Occurrence of β -casein fragments in cold-stored and curdled river buffalo (*Bubalus bubalis* L.) milk

A. Di Luccia,*¹ G. Picariello,† A. Trani,* G. Alviti,* P. Loizzo,* M. Faccia,* and F. Addeo†‡

*Dipartimento Progettazione e Gestione dei Sistemi Agro-Zootecnici e Forestali (PROGESA), Università di Bari, Via G. Amendola 165/A, 70126 Bari, Italy

†Istituto di Scienze dell'Alimentazione (ISA) – Consiglio Nazionale delle Ricerche (CNR), Via Roma 52A/C, 83100 Avellino, Italy

‡Dipartimento di Scienza degli Alimenti, University of Naples "Federico II," Parco Gussone, Portici 80055, Italy

ABSTRACT

The safeguard of river buffalo Mozzarella cheese, a Protected Designation of Origin dairy product, has prompted an analytical study to trace the milk and curd used as raw material in cheesemaking. This is to prevent the illegal use of milk or curd from different geographical areas outside of those indicated in the official production protocol. For this purpose, we studied primary proteolysis occurring in fresh and frozen milk and curd to identify a molecular marker that could indicate the raw material used. Whole casein from frozen river buffalo milk was separated using cation-exchange chromatography and sodium dodecyl sulfate-PAGE, and a protein component with an estimated molecular weight of 15.3 kDa was detected. This protein component was revealed in fresh river buffalo milk as a faint electrophoresis band, which drastically increased in intensity in refrigerated and frozen milk as well as in curd and was found to be associated with β -CN through hydrophobic interaction. By using matrix-assisted laser desorption/ionization-time of flight peptide mass mapping, this component was identified as the C-terminal fragment f(69–209) of β -CN (expected molecular weight of 15,748.8 Da). β -Casein f(69–209), originating from the early hydrolysis of Lys₆₈-Ser₆₉ by plasmin, has no counterpart in bovine milk. The increased rate of hydrolysis by plasmin toward the cleavage site Lys₆₈-Ser₆₉ has to be ascribed to the elevated proline content of the peptide 61–73. The favored production of β -CN f(69–209) has also drawn attention to the complementary proteose peptone β -CN f(1–68) that is presumed to play a physiological role in inducing milk secretion similar to that of β -CN f(1–29). The higher in vivo and in vitro production rate, compared with γ ₁-CN formation, indicates that β -CN f(69–209) and its complementary fragment are candidate molecular markers to evaluate milk and

curd freshness. We used indirect ELISA analysis based on the determination of remaining nonhydrolyzed β -CN to perform a quantitative evaluation of proteolysis.

Key words: γ -casein, river buffalo milk, frozen milk, curd

INTRODUCTION

Proteolysis in milk occurs mainly by plasmin (EC 3.4.21.7), which is a blood enzyme transported from plasma across mammary epithelial cells; its activity is determined by interactions between the inactive precursor, plasminogen, and plasminogen activators, associated with casein micelles or somatic cells (Politis et al., 1992). Thus, plasmin is concentrated in rennet-coagulated cheese curds, whereas inhibitors of plasminogen activators and plasmin are mainly lost in the whey (Kelly and McSweeney, 2003). Most of the existing studies on the plasminogen activation system have been carried out on bovine milk (Benfeldt et al., 1995), and available information is inadequate for milk of other dairy species.

In addition to breed (Richardson, 1983a), mammary physiology (Nicholas et al., 2002) and pathology (Zachos et al., 1992), storage (Richardson, 1983b), heat treatment, and pH (Richardson, 1983b; Burbrink and Hayes, 2006) are among the main factors that affect the plasmin system in milk and dairy products (Crudden et al., 2005). Plasmin activity continues during cold storage, leading to changes in the coagulation properties of milk, and plasmin survives high-temperature treatment during the processing of dairy products (Richardson, 1983b).

