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Proteomic Comparison of Equine and Bovine Milks on Renneting

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ABSTRACT: Rennet-induced coagulation of bovine milk is a complex mechanism in which chymosin specifically hydrolyzes κ-casein, the protein responsible for the stability of the casein micelle. In equine milk, this mechanism is still unclear, and the protein targets of chymosin are unknown. To reveal the proteins involved, the rennetability of equine milk by calf chymosin was examined using gel-free and gel-based proteomic analysis and compared to bovine milk. RP-HPLC analysis of bovine and equine milks showed the release of several peptides following chymosin incubation. The hydrolyses of equine and bovine casein by chymosin were different, and the major peptides produced from equine milk were identified by mass spectrometry as fragments of β-casein. Using two-dimensional electrophoresis, equine β-casein was confirmed as the main target of calf chymosin over 24 h at 30 °C and pH 6.5. The gel-based analysis of equine milk discriminated between the different individual proteins and provided information on the range of isoforms of each protein as a result of post-translational modifications, as well as positively identified for the first time several isoforms of κ-casein. In comparison to bovine milk, κ-casein isoforms in equine milk were not involved in chymosin-induced coagulation. The intensity of equine β-casein spots decreased following chymosin addition, but at a slower rate than bovine κ-casein.

KEYWORDS: *equine milk, rennet coagulation, κ-casein, proteomic, bovine milk*

INTRODUCTION

The milk from all species studied to date contains a heterogeneous mixture of proteins within which a few primary proteins dominate. In bovine milk these proteins are α_{s1}-, α_{s2}-, β-, and κ-caseins, β-lactoglobulin, and α-lactalbumin with relative proportions of ∼30:10:30:12:10:4, respectively.[1] Proportions of these proteins vary greatly among species but, apart from whey acidic protein (WAP), which has been identified in several species[2] but not in ruminant or human milk,[3] no major protein, other than one of these families, has yet been found in the milk of any species. The protein content of mature equine milk is lower than that of bovine milk, but the principal classes of proteins, that is, caseins and whey proteins, are similar in both milks.[4−7]

The milk proteome is extremely complex due to post-translational modifications of proteins and the presence of many genetic variants.[8−10] All milk proteins exhibit genetic polymorphism, usually due to the substitution of one or two amino acids, which do not have significant effects on protein functionality. Microheterogeneity of milk proteins also results from post-translational phosphorylation, glycosylation, or proteolysis.

Whereas the principal proteins of equine milk have been fairly well characterized, the presence of κ-casein in equine milk has been an issue of debate for several years, with several authors[11−14] reporting its absence. However, other studies[15−19] reported its presence, albeit at a low concentration, and Lenasi et al.[20] presented the entire cDNA sequence for equine κ-casein.

In bovine milk, κ-casein is located predominately on the surface of casein micelles and plays an important role in the formation, stabilization, and aggregation of the micelles and alters the manufacturing properties and digestibility of milk. The presence of a glycan moiety in the C-terminal region of κ-casein enhances its ability to stabilize the micelle, by electrostatic repulsion, and may increase the resistance by the protein to proteolytic enzymes and high temperatures.[21,22] It has been reported[13] that equine κ-casein is more highly glycosylated than bovine κ-casein, which could significantly contribute to equine micelle stability. Ochirkhuyag et al.[13] and Doreau and Martin-Rosset[23] concluded that the steric stabilization of equine casein micelles by κ-casein may be aided by unphosphorylated β-casein on the surface of the micelle, thus compensating for the low κ-casein content.

Chymosin (EC 3.4.23.4) is a neonatal gastric aspartal proteinase that hydrolyxes the κ-casein of bovine milk into a soluble glycopeptide [caseinomacropeptide (CMP): amino acid residues 106−169] and an insoluble part (para-κ-casein: amino acid residues 1−105), which is crucial for the production of cheese and for the nutrition of newborns.[24,25] The non-enzymatic secondary stage of the coagulation of milk by chymosin involves the aggregation and gelation of para-κ-casein under the influence of Ca^{2+}.[26] Apart from cleavage of the Phe_{105}−Met_{106} bond of κ-casein, chymosin causes limited hydrolysis of κ-casein.[25] Calf chymosin hydrolyzes the Phe_{97}−Ile_{98} bond of equine κ-casein[19] and slowly hydrolyzes the Phe_{105}−Ile_{106} bond of human κ-casein.[27] Although the CMPs released from equine and human κ-casein are less hydrophilic than bovine CMP, in all species CMP is believed to inhibit

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gastric acid secretion following milk intake[28−30] and to have antibacterial activity.[31,32] The sequence 97−116 of κ-casein is highly conserved across species, suggesting that the limited proteolysis of κ-casein and subsequent coagulation of milk are of major biological significance.[28] The other proteins in bovine milk, α_{s1}-, α_{s2}-, and β-caseins and α-lactalbumin, are hydrolyzed by chymosin but at a much slower rate than κ-casein.[33,34]

Over the past decade, milk from nonbovine mammals (goat, donkey, horse, and camel) have been studied, generally as a means of identifying the best substitute for human milk in infant nutrition.[35−37] Milk proteins have been analyzed extensively using proteomic techniques.[38] Two-dimensional electrophoresis (2-DE) allows simultaneous detection and quantification of several thousand protein spots in the same gel[38−40] when combined with mass spectrometry analysis, and it has been used for the separation and characterization of the proteins in bovine milk or colostrum[41,42] or for identification of specific proteins and isoforms thereof.[43−45] To date, analysis of the effects of various processing treatments on bovine milk proteins using proteomics has been confined to exploring the changes in disulfide bonds in milk proteins after heating or pressure treatment.[45−48] Caseins in human milk have been resolved using 2-DE,[49] and a proteomic comparison between preterm and term human milk has been reported.[50] Recently, Hinz et al.[14] compared the milk of several species, including equine milk, using proteomics. The proteins of equine colostrum and milk have been analyzed by electrophoretic and immunological methods,[39] whereas Egito et al.[13] characterized the casein fraction of equine milk using 1- DE and 2-DE of samples purified by anion exchange chromatography and reverse phase high-performance liquid chromatography (RP-HPLC). Various mass spectrometric approaches have been used to characterize the proteins of asinine[51−56] and equine[17,57,58] milks.

The aim of this study was to examine the rennetability of equine milk by calf chymosin using RP-HPLC and 2-DE followed by mass spectrometry with comparative analysis of a renneted bovine milk sample. The presence and implication of equine κ-casein in equine milk coagulation were also investigated.

