Two-dimensional cartography of equine β -casein variants achieved by isolation of phosphorylation isoforms and control of the deamidation phenomenon

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ABSTRACT

Because of variable degrees of phosphorylation, alternative splicing, and probable instability resulting from nonenzymatic deamidation, equine β -casein presents a complex pattern by 2-dimensional electrophoresis that needs clarification. β -Casein prepared from Haflinger mare's milk by hydrophobic interaction chromatography was fractionated by ion-exchange chromatography according to the degree of phosphorylation. Isoforms were identified by mass spectrometry; they corresponded to the full-length protein having 3 to 7 phosphate groups and to the splicing variant involving exon 5 and containing 4 to 7 phosphate groups. Investigations of nonenzymatic deamidation showed that β -casein did not deaminate spontaneously in stored milk and during the different steps of chromatography, but deamidation could occur when 2-dimensional electrophoresis was performed, increasing the β -casein pattern complexity. This phenomenon was strongly minimized when the first dimension step was carried out at 10°C instead of at room temperature. Finally, spot attribution on 2-dimensional pattern of β -casein was achieved by mixing each phosphorylation isoform in its native state with the whole β -casein fraction.

Key words: equine β -casein, deamidation, phosphorylation, two-dimensional cartography

INTRODUCTION

In milk, caseins are phosphorylated proteins forming colloidal micelles stabilized by phosphocalcium bridges (for review, see Ginger and Grigor, 1999). In the equine species, the components α_{S1} -CN, β -CN, and κ -CN have been already identified, whereas α_{S2} -CN remains to be characterized. Particularly, 3 isoforms of β -CN have been identified. The β -CN isoform 1 found in Haflinger mare's milk (Swiss-Prot accession number: Q9GKK3;

Girardet et al., 2006) corresponds to the protein produced from a full-length mRNA. Isoform 2 is produced from an mRNA lacking exon 5 (alternative splicing of the exon) that results in the absence of the 27 to 34 region (β -CN $^{\Delta 5}$). It was found in Welsh pony mare's milk by Miranda et al. (2004). Finally, in isoform 3, the region 50 to 181 encoded by the main part (75%) of exon 7 is deleted (Figure 1). This region is removed by using a cryptic splice site (Lenasi et al., 2006; Miclo et al., 2007). Isoform 3 [also called low molecular mass (M_r) β -CN; Miclo et al., 2007] has been characterized in Haflinger mare's milk. Moreover, Lenasi et al. (2006) highlighted the presence of another mRNA for which both exon 5 and exon 8 are alternatively spliced. The corresponding protein has not yet been found in equine milk.

The equine β -CN displays a variable degree of phosphorylation, from 3 to 7 phosphate (P) groups (3P to 7P; Girardet et al., 2006), unlike its bovine counterpart (Swiss-Prot accession number: P02666) that is fully phosphorylated on its 5 potential phosphorylation sites (Bandura et al., 2002). Similar to the equine protein but to a lesser extent, the human β -CN (Swiss-Prot accession number: P07498) presents a variable phosphorylation degree from 0P to 5P (Greenberg et al., 1984).

The Asn-135 residue of the equine β -CN undergoes a nonenzymatic deamidation process spontaneously occurring during incubation at 37°C and at slightly basic pH (Girardet et al., 2006). Deamidation of an Asn residue is one of the major routes of degradation of peptides and proteins. The mechanism of reaction is illustrated in Figure 2 (Xie and Schowen, 1999). The Asn-135 residue located within Asn-Gly sequence readily deamidates as demonstrated for several proteins containing the characteristic Asn-Gly sequence (particularly, the so-called o'clock molecules; Robinson and Robinson, 2004) submitted to physiological conditions (Robinson and Robinson, 2004). The bovine and human β -caseins do not, however, contain any Asn-Gly sequence.