Plasmin is an alkaline serine proteinase that preferentially cleaves Lys-X peptide bonds, primarily hydrolyzing β -CN to γ -caseins. These β -CN fragments (f) are a heterogeneous fraction (Eigel, 1977) composed of f(29–209), f(106–209), and f(108–209) peptides designated as γ ₁-, γ ₂-, and γ ₃-CN, respectively. Trieu-Cuot and Addeo (1981) reported that a further fragment, named compound D, was produced in water buffalo milk because of the action of plasmin on β -CN, possibly

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¹Corresponding author: a.diluccia@agr.uniba.it

by cleavage at the Lys₆₈-Lys₆₉ peptide bond. On the basis of γ -CN, ovine and goat milks are also distinguishable from bovine and river buffalo milk because their β -CN both comprise 207 amino acids, with deletion of 2 residues (either Pro₁₇₉-Tyr₁₈₀ or Tyr₁₈₀-Pro₁₈₁); consequently, the derived γ -CN are 2 amino acids shorter than their bovine and river buffalo counterparts (Roberts et al., 1992). Sites susceptible to hydrolysis by plasmin also occur in α_{s1} - and α_{s2} -CN, whereas κ -CN, β -LG, and α -LA are resistant to hydrolysis (Kelly and McSweeney, 2003).

Some authors proposed to use γ -CN formation and its secondary proteolysis products as markers of cheese ripening and quality by defining peptide profiles for Grana Padano cheese (Gaiaschi et al., 2001). In fresh cheeses such as Mozzarella, primary proteolysis of α_{s1} - and β -CN mainly occurs during storage, and the levels of α_{s1} fragments and γ -CN (Farkye et al., 1991) increase progressively. Although classical analytical techniques adequately define the proteolytic patterns occurring in dairy products, they are of limited use in quantifying the early proteolytic products of plasmin action. To monitor the proteolysis phenomena in Swiss-type cheese, Senocq et al. (2002) and Dupont et al. (2003) overcame this problem by producing anti-peptide antibodies against major cleavage sites of plasmin in β -CN sequence. Such an approach can also be applied to the quantitative evaluation of proteolysis during preservation processes of raw matters. This is crucial in the production of Protected Designation of Origin (PDO) dairy products where the origin of raw matter is controlled; for example, in the case of Mozzarella cheese from river buffalo milk (Mozzarella di Bufala). In fact, increased consumer demand, limited availability of buffalo milk in summer, and the increased economic profits can all make Mozzarella cheese a target for adulteration. Thus, frozen curds can be imported at a lower cost from geographical areas outside that of PDO Mozzarella cheese production. Therefore, to protect consumers against deceit and safeguard the link of PDO products with local geographical areas, the emerging analytical challenge is to trace the "curd history" in river buffalo dairy products.

On the basis of the above considerations, refrigerated and frozen river buffalo milk, curd, and Mozzarella cheese were examined to study the profile of caseins in these dairy samples and to evaluate the possible use of γ -CN as molecular markers to guarantee raw milk freshness in the manufacture of river buffalo Mozzarella cheese. In addition, the evolution of river buffalo β -CN fragments was also investigated by comparison to that of the better-known bovine counterparts.

MATERIALS AND METHODS

Raw whole milk samples were collected from local dairy farms; to prevent undesired proteolysis, phenyl methyl sulfonyl fluoride (Sigma, St. Louis, MO) was added at a final concentration of 1 mM, and the samples were refrigerated during transportation to the laboratory. All samples were skimmed by centrifugation at $4,500 \times g$ at 4°C for 30 min and subdivided into 10-mL portions. An aliquot of the samples was processed immediately and the remaining portions were stored at -20°C. Fresh bovine and PDO river buffalo Mozzarella cheese samples, produced by biological acidification, were taken from the local dairy factories of the Campania region (Italy). Three different samples for each species (bovine and river buffalo) were analyzed. All reagents were of analytical grade or better. Acetonitrile (AcN), formic acid, acetone, dithiothreitol (DTT), iodoacetamide, α -cyano-4-hydroxycinnamic acid, and 2,5-dimethoxy-4-hydroxycinnamic acid were purchased from Sigma. Trypsin (sequencing grade) was supplied by Sigma, and alkaline phosphatase (4,000 U) from calf intestine was purchased from Roche (Roche Diagnostics, GmbH, Mannheim, Germany).

Precipitation of Caseins

Whole casein was separated from fresh and frozen bovine and river buffalo skimmed milk by isoelectric precipitation at pH 4.6 by addition of acetic acid. The pellet was stored at -80°C until analysis.