MATERIALS AND METHODS

Milk Supply. Equine milk was obtained from Orchid's Paardenmelkerij (Zeeland, The Netherlands) from a bulk supply collected from five milkings over 24 h, from a herd of multiparous New Forest and New Forest/Arabian mares (n > 100) in midlactation, physically separated by day from their foals. The milk was defatted by centrifugation at 1000g using a Sorvall RC 5B centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 20 °C for 20 min, followed by filtration through glass wool to remove fat particles. Raw whole bovine milk was obtained from a local dairy farm and was defatted by centrifugation at 2000g for 20 min at 20 °C, followed by filtration through glass wool. Sodium azide (0.5 g L^{-1}) was added to the skimmed milks to prevent microbial growth. All chemicals used were of reagent grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Renneting Equine and Bovine Milks. Skimmed equine and bovine milks were adjusted to pH 6.5 and tempered at 30 °C for 20 min in thin-walled glass tubes in a thermostatically controlled water bath. A 1:10 (v/v) aqueous dilution of fermentation-produced chymosin (10 μL mL^{-1}; Maxiren 180; 180 international milk-clotting units (IMCU) mL^{-1}; DSM Food Specialties, Delft, The Netherlands) was added. Aliquots (1 mL) of renneted bovine milk were removed after 4, 10, 15, 20, 40, 60, 90, and 240 min and from renneted equine milk after 20, 30, 60, 90, 120, and 240 min and at 24 h. Renneted milk samples were diluted (1:1) immediately with 4% trichloroacetic acid (TCA) in Eppendorf tubes, vortexed for 1 min, and then held at 20 °C for 30 min. The samples were centrifuged at 5000g for 10 min in a microcentrifuge (Sigma 1-15, Sigma Laborzentrifuge, Osterode am Harz, Germany). Supernatants were removed carefully and diluted 1:1 with buffer (0.1 M bis-Tris, pH 7.0, 8 M urea, 45 mM citrate) to a final pH of 3.0.[59] Samples were filtered through 0.45 μm filters (Millex-HV, PVDF, 13 mm; Millipore Corp., Billerica, MA, USA) prior to injection onto the RP-HPLC column.

RP-HPLC Analysis of Renneted Equine and Bovine Milk. RP-HPLC was preformed according to the method of Vasbinder et al.[59] for RP-HPLC analysis of CMP. Analysis was carried out using a Waters 626 nonmetallic HPLC system (Waters Corp., Milford, MA, USA) consisting of a Waters 486 UV−vis detector, a Waters 717$^+$ autosampler, a Waters 626 pump with a 600S controller, and an online Degasys DG-2410 degassing unit (Sanwa Tsusho Co., Tokyo, Japan). A Varian Pursuit XRs C18 RP-HPLC column [250 × 4.6 mm, 5 μm particles size, 300 Å pore size (Varian Inc., Lake Forest, CA, USA)] was used. A wash step of 15 min at 70% solvent B followed by a 15 min equilibration with 85% solvent A was included between analyses of samples.

Identification of κ-Casein in Equine Milk. To determine if equine milk contained κ-casein, skimmed equine milk was analyzed by RP-HPLC according to the method of Miranda et al.[48] Prior to analysis, the milk was mixed 1:2 (v/v) with 0.1 M bis-Tris buffer, pH 8.0, containing 8 M urea, 1.3% trisodium citrate, and 0.3% DTT. Chromatographic equipment was as described above, and a Microsorb C4 RP-HPLC column (250 × 4.6 mm, 300 Å pore size, 5 μm particle size) from Varian maintained at 40 °C was used. A 20 min wash step with 60% solvent B was included after protein separation followed by 10 min at 100% solvent A before injection of subsequent samples. A fraction was collected manually in the κ-casein region identified by Miranda et al.,[48] precipitated using TCA/acetone and dephosphorylated as follows: 10 mL of sample was cooled on ice, 750 μL of 100% TCA was added, and the mixture was held on ice for 2 h. The sample was centrifuged at 10000g for 10 min at 4 °C. The supernatant was removed, and 2 mL of acetone (precooled on ice) was added to the pellet. The sample was vortexed for 2 min, followed by centrifugation at 10000g (10 min at 4 °C). The supernatant was removed, and the pellet was washed again with 2 mL of ice-cold acetone; this step was repeated once. Finally, the pellet was dissolved in 45 μL of distilled water, and 2 μL of 20% SDS was added. Prior to electrophoresis, two samples (15 μL each) were prepared as follows: For sample 1, 15 μL was added to 15 μL of a 2× electrophoresis loading buffer. Sample 2 was dephosphorylated using the lambda protein phosphatase (lambda PP) and buffer system from New England Biolabs (Hitchin, Herts., UK); 2 μL of $MnCl_2$, 2 μL of PPase buffer, and 2 μL of phosphatase were added to 15 μL of sample, and the mixture was incubated overnight at 30 °C; 20 μL of single-strength electrophoresis buffer was added to 20 μL of the dephosphorylated sample solution. Samples were denatured in denaturing NuPAGE sample buffer[60] and separated with a Bis-Tris−MOBS buffer system (Invitrogen, Paisley, UK). The selected bands were then

