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Occurrence of qualitative and quantitative polymorphism at donkey beta-Lactoglobulin II *locus*

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ABSTRACT

With the use of a proteomic approach a genetic screening of 70 individual donkey milk samples, from two autochthonous breeds reared in Italy, was carried out to determine the genetic polymorphism at donkey β -Lg *loci*. The achieved results showed the occurrence of the new β -Lg II E genetic variant, differing from β -Lg II D reference for D Asp²/E Asn², D Arg¹⁸/E Lys¹⁸ and D Val²⁵/E Ala²⁵ amino acid substitutions with a *M*_r 18 256 Da. Moreover a quantitative polymorphism seemed to affect the donkey β -Lg II *locus* since four phenotypes, with a relative quantitative percentage respect to total whey protein ranging from 0% to 3.65%, were detected by HPLC analysis.

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1. Introduction

The increased incidence of allergies and/or intolerances to bovine milk in newborns enhanced the production of infant *formulae* based on either bovine or soy proteins hydrolysates. However, peptides with a molecular size able to trigger an immune response in predisposed subjects can survive the production process and soy hydrolysates seem to cause a negative sensorial acceptance in infant consumers (Businco et al., 2000; Muraro, Giampietro, & Galli, 2002). Basically, allergies and/or intolerances to bovine milk are mainly depending on milk proteins otherwise present at very lower amount (α _{s1}-CN) or not expressed (α _{s2}-CN and β -Lg) in human milk (Docena, Fernandez, Chirido, & Fossati, 1996). To this aim, the milk from mammalian species having a more similar composition to human milk could be usefully employed. The sensitization to cow allergy, measured as anti-goat β -Lg antibodies, was found significantly decreased in guinea pigs fed on goat milk containing traces of α _{s1}-CN (Bevilacqua et al., 2001). Nevertheless, the availability of these types of milk requires a genetic screening to select the animals carrying weak or null alleles at α _{s1}-CN *locus*. On the contrary, due to natural similarity to human milk, the positive effects of donkey milk in nutrition of cow's milk protein allergy infants have been reported (Monti et al., 2007). In addition to *in vivo* beneficial healthy effects, important bioactivities of donkey milk were found by *in vitro* assays, such as a potent cytotoxicity causing tumor cell death by apoptosis (Mao et

al., 2009) and an inhibitory effect against several bacterial species *in vitro* (Tidona et al., 2011; Zhang, Zhao, Jiang, Dong, & Ren, 2008). These effects were positively related to the higher level of lysozyme (Lyz) (Salimei et al., 2004) in donkey milk than in cow counterpart (Guo et al., 2007; Vincenzetti et al., 2008).

To date, the composition of donkey milk in terms of TN (total nitrogen), CN (casein nitrogen), WPN (whey protein nitrogen), NPN (non protein nitrogen) and lactose content, as well as of CN/WPN ratio, has been the "scientific rationale" of its close similarity to human counterpart (Guo et al., 2007). Even though the quantitative level of TN is lower than cow milk (1.5–1.8% vs 3.1–3.8%), the WPN amount in both milks is very similar (0.49–0.80% vs 0.55–0.70%) (Guo et al., 2007).

From a qualitative point of view, very recently, the four casein fractions, α _{s1}, α _{s2}, β and κ -CN were found constitutive of asinine CN (Chianese et al., 2010). Each of them showed a high compositional heterogeneity due to concomitant presence of deleted non allelic forms and discrete phosphorylation (α _{s1}, α _{s2}, β -CN) or glycosylation (κ -CN) degree as in goat and sheep milks (Ferranti et al., 1997; Ferranti, Lilla, Chianese, & Addeo, 1999).

More scientific data were available on donkey whey proteins (WPs), consisting of α -La, β -Lg, blood serum albumin (BSA), lactoferrin (Lf) and Lyz as in ruminants (Martin, Szymanowska, Zwierzchowski, & Leroux, 2002) and horse milk (Uniacke-Lowe, Huppertz, & Fox, 2010). The primary structures of donkey WPs, as expected, showed a higher identity with horse than bovine counterparts. Donkey β -Lg, for instance, was consisting of two components, the major β -Lg I and the minor β -Lg II; the former is 162 a.a. residues

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long, as in ruminants milk, and the second 163 a.a. for the additional glycine residue between the 116th and the 117th a.a., as in mare milk (Halliday, Bell, & Shaw, 1991) (Table 1). The homology of donkey β -Lg I A with B variants accounted for 98.1% and each of them was 98.8 and 99.8% identical to horse counterpart, respectively. In the same way the homology of donkey β -Lg I with donkey β -Lg II and cow counterpart accounted for the 65.6% and 56.0%, respectively (<http://expasy.org>).

