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# STUDY OF THE SPONTANEOUS DEAMIDATION OF CAMEL'S MILK $\alpha$ -LACTALBUMIN

**Saliha Si Ahmed Zennia**<sup>1\*</sup>, Abderrahmane Mati<sup>1</sup>, Jean-Michel Girardet<sup>2</sup>

<sup>1</sup>*Université Mouloud Mammeri, Laboratoire de Recherche de Biochimie Analytique et Biotechnologies (LABAB), Tizi Ouzou, Algérie*

<sup>2</sup>*Université de Lorraine, Unité de Recherche Animal et Fonctionnalités des Produits Animaux (UR AFPA), Vandœuvre-lès-Nancy, F-54506, France*

\*E-mail address: [sami\\_science2005@yahoo.fr](mailto:sami_science2005@yahoo.fr)

## ABSTRACT

Many proteins are susceptible, both in vivo and in vitro, to non enzymatic deamidation that can lead to alterations in their structure and biological functions. Aspartyl and isoaspartyl residues are the primary products of this non-enzymatic reaction that proceeds through the succinimide pathway with a metastable cyclic imide as an intermediate.

Camel's milk  $\alpha$ -lactalbumin was purified by FPLC on ion exchange column (Mono Q) and the pure fraction of the  $\alpha$ -lactalbumin was dialysed and freeze-dried.

Purified  $\alpha$ -lactalbumin was incubated for 80h at 37°C in deamidation buffer (150 mM phosphate buffer, pH 7.4 or 8.4). Protein concentration was 15 mg mL<sup>-1</sup>. At specific time points, 150  $\mu$ L aliquots were withdrawn and the kinetics of spontaneous deamidation of the  $\alpha$ -lactalbumin was followed by FPLC which shows the presence of two isoformes, this result was confirmed by electrophoresis analysis on Alkaline-PAGE. The pHi of the two isoformes was estimated by a two-dimensional isoelectrophoresis.

To compare the rate of camel  $\alpha$ -lactalbumin deamidation, the half-life of native  $\alpha$ -lactalbumin was calculated at pH 7.4 and 8.4, the result shows that the deamidation of camel  $\alpha$ -lactalbumin is faster at elevated pH.

**Key words:** Spontaneous deamidation ; Camel milk ;  $\alpha$ -lactalbumin ; electrophoresis.

## 1. Introduction

$\alpha$ -Lactalbumin ( $\alpha$ -La) is the major whey soluble protein in *Camelus dromedarius* milk with an average concentration of *ca.* 7.2 g/L, five-fold higher than in cow's milk (Kappeler, 1998).

Camel  $\alpha$ -La consists of 123 amino acid residues forming a compact globular structure stabilized by four disulfide bonds. Its theoretical isoelectric point (pHi) is 5.01 and displays a molecular mass of 14,422.3 Da (average mass of the non-reduced form). The biological function of  $\alpha$ -La is to participate in lactose biosynthesis as the non-catalytic regulatory subunit of the lactose synthase complex.

The deamidation of Asn residues can alter the structure of some proteins and thereby contributes to change the biological activity of the protein. It is a well-characterized mechanism (Catak et al., 2006) which proceeds through a succinimide ring intermediate.

The aim of this work was (i) to unveil that nonenzymatic deamidation of camel  $\alpha$ -La could occur under certain conditions of pH and temperature and was responsible of the generation of two novel isoelectric forms of  $\alpha$ -La, (ii) to localize on the sequence the sites of deamidation, and (iii) to investigate the differential kinetics of deamidation of the potential deamidation sites.

## 2. Materials and methods

### 2.1. Preparation of camel whey soluble proteins

Milk samples were collected from a herd of *Sahraoui* dromedary females (*Camelus dromedarius*) and immediately stored at  $-20\text{ }^{\circ}\text{C}$  until used. Whey proteins were prepared according to Si Ahmed et al., (2013). Briefly the milk was skimmed by centrifugation (3500 g at  $32\text{ }^{\circ}\text{C}$  for 30 min) and the whole casein was precipitated at pH 4.3 with 1 M HCl (the minimal solubility of the camel caseins was reached for pH 4.3 instead of pH 4.6 for bovine caseins). Milk whey was neutralized with 1 M NaOH, dialyzed against ultra-pure water (resistivity of  $18.2\text{ M}\Omega\text{ cm}$  at  $25\text{ }^{\circ}\text{C}$ ) in the presence of thymol at  $4\text{ }^{\circ}\text{C}$  for 72 h, and the whey soluble proteins were lyophilized.

