



# Peptidomic approach based on combined capillary isoelectric focusing and mass spectrometry for the characterization of the plasmin primary products from bovine and water buffalo $\beta$ -casein

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## ABSTRACT

The main peptides produced by hydrolysis of water buffalo  $\beta$ -casein with plasmin were characterized by capillary electrophoresis and mass spectrometry and compared with their bovine homologous. A novel breakdown product arising from the hydrolysis of water buffalo  $\beta$ -casein, originated by the presence of a plasmin-sensitive Lys bond at position 68 was identified, which was not present in bovine  $\beta$ -casein. On the basis of this evidence, an improved procedure for the detection and the differentiation of the products of plasmin hydrolysis of bovine and water buffalo  $\beta$ -casein by capillary isoelectric focusing was set-up. In the experimental conditions, the  $\gamma$ -casein from the two species was efficiently separated. Comparison of the capillary electropherograms with those obtained by ultra-thin-layer isoelectric focusing, the reference method for routine analysis of plasmin digests of casein, suggests that capillary electrophoresis isoelectric focusing may constitute a successful alternative to the traditional slab gel electrophoresis analysis of plasmin digests of casein either for basic structural studies or for applications in the quality assessment of dairy products.

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## 1. Introduction

There is a general agreement that the main indigenous protease in bovine milk is plasmin [1]. Recent evidence showed that its inactive precursor, the plasminogen, is not synthesized in the mammary gland. Berglund et al. [2] observed the plasminogen expression in bovine liver tissue. Plasmin preferentially cleaves Lys-X and Arg-X bonds [3]. The milk proteins that most rapidly undergo cleavage by plasmin are  $\beta$ -casein (CN) and  $\alpha$ s<sub>2</sub>-CN [4,5]. In bovine  $\beta$ -CN there are three main plasmin-sensitive bonds: Lys28-Lys29, Lys105-His106, and Lys107-Glu108 giving rise to C-terminus  $\gamma$ <sub>1</sub>-CN (29–209),  $\gamma$ <sub>2</sub>-CN (106–209),  $\gamma$ <sub>3</sub>-CN (108–209), and to the complementary N-terminus fragments, namely proteose peptones (PPs) as  $\beta$ -CN (1–28) (PP 8 fast),  $\beta$ -CN (29–105),  $\beta$ -CN (29–107) (PP8 slow) and  $\beta$ -CN (1–105),  $\beta$ -CN (1–107) (PP 5) [6].

Plasmin and its precursor pass from the blood to milk where its activators and inhibitors are also present. As a consequence, the level of plasmin in freshly drawn milk is negligible but increases owing to plasminogen activation during milk storage [7]. The

enzyme activity increases, also, with stage of lactation, severity of mastitic infection and lactation number [8–10].

In dairy industry, an increased level of activity of plasmin is detrimental for milk quality due to consistent decrease of intact CN caused by the proteolytic phenomena. On the other hand, both the lower susceptibility of  $\gamma$ -CNs to the action of enzymes released from lactic acid bacteria (LABs) and their thermostability at the temperature of curd cooking in Parmigiano Reggiano Protected Denomination of Origin (PDO) cheese [11–13], justified their high quantitative level in the CN fraction after 24 months of cheese ripening. For these reasons, the ratio  $\gamma$ -CN/ $\beta$ -CN in Parmigiano Reggiano cheese, determined by urea-polyacrylamide gel electrophoresis (urea-PAGE), has been used to calculate the cheese ripening time and then, as a quality control index for ripened Parmigiano Reggiano PDO cheese [14].

The analysis of the main products of plasmin activity (i.e.  $\gamma$ -CNs) is also an important tool for detection of the adulteration of goat's, ewe's and water buffalo's milk due to the addition of cow milk which is not allowed in the manufacturing of several typical dairy products. Infact, according to the EEC/EU Regulation No. 213/2001 [15] this kind of adulteration can be detected by electrophoretic analysis of CN samples after plasmin hydrolysis. The method consists in the addition of plasmin to CN samples under the

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appropriate conditions, followed by analysis of the protein digest by means of ultra-thin-layer isoelectric focusing (utlIEF) gel electrophoresis in order to separate bovine  $\gamma_2$ - and  $\gamma_3$ -CN from the homologous goat's, ewe's and water buffalo's CNs and to quantify them using a densitometric procedure.

Capillary electrophoresis (CE) is an alternative to the traditional slab gel electrophoresis because it allows to perform automated analyses with very high resolution and very small sample and buffer requirement. Recently, a number of studies have demonstrated that CE is a suitable technique for analysing cows', ewes' and goats' milk proteins [16–18] and the peptides produced from their proteolysis during ripening in dairy products [19,20]. CE has been used to study the proteolysis of CNs permitting the simultaneous determination of changes in CN profiles due to proteolysis and the appearance of degradation products without limitations in molecular size [21,22].

Otte et al. [23] using CE on plasmin digests of CN from bovine milk and cheese, where separation of proteins was performed on the base of positive net charge at pH 2.5, showed that  $\gamma$ -CNs migrated well ahead of the  $\beta$ -CNs in a position close to that of the  $\alpha_{s1}$ -CNs, in agreement with the mobility observed in conventional gel electrophoresis. In similar pH conditions, Recio et al. [16] identified the  $\gamma$ -CNs arising from the action of plasmin on the different genetic variants of  $\beta$ -CN. This result was confirmed by further studies [24]. Molina et al. [17] used the same procedure to study  $\gamma$ -CNs in ewe, goat, and cow milk, showing that the  $\gamma_2$ -CNs from the different species had the same migration time. The same behaviour was observed for  $\gamma_3$ -CNs. By comparison of the protein primary structure, it was concluded that they comigrated because they have the same net charge in the experimental conditions [25]. No such study has been published on water buffalo CN so far.

