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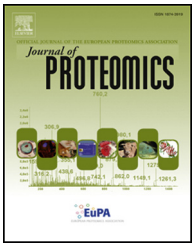

Peptidome characterization and bioactivity analysis of donkey milk

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ABSTRACT

Donkey milk is an interesting commercial product for its nutritional values, which make it the most suitable mammalian milk for human consumption, and for the bioactivity associated with it and derivative products. To further mine the characterization of donkey milk, an extensive peptidomic study was performed. Two peptide purification strategies were compared to remove native proteins and lipids and enrich the peptide fraction. In one case the whole protein content was precipitated by organic solvent using cold acetone. In the other one the precipitation of the most abundant milk proteins, caseins, was performed under acidic conditions by acetic acid at pH 4.6, instead. The procedures were compared and proved to be partially complementary. Considered together they provided 1330 peptide identifications for donkey milk, mainly coming from the most abundant proteins in milk. The bioactivity of the isolated peptides was also investigated, both by angiotensin-converting-enzyme inhibitory and antioxidant activity assays and by bioinformatics, proving that the isolated peptides did have the tested biological activities.

Biological significance
The rationale behind this study is that peptides in food matrices often play an important biological role and, despite the extensive study of the protein composition of different samples, they remain poorly characterized. In fact, in a typical shotgun proteomics study endogenous peptides are not properly characterized. In proteomics workflows one limiting point is the isolation process: if it is specific for the purification of proteins, it often comprises a precipitation step which aims at isolating pure protein pellets and remove unwonted interferent compounds. In this way endogenous peptides, which are not effectively precipitated as well as proteins, are removed too and not analyzed at the end of the process. Moreover, endogenous peptides do often originate from precursor proteins, but in phenomena which are independent of the shotgun digestion protocol, thus they can be obtained from cleavage specificities other than trypsin's, which is the main proteolytic enzyme employed in proteomic experiments. For this reason, in the end, database search will not be effective for identification of these peptides, thus the need to provide different workflows for peptide analysis. In the work presented in this paper this issue is considered

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for the first time for the analysis of the peptides isolated in donkey milk samples, which have been chosen for its nutritional interest. This study provides additional knowledge on this milk, already characterized by traditional proteomics studies and peptidomic studies after simulated digestion. This type of study is not just a description of the naturally occurring peptidome of a sample, but also represents a starting point to discover and characterize those naturally occurring peptides responsible for the observed bioactivities of biological samples, as in the case of donkey milk, which would remain uncharacterized by other approaches. In this paper an analytical protocol was described for the efficient isolation and purification of peptides in donkey milk, assessing the effect of the purification protocol on the final identifications. Purified peptide samples were also checked to empirically elucidate any ACE inhibitory or antioxidant activity. Finally, the peptidomic results were also further mined by a bioinformatic-driven approach for bioactive peptide identification in the donkey milk samples.

In our opinion, the main strengths of this study are related to the improved analytical workflow (either as purification protocol comparison or analytical platform development) which provides a high number of identified peptides, for which the biological significance as potential bioactive peptides has also been investigated.

1. Introduction

Donkey (*Equus asinus*) is a member of the horse family, which has been used as a working animal since antiquity and nowadays in pet therapy or food production. In particular, commercial donkey milk is a valuable product and can be used in multiple applications, to manufacture dairy products as well as cosmetics and soaps.

One of the main important features of donkey milk resides on its resemblance to human milk, with similar lactose and mineral contents, fatty acid and protein profiles, which make it the most appropriate mammalian milk for infant consumption [1,2], and in those cases in which other milk types cannot be employed, such as in presence of cow milk allergies in children and adults [3,4]. In particular, donkey milk is the best candidate as substitution of human milk for clinical tolerability, palatability and nutritional adequacy for children affected by a cow's milk protein allergy, furnishing additional physiological functions as well, such as providing antibacterial substances, digestive activity molecules, growth factors and hormones [5].

Apart from the above mentioned properties, donkey milk is receiving increasing attention due to other interesting biological activities, such as the antioxidant activity [6], the immuno-stimulating ability and anti-inflammatory effects, which may be useful in the treatment of immune-related diseases in humans and prevent atherosclerosis [7]. Moreover other interesting activities have been reported, such as the antimicrobial properties, due to the high concentration of lysozyme and lactoferrin [8], the antiviral activity [9], and the antiproliferative effect on A549 human lung cancer cells [10].

