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Release of angiotensin converting enzyme-inhibitory peptides during *in vitro* gastro-intestinal digestion of camel milk

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1 **Abstract**

2 The objective of the present study was to identify the angiotensin-converting enzyme (ACE)-
3 inhibitory peptides released from camel milk after simulated gastro-intestinal digestion. The
4 hydrolysis degree increased during digestion. The highest ACE-inhibitory activity was found in the
5 post-pancreatic <3kDa fraction. Peptides responsible for the biological activity were isolated by
6 reverse phase HPLC and identified by mass spectrometry. Among the identified sequences, 17 were
7 identical to known bioactive peptides with ACE-inhibitory activity. Based on previous structure-
8 activity relationship studies, the sequence of some peptides allowed us to anticipate the presence of
9 biological activities. The anti-hypertensive tripeptide isoleucine-proline-proline (IPP) was identified
10 and quantified in digested camel milk. The amount of released IPP was 2.56 ± 0.15 mg L^{-1} of milk.
11 For the first time, we showed that IPP is released during the gastro-intestinal digestion of camel
12 milk κ-casein. This research provides the basis to increase the exploitation of the health benefits of
13 camel milk.
14

15 **1. Introduction**

16 Previous research has recommended the potential use of camel milk as an alternative in case of cow
17 milk allergy and in the manufacture of infant formula (El-Agamy, Nawar, Shamsia, Awad &
18 Haenlein, 2009). In human milk as well as in camel milk, α-lactalbumin is the main whey protein,
19 whereas β-lactoglobulin is absent. The latter protein is the principal responsible for the allergic
20 reactions to cow milk in adult and children (El-Agamy et al., 2009).
21 Milk proteins possess unique properties and contain in their sequences encrypted peptides that show
22 potential physiological properties and health benefits. These peptides are inactive when included in
23 the protein sequence but can be released after hydrolysis (by enzymes or microorganism) displaying
24 their biological properties (Pihlanto, 2006). They can be released from both caseins (especially β-
25 casein) and whey proteins and have been identified in milk of several species (Korhonen &
26 Pihlanto, 2006).
27 The claimed biological activities of these peptides include antimicrobial, angiotensin-converting
28 enzyme (ACE)-inhibitory, anti-hypertensive, antithrombotic and antioxidative activities (Nagpal et
29 al., 2011). The main bioactive peptides studied are those with ACE-inhibitory activity (Phelan &
30 Kerins, 2011). ACE is a dipeptidyl carboxypeptidase that catalyses, *in vivo*, the conversion of the
31 plasmatic peptide angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE
32 plays an important role in blood pressure regulation and drugs that inhibit ACE are commonly
33 prescribed for the treatment of hypertension or related cardiovascular diseases (Acharya, Sturrock,
34 Riordan & Ehlers, 2003). Several peptides that inhibit ACE were reported after enzymatic
35 hydrolysis of milk proteins and after milk fermentation with Lactobacillus (Hernández-Ledesma,
36 García-Nebot, Fernández-Tomé, Amigo & Recio, 2014). Among ACE-inhibitory peptides derived
37 from bovine caseins in milk fermented with Lactobacillus, valine-proline-proline [VPP; β-casein
38 f(84–86)] and isoleucine-proline-proline [IPP; β-casein f(74–76)] have been the most studied
39 (Solieri, Rutella & Tagliazucchi, 2015). Other ACE-inhibitory peptides have been discovered in
40 enzymatic hydrolysates of milk caseins and whey proteins (Phelan & Kerins, 2011). Digestive