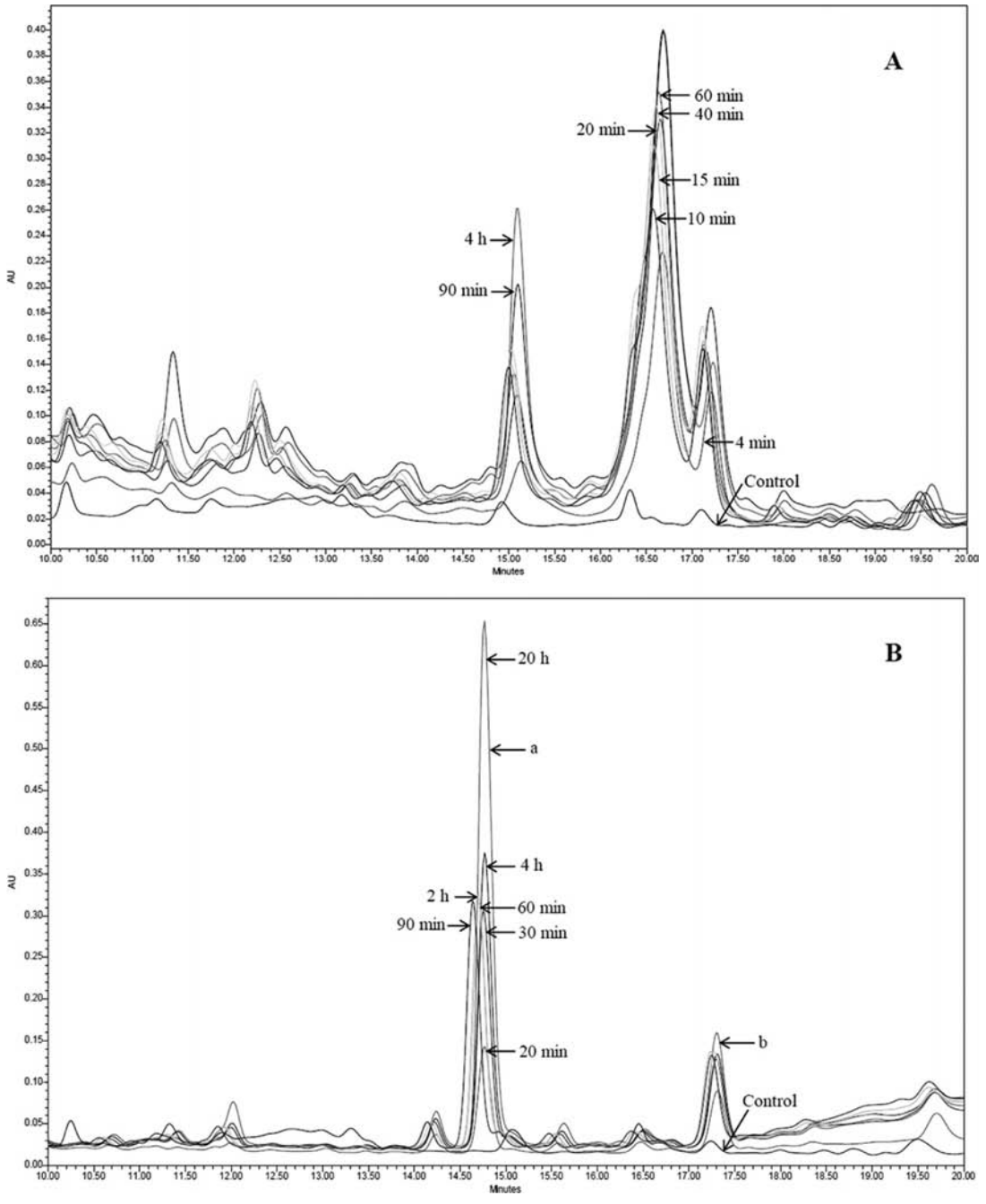

Figure 1. C18 RP-HPLC chromatograms of bovine (A) and equine (B) milks renneted with 10 μL mL^{-1} of 1:10 (v/v) Maxiren 180 at pH 6.5 and 30 °C. Fractions a and b were collected for MALDI-TOF MS/MS analysis.

in-gel digested with trypsin and subjected to nano-LC-MS/MS analysis as described below.

Two-Dimensional Electrophoresis Analysis of Renneted Equine and Bovine Milk. Samples of renneted equine milk were taken at 1, 2, 4, 8, and 24 h. Aliquots of renneted bovine milk were removed at 2 min intervals up to 16 min and at 20, 24, and 60 min. Renneted milk samples were diluted (1:10) immediately with solubilization buffer [9 M urea, 40 g L^{-1} 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 g L^{-1} Triton X100, and 65 mM dithiothreitol (DTT)] according to the method of Chevalier et al.[49] The protein content of 100 μL of each sample was determined by the Bio-Rad Protein Assay (No. 500-0002, Bio-Rad Laboratories, Hercules, CA, USA), which is based on the method of Bradford[61] and uses bovine serum albumin (BSA) as standard. Analysis was carried out in at least duplicate at each time point for both equine and bovine milks. Analytical 2-DE was carried out on renneted equine and bovine milks with 100 μg of protein using 7 cm immobilized pH gradient (IPG) strips (ReadyStrip, Bio-Rad, Hercules, CA, USA) with a linear pH gradient from 3 to 10 (Bio-Rad), and preparative 2-DE was carried out on equine and bovine milks with 300 μg of protein using 17 cm IPG strips and a linear pH gradient from 3 to 10 (Bio-Rad). The 7 or 17 cm IPG strips were rehydrated in the protein solubilization buffer solution. Isoelectric focusing was carried out according to the method of Armaforte et al.[38] using a Protean IEF Cell isoelectric focusing system (Bio-Rad). The IPG strips were then embedded using 6 g L^{-1} low melting point

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Table 1. Mass Spectrometry Results for Fractions a and b from C18 RP-HPLC Analysis of Equine Milk Renneted with Maxiren 180 at pH 6.5 and 30 °C; Analysis Carried out Using MALDI-TOF MS/MS

equine protein	obsd MW (Da)	theor MW (Da)	score	sequence[a]	amino acids[b]
			Fraction a		
β-casein	2336.289	2336.274	141	L-GPTGELDPATQPIVAVHNPVI-V	203−225
β-casein	1593.759	1593.867	48	V-APFPQPVVPYPQ-R	175−188
			Fraction b		
β-lg I	1677.872	1677.941	61	L-RPTPEDNLEIIL-R	45−58

[a]Cleavage sites indicated by hyphens. [b]Peptides identified by mass spectrometry include the signal peptide (amino acids 1−15 for β-casein and 1−18 for β-lg).

agarose on top of a 12.5% acrylamide gel, and sodium dodecyl sulfate−gel electrophoresis (SDS-PAGE) was carried out using a Criterion Dodeca Cell electrophoresis unit (Bio-Rad) for the 7 cm strips or a Protean II xi Cell electrophoresis unit (Bio-Rad) for the 17 cm strips. Gels were stained using colloidal Coomassie blue as described by Chevalier et al.[62] and stained gels digitized at 300 dpi using a GS-800 densitometer (Bio-Rad).

Image Analysis of 2-DE Gels. 2-DE gels were analyzed using Progenesis SameSpots V. 4.0 image analysis software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). The same gel area was selected from duplicate gels of each time point after rennet addition. Gel areas were compared, and the protein spot volumes were quantified as a mean of each replicate; spot quantities of all gels were normalized to remove non-expression-related variations in spot intensity as previously described.[63] To normalize the raw protein spot volume, each spot was expressed relative to the total volume of all spots on that image. Statistical analysis separated proteins that significantly increased or decreased ($p < 0.05$) after the treatments.[64]

In-Gel Digestion. Protein spots were selected for further analysis from preparative 2-DE and excised using a ∼2 mm diameter punch; gel pieces were transferred to 1.5 mL Eppendorf tubes. The gel spots were washed sequentially with Milli-Q water (Millipore Corp., Billerica, MA, USA), 25 mM ammonium bicarbonate, acetonitrile/25 mM ammonium bicarbonate (1:1, v/v), and acetonitrile. Gel fragments were dried using a Speed-Vac (GMI Inc., Ramsey, MN, USA), and the proteins were digested by the addition of sequencing grade trypsin (Promega, Charbonnieres, France) at 12.5 μg μL^{-1} in 25 mM ammonium carbonate, which was added on ice. After 15 min of gel rehydration, the proteins were digested at 37 °C for 2 h. Digested protein fragments were identified either by MALDI-TOF or by nano-LC MS/MS.

Protein Identification by MALDI-TOF Mass Spectrometry. Digested protein fragments were extracted by the addition of 20 μL of 0.1% TFA and sonicated for 15 min. The supernatants were transferred to 500 μL polypropylene microcentrifuge tubes. Peptide extraction was carried out by the addition of 20 μL of a 3:2 (v/v) acetonitrile/TFA solution and sonicated for 15 min. Supernatants were concentrated using a Speed-Vac to a final volume of 10−20 μL. Peptides were simultaneously desalted and concentrated using C18 Zip-Tip microcolumns, and supernatants were spotted directly on an AnchorChip MALDI target with α-cyano-4-hydroxycinnamic acid matrix solution and allowed to cocrystallize. Crystallized samples were washed with 0.1% TFA in water and recrystallized from 6:3:1 (v/v/v) ethanol/acetone/0.1% TFA in water. Peptide mass fingerprinting (PMF) was done using an UltraFlex MALDI-TOF/TOF mass spectrophotometer (Bruker, Bremen, Germany). The following parameters were used for the database search: taxonomy restricted to mammalia, one missed cleavage allowed, carbamidomethylation of cysteines as fixed modification, a mass tolerance of 30 ppm for the parent ion, and a mass tolerance of 0.6 Da for fragment ions. A minimum of five peptides matching the protein was used to validate the PMF identification, and peptide was validated if the p value was <0.05.