### 2.2. In vitro chemical deamidation of $\alpha$ -lactalbumin

The whey proteins (15 mg/ml) were solubilized in 150 mM sodium phosphate buffer, pH 7.4 or 8.4, containing 0.02% sodium azide. The protein solution was then filtered at  $0.45\text{ }\mu\text{m}$  and incubated at  $37\text{ }^{\circ}\text{C}$ . Volumes of  $50\text{ }\mu\text{L}$  were taken from the protein solution as a function of time and were diluted four times with ultra-pure water.

### **2.3. Anion-exchange fast protein liquid chromatography (FPLC)**

The diluted samples were separated by anion-exchange chromatography with an ÄKTA-FPLC system (GE Healthcare, Uppsala, Sweden) using an analytical Mono Q HR 5/5 column (50-mm length x 5-mm internal diameter; 10-nm particle size). Volumes of 100 µl of diluted samples (3.75 mg/ml) were loaded after filtration at 0.45 µm onto the column equilibrated in 20 mM trishydroxymethylaminomethane/HCl (Tris/HCl) buffer, pH 7.4, containing 0.02% sodium azide. A linear gradient from 0 to 0.4 M NaCl was applied from 5 min after injection to 35 min, at a flow rate of 1 ml/min and the column was washed with 1.0 M NaCl for 10 min. Detection was monitored at 280 nm. The relative proportions of the different forms were determined by peak area measurements (arbitrary units). The analytical FPLC separations were performed in triplicates.

### **2.4. Alkaline and two-dimensional electrophoreses**

Vertical polyacrylamide gel electrophoresis at pH 8.9 (alkaline PAGE) was carried out with Mini-ProteanTetracell (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 200 V and 4 °C for 45 min according to the method of Pâquet et al.,(1988). Volumes of 10 µl of the diluted samples were mixed with 20 µl of loading buffer (0.38 M Tris/HCl, pH 8.9, containing 50% glycerol and 0.01% bromophenol blue) and 10 µl of this mixture (corresponding to *ca.* 12.5 µg total protein) were loaded in the gel. After electrophoresis, proteins were stained by Coomassie blue R-250.

Two-dimensional electrophoresis (2-DE) was according to a previous work (Mateos et al., 2009). The first dimension consisted to an isoelectric focusing (IEF) performed using a pre-cast immobilized pH gradient gel strip (IPG-strip) at 4% polyacrylamide in the pH range of 4–7 (IPG ReadyStrip pH 4–7, length 11 cm, Bio-Rad). The IPG-strips were placed in a Protean IEFCell (Bio-Rad) and rehydrated with each protein solution for 15 h at 10 °C under active conditions (constant voltage of 50 V). The second dimension consisted to a sodium dodecyl sulfate (SDS)-PAGE composed of a 5% polyacrylamide stacking gel and a 15% polyacrylamide resolving gel. The proteins were revealed after migration at 4 °C by Coomassie Blue R-250.

### **2.5. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS)**

#### **2.5.1. Exact mass determination**

Samples ( $\alpha$ -La A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub>) were dissolved in a mixture of 50% methanol and 1% formic acid in water and injected on a Linear Trap Quadrupole (LTQ)-FT-ICR mass spectrometer (Thermo Scientific, San Jose, CA, USA) using TriVersaNanoMate source (Advion Biosciences, Ithaca, NY, USA). The isotopic distribution of the camel  $\alpha$ -La was calculated using the open source 'Molecular Weight Calculator 6.49' software.

### 2.5.2. Localization of the deamidation sites

Samples ( $\alpha$ -La A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub>) were dissolved in ammonium bicarbonate at 1 mg/ml. After reduction/alkylation (dithiothreitol at a final concentration of 10 mM, 2 h, 37 °C / iodoacetamide at final concentration of 50 mM, 30 min in the dark), 2.5 ng chymotrypsin (EC 3.4.21.1; Sequencing grade; from bovine pancreas; Roche Diagnostics, Meylan, France) in 150 mM ammonium carbonate was added and proteins were incubated overnight at 37 °C while shaking. Then, the reaction was stopped with 10  $\mu$ l of 10% aqueous formic acid and the peptides purified by using C<sub>18</sub> Tips (Ziptip; Millipore Co., Billerica, MA, USA) according to the manufacturer's instructions.