SDS-PAGE

All samples were solubilized in 8 M urea and analyzed by Bio-Rad DC-protein assay (BioRad Laboratories s.r.l., Milan, Italy). An aliquot of this solution was added to a sample buffer containing 0.6 M Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, and 1% β -mercaptoethanol to obtain a final protein concentration of 4 mg/mL. Ten microliters of this solution was applied to an 8 to 18% polyacrylamide pore gradient gel (1 $\times$ 180 $\times$ 160 mm), and electrophoresis was carried out in a Hoefer SE 600 electrophoresis unit (Amersham Biosciences Europe GmbH, Uppsala, Sweden) essentially according to Laemmli (1970) and Sheele (1975). The gels were blue silver stained according to Candiano et al. (2004). Prestained SDS-PAGE standards (Bio-Rad) were used as protein molecular mass markers. Molecular weights (MW) were estimated by means of the TotalLab TL100 software (NonLinear Dynamics, Newcastle upon Tyne, UK).

Ion Exchange Chromatography

Ion-exchange chromatography (IEC) was performed using a SP Sepharose Fast Flow strong cation exchange resin (Amersham Biosciences) packed in a 26- × 230-mm column as described by Andrews et al. (1985). Three grams of caseins, obtained by isoelectric precipitation, was solubilized in 40 mL of formate buffer containing 0.2% β -mercaptoethanol and loaded onto the column. Elution was performed with a gradient from 0.24 to 0.50 M NaCl in formate buffer at a flow rate of 3.0 mL/min. The effluent was monitored by UV detection at 280 nm, and peaks were collected manually, dialyzed, and freeze-dried.

Hydrolysis of Bovine and River Buffalo β -CN by Bovine Plasmin

Hydrolysis of pure bovine and buffalo β -CN by bovine plasmin (Roche) was performed as described by Eigel (1977). Sample aliquots were taken from the reaction mixture after 2, 4, 16, and 32 min. The enzymatic reaction was stopped by adding an equal volume of SDS-PAGE sample buffer.

Extraction of Proteins from Mozzarella Cheese

Samples of bovine and river buffalo Mozzarella cheese were minced and lyophilized. Aliquots (100 mg) were dissolved in 1 mL of aqueous AcN (70%, vol/vol) containing 0.1% trifluoroacetic acid (TFA, vol/vol), shaken in a vortex mixer for 15 min, and sonicated for 45 min. Protein solutions were centrifuged (Minifuge, Heraeus, Osterode, Germany) at 8,000 × *g* for 15 min at 4°C, and supernatants were concentrated in a Speed vac (Savant Instruments Inc., Holbrook, NY) until one-fifth of their initial volume remained. Water was added up to 1 mL and the solutions were defatted by centrifugation (15 min, 8,000 × *g* at 4°C). The protein solution was lyophilized and redissolved in 100 μ L of aqueous AcN (50%, vol/vol) containing 1% formic acid (vol/vol) for MS analysis.

Reversed-Phase HPLC of IEC Fraction 2

Reversed-phase (RP) HPLC separations were carried out using a Beckman Gold System (Beckman, Berkeley, CA) equipped with a 250 mm × 4.6 mm internal diameter C-18 reversed-phase column (5 μ m 218TP54, Vydac, Hesperia, CA). Lyophilized caseins from the IEC fraction (1 mg) were dissolved in 0.5 mL of 0.1% TFA and 10 mM DTT and incubated for 1 h at 37°C; 100 μ L of the resulting solution was used for each injection. Solvent A was 0.1% TFA in water (vol/vol)

and solvent B was 0.1% TFA in 95% AcN (vol/vol). For elution, a linear gradient from 35 to 50% solvent B was applied over 60 min at a flow rate of 1 mL/min. Column effluent was monitored at both 220 and 280 nm, and protein fractions were collected manually and used for MS analysis or for tryptic digestion after dephosphorylation.

Dephosphorylation of Casein and Tryptic Digestion of RP-HPLC Fractions

Chromatographic fractions from RP-HPLC were concentrated, lyophilized, and resuspended in ammonium bicarbonate buffer (0.4% wt/vol at pH 8.5) to a protein concentration of 1 mg/mL. An aliquot of each fraction, corresponding to 10 μ g of protein, was incubated overnight with alkaline phosphatase (0.05 μ g) at 37°C and then incubated in a boiling water bath for 5 min. Tryptic digestion was carried out for 6 h at 37°C with an enzyme:substrate ratio of 1:100 (wt/wt), and the reaction was stopped by overnight freeze-drying. Lyophilized peptides were redissolved in 50 μ L of AcN, water, and formic acid (50:45:5 vol/vol/vol) for MS analysis.