Given the strong correlation between nutrition and health, the characterization of the main constituent of food is of fundamental importance. In this context proteins are key nutrients and some also display a bioactivity in their native form. In some other cases, however, the bioactivity is cryptic and latent until proteolytic release of the active peptides. Bioactive peptides can be part of the endogenous peptidome of food or they can be released by enzymatic activity during gastrointestinal digestion or produced during ripening and fermentation. Milk, as well as dairy products as a whole, is one of the major sources of biologically active peptides [11]. In the case of milk bioactive peptides, the manifestation of latent bioactivities encrypted in proteins depends on where the proteolysis occurs (mammary gland or gastrointestinal tract) and may require the synergistic action of the bioactive peptides and other agents (such as lipids, sphingolipids, oligosaccharides). One of the major proteins responsible for bioactive peptide release is casein, together with the other main constituents of milk [12]. However, the most abundant milk proteins (α_{S1}-, β-, and κ-casein, β-lactoglobulin) have little or no bioactivity in their native state, with the exception of α-lactalbumin and lactoferrin. The latter exemplifies the complexity of the bioactivity in milk, because it has bioactivities in the native form (iron-binding, immunoregulation) and after hydrolysis into peptides (releasing bactericidal, anti-inflammation and immunoregulatory peptides).

Provided the importance and the interest for donkey milk for human consumption, the characterization of the protein and peptide content of this food matrix is significant. The proteomic profile of donkey milk has been elucidated over the years [13–23], as well as the analysis of the potentially bioactive peptides released after simulated hydrolysis in gastrointestinal conditions [1]. However, a comprehensive peptidomic analysis of commercial donkey milk is still lacking and would be useful, to provide a more complete overview of the nutritional potential. This is pursued in the present work, where two methods for peptide isolation from commercial donkey milk are investigated and compared. In one case all proteins were precipitated in cold acetone, whereas in the second one only caseins, the main constituents of milk, were precipitated at their isoelectric point (pI). The supernatants containing the peptides were then purified by C18 SPE and analyzed by reversed phase nanoHPLC with direct injection into a Orbitrap mass spectrometer for peptide sequencing. Finally, two of the most important biological activities, namely angiotensin-converting-enzyme (ACE) inhibition and antioxidant activity, were tested on the purified peptides. In

addition, the list of identified peptides were searched in databases including known bioactive peptides (BIOPEP, http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/ and PeptideDB, http://peptides.be/).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, reagents, and organic solvents of the highest grade available were purchased from Sigma-Aldrich (St. Luis, MO, USA) unless otherwise stated. ACE from porcine kidney was purchased from Sigma-Aldrich (St. Luis, MO, USA). Deionized water was prepared with an arium 611 VF system from Sartorius (Göttingen, Germany). SPE C18 cartridges were by BOND ELUT (Varian, Palo Alto, CA, USA). Commercial donkey milk was purchased from a local farm (Azienda Agricola Mariucci, Rignano Flaminio, Rome, Italy).

2.2. Donkey milk delipidation

Donkey milk samples were centrifuged at 3380 ×*g* for 30 min at 4 °C, the upper milk fat layer was removed, the defatted milk aliquoted and frozen at −80 °C for further processing.

2.3. Protein precipitation

Two different protocols for protein precipitation were chosen, *i.e.* employing cold acetone or acetic acid at pH 4.6. For each precipitation procedure three experimental replicates were performed.

2.3.1. Protein precipitation by cold acetone

For the first protocol all proteins present in the samples were precipitated. An aliquot of milk sample (1.5 mL) was placed in an acetone-compatible tube, and four volume of cold (−20 °C) acetone were added (6 mL). The tube was vortex shacked and incubated overnight at −20 °C. The precipitated proteins were collected by centrifugation (9400 ×*g*, 10 min at 4 °C), the supernatant was dried down using a Speed-Vac SC 250 Express (Thermo S 164 avant, Holbrook, NY, USA) and solubilized in 1.5 mL of ddH_2O with 0.1% of TFA.

2.3.2. Protein precipitation by acetic acid

In the second protocol, only caseins were precipitated. Milk aliquots were added with 2 mol L^{-1} acetic acid to the final pH value of 4.6 (pI); then samples were centrifuged for 15 min at 4 °C and 3380 ×*g*, and the supernatant divided into 1.5 mL aliquots. Before C18 SPE peptide purification, each aliquot was added with TFA to reach the final 0.1% (*v*/*v*) concentration.

2.4. Peptide solid phase extraction

All samples were purified by SPE onto C18 cartridges, previously conditioned with acetonitrile (ACN). After loading, peptides were rinsed with 0.1% TFA aqueous solution and then eluted with ACN/ddH_2O (70/30, *v*/*v*) with 0.1% TFA, and dried in the Speed-Vac. Samples were reconstituted with 50 μL of either 0.1% formic acid (HCOOH) aqueous solution for nanoHPLC–MS/MS analysis, TRIS HCl buffer (50 mmol L^{-1} TRIS HCl, pH 8.3, 300 mmol L^{-1} NaCl) for the ACE assay or ddH_2O for the antioxidant assay. All samples were stored at −80 °C until use.

