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Poppea's bath liquor: The secret proteome of she-donkey's milk

Vincenzo Cunsolo^{a,*}, Vera Muccilli^a, Elisa Fasoli^b, Rosaria Saletti^a,
Pier Giorgio Righetti^b, Salvatore Foti^a

^aDepartment of Chemical Sciences, University of Catania, Viale A. Doria 6, 95125 Catania, Italy

^bDepartment of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Via Mancinelli 7, Milano 20131, Italy

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ABSTRACT

Donkey's milk is today categorized among the best mother's milk substitute for allergic newborns, due to its much reduced or absent allergenicity, coupled to excellent palatability and nutritional value. However, up to the present, only a handful of proteins had been characterized, just about the standard eight to ten major ones known in all types of milk. By exploiting the combinatorial peptide ligand library technology, and treating large volumes (up to 300 mL) of defatted, de-caseinized (whey) milk, we have been able to identify 106 unique gene products, by far the largest description so far of this precious nutrient. Due to poor knowledge of the donkey's genetic asset, only 10% of the proteins could be identified by consulting the data base of *Equus asinus*; the largest proportion (70%) could be identified by homology with the proteins of *Equus caballus*.

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1. Introduction

The wide use of cow's milk (CM) in human diet has shown that a considerable percentage of subjects are allergic to some of its protein components. Particularly CM, used as substitute of breast feeding when mother's milk is not available or advisable, represents the main source of allergens in infants [1]. Successful therapy in cow's milk proteins allergy (CMPA) depends upon completely eliminating cow's milk protein (CMP) from the child's diet. Therefore, as milk represents the main source of nutrition for infants, a search to find a valid alternative has a high priority. Ideally, the substituting food should be hypo- or anallergenic, non-cross-reactive with CM, nutritionally adequate and palatable. Hypoallergenic milk (HA) formulas produced by pharmaceutical companies, represent the preferred choice in the treatment of CMPA, even if it has been demonstrated that allergenicity in these products is reduced, but never completely suppressed [2]. In addition, this milk is expensive, has poor palatability and, in some case, is

nutritionally not adequate. As an alternative to HA formulas, the possibility of using soybean-derived formulas or milk from goat and sheep has been examined. However, it has been shown that goat's and sheep's milk are contraindicated since both in vitro and in vivo cross-reactions with CMP are documented [3].

The legendary cosmetic and therapeutic properties of equine milk are widely known. In fact, Cleopatra, Poppea and other privileged ladies of ancient times were taking their baths in donkey's milk (DM) in order to keep their skin fresh and shiny. In addition, Hippocrates as well as Pliny the Elder believed that DM could act therapeutically in numerous cases, such as liver problems, infectious diseases, fevers, asthma, etc. [4,5].

Nowadays, clinical studies have clearly demonstrated that DM may be very useful in the prevention of atherosclerosis, being a strong vasodilator and an effective antimicrobial agent [6] and represents, for a large number (83–88%) of the investigated subjects, the unique safe and resolving "natural" treat-

* Corresponding author. Tel.: +39 095 7385041; fax +39 095 580138.
E-mail address: vcunsolo@unict.it (V. Cunsolo).

ment in both IgE-mediated and non-IgE-mediated CMPA, providing nutritional adequacy and good palatability [7,8]. Although the mechanism of this tolerance has not yet been fully clarified, it is reasonable to hypothesize that the reduced allergenic properties of DM can be related to structural differences of its protein components with respect to bovine milk. The high lactose content, the characteristic protein profile with a lower casein content (~50%) with respect to cow's milk (~80%) and the relevant percentage of essential amino acids make this milk a potential new dietetic food and a promising breast milk substitute [9].

Consequently, DM has recently aroused scientific and clinical interest, above all among pediatric allergologists. Notwithstanding this renewed growing interest, it should be underlined that, although the composition, physico-chemical and nutritional properties of DM are well known, characterization at molecular level is still scanty and regards only the most abundant proteins [10,11], whereas nothing is known about the minor or trace protein components. As a consequence, up to the present, the molecular basis for the demonstrated absence of allergy of this milk for a large number of CMA subjects, as well as the residual allergy for a minor number of subjects, remains unknown.

Recent technological advances for proteomic analysis have resulted in new powerful tools for the investigation and characterization of complex protein mixtures. In the current approach, the characterization of protein mixtures is usually performed by coupling highly efficient separation techniques, such as reversed-phase high performance liquid chromatography (RP-HPLC) or two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with MALDI (Matrix-Assisted Laser Desorption/Ionization) or ESI (Electrospray Ionization) mass spectrometry in order to obtain experimental data suitable for searching protein, genomic or ESTs (Expressed Sequence Tags) databases with appropriate software programs. However, it should be noted that the proteins present in most biological mixtures are expressed over a vast concentration range (up to 10–12 orders of magnitude in human sera) and that the most abundant proteins complicate the detection of low-abundance or trace components, including also allergen species, which could remain “hidden”.

Classical approaches such as pre-fractionation and immuno-depletion methodologies are frequently used to remove the most abundant proteins. Unfortunately, these methods not only are unsuccessful in concentrating trace components, which could remain below the detection limits of analytical approaches, but also may cause a non-specifically depletion of other components (including low-abundance species). In the case of immuno-depletion, then, the situation is less than brilliant: according to Tu et al. [12] “untargeted proteomic analyses using current LC-MS/MS platforms with immuno-depletion (MARS6) cannot be expected to efficiently discover low-abundance, disease-specific biomarkers in plasma”, since the increment in detection of these trace components after such a treatment results in a meager 25% increase, accounting for only 5–6% of total protein identifications in depleted plasma. An even harsher judgment was given by Zhi et al. [13] who candidly admitted that, whereas in neat serum they could detect 568 proteins, in MARS-14 depleted serum they could only identify 497 proteins, i.e. 10% less!

Recently, the characterization of minor components in complex protein systems, has been revolutionized by the introduction of the combinatorial peptide ligand libraries technology (CPLL) [14,15]. This new methodology, commercially named ProteoMiner, is based on the use of hexa-peptide baits to capture and normalize the relative concentrations of the components of the proteome under investigation. The major advantage of this technique, which has recently been applied to the exploration of cow's whey proteome [16], in comparison with other pre-fractionation methods, is that it not only diminishes the concentration of the more abundant proteins, but also concentrates low-abundance and even trace components, thus providing access to the “invisible” proteome. In addition, the loss of low-abundance species that may be accidentally eliminated by co-depletion using immuno-subtraction methods, is avoided.

Here, we report the application of the CPLL technology to the investigation of the “hidden” proteome of DM. Protein identification was performed by coupling the CPLL technology, SDS-PAGE, nLC-nESI MS/MS and database searching via the MASCOT engine.

2. Materials and methods

2.1. Materials

ProteoMiner (combinatorial hexapeptide ligand library beads, PM), molecular mass standards, SDS-PAGE precast gels and electrophoresis apparatus for one-dimensional electrophoresis were from Bio-Rad Laboratories, Hercules, CA. β -mercaptoethanol, dithiothreitol (DTT), De-Streak [Bis-(2-hydroxyethyl)disulphide, $(\text{HOCH}_2\text{CH}_2)_2\text{S}_2$] and all other chemicals used all along the experimental work were current pure analytical grade products and purchased from Sigma-Aldrich, St Louis, MO. Modified porcine trypsin was obtained from Promega (Madison, WI, USA).

2.2. Milk sample ProteoMiner treatment

Individual milk sample was collected in Eastern Sicily (ASILAT srl farm, Milo, Catania) from a Ragusano breed donkey at middle lactation stage. After milking, the sample was immediately frozen and stored at -20°C until used. Three hundred milliliters of milk was defatted by centrifugation (3500 rpm, 30 min) at 4°C and the fat layer was carefully removed. The casein fraction was precipitated from the skimmed milk at pH 4.3 with sodium acetate/acid acetic buffer and separated from the supernatant by centrifugation (8500 rpm, 30 min) at 4°C . The clear supernatant (whey fraction) was divided into three, 100 mL aliquots, separately titrated to three pH values (pH 4.0, 7.2 and 9.3), as per Fasoli et al. [17], and subsequently added, for all treatments, with 100 μL of CPLL beads (ProteoMiner). Protein adsorption was implemented via gentle shaking for 3 h at room temperature; then the beads were collected by filtration. The latter were then washed twice with proper buffers (10 mM acetate buffer at pH 4.0, phosphate at pH 7.0 and 10 mM Tris buffer at pH 9.3). The captured proteins from each peptide library sample were then desorbed by using a solution composed of 4% SDS, 2% DTT for 15 min, under boiling conditions (as per Candiano et al. [18]). Desorption was carried

out twice with 100 μ L each and the two eluates pooled. Such extractions were done in triplicate.

2.3. 1D-SDS-PAGE analysis

Twenty μ L of each bead eluate, in the presence of Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH approx. 6.8), was loaded onto the gel. The SDS-PAGE gel was composed by a 4% polyacrylamide stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS) cast over a 8–18% resolving polyacrylamide gel (in 375 mM Tris-HCl, pH 8.8, 0.1% SDS buffer). The cathodic and anodic compartments were filled with Tris-glycine buffer, pH 8.3, containing 0.1% SDS. Electrophoresis was at 100 V until the dye front reached the bottom of the gel. Staining and destaining were performed with colloidal Coomassie Blue and 7% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a VersaDoc imaging system (Bio-Rad).

2.4. 2D-PAGE analysis

The desired volume of each non-treated sample and CPLL eluates was solubilized in the “2-D sample buffer” (7 M urea, 2 M thiourea, 3% CHAPS, 40 mM Tris) to a final concentration of 2 mg/mL protein and the disulphide bridge reduction allowed to proceed at room temperature for 60 min by addition of TCEP [Tris(2-carboxyethyl)phosphine hydrochloride] at final concentration of 5 mM. For protecting reduced –SH groups, 150 mM De-Streak [Bis-(2-hydroxyethyl)disulphide, (HOCH₂CH₂)₂S₂] (diluted directly from the stock 8.175 M, Sigma-Aldrich) was added to the solution, followed by 0.5% pH 3–10 Ampholine (diluted directly from the stock, 40% solution) and a trace amount of bromophenol blue. Seven-cm long IPG strips (Bio-Rad), pH 3–10 L, were rehydrated with 150 μ L of protein solution, for 4 h. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell (Bio-Rad Laboratories) in a linear voltage gradient from 100 to 1000 V for 5 h, 1000 V for 4 h, followed by an exponential gradient up to 5000 V, for a total of 25 kV/h. For the second dimension, the IPGs strips were equilibrated for 25 min in a solution containing 6 M urea, 2% SDS, 20% glycerol, 375 mM Tris-HCl (pH 8.8) under gentle shaking. The IPG strips were then laid on 8–18% acrylamide gradient SDS-PAGE gel slabs with 0.5% agarose in the cathodic buffer (192 mM glycine, 0.1% SDS and Tris-HCl to pH 8.3). The electrophoretic run was at 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 15 mA/gel until the dye front reached the gel bottom. Gels were incubated in a colloidal Coomassie Blue solution and destaining was performed in 7% acetic acid till clear background, followed by a rinse in pure water. The 2-DE gels were scanned with a Versa-Doc image system (Bio-Rad), by fixing the acquisition time at 10 s; the relative gel images were captured via the PDQuest software (Bio-Rad Laboratories). After filtering the gel images for removing the background, spots were automatically detected, manually edited and then counted.

