



Donkeys' milk protein fraction investigated by electrophoretic methods and mass spectrometric analysis

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ABSTRACT

Donkeys' milk has attracted a growing interest in human nutrition, particularly in the dietotherapy of patients affected by cows' milk protein allergy. In this paper, donkeys' milk protein fractions were analysed by isoelectric focusing (IEF) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Four different IEF patterns were observed out of 51 individual milk samples. Thirty-six samples presented a common pattern, used as a reference, whereas 15 were characterized by the presence or absence of protein bands. A single sample, lacking two bands of the casein fraction, showed an apparent reduced amount of caseins, by SDS-PAGE as well as by chromatographic analysis. Mass spectrometry and high-performance liquid chromatography were used to characterize the IEF patterns. Compared with the reference, one pattern lacked two α_{s1} -casein bands, another lacked two β -lactoglobulin II bands and one accounted for a different β -lactoglobulin II variant. No signals related to putative α_{s2} - and κ -casein were observed.

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1. Introduction

Milk is a physiologically and nutritionally balanced secretion, suitable to every requirement of newborns, hence human milk is the best food for babies with healthy mothers who have appropriate nutritional stores. When breast-feeding is not possible or after weaning, finding an adequate alternative nourishment becomes mandatory.

Cows' milk is widely used as a substitute for human milk but, in an increasing number of cases, it can lead to an abnormal immunological response. In this respect, cows' milk protein allergy (CMPA) is the most common food allergy, affecting about 3% of children in the first three years of life (Sampson, 2004). Hypoallergenic alternatives to breast milk have been proposed through the years and include extensively hydrolysed formulas (eHF), amino acid formulas (AAF) and soy formulas (SF). Extensively hydrolysed formulas, industrial milk protein-based products treated with proteolytic enzymes, are recommended worldwide as the first choice for the dietotherapy of CMPA patients (American Academy of Pediatrics Committee on Nutrition, 2000; Høst et al., 1999). Nonetheless babies can still suffer from allergies even when fed eHF

that may contain residual allergenic epitopes; poor palatability may also be a problem (Høst and Halken, 2004; Ragno et al., 1993).

Amino acid formulas are the only breast milk substitute considered to be non-allergenic, but the unpleasant bitter taste often precludes their use in infant diet (Høst and Halken, 2004). Soy formulas, although providing a moderate palatability and in many cases good nutritional benefits, are not generally recommended before the age of 6 months for infants with IgE-associated CMPA symptoms (Bellioni-Businco et al., 1999). A high frequency of soy protein allergy also has been reported in these infants (American Academy of Pediatrics Committee on Nutrition, 2000).

In the last few years, milk from non-bovine mammals (goat, donkey, mare, camel) has been studied to identify the best natural substitute for human milk (Businco et al., 2000; El-Agamy et al., 1997; Muraro et al., 2002; Restani et al., 2002). Donkeys' milk was found to be a valid alternative to both IgE-mediated and non-IgE-mediated cows' milk protein allergy, with favourable effects on palatability and weight–height gain in an increasing number of clinical trials (Carroccio et al., 2000; Iacono et al., 1992; Monti et al., 2007), although the mechanism of this tolerance, likely related to the protein composition, has not yet been fully clarified. The high lactose content, the characteristic protein profile with a low casein content, and the relevant percentage of essential amino acids, make this milk a potential new dietetic food and a breast milk substitute (Guo et al., 2007).

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Nowadays, in Italy, the consumption of donkeys' milk is gradually increasing thanks to the interest of those consumers searching for a natural product to replace human or bovine milk when needed. In the last few years, the number of farms breeding donkeys has notably risen and in 2006 the Italian Breeders Association (AIA) introduced functional controls on donkey milk production. According to the Italian law, this product can only be sold directly from the farm as raw milk (Regio Decreto 1929 n° 994, paragraph 15, 43). Notwithstanding this renewed growing interest, donkeys' milk has not been completely investigated and, in particular, the data available for its protein component are still poor (Cunsolo et al., 2007a; Herrouin et al., 2000; Miranda et al., 2004; Vincenzetti et al., 2005).

Over the last 20 years, the use of mass spectrometric methods has clearly proven to be a powerful tool for the detection and characterization of different components in complex protein mixtures. *Equidae* milk protein fraction was also investigated and characterized by different mass spectrometric approaches (Cunsolo et al., 2006; Girardet et al., 2006; Iametti et al., 2001; Miclo et al., 2007; Neveu et al., 2002).

The aim of this work was to investigate the protein fraction of individual donkey milk samples produced in Sicily. A combined approach, coupling different electrophoretic methods, reverse phase high-performance liquid chromatography (RP-HPLC), trypsin digestion and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis, was used to characterize isoelectrophoretic protein patterns.

2. Materials and methods

2.1. Sample preparation

Individual milk samples were collected from 51 Ragusano breed donkeys at middle lactation stage from three farms in Sicily. Milk samples, kept cool at 4 °C, were defatted by centrifugation (1700 × g, 30 min) at 15 °C; the fat layer was solidified at –20 °C for 15 min and removed. Separation of the protein fraction into caseins and whey proteins was conducted according to a modified method from Ochirkhuyag et al. (2000). Specifically, whole casein was obtained from skimmed milk by isoelectric precipitation (pH 4.2) at 22 °C, using 1 mol L⁻¹ HCl. The precipitate was washed twice with distilled water at pH 4.2, solubilized at pH 7 by addition of 1 mol L⁻¹ NaOH, precipitated again at pH 4.2 with 1 mol L⁻¹ HCl and washed three times with distilled water. Finally, the whole casein was solubilized at pH 7, freeze-dried and stored at –20 °C. The supernatant, containing the whey proteins, was recovered by centrifugation (2800 × g, 30 min) at room temperature and subsequently stored at –20 °C.