41 enzymes and combinations of different proteinases such as alcalase and thermolysin have been
42 utilized to successfully generate bioactive peptides from various milk proteins. For example, two
43 peptides derived from bovine αS1-casein (RYLGY and AYFYPEL) after hydrolysis with pepsin,
44 were able to reduce blood pressure *in vivo* when administered in spontaneously hypertensive rats
45 (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Three potent ACE-inhibitory peptides (LLF,
46 LVRT and LQKW) were purified from caprine β-lactoglobulin hydrolysed with thermolysin
47 (Hernández-Ledesma, Recio, Ramos & Amigo, 2002).
48 In a recent series of two papers, Picariello and co-workers (Picariello et al., 2010; Picariello et al.,
49 2013) *in vitro* studied the release of bioactive peptides after digestion of bovine milk caseins and
50 whey proteins. They found that some ACE-inhibitory peptides (such as the sequence HLPLP from
51 β-casein and the sequences GLDIQK and VLDTDYK from whey proteins) were released and stable
52 under gastro-intestinal conditions and were able to across Caco-2 cells monolayers. ACE-inhibitory
53 peptides (HLPLP and WSVPQPK) may be also released from human milk after *in vitro* gastro-
54 intestinal digestion (Hernández-Ledesma, Quirós, Amigo & Recio, 2007). In addition, *in vitro*
55 gastro-pancreatic digestion of donkey milk resulted in the release of a potent ACE-inhibitory
56 peptide (VAPFPQPVVP) corresponding to the fragment f(176-185) of β-casein (Bidasolo, Ramos
57 & Gomez-Ruiz, 2012).
58 Only a few studies on the antioxidant and ACE-inhibitory activities of camel milk protein-derived
59 peptides have been performed. Salami et al. (2011) and Moslehishad et al (2013) found that camel
60 milk proteins released ACE-inhibitory and antioxidant compounds after the treatment with digestive
61 proteases or after fermentation with *Lactobacillus rhamnosus*. However, the sequence of bioactive
62 compounds involved in the effect has not been revealed.
63 Caseins are, quantitatively, the most important proteins in camel milk. The sequence homology
64 between bovine and camel milk caseins varies from 53% for αS1-casein to 75% for β-casein.
65 Although many bioactive sequences are not conserved in camel milk caseins, some portions capable

66 of releasing peptides with ACE-inhibitory activities are present in the sequence of camel caseins
67 (Kappeler, Farah & Puhan, 1998).
68 In this study, therefore, we used an *in vitro* digestion procedure mimicking the chemical and
69 physical condition of the gastro-intestinal tract to process skimmed camel milk. The digested
70 sample was characterized for the ACE-inhibitory activity and then further separated with high
71 performance liquid chromatography (HPLC) and the different fractions characterized for their
72 ACE-inhibitory activity. The fractions with the highest activity were then analyzed with mass
73 spectrometry (MS) with the aim to identify the bioactive peptides.

74 **2. Materials and methods**

75 *2.1. Materials*

76 Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine α-amylase, pepsin from
77 porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP), ACE from rabbit lung, mucin II
78 and III, bovine serum albumin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), sodium dodecyl sulphate
79 (SDS), dithiothreitol (DTT), lysozyme and urea were supplied by Sigma (Milan, Italy). The
80 tripeptide isoleucine-proline-proline (IPP; 95% purity) was synthesized by DBA (Milan, Italy).
81 Amicon Ultra-4 regenerated cellulose 3 kDa were supplied by Millipore (Milan, Italy). The whole
82 camel milk was obtained from farms at El-Alamin and Sidi-Barani areas around Alexandria
83 (Egypt). All electrophoretic, HPLC and MS reagents were from Bio-Rad (Hercules CA, U.S.A.).
84 All the other reagents were from Carlo Erba (Milan, Italy).
85

86 *2.2. Chemical analysis and camel skimmed milk preparation*

87 Whole camel milk was defatted, to obtain skimmed camel milk, by centrifugation at 2000*g* for 20
88 min at room temperature. Skimmed milk sample was analyzed for pH, total solids, fat and ash
89 according to Ling (1963), lactose by phenol-sulfuric acid method (Marier & Baulet, 1959), nitrogen
90 fractions, *i.e.* the total nitrogen, non-casein nitrogen by micro-Kjeldahl (Rowland, 1938).
91

92 *2.3. In vitro gastro-intestinal digestion*

93 For the *in vitro* digestion, the protocol, developed within the COST Action FA1005 and further
94 validated for milk (Kopf-Bolanz et al., 2012), was followed.
95 Simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were
96 prepared according to Kopf-Bolanz et al. (2012). SIF was prepared by mixing pancreatic (PF) and
97 bile (BF) fluids. Skimmed camel milk (9 mL) was added to 12 mL of SSF containing 150 U mL^{-1} of
98 porcine α-amylase and incubated for 5 min (oral phase). Gastric digestion was performed by adding
99 24 mL of SGF. The pH was adjusted to 2.0 with HCl 6 mol L^{-1} and supplemented with porcine