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Figure 1. Exon modular structure of the equine β -casein determined by Miranda et al. (2004). The putative phosphorylation sites are indicated with an asterisk according to Miclo et al. (2007). The nonenzymatic deamidation site identified by Girardet et al. (2006) is double underlined. Other hypothetical deamidation sites, which would undergo peptide bond cleavage, are single underlined. The exons that can be alternatively spliced are framed with a dotted line. The splicing sites involved in the low molecular mass (M_r) β -casein generation are indicated by arrows. Exon 9, which would be only translated in the case of exon 8 splicing, is indicated in italics (translated according to cDNA structure determined by Lenasi et al., 2003).

Urea-PAGE and 2-dimensional electrophoresis (2-DE) profiles of pure equine β -CN are difficult to interpret because of the complexity of this protein, as there is the presence of phosphorylation variants, exon splicing variants, or both with a possibility of deamidation. The aim of this study was, therefore, to 1) elucidate the complexity of electrophoretic patterns of β -CN purified from Haflinger mare's milk by hydrophobic interaction chromatography (HIC) and 2) determine if nonenzymatic deamidation might take place in stored milk or during the successive purification steps, or did not occur as easily as expected. To carry out the present work, each β -CN phosphorylation variant was isolated by ion-exchange chromatography (IEC) before the deamidation phenomenon undergone by the individually separated isoforms was studied in vitro. Thus, the 2-DE cartography of β -CN has been achieved.

MATERIALS AND METHODS

Preparation of Equine Sodium Caseinate

Haflinger mare's milk was obtained from Jum'vital (Volmunster, France). The milk was stored at -20°C until used. Just after thawing, 0.05% (vol/vol) toluene was added to the milk to prevent microorganism development. Skimmed milk was obtained by centrifugation at $4,000 \times g$ at 4°C for 30 min and toluene was added again. Sodium caseinate was prepared by isoelectric precipitation at pH 4.2 as previously reported (Egito et al., 2002).

HIC

Sodium caseinate was separated by HIC using a TSK Phenyl-5PW column (21.5×150 mm; Interchim, Montluçon, France) connected to a fast protein liquid chromatography (FPLC) system (ÄKTA-FPLC, Amersham Pharmacia, Uppsala, Sweden) according to the method used by Tauzin et al. (2003) to fractionate bovine caseins. The column was equilibrated in 480 mM PBS buffer, pH 6.4, containing 2.5 M urea, at a flow rate of 6 mL/min. Sodium caseinate (10 mg/mL) was dissolved in the PBS buffer and filtered at $0.45 \mu\text{m}$, and a volume of 10 mL was loaded onto the column. A decreasing linear gradient from 480 to 37 mM PBS buffer was applied for 47 min followed by an isocratic step performed for 12 min in 37 mM PBS buffer, pH 6.4, containing 2.5 M urea. Absorbance was recorded at 280 nm. The collected fractions were dialyzed in distilled water for 48 h in the presence of thymol and then freeze-dried.

IEC

The whole β -CN obtained by HIC was fractionated by IEC using a TSK DEAE-5PW column (7.5×75 mm, Amersham Pharmacia) connected to an ÄKTA-FPLC system according to the method used by Aoki et al. (1992) to separate phosphorylation isoforms of human β -CN. Protein sample (15 mg/mL) was dissolved in the imidazole buffer and filtered at $0.45 \mu\text{m}$. The column was equilibrated in 20 mM imidazole buffer, pH

Figure 2. The mechanism of Asn deamidation reaction at neutral and basic pH (according to Xie and Schowen, 1999). The α -amino of the residue C-flanking to the Asn makes a nucleophilic attack on the Asn side chain to form a succinimide intermediate. The cyclic imide undergoes hydrolytic attack at both carbonyl centers, leading to the iso-Asp and Asp products in a ratio of 2:1 to 4:1.