2.5. NanoHPLC-MS/MS analysis

NanoHPLC coupled to MS/MS analysis was performed on a Dionex Ultimate 3000 (Dionex Corporation Sunnyvale, CA, U.S.A.) directly connected to a hybrid linear ion trap–Orbitrap mass spectrometer (Orbitrap Elite, Thermo Scientific, Bremen, Germany) by a nanoelectrospray ion source. Peptide mixtures were enriched on a 300 μm ID × 5 mm Acclaim PepMap 100 C18 (5 μm particle size, 100 Å pore size) precolumn (Dionex Corporation Sunnyvale, CA, U.S.A.), employing a premixed mobile phase made up of ddH_2O/ACN 98:2 (*v*/*v*) containing 0.1% (*v*/*v*) HCOOH, at a flow-rate of 10 μL min^{-1}. Then, peptide mixtures were separated by RP chromatography using a LC system equipped with a 25 cm long fused silica nanocolumn, 75 μm ID, in-house packed with Acclaim-C18 2.2 μm microparticles, and outlet Kasil frit. The LC gradient was optimized to detect the largest set of peptides using ddH_2O/HCOOH (99.9:0.1, *v*/*v*) as mobile phase A and ACN/HCOOH (99.9:0.1, *v*/*v*) as mobile phase B. After an isocratic step at 10% B for 10 min, B was linearly increased to 15% within 2 min and then to 35% within 50 min; afterward, phase B was maintained at 35% for 5 min, and increased to 75% within the following 5 min. Then, phase B was maintained at 75% for 10 min to rinse the column. Finally, B was lowered to 10% over 5 min and the column re-equilibrated for 20 min (102 min total run time). MS spectra of eluting peptides were collected over an m/z range of 350–1700, using a resolution setting of 60,000 (full width at half-maximum at m/z 400), operating in the data-dependent mode to automatically switch between Orbitrap-MS and linear ion trap-MS/MS acquisition. MS/MS spectra were collected for the 20 most abundant ions in each MS scan. Rejection of +1, and unassigned charge states was enabled. All MS/MS spectra were collected using normalized collision energy of 30%, and an isolation window of 2 m/z. Ion trap and Orbitrap maximum ion injection times were set to 100 and 200 ms, respectively. Automatic gain control was used to prevent overfilling of the ion traps and was set to 1×10^6 for full FTMS scan, and 1×10^4 ions in MS^n mode for the linear ion trap. To minimize redundant spectral acquisitions, dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s, with exclusion duration of 70 s. In order to increase the number of identified peptides, three technical replicates (nanoHPLC-MS/MS runs) were performed for each of the three experimental replicates.

2.6. Database search and peptide identification

All raw files from Xcalibur software (version 2.2 SP1.48, Thermo Fisher Scientific) were analyzed together using the MaxQuant software [24] (version 1.5.1.2). The derived peak list was searched with the built-in Andromeda search engine [25] against the proteome of *Equus* genus downloaded from Uniprot (http://www.uniprot.org/) on 10-06-2014 (28,637 sequences with 14,264,687 residues) and a file containing 247 frequently observed contaminants, such as human keratins,

bovine serum proteins, and proteases. Unspecific digestion was chosen and the minimum required peptide length was set to 5 amino acids. Neither fixed nor variable modifications were set. As no labeling was performed, multiplicity was set to 1. During the main search, parent masses were allowed an initial mass deviation of 4.5 ppm and fragment ions were allowed a mass deviation of 0.5 Da. Peptide-spectrum match and protein identifications were filtered using a target-decoy approach at a false discovery rate of 1%. The second peptide feature was enabled. The match between runs option was also enabled with a match time window of 0.7 min and an alignment time window of 20 min. Each peptide identification was accepted if detected in at least six technical replicates in a single purification protocol (9 total runs).

Moreover, the lists of identified peptides were analyzed with two free databases that included known bioactive peptides, BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/) and PeptideDB (http://peptides.be/).

2.7. ACE inhibition and antioxidant activity assay

The activity of ACE was determined using hippuryl-His-Leu hydrate as the substrate [26] with the modification of Mehanna and Dowling [27]. The assay was conducted in a TRIS buffer (50 mmol L^{-1}, pH 8.3) containing 300 mmol L^{-1} NaCl. The same buffer was used to dilute the peptide samples, enzyme and substrate. The initial assay volume consisted of 50 μL of the substrate (5 mmol L^{-1}), 50 μL of ACE solution containing 1 mU of declared enzyme activity and 50 μL of assay sample. The mixture was incubated at 37 °C for 90 min. The reaction was quenched by adding 250 μL of 1 mol L^{-1} HCl and the resulting hippuric acid was extracted with 1.5 mL of ethyl acetate, centrifuging it for 15 min at 2500 ×*g* and 25 °C. After centrifugation, 1 mL of the organic layer was dried down. The hippuric acid was redissolved in 3 mL ddH_2O, and the absorbance value was determined at 228 nm by a UV/visible spectrophotometer (V-530, Jasco, Easton, U.S.A.).