The aim of this work was to study the plasmin primary products from bovine and water buffalo  $\beta$ -CN using an innovative peptidomic approach based on the combination of capillary isoelectric focusing (cIEF) and mass spectrometry. Furthermore, the CE-based approach, being more sensitive and easy to perform when compared to the traditional gel electrophoretic analysis, could improve the application of the isoelectric focusing protocols of analysis of CN plasmin digests as reported in EEC/EU Regulation No. 213/2001 [15].

## 2. Experimental

### 2.1. Milk and CN samples

Individual and bulk milk samples analysed were from Mediterranean water buffaloes (*Bubalus bubalis* L.) reared in Southern Italy area. Whole CN was prepared by acid precipitation of individual skimmed milk as previously described [26].

### 2.2. Isolation of CN fraction by ion-exchange chromatography

Ion-exchange chromatography purification of  $\beta$ -CN samples from either cow or water buffalo was carried out on a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). Whole CN samples were fractionated on a Q-Sepharose Fast Flow anion-exchange column (Pharmacia, Uppsala, Sweden), scaling up the analytical procedure described by Andrews et al. [27] with slight modifications. Briefly, whole CN, at a concentration of 10 g/100 ml in 9 M urea and 0.5% 2-mercaptoethanol, was applied onto a XK 16/40 (240 mm height  $\times$  16 mm I.D.) Amersham Pharmacia Biotech column equilibrated in 25 mM Tris-Cl, 4.5 M urea, 1% 2-mercaptoethanol, pH 8.5, at a flow rate of 3 ml/min. Elution was carried out by a linear sodium chloride gradient from 0 to 0.35 M in 4.5 h. Pooled fractions were extensively dialyzed on cut-off 10 kDa membranes, and then lyophilized.

### 2.3. Hydrolysis of $\beta$ -CN with plasmin

Samples of either  $\beta$ -CN or whole CNs were dissolved 0.2 M ammonium bicarbonate, pH 8.0, and hydrolysed with plasmin (Boehringer, Mannheim, Germany) at 37 °C using a 1:1000 E:S ratio. The reaction was stopped after 1 h by precipitation with 12% trichloroacetic acid (TCA). After centrifugation at 12,000 rpm for 5 min, the precipitate was stored frozen.

### 2.4. Ultra-thin layer isoelectric focusing (utlIEF) analysis

For utlIEF analysis, samples were dissolved in 9 M urea and 0.5%  $\beta$ -mercaptoethanol at a concentration of 2%, and analysed using a PhastSystem electrophoresis system (Amersham Biosciences, Uppsala, Sweden) as previously reported [28].

### 2.5. Capillary isoelectric focusing (cIEF)

cIEF analysis was carried out using a BioFocus 3000 System (Bio-Rad). The cIEF-buffer was 8 M urea, 0.75% (v:v) TEMED, 0.2% (w:v) hydroxypropylmethylcellulose (Sigma-Aldrich, Milan, Italy), 2.743% (w:v) Ampholine (Pharmacia LKB, Uppsala, Sweden), citric acid 0.5 M, pH 8.5. The pH gradient 2.5–9.0 was obtained by mixing Ampholine 2.5–5.0, 4.2–4.9, 5.0–8.0, 7.0–9.0 and 5.0–7.0 in the following volume ratio of 1.0:1.0:1.4:1.4:2.0. The focusing anolyte was 20 mM phosphoric acid; the focusing catholyte was 40 mM sodium hydroxide; the mobilization anolyte was 20 mM phosphoric acid and the mobilization catholyte was a zwitterion solution from Bio-Rad (cat. nos. 148–5030).

Whole CN and FPLC-purified CN fractions were diluted in the cIEF-buffer to a final concentration of 0.043% (w/v) and 0.010% (w/v), respectively. 2-Mercaptoethanol was also added to a final concentration of 0.15% (v/v). After filtering on 0.22  $\mu$ m Millex-GS MCE (Millipore) the solution was loaded in the Biofocus System. The separation was carried out using a hydrophobic coated capillary (J&W DB-1, 126–1013, 240 mm, 50  $\mu$ m). Sample loading was carried out at 14 kV focusing voltage for 240 s and mobilization was carried out at the same voltage for 50 min. The capillary temperature was 25 °C and detection wavelength was 280 nm.

### 2.6. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

MALDI-TOF-MS experiments were carried out on a PerSeptive Biosystems (Framingham, MA, USA) Voyager DE-PRO instrument equipped with a  $N_2$  laser (337 nm, 3 ns pulse width). Each spectrum was taken with the following procedure: 1- $\mu$ l aliquot of sample solution was loaded on a stainless steel plate together with 1  $\mu$ l of matrix  $\alpha$ -cyano-4-hydroxycinnamic acid (10 mg in 1 ml of aqueous 50% acetonitrile) or 1  $\mu$ l of sinapinic acid (10 mg in 1 ml of water). For each mass spectrum acquisition was performed in the positive linear mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards (PerSeptive Biosystems).