100 pepsin (1115 U mL^{-1} of SGF). The gastric bolus was further incubated for 120 min (gastric phase).
101 The intestinal digestion was carried out by adding to the gastric bolus 36 mL of SIF (24 mL of PF
102 and 12 mL of BF), adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was
103 further incubated for 120 min (pancreatic phase). All incubations were performed at 37°C on a
104 rotating wheel (10 rpm). The digested samples were cooled on ice and immediately frozen at –80°C
105 for further analysis. The digestions were performed in triplicate.
106 A control digestion, which included only the gastro-intestinal juices and enzymes and water in place
107 of milk, was carried out to consider the possible impact of the digestive enzymes in the subsequent
108 analysis.
109
110 *2.4. Determination of protein hydrolysis during the digestion*
111 The determination of protein hydrolysis in the digested samples was carried out by measuring the
112 peptide concentration by the TNBS method using leucine as standard (Adler-Nissen, 1979). The
113 absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Orlando
114 FL, U.S.A.).
115 The hydrolysis degree (DH) was calculated as reported in equation (1):
116 $$\mathbf{DH} = (\mathbf{h}/\mathbf{h}_{tot}) \cdot \mathbf{100} \quad (1)$$
117 where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of
118 α-amino groups formed at the different stages of the simulated digestion, and $\mathbf{h}_{tot}$ is the hydrolysis
119 equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the
120 individual amino acids in 1 g of protein and considering caseins as the only proteins in milk).
121 According to Adler-Nissen (1979), the $\mathbf{h}_{tot}$ value was fixed at 8, which is the value calculated for
122 caseins.
123 Hydrolysis degree data were subtracted with the data obtained in the control digestion.
124

125 *2.5. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)*

126 Samples taken at different times of digestion were subjected to SDS-PAGE electrophoresis using
127 17% polyacrylamide separating gel, to evaluate the changes in protein profile during the hydrolysis
128 (Helal, Tagliazucchi, Verzelloni & Conte, 2014). Samples were diluted to similar end dilutions in
129 Laemmli buffer (0.05 mol L^{-1} Tris, pH 6.8, containing 2% SDS, 0.1 mol L^{-1} DTT, and 0.025%
130 Bromophenol Blue). Vials were heated in boiling water for 4 min, and 5 μL of each sample
131 (corresponding to 5 μg of undigested milk proteins) were loaded into the gel. The running condition
132 were 200 V, 60 mA and 1 hour. The gel was stained by incubation in Coomassie staining solution
133 for 1 hour with gentle shaking. Coomassie staining solution was prepared by dissolving 0.05 g of
134 Coomassie Brilliant Blue R-250 in 100 mL of a methanol/water/acetic acid solution (40/50/10).

135

136 *2.6. Fractionation of post-pancreatic digested samples*

137 Post-pancreatic digested samples (4 mL) were subjected to ultrafiltration with Amicon Ultra-4
138 nominal cut-off 3 kDa, at 7500*g* for 120 min at 4°C. At the end of the separation the filtrate,
139 containing low molecular weight peptides, were freeze dried and re-filled to 4 mL (original volume)
140 with potassium phosphate buffer (0.1 mol L^{-1}; pH 7). The peptide content of the fractionated
141 samples was determined by using the TNBS method as described in paragraph 2.4 and expressing
142 the results as mg of leucine equivalent mL^{-1}.

143

144 *2.7. Measurements of ACE-inhibitory activity*

145 ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983)
146 using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate.
147 For the control assay, 350 μL of 1.6 mmol L^{-1} FAPGG dissolved in the reaction buffer (Tris-Cl 100
148 mmol L^{-1} pH 8.2 and containing 0.6 mol L^{-1} of NaCl) were mixed directly in cuvette with 330 μL of
149 reaction buffer. The solution was kept at 37°C for 5 min before the addition of 20 μL of ACE
150 solution (so that the final activity of the enzyme in the assay was 50 mU mL^{-1}).