The genetic polymorphism occurred at donkey β -Lgs loci gave rise to A and B β -Lg I genetic variants (Cunsolo, Saletti, Muccilli, & Foti, 2007; Godovac-Zimmermann et al., 1988, 1990; Herrouin et al., 2000; Zuccaro et al., 2010) (Table 1). Unlike ruminants β -Lgs and with the exception of donkey β -Lg II variant A (each having five Cys residues per molecule of protein), only four Cys residues per molecule of protein were found in donkey β -Lg II variants. They were arranged in two disulphide bridges at the same chain position (namely Cys¹⁰⁶–Cys¹²⁰ and Cys⁶⁶–Cys¹⁶¹) (Cunsolo, Costa, et al., 2007) of the dairying species counterparts. Another interesting structural characteristic differentiating donkey β -Lgs with respect to ruminants was the maintenance of a monomeric structure at the pH of milk (Table 1).

So far, donkey α -La resulted monomorphic differently from the three genetic variants A, B and C occurring in horse counterpart (Giuffrida et al., 1992). Since the knowledge about the genetic profile of donkey whey proteins of animals reared in Italy is very scarce, the present research was aimed to filling this gap and to relating it to the milk nutritional quality. In particular, whey proteins extracted from individual donkey milks, were separated in the constitutive components by means of one- and/or two-dimensional immuno-electrophoresis, RP-HPLC, coupled to MALDI-TOF-MS and ESI-Q-TOF-MS/MS analysis.

2. Materials and methods

2.1. Materials

All chemicals were of the highest purity commercially available and used without further purification. Tetra-methyl-ethylene-diamine (TEMED), ammonium persulphate, glycine, urea, acetic acid, sodium acetate, ammonium bicarbonate (AMBIC), HPLC grade H₂O, formic acid (FA), Trifluoroacetic acid (TFA) and CH₃CN were purchased from Carlo Erba (Milan, Italy). Sinapinic acid (SA), α -cyano-4-hydroxycinnamic acid (CHCA), iodoacetamide (IA), dithiothreitol (DTT) were obtained purchased from Aldrich (St. Louis, MO, USA). Modified trypsin, sequencing grade, was from Promega (Madison, WI, USA). Dialysis tubing with molecular weight cut off (MWCO) 12–14,000 Da was from Medicell International Ltd (London, UK). Amicon® Ultra centrifugal filters 3.000 MWCO and ZipTip™ C₁₈ micro columns were obtained from Millipore (Bedford, MA, USA). Acrylamide, Bis acrylamide, Ampholine buffers were from GE Healthcare Amersham Bioscience (Buckinghamshire,

UK). Coomassie Brilliant Blue G 250 was purchased from Bio-Rad (Richmond, CA, USA).

2.2. Sample preparation

Individual donkey milk samples (70) were collected from two breeds reared in Italy (Sicilia and Abruzzo) and the whey proteins fraction was isolated by acidification at pH 4.6 of defatted milk (Aschaffenburg & Drewry, 1959). The supernatant, containing the whey proteins, was recovered by centrifugation (2500 $\times$ g, 30 min) at room temperature and subsequently dialysed in tubing with molecular cut off 12,000–14,000 Da, individual whey proteins samples were freeze-dried and stored at -20°C before use.

2.3. Ultra thin layer isoelectric focusing (UTLIEF) and immunoblotting analysis

UTLIEF analysis was that early reported (Chianese et al., 2010). Briefly, individual whey proteins samples (20 g L⁻¹) were dissolved in 9 M urea solution, containing 2-mercaptoethanol (1 mL L⁻¹). This procedure was performed for 124 $\times$ 258 $\times$ 0.25 mm gel slabs in a 4.2–9.0 pH gradient mixing 1% (v/v) Ampholine buffers: 4.2–4.9 (350 μ L), 4.5–5.4 (350 μ L), 5.0–7.0 (150 μ L), 7.0–9.0 (150 μ L).

For immunoblotting analysis, the whey proteins separated by UTLIEF analysis were transferred by capillary diffusion from the gel onto a nitrocellulose membrane (0.45 μ m, Trans-Blot, Bio-Rad, Richmond, CA 94804, USA) and immunostained using polyclonal antibodies against bovine α -La and β -Lg as primary antibodies. These were prepared according to the following procedure: bovine α -La and β -Lg, fractionated by RP-HPLC as described in Section 2.4, were used as antigen by Primm (Milan, Italy) to prepare rabbit polyclonal antisera against bovine α -La and β -Lg. Finally, the antisera were filtered using 0.45-mm filters (Millipore, Bedford, MA), divided into aliquots of 1 mL, and stored at -20°C .