### 2.7. Liquid chromatography-electrospray mass spectrometry (LC/ESI-MS) analysis

Liquid chromatography-electrospray mass spectrometry (LC/ESI-MS) analysis was performed using an HP 1100 modular system (Agilent Technology, Palo Alto, CA, USA), on-line connected with a Platform single quadrupole mass spectrometer (Waters, Manchester, UK). CN digests were loaded on a 218TP52, 5  $\mu$ m Vydac C18, 250 mm  $\times$  2.1 mm I.D. (Vydac, Hesperia, CA, USA) column. Solvent A was 0.3 ml trifluoroacetic acid/l water and solvent B 0.2 ml trifluoroacetic acid/l acetonitrile. Dried sample

was dissolved in 10  $\mu$ l solvent A and injected on the HPLC column equilibrated with the solvent A. A linear gradient from 0% to 60% solvent B was applied over 60 min with a flow rate of 50  $\mu$ l/min. The column effluent was split 1:25 to give a flow rate of about 4  $\mu$ l/min into the electrospray nebulizer. The bulk of the flow was run through the detector for peak collection as measured by following the absorbance at 220 nm. The electrospray mass spectra were scanned from 1800 to 400  $m/z$  at scan cycle of 5 s/scan. The source temperature was 100°C and the cone voltage was 40 V. Mass values were reported as average masses. Mass scale calibration was carried out with horse heart myoglobin.

Signals recorded in the mass spectra of peptides were associated with the corresponding  $\beta$ -CN peptides on the basis of the molecular mass, taking into account the enzyme specificity and the reported amino  $\beta$ -CN sequence from the bovine and water buffalo species [25,29].

## 2.8. Sequence analysis

Automated Edman degradation was performed using an Applied Biosystems mod. 477A Protein Sequencer with on-line phenylthiohydantoinyl amino acid (Pth-Xaa)-HPLC analyser.

## 3. Results and discussion

### 3.1. cIEF analysis of bovine and water buffalo CN

The first step in developing a cIEF-based procedure for analysis of cow (C) and water buffalo (WB) CN consisted in protein isolation from milk by isoelectric precipitation. In Fig. 1, the cIEF elution profiles of the resulting bovine and buffalo CN (a previously standardised sample containing  $\alpha_{s1}$ -CN BC,  $\kappa$ -CN AB and  $\beta$ -CN A<sub>1</sub>A<sub>2</sub> at the heterozygous state) in the pH range 3.5–9.0, is shown in comparison with the corresponding “traditional” utlIEF pattern carried out according to the EEC official method in the same pH range.

In both species, the CN fractions eluted from capillary column are in the order:  $\kappa$ -CN,  $\alpha_{s2}$ -CN,  $\beta$ -CN and  $\alpha_{s1}$ -CN. Each CN fraction produced several peaks on the electropherogram either due to differently phosphorylated components or to the co-presence of the genetic variants. In the cow sample, the four peaks of  $\alpha_{s1}$ -CN, the genetic variants B and C in heterozygous state are eluted each consisting in the two phosphorylated components  $\alpha_{s1}$ -CN with 8 and 9 phosphate groups, respectively. Also  $\beta$ -CN and  $\kappa$ -CN were found in heterozygous state  $\beta$ -CN A<sub>1</sub>A<sub>2</sub> and  $\kappa$ -CN AB, which are the most common phenotypes at those CN loci. The genotype of  $\alpha_{s2}$ -CN was the most common variant  $\alpha_{s2}$ -CN A, which was consisting in almost four phosphorylated components (11P–13P). The genotype of the four CN fraction in Mediterranean buffalo breed, instead, to date is reported to be very monomorphic at all four CN loci, as shown in the figure where the heterogeneity of  $\alpha_{s1}$  and  $\alpha_{s2}$ -CN was due to the phosphorylation degree only.

Furthermore, comparing the two cIEF profiles of bovine and buffalo CNs a similar elution time is shown between: buffalo  $\alpha_{s1}$ -CN and bovine  $\alpha_{s1}$ -CN B, buffalo  $\beta$ -CN and bovine  $\beta$ -CN A<sub>2</sub>, buffalo  $\kappa$ -CN and bovine  $\kappa$ -CN B, respectively. The most common bovine  $\alpha_{s2}$ -CN A, instead had a higher elution time compared to the buffalo counterpart. The same results were obtained comparing the utlIEF profiles of the two species shown in Fig. 1. All electrophoretic patterns were consistent with the amino acid differences present in the primary structure of buffalo  $\alpha_{s1}$ -CN and  $\beta$ -CN with respect to bovine counterpart as reported by Ferranti et al. [29] and  $\kappa$ -CN reported by Das et al. [30]. Finally, the close correspondence existing between the component elution order in the cIEF electropherogram and the one on the utlIEF gel, as outlined in Fig. 1 must be emphasized. In conclusion, both the two analytical approaches

provide a very efficient separation of the four CN fractions  $\alpha_{s1}$ ,  $\alpha_{s2}$ ,  $\beta$  and  $\kappa$  in the two ruminant species.

### 3.2. MALDI-TOF-MS characterization of $\beta$ -CN from bovine and water buffalo

The  $\beta$ -CN fraction from either bovine or water buffalo individual milks, purified by ion-exchange chromatography according to Andrews et al. [27] was analysed using MALDI-TOF-MS in order to confirm their identity. The results (data not shown) confirmed the CN origin of these fractions and particularly that bovine  $\beta$ -CN in the sample consisted of a mixture of the two common variants A<sub>1</sub> (MW 24,022 Da) and A<sub>2</sub> (MW 23,982 Da). This polymorphism could not be detected by ion exchange chromatography because, in the experimental conditions employed (pH 7.0), the substitution His-Pro at position 67 [25,31] did not induce any net charge difference in the two genetic variants.