151 For the inhibition assay, variable amounts of sample were added in place of the buffer.

152 The reaction was followed at 345 nm for 10 min using a Jasco V-550 UV/Vis spectrophotometer

153 (Orlando FL, U.S.A.).

154 Results are expressed as IC_{50} that is defined as the concentration of peptides required to inhibit 50%

155 of the enzymatic activity.

156

157 *2.8. HPLC analysis of peptides*

158 HPLC separation of the fractionated (<3 kDa) post-pancreatic digested camel milk was performed

159 with a Jasco HPLC system equipped with a 250mm x 4.6mm reversed phase column Hamilton

160 HxSil C18 (Hamilton, Reno, NV, USA) as described by Tagliazucchi, Martini, Bellesia & Conte

161 (2015). A linear gradient of solvent B (acetonitrile-trifluoroacetic acid 0.027%) in A (water-

162 trifluoroacetic acid 0.037%) ranging from 0% to 45% in 115 min with a flow rate of 0.5 mL min^{-1}

163 was used to separate the peptides contained in the low molecular weight fractions of digested milk.

164 The PDA detector was set at 214 nm. Eight major fractions were collected. The volume of sample

165 loaded into the HPLC system was 20 µL. To obtain enough amount of peptidic fractions for the

166 assays, the injection was repeated 10 times. At the end of the collection procedure, the tubes

167 corresponding at each specific fraction were mixed together, freeze-dried and re-filled to the

168 original volume (200 µL) with a potassium phosphate buffer (0.1 mol L^{-1}; pH 7).

169 These fractions were characterized for their peptide content (paragraph 2.4) and ACE-inhibitory

170 activity (paragraph 2.7).

171

172 *2.9. Peptide profile determination with nanoflow liquid chromatography accurate mass quadrupole*

173 *time-of-flight mass spectrometry with electrospray ionization (LC-ESI-QTOF MS)*

174 The fractions with the highest ACE-inhibitory activity were subjected to QTOF MS/MS analysis for

175 peptide identification. Nano LC/MS and tandem MS experiments were performed on a 1200 Series

176 Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS

177 via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic
178 separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and
179 a 43mm × 75µm analytical column, both packed with a Zorbax 300SB 5 µm C18 phase (Agilent
180 Technologies). The mobile phases composition and the gradient were the same as reported by
181 Tagliazucchi et al. (2015). The mass spectrometer was tuned, calibrated and set with the same
182 parameters as reported by Dei Più et al. (2014).
183 For peptide identification and sequencing, MS/MS spectra were converted to .mgf and *de novo*
184 peptide sequencing was performed using Pepnovo software
185 (http://proteomics.ucsd.edu/ProteoSAFe/). The following parameters were considered: enzyme,
186 none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable modification,
187 oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a single peptide 3.
188 A search for the biological activity of peptides identified was carried out through the BIOPEP
189 database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php). Confirmation of peptides
190 sequence in camel milk proteins was performed using Peptide Match
191 (http://research.bioinformatics.udel.edu/peptidematch/index.jsp).
192
193 *2.10. Identification and quantification of IPP in fraction F4*
194 IPP in fraction F4 was identified by comparing the retention time and fragmentation spectra of a
195 synthetic standard tripeptide. IPP was quantified in fraction F4 using the method reported in Solieri
196 et al. (2015). The synthetic tripeptide IPP was solubilized at 5 g L^{-1} in 0.1 mmol L^{-1} potassium
197 phosphate buffer (pH 7.0), and then diluted 1:1000 with solvent A (H_2O/acetonitrile/formic acid,
198 96.9:3:0.1, v/v/v), to obtain the 5 mg L^{-1} solution. Subsequent dilutions were made in the same
199 solvent A to yield the following concentrations: 1, 5, 10, 20, 50 and 100 µg L^{-1}. Each solution
200 contained the internal standard EGVNDNEEGFFSAR at the concentration of 50 µg L^{-1}. The
201 calibration curve was constructed from the peak area of the peptide relative to the peak area of the

202 internal standard *versus* concentration. The concentrations of IPP was calculated using the

203 following linear equation:

204 y = 12585x -3945 (R^2 = 0.9940).