Protein Identification by Nano-LC Mass Spectrometry. When low-intensity protein spots could not be identified by MALDI-TOF mass spectrometry, nano-LC MS was used. Protein digests were extracted using formic acid and peptides were analyzed using an ion trap mass spectrometer (Esquire HCT plus; Bruker, Billerica, MA, USA) coupled to a nanochromatography system (HPLC 1200, Agilent, Santa Clara, CA, USA) interfaced with an HPLC-Chip system (Chip Cube, Agilent). Samples were first loaded onto the 4 mm enrichment cartridge at a flow rate of 4 μL min^{-1} using 0.1% formic acid. After preconcentration, peptides were separated on the column (75 μm diameter, 43 mm length) at a flow rate of 0.3 μL min^{-1} using a 15 min linear gradient from 3 to 80% acetonitrile in 0.1% formic acid and eluted into the mass spectrometer. The tandem mass spectrometer was an Esquire HCT+ (Bruker Daltonik GmBH, Bremen, Germany) operating in positive ion mode. The automated data-dependent acquisition parameters were chosen such that only doubly and triply charged precursor ions were selected for CID, excluding singly charged ions. MS/MS raw data were analyzed using Data Analysis software (Bruker Daltonik GmbH) to generate the peak lists. MASCOT search engine software (Matrix Science, London, UK) was used to search the Swissprot database (release 20100121). The following parameters were used for the database search: mass tolerance of 0.6 Da for parent and fragment ions, one missed cleavage allowed, carbamidomethylation of cysteines as fixed modification, peptide charge fixed at 2+ and 3+, and taxonomy restricted to mammalia. A protein was validated once it showed at least one peptide with $p < 0.05$.

RESULTS AND DISCUSSION

RP-HPLC of Renneted Equine and Bovine Milk. Chromatograms of 2% TCA extracts of equine and bovine milks renneted using Maxiren 180 and analyzed using C18 RP-HPLC are shown in Figure 1. Compared to a control bovine milk, renneted bovine milk contained peptides eluting at ∼15 and ∼16−17.5 min, which increased significantly in height about 4 min after rennet addition (Figure 1A) and continued to increase up to 240 min. Renneted equine milk (Figure 1B) had a different RP-HPLC profile from that of bovine milk, and no peptides appeared until ∼20 min after rennet addition. The time after rennet addition was therefore adjusted for equine milk, and samples for RP-HPLC were taken for up to 20 h.

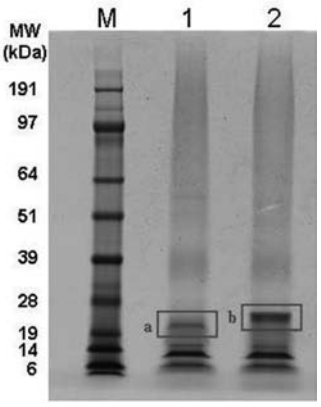

Figure 2. Electrophoretogram of fraction collected from C4 RP-HPLC analysis of equine milk. Lanes: M, molecular weight marker; 1, untreated sample; 2, sample treated with phosphatase. Bands a and b were excised for identification by nano-LC-MS/MS.

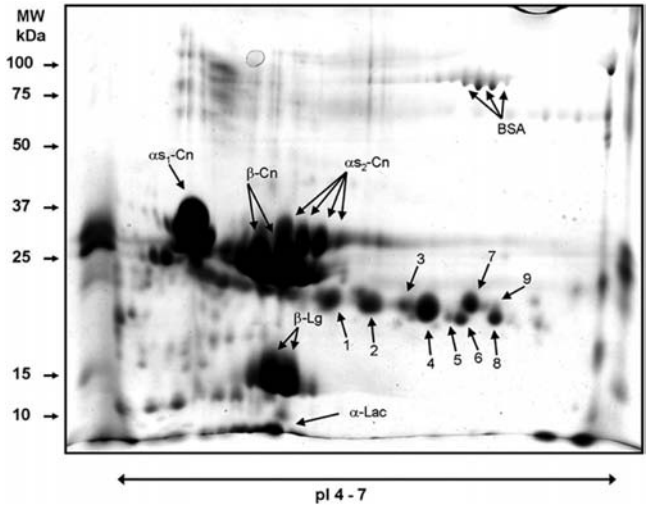

Figure 4. 2-DE preparative gel of a control bovine milk sample (~300 μg of proteins) under reducing conditions using pH 4–7 p*I* range for the first dimension and a 12% acrylamide gel for the second dimension. The numbered spots were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprint and/or nano-LC MS/MS (results in Table 2). Proteins identified in figure from Chevalier and Kelly.[45]

Egito et al.[19] reported that calf chymosin readily cleaves equine β-casein at Leu_{190}–Tyr_{191}, and the fragments produced were resistant to hydrolysis even after 24 h of incubation with chymosin. Egito et al.[19] fractionated whole equine casein using affinity chromatography on agarose wheat germ agglutinin (WGA) and demonstrated the relatively slow hydrolysis of equine κ-casein (WGA-bound fraction) by calf chymosin at the bond Phe_{97}–Ile_{98}. Fractions labeled a and b (Figure 1B) in this study were collected and analyzed by MALDI-TOF MS/MS, and the faster eluting peak, a, was identified as originating from equine β-casein (Table 1), which was hydrolyzed quickly by calf chymosin in agreement with Egito et al.[19] Peptides from equine κ-casein were not identified in either fraction by MS.

Identification of κ-Casein in Equine Milk. The fraction containing κ-casein, isolated using C4 RP-HPLC and identified according to Miranda et al.,[48] was collected and treated with phosphatase. The electrophoretograms of the enzymatically treated samples are shown in Figure 2. The phosphatase-treated sample had one band of ~24000 Da, which was selected for MS analysis along with a band of ~19000 Da from the control sample. Using MALDI-TOF and MALDI TOF/TOF analysis, no result was obtained by PMF, and multiple TOF-TOF analyses identified equine lysozyme; however, using nano-LC MS/MS a peptide (YIPIYYVLNSSPR) from equine κ-casein was identified in band b (Figure 3). Band a (Figure 2) was from a control equine milk, which was not dephosphorylated, and κ-