2.5. In-gel digestion of 1D-SDS-PAGE slices

The four sample lanes (E4, E7, E9 and Ctrl) of SDS-PAGE gels were manually cut in 26 pieces along the migration path. The excised gel pieces were transferred to 1.5 mL microcentrifuge

tubes, washed three times with H₂O/CH₃CN (1:1; v:v) at 37 °C, shrunk in “acetonitrile (ACN)” and dried under vacuum. Then, proteins were reduced by 10 mM DTT and alkylated by 55 mM iodoacetamide. Gel pieces were shrunk in ACN and dried under vacuum. Finally, alkylated proteins were subjected to in-gel digestion using modified porcine trypsin. After soaking trypsin into the gel pieces, the supernatant containing an excess of enzyme was discarded and the gel pieces were covered with 50 μ L of 50 mM ammonium bicarbonate. Digestion was allowed to proceed overnight at 37 °C. After in-gel digestion, the digested solution was transferred into a clean 0.5 mL tube. The peptides were extracted from gel pieces with 0.1% “trifluoroacetic acid (TFA)” solution and subsequently with ACN. This extraction procedure was repeated three times. The total extracts were pooled, joined with the first supernatant, lyophilized and re-dissolved in 20 μ L of 0.1% TFA.

2.6. Capillary RP-HPLC/nESI-MS/MS analysis

Capillary RP-HPLC/nESI-MS/MS analysis of the in-gel enzymatic digests was performed using an Ultimate 3000 LC system combined with an autosampler and a flow splitter 1:100 (Dionex Corporation, Sunnyvale, CA, USA), coupled on-line with a linear ion trap nano-electrospray mass spectrometer (LTQ, Thermo Fischer Scientific, San Jose, CA). Ionization was performed with liquid junction using an uncoated capillary probe (20 mm i.d.; New Objective, Woburn, MA, USA). Ten microliters of tryptic digest for each band was loaded onto a μ -pre-column C18 (0.3 mm $\times$ 5 mm, 100 Å, 5 μ m, PepMap, Dionex) equilibrated with 0.5% “formic acid (FA)” aqueous solution at a flow rate of 20 μ L/min for 4 min. Then, the solution was switched onto a reversed-phase C18 column (0.18 $\times$ 150 mm, 300 Å, 5 μ m, BioBasic, ThermoFisher Scientific) and peptides were separated by elution at room temperature with a linear gradient of solvent B (ACN+0.5% FA) in A (H₂O+0.5% FA) from 20% to 55% in 40 min at a flow rate of 1.5 μ L/min. The nano-ESI source operated under the following conditions: source temperature 220 °C, spray voltage 2 kV, and capillary voltage 38 V. Repetitive mass spectra were acquired in positive ion mode in the m/z range 250–2000. Characterization of peptide ions was performed by the data-dependent method as follows: i) full scan MS in the m/z range 250–2000; ii) zoom scan of the five most intense ions (isolation width: 1.5 Da); MS/MS analysis of the five most intense ions (normalized collision energy: 24 a.u., activation Q: 0.250). Mass calibration was made using a standard mixture of caffeine (Mr 194.1 Da), MRFA peptide (Mr 423.6 Da) and Ultramark (Mr 1621 Da). Data acquisition was performed using the Excalibur v. 1.4 software (ThermoFisher Scientific).

2.7. Protein identification via MASCOT engine

MS/MS data were used to perform protein identifications by searching in a non-redundant protein sequence database (“nrNCBI”, updated May 2010) using the MOWSE algorithm as implemented in the MS search engine MASCOT server (Matrix Science, London, UK, version 2.3). MASCOT was set up to search against the Mammalia taxonomy (778,778 entries) of nrNCBI database assuming the digestion enzyme was trypsin with a maximum of 2 missed cleavages allowed. MASCOT was

searched with a fragment ion mass tolerance of 0.6 Da and a parent ion mass (PIM) tolerance of ± 2 Da. Cysteine alkylation was specified as fixed modification, whereas oxidation of methionine, transformation of N-terminal glutamine and N-terminal glutamic acid residue in the pyroglutamic acid form, phosphorylation of serine, threonine and tyrosine were specified as variable modifications.

In MASCOT searches, proteins scores (as MudPIT score) were derived from ion scores as a non-probabilistic basis for ranking protein hits, disregarding all individual peptides with scores below 20 (reports of all MASCOT searches are available on the supplementary material online) [19]. Taking into account the extremely low amount of database entries from *Equus asinus*, all protein hits derived from MASCOT search were manually checked. In particular, doubtful and low MASCOT scoring MS/MS spectra were also subjected to manual interpretation assisted by the PepNovo software (<http://proteomics.ucsd.edu/Software/PepNovo.html>) in order to confirm or disregard peptide sequence assignment and therefore protein identification.

Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The assignment of putative roles into known eukaryotic biosynthetic pathways was performed using the UniProt (Universal Protein Resource) database (www.uniprot.org/).

3. Results

Fig. 1 shows the SDS-PAGE profiling of the control (Ctrl) as well as of the various fractions eluted from the ProteoMiner beads.

From left to right the three lanes of CPLL eluates obtained at pH 9.3, 7.2 and 4.0, respectively, are reported. It is seen that in all CPLL treatments many more components are visible, with Mr values covering the entire spectrum from ca. 10 kDa up to 250 kDa. On the contrary, in the control, only two mass regions seem to be populated: from 10 to 25 kDa and then again from 65 to 80 kDa.

Two-dimensional map analyses confirmed this trend: in the control, only barely about 100 spots are visible, whereas in all eluates several hundred spots can be appreciated (Fig. 2). Additionally, in the control the strongest component appears to be residual caseins, which form a spot at around 25 kDa plus a heavy smear reaching up to 75 kDa, probably representing poorly-soluble aggregates. Conversely, in the various eluates, the level of residual caseins is strongly lowered, except in the pH 4.0 capture, in which caseins are still visible as a strong band. Probably in this last capture the level of casein captured was magnified also by the fact that adsorption occurred at a pH value quite close to the pI of casein, this favoring perhaps its adsorption/precipitation onto the CPLL beads. Nevertheless, it can be appreciated that even this capture at pH 4.0, which seems to be the one exhibiting the lowest number of spots, has the unique advantage of capturing a population of rather acidic proteins (extending down to pH 3.0), which are not visible on the capture at the two higher pH values (7.2 and 9.3). By the same token, the capture at pH 9.3 (which seems to be the one richest in spot number) contains a more abundant population of alkaline species, a behavior that had been predicted by Fasoli et al. [17].

As stated in the relevant section in **Materials and methods**, the four sample lanes (E4, E7, E9 and Ctrl) of SDS-PAGE gels were manually cut in 26 pieces along the migration path, all

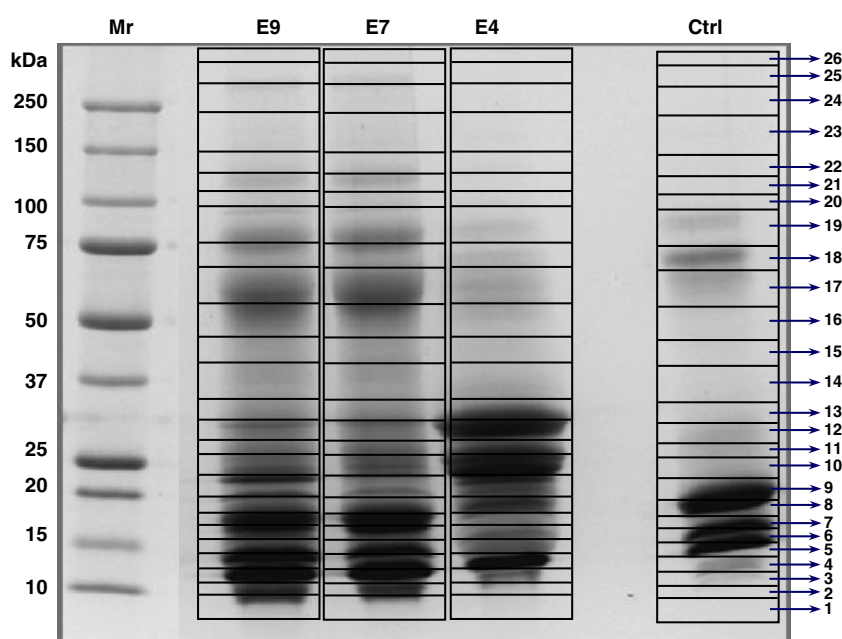


Fig. 1 – SDS-PAGE profiling of donkey's milk. From left to right: Mr: molecular mass markers; E9, E7, E4: 4% SDS eluates of donkey's milk treated by ProteoMiner beads at pH 9.3, 7.2 and 4.0, respectively; Ctrl: control, untreated donkey's milk. In all cases, a 20 μ L volume was seeded into the gel pockets. Staining with Coomassie Brilliant Blue. The broken horizontal lines represent the slicing position with slice number, prior to proteins digestion and LC-MS/MS analysis.

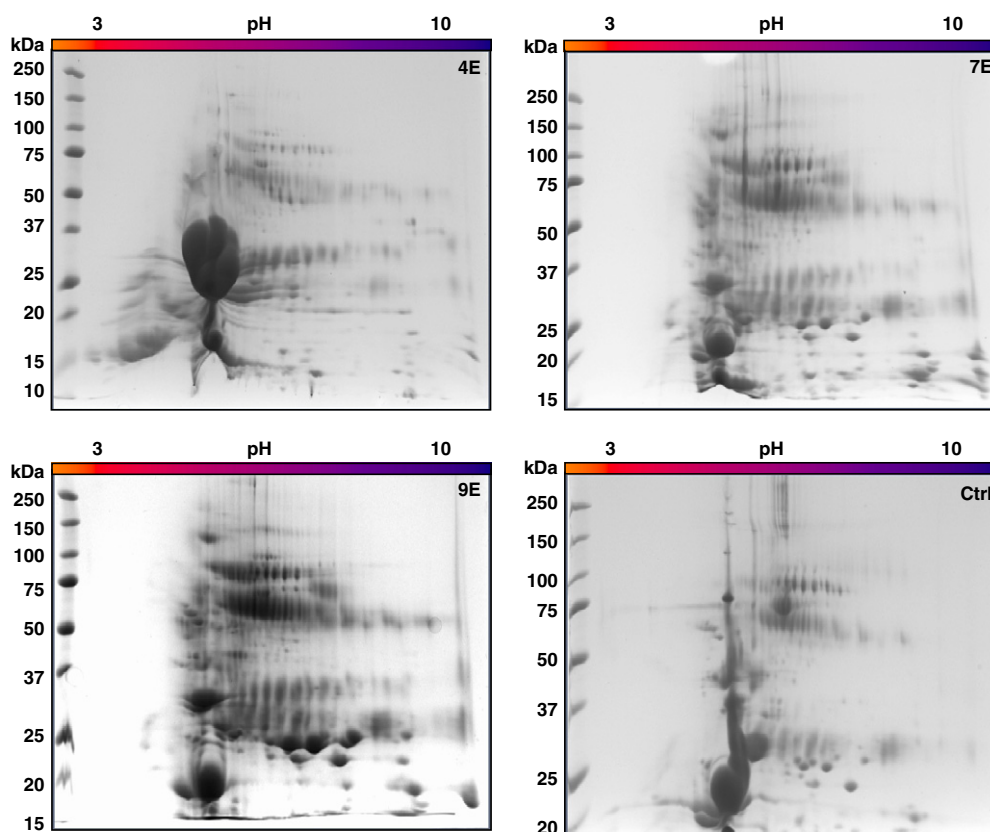


Fig. 2 – Two dimensional map analysis of the three eluates and of control donkey's milk of Fig. 1. In all cases, a total of 150 μ g protein was applied to each IPG strip, in the pH interval 3–10. Staining with Coomassie Brilliant Blue.

proteins digested and the species identified by MS. This approach allowed discovering and identifying a total of 106 unique gene products (Table 1), most of which (82%) were exclusively found in the whey proteome after ProteoMiner treatment. As shown in Table 1, among the 106 identified proteins, 18 components were well known milk proteins (caseins, alpha-lactalbumin, beta-lactoglobulins, lysozyme, serum albumin and lactoferrin), whereas the others (83%) were not described in previous proteomic studies, and therefore represent the most comprehensive list of donkey's whey proteins at present. Seventy percent of identified proteins were from *Equus caballus*, 7% were from *Homo sapiens*, 3% from *Bos taurus*, 9% were from other mammalian and only 11 proteins (10%) were from *E. asinus*.