Mass Spectrometry

Peptide Mass Mapping by Matrix-Assisted Laser Desorption Ionization-Time of Flight. Manually excised bands from Coomassie-stained gels of a component with an estimated molecular mass of 15.3 kDa and of β -casein were destained, reduced, and alkylated by carboxymethylation and then digested in situ by trypsin (Sigma) overnight at 37°C, according to the procedure of Shevchenko et al. (1996). Peptides resulting from digestion were extracted 3 times with 40 μ L of AcN, water, and formic acid solution (50:45:5, vol/vol/vol) and then concentrated by vacuum centrifugation for MS analysis. Finally, peptide mixtures were dissolved in aqueous 50% (vol/vol) AcN containing 1% (vol/vol) formic acid. All steps of the excision procedure, destaining, reduction/alkylation, and peptide extraction were carried out under a laminar flow hood to avoid contamination by keratins. Mass spectrometry experiments were carried out on a PerSeptive Biosystems (Framingham, MA) Voyager DE-Pro instrument equipped with a N₂ laser (337 nm, 3 ns pulse width). Peptide mixtures from each digested spot (1 μ L) or from tryptic digestion of RP-HPLC fractions were loaded onto a stainless steel plate, together with 1 μ L of matrix, prepared by dissolving 10 mg of α -cyano-4-hydroxycinnamic acid in 1 mL of aqueous 50% (vol/vol) AcN containing 0.1% (vol/vol) TFA. Typically, 250 laser pulses were acquired for each spectrum and

the mass spectra were acquired in the reflector mode with delay extraction; the accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards (Applied Biosystems, Framingham, MA).

Protein Identification. The matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) spectra were analyzed using the Data Explorer 5 software supplied with the instrument. The measured molecular masses were compared with those generated by theoretical hydrolysis of caseins performed using the Peptide Tools software version A.01.02 (Agilent Co., Palo Alto, CA).

MALDI-TOF MS of Proteins Extracted from Mozzarella Cheese. Mass spectrometry measurements of nonhydrolyzed protein and protein extracted from bovine and river buffalo Mozzarella cheese were carried out using the above instrument. In this case, sinapinic acid [3,5 dimethoxy-4-hydroxycinnamic acid, prepared by dissolving 10 mg of crystalline powder in 1 mL of aqueous 50% (vol/vol) AcN containing 0.1% TFA] was used as matrix. Matrix (1 μ L) was mixed directly with the sample protein solutions (1 μ L) on the MALDI target, and mixtures were air-dried before analysis. Spectra were acquired in linear positive ion mode and the accelerating voltage was 25 kV. Mass calibration was performed internally using the signals of α -LA and β -LG in river buffalo Mozzarella cheese extracts. These 2 components were present in relatively high amounts in all 3 river buffalo Mozzarella cheese samples analyzed. Spectra of protein extracts from bovine Mozzarella cheese were calibrated externally according to measurement of river buffalo α -LA and β -LG (singly and doubly charged) and dephosphorylated β -CN. To check repeatability, each sample was analyzed in triplicate (at least).

RESULTS

Chromatography and Electrophoretic Analyses of Casein from Frozen River Buffalo Milk

Sodium dodecyl sulfate-PAGE of whole casein from fresh and frozen river buffalo milk revealed a band with an estimated MW of 15.3 kDa (Figure 1, panel a). This band was faint in fresh milk, but intense in frozen milk. To identify this component, casein from frozen milk was fractionated by cation IEC at pH 4.0 (Figure 1, panel b), and the composition of the separated protein fractions was analyzed by SDS-PAGE (Figure 2). Based upon electrophoretic mobility and IEC retention times, the first and second peaks were identified as β -CN, and the remaining peaks were κ - and α _S-caseins. The anomalous chromatographic behavior of β -CN was

attributed to hydrophobic interaction of the additional 15.3 kDa component (component A in Figure 2) with β -CN; the interaction increased the net positive charge by masking hydrophobic portions where the 2 polypeptides interacted and consequently increased the IEC elution time. This hypothesis was supported by RP-HPLC analysis (Figure 3) of the second IEC fraction, which was split into 2 components as the hydrophobic condition changed. The peak at 36.9 min was identified by MALDI-TOF MS analysis as β -CN (4- and 5-fold phosphorylated) whereas that at 39.8 min exhibited a molecular mass of 15,748.8 Da (inset of Figure 3). It was also possible to establish that river buffalo β -CN and α _S-CN comigrated in SDS-PAGE, revealing a very similar electrophoretic mobility.