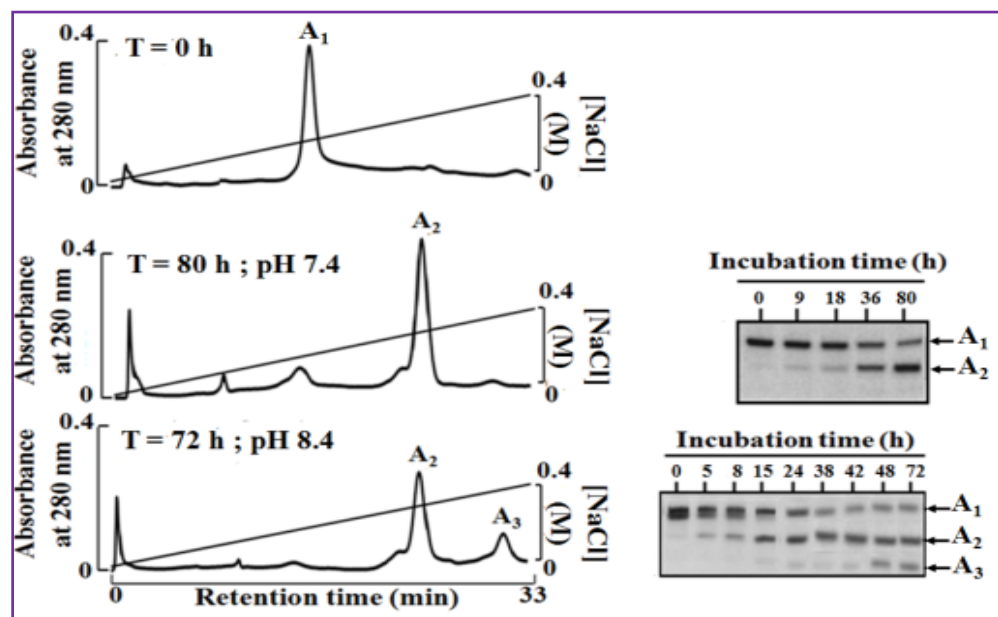
A volume of 2  $\mu$ l of peptide solution was injected on a capillary reversed-phase column (nanoC<sub>18</sub> Acclaim PepMap100, 75- $\mu$ m internal diameter x 15-cm length, 10-nm particle size; Dionex-Thermo Scientific, Chelmsford, MA, USA), at a constant flow rate of 220 nl/min, with a gradient 2–40% buffer B in buffer A in 45 min (buffer A: water/acetonitrile/trifluoroacetic acid 98:2:0.1, buffer B: water/acetonitrile/trifluoroacetic acid 10:90:0.1). Coupling with mass spectrometer was done using a TriVersaNanoMate (Advion). The mass spectrometry (MS) analysis was performed on a FT-ICR mass spectrometer (LTQ-FT Ultra; Thermo Scientific) with the top 7 acquisition method (MS resolution of 60,000 Da, mass-to-charge ratio range of 500–2000 Th), followed by 7 MS/MS (LTQ) on the 7 most intense peaks, with a dynamic exclusion for 90 s. The raw data were processed using Xcalibur 2.0.7 software. All the data were submitted to Mascot search engine version 2.3 (Matrix Science, London, United Kingdom) with the *Camelus dromedarius*  $\alpha$ -La sequence (Swiss-Prot accession number P00710) as reference. Proteome Discoverer 1.3 (ThermoScientific) and Mascot were used to search the data and filter the results. The following parameters were used: chymotrypsin, up to 2 miss cleavages; MS tolerance 10 ppm; MS/MS tolerance 0.5 Da.