The low number of identified proteins from *E. asinus* can be explained by the extremely low amount of database entries from this specie, whose genome is still unsequenced. On the other hand, the large presence of known homologous proteins from the closest related specie (*E. caballus*), allowed the cross-species identification for most of the proteins investigated. By searching the UniPROT database and literature data all proteins listed in Table 1 were grouped into ten putative molecular function categories (Fig. 3). The most abundant classes of identified components are represented by proteins belonging to the categories of "protein binding" (~28%), "ion binding" (~19%), "enzyme inhibitors" (~17%) and "hydrolase" (~10%).

4. Discussion and conclusions

Bovine milk proteins, present at a concentration of 32 g/L, are generally classified as caseins (80% of the total milk protein content), whey proteins (16%), peptones/low molecular mass peptides (3%) and milk fat globule membrane (MFGM) proteins (1%). Considering that just height major proteins constitute the vast majority of bovine (but also human) milk (α s1-, α s2-, β -, κ - and γ -caseins) together with β -lactoglobulin, α -lactalbumin and serum albumin that infiltrates from blood to the mammary gland, this leaves little room for searching for the low-abundance proteome, which in principle might be constituted by hundreds of species. In fact the discovery of the "minority proteome" has been painfully slow, considering that in 2007 Conti et al. [20] compiled an extensive review on the proteomics of human milk and listed a total of barely 73 unique gene products (UGP), by summing up all species found not just only in mature milk, but also in colostrum, whey, skimmed milk and milk fat globule proteins. A quantum jump was made in 2009 when D'Amato et al. [16], by using the CPLL methodology, at once reported a total discovery of 149 UGPs in the whey fraction alone, by far the most extensive discovery at that date. These data were expanded later on by the findings of Le et al. [21] who, in colostrum and mature bovine whey reported a grand total of 293 UGPs, a most remarkable deed, indeed, which however came at the expenses of a most laborious protocol,

Table 1 – List of the identified proteins in donkey's milk.

Identified proteins (general index; species)	Mr protein	PIM ^a (charge)	Experimental Mr	Theoretical Mr	Delta Mr Exp–Theor	Peptide sequence	Origin
Alpha s1 casein ^b (gi 238055131; <i>Equus asinus</i>)	24 kDa	838.6 (2+)	1675.2	1674.8	0.4	EDMLYQHTLEQLR	E4–E9
		1021.2 (2+)	2041.4	2041.0	0.4	IPREDMLYQHTLEQLR ^c	
		820.7 (2+)	2459.4	2460.2	–0.8	KFPSFALHTPREYINELNR ^b	
		923.2 (2+)	1844.4	1843.9	0.5	LIASENSEKTDIPEW	
		1210.9 (2+)	2419.8	2419.3	0.6	LSKYNQLQLQAIYAQEQLIR ^c	
		859.2 (3+)	2574.6	2575.4	–0.8	RLSKYNQLQLQAIYAQEQLIR	
Alpha s1 casein precursor (gi 30794348; <i>Bos taurus</i>)	23 kDa	1046.4 (2+)	2090.7	2091.1	–0.3	YNQLQLQAIYAQEQLIR	Ctrl
		1158.8 (2+)	2315.6	2315.1	0.4	EPMIGVNQELAYFYPELFR	
		693.2 (2+)	1384.4	1383.7	0.7	FFVAPFPEVFGK	
		821.8 (2+)	1641.6	1640.8	0.8	FFVAPFPEVFGKEK	
		669.8 (2+)	1337.6	1336.6	1.0	HIQKEDVPSEK	
		1118.7 (2+)	2235.4	2234.2	1.3	HPIKHQGLPQEVNLNLLR	
Alpha s1 casein precursor ^d (gi 229487207; <i>Equus asinus africanus</i>)	25 kDa	880.8 (2+)	1759.6	1758.9	0.7	HQGLPQEVNLNLLR	Ctrl–E4–E7–E9
		634.8 (2+)	1267.6	1266.6	1.0	YLGYLEQLLR	
		856.7 (2+)	1711.4	1711.8	–0.4	FPSFALEYINELNR ^d	
		920.2 (2+)	1839.4	1839.9	–0.5	KFPSFALEYINELNR ^d	
		807.5 (3+)	2419.5	2419.3	0.2	LSKYNQLQLQAIYAQEQLLR	
		859.2 (3+)	2574.6	2575.4	–0.8	RLSKYNQLQLQAIYAQEQLLR	
Alpha s2 casein (gi 218390193; <i>Equus asinus</i>)	28 kDa	1046.8 (2+)	2091.6	2091.1	0.5	YNQLQLQAIYAQEQLLR	Ctrl–E4–E7–E9
		1151.4 (2+)	2300.8	2300.2	0.6	INQFYKLNFLQYLQALR	
		713.3 (2+)	1424.6	1424.7	–0.1	LLNKINQYYEK	
		689.8 (2+)	1377.6	1377.7	–0.1	LNFLQYLQALR	
		748.7 (2+)	1495.4	1495.7	–0.3	TELTEEEKNYLK	
		1259.9 (2+)	2517.8	2517.3	0.5	TGASPFPIVNTTEQLTSEEIPK	
Alpha s2 casein (gi 71982842; <i>Bubalus bubalis</i>)	26 kDa	652.2 (2+)	1302.4	1302.7	–0.3	TNSYQIIPVLR	Ctrl
		684.8 (2+)	1367.6	1366.6	1.0	ALNEINQFYQK	
Alpha s2 casein B precursor (gi 254826592; <i>Equus asinus</i>)	18 kDa	1433.8 (2+)	2865.6	2865.4	0.2	FPQYLQYLYQGPIVLPWDQVER ^c	E4–E9
		1328.2 (2+)	2654.4	2654.2	0.2	FPSPQYFQAVHPQIQIPMSPWNR ^{c,e}	
		461.7 (2+)	921.4	921.5	–0.1	QLVKINPK (36)	
Alpha-1-antiproteinase 2 precursor (gi 167621418; <i>Equus caballus</i>)	47 kDa	973.3 (3+)	2916.9	2915.3	1.6	TVDMGSTEIFPEEIELSDEEKNYLK ^{e–f}	Ctrl–E4–E7–E9
		1007.2 (2+)	2012.4	2013.0	–0.6	DLDKDTVLALVNYIFFK	
		776.7 (2+)	1551.4	1551.8	–0.4	FNRPFVLIHYDR	
		904.8 (3+)	2711.4	2711.2	0.2	GTWEKPFPEYTTTEQDFHVDEK	
		799.3 (2+)	1596.6	1596.8	–0.2	IAPNLADFAFSLYR	
		894.6 (3+)	2680.8	2680.5	0.3	IVDLVKDLDKDTVLALVNYIFFK	
Alpha-1-antitrypsin (gi 197631757; <i>Equus caballus</i>)	47 kDa	599.1 (2+)	1196.2	1196.6	–0.4	LQHLEDTLTK	E4–E9
		1061.0 (2+)	2120.0	2120.1	–0.1	LSISGTYYDLTSILPELGITK	
		893.6 (2+)	1785.2	1785.9	–0.7	QADLSGITEEVPLTVSK	
		731.6 (2+)	1461.2	1461.7	–0.5	QINDYVEKGTQGK	
		1006.2 (2+)	2012.4	2013.0	–0.6	DLDKDTVLALVNYIFFK	
		879.5 (3+)	2635.5	2635.2	0.3	GTWEKPFPESTTEQDFHVDEK	
		632.7 (2+)	1263.4	1263.7	0.3	HSSFVNVLHPK	
		799.2 (2+)	1596.4	1596.8	0.4	IAPNLADFAFSLYR	
		599.2 (2+)	1196.4	1196.6	0.2	LQHLEDTLTK	