2.2. Electrophoretic analysis

Isoelectric focusing (IEF) analysis with carrier ampholytes, using ultra thin (0.3 mm) polyacrylamide gels containing urea, was performed using a Multiphor II Flatbed Electrophoresis System (Amersham Biosciences, Uppsala, Sweden) according to a modified method of Erhardt (1989). Gels of pH 2.5–10 and 2.5–6 gradients were ran at a constant temperature of 13 °C and stained with Coomassie Blue R350. Specifically, a pH 2.5–10 gradient was obtained by mixing 3–10, 2.5–5, 4.2–4.9 and 5–6 Ampholine™ (Amersham Biosciences, Uppsala, Sweden) in a 2.1:3.3:2.3:2.3 volume ratio, whereas a pH 2.5–6 gradient was obtained by mixing 2.5–5, 4.2–4.9, 4.5–5.4 and 5–6 Ampholine™ in a 3.5:2.4:1.7:2.4 volume ratio. IEF runs were performed on skim milk, as well as on casein and whey protein fractions. Gels were pre-focused at a constant value of 3000 V and at the maximum limit of 20 mA for 180 V h⁻¹; the subsequent two running steps were set at 3000 V and 30 mA, for 300 V h⁻¹ and 3000 V h⁻¹, respectively.

Images of IEF gels were acquired by an office scanner and analysed by Image-Pro Plus software ver. 6.0 (MediaCybernetics, Bethesda, MD, USA). The individual samples were grouped into different patterns according to the occurrence of proteins bands.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a standard vertical gel electrophoretic apparatus (Protein® II xi Cell–BioRad, Hercules, CA, USA) using 14% polyacrylamide gels which were stained with Coomassie Blue R250 (Grosclaude et al., 1987).

2.3. In-gel digestion of protein bands

Selected pieces were excised from Coomassie Blue-stained IEF gels, washed, in-gel reduced with dithiothreitol, S-alkylated with iodoacetamide, and subjected to in-gel trypsin digestion according to Shevchenko et al. (1996). The gel pieces were swollen in a digestion buffer containing 50 mM NH₄HCO₃ and 12.5 ng μL⁻¹ trypsin (modified porcine trypsin, sequencing grade, Promega, Madison WI, USA) in an ice bath. After 30 min the supernatant, containing an excess of trypsin, was removed and discarded and 50 μL of 50 mM NH₄HCO₃ was added to the gel pieces. Digestion was allowed to proceed overnight at 37 °C. The enzymatic reaction was stopped by cooling the gel pieces and supernatant solution at –24 °C.

2.4. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry analysis

Supernatant solutions (10 μL) containing tryptic peptides were used for micro-purification (desalting/concentration) of peptides with a homemade 5-mm nanocolumn packed with C18 resin (POROS R2; PerSeptive Biosystems, Foster City, CA, USA) in a constricted GELoader tip (Eppendorf Scientific, Westbury, NY, USA), according to Gobom et al. (1999). A syringe was used to force liquid through the columns by applying gentle air pressure. The columns were washed with 10 μL 0.1% trifluoroacetic acid (TFA) and the bound peptides subsequently eluted directly onto the MALDI target with 0.6 μL matrix solution (10 mg mL⁻¹ α-cyano-4-hydroxycinnamic acid in 70%, v/v CH₃CN and 0.1%, w/v TFA).

MALDI mass spectra were acquired in positive ion reflectron-delayed extraction mode over a *m/z* range of 600–4000, using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a UV nitrogen laser (337 nm), and 150 shots were averaged to improve the S/N ratio. The *m/z* software (Proteometrics, New York, NY, USA) was used to analyze MS spectra. MALDI-TOF spectra were internally calibrated using porcine trypsin autolysis products (*m/z* 842.51 and 2211.10). When trypsin autolysis peaks could not be detected, bovine β-lactoglobulin (Aldrich, St. Louis, MO, USA), tryptic peptides (*m/z* 837.48, 2313.26 and 2707.38) were used for external mass calibration. So-called project-specific contaminants (peaks present in all mass spectra, for the most part originating from contaminating human keratin or the trypsin added for digestion of proteins) were identified and eliminated from the MS spectra using the software PeakEraser (Peri et al., 2001; <http://www.welcome.to/GPMaw>).

Protein identification was performed by searching the MALDI-MS spectra against a non-redundant protein sequence database (NCBI nr) via MASCOT Peptide Mass Fingerprint tool of the MASCOT program (<http://www.matrixscience.com>), limited to the Mammalia taxonomy. The following parameters were used for a standard database search: monoisotopic mass accuracy, 80 ppm; missed cleavages, 2; complete carbamidomethylation of cysteines, partial oxidation of methionines and partial transformation of N-terminal glutamine and N-terminal glutamic acid residues in the pyroglutamic acid form. The Probability Based Mowse Score of proteins was obtained by $[-10 \times \log(P)]$, where P is the probability that the observed match is a random event. For positive

identification, the protein score had to be over the significance threshold level ($P < 0.05$). In addition, significant hits were carefully inspected manually to eliminate possible false positives, based on criteria such as mass error distribution and the likelihood of missed cleavages.