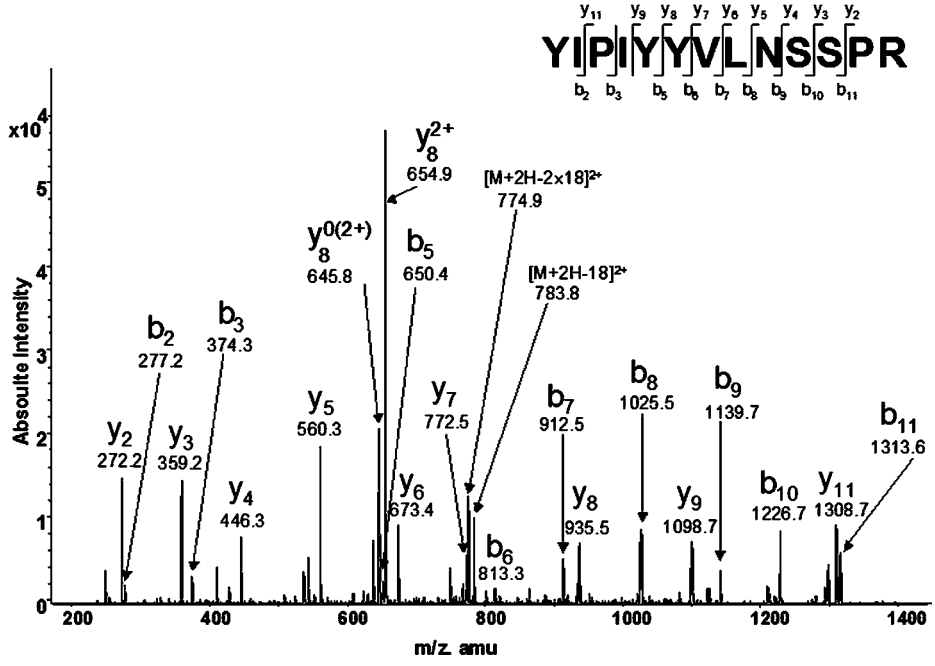

Figure 3. Nano-LC MS/MS spectra of fragmentation process of the YIPIYYVLNSSPR peptide (with m/z 792.9) from equine κ-casein; b^0, b-ion with loss of water (−18 Da); $[M + 2H - 18]^{2+}$, precursor ion with neutral loss of one molecule of water; $[M + 2H - 2 \times 18]^{2+}$, precursor ion with neutral loss of two molecules of water.

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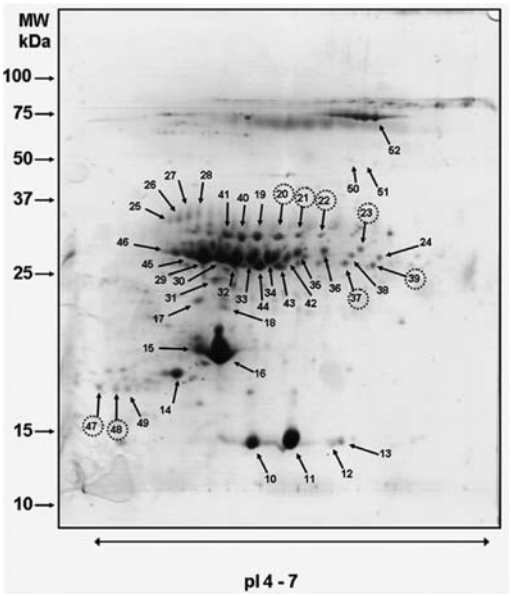

Figure 5. 2-DE preparative gel of equine milk (~300 μg of proteins) under reducing conditions using a 17 cm pH 4−7 p*I* range in the first dimension and a 12% acrylamide gel for the second dimension. The most abundant spots are indicated with arrows, and they were submitted to mass spectrometry identification by MALDI-TOF peptide mass fingerprinting and/or nano-LC/MS-MS (results in Table 3). Gel spots with dashed circles were those identified in subsequent analysis as having decreased following renneting with Maxiren 180 at pH 6.5.

casein was not identified therein. The κ-casein peptide, YIPIYYVLNSSPR, identified in band b, contained several potential phosphorylation sites; the lower ion efficiency of phosphorylated peptides in complex samples makes it difficult for MS identification. Using NetPhos 2.0, 11 phosphorylation sites in equine κ-casein (2 serine, 6 threonine, 3 tyrosine) were predicted, which scored >0.5 in the NetPhos scoring system. In the sequence YIPIYYVLNSSPR, identified from equine κ-casein, the serine residue highlighted in bold scored 0.947 on the NetPhos scale, which indicated a high probability of phosphorylation.

Two-Dimensional Electrophoresis Analysis of Equine and Bovine Milks. Preparative 2-DE gels of skimmed bovine and equine milk samples, performed under reducing conditions, are shown in Figures 4 and 5, respectively. Protein spots in the bovine sample were identified according to the method of Chevalier and Kelly;[45] the spots in the κ-caseins region were identified by MALDI-TOF with PMF, and the results are shown in Table 2. In this region, five κ-caseins, that is protein spots 1, 2, 3, 4, and 6, were identified by MALDI-TOF/TOF with PMF (Table 2). It has been reported that up to 10 isoforms of κ-casein with p*I* values from 4.47 to 5.81 can be identified by 2-DE gels of bovine milk.[43] If α_s- and β-caseins are removed, up to 16 gel spots can be identified as κ-casein isoforms arising from post-translational modifications such as phosphorylation and glycosylation.[44] In this study, within the region of the κ-casein isoforms, several β-casein isoforms were identified (Table 2), which have been reported to migrate to similar regions on a 2-DE gel as the more acidic κ-casein isoforms.[44] Holland et al.[44] and Chevalier and Kelly[45] identified the principal κ-casein spots in bovine milk, but in the present study the effect of added chymosin over time was considered for only the major isoforms.

The most abundant protein spots in equine milk isolated by preparative 2-DE were identified using peptide mass fingerprinting (PMF) with MALDI-TOF or nano-LC-MS/MS. Equine κ-casein was identified in spots 25, 26, and 27, and some κ-casein comigrated with β-casein in spots 40 and 41 (Figure 5 and Table 3). By comparison with bovine κ-casein, the isoelectric points of equine κ-caseins were significantly

Table 2. Identification of the Protein Spots in the κ-Casein Region of Bovine Milk by 2-DE with Peptide Mass Fingerprinting (PMF) Using MALDI-TOF or TOF/TOF[a]

spot	bovine protein	accession no.[b]	obsd p*I*	obsd MW (kDa)	theor p*I*	theor MW (kDa)	score	no. of matching peptides	sequence[c]	amino acids[d]
1	κ-casein	P02668	5.23	20	5.93	18.9	100	2	K-YIPIQYVLSR-Y	45−56
									R-SPAQILQWQVLSNTVPAK-S	89−108
2	κ-casein	P02668	5.45	20	5.93	18.9	76	1	R-SPAQILQWQVLSNTVPAK-S	89−108
3	κ-casein	P02668	5.66	20	5.93	18.9	46	1	K-YIPIQYVLSR-Y	45−56
4	κ-casein	P02668	5.78	19	5.93	18.9	108	1	R-SPAQILQWQVLSNTVPAK-S	89−108
5	κ-casein	P02668	5.94	19	5.93	18.9	55	1	K-YIPIQYVLSR-Y	45−56
6	β-casein fragment	P02666	5.98	18	5.13	23.6	151	4	K-VLPVPQK-A	184−192
									K-AVPYPQR-D	191−199
									K-FQSEEQQQTEDELQDK-I	48−64
									R-DMPIQAFLLYQEPVLGPVR-G	198−218
7	κ-casein	P02668	6.03	20	5.93	18.9	159	2	K-YIPIQYVLSR-Y	45−56
									R-SPAQILQWQVLSNTVPAK-S	89−108
8	β-casein fragment	P02666	6.17	18	5.13	23.6	111	4	R-GPFPIIV	217−224
									K-VLPVPQK-A	184−192
									K-AVPYPQR-D	191−199
									R-DMPIQAFLLYQEPVLGPVR-G	198−218
9	β-casein fragment	P02666	6.17	19	5.13	23.6	50	2	K-VLPVPQK-A	184−192
									K-AVPYPQR-D	191−199