## 3. Results and discussion

### 3.1. Unveiling of nonenzymatic deamidation of camel $\alpha$ -Lactalbumin

To investigate the stability of camel  $\alpha$ -La, the whey proteins were first incubated at 37 °C as a function of time in 150 mM phosphate buffer adjusted either at pH 7.4 or 8.4. Then, aliquots were collected during the incubation period and analyzed either by anion-exchange chromatography or alkaline electrophoresis (**Fig. 1**). The chromatographic conditions, especially the ionic strength linear gradient, have been previously optimized to obtain the best resolution of the separation of the camel  $\alpha$ -La. The native form of  $\alpha$ -La was noted A<sub>1</sub> and was identified by SDS-PAGE according to Hinz et al., (2012). The other whey proteins were mainly eluted in the

column washing volume with a NaCl concentration of 1.0 M. Under physiological conditions (37 °C, pH 7.4), a supplementary electrophoretic band (noted A<sub>2</sub>) displaying greater migration rate than A<sub>1</sub>, and an additional chromatographic peak with greater retention time appeared in the course of the incubation at the expense of A<sub>1</sub>. When the pH value was increased to 8.4, another novel band (A<sub>3</sub>) with the fastest migration rate, and another novel peak of A<sub>3</sub> much more retained on the column, appeared on the electrophoretic and chromatographic patterns, respectively. Thus, a second isoform of α-La was generated under the alkaline conditions, whereas the relative content of A<sub>1</sub> (and of A<sub>2</sub> in a second time after its generation) had diminished. This result suggested that negative charges were generated spontaneously during the incubation leading to the formation of two α-La isoforms A<sub>2</sub> and A<sub>3</sub> and supported the hypothesis that the instability of the camel α-La A<sub>1</sub> was caused by a deamidation process.

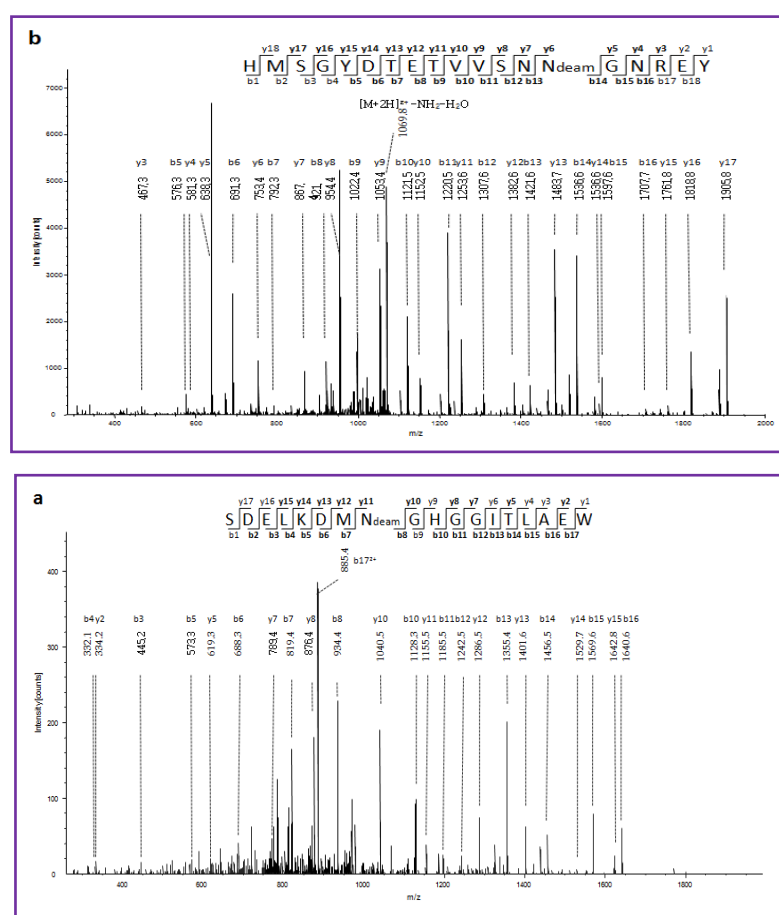


**Fig. 1.** Formation of the A<sub>2</sub> and A<sub>3</sub> isoforms from the native camel α-lactalbumin A<sub>1</sub> highlighted by anion-exchange ÄKTA-FPLC and SDS-PAGE as a function of time of incubation at 37 °C, either at pH 7.4 or at pH 8.4 in 150 mM phosphate buffer.