205

206 *2.11. Statistical analysis*

207 All data are presented as mean ± standard deviation (SD) for three replicates for each prepared

208 sample. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using

209 Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered

210 significant with $P < 0.05$.

3. Results and Discussion

3.1. Assessment of protein hydrolysis during simulated digestion

The chemical composition of skimmed camel milk expressed as percentage (w/w) was: total solids 13%, total proteins 3.48%, caseins 2.68%, whey proteins 0.80%, lactose 4.87%, fat 0.05%. The pH was 6.61.

The hydrolysis of camel milk proteins during the *in vitro* digestion was characterized by assessing the DH value and through SDS-PAGE analysis.

As expected, simulated salivary digestion did not increase the hydrolysis degree. Un-digested camel milk showed a DH of 2.7 ± 0.1, which was 2.9 ± 0.1 after 5 minutes of salivary digestion ($P >$ 0.05). Instead, a significant increase (P <0.0001) in the DH was found already after 30 minutes of gastric digestion (**Figure 1**). After that, peptic activity resulted in a further but not significant increase in DH during the 120 minutes of gastric digestion. Pancreatic digestion had a deep effect on protein hydrolysis (**Figure 1**) which increased significantly ($P < 0.0001$) from 20.5% (post-gastric sample) to 69.6% (post-pancreatic sample). The greatest increase was observed after 30 minutes of pancreatic digestion ($P < 0.0001$ respect to the post-gastric sample; 120 min of digestion). Subsequently, for the remaining 90 minutes of pancreatic digestion the value remained almost constant.

A comparison with the data reported by Kopf-Bolanz et al. (2012) showed that camel milk proteins were more prone to hydrolysis by gastro-intestinal proteases respect to bovine milk**.** Complete digestion of bovine milk resulted in a DH of 54 respect to the value of 69.6 found for camel milk. These results are also in agreement with Salami et al. (2008) who found that the extent of hydrolysis of camel caseins with pancreatic enzymes was greater than that of bovine caseins.

A comparison of the protein pattern of the digested samples revealed that salivary juice had no effect on protein (**Figure 2A, lane 2**) as compared to the un-digested camel milk (**Figure 2A, lane 1**). The addition of gastric juice without pepsin caused a decrease in the intensity of the protein

237 bands probably related to precipitation at acidic pH values (**Figure 2A, lane 3**). All caseins and
238 major camel milk proteins were completely digested within 5 minutes by the gastric juice with the
239 exception of α-lactalbumin (**Figure 2A, lanes 4-8**) suggesting that pepsin alone was not able to
240 digest this whey protein. However, the addition of the intestinal fluid resulted in the hydrolysis of α-
241 lactalbumin (**Figure 2B, lanes 3-7**), indicating that the presence of pancreatic enzymes and bile salt
242 is basic for α-lactalbumin digestion. These results are in accordance with those of Salami et al.
243 (2008) showing that camel α-lactalbumin was slowly hydrolyzed by trypsin but it was more
244 extensively hydrolyzed by chymotrypsin.
245

246 *3.2. ACE-inhibitory activity and RP-HPLC fractionation of < 3 kDa fraction of digested camel milk*
247 Peptidic fraction was further extracted from the post-pancreatic digested sample through
248 ultrafiltration with a cut-off of 3 kDa. Hereinafter, we concentrated on the < 3 kDa fractions of
249 digested camel milk because it is likely to contain peptides that are potentially less susceptible to
250 further digestion by brush border proteases and may be absorbed at intestinal level reaching the
251 blood stream. The total amount of peptides found in the < 3 kDa fraction was calculated resulting in
252 a value of 21.74 mg mL^{-1}.
253 The post-pancreatic peptidic fraction was used for the determination of the ACE-inhibitory activity
254 of the peptides produced during the digestion. The IC_{50} value was 1771.4 ± 3.1 μg of peptides mL^{-1}.
255 With the aim of identifying putative active peptides, the < 3 kDa fraction of the samples obtained
256 during simulated digestion was loaded on the HPLC C18 column and peptides were detected at 214
257 and 280 nm with a photodiode array detector.
258 The chromatogram presented in **Figure 3** shows most peptides eluting between 6 and 45 min,
259 whereas no additional peptides were found after 60 minutes of elution. As shown in **Figure 3**, eight
260 fractions (F1–F8) were collected.
261 The peptide content of collected fractions ranged from 0.71 to 11.10 mg mL^{-1} (**Table 1**). The yield
262 of the eight collected fractions was estimated (**Table 1**). Fractions from F3 to F8 showed a similar