2.4. Reversed-phase high performance liquid chromatography (RP-HPLC)

Whey protein samples were fractionated by RP-HPLC on a 214TP54, 5 μ m Vydac C₄, 250 $\times$ 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) at a detection wavelength of 220 nm. Solvent A was 0.1% TFA in ultra-pure water (v/v) and solvent B 0.1% (v/v) TFA in CH₃CN. Two hundred microliters of a solution containing 1 mg (whey protein sample) mL⁻¹ (solvent A) were loaded onto a C₄ column, equilibrated with solvent A. The elution program involved a gradient from 30 to 50% of solvent B in 45 min, with a flow rate of 1 mL min⁻¹. Each eluted whey protein fraction was manually collected, freeze-dried, and stored at -20°C .

Table 1
Physico-chemical characteristics of donkey α -La and β -Lg I, II genetic variants.

Protein	Genetic variant		Amino acid substitutions				Amino acid residues	Molecular mass (Da)	Number and positions of disulphide bridges		
β-Lg I	^a A	E ³⁶	S ⁹⁷	V ¹⁵⁰			162	18,528	2 (66–160; 106–119)		
	^b B	S ³⁶	T ⁹⁷	I ¹⁵⁰			162	18,514	2 (66–160; 106–119)		
β-Lg II	^c A				D ⁹⁶	C ¹¹⁰	M ¹¹⁸	D ¹⁶²	163	18,263	2 (66–161;106–120)
	^d B					P ¹¹⁰	T ¹¹⁸		163	18,227	2 (66–161;106–120)
	^d C				E ⁹⁶	P ¹¹⁰	T ¹¹⁸		163	18,241	2 (66–161;106–120)
	^e D	D ²	R ¹⁸	V ²⁵		P ¹¹⁰		G ¹⁶²	163	18,311	2 (66–161;106–120)
	^f E	N ²	K ¹⁸	A ²⁵		P ¹¹⁰		G ¹⁶²	163	18,256	2 (66–161;106–120)
	^g A								123	14,222	4(6–120;28–111;61–7;73–91)
α-La											

^a SWISS-PROT database (<http://au.expasy.org/tools>).

^b Godovac-Zimmerman et al., 1988.

^c Godovac-Zimmerman et al., 1990.

^d Herrouin et al., 2000; Zuccaro et al., 2010.

^e Cunsolo et al., 2007.

^f Present research.

^g Giuffrida et al., 1992.

2.5. LC/ESI-MS analysis

The screening on individual whey samples to discriminate β -Lg II A from B variant, otherwise comigrating in UTIEF analysis, was carried out using a single quadrupole instrument LC ESI-MS (HP1100-MSD, Agilent Technologies, Santa Clara, CA, USA) and a 214TP54, 5 μ m Vydac C₄, 250 $\times$ 2.1 mm internal diameter column and the same chromatographic conditions previously described for RP-HPLC analysis. The elution program involved a gradient from 37% to 50% of solvent B in 30 min, at a flow rate of 0.2 mL min⁻¹. Each β -Lg II component, ionising in positive mode was recorded in m/z range 800–2400.

2.6. β -Lg alkylation and trypsin enzymatic digestion

The isolated β -Lg II fraction, collected by RP-HPLC, was dried using a Savant concentrator (Speed-Vac, Milan, Italy), lyophilized, denatured and alkylated before trypsin hydrolysis. Briefly, isolated donkey β -Lg II fraction was denatured at room temperature, in a guanidine buffer (6 M guanidine in 0.5 M Tris-HCl with 1 mM EDTA) at pH 8. Subsequently, disulfide bridges were reduced by the addition of 10 mM L⁻¹ DTT for 60 min at 56 °C. SH groups were subsequently alkylated with 55 mM L⁻¹ IA in the above guanidine buffer, and maintained for 30 min in the dark. With the aim to remove reagents, the alkylated whey protein was ultra-filtered on Amicon® Ultra filters 3000 MWCO, centrifuged at 4000 g for 25 min and washed with 50 mM NH₄HCO₃ pH 7.4. The retentate solution containing the purified alkylated β -Lg II, was added of trypsin at 1:50 (w w⁻¹) and incubated overnight (approximately 14 h), at 37 °C. The digestion was stopped by frozen (–20 °C).