2.5. Analytical reversed phase high-performance liquid chromatography

Analytical reversed phase high-performance liquid chromatography (RP-HPLC) was carried out on a Varian 9010 (Sunnyvale, CA, USA), equipped with a Varian 9050 Ultraviolet (UV) detector. Data were acquired with a PC using the software system "Peak Simple II" (SRI Instruments, Torrance, CA, USA). The solutions of whey fraction (dissolved in sodium acetate/acetic acid buffer) and phosphorylated casein fraction (solubilized in 50 mM NH_4HCO_3 , pH 8.2) were filtered on Micro-spin filters (Alltech, Milan, Italy) and loaded onto a reversed phase analytical Vydac C4, column (4.6×250 mm, 300 Å, 5 μm). The column was eluted at 50 °C with a linear gradient of solvent B ($\text{CH}_3\text{CN} + 0.05\%$ TFA) in A ($\text{H}_2\text{O} + 0.05\%$ TFA) from 35% to 55% over 60 min. Flow rate was 1 mL min^{-1} . Peaks were detected by their absorption at 224 nm, collected manually and freeze-dried.

2.6. Tryptic digestion of the collected chromatographic peaks

The collected chromatographic peaks were dissolved in 50 mM NH_4HCO_3 , pH 8.2, at a concentration of approximately 1 $\mu\text{g mL}^{-1}$, and subjected to enzymatic digestion with modified porcine trypsin. Specifically, modified porcine trypsin, dissolved in 50 mM NH_4HCO_3 , pH 8.2, was added at a molar enzyme to substrate ratio of 1:50 and the solution was incubated at 37 °C for 4 h. The digestion was stopped by cooling in liquid nitrogen.

3. Results

3.1. Electrophoresis

The first screening by IEF was carried out on skim milk. The protein fraction of the analysed milk samples was classified according to the occurrence of protein bands. On the basis of this criterion four different IEF patterns, named A, B, C and D, were observed (Fig. 1).

The IEF pattern A was the most common (36 samples; 70.6%) and it was used as a reference for the comparison with the other patterns. Pattern B (1 sample; 2%) and C (12 samples; 23.5%) appeared anomalous: pattern B lacked two bands, identified as A1 and A2, whereas pattern C lacked two bands, identified as A3 and A4 (Fig. 1a). Pattern D (2 samples; 3.9%), with respect to the reference, lacked band A3 and showed a different band, identified as A5 (Fig. 1b).

To identify which fraction each band belonged to, separated casein and whey protein fractions of the reference sample were also investigated by IEF. This analysis revealed that A1 and A2 bands belonged to the casein fraction, whereas A3 and A4 belonged to the whey protein fraction (Fig. 1a). The two samples showing pattern D were also separated into caseins and whey proteins; the IEF analysis of these fractions revealed that A5 band belonged to whey proteins (Fig. 1b).

SDS-PAGE analysis highlighted a pattern similar to that reported by other authors for donkey (Guo et al., 2007; Salimei et al., 2004) and mare milk (Bonomi et al., 1994; Pagliarini et al., 1993), and revealed the most abundant whey proteins such as β -lactoglobulin, lysozyme and α -lactalbumin, as well as the minor components, lactoferrin, serum albumin and immunoglobulins. Conversely, the method did not provide for high resolution of the casein sub-fractions, which co-migrated in a unique large band. However, the assay showed a remarkable reduction in total casein in the IEF pattern B of the anomalous milk sample in comparison with the reference (data not shown).

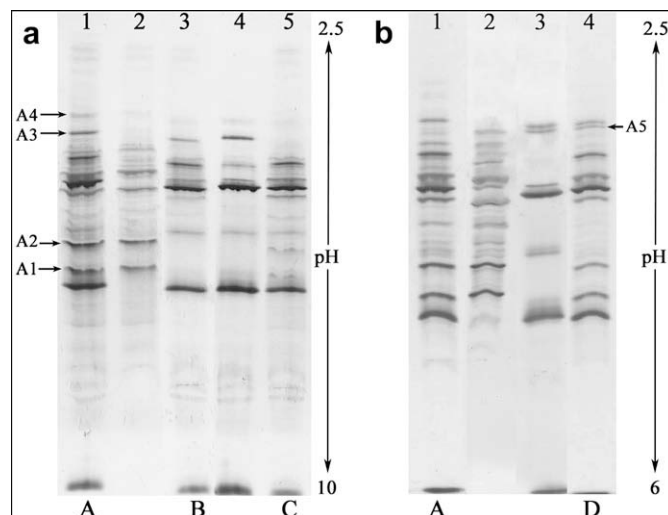


Fig. 1. Isoelectric focusing of donkey skim milk and protein fractions. Panel (a): lane 1, reference milk sample, pattern A; lane 2, casein fraction of reference sample; lane 3, pattern B; lane 4, whey protein fraction of reference sample; lane 5, pattern C. Panel (b): lane 1, reference milk sample, pattern A; lane 2, casein fraction of pattern D; lane 3, whey protein fraction of pattern D; lane 4, pattern D.