The inhibition activity (IA) was calculated using the following equation:

$$IA_{(\%)} = [(A_c - B_c) - (A_s - B_s)]/(A_c - B_c) \times 100$$

where: A_c is the absorbance of the control sample (enzyme with substrate,) B_c is the absorbance when the stop solution was added before the reaction occurred in the control sample (blank control sample, substrate with HCl and enzyme), A_s is the absorbance of the reaction mixture (peptide sample, substrate and enzyme), B_s is the blank of the sample (peptide sample, substrate with HCl and enzyme).

For the antioxidant activity, a solution 2.5 mmol L^{-1} containing 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used. The DPPH radical-scavenging activity was measured according to the method of Huang and Mau [28]. The DPPH solution was diluted with methanol to obtain a final 0.125 mmol L^{-1} concentration. An aliquot of 2 mL of donkey milk peptide sample was mixed with 2 mL of methanol solution containing 0.125 mmol L^{-1} DPPH radicals. The mixture was kept for 60 min in the dark, and the absorbance was determined at 517 nm. A solution methanol/ddH_2O, 50/50 (v/v) was used as a blank. Scavenging DPPH activity was calculated according to the following equation:

$$AA\% = [(A_b - A_s)/(A_b)] \times 100$$

where A_s is the absorbance of the peptide sample and A_b is the absorbance of the blank.

3. Results and discussion

3.1. Method development

The analysis of donkey milk samples provided a total of 1330 identified peptides, divided into 1104 peptides for the acetone precipitation protocol and 984 peptides for the precipitation at pH 4.6. Slightly more than half of the identifications (57%) were common to both protocols, whereas the acetone precipitation protocol provided the largest unique contribution, with 26% of the total identifications, and the precipitation in acidic conditions provided an additional minor contribution, with 17% of the total identifications (Fig. 1).

In the experiment the two precipitation protocols were compared because concentration and purification of peptide samples are important for optimizing the conditions for the following nanoHPLC–MS/MS analysis. Interfering compounds must be removed for working in optimized conditions to maximize the final peptide chromatographic separation and MS/MS sequencing, thus in the case of peptide analysis lipids and whole proteins are both interfering compounds. Therefore the protocols both comprised a delipidation step and a protein precipitation one. The former was the same for both protocols and consisted in a simple centrifugation to remove the lipid content. This operation is performed at a relatively high speed, which is suitable to eliminate not only lipids, but also α_{1S}-casein [29]. After lipid removal, the samples were subjected to two different protein precipitation methods, with different efficiency in peptide extraction and purification. In the first case the whole protein content was organic solvent precipitated using cold acetone. In the second case only the most abundant proteins, caseins, were precipitated, by acidification to pI. This choice relied on the fact that there is no universal protein precipitation method suitable for all samples. Cold acetone precipitates almost all proteins, but

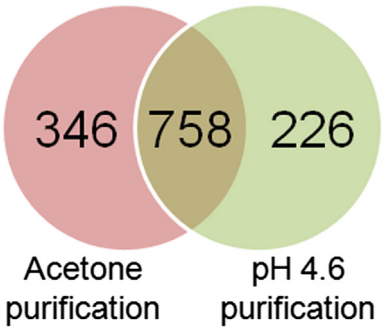

Fig. 1 – Venn diagram depicting the distribution of the identified peptides for the two precipitation protocols.

few peptides might precipitate as well, whereas the precipitation at pI is selective for caseins, thus some of the less abundant proteins remain in the supernatant and might impair the peptide identification. For these reasons the employ of both protocols in separate samples could be useful to maximize peptide identification in donkey milk samples. Moreover, the analysis of precipitation supernatants pointed out that for peptide analysis a dedicated workflow was necessary. In fact, these peptides are not analyzed in typical proteomics protocols, since the supernatant are discarded and only precipitated purified proteins are further analyzed. Besides, even in the case peptides could be recovered in the protein pellet, the following sample processing and, in particular, data management, was not suitable for their final identification; because peptides are likely to originate from proteins but with cleavage specificity which is different from that of the enzyme employed for digestion (in most of the case, trypsin). Additionally, also considering peptidomic studies mining bioactivity and that investigate the peptidome profile after a certain event, such as gastrointestinal digestion simulation, these peptides are not be characterized as well, because typical workflows always focus on protein isolation before simulated digestion [1]. Thus, the strategy as proposed in the described workflow would allow for a more comprehensive characterization of the peptidome of a sample. The peptide identifications pointed out that the two protocols provided partially overlapping results, but neither of them was sufficiently effective for a complete characterization. Therefore a more complete analytical platform could be obtained by merging the results from each single protocol.