MALDI-TOF MS Mass Mapping

To identify the component with estimated MW of 15.3 kDa in SDS-PAGE and measured molecular mass of 15,748.8 Da, peptide mass mapping by MALDI-TOF MS of trypsin-hydrolyzed species was performed. In Figure 4 the MALDI-TOF spectrum of the in-gel-digested protein component A (Figure 4 b) is compared with the spectrum of tryptic peptides derived from dephosphorylated β -CN (Figure 4 a), and the mass signals matching the theoretical peptides of β -CN are assigned. The 2 peptide maps overlapped substantially, demonstrating that component A is clearly derived from β -CN. Apart from the signals at m/z 1,252.7 and 971.5, due to κ -CN-derived impurities (peptide 1–10, with N-terminal pyro-glutamic acid, and peptide 98–105, respectively), 3 signals in the spectrum of Figure 4 b that occurred in the peptide pattern of β -CN were missing: m/z 3,139.9 corresponding to the dephosphorylated peptide 1–28, m/z 3268.0 corresponding to the dephosphorylated peptide 1–29, and m/z 4,233.6 corresponding to the tryptic peptide 33–68.

In the spectrum of peptides derived from component A (Figure 4 b), several strong signals were generated from the C-terminal moiety of β -CN and were common to those found also in the tryptic digest of β -CN. The above data demonstrated that component A is a C-terminal fragment of β -CN. The measured molecular mass of component A, isolated by RP-HPLC, was 15,748.8 Da, close to the theoretical m/z of 15,749.8 Da (as MH^+) of β -CN fragment 69–209; in addition, this portion of β -CN was not phosphorylated, which is consistent with the observation that its molecular mass did not change after alkaline phosphatase treatment (data not shown). The MALDI-TOF peptide maps obtained from in-gel tryptic digestion of the 15.3 kDa component band gave rise to a pattern overlapping with that obtained from in-solution tryptic digestion of

Figure 1. a) Sodium dodecyl sulfate-PAGE electrophoretograms of fresh (1) and frozen (2) river buffalo milk; b) cation exchange chromatography (cationic ion-exchange chromatography) separation of casein from frozen buffalo milk. Collected fractions were analyzed by SDS-PAGE (see Figure 2).

the protein component at m/z of 15,748.8 isolated by RP-HPLC (data not shown). Therefore, component A was conclusively identified as β -CN f(69–209).

Formation of Bovine and River Buffalo γ -CN

To establish if β -CN f(69–209) actually originated from β -CN as consequence of plasmin activity, *in vitro* plasmin hydrolysis was carried out on pure β -CN. In Figure 5, the kinetics of formation of river buffalo γ -CN are compared with that of the bovine counterparts using SDS-PAGE. Hydrolysis of river buffalo β -CN produced β -CN f(69–209), which was not detected in the bovine counterpart.

On hydrolysis of bovine β -CN, the formation of different fragments occurred principally with production of γ_1 , γ_2 , and γ_3 as well as β -CN f(49–209) and β -CN f(98–209), although these last 2 were already visible by analyzing pure β -CN. After 2 min and with increasing time up to 16 min of plasmin treatment, an increase in all the β -CN fragments was observed. At 32 min, an almost complete disappearance of β - and γ_1 -CN was observed, whereas γ_2 - and γ_3 -CN continued to increase. In 32 min, hydrolysis of river buffalo β -CN by plasmin produced a slower rate of decrease in γ_1 -CN than that of β -CN and an increase of all other γ fragments. The

formation of β -CN f(69–209) was already evident after 2 min, confirming that it was an early product of plasmin activity.