8.0, containing 3.3 *M* urea and 0.08 *M* NaCl, at a flow-rate of 0.5 mL/min. The sample (volume of 1 mL) was injected onto the column. A linear gradient from 0.08 to 0.15 *M* NaCl was applied for 80 min followed by an isocratic step at 0.28 *M* NaCl for 15 min at a flow rate of 0.5 mL/min. Absorbance was recorded at 280 nm. The collected fractions were dialyzed and freeze-dried.

In Vitro Study of Nonenzymatic Deamidation

The IEC fractions corresponding to the 5P and 6P variants of β -CN were solubilized at 1 mg/mL in 150 mM PBS buffer, pH 8.4, containing 0.02% (wt/vol) sodium azide and 10% (vol/vol) of protease inhibitor cocktail (a commercial mixture containing 4-[2-aminoethyl]-benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin hydrochloride, N-[trans-epoxysuccinyl]-L-leucine-4-guanidinobutylamide, EDTA disodium salt, and leupeptin hemisulfate salt; the mixture, packaged in a 10-mL serum vial, was diluted in 100 mL of water before use; Sigma, St. Louis, MO). The β -CN 5P and β -CN 6P samples were filtered at 0.45 μ m and incubated at 37°C for 24 and 48 h to allow the nonenzymatic deamidation process. Then, each sample was loaded in a dialysis cell (FastDialyzed Uptima, Interchim) composed of a 1-mL sample chamber and a dialysis membrane with a 10-kDa cut-off. The dialysis occurred at 4°C for 1 h before

freeze-drying. The native and deamidated caseins were finally analyzed by urea-PAGE and 2-DE as described below.

Electrophoretic Methods

The urea-PAGE migration was performed with a vertical electrophoresis apparatus model 2001 (Amersham Pharmacia) according to Ng-Kwai-Hang and Kroeker (1984). Protein samples (2 mg/mL) were solubilized in 75 mM Tris-buffered saline (TBS) buffer, pH 8.9, containing 4 *M* urea, 5% (vol/vol) β -mercaptoethanol, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue. A sample volume of 20 μ L was loaded onto the gel. After migration, proteins were fixed by 12% (wt/vol) TCA and stained with Coomassie Brilliant Blue R-250.

The 2-DE separation, coupling isoelectric focusing (IEF) and SDS-PAGE, was adapted from the method of Herbert et al. (1988). For the first dimension (IEF), 50 μ g of protein sample was dissolved in 200 μ L of sample solution containing 8 *M* urea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 2 mM tributyl phosphine, 40 mM Tris, 0.2% (wt/vol) ampholytes 3–10 (Bio-Lytes 3–10; Bio-Rad, Hercules, CA), and 0.001% (wt/vol) bromophenol blue. The pH of the sample solution was 7.7. An immobilized

pH gradient gel (IPG) strip with a pH linear gradient range of 4 to 7 (IPG ReadyStrip pH 4–7, 11-cm length; Bio-Rad) was placed in the Protean IEF Cell (Bio-Rad) and was rehydrated with the protein solution for 15 h at 10°C under active conditions (constant voltage of 50 V). The IEF migration was then performed in 3 steps at 10°C. First, a low voltage (250 V for 15 min) was required to remove excess salts. Second, a voltage ramp from 250 to 8,000 V for 150 min was applied with a current limit of 50 μ A per IPG strip. Third, the voltage was kept constant at 8,000 V for 5 h. For the second dimension (SDS-PAGE), the IPG strip was equilibrated for 15 min in 125 mM TBS buffer, pH 6.8, containing 6 M urea, 2% (wt/vol) SDS, 20% (vol/vol) glycerol, and 5 mM tributyl phosphine. The IPG strip was then embedded on the top of an SDS-PAGE gel using molten agarose. The SDS-PAGE gel was composed of a 5% (wt/vol) polyacrylamide stacking gel containing 125 mM TBS buffer, pH 6.8, and 0.1% (wt/vol) SDS and of a 15% (wt/vol) polyacrylamide resolving gel containing 380 mM TBS buffer, pH 8.8, and 0.1% (wt/vol) SDS. Proteins were detected in gel by Coomassie Brilliant Blue R-250 staining after protein fixation by 12% (wt/vol) TCA.