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

MALDI-TOF-MS experiments were carried out on a Voyager DEPRO mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with delay extraction technology and N₂ laser at 337 nm. Mass spectra were acquired both in positive linear or in reflector mode and 10 mg mL⁻¹ SA and CHCA both dissolved in 50% ACN/0.1% TFA, were used as matrices for the analysis of proteins and peptides, respectively. MALDI-TOF analysis of intact proteins were obtained in linear positive ion mode over the m/z range 10,000–30,000 and were averaged from about 150 laser shots. The mixtures of tryptic peptides were subjected to a desalting/concentration step with Zip-Tip C18

microcolumns prior to analysis by MALDI-TOF. Spectra were obtained in reflectron positive ion mode over an m/z range 600–4000 and were averaged from about 150 laser shots. External calibration was performed by acquiring separate spectra of a mixture of standard peptides (PerSeptive Biosystems).

2.8. Electrospray quadrupole-time of flight-mass spectrometry (ESI-Q-TOF-MS/MS) analysis

Tandem MS (MS/MS) data were obtained using a Q-STAR instrument (Applied Biosystems, Foster City, CA) equipped with a nanospray interface (Protana, Odense, Denmark). Dried samples were resuspended in 0.1% TFA, desalted by using ZipTip C₁₈ micro columns, and sprayed from gold-coated “medium-length” borosilicate capillaries. The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by using the quadrupole mass filter and then induced to fragment by collision. The collision energy was 20 to 40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst 5 software (Applied Biosystems) as well as the deconvoluted MS/MS spectrum manually interpreted.

3. Results and discussion

3.1. Determination of donkey β -Lgs genetic polymorphism by UTIEF and immunoblotting analysis

The most representative UTIEF profiles of individual donkey whey proteins are shown in Fig. 1; after Coomassie blue staining (Panel A) the main whey proteins, α -La and β -Lg I, were identified according to known theoretical pI value and after immunoblotting with α -La and β -Lg specific polyclonal antibodies (Panel B). On the basis of the increasing pI value β -Lg II, β -Lg I and α -La focalised in A, B and C zone of UTIEF pH gradient, respectively. The composition of each electrophoretic zone was heterogeneous for the presence of almost two components recognised by each specific polyclonal antibody. This was the case of B and C zones where β -Lg I and α -La were “escorted” by a and b satellite bands, respectively, while four β -Lg II phenotypes, numbered in order of decreasing pI value in Fig. 1, were immunostained in A zone. Tackling into account the known theoretical pI values, A and B genetic variants of β -Lg II focalised in band 2 (pI=4.70), β -Lg II C in band 1 (pI=4.72) and β -Lg II D (pI=4.64) in band 4. Since, to date, none genetic variant having a pI value intermediate between B and D β -Lg II was reported,

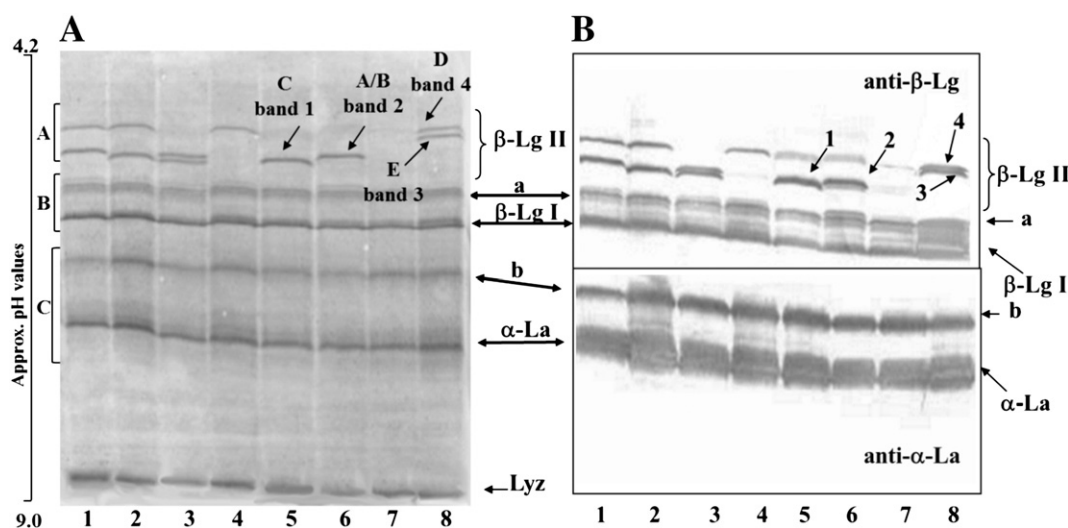


Fig. 1. Ultra-thin layer polyacrylamide gel electrophoresis (UTIEF) in pH gradient 4.2–9.0 of individual whey proteins samples containing representative donkey phenotypes detected by double staining with CBB (Panel A) and polyclonal antibodies against α -La and β -Lg (Panel B).