To verify the formation rate of β -CN f(69–209) *in vivo*, river buffalo milk was stored at 4°C or –20°C and analyzed after 1, 2, 4, 10, 15, and 30 d by SDS-PAGE (Figure 6). The electrophoresis patterns already showed the presence of β -CN f(69–209) in refrigerated milk samples 1 d after milking and the formation of a further band (with an estimated MW of 13 kDa) in samples kept for 2 d at 4°C. This band intensified at 4 d and was absent when the milk sample was stored at –20°C. This protein band was identified by MALDI-TOF mass mapping as the N-terminal moiety of κ -CN (data not shown) and its estimated MW indicated that it was *para*- κ -CN. Analogously, the formation of γ_1 -CN was established by MALDI-TOF identification of a band with an estimated MW of 23 kDa (data not shown). The intensity of this band increased with time of storage up to 32 d at –20°C. In Figure 7, the electrophoretic profiles of bovine and river buffalo frozen milk, as well as river buffalo cheese curds, are shown. In all profiles of river buffalo dairy products, β -CN f(69–209) was well able to differentiate the 2 species, because it was absent in bovine milk. The profiles of curds also showed the occurrence of *para*- κ -casein. The latter was

also present in lane 6, corresponding to that of river buffalo milk stored for 1 yr.

MALDI-TOF Analysis of Protein Extracts from Mozzarella Cheese

The protein composition of river buffalo Mozzarella cheese was compared with its bovine counterpart by MALDI-TOF analysis to confirm the effective formation of β -CN f(69–209). In the resulting spectra (Figure 8), the intense signal with m/z of 15,748.5 Da was exclusively present in the protein extract from river buffalo Mozzarella cheese (Figure 8, panel b), whereas no corresponding signal was detected in the protein extract from bovine Mozzarella cheese (Figure 8, panel a). Furthermore, the detection of intact phosphorylated β -CN f(1–68) by MALDI-TOF analysis (data not shown) demonstrated the earlier cleavage of the Lys₆₈-Ser₆₉ with respect to γ_1 -CN formation. A relatively high level of whey proteins was observed in river buffalo Mozzarella cheese because it is stored in the typical brine containing diluted acid whey. Signals of *para*- κ -caseins, originating from the specific hydrolysis of Phe₁₀₅-Met₁₀₆ by rennet, were among the most prominent. Pyro-glutamate, as the N-terminal amino acid, explained the measured masses of 12,267.4 Da (theoretical m/z of 12,268.1) and 12,346.7 Da (theoretical m/z of 12,349.6) for bovine and river buffalo caseins, respectively. The γ_2 - and γ_3 -CN were also detected in both samples; it is noteworthy that γ_1 -CN was only present in the spectrum of bovine Mozzarella cheese samples. Consistent with electrophoretic data, river buffalo γ_1 -CN was produced at very small amounts compared with the bovine counterpart, and MALDI-TOF detection was further reduced because of phosphorylation. Finally, the intense signals at m/z 9,449.8 and 9,407 were assigned to the C-terminal fragments generated from cleavage of the Leu₁₂₅-Thr₁₂₆ site of bovine and buffalo β -CN, respectively, by means of pepsin.

DISCUSSION

Chromatographic and electrophoretic investigation of cold-stored river buffalo milk, curd, and Mozzarella cheese revealed an additional major γ -CN, identified as β -CN f(69–209). The extensively studied pathway of γ -CN production in bovine milk and dairy products was selected here as the reference model. Although bovine and river buffalo β -CN showed high sequence homology (97.6%; they differ by 5 amino acids out of 209), different production of γ -CN was observed in the respective milks. In particular, β -CN f(69–209) had no counterpart in plasmin digests of bovine β -CN, which has Asn₆₈-Ser₆₉ instead of Lys₆₈-Ser₆₉ at the cleavage

Figure 2. Sodium dodecyl sulfate-PAGE electrophoretograms of fractions collected from cationic ion-exchange chromatography. Protein bands correspond to whole casein of frozen buffalo milk; lane 1 = β -CN; lane 2 = β -CN with the additional 15.3 kDa component; lanes 3, 4, and 5 = κ -CN; lanes 6 and 7 = α_s -CN.