Mass Spectrometry Analysis

The determination of the molecular mass of the equine β -CN isoforms was performed by liquid chromatography electrospray mass spectrometry (LC/ESI-MS) on a PE-Sciex API III⁺ triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) or on an ESI nanospray source coupled with a hybrid quadrupole time-of-flight (Q/TOF) mass spectrometer Q/Star XL (MDS Sciex, Toronto, Canada) as described previously (Girardet et al., 2006). Mass determination of proteins was done with a precision of 0.01 and 0.001% for API III⁺ triple quadrupole mass spectrometer and hybrid Q/TOF mass spectrometer Q/Star XL, respectively. The instrument mass-to-charge ratio scale for the API III⁺ was calibrated with the ions of the ammonium adduct of polypropylene glycol. The Q/Star XL was calibrated for ESI with a multi-point calibration using fragment ions that resulted from collision-induced decomposition of a peptide from bovine β -CN (β -CN 193–209). The API III⁺ worked online with a conventional HPLC on narrow-bore columns and collected peaks were infused onto the Q/Star XL with nano-ESI needle (Proxeon Biosystems, Odense, Denmark). All protein mass spectra were obtained from the signal averaging of multiple scans. The reproducibility determined in triplicate for measurements of molecular mass of an entire protein was ± 1 Da.

RESULTS AND DISCUSSION

Fractionation of β -CN Isoforms

Girardet et al. (2006) have putatively attributed 2-DE spots to the 3P to 7P phosphorylated isoforms of β -CN. Furthermore, β -CN could undergo a nonenzymatic deamidation process (Girardet et al., 2006). So, it might be possible that a given spot corresponded to a deamidated isoform, to a native isoform, or to a mixture of these 2 forms. Moreover, it was difficult to distinguish a native isoform from its deamidated counterpart only on the basis of the mass criterion (the mass difference between the 2 forms is only 1 Da). Then, the spot identification on the 2-DE profile of the whole β -CN would be difficult to carry out. In this context, separation of each phosphorylation isoform was crucial to evaluate the ability of the different β -CN isoforms to deamidate and to perform the 2-DE spot attribution.

Equine sodium caseinate was fractionated by HIC into 4 main fractions (designated F1 to F4) that were analyzed by urea-PAGE (Figure 3). Identification of the different CN components on the electrophoretic profile was performed according to our previous works (Egito et al., 2002; Miclo et al., 2007). The full-length β -CN containing the 2 regions encoded by exons 5 and 7 was recovered in F2, whereas the low- M_r β -CN, which lacks 75% of the region encoded by exon 7, was contained in F1. The heterogeneous α_{S1} -CN was split in 2 fractions, F3 and F4, whereas κ -CN coeluted with α_{S1} -CN in F4.

The β -CN recovered in F2 was then fractionated by IEC into 5 main subfractions (F2.1 to F2.5) that were analyzed by urea-PAGE (Figure 4). One single band per subfraction was visible after Coomassie Blue staining with ascending migration rate correlated with the chromatographic elution order. Each subfraction was submitted to LC/ESI-MS analysis for protein identification (Figure 5 and Table 1). A trace amount of β -CN 3P was found in F2.1. The β -CN 4P to 7P isoforms were recovered in F2.2, F2.3, F2.4, and F2.5, respectively, in agreement with the increase in phosphorylation level. Contamination by compounds of the previous fraction was observed for fractions F2.3, F2.4 and F2.5 (Figure 5), which is probably due to a relatively low chromatographic resolution.