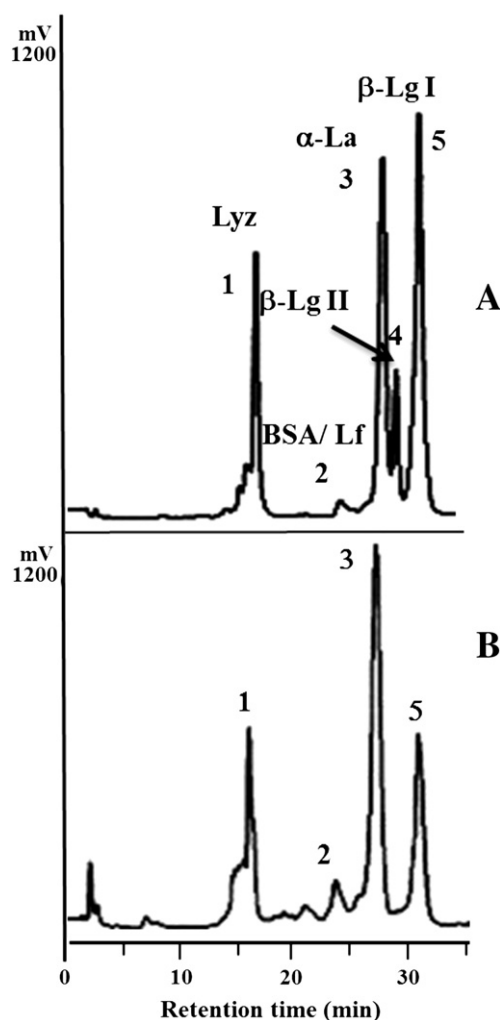


Fig. 2. RP-HPLC profiles of representative donkey whey protein samples (profiles a and b) containing a different quantitative level of β -Lgs and α -La.

the occurrence of a new variant E was supposed in band 3. Moreover a defective coding allele for β -Lg II determined the absence of expressed protein in lane 7. A similar milk sample was found in Sicilian (Criscione et al., 2009) and Sardinian (Conti, Napolitano, Lai, Pinna, & Godovac-Zimmerman, 1989) donkey breeds. In conclusion, from a genetic point of view, in examined donkey milk samples, α -La and β -Lg I were monomorphic and β -Lg II polymorphic, since known (B, C and D), new (E) variants and a defective quantitative allele occurred at this locus.

Table 2
Identified donkey whey proteins by LC/MS analysis.

Retention time (min)	Peak	Molecular Mass, Da		Protein identification
		Measured	Theoretical	
17.6	1	14,626	14,631	Lysozyme B
24.4	2	65,601	65,597	Serum Albumin
		75,536	75,420 ^a	Lactoferrin
27.5	3	14,210	14,214	α -La A variant
28.6	4	18,221, 18,239, 18,314, 18,256 ^b	18,227, 18,241, 18,311	β -Lg II B, C, D, E ^b variants
30.3	5	18,505	18,510	β -Lg I B variant

^a Horse Molecular Mass.

^b Present research.

3.2. Identification of donkey β -Lgs genetic variants by RP-HPLC coupled to LC/MS analysis

The screening, carried out by RP-HPLC analysis, on individual donkey whey protein samples allowed to select the *a* and *b* representative chromatograms (Fig. 2). By LC/MS analysis Lyz, BSA/Lf, α -La, β -Lg II and β -Lg I, in increasing order of elution time, were identified (Table 2). Comparing *a* and *b* chromatographic profiles, a different quantitative expression of β -Lgs and α -La can be observed; in particular, β -Lg II was lacking in *b*, while a different quantitative ratio α -La/ β -Lg I was assessed in each profile (*a* = 1 and *b* = 2, approximately). The use of LC/MS allowed to identify the four β -Lg II variants (B, C, D and E) otherwise co-eluting in the same chromatographic peak 4 or co-focalising at the same pI value of UTIEF pH gradient, as β -Lg II A and B (Table 2). These results confirmed the monomorphism of α -La and the occurrence of two A and B genetic variants for β -Lg I. We found a more complex polymorphism of β -Lg II for the occurrence