[a]Theoretical molecular mass and isoelectric point (p*I*) of proteins are based on the amino acid primary sequence without taking into account any post-translational and/or degradation modifications. Observed molecular mass and isoelectric points (p*I*) are those observed from the position of the corresponding spot on the two-dimensional electrophores. [b]Accession number corresponds to the Swiss-Prot accession number. [c]Cleavage sites indicated by hyphens. [d]Peptides identified by mass spectrometry include the signal peptide (amino acids 1−15 for β-casein and 1−18 for β-lg.

Table 3. Identification of the Most Abundant Spots from Two-Dimensional Gel of Equine Milk by Nano-LC-MS/MS[a]

spot	equine protein	accession no.[b]	obsd p*I*	obsd MW (kDa)	theor p*I*	theor MW (kDa)	score	no. of matching peptides
10	α-lactalbumin	P08334	5.25	14	4.95	14.2	424	8
11	α-lactalbumin	P08334	5.55	14	4.95	14.2	561	6
12	α-lactalbumin	P08334	5.89	14	4.95	14.2	401	9
	heart-type fatty acid-binding protein	Q9XSI5	5.89	14	5.92	13.9	315	6
13	α-lactalbumin	P08334	5.94	14	4.95	14.2	327	7
	heart-type fatty acid-binding protein	Q9XSI5	5.94	14	5.92	13.9	115	3
14	β-lactoglobulin II	LGHO2	4.78	18	4.71	18.3	245	5
15	β-lactoglobulin I	P08334	4.91	19	4.85	18.5	415	9
16	β-lactoglobulin I	P08334	5.02	20	4.85	18.5	522	11
17	β-lactoglobulin I	P08334	4.87	25	4.85	18.5	330	7
	β-casein	Q9GKK3	4.87	25	5.78	25.5	180	5
	α_{s1}-casein	Q95KZ7	4.87	25	5.57	24.7	114	3
18	β-lactoglobulin I	P08334	4.97	24	4.85	18.5	266	6
	α-lactalbumin	P08334	4.97	24	4.95	14.2	165	3
19	β-casein	Q9GKK3	5.3	28	5.78	25.5	264	7
20	β-casein	Q9GKK3	5.53	28	5.78	25.5	235	6
21	β-casein	Q9GKK3	5.73	28	5.78	25.5	223	6
22	β-casein	Q9GKK3	5.97	28	5.78	25.5	237	6
23	β-casein	Q9GKK3	6.31	29	5.78	25.5	252	7
24	α_{s1}-casein	Q95KZ7	6.53	26	5.57	24.7	329	7
25	κ-casein	P82187	4.72	33	8.03	18.8	138	3
26	κ-casein	P82187	4.75	33	8.03	18.8	181	4
27	κ-casein	P82187	4.79	33	8.03	18.8	61	1
28	β-casein	Q9GKK3	4.85	32	5.78	25.5	82	2
29	β-casein	Q9GKK3	4.9	26	5.78	25.5	212	6
	α_{s1}-casein	Q95KZ7	4.9	26	5.57	24.7	167	2
30	β-casein	Q9GKK3	5.02	27	5.78	25.5	252	6
	α_{s1}-casein	Q95KZ7	5.02	27	5.57	24.7	95	2
31	α_{s1}-casein	Q95KZ7	4.97	24	5.57	24.7	239	5
	β-casein	Q9GKK3	4.97	24	5.78	25.5	177	4
32	β-casein	Q9GKK3	5.1	26	5.78	25.5	168	5
	α_{s1}-casein	Q95KZ7	5.1	26	5.57	24.7	107	2
33	β-casein	Q9GKK3	5.31	26	5.78	25.5	259	7
	α_{s1}-casein	Q95KZ7	5.31	26	5.57	24.7	104	2
34	β-casein	Q9GKK3	5.44	26	5.78	25.5	232	5
	α_{s1}-casein	Q95KZ7	5.44	26	5.57	24.7	59	2
35	β-casein	Q9GKK3	5.75	26	5.78	25.5	194	4
	α_{s1}-casein	Q95KZ7	5.75	26	5.57	24.7	77	3
36	β-casein	Q9GKK3	5.98	26	5.78	25.5	267	7
37	β-casein	Q9GKK3	6.18	25	5.78	25.5	354	9
	α_{s1}-casein	Q95KZ7	6.18	25	5.57	24.7	207	5
38	α_{s1}-casein	Q95KZ7	6.23	26	5.57	24.7	245	5
	β-casein	Q9GKK3	6.23	26	5.78	25.5	225	6
39	α_{s1}-casein	Q95KZ7	6.48	25	5.57	24.7	454	8
	β-casein	Q9GKK3	6.48	25	5.78	25.5	208	6
40	β-casein	Q9GKK3	5.13	27	5.78	25.5	248	6
	κ-casein	P82187	5.13	27	8.03	18.8	180	3
41	β-casein	Q9GKK3	5.01	28	5.78	25.5	254	6
	κ-casein	P82187	5.01	28	8.03	18.8	115	2
42	β-casein	Q9GKK3	5.58	26	5.78	25.5	131	3
	α_{s1}-casein	Q95KZ7	5.58	26	5.57	24.7	118	2
43	α_{s1}-casein	Q95KZ7	5.53	26	5.57	24.7	96	1
	β-casein	Q9GKK3	5.53	26	5.78	25.5	61	1
44	α_{s1}-casein	Q95KZ7	5.38	25	5.57	24.7	282	3
45	β-casein	Q9GKK3	4.85	26	5.78	25.5	220	5
	α_{s1}-casein	Q95KZ7	4.85	26	5.57	24.7	145	3
46	β-casein	Q9GKK3	4.74	27	5.78	25.5	239	5
	α_{s1}-casein	Q95KZ7	4.74	27	5.57	24.7	93	2
47	β-casein	Q9GKK3	4.25	17	5.78	25.5	87	3
48	β-casein	Q9GKK3	4.36	17	5.78	25.5	64	1
49	β-casein	Q9GKK3	4.46	17	5.78	25.5	75	2

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Table 3. continued

spot	equine protein	accession no.[b]	obsd p*I*	obsd MW (kDa)	theor p*I*	theor MW (kDa)	score	no. of matching peptides
50	lactoferrin	O97668	5.93	51	8.32	75.4	793	15
51	lactoferrin	O97668	6.15	50	8.32	75.4	863	16
52	lactoferrin	O97668	6.17	75	8.32	75.4	49	2

[a]Theoretical molecular mass and isoelectric point (p*I*) of proteins are based on the amino acid primary sequence without taking into account any post-translational and/or degradation modifications. Observed molecular mass and isoelectric points (p*I*) are those observed from the position of the corresponding spot on the two-dimensional electrophoresis gel. [b]Accession number corresponds to the Swiss-Prot accession number.