The MALDI-TOF-MS results relative to the buffalo  $\beta$ -CN demonstrated, instead, that this protein was  $\beta$ -CN variant B (MW 23,982 Da) in the homozygous state. The presence of this single variant was expected since, as highlighted by Ferranti et al. [29], the  $\beta$ -CN A variant is only found in Venezuelan populations.

### 3.3. Comparison between utlIEF and cIEF analysis of bovine and buffalo $\beta$ -CN hydrolysates by plasmin

After hydrolysis with plasmin, the fraction of hydrolysed  $\beta$ -CN insoluble in 12% TCA was analysed using utlIEF in pH gradient (7.7–5.0) (Fig. 2A) and cIEF analysis (Fig. 2B). The utlIEF profiles of

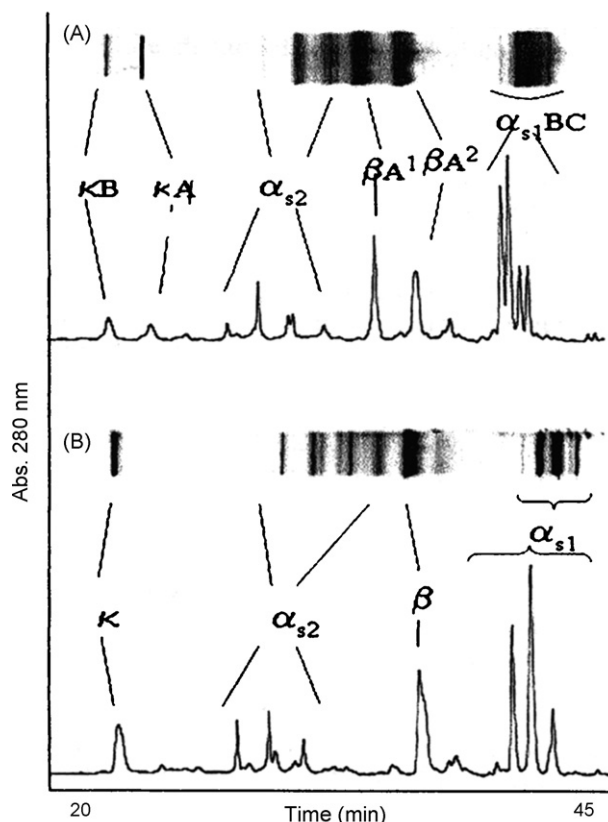


**Fig. 1.** Electropherogram comparison of bovine (A) and water buffalo (B) CN. Gel isoelectric focusing in the pH range 3.5–9.0 according to the EU official method (Commission Regulation No. 213/2001); cIEF in the pH range 3.5–9.0 (Biofocus 3000 System Bio-Rad); capillary coated 25 cm length  $\times$  50  $\mu$ m I.D. (Bio-Rad). Running buffer: 8 M urea, 0.75% TEMED, 0.2% HPMC, Pharmacia ampholytes 2.2%; Bio-Rad electric buffer. Focusing conditions: 14 kV, 240 min; mobilisation: 14 kV.

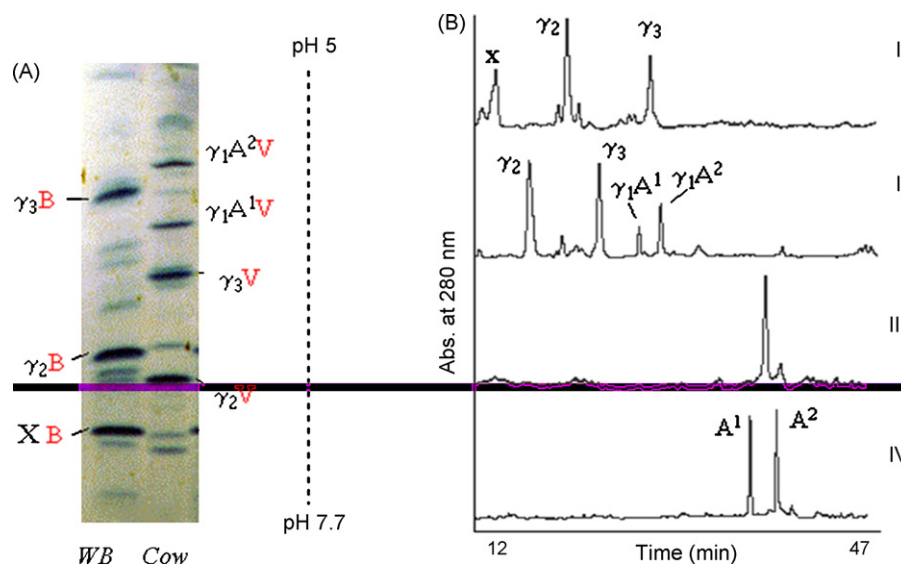


**Fig. 2.** (A) utlIEF analysis of the plasmin digests of FPLC purified whole cow and water buffalo  $\beta$ -CN, compared to (B) cIEF analysis of the plasmin digests of FPLC purified whole water buffalo (I) and cow (II) CN; (III): intact water buffalo  $\beta$ -CN; (IV): intact cow  $\beta$ -CN.

the peptides produced by plasmin action were composed by three main components for buffalo and four main components for cow, respectively. In the profile of the plasmin digest of bovine  $\beta$ -CN, the  $\gamma_1$ -CN  $A_1$  band was located in a more alkaline zone than the  $\gamma_1$ -CN  $A_2$  variant due to the already mentioned amino acid substitution His-Pro at position 67 [25,31]. In the profile of hydrolysed buffalo  $\beta$ -CN, a third main band was also found showing the highest pI value, indicated as “X” in the figure.