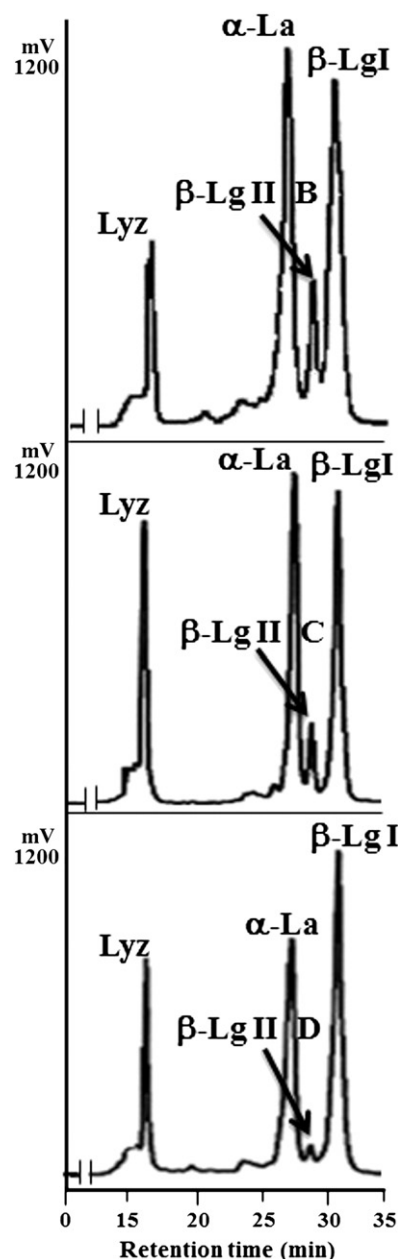


Fig. 3. RP-HPLC profiles of individual whey protein from donkey milk samples containing β -Lg II B, C and D variants.

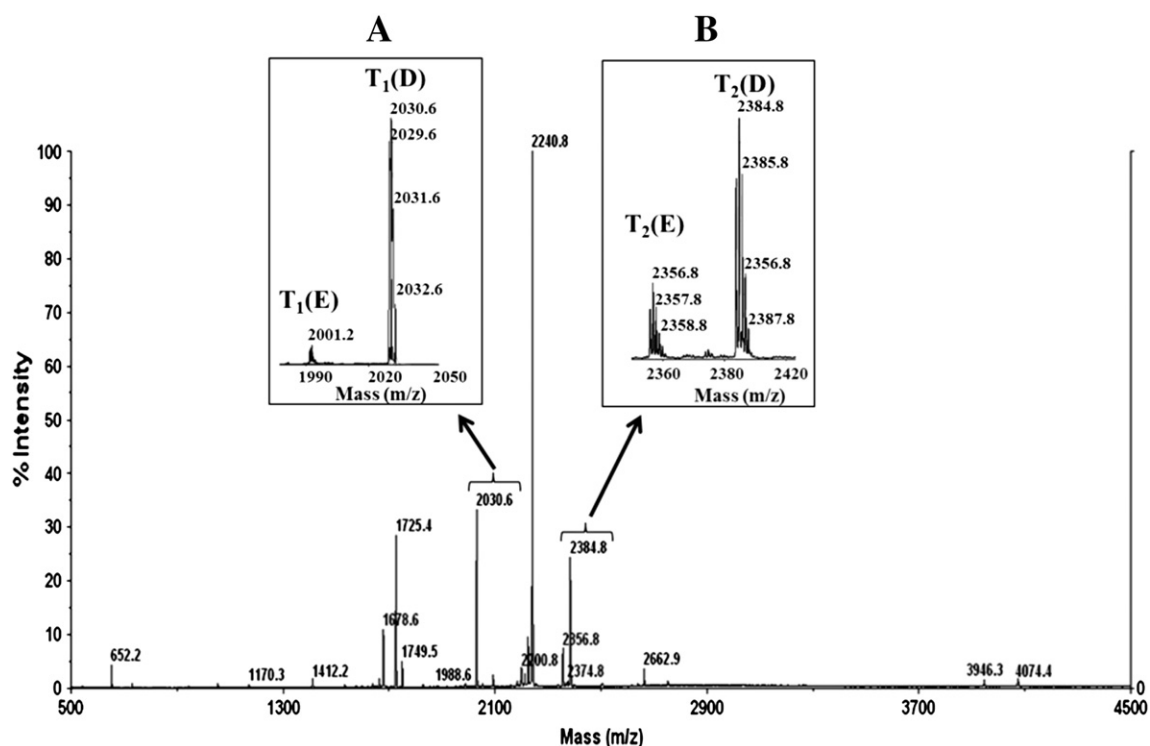


Fig. 4. MALDI TOF mass spectrum of the tryptic digest of β -Lg II DE. Inset in panel a: peptides 1–18 T_1 (D) and T_1 (E) in m/z range 1990–2050. Inset in panel b: peptides 19–40, T_2 (D) and T_2 (E) in m/z range 2360–2420.

of known (B, C and D) new (E) variants and null allele at this *locus*. Furthermore, the HPLC analysis of whey samples containing B, C and D β -Lg II variants (Fig. 3) showed a different peak area in the following decreasing order: BB (3.65%), CC (1.81%) and DD (0.76%); therefore these genetic variants could be expressed by quantitative alleles (strong, intermediate, weak and null).