Alpha-1-antitrypsin (gi 197631763; <i>Equus caballus</i>)	47 kDa	1061.1 (2+)	2120.2	2120.2	0.0	LSISGTYDLTSILPELGITK	E4-E9
		893.8 (2+)	1785.6	1785.9	0.3	QADLSGITEEVPLTVSK	
		1006.2 (2+)	2012.4	2013.0	-0.6	DLDKDTVLALVNYIFFK	
		632.7 (2+)	1263.4	1263.7	-0.3	HSSFVNVLHPK	
		799.2 (2+)	1596.4	1596.8	-0.4	IAPNLADFAFSLYR	
		610.7 (2+)	1219.4	1219.6	-0.2	LVDKFQEDVK	
Alpha-lactalbumin (gi 126000; <i>Equus asinus</i>)	15 kDa	599.2 (2+)	1196.4	1196.6	-0.2	LQHLEDTLTK	Ctrl-E4-E7-E9
		1061.1 (2+)	2120.2	2120.2	0.0	LSISGTYDLTSILPELGITK	
		893.8 (2+)	1785.6	1785.9	-0.3	QADLSGITEEVPLTVSK	
		973.3 (2+)	1944.6	1944.9	-0.3	EGIDYWLAHKPLCSEK	
		837.1 (2+)	1672.2	1672.6	-0.4	FLDDDLTDDVMCAK	
		901.2 (2+)	1800.5	1800.7	-0.2	FLDDDLTDDVMCAKK	
		1187.4 (2+)	2372.8	2373.1	-0.3	ILDSEGIDYWLAHKPLCSEK ^c	
		1251.4 (2+)	2500.8	2501.2	-0.4	KILDSEGIDYWLAHKPLCSEK	
		804.9 (2+)	1607.8	1607.9	-0.1	KQFTKCELSQVLK	
		795.7 (2+)	1589.4	1590.7	-1.3	MWCRDNQILPSR	
		532.8 (2+)	1063.6	1064.5	-0.9	NICGISCNK	
		1352.8 (2+)	2703.6	2703.1	0.5	NICGISCNKFLDDDLTDDVMCAK ^c	
Alpha-lactalbumin (gi 167643009; <i>Pusa hispida</i>)	17 kDa	871.2 (2+)	1740.4	1738.8	1.6	NNGKTEYGLFQINN	E4-E9
		664.1 (2+)	1326.2	1325.6	0.6	TEYGLFQINN	
		973.3 (2+)	1944.6	1944.9	-0.3	EGIDYWLAHKPLCSEK	
		844.1 (2+)	1686.2	1686.7	-0.4	FLDDDLTDDMICAK	
		899.9 (2+)	1797.9	1798.8	-0.9	FLDDDLTDDMICAKK	
		552.4 (2+)	1102.8	1103.6	-0.8	KCELSQVLK	
Alpha-lactalbumin B/C (gi 125992; <i>Equus caballus</i>)	15 kDa	1188.2 (2+)	2374.4	2375.1	-0.7	DLDSEGIDYWLAHKPLCSEK	Ctrl-E4-E7-E9
		829.0 (2+)	1656.0	1656.7	-0.7	FLDDDLTDDVMCAK	
		893.7 (2+)	1784.4	1784.7	-0.3	FLDDDLTDDVMCAKK	
		1188.2 (2+)	2374.4	2375.1	-0.7	KDL DSEGIDYWLAHKPLCSEK	
		788.1 (2+)	1574.2	1574.7	-0.5	MWCRDNQILPSR	
		532.8 (2+)	1063.6	1064.5	-0.9	NICGISCNK	
		907.7 (3+)	2720.1	2719.1	1.0	NICGISCNKFLDDDLTDDVMCAK	
		886.1 (2+)	1770.2	1770.9	-0.7	YLQTVTDYGDVLVEK	
Apolipoprotein A-II (gi 47115663; <i>Equus caballus</i>)	9 kDa	746.2 (2+)	1490.4	1490.8	-0.4	VQHSTLKEPLVK	E9
Beta-2-microglobulin (gi 75072805; <i>Equus asinus</i>)	13 kDa	937.8 (2+)	1873.6	1874.0	-0.4	KVMPFLKSPIVPFSEK ^e	E4
Beta-casein (gi 1293119; <i>Equus grevyi</i>)	16 kDa	1015.9 (2+)	2029.8	2030.1	-0.3	RKVMPLKSPIVPFSEK	E4
		516.1 (2+)	1030.2	1030.5	-0.3	SPIVPFSEK	
Beta-casein precursor (gi 270118591; <i>Equus asinus</i>)	27 kDa	797.7 (2+)	1593.4	1593.8	-0.4	VAPFPQPVVPYPQR	E4-E9
		588.2 (2+)	1761.4	1761.9	-0.5	VMPFLKSPIVPFSEK	
		1103.4 (2+)	2204.8	2205.1	-0.3	VAPFPQPVVPYPQRDTPVQA	
		620.1 (2+)	1238.2	1239.6	-1.4	EVEHQDKISR	
		937.8 (2+)	1873.6	1874.0	-0.4	KVMPFLKSPIVPFSEK ^e	
		685.2 (2+)	1368.4	1367.7	0.7	QILNPTNGENLR	
		1015.9 (2+)	2029.8	2030.1	-0.3	RKVMPLKSPIVPFSEK	
		516.1 (2+)	1030.2	1030.5	-0.3	SPIVPFSEK	
		797.7 (2+)	1593.4	1593.8	-0.4	VAPFPQPVVPYPQR	
		588.2 (2+)	1761.4	1761.9	-0.5	VMPFLKSPIVPFSEK	
		748.7 (2+)	1495.4	1495.7	-0.3	IFAEKTESPAEFK	
		1017.7 (2+)	2033.4	2033.9	-0.5	INYLDEDTVFALDTDYK ^g	
Beta-lactoglobulin-1 precursor ^g (gi 126723662; <i>Equus caballus</i>)	21 kDa						Ctrl-E4-E7-E9

(continued on next page)

Table 1 (continued)

Identified proteins (general index; species)	Mr protein	PIM ^a (charge)	Experimental Mr	Theoretical Mr	Delta Mr Exp-Theor	Peptide sequence	Origin
Beta-lactoglobulin-2 variant A (gi 125904; <i>Equus asinus</i>)	18 kDa	1040.9 (3+)	3119.8	3119.4	0.4	INYLDDEDTVFALDSTDYKNYLFLCMK	Ctrl-E4-E7-E9
		812.5 (2+)	1623.0	1623.8	-0.8	KIFAEKTESPAEFK	
		839.8 (2+)	1677.6	1677.9	-0.3	LRPTPEDNLEIILR	
		746.2 (3+)	2235.4	2235.1	0.3	LRPTPEDNLEIILREGENK	
		875.2 (2+)	1748.4	1747.8	0.6	NAATPGQSLVCQYLAR	
		504.6 (2+)	1007.2	1006.6	0.6	RALQPLPGR	
		684.6 (2+)	1367.2	1367.5	-0.3	TQMVDEEIMEK	
		837.0 (2+)	1672.0	1670.7	1.3	TQMVDEEIMEKFR ^e	
		1179.0 (2+)	2356.0	2355.1	0.9	WHSVAMAASDISLLDSESAPLR ^g	
		1120.8 (2+)	2239.6	2239.1	0.5	ALQPLPGHVQIIQDPSSGQER	
		585.1 (2+)	1168.2	1169.5	-1.3	EGANHVCVER	
		1015.1 (2+)	2028.2	2028.9	-0.7	TDIPQTMQDLDLQEVAGR	
		863.1 (2+)	1724.2	1724.8	-0.6	TEDPAVFTVNYQGER	
		927.1 (2+)	1852.2	1852.8	-0.6	TEDPAVFTVNYQGERK	
Cathepsin D (gi 115719; <i>Sus scrofa</i>)	38 kDa	946.3 (2+)	1890.6	1890.0	0.6	QTFGEATKQPGLTFIAAK ^h	E9
C-C motif chemokine 28 precursor [gi 22538811; <i>Homo sapiens</i>]	15 kDa	798.6 (2+)	1595.2	1595.8	-0.6	ADGDCDLAAVILHVK	E9
Cholinesterase (gi 21362409; <i>Equus caballus</i>)	66 kDa	730.1 (2+)	1458.2	1457.7	0.5	VLELTGNIDEAER	E9
CLN2 protein (gi 56122204; <i>Saimiri boliviensis</i>)	56 kDa	1100.3 (2+)	2198.6	2198.2	0.4	SVTLFGESAGAASVSLHLLSPR	E9
		737.7 (2+)	1473.4	1473.7	-0.3	LFGGNFAHQASVAR	
Clusterin precursor (gi 126352584; <i>Equus caballus</i>)	53 kDa	1507.0 (2+)	3012.0	3011.5	0.5	HQFRPSFPASSPYVTVVGTSFQNPFR	E4-E9
		706.1 (2+)	1410.2	1410.6	-0.4	ASSIMDELFQDR	
		987.3 (2+)	1972.6	1972.8	-0.2	EPQDTYYYSFSSPHR ^c	
		1263.4 (2+)	2524.8	2524.1	0.7	FTREPQDTYYYSFSSPHR ^c	
		658.8 (2+)	1315.6	1315.8	-0.2	KSLLGTLLEAKK	
		961.3 (2+)	1920.6	1921.0	-0.4	LFDSYPITVTVPVVSR	
		1059.3 (2+)	2116.6	2115.9	0.7	LPDQLPMTFSEGDNHDR ^{c,e}	
		764.7 (2+)	1527.4	1527.7	-0.3	LYDELLQSYQEK	
		687.2 (2+)	1372.4	1372.7	-0.3	NLIEQTNEERK	
		594.7 (2+)	1187.4	1187.7	-0.3	SLLGTLLEAKK	
Complement component C9 precursor (gi 126352550; <i>Equus caballus</i>)	63 kDa	728.2 (2+)	1454.4	1454.7	-0.3	AIEDYINEFSVR	E4
Gelsolin (gi 126352530; <i>Equus caballus</i>)	80 kDa	628.2 (2+)	1254.4	1253.7	0.7	AGKEPGLQIWR	E4-E7-E9
		1151.3 (2+)	2300.6	2301.1	-0.5	AQPQVAEGSEPDSEFWEALGGK	
		826.1 (2+)	1650.2	1650.7	-0.5	DSQDEEKTEALTSK	
		862.1 (2+)	1722.2	1721.8	0.4	EVQGFESATFLGYFK	
		955.6 (3+)	2863.9	2863.4	0.5	NWRDPDQTEGLGLAYLSSHIAHVER	
		904.6 (2+)	1807.2	1806.9	0.3	TPSAAYLWVGAGASEAEK	
Gelsolin isoform b (gi 77736201; <i>Bos taurus</i>)	80 kDa	1258.8 (2+)	2515.6	2514.3	1.2	VSNAGAGPMVSVLADENPFAQGALR ^e	E9
		628.2 (2+)	1254.4	1253.7	0.7	AGKEPGLQIWR	
		1151.3 (2+)	2300.6	2301.1	-0.5	AQPQVAEGSEPDSEFWEALGGK	
		915.2 (2+)	1828.4	1828.9	-0.5	QTQVSVLPEGGETPLFK	

