): $A1/A2 = -1.5 \log(\text{time}) + 3.3$, $r = 0.98$; ($\blacksquare$): $A1/A2 = -1.2 \log(\text{time}) + 2.3$, $r = 0.99$.

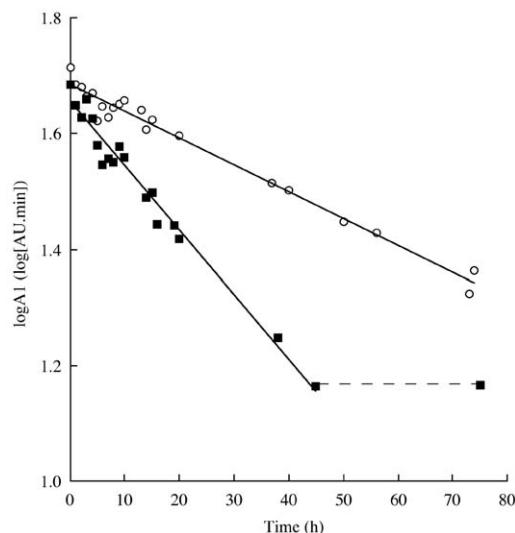


Fig. 8. Peak area of α -lactalbumin A1 (determined by FPLC) as a function of incubation time at 37°C in different buffers at pH 7.4. Equine whey soluble proteins (15 mg mL⁻¹) were solubilised in 150 mM Tris/HCl ($\circ$) or 150 mM sodium phosphate buffer ($\blacksquare$). The mathematical functions of the two curves are: ($\circ$) $\log A1 = 5 \times 10^{-3} (\text{time}) + 1.69$, $r = 0.99$; ($\blacksquare$) $\log A1 = 11 \times 10^{-3} (\text{time}) + 1.66$, $r = 0.99$ (in the range 0–45 h). AU: absorbance unit.

equivalent molarities of 150 mM, the degradation rate of α -LA was significantly higher in the presence of sodium phosphate buffer than in the presence of Tris/HCl buffer. The lowering of the Tris/HCl concentration from 150 to 20 mM promoted the α -LA degradation, which rate became close to that observed in the presence of sodium phosphate. The degradation of α -LA A1 followed a chemical reaction of first order with a kinetic constant 2.2-fold higher in the presence of 150 mM sodium phosphate than in the presence of 150 mM Tris/HCl (Fig. 8). The reaction rate slowed down after 45 h incubation time at 37°C in the presence of sodium phosphate, whereas it remained constant up to 75 h in the presence of Tris/HCl.

Rates of deamidation of amide residues depend upon primary and three-dimensional structures and solution properties, such as pH, temperature, ionic strength, and buffer ions (Robinson, 2002). It has been reported that Tris catalyses deamidation to a much lesser extent than most other buffers (Robinson, 2002). In particular, a 150 mM sodium phosphate buffer increases deamidation rates two- or three-fold as compared with those in a 150 mM Tris/HCl buffer, at pH 7.4 and 37°C (Robinson & Robinson, 2001a). The results obtained with the Tris/HCl and sodium phosphate buffers supported the hypothesis that the instability of the equine α -LA was caused by a deamidation process. On the other hand, the protein or peptide deamidation occurs at neutral and near-neutral pH (roughly 4–9; Xie & Schowen, 1999). Accordingly, the equine α -LA remained stable at pH 2.0:

no isoform A2 was generated by incubation at 37°C of equine α -LA solubilised in a solution containing 5% (v v⁻¹) acetonitrile and 0.1% (v v⁻¹) trifluoroacetic acid.

3.6. Deamidation rate of α -LA

Deamidation of the equine α -LA was clearly evidenced in vitro by the measurement of total ammonia released as a function of incubation time at 37°C (Fig. 9). In 150 mM sodium phosphate buffer, pH 7.4, the initial rate of the degradation reaction was 4.5 μ M ammonia liberated per hour. The reaction rate decreased after approximately 40–45 h incubation time, as it was observed by analytical FPLC in the case of the 150 mM sodium phosphate buffer (Fig. 8).