In addition to the main components, the presence of unknown minor peptides was also observed in both species. They were probably generated by the non-specific action of the plasmin on other Lys-X or Arg-X type bonds, which are less “exposed” and, therefore, produce peptide bands with a lower intensity.

The same number of components was observed in cIEF electropherogram (Fig. 2B, I and II) where three main peaks for buffalo and four main peaks for cow, in agreement with the results obtained using the gel technique (Fig. 2A) were present, respectively. By comparing the electrophoretic patterns obtained using the two techniques, it was possible to identify the main components of the cIEF electropherograms.

The above cIEF analysis was then applied to resolving mixtures of buffalo milk with 1%, 5%, 10% and 50% of cow milk added (Fig. 3). In these samples, the species-specific markers consisted of cow  $\gamma_1$ -CN derived from both the most common  $A_1$  and  $A_2$  genetic variants, and of the terminal products of plasmin action,  $\gamma_2$  and  $\gamma_3$ . Comparing the elution time and area of the peaks corresponding to the  $\gamma$ -CN components for each determined amount of cow milk added to buffalo milk, we could observe that all the four  $\gamma$ -CN components were positively related to the increase of added amount of cow milk to buffalo milk. Furthermore, from the results shown in Fig. 3 we can conclude that the species-specific markers of cow are detectable since 5% of cow milk addition. This level should be further decreased to make the sensitivity of the method comparable to the traditional one (0.5%) [15].

### 3.4. MALDI-TOF-MS and LC/ESI-MS analysis of the plasmin digest of bovine and water buffalo $\beta$ -CN

The peptide fraction insoluble in 12%TCA from the plasmin digests of bovine and water buffalo  $\beta$ -CN was analysed using MALDI-TOF-MS for detailed structural analysis.

The MALDI-TOF mass spectra are shown in Fig. 4, and the identification of peptides, obtained by combining MW measurement with the known enzyme specificity of plasmin, is reported in Table 1. MALDI-TOF-MS analysis showed that, in the experimental conditions employed for plasmin hydrolysis of bovine  $\beta$ -CN, very small

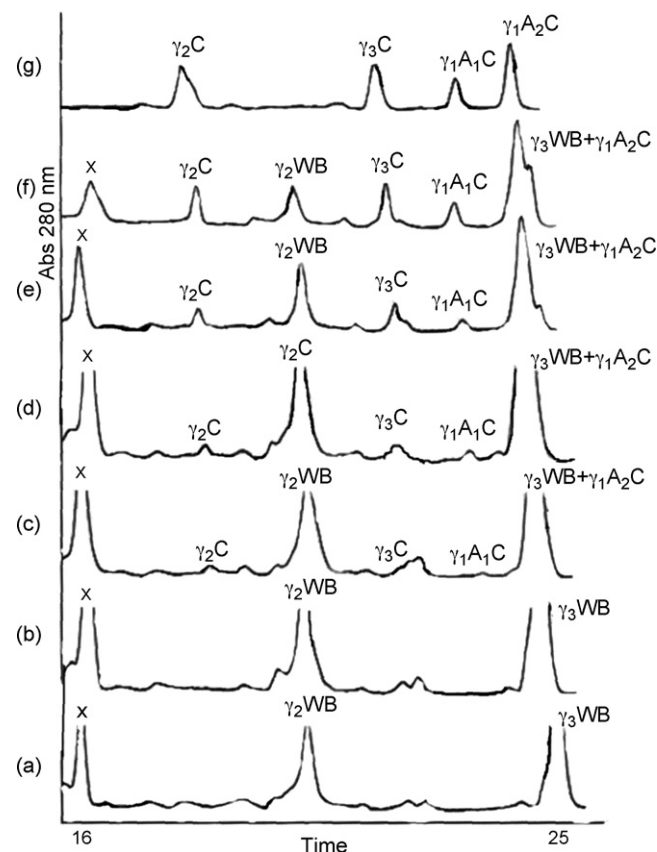


**Fig. 3.** cIEF electropherograms in the pH range 3.5–9.0 showing the separation of the  $\gamma$ -CN fractions from water buffalo (WB) milk (a), cow (C) milk (g), and water buffalo milk with 1% (b), 5% (c), 10% (d), 25% (e) and 50% (f) of cow milk added. Milk samples were treated with plasmin according to EU official method, Commission Regulation No. 213/2001.