Heat shock protein 70 kDa family, member 13 (gi 22137780; <i>Homo sapiens</i>)	52 kDa	904.6 (2+)	1807.2	1806.9	0.3	TPSAAYLWVGAGASEAEK	E9
		855.2 (2+)	1708.4	170.8.9	-0.5	KSGESQVLFETEISR	
Hypoxia up-regulated 1 (gi 29747953; <i>Mus musculus</i>)	111 kDa	842.1 (2+)	1682.2	1681.9	0.3	AANSLKAFIFETQDK	E9
		1048.7 (2+)	2095.4	2094.1	1.3	VLQLINDNTATALSYGVFR	
IgG Fc binding protein (gi 1944352; <i>Homo sapiens</i>) Immunoglobulin alpha constant heavy chain (gi 32331167; <i>Equus caballus</i>)	38 kDa	765.7 (2+)	1529.4	1529.7	-0.3	SPANCPLSCPANSR	E9 Ctrl-E4-E7-E9
		729.2 (2+)	1456.4	1456.7	-0.3	ADSGDPVVIACLIK	
		646.7 (2+)	1291.4	1291.7	-0.3	EKYLVFVKPLR	
		726.1 (2+)	1450.2	1449.8	0.4	EVLVLWLQGHEK	
		1119.8 (2+)	2237.6	2238.0	-0.4	GDVFSCMVGHEALPLSFTQK ^e	
		667.6 (2+)	1333.2	1333.7	-0.5	GFFPLGFPEPVK	
		793.2 (2+)	1584.4	1584.8	-0.4	KADSGDPVVIACLIK	
		1167.7 (2+)	2333.4	2334.1	-0.7	LSGKPTHVNVSVVMAEADGTCY	
		794.2 (3+)	2379.6	2378.1	1.5	RGDVFSCMVGHEALPLSFTQK ^e	
		518.2 (2+)	1034.4	1034.6	-0.2	YLVFKPLR	
		1068.8 (2+)	2135.6	2136.1	-0.5	CPAPPELLGGPSVFIFPPNP	
		708.1 (2+)	1414.2	1414.6	-0.4	FNWYMDGVEVR	
Immunoglobulin gamma 1 heavy chain constant region (gi 15020816; <i>Equus caballus</i>)	38 kDa	808.8 (2+)	1615.6	1614.8	0.8	IQHQDWLSGKEFK	Ctrl-E4-E7-E9
		1057.7 (2+)	2113.4	2114.0	-0.6	TPEVTCVVVDVDSQENPDVK	
		689.9 (2+)	1377.8	1377.7	0.1	VNNQALPQPIER	
		1095.1 (2+)	2188.2	2187.9	0.3	YSTTQAQDSDGSYFLYSK	
		808.7 (2+)	1615.4	1615.9	-0.5	FLGGPSVFIFPPNP	
		560.7 (2+)	1119.4	1119.6	-0.2	SKVSVTCLVK	
Immunoglobulin gamma 2 heavy chain constant region (gi 15026999; <i>Equus caballus</i>)	38 kDa	901.2 (2+)	1800.4	1801.0	-0.6	VPQVYVLPHPDELAK	Ctrl-E4-E9
		903.0 (2+)	1804.0	1805.0	-1.0	VVSVLPQHQDWLSGK	
		1056.7 (2+)	2111.4	2112.0	-0.6	YSTTPAQLDSDGSYFLYSK	
		799.2 (2+)	1596.4	1594.8	1.6	CKVNNQALPAPVER	
		717.5 (3+)	2149.5	2150.1	0.6	CPAPPELLGGPSVFIFPPKPK	
		1309.8 (2+)	2617.6	2617.2	0.4	DFYPTDITVEWQSNHEPEEGK	
Immunoglobulin gamma 3 heavy chain constant region (gi 32442221; <i>Equus caballus</i>)	39 kDa	774.6 (2+)	1547.2	1547.7	-0.5	TYICNV AHPASSTK	Ctrl-E9
		654.8 (2+)	1307.6	1306.7	0.9	VNNQALPAPVER	
		896.7 (2+)	1791.4	1790.9	0.5	VPQVYVLPAPHPDELAK	
		771.1 (2+)	1540.2	1540.7	-0.5	DFYPTDIDIEWK	
		940.8 (2+)	1879.6	1880.0	-0.4	EPQVYVLPAPHRDELAK	
		882.6 (2+)	1763.2	1762.8	0.4	SQTYICNV AHPASSTK	
Immunoglobulin gamma 4 heavy chain (gi 42528293; <i>Equus caballus</i>)	36 kDa	654.2 (2+)	1306.4	1306.7	-0.3	VNNKALPAPVER	Ctrl-E4-E9
		903.0 (2+)	1804.0	1805.0	-1.0	VVSVLPQHQDWLSGK	
		1072.2 (2+)	2142.4	2142.0	0.4	YSTTPAQLDSDGSYFLYSK	
		771.2 (2+)	1540.4	1540.8	-0.4	CSVTNKALPAPVER	
		560.7 (2+)	1119.4	1119.6	-0.2	NTVSVTCLVK	
		888.5 (2+)	1775.0	1775.0	0.0	VPQVYVLPAPHPDELAK	
Immunoglobulin gamma 5 heavy chain constant region (gi 18996195; <i>Equus caballus</i>)	36 kDa	903.0 (2+)	1804.0	1805.0	-1.0	VVSVLPQHQDWLSGK	Ctrl-E4-E9
		1331.3 (2+)	2660.6	2660.2	0.4	DFYPPDINVEWQSNEEPEPEVK	
		920.3 (2+)	1838.6	1837.9	0.7	FNWYVDGVEAHTATTK	
		560.7 (2+)	1119.4	1119.6	-0.2	NTVSVTCLVK	
		1029.2 (2+)	2056.4	2057.0	-0.6	QSSGLYSLSSMVTVPASSLK ^e	
		882.6 (2+)	1763.2	1762.8	0.4	SQTYICNV AHPASSTK	
Immunoglobulin gamma 6 heavy chain constant region (gi 18996197; <i>Equus caballus</i>)	36 kDa	1057.7 (2+)	2113.4	2114.0	-0.6	TPEVTCVVVDVDSQENPDVK	Ctrl-E4-E9

(continued on next page)

Table 1 (continued)

Identified proteins (general index; species)	Mr protein	PIM ^a (charge)	Experimental Mr	Theoretical Mr	Delta Mr Exp-Theor	Peptide sequence	Origin
Immunoglobulin J chain (gi 261291939; <i>Equus caballus</i>)	18 kDa	789.1 (2+)	1576.2	1575.8	0.4	VVSVLP IQHQDWR	Ctrl-E4-E7-E9
		1056.7 (2+)	2111.4	2111.9	-0.5	YSTTPAQLDGDGSYFLYSK	
		641.6 (2+)	1281.2	1280.6	-0.6	FVYHLSDLCK	
		868.2 (2+)	1734.4	1734.9	-0.5	IIPSPENPNEDILER	
		819.7 (2+)	1637.4	1637.8	-0.4	TKFVYHLSDLCKK	
Immunoglobulin mu heavy chain constant chain secreted form (gi 51831151; <i>Equus caballus</i>)	50 kDa	532.2 (2+)	1062.4	1062.6	-0.2	VPLTYGGQTK	E4-E7-E9
		1045.2 (2+)	2088.4	2089.0	-0.6	DGKPVESGFTTEEVQPQNK	
		824.2 (2+)	1646.4	1646.8	-0.4	GFSPPDV FVQWLQK	
		681.6 (2+)	1361.2	1361.7	-0.5	HPPSVYVLPPTTR	
		811.2 (2+)	1620.4	1620.9	-0.5	IFAIPPSFAGIFLTK	
		1151.0 (2+)	2300.0	2300.1	-0.1	LSCQVTNLGTYDSLISWTR	
		702.1 (2+)	1402.2	1402.7	-0.5	SQDIKNFPSVLR	
		1228.8 (2+)	2455.6	2456.0	-0.4	STGKPTLYNVSLVMSDMASTCY ^e	
		925.1 (3+)	2772.3	2772.4	-0.1	TPDLFPLVSCGPSLDESLVAVGCLAR	
		967.5 (3+)	2899.5	2899.3	0.2	TVDKSTGKPTLYNVSLVMSDMASTCY ^e	
Inter-alpha (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein) (gi 194221223; <i>Equus caballus</i>)	14 kDa	941.2 (2+)	1880.4	1880.9	-0.5	VTSMILTITESDWLNQK ^e	E9
		948.2 (2+)	1894.4	1895.0	-0.6	GGTNINDAVLLAVQLLER	
Inter-alpha-trypsin inhibitor (gi 55584152; <i>Equus caballus</i>)	14 kDa	829.1 (2+)	1656.2	1654.8	1.4	TVAACNLPIVQGPCR	E9
Kappa-casein (gi 168997371; <i>Equus asinus</i>)	20 kDa	610.2 (2+)	1218.4	1218.6	-0.2	NEPIYYQHR	E9
Kappa-casein precursor (gi 126352442; <i>Equus caballus</i>)	21 kDa	797.3 (2+)	1592.6	1592.9	-0.3	HPRPHPSFIAIPPK	E4-E9
		957.2 (2+)	1912.4	1912.0	0.4	TVKYIPIYYVLNSSPR	
Lactoferrin (gi 6136044; <i>Equus caballus</i>)	78 kDa	793.1 (2+)	1584.2	1583.8	0.4	YIPIYYVLNSSPR	E9
		687.8 (2+)	1373.6	1374.7	-1.1	GSQFQLNQLQGK	
		1045.2 (2+)	2088.4	2088.0	0.4	ADAVTL DGGLVYEAGLHPYK	
		752.2 (2+)	1502.4	1502.8	-0.4	KSGFQLNQLQGK	
		770.1 (2+)	1538.2	1538.7	-0.5	TSSFECIQIAAANK	
		961.6 (2+)	1921.2	1921.9	-0.7	VACASASTTEECIALVLK	
		989.3 (2+)	1976.6	1976.0	0.6	AGDVAFVKDVTVLQNTDGK	
		1099.3 (2+)	2196.6	2196.1	0.5	QSNKGKYAASSYLTLTPAQWK ^h	
Lambda-immunoglobulin (gi 291462; <i>Equus caballus</i>)	20 kDa	843.7 (2+)	1685.4	1684.9	0.5	VNGAAISQGVQTTKPSK	Ctrl-E7-E9
		850.2 (2+)	1698.4	1698.8	-0.4	YAASSYLTLTPAQWK	
		773.7 (2+)	1545.4	1544.7	0.7	QSNKGKYAASSYLTR	
Lambda-immunoglobulin (gi 291470; <i>Equus caballus</i>)	24 kDa	572.6 (2+)	1143.2	1143.5	-0.3	SYSSVSCQVK	Ctrl-E4-E7-E9
		738.6 (2+)	1475.2	1475.7	-0.5	VNDAVTTDGVQTTTR	
		516.1 (2+)	1030.2	1030.5	-0.3	YAASSYLTR	
		777.7 (2+)	1553.4	1552.8	0.6	ADELPPVSNLILDR	
LPS receptor (gi 53854319; <i>Equus caballus</i>)	38 kDa	890.1 (3+)	2667.3	2667.4	-0.1	ADELPPVSNLILDRNPYLDPEASK	E7-E9
		833.2 (2+)	1664.4	1662.9	1.5	GLIAALCPHKFPALR	
		915.3 (2+)	1828.6	1829.1	-0.5	LTVGAVQVPAPLLVALLR	
Lysozyme C variant B (gi 126613; <i>Equus asinus</i>)	15 kDa	764.1 (2+)	1526.2	1526.7	-0.5	DKDLSEYLASCNL	Ctrl-E4-E7-E9
		977.2 (2+)	1952.4	1951.9	0.5	HCKDKDLSEYLASCNL	
		674.3 (2+)	1346.6	1345.7	0.9	KVFSKCELAHK	
		1163.5 (3+)	3487.5	3487.5	0.0	LKAQEMDGGGYS LANWVCMAEYESNFNTR	
		804.2 (2+)	1606.4	1605.7	0.7	LLDDNIDDDISCAK	