Scatter plots were used to assess the analytical reproducibility and the correlation between the two purification protocols (Fig. 2). Scatter plots obtained reporting the technical replicates for the same type of procedure were a way to establish the reproducibility of the whole experiment (Fig. 2A, B). The plots showed that the reproducibility was good and points aligned with low degree of scattering. This was supported by the Pearson correlation values, which were also calculated. The acetone precipitation showed the best reproducibility (with coefficients ranging between 0.95 and

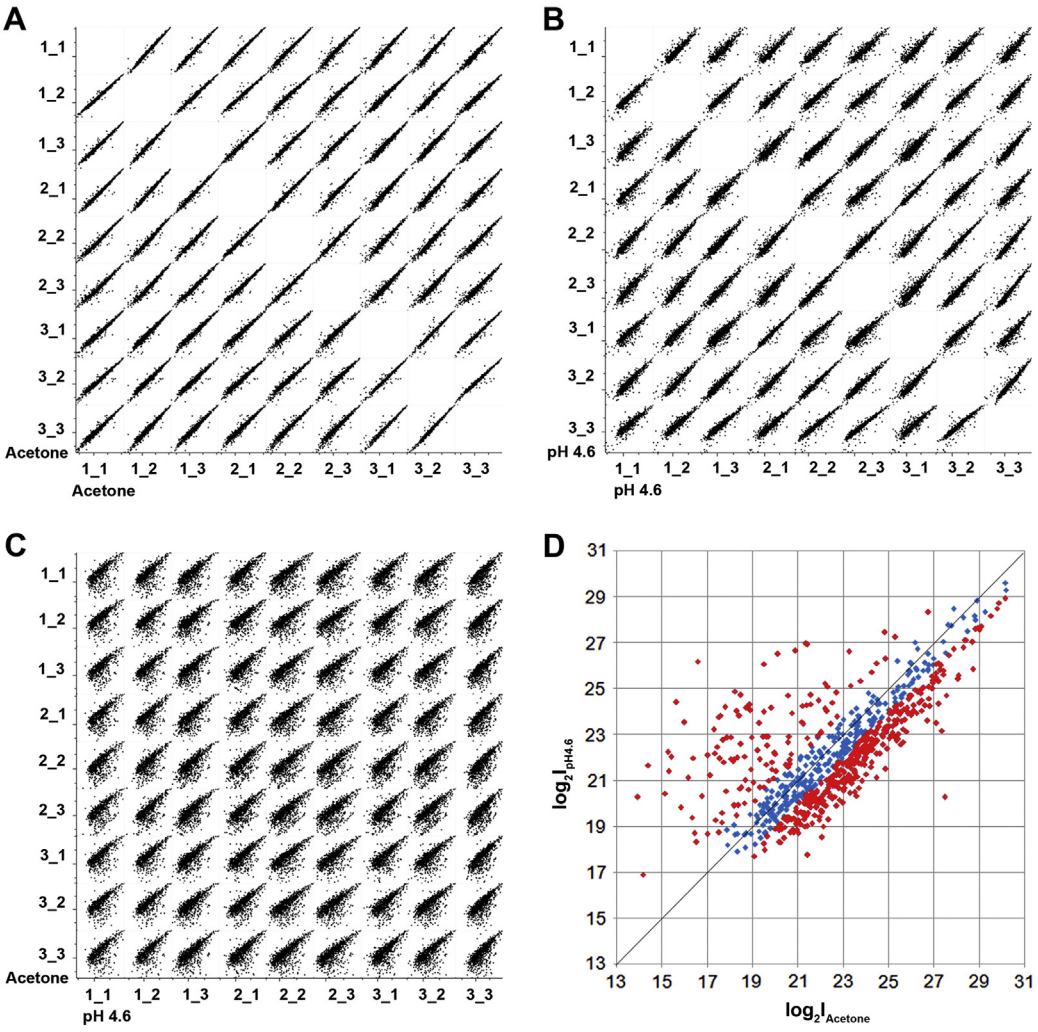

Fig. 2 – Scatter plots reporting the peptides identified in the technical replicates of: A) acetone precipitation *vs* each other; B) precipitation in acidic conditions *vs* each other; C) acetone precipitation *vs* precipitation in acidic conditions; D) Scatter plot of $log_2 I_{Acetone}$ *vs* $log_2 I_{pH\ 4.6}$ values reported for the758 peptides common to both purification protocols. Red point refer to peptides with $log_2 I_{Acetone} - log_2 I_{pH\ 4.6}$ either >1 or <–1.

0.99). Points were more scattered in the case of the precipitation at pH 4.6, instead (with coefficients ranging between 0.88 and 0.97). The same type of plot with replicates from different experiments graphically showed that even at technical replicate level the two precipitation protocols performed differently and identified different peptides (Fig. 2C). The correlation was very poor, indeed, with Pearson correlation coefficients raging between 0.61 and 0.75.