3.2. Mass spectrometry and reversed phase high-performance liquid chromatography

3.2.1. Characterization of pattern A

With the aim of identifying the differences between the reference and the other patterns, the protein profile of an individual milk sample showing pattern A was fully characterized by in-gel trypsin digestion, MALDI-TOF MS analysis and database searching. In order to obtain a higher resolution, the analysis was performed on the main protein bands of separated casein and whey protein fractions of the reference sample (Fig. 2).

The IEF profile of the reference pattern A (skim milk, caseins and whey proteins; Fig. 2), shows that the bands present in the casein fraction can be subdivided into two groups, exclusively belonging

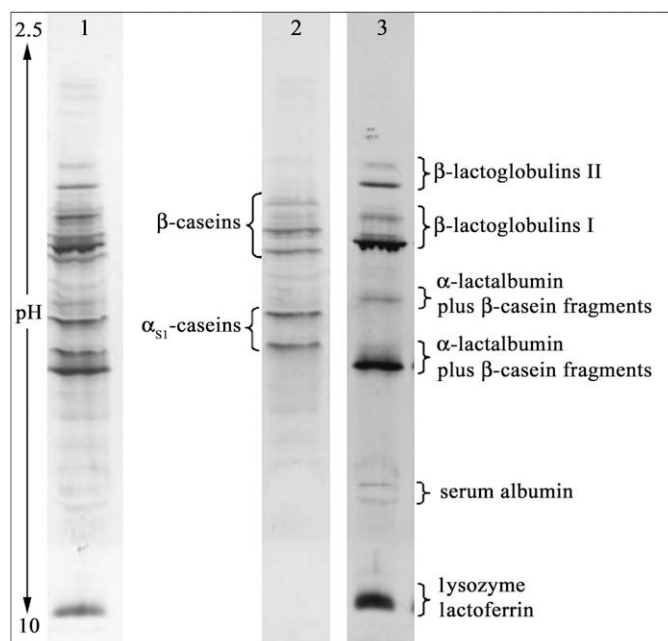


Fig. 2. Characterization of protein bands of the isoelectric focusing pattern of reference milk sample A. Lane 1, skim milk; lane 2, casein fraction; lane 3, whey protein fraction.

to β -casein (Lenasi et al., 2003) and α_{s1} -casein. It is worth noting that the different bands observed in the IEF pattern may be attributed to multi-phosphorylated isoforms of donkey β - and α_{s1} -casein, showing different pI. No signals related to a putative α_{s2} - and κ -casein were observed.

The characterization of the whey protein bands (Fig. 2, lane 3) allowed the identification of the main whey protein components, such as β -lactoglobulin II variants B and C (Herrouin et al., 2000), β -lactoglobulin I B (Herrouin et al., 2000), α -lactalbumin (Giuffrida et al., 1992) and lysozyme B (Herrouin et al., 2000), together with two minor components such as serum albumin (National Centre for Biotechnology Information NCBI accession number AAV28861) and lactoferrin (Cunsolo et al., 2007b). In addition, peaks belonging to tryptic fragments of donkey β -casein were identified in the MALDI mass spectra of the α -lactalbumin bands (data not shown); this result could be explained by the presence, in the whey fraction, of residual β -casein fragments, which arise from its partial digestion by endogenous proteases.

3.2.2. Characterization of pattern B

The absence of casein bands A1 and A2 characterizes the milk sample with IEF pattern B. Fig. 3 shows the MALDI mass spectra of the in-gel tryptic digests of these bands which were excised from the IEF gel of the casein fraction of a pattern A sample. Signals

present in the two MALDI mass spectra, which were almost identical, were used for the database search and allowed the identification of two horse milk α_{s1} -casein variants (NCBI accession numbers AAK83668 and AAL05435; Table 1). Protein identification was achieved by the matching of eleven signals, which are common to two horse milk α_{s1} -caseins. In detail, the signals at m/z 1675.9 and 1712.9 corresponded with the MH^+ of the theoretical tryptic fragments T17 and T8, respectively; the signals at m/z 786.4, 1832.0, 1841.0, 1845.0, 1997.1 and 2042.1 were due to the partial cleavages T(9 + 10), T(17 + 18), T(7 + 8), T(25 + 26), T(8 + 9) and T(16 + 17), whereas signals at m/z 769.4, 1691.9 and 1848.0 were consistent with the theoretical pyroglutamic form of the tryptic fragment T(9 + 10) and the methionine oxidized form of the tryptic fragments T17 and T(17 + 18), respectively.

Gel pieces from the protein-free region corresponding to the investigated A1 and A2 bands, were also excised from the gel of the anomalous pattern B for a parallel control of chemical noise and nonspecific protein background, and no signal of α_{s1} -casein was detected. This result demonstrates that the bands A1 and A2 belong to α_{s1} -casein from donkeys' milk.