		882.3 (2+)	1762.6	1761.8	0.8	LLDDNIDDDISCAKR	
						NANGSSDYGLFQLNNK	
MCH class I HLA-B*5104 (gi 28235; <i>Homo sapiens</i>)	41 kDa	815.2 (2+)	1628.4	1628.8	-0.4	FIAVGYYDDTQFVR	E9
Novel protein (gi 147223341; <i>Sus scrofa</i>)	52 kDa	603.2 (2+)	1204.4	1204.6	-0.2	KTECLSRALK	E9
Nucleobindin-1 precursor (gi 16758210; <i>Rattus norvegicus</i>)	53 kDa	786.2 (2+)	1570.4	1570.7	-0.3	EVWEELDGLDPNR	E9
		753.6 (2+)	1505.2	1505.8	-0.6	LVTLEEFLLASTQR	
		817.8 (2+)	1633.6	1633.9	-0.3	LVTLEEFLLASTQRK	
		560.6 (2+)	1119.2	1119.6	-0.4	RFEELAAAR	
		1448.0 (2+)	2894.0	2893.4	0.6	TFILHDINS DGVLDQELEALFTK ^c	
		967.2 (2+)	1932.4	1932.0	0.4	YLQEVINVLETDGHFR	
PHL E1F1 (gi 1050983; <i>Homo sapiens</i>)	15 kDa	801.1 (2+)	1600.2	1600.8	-0.6	FPSVSLQEASSFR	E9
Polymeric immunoglobulin receptor (gi 261291941; <i>Equus caballus</i>)	84 kDa	615.6 (2+)	1229.2	1229.6	-0.4	EKGTGSGALSSVR ^h	Ctrl-E4-E9
		760.1 (2+)	1518.2	1517.7	0.5	FDCALGPEVVNVAK	
		808.1 (2+)	1614.2	1613.8	0.4	GQCTTLISSNGYVSK	
		808.7 (2+)	1615.4	1615.8	-0.4	GVVGGSAVLCPYNPK	
		661.2 (2+)	1320.4	1320.8	-0.4	IVEGQPNLKVPK	
		879.6 (3+)	2635.8	2636.4	-0.6	SNVDLHVLKPELVYADLGSSVR	
		1030.7 (2+)	2059.4	2060.0	-0.6	SPIFGPPEIDSVETSTSIK	
		1086.2 (2+)	2170.4	2171.0	-0.6	TVNVELGETVQLTCHSPCK	
		867.2 (2+)	1732.4	1732.9	-0.5	VSISCPFTSANLPNVK (62)	
Predicted: alpha-2-macroglobulin (gi 194211673; <i>Equus caballus</i>)	164 kDa	739.1 (2+)	1476.2	1476.8	-0.6	ASFSVLGDILGSAIK	E9
Predicted: carboxypeptidase D (gi 194217390; <i>Equus caballus</i>)	14 kDa	890.1 (2+)	1778.2	1777.8	0.4	NFGTDPEVTDLVQSTR	E9
		884.7 (2+)	1767.4	1767.8	-0.4	SPDDAVFQQIALSYSK	
Predicted: fibronectin (gi 194211292; <i>Equus caballus</i>)	27 kDa	646.6 (2+)	1291.2	1291.6	-0.4	DLQFVEVTDVK	E4-E7-E9
		962.7 (2+)	1923.4	1923.9	-0.5	EESPLVGQQSTVSDVPR	
		1073.8 (2+)	2145.6	2146.1	-0.5	EINLAPDSTSVVVSGLMVATK ^e	
		646.1 (2+)	1290.2	1290.7	-0.5	GATYNIIVEALK	
		797.1 (2+)	1592.2	1591.8	0.4	GDSPASSKPISIDYR	
		779.9 (3+)	2336.7	2336.2	0.5	GLRPGVVYEGQLISVQHYGHK	
		931.2 (2+)	1860.4	1860.9	-0.5	HASLQTTSTGSGPFTDVR	
		911.2 (2+)	1820.4	1820.9	-0.5	ITIMWTPPEAVTGYR	
		1084.2 (2+)	2166.4	2167.0	-0.6	ITYGETGGNSPVQFTVPGSK	
		984.3 (2+)	1966.6	1967.0	-0.4	NTFAEVTGLSPGVTYHFK	
		790.2 (2+)	1578.4	1578.8	-0.4	QYNVGPSASQYPLR	
		846.2 (2+)	1690.4	1690.9	-0.5	SLQPGSEYTVTLVAVK	
		772.1 (2+)	1542.2	1542.7	-0.5	SYTITGLQPGTDYK	
		836.2 (2+)	2505.4	2505.2	0.2	TEIDKPSQM QVTDVQDNSISVR ^e	
		687.1 (2+)	1372.2	1372.6	-0.4	TFYQIGDSWEK	
		896.6 (2+)	1791.2	1790.8	0.4	TYLGNALVCTCYGGSR	
		815.2 (2+)	1628.4	1628.9	-0.5	VDVLPVNLPGHEGQR	
		716.2 (2+)	1430.4	1430.8	-0.4	VPGTSASATLTGLTR	
		797.6 (2+)	1593.2	1592.8	0.4	VTDATETTTISWR	
		953.2 (2+)	1904.4	1905.1	-0.7	VTWAPPSIELTNLLVR	
Predicted: granulin (gi 149723703; <i>Equus caballus</i>)	67 kDa	775.1 (2+)	1548.2	1546.7	1.5	SCEKEVDSAQPAAR	E4
Predicted: prosaposin (variant Gaucher disease and variant metachromatic leukodystrophy) (gi 149689950; <i>Equus caballus</i>)	60 kDa	799.2 (2+)	1596.4	1596.7	-0.3	ADVCEVCELLVK	E9
		1175.3 (2+)	2348.6	2349.0	-0.4	AGGDVCQDCIQMVTDIQNAVR ^e	
		875.1 (2+)	1748.2	1746.9	1.3	EIVDSYLPVILDMIK ^e	

(continued on next page)

Table 1 (continued)

Identified proteins (general index; species)	Mr protein	PIM ^a (charge)	Experimental Mr	Theoretical Mr	Delta Mr Exp-Theor	Peptide sequence	Origin
Predicted: similar to 45 kDa calcium-binding protein precursor (Cab45) (stromal cell-derived factor 4) (SDF-4) (gi 149758374; <i>Equus caballus</i>)	41 kDa	989.3 (2+)	1976.6	1976.2	0.4	VVSENVIPALELVEPIKK	E9
		730.7 (2+)	1459.4	1459.7	-0.3	TAEHFQEAI AESK	
		768.2 (3+)	2301.6	2300.0	1.6	VHFQAVDPDGDGHVSWDEYK	
Predicted: similar to adipophilin (adipose differentiation-related protein) (ADRP) (gi 194224863; <i>Equus caballus</i>)	48 kDa	811.0 (2+)	1620.0	1618.8	1.2	MVQLVSSGVENALTR ^e	E9
		1058.3 (2+)	2114.6	2114.1	0.5	SQATISQLHSTVN LIEFAR	
		1074.3 (2+)	2146.6	2147.0	-0.4	SELLVDQYLP L TEEELEK	
Predicted: similar to alpha-1-antitrypsin; serine protease inhibitor (gi 194225324; <i>Equus caballus</i>)	47 kDa	1007.3 (2+)	2012.6	2013.1	-0.5	DLDKDTVLALVNYIFFK	E9
		632.7 (2+)	1263.4	1263.7	-0.3	HSSFVNVLHPK	
		799.1 (2+)	1596.2	1596.8	-0.6	IAPNLADFAFSLYR	
		618.1 (2+)	1234.2	1233.7	0.5	LLDKFQEDVK	
		1061.0 (2+)	2120.0	2120.2	-0.2	LSISGTYDLT SILPELGITK	
		1086.7 (2+)	2171.4	2171.0	0.4	NLYHSEAFSINFGDTEEAK	
		1150.3 (2+)	2298.6	2299.1	-0.5	NLYHSEAFSINFGDTEEAK ^c	
		1007.3 (2+)	2012.6	2013.1	-0.5	DLDKDTVLALVNYIFFK	
Predicted: similar to alpha-1-antitrypsin; serine protease inhibitor (gi 194225326; <i>Equus caballus</i>)	47 kDa	799.1 (2+)	1596.2	1596.8	-0.6	IAPNLADFAFSLYR	E4-E9
		894.6 (3+)	2680.8	2680.5	0.3	IVDLVKDLDKDTVLALVNYIFFK	
		656.1 (2+)	1310.2	1310.7	-0.5	LSISGCYDLKR	
		610.6 (2+)	1219.2	1219.6	-0.4	LVDKFQEDVK	
		1230.4 (2+)	2458.8	2459.3	-0.5	TLLHTNVEFNRPFVLIYDR	
		558.1 (+2)	1114.2	1113.6	0.6	ALWSGAVTPGR	
Predicted: similar to alpha-1B-glycoprotein precursor (alpha-1-B glycoprotein) (gi 194216172; <i>Equus caballus</i>)	69 kDa	939.3 (+2)	1876.6	1876.9	-0.3	HWADLVLSYVGPQHAGK ^c	E4
		974.3 (+2)	1946.6	1946.9	-0.3	RGEQELLVPMSSAYPDR	
		968.4 (+2)	1934.8	1934.1	0.7	VQEASAQVLRPGVSLRPK ^c	
		827.6 (+2)	1653.3	1653.9	-0.6	AEHPFIVEEFVLPK	
Predicted: similar to alpha-2-macroglobulin precursor (alpha-2-M) (gi 194211675; <i>Equus caballus</i>)	16 kDa	762.9 (+2)	1523.8	1522.8	1.0	ASFVSLGDILGSAMR	E9
		897.3 (+2)	1792.6	1793.0	-0.4	DTIHKPLLVEPEGLEK	
		731.1 (+2)	1460.2	1459.7	0.5	IQEEGTEVELTGR	
		944.7 (+2)	1887.4	1888.0	-0.6	LLVYTILPDGEVVGDSAK	
		614.2 (2+)	1226.4	1226.6	-0.2	FRIEDSFSVK	
Predicted: similar to antithrombin protein (gi 149708147; <i>Equus caballus</i>)	53 kDa	641.1 (2+)	1280.2	1280.6	-0.4	SDLYVSDAFHK	E4
		670.7 (2+)	1339.4	1339.6	-0.2	TSDQIHFFFAK	
		838.3 (2+)	1674.6	1673.9	0.7	LKPLDFKENAEQSR	
		1146.3 (2+)	2290.6	2290.1	0.5	AFLEVNEEGSEAAASTAVVIAGR ^c	
		611.6 (2+)	1221.2	1221.6	-0.4	ASFLLAAIDEASK	
Predicted: similar to apolipoprotein A-I precursor (gi 149716548; <i>Equus caballus</i>)	30 kDa	651.6 (2+)	1301.2	1301.6	-0.4	DTEGLRQELNK	E9
		706.6 (2+)	1411.2	1411.7	-0.5	EYVAQFEASALGK	
		767.2 (2+)	1532.4	1531.8	0.6	LLDNWDSL GTTLGK	
		916.3 (2+)	1830.6	1830.9	-0.3	LREQLGPVTQDFWDKLEK	
		589.7 (2+)	1177.4	1178.7	-1.3	QGLLPVLES LK ^h	
		746.4 (2+)	1490.8	1490.7	0.1	QKVQPYLDDFQK ^h	
		711.2 (2+)	1420.4	1419.8	0.6	VKDLATVYVDAVK	
		619.1 (2+)	1236.2	1236.6	-0.4	WQEEVEVYR	
		924.2 (2+)	1846.4	1845.9	0.5	NPDEEGLFTVTASVVIR	
Predicted: similar to butyrophilin (gi 149754992; <i>Equus caballus</i>)	59 kDa	645.2 (2+)	1288.4	1288.6	-0.2	HYWEVEVGDR	E4-E9