It was well known that a similar quali-quantitative polymorphism was found at goat α_{s1} -CN *locus* and the related scientific studies reported in numerous research papers (Caroli et al., 2006; Marletta, Criscione, Bordonaro, Guastella, & D'Urso, 2007; Martin et al., 2002).

3.3. Characterization of the new β -Lg II E variant by MALDI/TOF and ESI-Q-TOF-MS/MS analysis

To characterize the new β -Lg II E variant, the whey proteins of sample containing the variant, were separated by RP-HPLC and the eluted peak 4 manually collected, was submitted to LC/MS analysis. Two components at Mr 18314 and Mr 18256 Da, were determined

in the peak sample. Of these, the first corresponded to expected β -Lg II D variant (Table 1) while the other one was assigned to the new genetic β -Lg II E variant. To identify the a.a. substitutions, β -Lg II E and D variants (co-eluted in peak 4) were digested by trypsin before MALDI TOF analysis. The obtained mass spectra (Fig. 4) gave rise to peptides with measured masses reported in Table 3; as expected, all signals were assigned to D variant except two new T_1 (E) and T_2 (E) peptides at Mr 2001.2 Da and 2356.8 Da, displayed in insets a and b. These peptides presented a molecular weight lower of 29 and 28 Da than T_1 (D) and T_2 (D) respectively. These achieved data were in close agreement with the 57 Da molecular mass difference measured between β -Lg II D and β -Lg II E.

The peptides T_1 (E) and T_2 (E) were submitted to ESI-Q-TOF-MS/MS analysis for sequencing (Fig. 5): the peptide T_1 (E) corresponded to sequence 1–18 with two amino acid substitutions, $D^{25} \rightarrow N^{25}$ and the C-terminal $R^{18} \rightarrow K^{18}$, while peptide T_2 (E) exchanged respect to peptide T_2 (D) for a.a. substitution $V^{25} \rightarrow A^{25}$. Overall, the β -Lg II E amino acid substitutions experimentally observed in this work were all

Table 3
MALDI-TOF MS identification of RP-HPLC peak 4 eluted at 28.6 min (Fig. 3) digested with trypsin.

Fragment	Position	Molecular mass, Da		Peptide sequences	β -Lg II variants
		Theoretical	Measured		
T_1	1–18	2029.9	2030.6	TDIPQTMQLDDQLQEVQGR	D
T_1	1–18	2000.2	2001.2 (–29 Da)	TNIPQTMQLDDQLQEVQGR	E
T_2	19–40	2384.2	2384.8	WHSVAMVASDISLLDSESAPLR	D
T_2	19–40	2355.8	2356.8 (–28 Da)	WHSVAMAASDISLLDSESAPLR	E
T_3	41–59	2240.2	2240.8	VYVEELRPTPEGNLEILR	D, E
T_4	60–69	1112.5		EGANHVCVER	D, E
T_5	70–75	671.4		NIVAQK	D, E
T_6	76–90	1724.8	1725.4	TEDPAVFTVNYQGER	D, E
T_8	92–125	3831.8	3945.7 ^a	ISVLDTDYAHYMFVCVGPPLPSAEHGMVCQYLAR	D, E
T_{7+8}	91–125	3959.8	4091.6 ^a	KISVLDTDYAHYMFVCVGPPLPSAEHGMVCQYLAR	D, E
T_{10}	129–136	977.4		VDEEVMEK	D, E
T_{10+11}	129–139	1368.5		VDEEVMEKFSR	D, E
T_{12}	140–160	2240.2		ALQPLPGHVQIIQDPGSGQER	D, E

^a Alkylated.