It is worth noting that as the amino acid sequence of donkeys' milk α_{s1} -casein is still unknown, band assignment was achieved by the horse milk counterparts (cross-species protein identification; Wilkins and Williams, 1997) sharing amino acid sequence

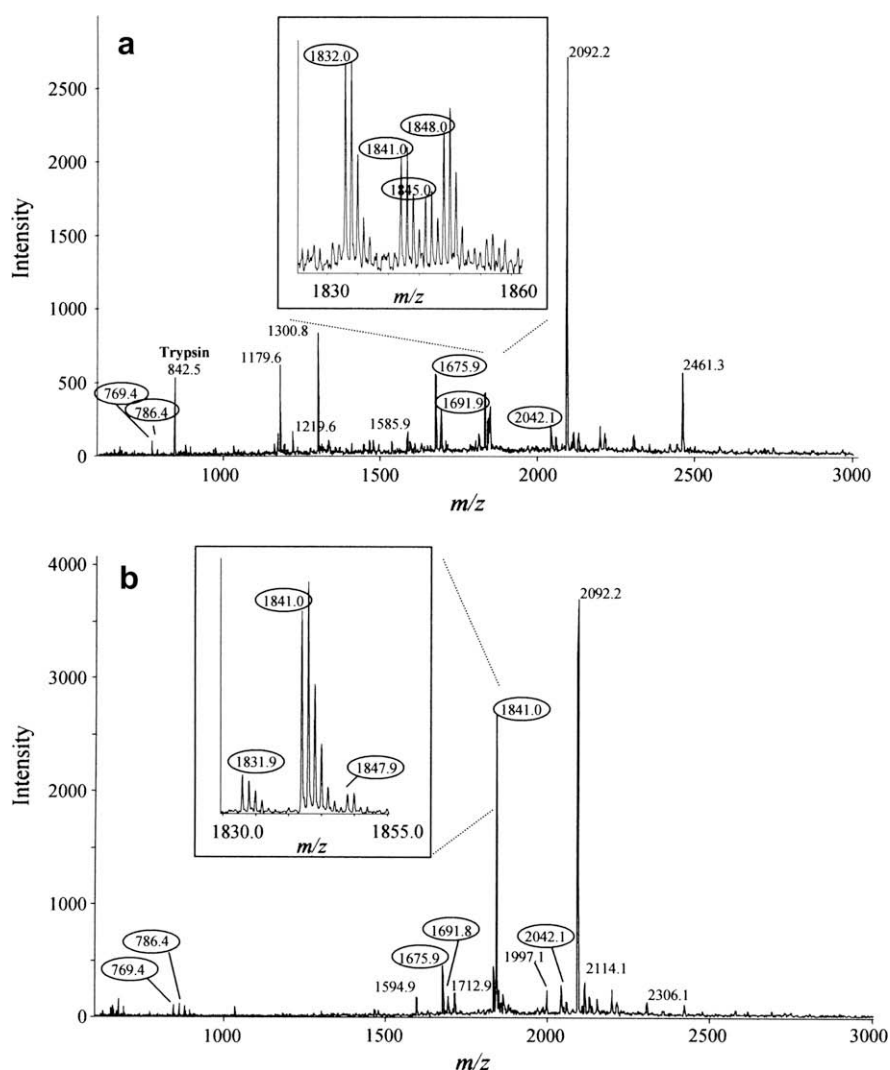


Fig. 3. Matrix-assisted laser desorption/ionization mass spectrometry spectra of the in-gel tryptic digests of the bands A1 (panel a) and A2 (panel b). The circled peaks correspond to the theoretical tryptic peptides of horse milk α_{s1} -casein.

Table 1
Sequence, position, calculated monoisotopic MH^+ and experimentally measured MH^+ of tryptic fragments of horse milk α_{s1} -casein (α_{s1} -Cn). The peaks matched (measured MH^+) are common to the sequence of the horse milk α_{s1} -Cn (NCBI^a Acc. No. AAL05435).

Fragment	Position	Calculated MH^+ monoisotopic	Measured MH^+ MALDI-MS	Sequence of horse α_{s1} -Cn (NCBI Acc. No. AAK83668)
T1	1–3	400.3	–	RPK
T2	4–7	522.3	–	LPHR
T3	8–19	1456.7	–	QPEIIQNEQDSR
T4	20–21	276.2	–	EK
T5	22–24	359.3	–	VLK
T6	25–26	304.2	–	ER
T7	27–27	147.1	–	K
T8	28–41	1712.8	1712.9	FPSFALEYINELNR
T(7 + 8)	27–41	1840.9	1841.0	
T9	42–43	303.2	–	QR
T(8 + 9)	28–43	1997.0	1997.1	
T10	44–47	502.3	–	ELLK
T(9 + 10)	42–47	786.5	786.4	
T11	48–49	276.2	–	EK
T12	50–51	275.2	–	QK
T13	52–55	528.2	–	DEHK
T14	56–84	3282.5	–	EYLIEDPEQQESSSTSSSEEVVPINTEQK
T15	85–85	175.1	–	R
T16	86–88	385.3	–	IPR
T17	89–101	1675.8	1675.9	EDMLYQHTLEQLR
T(16 + 17)	86–101	2042.0	2042.1	
T18	102–102	175.1	–	R
T(17 + 18)	89–102	1831.9	1832.0	
T19	103–105	347.2	–	LSK
T20	106–122	2066.1	–	YNQLQLQAIHAQEQLIR
T21	123–124	278.1	–	MK
T22	125–129	633.3	–	ENSQR
T23	130–133	531.3	–	KPMR
T24	134–181	5734.7	–	VVNQEQAYFYLEPFQPSYQLDVYPYAAWFHPAQIMQHVAYSFPFHD TAK
T25	182–190	990.5	–	LIASENSEK
T26	191–197	873.4	–	TDIIP EW
T(25 + 26)	182–197	1844.9	1845.0	

^a National Centre for Biotechnology Information.

similarity with respect to donkeys' milk α_{s1} -casein. It is further worth noting that in regard to the characterized amino acid regions (about 26%), donkey and horse milk α_{s1} -casein sequences are identical. As expected the similarity is only partial and the unassigned peaks in the MALDI mass spectra are probably due to tryptic peptides of donkey milk α_{s1} -casein, which contain amino acid substitutions (or deletions or insertions) with respect to the sequences of the horse milk α_{s1} -casein, and therefore cannot be assigned by the peptide mass fingerprinting search.