site of river buffalo β -CN (Trieu-Cuot and Addeo, 1981; Ferranti et al., 1998). The Asn₆₈ site is also conserved in the β -CN of ruminant species other than river buffalo. Trieu-Cuot and Addeo (1981) already suggested that β -CN f(69–209), named component D, could be present only in river buffalo whole caseins. Later, Angeletti et al. (1998) exploited the analytical potentiality of mass profiling by MALDI-TOF MS to detect possible adulteration in river buffalo milk and Mozzarella cheese. They found a characteristic signal in buffalo milk with a molecular mass of 15,790 Da, called “protein X,” and did not recognize this protein as a C-terminal moiety of β -CN. In river buffalo milk, in vitro and in vivo produc-

Figure 3. Reversed phase (RP)-HPLC chromatograms of the cationic ion-exchange chromatography fraction 2 showing 2 components. The matrix-assisted laser desorption ionization time of flight spectrum of the RP-HPLC fraction at 39.8 min (inset) confirmed the presence of the 15,748.8 Da protein component.

Figure 4. Matrix-assisted laser desorption/ionization time of flight mass spectra of peptides arising from tryptic digestion of a) dephosphorylated river buffalo β -CN and b) protein component A (m/z 15,748.8 Da), isolated by reversed-phase HPLC.

Figure 5. Sodium dodecyl sulfate-PAGE analysis of in vitro action of plasmin on bovine and river buffalo β -CN. Lane 1 = pure buffalo β -CN; lanes 2, 3, 4, and 5 = buffalo β -CN incubated with plasmin after 2, 4, 16, and 32 min, respectively; lane 6 = river buffalo β -CN containing β -CN fragment (69–209); lane 7 = molecular weight standards; lane 8 = pure bovine β -CN; lanes 9, 10, 11, and 12 = bovine β -CN incubated with plasmin for 2, 4, 16, and 32 min, respectively.

tion of β -CN f(69–209) was faster and more abundant than that of γ_1 -CN. This event accounted for a better exposition of protein surface containing plasmin cleavage site Lys₆₈-Ser₆₉ with respect to the main cleavage sites in bovine β -CN, Lys₂₈-Lys₂₉, Lys₁₀₅-His₁₀₆, and Lys₁₀₇-Glu₁₀₈, which are considered well exposed on the outer protein surface and located on a hydrophilic side, in particular on the long distorted arms, according to the structural studies on 3-dimensional modeling by Kumosinski et al. (1993).

The above suggestions are substantiated by electrophoretic analyses showing the presence of β -CN f(69–209) in cold-stored milk and curd. β -Casein f(69–209) was already detected in milk stored at 4°C 24 h after milking. Furthermore, its quantity increased in the stored milk. After 4 d of storage at 4°C, the formation of *para*- κ -CN was also observed, which could be due to contamination of milk with psychrotrophic bacteria (Kohlmann et al., 1991) or by cathepsin D activity (Larsen et al., 1996). An analogous result was obtained after 1 yr of storage at –20°C (Figure 7). Preservation of raw milk at –20°C for 1 mo (Figure 6) produced β -CN f(69–209), which increased more rapidly than that observed for γ_1 -CN, without formation of *para*- κ -CN. Our results concerning in vitro and in vivo plasmin action also demonstrated that differences in plasmin concentration or time of storage of milk and fresh milk products could produce different β -CN fragments by means of primary and secondary proteolysis. A quantitative determination by ELISA of γ -CN could offer an appropriate method for evaluating

the quality of milk and milk products. In fact, Dupont et al. (2003) studied plasmin activity on β -CN throughout ripening of Comtè cheese and proposed creation of antibodies specifically directed against the cleavage site of plasmin. The antibodies produced recognized their epitopes as long as the peptide bond was intact in the protein. The hydrolysis of the peptide bond by the enzyme led to failure of the antibody to recognize the epitope. The application of this strategy demonstrated that the Lys₂₈-Lys₂₉ peptide bond, monitored by anti-peptide β -CN f(20–39), is preferentially cleaved during ripening. During refrigeration and freezing of river buffalo raw milk, β -CN f(69–209) showed an interesting

Figure 6. Sodium dodecyl sulfate-PAGE analysis of in vivo action of plasmin in cooled and frozen river buffalo milk. Fresh milk cooled at 4°C was sampled after 1, 2, and 4 d (lanes 1, 3, and 4, respectively); frozen milk at –20°C was sampled after 2, 4, 10, 15, and 30 d (lanes 2, 5, 6, 7, and 8, respectively).