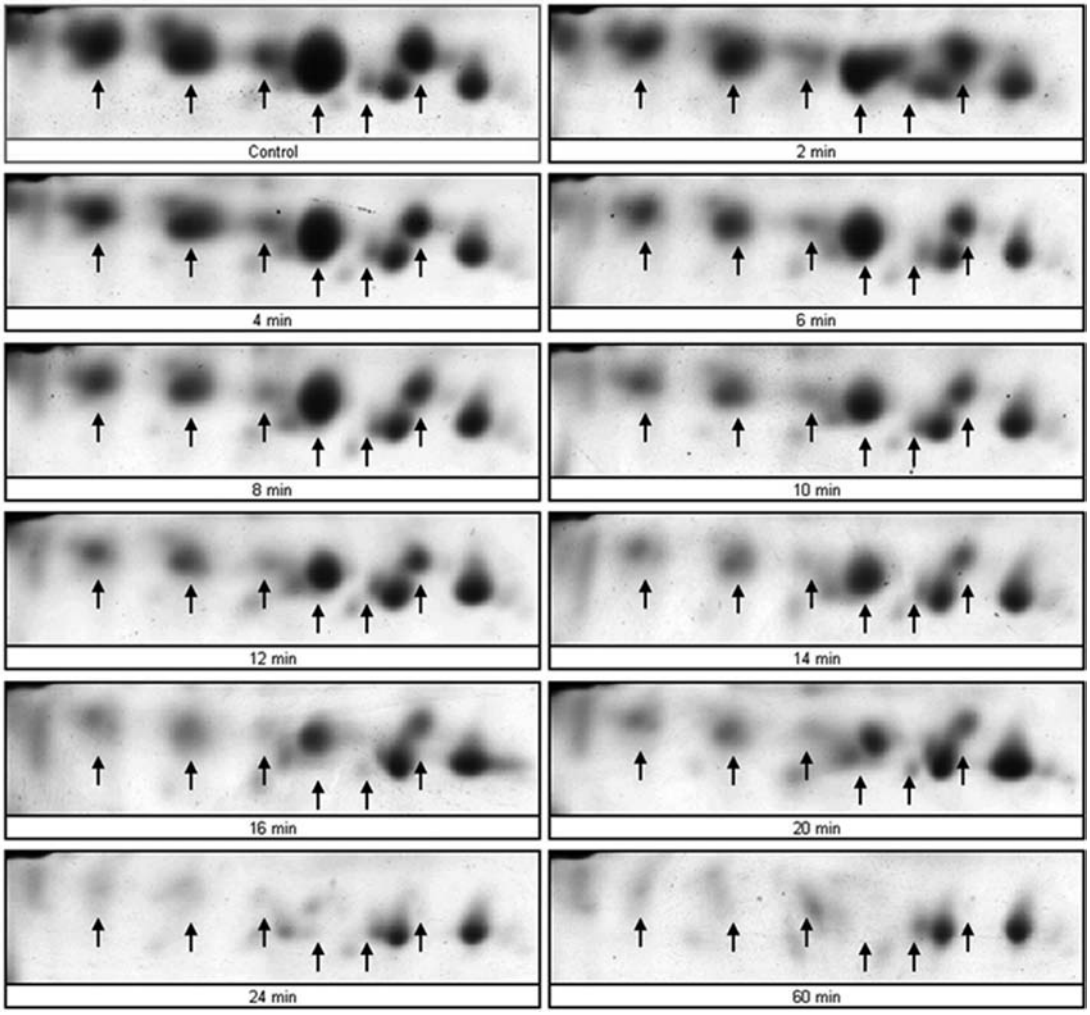

Figure 6. Image analysis of κ-casein modulated protein spots from 2-DE gels of bovine milk renneted over 60 min. Arrows indicate the κ-caseins that change in intensity (amount) over time.

lower than those of their bovine counterparts. Areas of the 2-DE gel of equine milk can be identified where isoforms of proteins are grouped; protein spots 20, 21, 22, 23, 28, and 29 form a cluster of β-casein isoforms, whereas protein spots 30, 31, 33, 34, and 35 represent isoforms of equine α_{s1}-casein (Figure 5 and Table 3). On the basis of spot intensities, approximately ∼35% of protein spots were β-casein isoforms, and a further 35% were α_{s1}-casein isoforms. Together with these two main milk proteins, other well-known milk proteins were identified including α-lactalbumin (4 spots), β-lactoglobulin (5 spots), and lactoferrin (3 spots). This is the first study in which a complete identification of the most abundant proteins in equine milk was achieved. Previously, 2-DE has been used to display the microheterogeneity of equine α_{s1}-[65] and β-caseins.[57,66] The 2-DE gel of equine milk highlighted the complexity of the milk proteome, and although very different from that of bovine milk in general appearance, it contained a multitude of protein spots. The proteome of bovine milk, and the milk of most species, is dominated by 6 gene products that constitute ∼95% of milk protein, but >150 protein spots can be detected using 2-DE of whole bovine milk. Many of the protein spots represent isoforms of the major gene products that have been produced by extensive post-translational modifications, including phosphorylation, glycosylation, and proteolysis.[43] D'Auria et al.[67] conducted a proteomic evaluation of milk from various species, including equine milk, as a means of identifying and evaluating the suitability of such milks for infant nutrition and highlighted the complex pattern of proteins of 2-DE electrophoretograms from human, equine, asinine, caprine, ovine, and bovine milks and reported that phylogenetically