With the aim of confirming the absence of α_{s1} -casein components in the milk sample with IEF pattern B, an RP-HPLC-UV analysis of the casein fractions of this milk sample versus a milk sample with IEF pattern A, was performed. The comparison of the two UV profiles (Fig. 4a) reveals that the main difference is the absence, in the chromatogram of IEF pattern B, of the peak I, eluting at 14 min in the chromatogram of IEF pattern A. Chromatographic peak I was manually collected during the HPLC run of the milk sample with IEF pattern A, subjected to trypsin digestion and analysed by MALDI-TOF MS (data not shown). The chromatographic fraction eluting at 14 min in the HPLC run of the milk sample with IEF pattern B was also manually collected and analysed for a parallel control of chemical noise and nonspecific protein background. Finally, the characterization of the tryptic mixtures revealed that the protein eluting as peak I belonged to the α_{s1} -casein family, which is lacking in the milk sample with IEF pattern B.

3.2.3. Characterization of pattern C

Anomalous pattern C was characterized by the absence of the bands A3 and A4 belonging to the whey protein fraction. These two bands were excised from the gel of the whey protein fractions of a milk sample showing the IEF pattern A. A database search of the signals present in the MALDI mass spectra of the bands A3 and A4 allowed the identification of two variants of donkey milk

β -lactoglobulin II (Fig. 5). In detail, signals present in the MALDI mass spectra at m/z 409.2, 672.4, 1170.5, 1725.9, 2030.0, 2240.2 and 2384.1 correspond to the theoretical tryptic peptides T11, T5, T4, T6, T1, T3 (or the isobaric fragment T12) and T2 of the donkey milk β -lactoglobulin II, respectively (Table 2).

In addition, the MALDI mass spectrum of the band A3 (Fig. 5a) shows a signal at m/z 3914.7, which corresponds to the theoretical tryptic fragment T8 of the variant B, whereas the MALDI mass spectrum of the band A4 (Fig. 5b) presents a signal at m/z and 3928.7 which corresponds to the theoretical tryptic fragment T8 of the variant C. Gel pieces from the protein-free region corresponding to the investigated A3 and A4 bands, were also excised from the gel of the anomalous pattern C for a parallel control of chemical noise and nonspecific protein background, and no signal of β -lactoglobulin II was detected. This finding showed the presence of the donkey milk β -lactoglobulin II B and C in the two bands A3 and A4, respectively, leading to the conclusion that both these proteins are clearly absent in the milk samples with IEF pattern C (Fig. 1a).

To confirm this result, the RP-HPLC-UV profiles of the whey fractions belonging to a milk sample with IEF pattern C and to a milk sample with IEF pattern A were compared. Fig. 4b shows that the main difference between the two UV profiles concerns the absence in the chromatogram of IEF pattern C of peak II, eluting at 22 min in the chromatogram of IEF pattern A. The characterization of this peak by trypsin digestion and MALDI-MS analysis (data not shown) allowed the identification of β -lactoglobulin II B and C variants, which are both absent in the corresponding chromatographic fraction in the HPLC run of the milk sample with IEF pattern C.

3.2.4. Characterization of pattern D

Finally, with the aim of identifying the protein migrating in the band A5, observed only in pattern D, this band was cut from the IEF gel of the whey protein fraction of a sample with pattern D, in-gel

