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Protein fraction heterogeneity in donkey's milk analysed by proteomic methods

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ABSTRACT - Donkey's milk is often well tolerated by patients affected by cow's milk protein allergy, probably thanks to its protein composition. This empirical evidence, confirmed by some clinical trials, needs to be better investigated. A preliminary survey on the protein fraction of donkey's milk was carried out: fifty-six individual milk samples have been collected and analysed by IEF and SDS-PAGE. Five different IEF patterns have been identified, showing a marked heterogeneity both in casein and whey protein fractions. A single IEF pattern showed an apparent reduced amount of casein fraction highlighted by SDS. Three of the five IEF patterns have been further investigated by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS).

Key words: Donkey, Milk proteins, Polymorphism, Proteomics.

INTRODUCTION - Equidae milk, characterized by a low casein-to-whey protein ratio (Zicker and Lonnerdal, 1994), can represent a good alternative to human milk in infant nutrition (Iacono *et al.*, 1992). Notwithstanding this, equidae milk have been poorly investigated: scarce data are available on protein fraction, except for whey proteins (Herrouin *et al.*, 2000; Miranda *et al.*, 2004). Sicily is the first region in donkey breeding and in the last few years the consumption of donkey's milk has arisen in importance. The aim of this work was to provide first information about milk protein variability on a large number of animals.

MATERIAL AND METHODS - Individual milk samples were collected from fifty-six lactating donkeys in three Sicilian farms and immediately cooled to 4°C. Milk samples were defatted, (3500rpm, 15°C, 30 min). Whole casein was obtained from skim milk by isoelectric precipitation (pH 4.6) at 22 °C, using 1 mol·L⁻¹ HCl and the supernatant, containing the whey proteins, was recovered by centrifugation at 4500 rpm at room temperature for 30 min and subsequently stored at -20°C (Ochirkhuyag *et al.*, 2000). Isoelectric focusing (IEF) in ultrathin polyacrylamide gels with carrier ampholytes was performed in Multiphor II Flatbed Electrophoresis System (Amersham Biosciences). Gels were stained with Coomassie Blue R350. SDS-PAGE was performed in a vertical standard gel electrophoretic apparatus (Protean® II xi Cell-BioRad) using 14% polyacrylamide gels which were stained with Coomassie Blue R250. Selected bands were excised from IEF gel of a reference sample (pattern A), washed, in-gel reduced by DTT, S-alkylated with iodoacetamide, and in-gel digested with porcine trypsin according to Shevchenko *et al.* (1996). An aliquot of the tryptic peptides was then micro-purified (Gobom *et al.*, 1999) for the further MALDI-MS analysis. The peptides were eluted with 0.6 µL of matrix solution (10 mg/mL α -cyano-4-hydroxycinnamic acid in 70% [v/v] CH₃CN and 0.1% [w/v] trifluoroacetic acid) and deposited directly onto the MALDI target. MALDI mass spectra were acquired in positive ion reflectron-delayed extraction mode, using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems) equipped with UV nitrogen laser (337 nm). The *m/z* software (Proteometrics, NY) was used to analyze MS spectra.

MALDI-TOF spectra were calibrated using bovine β -lactoglobulin tryptic mixture as external standard. MALDI-TOF peptide mass data were used to perform protein identifications by searching in a non-redundant protein sequence database with Mascot server ([http:// www.matrixscience.com](http://www.matrixscience.com)).

RESULTS AND CONCLUSIONS - Donkey's milk samples exhibited marked heterogeneity, showing five different IEF patterns. Most of the milk samples (36 samples - 64.3%) presented a common pattern, named A (Figure 1) and here used as a reference, whereas some others were characterized by the presence/absence of some protein bands. IEF was carried out on milk, caseins and whey proteins (fig. 1a) in order to identify which protein fraction the bands belonged to. Pattern B (1 sample - 1.8%) and C (12. - 21.4%) appeared to be defective, lacking some bands in the casein and in the whey protein fraction, respectively (Figure 1a). Pattern D (5 - 8.9%) and E (2 - 3.6%), on the contrary, showed the presence of split bands apparently belonging to casein and whey protein fraction respectively (Figure 1b). SDS-PAGE revealed a marked reduced amount of the casein fraction of the defective milk sample (IEF pattern B) compared

Figure 1. Isoelectric focusing of donkey's skim milk and protein fractions. a) 1: Reference milk sample, Pattern A; 2: Casein fraction of reference sample; 3: Pattern B; 4: Whey protein fraction of reference sample; 5: Pattern C. b) 1: Reference milk sample, pattern A; 2: Pattern D; 3: Pattern E.