Some minor proteins with characteristic molecular masses were observed on the mass spectrum of each IEC fraction. They were identified as β -CN lacking the region encoded by exon 5 (named β -CN $^{\Delta 5}$), because each β -CN $^{\Delta 5}$ phosphorylation isoform (4P to 7P) displayed a mass difference of 923 ± 1 Da (± 3 Da for the 7P component) with the full-length β -CN (Table 1). The β -CN $^{\Delta 5}$ variant with 3P was not detected by MS. The β -CN $^{\Delta 5}$ variants were present in very low amounts

Figure 3. Hydrophobic interaction chromatography of Haflinger mare's sodium caseinate. The sample (100 mg) was loaded onto a TSK Phenyl 5-PW column (Interchim, Montluçon, France). In the presence of 2.5 M urea, a linear gradient from 480 to 37 mM PBS buffer, pH 6.4, was applied for 47 min at a flow rate of 6 mL/min followed by an isocratic step at 37 mM sodium phosphate for 12 min. Composition of each collected fraction (F1 to F4) was checked by urea-PAGE (inset). For that, 40- μ g quantities of proteins were loaded in gel. Proteins were stained by Coomassie Brilliant Blue. The presence of κ -casein in gel was specifically detected using Schiff's reagent after periodic acid oxidation. eCN = equine sodium caseinate.

compared with the corresponding full-length variants. So, when β -CN 3P was present in trace amounts in Haflinger mare's milk, the β -CN $^{\Delta 5}$ 3P variant was undetectable. For a given phosphorylation degree, the full-length β -CN and the β -CN $^{\Delta 5}$ coeluted in the same fraction by IEC. From the urea-PAGE profile (Figure 4), each isoform of the full-length β -CN and of β -CN $^{\Delta 5}$ with the same phosphorylation degree seemed to comigrate in one common band. This was supported by the fact that the splicing of exon 5 undergoes the loss of 1 negative (Glu-27) and 1 positive (Lys-34) charge and does not change the global charge of the protein at pH 8.9.

The β -CN pre-mRNA is subjected to alternative splicing of exons. Indeed, 4 types of transcripts of β -CN of Lipizzan mare have been found that correspond to full-length β -CN, β -CN $^{\Delta 5}$, β -CN $^{\Delta 5,8}$, and to the β -CN that lacks most of the region encoded by exon 7 (Lenasi et al., 2006). Together with the full-length β -CN, the existence of the splicing variant β -CN $^{\Delta 5}$ is reported in Welsh pony mare's milk (Miranda et al., 2004), whereas only cDNA without exon 5 (but no cDNA corresponding to the full-length β -CN) has been characterized from

lactating mammary gland in Hannoverian mare (Lenasi et al., 2003). In Haflinger mare's milk, full-length β -CN (apparent molecular mass determined by SDS-PAGE of 32 kDa) and low- M_r β -CN (apparent molecular mass of 19 kDa) have been identified at the protein level in our previous works and the presence of β -CN $^{\Delta 5}$ (that may correspond to a band at 30 kDa) is suggested (Girardet et al., 2006; Miclo et al., 2007). Nevertheless, the β -CN $^{\Delta 5,8}$ proteic variant has never been found.

Study of Nonenzymatic Deamidation

In vitro experiments of nonenzymatic deamidation were carried out to determine electrophoretically if this phenomenon could readily occur in equine β -CN. Rates of deamidation of amide groups partly depend on experimental conditions such as pH, temperature, ionic strength, and buffer ions (Robinson, 2002). For example, a 150 mM PBS buffer increases deamidation rates 2- or 3-fold compared with a 150 mM TBS buffer at pH 7.4 and 37°C (Robinson and Robinson, 2001). In the present work, a first experiment of deamidation process has been undertaken on the major phospho-