To further understand the difference in method performance we investigated the intensities of the single peptides identified by both precipitation protocols. In particular, the logarithm values of the intensities for each peptide were used to calculate the difference in peptide abundance in the purified extracts from the two experimental procedures under investigation (Fig. 2D). Values along the diagonal line corresponded to peptides with comparable intensities; among the scattered points, the ones reported in red have difference values which are >1 or <−1 (*i.e.* twice as much abundances), indicating different intensities. In particular, 215 peptides (28% of the common peptides) had more intense signals for the precipitation at pH 4.6, whereas more than half as much, with 543 peptides (72% of the common peptides) had more intense signals for the acetone precipitation. This result indicated that for common peptides, the performance of the two protocols was different and the precipitation with acetone could recover larger amounts of peptides, thus would be more suitable for their isolation in donkey milk. The observed differences could be ascribed to the different precipitation conditions, which produce a precipitate with a different surface area and occur in media with different polarity (aqueous or organic solvent).

After this consideration, we evaluated the features of the identified peptides, namely the molecular weight (MW) and the grand average of hydropathicity (GRAVY) index value distribution (Fig. 3A, B, respectively). Considering the global peptide identifications, the MWs ranged between 908 and 3165 Da, with 76% of the peptides comprised between 1200 and 2000 Da. The same profile was observed considering the total identifications for each single precipitation protocols. A slightly different distribution was observed considering only the peptides which showed a significant difference in intensity, in particular for lower MWs. In fact, in the case of acetone precipitation (reported as acetone* in Fig. 3A), the range 1600–2000 Da was less represented (22% *vs* an average 28% value), whereas the range < 1200 Da was slightly enriched (13% *vs* 8%). Similarly, but with opposite trend, the same consideration for the precipitation in acidic conditions (reported as pH 4.6* in Fig. 3A) showed a richer 1600–2000 Da population (with 39% *vs* 28%) and a less represented 1200–1600 Da fraction (Fig. 3A).

As far as the hydrophobicity was concerned, the identified peptides had prevalently a hydrophilic nature, with 80% of the total identified peptides having a GRAVY values ≤ 0 and 40% of them with intermediate values (0 ≤ GRAVY ≤ −1, Fig. 3B). Only 20% had a more hydrophobic nature. As before, by the comparison of the two precipitation protocols, practically no difference was observed. On the contrary, peptides isolated in significant different amount by either one of two protocols showed a different distribution, instead; the acetone precipitation had 27% of the peptides with positive GRAVY index values (*vs* 20% of the total peptides and 9% of the pH 4.6* more enriched peptides) whereas the opposite trend was observed for more hydrophilic peptides, which were better purified by protein precipitation in acid conditions (with 91% *vs* 80% for the total identified peptides and 73% for acetone* more enriched peptides). Finally, the evaluation of the MW and GRAVY index of the identified peptides evidenced that the two purification protocols did not show overall differences in the chemical–physical properties of the purified peptides, with the exception of the peptides which are preferentially more enriched in one of the two procedures. Particularly, the acetone protein precipitation was more selective for smaller and hydrophobic peptides whereas the protein precipitation in acidic conditions performed better in purifying medium-size and more hydrophilic peptides.

Considering the total peptide identification distribution and their differences in chemical–physical properties according to the purification protocols, neither of two procedures was able to provide a comprehensive purification method for

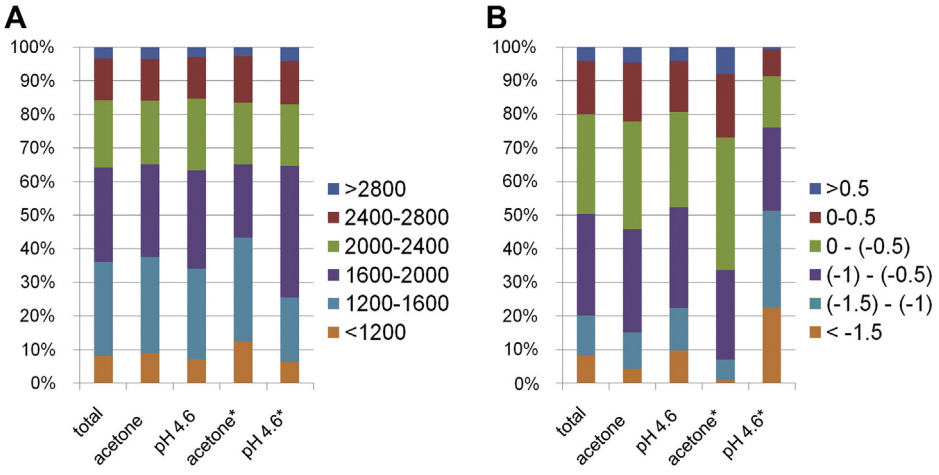

Fig. 3 – A) MW distribution (in Da) and B) GRAVY index values of the identified peptides reported according to the total number of peptides, the single procedures and the common peptides identified with a significant larger SC difference either in acetone (acetone*) or pH 4.6 (pH 4.6*) precipitation procedures.

peptides in donkey milk; therefore, due to the partially complementary information achievable by the two purifications, a better description could be provided by the combined use of both of them.