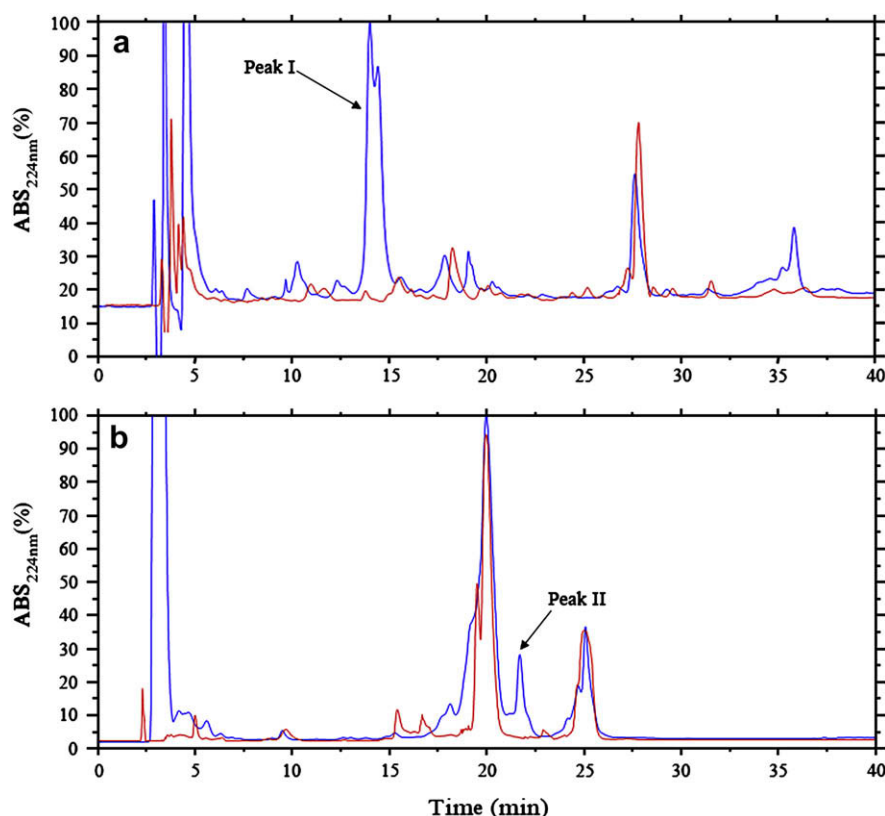


Fig. 4. Reverse phase high-performance liquid chromatography UV chromatograms of: panel (a) the casein fractions belonging to the milk sample with isoelectric focusing (IEF) pattern B (in red) and a milk sample with IEF pattern A (in blue); panel (b) the whey fractions belonging to a milk sample with IEF pattern C (in red) and a milk sample with IEF pattern A (in blue).

digested and analysed by MALDI-MS spectrometry. A database search of the MALDI mass spectrum (data not shown) of this band led to the identification of donkey β -lactoglobulin II variant D recently described by Cunsolo et al. (2007a) in the Ragusano breed. This finding permits us to conclude that the two milk samples characterized by pattern D, show the variants C and D of β -lactoglobulin II, and it is evident that band A3, present in pattern A, is replaced by band A5.

4. Discussion

In light of the preceding evidence, the protein fraction of the analysed donkeys' milk produced in Sicily exhibited a remarkable heterogeneity. A significant percentage of donkeys (>23%) produced milk lacking in β -lactoglobulin II and, in a rare case, a milk protein profile lacking α_{s1} -casein.

These findings are the first evidence of donkeys' milk protein polymorphism that is worthy of genomic investigation. Indeed the occurrence of such a polymorphism, if confirmed at the DNA level, represents an unquestionable advantage in donkey breeding since it constitutes a biodiversity index usable for conservation and selection strategy, as well as for exploiting the consumption of donkeys' milk in dietotherapy.

The variability associated with both the anomalous patterns (B and C), lacking single components (α_{s1} -casein and β -lactoglobulin II) could significantly affect the milk protein composition of donkeys' milk and, as a consequence, its allergenic properties. In fact, although it is not clear which are the major milk allergens, several studies indicate that α_s -caseins and β -lactoglobulin, scarce and absent in human milk respectively, seem to be the most relevant (Docena et al., 1996; Wal, 2002). Moreover, even the balance between caseins

and whey proteins seems to play an important role in the sensitization capacity of cows' milk, and its modification is able to reduce allergenicity of cows' milk (Lara-Villoslada et al., 2005).

To date, most of the studies carried out to measure the allergenicity of different types of milk use bulk, instead of individual and thus well-characterized milk. This bulk milk sampling strategy cannot highlight the specific role of single proteins, nor the impact of the polymorphism on allergenic traits. The identification of different donkey milk samples apparently lacking single protein components could promote a different and promising experimental approach, as already reported for goat milk without α_{s1} - or α_{s2} -casein (Bevilacqua et al., 2001; Marletta et al., 2004). Finally, the observed heterogeneity might also explain the unsolved cases observed in clinical trials and improve the already good toleration of donkeys' milk protein by using the individual and characterized milk in the treatment of CMPA patients. Certainly, the direct impact of this heterogeneity needs to be deeply investigated in order to gain new insights into the allergenic properties of donkey milk proteins, currently barely known and exploited.

The high glycosylation level of equine κ -casein (five glycosylation sites, according to Lenasi et al., 2003) makes its detection more complex (Egito et al., 2001), although it presents the most conserved sequence among casein genes in comparison with other species (human, camel, pig, cow, goat, sheep). Recently, a survey carried out by means of three different methodological approaches (ion-exchange chromatography, reverse phase HPLC and chromatofocusing) focused on the analysis of donkeys' milk protein fraction and found neither the presence of α_{s2} - nor κ -casein. The authors concluded that these caseins might be present in minute, and therefore undetectable, quantities, making their characterization difficult (Vincenzetti et al., 2008).