Figure 4. Fractionation by ion-exchange chromatography of Haflinger mare's β -casein phosphorylation isoforms. The sample (F2, 15 mg) was loaded onto a TSK DEAE 5-PW column (Interchim, Montluçon, France). A linear gradient from 0.08 to 0.8 M NaCl in 20 mM imidazole buffer, pH 8.0, containing 3.3 M urea, was applied for 70 min at a flow rate of 0.5 mL/min. Composition of each collected subfraction (F2.1 to F2.5) was checked by urea-PAGE (in inset). For that, 40- μ g quantities of proteins were loaded onto gel. Proteins were stained by Coomassie Brilliant Blue. XP = number of phosphate groups.

rylation isoforms of β -CN, the 5P and 6P isoforms, solubilized in 150 mM PBS buffer at pH 7.4. After 48 h of incubation at 37°C, about half of the β -CN 5P and 6P was deamidated and proteolytic degradation was found (urea-PAGE profiles not shown). It is indeed reported that deamidation tends to open the structure of proteins, consequently making them more sensitive to proteolytic enzymes (Robinson and Robinson, 2004). A second experiment was thus carried out in the presence of high concentrations of a bacteriostatic agent and protease inhibitors and the pH was increased to 8.4 (because a more basic pH accelerates the deamidation of Asn residues; Robinson and Robinson, 2004). Approximately half of the 5P and 6P isoforms were deamidated after 24 h of incubation at 37°C and the greatest part after 48 h of incubation (Figure 6). During the course of the deamidation process, one additional band appeared on urea-PAGE profile of F2.3 and F2.4 and displayed a migration rate faster than the original band corresponding to 5P variant for F2.3 and 6P variant for F2.4. These 2 newly generated bands seemed to correspond to the deamidated (d) counterparts, named 5P/d and 6P/d, as they display one supplementary negative charge per molecule (Girardet et al., 2006), which could cause the faster migration in gel. In addition,

these new bands were not confused with bands of other native isoforms. Indeed, β -CN 5P/d migrated in a distinct band located between those of β -CN 5P and β -CN 6P, and β -CN 6P/d was between β -CN 6P and β -CN 7P (Figure 6). The fact that the bands of the 5P/d and 6P/d isoforms were absent originally showed that the deamidation process did not occur in stored milk or during the different chromatography and dialysis steps. On the other hand, proteolysis occurred to a slight extent and was detectable in gel after 48 h of incubation by Coomassie Blue staining (not shown on Figure 6) despite the use of a bacteriostatic agent and protease inhibitors.

2-DE Cartography of β -CN

To achieve 2-DE cartography, the β -CN purified by HIC and the F2.2 to F2.5 subfractions were characterized by 2-DE. When the IPG strip rehydration was performed overnight at slightly basic pH of 7.7 and at room temperature (approximately 22°C) before IEF migration (also performed at 22°C), it was observed (data not shown) that the 2-DE profile of each IEC subfraction presented 2 distinct spots per apparent molecular mass (30 and 32 kDa). These spots might be

Figure 5. Reconstructed masses from liquid chromatography electrospray ionization mass spectrometry of Haflinger's mare β -casein isoforms contained in subfractions F2.2 to F2.5. β -CN = full-length β -casein; Δ^X = deletion of the region encoded by exon X; XP = number of phosphate groups; cps = count per second.

the result of deamidation phenomenon (appearance of one negative charge that shifted the original spots) that seemed to appear during the electrophoretic process, as no deamidation was detected by urea-PAGE after IEC. The only parameter that could be changed to try to minimize this deamidation phenomenon without modifying the suitable composition of the sample solution

was the temperature. So, the rehydration (at pH 7.7) and the IEF migration (between pH 4.0 and 7.0) were performed at 10°C instead of 22°C for each chromatographic fraction. Thus, for each apparent mass of 30 and 32 kDa, one main spot was detected by Coomassie Blue staining on the F2.2 to F2.5 profiles (Figure 7a). Each main spot at 32 and 30 kDa corresponded to full-length

β-CN and to β-CNΔ5, respectively, with these isoforms being identified by LC/ESI-MS analysis (Figure 5).