### 3.2. FT-ICR-MS/MS spectra of the deamidated camel $\alpha$ -lactalbumin

Mass spectrometry analysis was then performed with A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> after their purification by ÄKTA-FPLC to prove that the two newly generated isoforms were the result of a deamidation process. As deamidation of one Asn residue raises the molecular mass of the protein by only 0.984 Da ( $-\text{NH}_2$  vs.  $-\text{OH}$ ), FT-ICR-MS has been chosen. The native  $\alpha$ -La (A<sub>1</sub>) displayed an experimental molecular mass of 14,421.8659 Da (**Fig. 2a**), whereas the deamidated isoforms A<sub>2</sub> and A<sub>3</sub> had masses of 14,422.8569 Da (**Fig. 2b**) and 14,423.8479 Da (**Fig. 3c**), respectively.

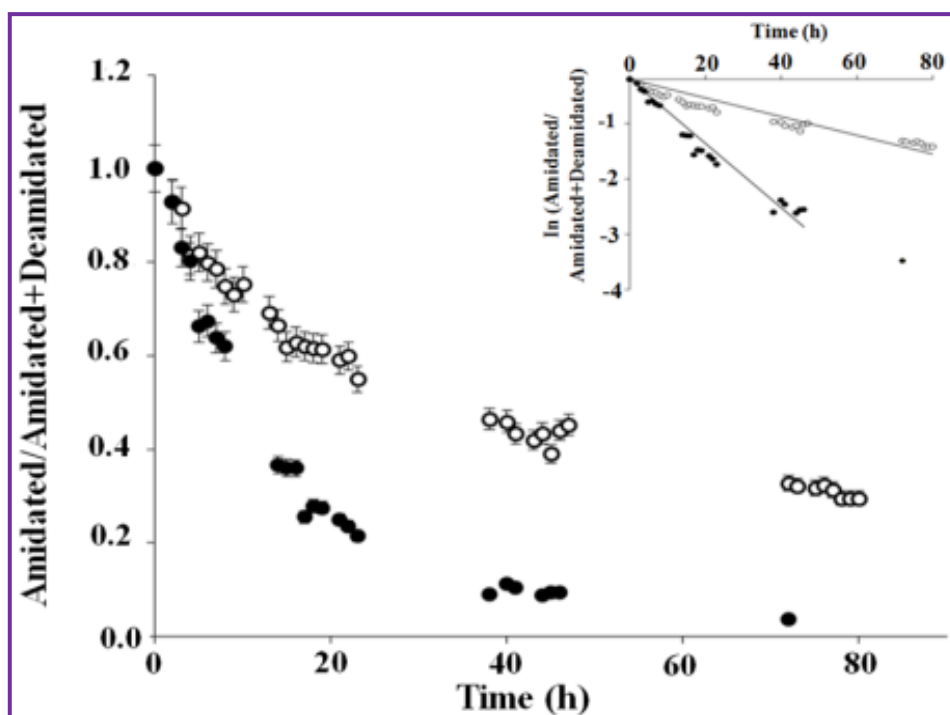
The mass difference was 0.991 Da between A<sub>2</sub> and A<sub>1</sub> and also between A<sub>3</sub> and A<sub>2</sub>, a value slightly higher than 0.984 Da. According to Robinson et al. (2009), a mass difference of 1.000 Da arises from the different nuclear binding energies of the amine group vs. the hydroxyl group. Thus, we provided evidence that A<sub>2</sub> contained one deamidated residue and A<sub>3</sub> two deamidated residues in comparison to the native isoform A<sub>1</sub>.



**Fig. 2.** FT-ICR-MS/MS spectra of the deamidated camel  $\alpha$ -lactalbumin chymotryptic peptides 9–26 (1972.89 Da; a) and 32–52 (2172.90 Da; b). The sequences of the peptides are displayed with the *b* and the *y* fragment ions observed on the spectra (in bold characters).

### 3.3. Kinetics analysis of deamidation as a function of pH

Kinetics of nonenzymatic deamidation of the camel  $\alpha$ -La was investigated by determining the relative proportion of native  $\alpha$ -La that underwent deamidation in course of time at pH 7.4 and at pH 8.4. The buffer system was properly chosen in order to promote the deamidation reaction. Indeed, the negatively-charged phosphate ions increase the reaction rate *ca.* three-fold as compared with the Tris molecules at 150 mM and pH 7.4 (Robinson & Robinson, 2001b). Whatever the pH, i.e. near-neutral or basic, the relative proportion decreased according to a logarithmic function of time of incubation (**Fig. 3**). The deamidation rate of  $\alpha$ -La was, however, significantly faster at pH 8.4 than at 7.4.