The rate of deamidation is strongly influenced by the nature of the side-chain of the adjacent carboxyl residue, with Asn-Gly, Asn-Ser (Geiger & Clarke, 1987; Tyler-Cross & Schirch, 1991; Robinson & Robinson, 2001b), Asn-His (Robinson & Robinson, 2001b) or Gln-Gly (Robinson, 2002) being the most susceptible sequences. Only one putative deamidation site is present in the primary structure of the equine α -LA (⁴⁵Asn-Gly⁴⁶; Fig. 3). This supports the hypothesis of deamidation of a single side-chain that would generate the isoform A2. On the other hand, it is worthwhile to note that the donkey's α -LA isoforms also possess the ⁴⁵Asn-Gly⁴⁶ sequence. No such deamidation sites are present in the bovine protein. This is probably the reason why the bovine α -LA seems to be more stable than the equine protein. It has been reported that the Asn-Gly peptide

bond obstructs microsequencing of equine α -LA peptides containing residues 45–46 by causing a great decline in yield at the Asn⁴⁵ according to an unexplained mechanism (Kaminogawa et al., 1984). This unknown mechanism might be spontaneous deamidation of Asn⁴⁵. No drastic decrease in sequencing yield is reported at other Asn or Gln residues of equine α -LA.

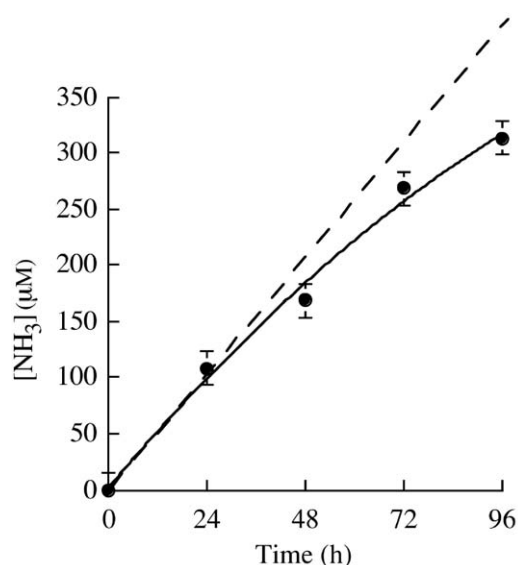


Fig. 9. Ammonia concentration liberated by protein deamidation as a function of incubation time at 37°C. Equine whey soluble proteins (15 mg mL^{-1}) were solubilised in 150 mM sodium phosphate buffer, pH 7.4. Total ammonia released after incubation at 37°C was measured at 340 nm by an NADPH-glutamate dehydrogenase method. The equation of the curve was $[\text{NH}_3] = -11 \times 10^{-3} (\text{time})^2 + 4.3 (\text{time}) + 1.8$, $r = 1.0$; $n = 3$. The initial deamidation rate (dotted line) was determined from the equation $[\text{NH}_3] = 4.5 (\text{time})$.

3.7. Effect of deamidation on the secondary and tertiary structures of α -LA

Far- and near-UV CD spectra of the isoforms A1 and A2 were compared to investigate the effects of deamidation on the conformation of α -LA (Fig. 10). The secondary structure content of the native α -LA A1 was determined from the far-UV CD spectrum (ca. 26% α -helices and ca. 18% β -strands) and was very similar to the data determined from the X-ray crystallographic study of the bovine α -LA (26% α -helices and 15.5% β -strands; Chrysina, Brew, & Acharya, 2000). The deamidation did not induce a large alteration of the far-UV CD spectrum of the equine α -LA, as the deamidated form A2 kept a high content of secondary structure (ca. 30% α -helices and ca. 12% β -strands). However, the α -helix content of the deamidated form was higher than that of the native form A1, whereas the β -strand content was lower. The deamidation did not alter the tertiary structure, as the near-UV CD spectra of A1 and A2 were very similar: the microenvironment of the non-polar side-chains (Tyr and Trp) remained unchanged between the native and the modified isoforms of α -LA.