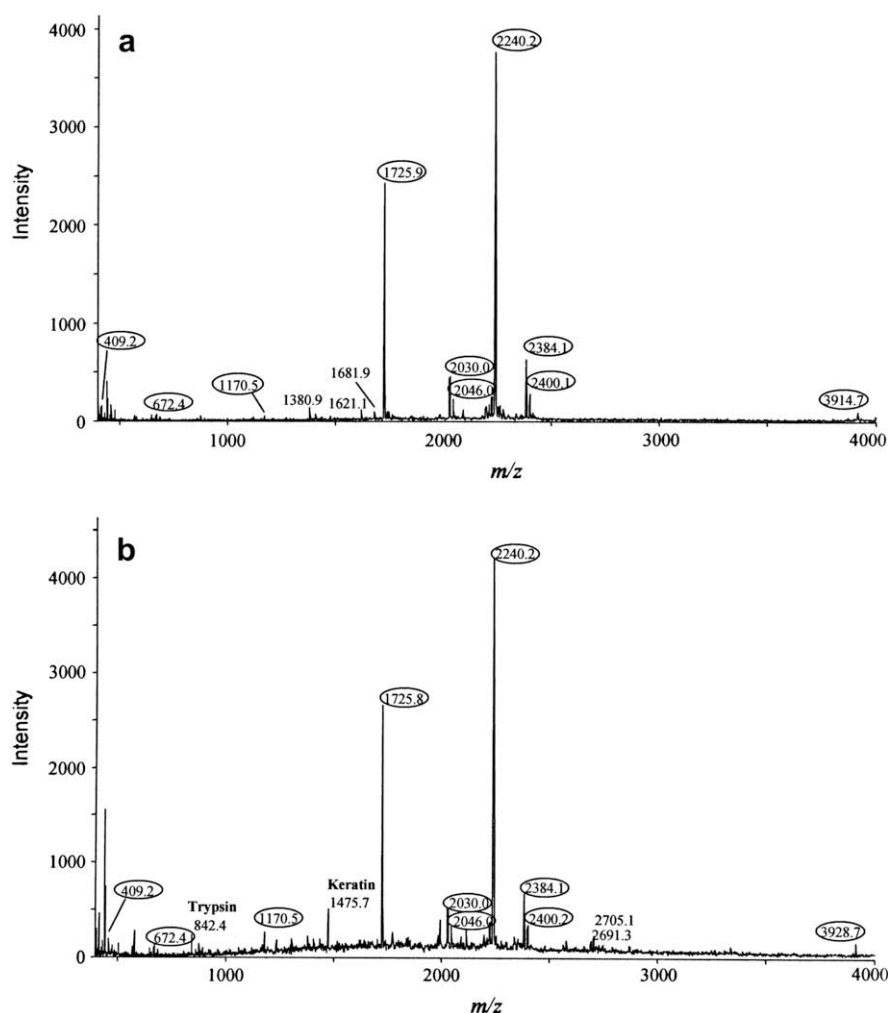


Fig. 5. Matrix-assisted laser desorption/ionization mass spectrometry spectra of the in-gel tryptic digests of the bands A3 (panel a) and A4 (panel b). The circled peaks correspond to the theoretical tryptic peptides of donkey β -lactoglobulin II.

In mare milk the characterization of α_{s1} -, β - and κ -casein has been reported: α_{s2} -like casein was identified, but not satisfactorily characterized, probably due to the very low level found (Egito et al., 2002). Moreover the absence of sequence data on α_{s2} -casein, even in horse milk, makes its detection in donkey milk more difficult, by means of the Peptide Mass Fingerprint tool.

In this study MALDI-TOF analysis provided signals corresponding to all the components of the whey protein fraction and β - and α_{s1} -casein; no signals were observed relating to the presence of putative α_{s2} - and κ -casein. Further investigation is needed to fully characterize the donkey milk casein fraction. To our knowledge the identification of α_{s2} - and κ -casein in donkeys' milk has not yet been

Table 2

Sequence, position, calculated monoisotopic MH^+ and experimentally measured MH^+ of tryptic fragments of donkey β -lactoglobulin II B.

Fragment	Position	Calculated MH^+ monoisotopic	Measured MH^+ MALDI-MS	Sequence of donkey β -lactoglobulin II B
T1	1–18	2030.0	2030.0	TDIPQTMQDLDLQEVAGR
T2	19–40	2384.2	2384.1	WHSVAMVASDISLLDSSEAPLR
T3	41–59	2240.2	2240.2	VYVEELRPTPEGNLEILR
T4	60–69	1170.5	1170.5	EGANHVCVER
T5	70–75	672.4	672.4	NIVAQK
T6	76–90	1725.8	1725.9	TEDPAVFTVNYQGER
T7	91–91	147.1	–	K
T8	92–125	3914.8	3914.7	ISVLD <u>TD</u> YAHYMF ~~F~~ CVGPPLPSAEHGTVCQYLAR
T8 ^a	92–125	3928.8	3928.7	ISVLE <u>TD</u> YAHYMF ~~F~~ CVGPPLPSAEHGTVCQYLAR
T9	126–128	376.2	–	TQK
T10	129–136	978.4	–	VDEEVMEK
T11	137–139	409.2	409.2	FSR
T12	140–160	2240.2	2240.2	ALQPLPGHVQIIQDPSGGQER
T13	161–163	383.1	–	CGF

^a Characteristic tryptic fragment of β -lactoglobulin II C differing by the D $\rightarrow$ E (underlined) amino acid substitution with respect to the corresponding tryptic fragment of variant B.

reported. The apparent absence of α_{s2} -casein in donkeys' milk, and the fact that its presence was not demonstrated in human milk, could perhaps explain the natural hypoallergenicity showed by donkeys' milk in most CMPA patients.

5. Conclusions

This study demonstrates that donkeys' milk proteins show phenotypic variability. This initial evidence of polymorphism, highlighted with a representative sample of the Ragusano breed, suggests extending the survey to include a larger number of animals and different breeds.

The methodological approach reported here, coupling IEF and MALDI-MS analyses, proved to be effective in characterizing the milk protein fraction at the phenotypic level. Although the electrophoretic analysis has limitations in fully detecting protein fractions, the use of IEF, especially in combination with RP-HPLC, may be useful as a screening approach for individual milk samples.

Further study should be carried out in order to assess the origin of the observed variability by sequencing milk protein genes to confirm the occurrence of genetic polymorphism. Moreover, since donkeys' milk is mainly consumed by CMPA patients as a hypoallergenic food, it is compelling to test the well-characterized individual milk samples for their own allergenic properties.

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