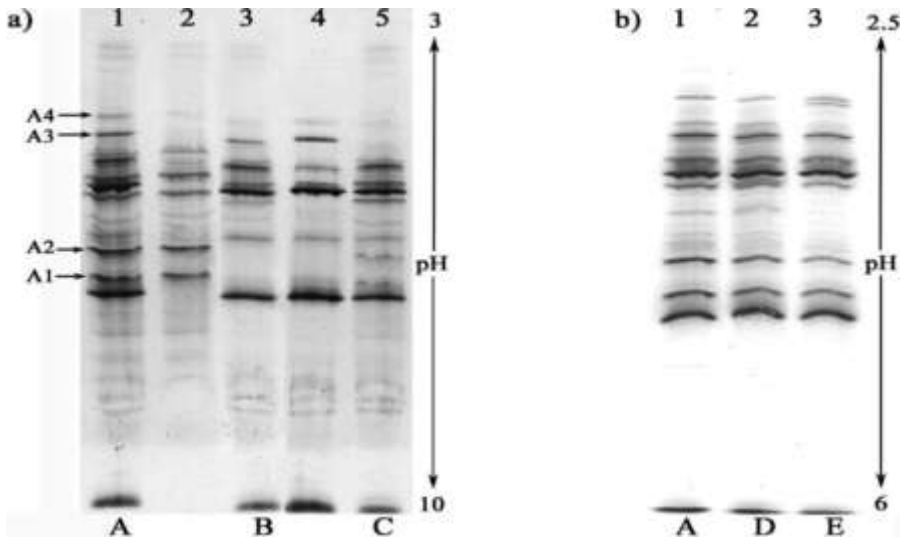
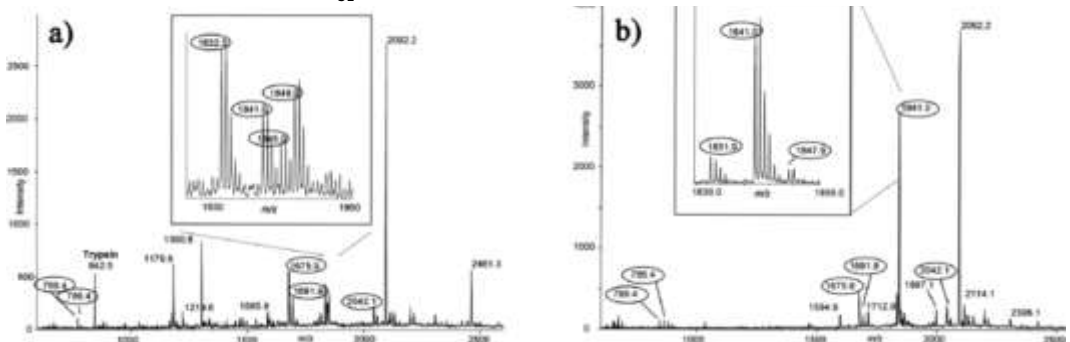


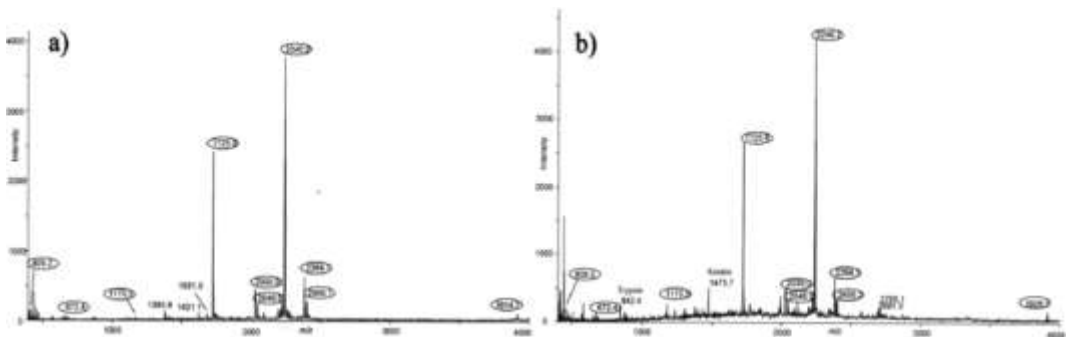
Figure 2. MALDI mass spectra of the in-gel tryptic digests of the bands A1 (Figure 2a) and A2 (Figure 2b). The circled peaks correspond to the theoretical tryptic peptides of horse α_{s1} -caseins.



to the reference. Three milk samples (reference pattern A and defective patterns B and C) were fully characterized by Mass Spectrometry analysis. The MALDI mass spectra of the in-gel tryptic digests of the bands A1 and A2 extracted from pattern A and which are absent in pattern B, are shown in figure 2a - b.

Signals in the two MALDI mass spectra are almost coincident and correspond to the theoretical tryptic peptides of two horse α_{s1} -caseins variants recorded in database (Acc. N. gi19031195 and gi15723738). Because of a good homology (30%), the two IEF bands A1 and A2 could be attributed to still unknown donkey's α_{s1} -casein variants. The MALDI mass spectra of the in-gel tryptic digests of the bands A3 and A4, absent in pattern C, allowed to identify two variants of donkey's β -lactoglobulin. In detail, signals present in the MALDI mass spectrum of the bands A3 (Figure 3a) correspond to the theoretical tryptic peptides of the donkey's β -lactoglobulin II variant B, whereas the peaks present in the MALDI-MS spectrum of the bands A4 (Figure 3b) correspond to the theoretical tryptic peptides of the donkey's β -lactoglobulin II variant C. This result leads to the conclusion that the two IEF bands A3 and A4 could be attributed to the donkey's β -lactoglobulin II variant B and variant C, respectively.

Figure 3. MALDI-MS spectra of the in-gel tryptic digests of the bands A3 (Figure 3a) and A4 (Figure 3b). The circled peaks correspond to the theoretical tryptic peptides of β -Lactoglobulins.



The methodological approach here reported, coupling IEF and MALDI-MS analysis, provide evidence of efficiency in detecting the variability of principal protein in donkey's milk at phenotypic level. Further investigations are needed at molecular level to confirm the occurrence of genetic polymorphism in donkey's milk protein genes. Each of the different milk protein pattern should be used in immunological test (*in vitro* and *in vivo*) in order to assess their allergenic capacity. The occurrence of polymorphism, at casein and whey protein fraction, could be useful to better exploit donkey breeding and particularly to promote donkey's milk utilization in dietotherapy.

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