Predicted: similar to C4b-binding protein alpha chain precursor (C4bp) (proline-rich protein) (PRP) (gi 149708028; <i>Equus caballus</i>)	68 kDa	768.2 (2+) 828.1 (2+) 1005.8 (2+)	1534.4 1654.2 2009.6	1534.7 1654.8 2009.0	-0.3 -0.6 0.6	QEDSFEEAIVQLK GVDWSDPLPVCVIAK YQRPTKEDVYNVGTVLK ^c	E9
Predicted: similar to cathepsin B (gi 149698064; <i>Equus caballus</i>)	38 kDa	1025.0 (2+) 775.9 (3+)	2048.0 2324.7	2047.9 2324.2	0.1 0.5	NGPVEAAFTVYSDFLQYK SRPYFQPLSDELVNYVNKR	E9
Predicted: similar to cathepsin L (gi 149755226; <i>Equus caballus</i>)	38 kDa	715.7 (2+)	1429.4	1429.8	-0.4	VFLEPLFLEVPK	E9
Predicted: similar to cathepsin Z (gi 194224588; <i>Equus caballus</i>)	36 kDa	668.7 (2+)	1335.4	1335.7	-0.3	NVDGINYVSVTR	E4
Predicted: similar to ceruloplasmin precursor (ferroxidase) (gi 149729967; <i>Equus caballus</i>)	123 kDa	775.1 (2+)	1548.2	1548.7	-0.5	TESSTVTPTSPGETR	E9
Predicted: similar to complement C3 precursor (gi 194212541; <i>Equus caballus</i>)	187 kDa	1072.2 (2+)	2142.4	2142.1	0.3	ILLQGTTPVAQMAEDAIDGER ^e	E9
Predicted: similar to complement component 2 (gi 149732356; <i>Equus caballus</i>)	38 kDa	741.7 (2+)	1481.4	1481.9	-0.5	LAVDNIKELLNIK	E9
Predicted: similar to complement component C4A (gi 149732359; <i>Equus caballus</i>)	19 kDa	771.1 (2+) 854.9 (2+) 956.2 (2+) 771.1 (2+) 1249.3 (2+) 807.2 (2+)	1540.2 1707.8 1910.4 1540.2 2496.6 1612.4	1540.8 1707.9 1910.8 1540.8 2496.3 1611.9	-0.6 -0.1 -0.4 -0.6 0.3 0.5	FVSSPFLDLSNTK HFVPGDPFLLQALVR LLSTLCSADVQCQCAEGK VLSLAQEQQVGGSPK VTASDPLDALGSEGALSPGGTSLLR YIYGKPVQGVAYVR	E9
Predicted: similar to complement regulator factor H (gi 194227377; <i>Equus caballus</i>)	110 kDa	1061.1 (2+) 1084.2 (2+) 741.1 (2+) 1005.6 (2+)	2120.2 2166.4 1480.2 2009.2	2119.9 2167.1 1478.7 2008.9	0.3 -0.7 1.5 0.3	ECDAADGWTNDIPLCEVVK LISSALELDQEYTFGQVVR SCGPPPQLPNGEVK YYYYYCNQNFVTPSK	E9
Predicted: similar to complement regulator factor H (gi 194227381; <i>Equus caballus</i>)	110 kDa	741.1 (2+)	1480.2	1478.7	1.5	SCGPPPQLPNGEVK	E9
Predicted: similar to corticosteroid binding protein (gi 194225322; <i>Equus caballus</i>)	46 kDa	966.2 (2+)	1930.4	1930.9	-0.5	GTWTQLPQCIATDELAK	E4
Predicted: similar to C-reactive protein (gi 149755947; <i>Equus caballus</i>)	25 kDa	806.7 (2+)	1611.4	1611.8	-0.4	LFLSSTYDLGDILR	E4
Predicted: similar to Equ c1 (gi 194225647; <i>Equus caballus</i>)	21 kDa	769.2 (2+) 999.8 (2+) 623.7 (2+)	1536.4 1997.6 1245.4	1536.8 1998.0 1245.7	-0.4 -0.4 -0.3	AYGEVFIKPLWS GAYSVSVGGADVFFKLPER KQNNEILLFK	E9
Predicted: similar to Fc fragment of IgG binding protein (gi 194685619; <i>Bos taurus</i>)	331 kDa	778.7 (2+)	1555.4	1555.8	-0.4	DRPYQVLEFYAR	E9
Predicted: similar to fibrinogen gamma chain (gi 194208381; <i>Equus caballus</i>)	52 kDa	1097.2 (2+)	2192.4	2193.1	-0.7	ISEFGDSTYIIVYLANFNK	E9
Predicted: similar to haptoglobin precursor (gi 149699777; <i>Equus caballus</i>)	39 kDa	606.1 (2+)	1210.2	1210.6	-0.4	VAYDLVYNVR	E9
Predicted: similar to heat shock 70 kDa protein 5 (gi 194225828; <i>Equus caballus</i>)	83 kDa	770.2 (2+)	1538.4	1538.8	-0.4	IAIGEGQQFHLGAAK	E9
Predicted: similar to inter-alpha (globulin) inhibitor H1 (gi 149728587; <i>Equus caballus</i>)	100 kDa	592.7 (2+) 936.3 (2+)	1183.4 1870.6	1183.6 1871.0	-0.2 -0.4	VPSILDWVQK VVFHPDYQEVDIGLIK ^c	E4-E9
Predicted: similar to inter-alpha globulin inhibitor H2 polypeptide (gi 194227183; <i>Equus caballus</i>)	107 kDa	1083.4 (2+)	2164.8	2164.0	0.8	IEIESFYEGEDFSETLTR	E4-E9
Predicted: similar to 1-amino acid oxidase (gi 194207596; <i>Equus caballus</i>)	51 kDa	1144.2 (2+) 868.1 (2+) 781.1 (2+)	2286.4 1734.2 1560.2	2286.2 1734.8 1560.9	0.2 -0.6 -0.7	ALQMSLAYQFVTPLTSMTIR ^{c,e} HKQYYEGSEIVVAGR LWAYLTIQELLAK	E7-E9
		791.7 (2+)	1581.4	1581.8	-0.4	IQPSGGTNINEALLR	E9
		878.5 (3+)	2632.5	2632.3	0.2	TILDDLRTEDQFSVVDNFHNVR	E9
		1080.3 (2+)	2158.6	2159.1	-0.5	ALQDAGHQVTVLEASDHVGGR	E4-E9
		607.1 (2+)	1212.2	1212.7	-0.5	ALSASLKPGTIR	E4-E9

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Table 1 (continued)

Identified proteins (general index; species)	Mr protein	PIM ^a (charge)	Experimental Mr	Theoretical Mr	Delta Mr Exp-Theor	Peptide sequence	Origin
		816.2 (2+)	1630.4	1630.8	-0.4	ANPELLGYSVNPTEK	
		573.1 (2+)	1144.2	1143.6	0.6	GGVSITDRPSR	
		746.9 (3+)	2237.7	2338.2	-0.5	HDQVVDIVLEDLAAVHQIPK	
		942.6 (2+)	1883.2	1883.1	0.1	LITFKPALSPDKVDALR	
		710.2 (2+)	1418.4	1417.7	0.7	NIQAAVDQEAMGR ^e	
		1229.3 (2+)	2456.6	2456.3	0.3	VDEPNSELHTLTADFVIISASAK ^c	
		767.1 (2+)	1532.2	1532.7	-0.5	VILACNEPFWER	
Predicted: similar to long palate lung and nasal epithelium protein 5 (gi 194224317; <i>Equus caballus</i>)	54 kDa	768.7 (2+)	1535.4	1535.8	-0.4	APRPDHLAPQPLPK	E9
Predicted: similar to nucleobindin 1 (gi 194215746; <i>Equus caballus</i>)	42 kDa	1055.7 (2+)	2109.4	2109.0	0.4	VSKDQLETDISDLFSEQR	
		765.4 (3+)	2293.2	2293.2	0.0	AKMDAQEPNVQLDHLSELLK ^e	E4-E9
		619.6 (2+)	1237.2	1237.6	-0.4	ELDFVSHHVR	
		744.6 (2+)	1487.2	1487.7	-0.5	ELQQAVALQMEQR ^e	
		753.6 (2+)	1505.2	1505.8	-0.6	LVTLEEFLLASTQR	
		560.7 (2+)	1119.4	1119.6	-0.2	RFEELAAAR	
		676.1 (2+)	1350.2	1350.7	-0.5	YLESLGEEQRK	
		966.7 (2+)	1931.4	1931.9	-0.5	YLQEVINVLETGDHFR	
Predicted: similar to nucleobindin 1 precursor (CALNUC) isoform 1 (gi 73947136; <i>Canis familiaris</i>)	53 kDa	619.6 (2+)	1237.2	1237.6	-0.4	ELDFVSHHVR	E9
		744.6 (2+)	1487.2	1487.7	-0.5	ELQQAVALQMEQR ^e	
		753.6 (2+)	1505.2	1505.8	-0.6	LVTLEEFLLASTQR	
		1048.2 (2+)	2094.4	2095.0	-0.6	MDAQEPNVQLDHLSELLK ^e	
		560.7 (2+)	1119.4	1119.6	-0.2	RFEELAAAR	
		676.1 (2+)	1350.2	1350.7	-0.5	YLESLGEEQRK	
		966.7 (2+)	1931.4	1931.9	-0.5	YLQEVINVLETGDHFR	
Predicted: similar to nucleobindin-2 precursor (gi 149719485; <i>Equus caballus</i>)	50 kDa	784.6 (2+)	1567.2	1567.7	-0.5	QVIDVLETDNHFR ^h	E9
Predicted: similar to peroxiredoxin-4 (Prx-IV) (thioredoxin peroxidase AO372) (gi 109130180; <i>Macaca mulatta</i>)	31 kDa	895.2 (2+)	1788.4	1788.8	-0.4	IEAPDTGLYYDEYLLK	
Predicted: similar to prothrombin (gi 194217874; <i>Equus caballus</i>)	72 kDa	813.2 (2+)	1624.4	1623.7	0.7	DYGVYLED SGHTLR	E9
		643.1 (2+)	1284.2	1284.7	-0.5	DNLDRDIALLK	E4-E7-E9
		887.7 (2+)	1773.4	1772.9	0.5	KSPQELLCGASLISDR	
		787.1 (2+)	1572.2	1572.7	-0.5	TTDEDFPLFFDVK	
Predicted: similar to PWP1-interacting protein 4 (gi 149731196; <i>Equus caballus</i>)	41 kDa	964.3 (2+)	1926.6	1926.9	-0.3	FQDLGAAYEVLSDSEKR	E4
Predicted: similar to secreted phosphoprotein 1 isoform 1 (gi 149701529; <i>Equus caballus</i>)	25 kDa	912.2 (2+)	1822.4	1822.9	-0.5	AVLVAQGLHVASDWDSDR	E4-E7-E9
Predicted: similar to stress 70 protein	52 kDa	911.6 (2+)	1821.2	1821.8	-0.6	VSQEFPSQEFHSSEK	
chaperone microsome-associated 60 kDa protein precursor (microsomal stress 70 protein ATPase core) (gi 149742236; <i>Equus caballus</i>)		752.1 (2+)	1502.2	1502.7	-0.5	IFTPEELEAEIGR	E9
Predicted: similar to transforming growth factor beta-3 precursor (TGF-beta-3) (gi 149737393; <i>Equus caballus</i>)	48 kDa	855.2 (2+)	1708.4	1708.8	-0.4	KSGESQVLFFETEISR	
Predicted: similar to transforming growth factor-beta-induced protein ig-h3 precursor (gi 194219905; <i>Equus caballus</i>)	73 kDa	849.2 (2+)	1696.4	1695.8	0.6	GTAEWLSFDVTDTVR	E4-E9
		1011.3 (2+)	2020.6	2021.0	-0.4	FSMLVAAIQSAGLTETLNR	E4-E9
		704.1 (2+)	1406.2	1406.7	-0.5	GDELADSALEIFK	
		726.7 (2+)	1451.4	1451.8	-0.4	ILGDPALRDLK	
		860.8 (2+)	1719.6	1719.0	0.6	NLLLNHIKDLQASK	