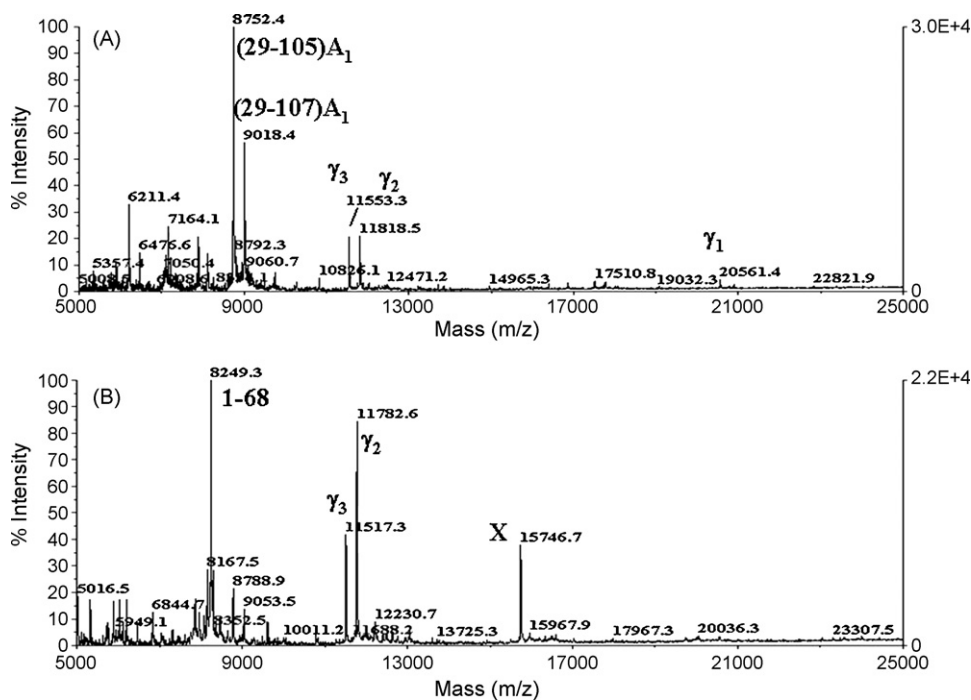


Fig. 4. MALDI-TOF-MS analysis of plasmin hydrolysates of cow (A) and water buffalo (B)  $\beta$ -CN.

amounts of PPs 29–107 and 29–105 and of small size non-specific fragments were formed together with the  $\gamma$ -CNs ( $\gamma_1$ -CN A<sub>1</sub>,  $\gamma_1$ -CN A<sub>2</sub>,  $\gamma_2$ -CN and  $\gamma_3$ -CN). In the case of hydrolysed buffalo  $\beta$ -CN, on the other hand,  $\gamma_1$  was absent, while the most abundant components were  $\gamma_2$ - and  $\gamma_3$ -CN, and a novel peptide at 15,476 Da. A peptide with this molecular weight had been detected previously by Cozzolino et al. [32,33] in the analysis of water buffalo CNs by MALDI-TOF-MS.

To characterize the peptide 15,476 Da, the 12% TCA insoluble fraction of the plasmin digest of water buffalo  $\beta$ -CN was analysed by LC/ESI-MS analysis (Fig. 5) in comparison with its bovine homologous (results in Table 2). Automated sequence determination of the purified peptide fraction showed that the peptide at 15476 Da had the N-terminal sequence Ser-Leu-Pro-Gln-Asn. This information, combined with that on the molecular mass, allowed to unambiguously identify the peptide as  $\beta$ -CN (69–209).

**Table 1**  
Peptides identified in the plasmin hydrolysate of bovine and water buffalo beta casein by MALDI-TOF-MS analysis

| Water buffalo |                        |        | Cow     |                            |
|---------------|------------------------|--------|---------|----------------------------|
| MW (Da)       | Peptide                | Notes  | MW (Da) | Peptide                    |
| 15,747        | 69–209 ( $\gamma_4$ )  |        | 20,521  | $\gamma_1$ -A <sub>2</sub> |
| 11,782        | 105–209 ( $\gamma_2$ ) |        | 20,561  | $\gamma_1$ -A <sub>1</sub> |
| 11,517        | 107–209 ( $\gamma_3$ ) |        | 11,822  | $\gamma_2$                 |
| 8,751         | 1–68                   | Lys 68 | 11,556  | $\gamma_3$                 |
| 4,483         | 170–209                |        | 10,829  | 114–209                    |
| 4,218         | 133–169                |        | 9,021   | 29–107A1                   |
| 3,722         | 177–209                |        | 8,981   | 29–107A2                   |
| 2,910         | 184–209                |        | 8,756   | 29–105A1                   |
|               |                        |        | 8,716   | 29–105A2                   |
|               |                        |        | 6,559   | 49–107A1                   |
|               |                        |        | 6,519   | 49–107A2                   |
|               |                        |        | 6,294   | 49–105A1                   |
|               |                        |        | 6,254   | 49–105A2                   |
|               |                        |        | 4,484   | 170–209                    |
|               |                        |        | 4,218   | 133–169                    |
|               |                        |        | 3,782   | 177–209                    |
|               |                        |        | 2,910   | 184–209                    |

A further confirmation of this peptide identity is provided by the finding, by both MALDI-TOF-MS and LC/ESI-MS analysis, of the complementary peptide 1–68 (MW 8752) of  $\beta$ -CN, carrying 5 phosphate groups, which was present only in the water buffalo digest, and absent in the bovine one (Figs. 3 and 4, Tables 1 and 2).

By comparing the primary structure (Fig. 6) [25,29,31] of the  $\beta$ -CNs from the different species, it can be seen that the buffalo  $\beta$ -CN has a Lys residue in position 68, while the homologous bovine  $\beta$ -CN has an Asn residue. This difference would justify the production of the fragment 69–209 in buffalo  $\beta$ -CN only. Moreover, the absence of  $\gamma_1$ -CN (29–209) in the plasmin digest, is suggestive either that this fragment is not formed or, most probably, it is rapidly cleaved off at position 68 of the protein sequence soon after formation. The identification of peptide 1–68 seems to support the first possibility.