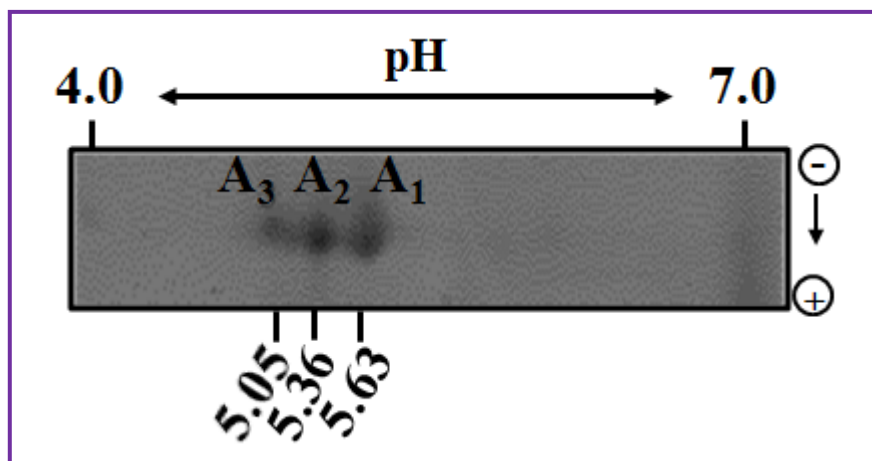
**Fig. 3.** Time-course analysis of deamidation of camel  $\alpha$ -lactalbumin incubated at 37 °C in 150 mM phosphate buffer either at pH 7.4 (empty circles) or pH 8.4 (filled circles). Each point was determined from triplicate peak area measurements  $\pm$  standard deviation.



### 3.4. Characterization of the $\alpha$ -lactalbumin isoforms by 2-DE

The purified  $\alpha$ -La isoforms A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub> were subjected individually to 2-DE analysis in a pH range of 4–7. Each isoform consisted in a single visible spot at apparent molecular mass of *ca.* 14 Da and no spot at *ca.* 16 kDa was observed (even when the whole whey proteins were analyzed by 2-DE; result not shown) suggesting that no *N*-glycosylated form of the camel  $\alpha$ -La was present in a detectable amount (the molecular mass of an *N*-glycan chain is *ca.* 2000 Da). Although no usual potential *N*-glycosylation site (Asn-Xxx-Ser/Thr) is present along the camel sequence, the occurrence of a glycan moiety was not, however, discarded. Indeed, the camel sequence contains the triplet Asn<sup>71</sup>-Ile-Cys<sup>73</sup> that is partly *N*-glycosylated (1%) in the case of the human  $\alpha$ -La (Giuffrida et al., 1997) and of the equine  $\alpha$ -La (the unglycosylated and *N*-glycosylated forms co-elute by anion-exchange FPLC; Girardet et al., 2004). But, in a former study, Ochirkhuyag et al. (1998) have not found any camel  $\alpha$ -La glycoform after glycoprotein specific staining.

The three 2-DE gels were then superposed for an optimal visualization of the shifts of the spots observed according to the degree of deamidation (**Fig. 4**).



**Fig. 4.** Superposition overview of the 2-DE profiles of the purified camel  $\alpha$ -lactalbumin isoforms A<sub>1</sub>, A<sub>2</sub>, and A<sub>3</sub>. The spots were stained with Coomassie blue. The different pHs are indicated on the Figure.

### 3. Conclusion

The present study has shown that the two variants A and B of  $\alpha$ -La reported in former studies corresponded in fact to the isoform spontaneously deamidated at Asn<sup>45</sup> at near-neutral pH and to the native protein with respect to the pHi values. Moreover, a third isoform was generated at basic pH and was the result of a second nonenzymatic deamidation at Asn<sup>16</sup>. The probable formation of isoAsp during the deamidation process was not studied in this work, but the chemical instability of the camel  $\alpha$ -La or of some of its peptides occurring in the gut during the postprandial phase might lead to formation and accumulation of isoAsp residues, the latter being able to decrease the biological activity and elicit auto-immunity (Aswad, Paranandi, & Schurter, 2000).

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