A 2-DE analysis of the main IEC subfractions, F2.3 and F2.4, was performed after deamidation of β-CN 5P and β-CN 6P by a 48-h incubation at 37°C and at pH 8.4 (Figure 7b). For the 2 fractions analyzed after deamidation (designated F2.3d and F2.4d), 2 spots were visible at each apparent molecular mass of 32 and 30 kDa, whereas 1 spot was visible initially. The additional spots were more acidic than the initial spots and seemed to correspond to deamidated forms displaying an additional negative charge. Thus, this in vitro experiment showed that IEF performed at room temperature could cause a nonenzymatic deamidation process. Deamidation had to be avoided during the 2-DE analysis, as confusion between native and eventual deamidated forms could be possible on the 2-DE profile of the whole β-CN. Indeed, the 5P/d and 6P isoforms corresponded to a same spot by superposition of the F2.3d and F2.4 profiles, and the spots of the 6P/d and 7P isoforms of the F2.4d and F2.5 profiles were also confused (Figure 7). So, the IEF migration had to be performed at 10°C to avoid the deamidation reaction during the 2-DE analysis. The slight proteolysis occurring during the in vitro deamidation experiment and observed by urea-PAGE also led to the presence of minor spots visible on the 2-DE profiles of F2.3d and F2.4d (Figure 7b). These spots displayed low apparent Mr distributed between 17 and 28 kDa and might correspond to autolysis of the 5P and 6P isoforms. It is reported that the deamidation reaction of an Asn residue can generate cleavage of the adjacent peptide bond especially in the Asn-Pro sequence (Robinson and Robinson, 2004). The cleavage of the Asn-Pro bond is known to occur at acid pH (Landon, 1977) as well as

Figure 6. Urea-PAGE monitoring of deamidation process of equine β-casein 5P and 6P isoforms isolated in the F2.3 and F2.4 subfractions, respectively. The reaction was carried out by incubation at 37°C in 150 mM PBS buffer, pH 8.4, containing 0.02% (wt/vol) sodium azide and 10% (vol/vol) protease inhibitor cocktail for 24 and 48 h. Fraction F2.5 containing native β-casein 7P was loaded onto the gel for migration comparison. P = phosphate group; XP = number of phosphate groups; d = deamidated; 0, 24, and 48 = time of incubation (h).

at physiological pH (Geiger and Clarke, 1987; Tarelli and Corran, 2003). Cleavage of other peptide bonds can occur in PBS buffer at near-neutral pH, but to a lesser extent, and mainly concerns Asn-Leu sequence (Geiger and Clarke, 1987; Tyler-Cross and Schirch, 1991). The equine β-CN contains 2 Asn-Pro sequences at Asn132-Pro133 and Asn222-Pro223 and 1 Asn-Leu sequence (Asn138-Leu139) that might be cleaved during the deamidation process (Figure 1). Thus, the degradation products observed by 2-DE might be generated by the deamidation process and peptide bond cleavage occurring on Asn-Pro or Asn-Leu or on both. Bovine β-CN, which does not contain any Asn-Gly or Asn-Pro sequences and contains only one Asn-Leu sequence, seems to be highly stable. Indeed, after 96 h of incubation at pH 7.4 and 37°C, the assay of release of ammonia shows that less than 2% of bovine β-CN is deamidated compared with 80% for the equine β-CN (Girardet et al., 2006).