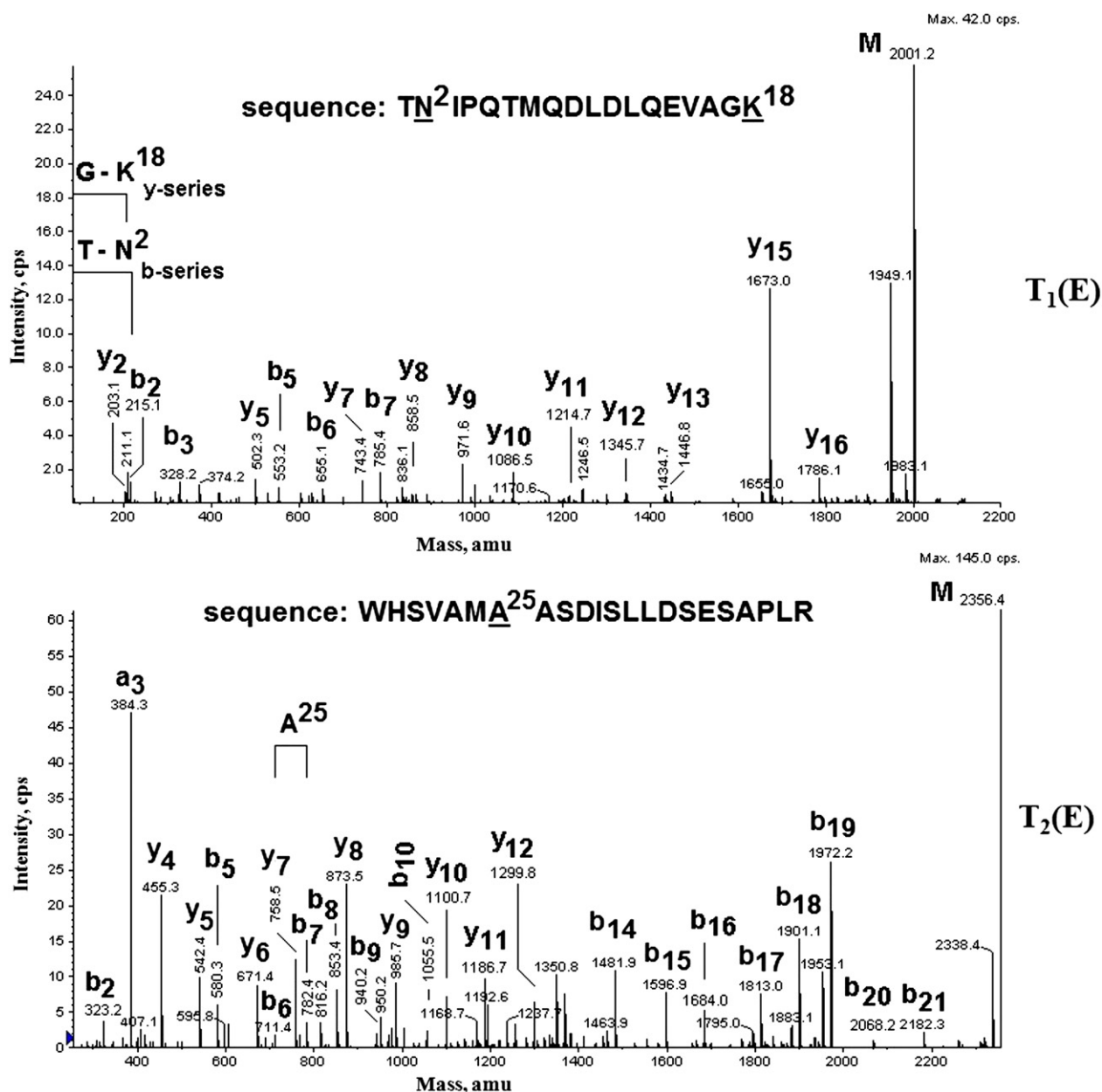


Fig. 5. ESI-Q-TOF-MS/MS fragmentation of T₁ (1–18) and T₂ (19–40) tryptic peptides of β -Lg E variant.

confined in the N-terminal area of the protein sequence that was known to be conserved among the other β -Lg II genetic variants.

4. Conclusion

Three a.a. substitutions characterized β -Lg II E variant in comparison with D variant: D β -Lg II Asp² $\rightarrow$ E β -Lg II Asn², D β -Lg II Arg¹⁸ $\rightarrow$ E β -Lg II Lys¹⁸ and D β -Lg II Val²⁵ $\rightarrow$ E β -Lg II Ala²⁵. These results indicated a direct phylogenetic origin of E from D variant. Moreover, since the percentage area value of β -Lg II DE was very close to that of CD HPLC profile counterpart (results not shown) it can be hypothesized that β -Lg II E is expressed by an intermediate allele. These data, of course, require further experimental results for their validation.

From a nutritional point of view the minor content of β -Lgs in donkey milk in addition to quantitative genetic polymorphism occurred at β -Lg II locus can be related to the tolerance mechanism of donkey milk in the allergic patients to bovine milk.

Thus, the proteomic methodology is a powerful tool to underline the genetic biodiversity inter and/or intra species. In particular, the sensibility of based separative technique as UTLIEF was very high and able to discriminate between β -Lg II D and E variants and between β -Lg II C and B/A variants differing each other for 0.01 pH unit.

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