3.5. Localisation of component X as  $\beta$ -CN (69–209) along the electrophoretic profiles of plasmin hydrolysates of water buffalo  $\beta$ -CN

As expected, the cIEF analysis of the hydrolysed buffalo  $\beta$ -CN also revealed an extra peak (Fig. 2B), not occurring in the bovine counterpart. Given its abundance, this component could correspond to peptide 69–209 of  $\beta$ -CN. In fact, in both the MALDI-TOF-MS and LC/ESI-MS analysis of the plasmin hydrolysed buffalo material, it was found in amount comparable to the buffalo  $\gamma$ -CN. The identity of the peptide was confirmed by cIEF analysis of the peptide purified through LC/ESI-MS analysis of Fig. 5 showed that the peptide comigrated with the unknown component.

In order to further support this hypothesis through the use of the Expert Protein Analysis System (ExPASy) program developed by Swiss Institute of Bioinformatics (SIB), the theoretical isoelectric point (pI) of buffalo peptide 69–209 and of all the bovine and buffalo  $\gamma$ -CNs identified were calculated, as reported in Table 2. The presence of phosphorylated serine residues ascertained by mass spectrometry analysis was also taken into account. These data were used to construct a “theoretical” utlIEF profile in order to carry out

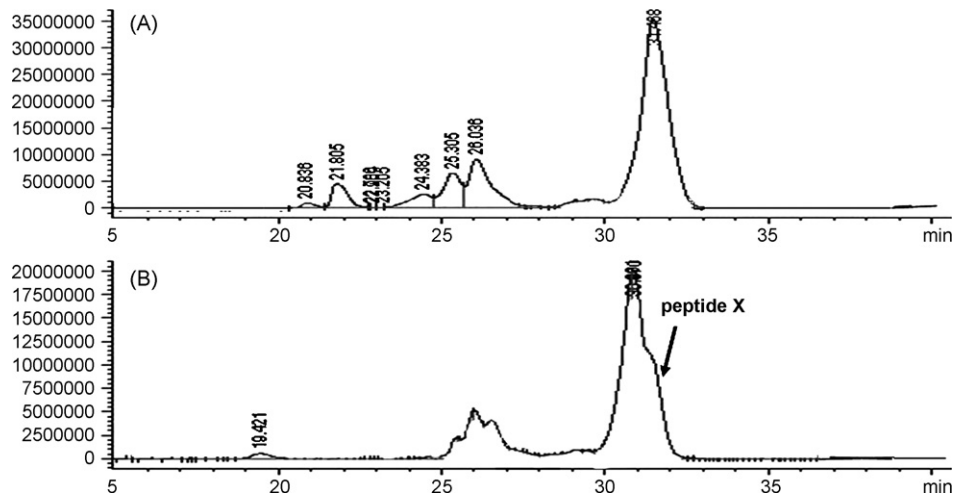


Fig. 5. LC/ESI-MS analysis of plasmin digest of cow (A) and water buffalo (B)  $\beta$ -CN.

Table 2

Peptides identified in the plasmin hydrolysate of bovine and water buffalo beta casein by LC/ESI-MS analysis

| Water buffalo |         |                        |                         |                            | Cow      |         |                            |                            |                 |
|---------------|---------|------------------------|-------------------------|----------------------------|----------|---------|----------------------------|----------------------------|-----------------|
| tR (min)      | MW (Da) | Peptide                | Notes                   | Calculated pI <sup>a</sup> | tR (min) | MW (Da) | Peptide                    | Calculated pI <sup>a</sup> | Notes           |
| 20.6          | 2,237   | 49–68                  |                         | 8.60                       | 19.2     | 7,220   | 30–91                      | 4.69                       | 1P <sup>b</sup> |
| 21.6          | 4,809   | 29–68 P                | Lys 68, 1P <sup>b</sup> | 4.49                       | 23.3     | 7,182   | 30–91                      | 4.49                       | 1P <sup>b</sup> |
|               | 4,681   | 30–68 P                | Lys 68, 1P <sup>b</sup> | 4.25                       |          | 7,054   | 29–91                      | 4.29                       | 1P <sup>b</sup> |
|               | 4,310   | 33–68 P                | Lys 68, 1P <sup>b</sup> | 4.08                       | 25.3     | 9,021   | 29–107A <sub>1</sub>       | 5.46                       | 1P <sup>b</sup> |
|               | 4,264   | 69–107                 | MetOx                   |                            |          | 8,981   | 29–107A <sub>2</sub>       | 5.57                       | 1P <sup>b</sup> |
|               | 3,997   | 69–105                 | MetOx                   |                            |          |         |                            |                            |                 |
| 24.9          | 4,247   | 69–107                 |                         | 9.52                       | 25.8     | 8,756   | 29–105A <sub>1</sub>       | 4.89                       | 1P <sup>b</sup> |
| 25.9          | 3,982   | 69–105                 |                         | 8.22                       | 26.3     | 8,716   | 29–105A <sub>2</sub>       | 4.68                       | 1P <sup>b</sup> |
| 26.2          | 8,752   | 1–68                   | 5P <sup>b</sup>         | 3.89                       | 28.2     | 8,118   | 106–176                    | 6.44                       |                 |
| 26.7          | 8,774   | 29–105                 | 1P <sup>b</sup>         | 4.91                       | 28.9     | 4,483   | 170–209                    | 8.47                       |                 |
|               | 6,201   | 49–105                 |                         | 9.40                       | 29.3     | 3,721   | 177–209                    | 6.11                       |                 |
| 28.8          | 4,483   | 170–209                |                         | 8.47                       | 30.4     | 20,521  | $\gamma_1$ -A <sub>2</sub> | 5.62                       | 1P <sup>b</sup> |
| 29.5          | 3,722   | 177–209                |                         | 6.11                       |          | 20,561  | $\gamma_1$ -A <sub>1</sub> | 5.47                       | 1P <sup>b</sup> |
| 30.5          | 11,782  | 106–209 ( $\gamma_2$ ) |                         | 6.30                       | 30.5     | 11,822  | $\gamma_2$                 | 6.44                       |                 |
| 31.4          | 15,747  | 69–209 ( $\gamma_4$ )  |                         | 6.80                       | 31.4     | 11,556  | $\gamma_3$                 | 5.82                       |                 |
| 31.8          | 11,517  | 108–209 ( $\gamma_3$ ) |                         | 5.49                       |          |         |                            |                            |                 |