When bovine α -LA is deamidated by action of protein-glutaminase, whose targets are four out of six Gln residues of α -LA, the stability of the tertiary structure of α -LA is closely related to the degree of enzymatic deamidation (Gu, Matsumura, Yamaguchi, & Mori, 2001). The secondary structure content is kept high although the far-UV CD spectra of the polydeamidated form of the bovine α -LA display a slight increase of negative intensity (Gu et al., 2001). In the present study, an increase of negative intensity between the far-UV spectra of A1 and A2 was also observed and led to increase the α -helix

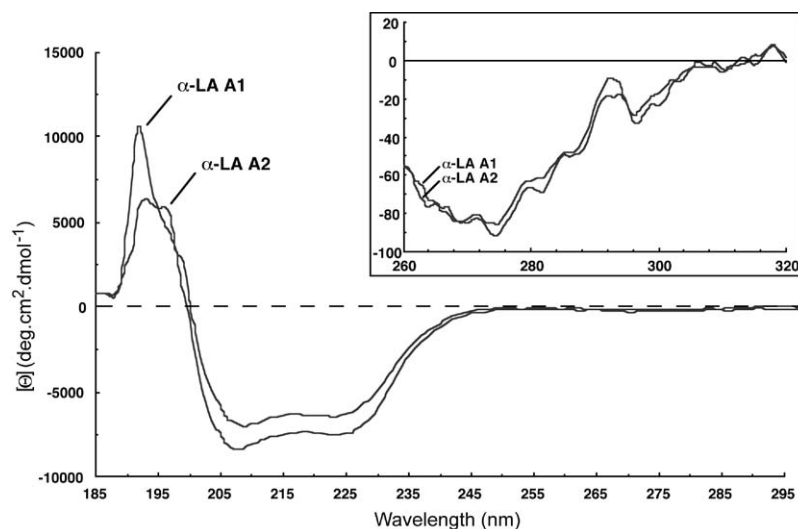


Fig. 10. CD spectra of equine-lactalbumins A1 and A2 ($1.6 \times 10^{-5} \text{ M}$ in 10 mM sodium phosphate buffer, pH 7.1) determined in the far-UV region and in the near-UV region (insert). $[\theta]$ mean residue ellipticity.

content in the deamidated form. However, the chemical modification of probably only one Asn residue was not enough important to induce some alteration in the tertiary structure of the equine α -LA.

In conclusion, the equine α -LA underwent non-enzymatic alteration, identified as deamidation of probably only one Asn residue. It was worthwhile to note that the probable site of this chemical degradation (Asn⁴⁵) corresponded to the usual *N*-glycosylation site found in α -LAs of most of the mammals. However, the region ⁴⁵Asn-Gly Lys⁴⁷ of the equine α -LA sequence did not satisfy the sequence requirements, namely Asn-Xaa-Ser/Thr, for attachment of carbohydrate to the Asn residue. Nevertheless, approximately 1% of the whole equine α -LA was found to be *N*-glycosylated probably to Asn⁷¹ of the unusual signal sequence ⁷¹Asn-Ile-Cys⁷³, as in the case of human α -LA.

No significant incidence of deamidation on the protein conformation was detected, as this degradation process did not alter notably the secondary and tertiary structures of the equine α -LA and did not lead to the release of calcium by the modified form. The reaction of deamidation proceeds via a succinimide derivative, which generates by spontaneous hydrolysis either an α -linked Asp or a β -linked isoAsp residue in an approximate yield of the α - to the β -linked product of 1:2 (Aswald, Paranandi, & Schurter, 2000) or 1:3 (Meinwald, Stimson, & Scheraga, 1986). Formation of isoAsp can decrease the biological activity of a protein, alter its susceptibility to proteolysis, and elicit autoimmunity (Aswald et al., 2000). Therefore, deamidation is a very important chemical degradation process, that must be taken into account for structure-function relationship studies of dietary proteins.

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