3.2. Origin of the peptides in commercial donkey milk

If the global peptide identifications were considered, then it was interesting to ascribe the different peptides to the original proteins (or protein groups in the case of shared peptides) that they came from. The peptides isolated in this experiment came from 64 protein groups, identified for different species belonging to the *Equus* genus. As already assessed for milk by different mammals [30], also in the case of donkey milk the majority of the peptides originated from the most abundant milk proteins, in particular caseins. Indeed, the most representative proteins were β-casein, with 426 peptides ascribed to its sequence (32% of the total identifications), and α_{S1}-casein, with 218 ascribed peptides (16% of the total identifications). However, the third most abundant protein was serum amyloid A protein group, to which 139 peptides (10%) were ascribed. This is an acute phase protein the concentration of which increases with inflammation. In particular, it has been suggested as a possible marker of mastitis in cows [31]. The following most represented protein group originating the peptides insolated in the experiment was made up of perilipin and adipophilin-like protein group, with 83 derived peptides (6%), which are lipid transport and storage proteins hypothesized to play a pivotal role in both formation and secretion of milk lipids [32]. Other minor protein groups were α_{S2}-casein, with 80 ascribed peptides (6%), β-lactoglobulin-1, with 49 peptides (4%), α_{S2}-casein B, with 45 peptides, κ-casein, with 26 peptides, lysozyme C, with 9 peptides, and fibrinogen α chain, with 4 peptides.

In order to understand the origin of the identified peptides, we counted how many times the single amino acid making up the peptides was found in the primary sequence of the most represented proteins. Results are reported in the heat maps (Fig. 4). In the case of β-casein (D2EC27, 1–241 amino acids) the heat map graphically showed that the identified peptides were almost equally distributed along the protein primary sequence; however, most of them belonged to the central part, being concentrated in the regions determined by the amino acids E_{58}–V_{80} (with amino acids identified 23–41 times), K_{128}–L_{212} (with amino acids identified 26–86 times) and in the C-terminus region, in particular between T_{222} and V_{239} (27–46 times). All other regions contained less identified peptides or sequence motives not subjected to proteolysis at all.

The same analysis for the second most abundant protein provided a different overview. In this case it was a protein group comprising two α_{S1}-caseins, one for *Equus asinus* (P86272, 1–202 amino acids) and one for *Equus asinus africanus* (C3W972, 1–212). In this case some peptides were not unique of a single proteins, thus the software ascribed them to both proteins; the two proteins are two isoforms which differ for the pentapeptide HTPRE (P86272, H34–E38) and a two amino acid substitutions (Q104 → A114, and I126 → L137 for P86272 and C3W972, respectively). However, in this case such peptides were reported only in the heat map for P86272, referring to domesticated donkeys, and therefore were much closer to the animals used to produce the milk samples.

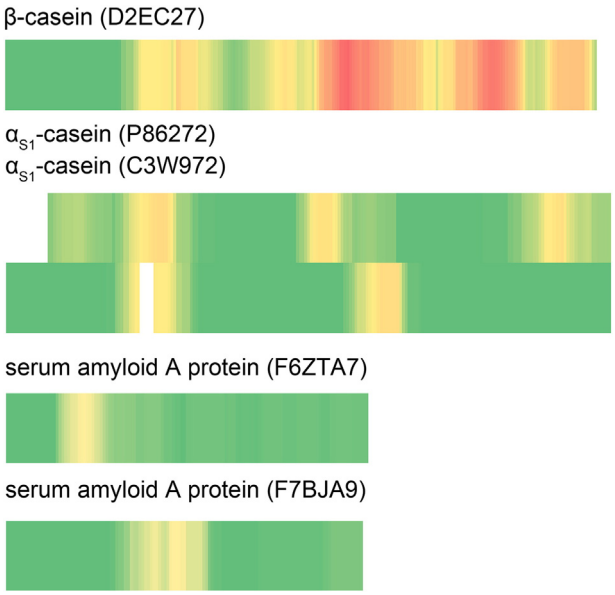

Fig. 4 – Heat maps graphically displaying the occurrence of the different amino acids in the primary sequence of the most represented parent protein of the identified peptides. Green regions indicate low occurrence, red regions frequently appearing residues.

Looking at the peptide distribution along the former protein, the proteolytic activity here was more extensive, with several cleavage sites occurring along the whole protein primary sequence, namely in the N–terminus and the initial part (R_1–L_{51}, residues occurring up to 37 times), the central part of the protein (R_{90}–L_{125}, residues occurring up to 34 times) and the C–terminus (H_{158}–W_{202}, residues occurring up to 35 times).