Table 1. Molecular masses (Mr) of the different ion-exchange chromatography subfractions of equine β-casein determined by liquid chromatography electrospray ionization mass spectrometry

Subfraction	Observed Mr ¹	Identification ²	Theoretical Mr ³
F2.1	25,832	β-CN 4P	25,831
	25,751	β-CN 3P	25,751
	24,908	β-CNΔ5 4P	24,908
F2.2	25,831	β-CN 4P	25,831
	24,908	β-CNΔ5 4P	24,908
F2.3	25,911	β-CN 5P	25,911
	25,832	β-CN 4P	25,831
	24,988	β-CNΔ5 5P	24,988
	25,991	β-CN 6P	25,991
F2.4	25,912	β-CN 5P	25,911
	25,069	β-CNΔ5 6P	25,068
	26,072	β-CN 7P	26,071
F2.5	25,991	β-CN 6P	25,991
	25,151	β-CNΔ5 7P	25,148

1Mr ± 1 (±3 with β-CNΔ5 7P).
2ΔX = deletion of the region encoded by exon X; P = phosphate group.
3Average mass calculated with ProtParam tool (expasy.org/tools/protparam.html).

Figure 7. Two-dimensional electrophoresis (a) of the whole equine β -casein recovered in the F2 fraction by hydrophobic interaction chromatography and of its F2.2 to F2.5 ion-exchange chromatography subfractions, (b) of deamidated F2.3 and F2.4 (named F2.3d and F2.4d) subfractions. See text for deamidation reaction conditions. Quantities of 50 μ g of protein were loaded in the immobilized pH gradient strips. Proteins were stained by Coomassie Brilliant Blue. XP = number of phosphate groups; Δ^x = deletion of the region encoded by exon X; d = deamidated; M = molecular mass standards. The small arrow indicates the spot of β -CN 5P on each 2-dimensional profile by superposing the profile of F2.3. The dashed arrows show that the 6P and 5P/d spots and the 7P and 6P/d spots have the same positions on the 2-dimensional gel.

Figure 8. Two-dimensional electrophoresis of each of the F2.2 to F2.5 ion exchange chromatography subfractions mixed in a 1:1 mass ratio with the whole β -casein recovered in the F2 fraction by hydrophobic interaction chromatography. Quantities of 50 μ g of total protein were loaded in immobilized pH gradient strips. Proteins were stained by Coomassie Brilliant Blue. XP = number of phosphate groups, Δ X = deletion of the region encoded by exon X. Putative identification of the 3P variants is shown in italics.

To precisely attribute one spot to one phosphorylation isoform, each IEC subfraction was mixed with the F2 HIC fraction containing all the isoforms of β -CN in a 1:1 mass ratio. By comparison with the 2-DE profile of F2 alone, the spot displaying an increasing intensity was attributed to a suitable phosphorylation isoform previously identified by LC/ESI-MS analysis (Figure 8). Thus, as expected, the spot attribution showed that more the isoform is phosphorylated, more acidic it is. So, the less acidic spot at 32 kDa on the F2 profile might correspond to the full length β -CN 3P previously identified by LC/ESI-MS (Figure 8). In the same manner, the β -CN Δ 5 3P might correspond to the minor spot at 30 kDa, having the less acid isoelectric point although no β -CN Δ 5 3P was found by LC/ESI-MS analysis.

CONCLUSIONS

The individualization of each phosphorylation isoform of equine β -CN by IEC was required to establish with precision the 2-DE cartography of the various phosphorylation and splicing variants, taking into account the potential of the equine β -CN to undergo nonenzymatic deamidation. The LC/ESI-MS analysis of the

individual isoforms revealed the presence of β -CN Δ 5 4P to 7P in Haflinger mare's milk, and the 2-DE analysis suggested the presence of β -CN Δ 5 3P. In the present work, nonenzymatic deamidation did not occur in stored milk or during the different steps involved in the preparation of β -CN samples. Nevertheless, the occurrence of a probable nonenzymatic deamidation reaction was observed during the 2-DE process performed at room temperature. This reaction was avoided by decreasing the temperature to 10°C. Finally, the location of each phosphorylated isoform on a 2-DE profile was determined with precision by a method consisting of intensity enhancement of each spot by enrichment of the whole β -CN with each of the purified native isoforms. Investigations are now in progress to locate the phosphorylated residues of each phosphorylation variant.

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