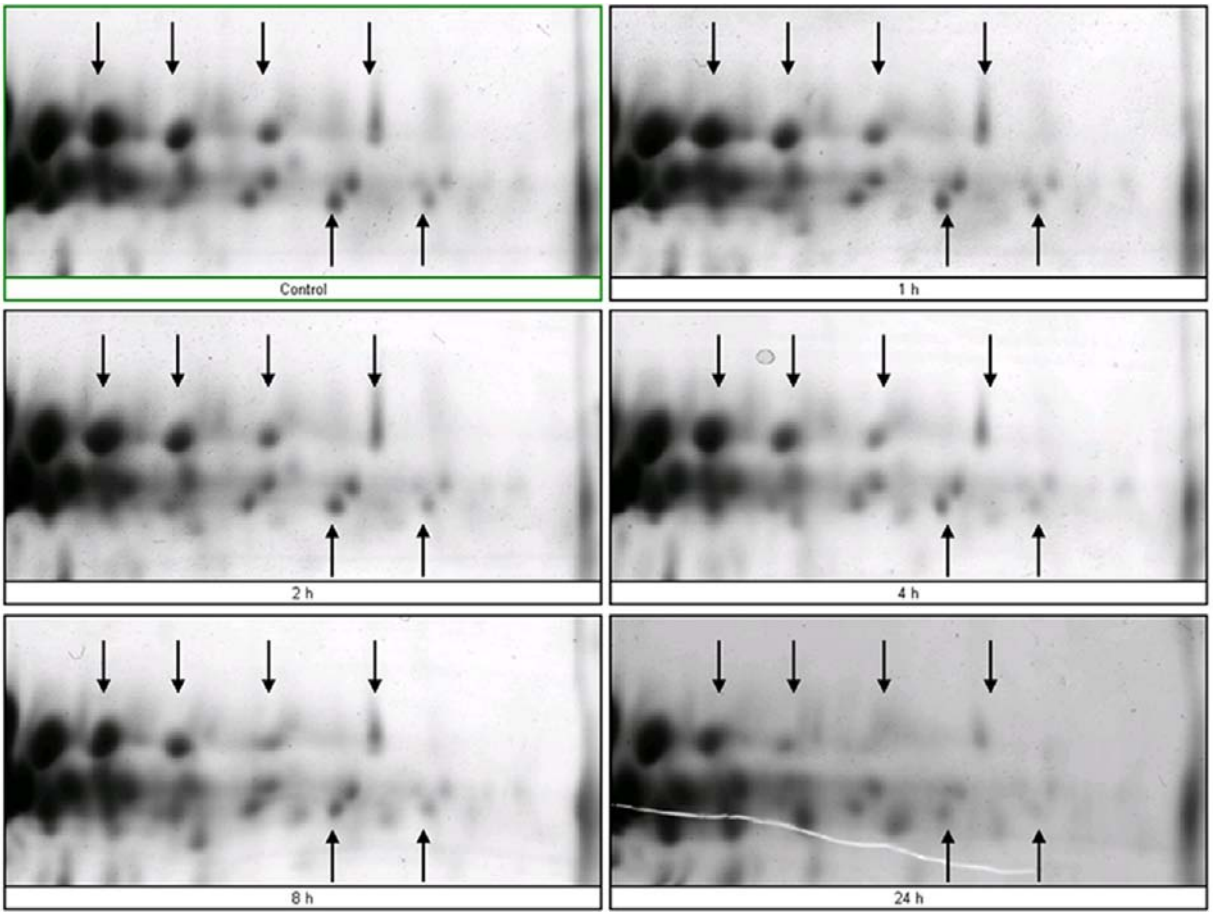

Figure 7. Image analysis of β-casein modulated protein spots from 2-DE gels of equine milk renneted over time. Arrows indicate the β-caseins that change in intensity (amount) over 24 h.

related species such as sheep/goats and horses/donkeys had quite similar protein expressions. Human milk protein expression was reported to be quite similar to both those of the horse and donkey.[67]

Effect of Renneting on Protein Spot Intensity in Equine and Bovine Milk. Two-dimensional analytical gels of renneted bovine and equine milk samples were scanned using SameSpots V4.0 software, and protein spots that changed in intensity over time were identified. The areas of the gels where protein spots susceptible to chymosin hydrolysis were identified and selected for further analysis. In the case of renneted bovine milk, six κ-casein spots decreased over time after chymosin addition (Figure 6). The result showed the decrease in intensity of bovine κ-caseins with time after chymosin addition (Figure 6). Bovine β-caseins were not significantly affected by chymosin during the time course of this experiment.

The proteins in equine milk that were hydrolyzed by chymosin over 24 h were identified as β-caseins only, that is, protein spots 20, 21, 22, 23, 47, and 48 as well as 37 and 39, which were identified as mixtures of β- and α_{s1}-caseins (Figure 3 and Table 3). Image analysis was performed on the 2-DE gel area where the most significant changes in protein spot intensities were detected (Figure 7), which showed the decrease in equine β-casein over 24 h after chymosin addition with the most significant decrease having taken place ~4 h after chymosin addition. According to the picture analysis, no change was observed over time with spots identified as κ-casein. This result was in agreement with the C18-RPLC analysis carried out on renneted equine milk (see RP-HPLC of Renneted Equine and Bovine Milk), where the peptide that increased in intensity following chymosin hydrolysis was identified as a peptide from β-casein. Egito et al.[19] reported the slow hydrolysis of equine κ-casein by chymosin with peptides generated ~10−20 min after chymosin addition when analyzed by C18 RP-HPLC.

Because equine milk contains a low level of κ-casein,[16,18,19,68,69] it has been suggested that the steric stabilization of equine casein micelles by κ-casein may be aided by unphosphorylated β-casein;[8] it is evident from this study that β-casein plays a significant role in equine casein micelle structure, and because it is hydrolyzed preferentially by chymosin, rather than κ-casein, it is likely that a significant proportion has an exposed location. It is not known whether phosphorylated or unphosphorylated forms of equine β-casein may stabilize the casein micelle. Phosphorylated forms would be hydrophilic and, in theory, could contribute to micellar stability, but they may be susceptible to precipitation by calcium. The significance of casein micelle phosphate groups is discussed by Yoshikawa et al.[70]

Unlike bovine κ-casein, the amino acid sequence of equine κ-casein does not have a distinctly hydrophilic C-terminal domain, particularly due to the absence of a strong hydrophilic region at residues 110−120, which, in the case of bovine casein micelles, protrudes from the micellar surface providing steric stabilization. However, equine κ-casein is reported to be heavily glycosylated,[13] which could compensate for the lack of

hydrophilic amino acid residues. The high level of glycosylation of equine κ-casein means the molecular mass of equine κ-casein prior to post-translational modification is ∼18.8 kDa,[14] whereas after post-translational modification it is ∼25.3 kDa,[28] suggesting that carbohydrate moieties represent ∼35% of total mass of equine κ-casein. For bovine κ-casein, glycosylation increases the mass of κ-casein by <5%.[71] The degree of glycosylation of κ-casein can have considerable effects on the colloidal stability of casein micelles.[72,73]

Conclusion. Isoforms of equine κ-casein were unequivocally observed and identified in several spots of a 2-DE map by MS. Also, a peptide from equine κ-casein was separated by C18 RP-HPLC and identified by MS analysis. This study demonstrated the ability of 2-DE coupled with mass spectrometry and image analysis to follow the effects of renneting on milk proteins over time. 2-DE and MS allowed the simultaneous evaluation of the relative abundance and modification of the endogenous proteins in equine and bovine milk following hydrolysis by calf chymosin. Equine milk was susceptible to hydrolysis by calf chymosin over 24 h at 30 °C and pH 6.5, but the peptides produced were from equine β-casein, which was also confirmed by MS analysis of C18 RP-HPLC peptides. Whereas equine β-casein was hydrolyzed by chymosin, hydrolysis occurred at a much slower rate compared to that of bovine κ-casein. In this study, equine κ-casein was not susceptible to hydrolysis by calf chymosin. The hydrolysis pattern of equine caseins by calf chymosin is not necessarily unique, and other studies have shown that the milk of a particular species is preferentially coagulated by chymosin from that species.[74−79] The isolation of equine chymosin and its ability to coagulate equine milk as well as its hydrolysis of equine caseins warrants investigation.

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Notes

The authors declare no competing financial interest.

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