<sup>a</sup> pI: calculated isoelectric point value of the peptide.

<sup>b</sup> P: phosphate group.

|     |     |     |     |     |
|-----|-----|-----|-----|-----|
| 1   | 10  | 20  | 30  | 40  |
| R   | E   | L   | E   | E   |
| R   | E   | L   | E   | E   |
| 41  | 50  | 60  | 70  | 80  |
| T   | E   | D   | E   | L   |
| M   | E   | D   | E   | L   |
| 81  | 90  | 100 | 110 | 120 |
| P   | V   | V   | V   | P   |
| P   | V   | V   | V   | P   |
| 130 | 140 | 150 | 160 |     |
| E   | S   | Q   | S   | L   |
| E   | S   | Q   | S   | L   |
| 170 | 180 | 190 | 200 |     |
| S   | V   | L   | S   | L   |
| S   | V   | L   | S   | L   |
| 201 | 209 |     |     |     |
| V   | R   | G   | P   | F   |
| V   | R   | G   | P   | F   |

Fig. 6. Comparison of cow (A) and water buffalo (B)  $\beta$ -CN amino acid sequence.

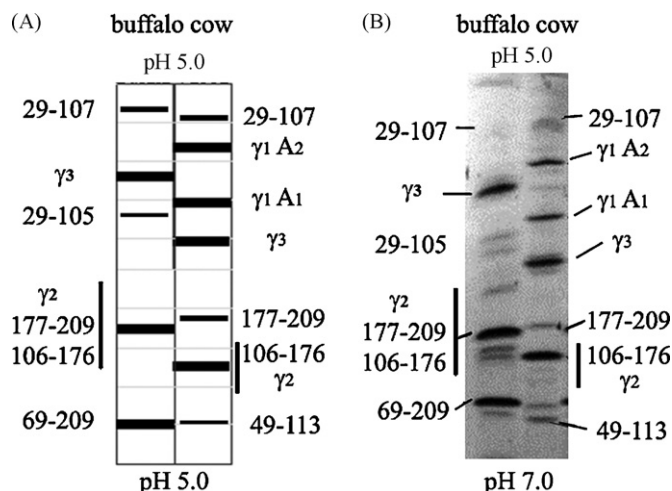


**Fig. 7.** Comparison of predicted (A) and experimental (B) utlIEF profiles of cow and water buffalo  $\beta$ -CN plasmin digests.

a comparison with the real utlIEF profile (Fig. 7). The “theoretical” profile was obtained assuming a hypothetical linear type variation of in the pH range  $5.0 < \text{pH} < 7.7$ .

The comparison shows that the two profiles coincide and that the peptide 69–209, with an estimated *pI* of 6.8, was located in a more alkaline zone of the theoretical electropherogram (Fig. 6, II) than the bovine  $\gamma_2$ . This evidence confirmed the identification of the unknown band as peptide 69–209.

We therefore propose that peptide 69–209, being produced by plasmin proteolysis of water buffalo  $\beta$ -CN, similarly to  $\gamma_1$ -,  $\gamma_2$ -, and  $\gamma_3$ -CN, can be referred to as  $\gamma_4$ -CN. This component is peculiar of water buffalo  $\beta$ -CN, and can be considered a molecular marker of the presence of water buffalo CN.

#### 4. Conclusions

The primary aim of any PDO label is to protect the typical products of a given area against saled similar non-typical products. The cIEF method developed made it possible to separate and identify all the  $\gamma$ -CNs produced by hydrolysis of the two species and then this procedure could be used for the detection of fraudulent addition of cow milk to buffalo milk in the production of Buffalo Mozzarella cheese. By comparing the two techniques used, utlIEF and cIEF, it can be seen that this latter is faster as it does not involve staining and requires shorter processing times; however it has to be outlined

that, at this stage, the lowest quantity of cow milk added was 5% and further work is needed to decrease this sensitivity threshold.

Finally, the results of this study suggest that the cIEF, in combination with mass spectrometry for structural confirmation when necessary, may be a useful analytical tool for studying the products of enzymatic hydrolysis of  $\beta$ -CN and the genetic polymorphism of individual CN fractions.

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