Predicted: similar to tripeptidyl peptidase 1 (gi 194213729; <i>Equus caballus</i>)	58 kDa	646.4 (2+)	1290.8	1289.7	1.1	SLQGDKLEVSSK	E9
		664.2 (2+)	1326.4	1326.7	-0.3	SPYQLVLQHSR	
		1035.3 (2+)	2068.6	2069.0	-0.4	TLFELAADSVDSTAVDLFR	
		893.0 (3+)	2675.0	2675.4	-0.4	VISTVTNNIQHIEIDTFETLR	
		928.2 (2+)	1854.4	1854.0	0.4	YHVGDEILVSGGIGALVR	
Predicted: similar to tripeptidyl peptidase 1 (gi 291384507; <i>Oryctolagus cuniculus</i>)	62 kDa	759.2 (2+)	1516.4	1516.8	-0.4	ILSGHPPLGFLNPR	E9
		1507.0 (2+)	3012.0	3011.5	0.5	HQFRPSFPASSPYVTTVGGTSFQNPFR ^c	
		628.1 (2+)	1254.2	1254.6	-0.4	LVQAVSDPSSPR	
Predicted: similar to vitronectin precursor (serum-spreading factor) (S-protein) (V75) (gi 194217342; <i>Equus caballus</i>)	55 kDa	727.2 (2+)	1452.4	1452.7	-0.3	QAEALLSGAEFHR ^h	E9
Predicted: sperm-membrane associated protein P47 (gi 194206303; <i>Equus caballus</i>)	46 kDa	1001.2 (2+)	2000.4	2000.0	0.4	LIQDVWGIEGPIDAAFR	E9
		1033.3 (2+)	2064.6	2064.0	0.6	DAGDSKDKVFGNVDNSGLK	
		706.2 (2+)	1410.4	1410.7	-0.3	DFGHIQYVAAYK	
		553.2 (2+)	1104.4	1104.6	-0.2	ILPVAWHNR	
		760.5 (2+)	1519.0	1518.7	0.3	LVPVACHHGCTLR	
		939.7 (2+)	1877.4	1877.9	-0.5	TWGLNAFSWYPFYAR	
		624.9 (2+)	1247.8	1247.6	0.2	VFVGNVDNSGLK	
		854.6 (2+)	1707.2	1707.8	-0.6	VNMFDPVLEVQYVR	
Proline-rich protein 4 isoform 1 (gi 154448886; <i>Homo sapiens</i>)	15 kDa	865.7 (2+)	1729.4	1728.8	0.4	FPSVSLQEASSFFQR	E9
Serotransferrin precursor (gi 126352628; <i>Equus caballus</i>)	80 kDa	879.1 (2+)	1756.2	1756.8	-0.6	AVSNFFAGSCVPCADR	E4
		747.7 (2+)	1493.4	1493.8	-0.4	DLLFKDSALGFLR	
		838.8 (3+)	2513.4	2512.2	1.2	DLLFRDDTQCLANLQPTTTYK	
		1194.8 (2+)	2387.6	2387.2	0.4	IPPAMDTWLYLGYEYVTAIR ^e	
		1085.8 (2+)	2169.6	2170.1	-0.5	IVKGEADAMSLDGGFIYIAGK ^e	
Serpine peptidase inhibitor, clade I (neuroserpin), member 1 (gi 149731078; <i>Equus caballus</i>)	46 kDa	696.1 (2+)	1390.2	1389.7	0.5	NSNFQLNQLQGK	E9
		1105.3 (2+)	2208.6	2209.2	-0.6	SAGWNIPIGLLYWQLPEPR	
		839.2 (2+)	1676.4	1675.9	0.5	FTVEQEIDLKDVVK	
		841.1 (2+)	1680.2	1680.8	-0.6	VDFSQNVAVANYINK	
		975.8 (2+)	1949.6	1950.0	-0.4	DFDALTHLALINAVYFK	
Serum albumin (gi 76363596; <i>Equus asinus</i>)	70 kDa	805.1 (2+)	1608.2	1608.8	-0.6	DVFLGTFLYEYSR	Ctrl-E4-E9
		804.7 (2+)	1607.4	1606.8	0.6	LPCSENHLALALNR	
		798.2 (2+)	1594.4	1594.9	-0.5	KAPQVSTPTLVEIGR	
		728.2 (2+)	1454.4	1454.8	-0.4	RHPDYSVSLLLR	
		627.2 (2+)	1252.4	1252.7	-0.3	TVLGNFSFAVAK	
Trypsin inhibitor (gi 33985; <i>Homo sapiens</i>)	107 kDa	791.7 (2+)	1581.4	1581.8	-0.4	IQPSGGTNINEALLR	E9
		1061.7 (2+)	2121.4	2121.1	0.3	VVNSSPQPQNVVFDVQIPK	

^a Precursor Ion Mass.

^b MS/MS data allowed to identify the variant A [20].

^c Peptide also detected as triply charged.

^d MS/MS data allowed to identify the variant A⁵⁵ [20].

^e Methionine residue presents as methionine sulfoxide.

^f Serine residue presents as phosphoserine residue.

^g MS/MS data allowed to identify the variant B from donkey [21], which is not reported in the database.

^h N-terminus as pyro-glutamic form.

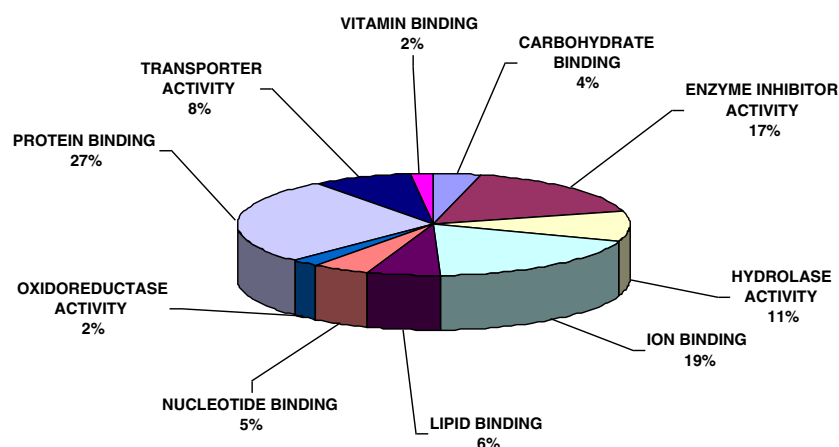


Fig. 3 – Gene Ontology (GO) functional classification of all proteins listed in Table 1.

contemplating a mixed-bed ion-exchanger eluted with two different salt gradients, either of NaCl or of the chaotrope NaClO₄. In turn, a grand total of 30 fractions were collected and had to be individually analyzed by trypsinization and mass spectrometry. Finally, by compiling the wealth of data present in the literature dealing with massive proteomic analyses of milk fractions and independent targeted studies on specific groups of proteins, D'Alessandro et al. [22] were able to tabulate an exhaustive list of 573 non-redundant annotated protein entries. This inventory was exploited for integrated in silico studies, including functional GO term enrichment, multiple pathway and network analyses. Most of the milk proteins could thus be grouped under pathways and networks referring to nutrient transport, lipid metabolism and objectification of the immune system response, as it would be expected from a nutrient meant for newborns.

Notwithstanding the major progresses made in bovine milk analysis, little was known up to the present on donkey's milk, although this nutrient is now well recognized as essentially non-allergenic and thus as a promising breast milk substitute. Considering that only the major proteins had been extensively characterized, the present report that lists a grand total of 106 UGPs appears to be by far the most extensive profiling of this milk. However, it is important to highlight that the list of the identified components reflects the protein composition of the individual she-donkey's milk here investigated and geographic and/or breed differences could determine variations in milk proteins composition. As stated in the [Result](#) sections, taking into account the very low database entries from donkey and considering that the conventional protein identification approach here employed is a highly stringent search, which allows to identify only known (i.e. present in a database) proteins, or unknown proteins sharing identical peptides with related database sequences, the identification of all these low-abundance species required a most laborious consulting of all databases. It turned out, in fact, that only 10% of the identified proteins were obtained from the scanty database of *E. asinus*, while 70% were homologous to those of *E. caballus*, which is the closest related specie to donkey.

On the contrary, the genetic distance between cow and donkey led to a low number of cross-species identifications (3%). In fact, milk proteins from these two species share low-

sequence similarity, as recently reported by Cunsolo et al. [23]. Sequence characterization of donkey's α s1-CN, indeed, revealed that it shares a low sequence homology (41% of identity and 57% of similarity) with the bovine counterpart, one of the major cow's milk allergens. Particularly, this comparison highlighted that the IgE-binding linear epitopes of cow's α s1-CN and the corresponding domains present in donkey's α s1-CN had remarkable differences in their amino acid sequences, which could be related to the already demonstrated low allergenic properties of donkey's milk.

By grouping all of the identified proteins in donkey's milk on the basis of their putative molecular function ([Fig. 3](#)) it was possible to reveal that the most abundant classes are represented by proteins belonging to the categories of "protein binding", "ion binding", "enzyme inhibitors" and "hydrolase" and therefore involved in the nutrient transport and the immune system response. Although the components detected in donkey's milk are significantly lower with respect to those known in bovine milk [22] and an exhaustive comparison between these two proteomes was not really performed, a preliminary evaluation of the qualitative composition of cow's and she-donkey's milk proteome does not reveal significant differences. Therefore, as above reported, the already demonstrated low allergenic properties of donkey's with respect to cow's milk seem to be mainly related to the remarkable differences in the primary structure of their proteins, which determine deep divergences between the amino acid sequences of IgE-binding linear epitopes of cow's milk allergens and the corresponding domains present in donkey's milk proteins.

More work will have to be done in identifying more components and in studying the nutritional properties of all the new species reported in [Table 1](#); what is missing, at present, is an understanding of the reasons why our Roman ancestors valued so much donkey's milk, as to convince Poppea and other ladies of the Roman aristocracy to take frequents baths in this precious liquid. A cruel act, considering the scarcity of this milk, and an act that must have resulted in a thinning of the newborn donkey population, deprived of this precious nutrient, in the Lazio region around Rome. Yet some light has been recently shed by the interactomic study of D'Alessandro et al. [22]. These authors have observed another functional family statistically significant, which included proteins

involved in the induction of cellular proliferation processes as well as in anatomical and hematological system development. Paradoxically, then, maybe Poppea and other ladies, without much knowledge of proteomics and/or interactomics, used donkey's milk to rejuvenate their skin. We hope that producers of beauty creams and lotions will never find out, otherwise there might ensue another hecatomb of newborn donkeys!

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Appendix A. Supplementary data

Supplementary data to this article can be found online at [doi:10.1016/j.jprot.2011.05.036](https://doi.org/10.1016/j.jprot.2011.05.036).

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