Figure 7. Sodium dodecyl sulfate-PAGE electrophoretograms of milk and curd stored at -20°C ; lane 1 = molecular weight standards; lanes 2, 3, and 4 = bovine milk after 3, 6, and 5 mo, respectively; lanes 5, 6, and 7 = river buffalo milk after 3 mo, 1 yr, and 6 mo, respectively; lanes 8 and 9 = bovine curd and lanes 10, 11, and 12 = river buffalo curd after 1 yr of storage. River buffalo milk was characterized by the presence of β -CN fragment (69–209); curds were distinguished by the presence of *para*- κ -CN.

trend of production similar to that of γ_1 -CN in bovine milk, which are both derived from the 2 most sensitive peptide bonds. This led to the conclusion that an indirect ELISA for the quantitative determination of hydrolyzed river buffalo β -CN applied to the peptide bond Lys₆₈-Ser₆₉ may be suitable to establish the occurrence of proteolysis in river buffalo milk and evaluate the quality of the raw material. Moreover, it is possible that in milk with increased plasmin activity (e.g., in cases of mastitis or in stored milk), γ -CN could be produced in different concentrations and, probably, further fragments as in the case of the *in vitro* experimental condition. The findings about the hydrolysis of the site Lys₆₈-Ser₆₉ by plasmin offer 2 considerations. First, the cleavage site falls in a strategic zone of β -CN, releasing β -casomorphins upon gastrointestinal hydrolysis; this may account for the production of some biologically active peptides, implicating a possible specific physiological role in river buffalo milk compared with the milk of other ruminant species (Petrilli et al., 1984). The second concerns the delivering of β -CN f(1–68) in the mamma-

ry gland cell. Brown et al. (1995) found β -CN f(1–28), the N-terminal complement of γ_1 -CN, to be negatively correlated with yield of bovine, sheep, and goat milk in the late phase of lactation. This result supported the concept that β -CN f(1–28) plays a role in the regulation of milk secretion (Silanikove et al., 2000) by acting as a potent blocker of K^+ channels in the apical membrane of mammary epithelial cells. Silanikove et al. (2000) also noted the presence of a phosphorylated peptide that was responsible for changes in K^+ permeability in bovine and goat udders. The production of β -CN f(1–28) during milk incubation in the udder represents between 8 and 12% of the total proteose peptone fraction in whey and matches the formation of γ_1 -CN as an ideal candidate marker for negative feedback control of milk secretion. Therefore, occurrence of small amounts of β -CN f(69–209) even in fresh milk raised a question about early proteolytic action in the mammary gland cell: could β -CN f(69–209) be considered the molecular effector responsible for negative feedback control in river buffalo milk secretion?

Figure 8. Matrix-assisted laser desorption/ionization time of flight spectra of protein extract from a) bovine and b) river buffalo Mozzarella cheese. To simplify, the first decimal figure in the molecular mass values has been omitted.

CONCLUSIONS

River buffalo milk is characterized by a further γ -CN defined here: β -CN f(69–209). This fragment was previously known as “component D” by Trieu-Cuot and Addeo (1981) and was named “protein X” by Angeletti et al. (1998) but has not been precisely identified until now. Also, the reasons for its occurrence in river buffalo milk were not completely understood previously. Our results demonstrate that the formation of this fragment is early with respect to that of γ ₁-CN, because of conformational exposition of the plasmin cleavage site Lys₆₈-Ser₆₉, whereas the complementary proteose peptone β -CN f(1–68) could have a physiological role in the control of milk secretion such as occurs for bovine, ovine, and caprine proteose peptone β -CN f(1–28).

The amount of β -CN f(69–209) increased as river buffalo milk freshness decreased, but could also increase in mastitic milk as occurs for γ ₁-CN of other ruminant species. The monitoring of quantitative formation of β -CN f(69–209) by indirect ELISA could easily allow

evaluation of the conservation time and sanitary condition of fresh milk and could distinguish between fresh and frozen curd. In the latter case, it should be possible to detect curds coming from areas different from that of production. The development of these studies might yield a powerful tool to guarantee the quality of dairy products to consumers and to defend protected dairy production.

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