A similar situation was found for serum amyloid A protein, in which a protein group was found. Here the identification was done by homology to *Equus caballus*, however. In this group we considered the two most recurring proteins, namely F6ZTA7 (1–130) and F7BJA9 (1–128), and considered the peptides found in their primary sequence (Fig. 4). In this case, as before, the degree of similarity was high, with only 26 amino acids differing in the primary sequence of two after alignment. However, the most intensely colored parts fell into different regions of the two primary sequences. In F6ZTA7 the most intense region was between R_{19} and Y_{47} (with residues occurring up to 49 times), whereas for F7BJA9 the region that is more prone to proteolysis was the one between N_{44} and A_{71} (with residues occurring up to 23 times).

In order to further investigate the origin of the peptides identified in the experiment, we looked for sequence cleavage specificity. For the analyzed donkey milk samples the peptide mixture can be very complex, more than the one obtained by either by a tryptic digestion typical of proteomics experiments [11] or by *in vitro* simulated enzymatic digestion. In fact, peptides are released by the action of various unspecific and specific endogenous proteases. Moreover, commercial milk is subjected to food-processing, such as pasteurization, which can further complicate the peptidomic profile by unspecific protein hydrolysis. The results are outlined in Table 1. For each peptide we considered the first and last amino acid,

Table 1 – Number of times which the different amino acids, the N-terminus and the C-terminus have been found as first and last amino acid of each identified peptides or in the adjacent positions.

	Amino acid before	First amino acid	Last amino acid	Amino acid after
A	134	104	115	85
C	4	0	0	1
D	33	75	39	27
E	39	99	27	71
F	38	83	54	33
G	39	56	31	45
H	21	43	36	29
I	67	54	24	61
K	225	57	189	100
L	122	93	130	158
M	22	30	17	31
N	32	72	59	49
P	104	50	47	41
Q	40	58	69	92
R	133	75	180	123
S	94	162	75	116
T	44	77	33	52
V	88	82	109	98
W	6	13	27	11
Y	40	45	67	43
N-terminus	2	0	0	0
C-terminus	0	0	0	61

and then the amino acids in the adjacent positions, either preceding or following in the primary sequence of the most probable protein to which the peptides were attributed. From this it was possible to observe that the distribution was not the same for the different amino acids.

As observed in other milk peptide profiles [30] also in the case of donkey milk there was a clear preference for hydrolysis after lysine (K) and arginine (R), which were the most occurring residues both as the last one in the identified peptides and the amino acid before in the primary sequence. This was consistent with the action of plasmin, which has a high preferential cleavage at the carboxyl side of lysine and arginine residues and which has been reported as one of the endogenous proteases in milk [30]. However, for the same consideration, other residues were also frequently occurring, in particular alanine (A) and leucine (L). Some peptides were derived from the extremities of the parent proteins, in particular the major part came from the C-terminus, with 61 peptides, and only 2 from the N-terminus. In addition, it should be noted that several peptides differed for the loss of the C-terminal amino acid, consistent with the action of exopeptidases and carboxypeptidases.

3.3. *Bioactivity of peptides in commercial donkey milk*

Two bioactivity assays were performed, pooling together the purified peptides by acetone protein precipitation and pH 4.6 casein precipitation. Both assays showed the presence of the investigated bioactivities, with an antioxidant activity of 35% and ACE inhibitory activity of 67%. Moreover, all of the identified peptides were submitted to search in the BIOPEP and PeptideDB databases, which contain a list of biologically active and validated peptide sequences, in order to find out if any already established bioactive peptide was to be found. Among all the identified peptides we found one for which the bioactivity has been reported before, the peptide TKTEEGEFISEGGGVR. This fibrinopeptide belongs to fibrinogen α chain, one of the precursor proteins identified in the experiment, to which 4 peptides were ascribed. This peptide has a major function in hemostasis as one of the primary components of blood clots. Maternal fibrinogen is essential for successful pregnancy. Fibrin deposition is also associated with infection. May also facilitate the immune response *via* both innate and T-cell mediated pathways.

4. Conclusions

The present work described the development of a workflow for the analysis of peptides in commercial donkey milk samples, also trying to determine the possible bioactivity by specific assays and bioinformatics. These peptides were purified from protein precipitation supernatants, obtained after cold acetone precipitation or precipitation of caseins at pI. The tested protocols resulted complementary and provided 1330 total identifications. These peptides are not analyzed in typical proteomics workflows nor simulated digestion peptidomic analyses, thus the described protocols can be efficiently combined to provide an analytical platform for the comprehensive description of the peptide profile in donkey milk samples.

Conflict of interest statement

The authors declare no conflict of interest regarding the material discussed in the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.01.020.

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