263 yield ranging from 2 and 5%. Fraction F1 showed the highest yield of approximately 51% (**Table**
264 **1**). The sum of the peptide concentration in the collected fractions resulted in 18.69 mg mL^{-1}, giving
265 a total yield of 86%.
266 All fractions exerted some ACE-inhibitory activity. **Table 1** shows the ACE-inhibitory activity,
267 expressed as IC_{50} values, of the peptidic fractions isolated by RP-HPLC. Low IC_{50} values, which
268 means high ACE-inhibitory activity, were found in fractions F1, F2 and F4. The lowest IC_{50} values
269 of 37.2 ± 0.3 and 38.6 ± 0.4 µg peptides mL^{-1} were found in fractions F4 and F1, respectively,
270 which were about thirty times lower than the IC_{50} value of the post-pancreatic < 3 kDa permeate.
271
272 *3.3. NanoLC-ESI-QTOF-MS/MS analysis of the HPLC collected fractions*
273 On the basis of ACE-inhibitory activities of the collected HPLC fractions, F1, F2 and F4 were
274 selected and analyzed with nanoflow LC-ESI-QTOF MS to identify the peptides present in these
275 fractions. **Figure 4** shows the full MS spectra of the fractions F1, F2 and F4. Each peak was
276 selected for peptide identification by MS/MS ion scan using de novo sequencing software. Results
277 from peptide identification were subjected to a manual evaluation, and the validated peptide
278 sequences were responsibly for the majority of the most intense peaks in the MS spectra.
279 In the lowest part of the MS spectra of fraction F1 (**Figure 4A**) the most intense signals were
280 identified as the amino acids T (m/z=120.0807), (iso)leucine (Lx; m/z=132.1014) and
281 phenylalanine (F; m/z=166.0861). An additional signal at m/z of 182.0805 was assigned to the
282 aromatic amino acid tyrosine (Y). In the peptidic part of the spectra the most intense signals
283 corresponded to the dipeptide SLx (m/z 219.1330) and the tripeptides VPT (m/z= 316.2101) and
284 GS(phospho)Q (m/z= 371.2290). The list of compounds identified in fraction F1 is shown in **Table**
285 **2** together with the MS data, the protein precursor and the potential bioactivity.
286 In fraction F2 (**Figure 4B**), phenylalanine still represented the most intense signal (m/z=166.0854).
287 An additional amino acid (threonine) was identified (m/z=120.0792). The peptides which gave the
288 most intense signals in this fraction were the tripeptide NPT (m/z=331.1643) and the dipeptide PLx

289 (m/z=229.1554). The longest identified peptide was the β-casein-derived tetrapeptide LQPK
290 (m/z=485.3254). **Table 3** reports the complete list of identified compounds together with the MS
291 and bioactivity data.
292 In the fraction F4 (**Figure 4C**), the dominant signal was tryptophan (W; m/z=205.0942). The
293 peptides PLx and LxP (m/z= 229.1504), VPY (m/z=378.1953) and LQSP (m/z=444.2396) also
294 gave intense signals in the MS spectra. All the identified compounds are reported in **Table 4**.
295 Interestingly, all the amino acids identified in the three fractions (threonine, tyrosine, phenylalanine,
296 tryptophan and (iso)leucine) are essential for humans, suggesting that camel milk represents a good
297 source of essential amino acids.
298
299 *3.4. Identification of ACE-inhibitory peptides in HPLC fractions F1, F2 and F4*
300 A dipeptide with potent ACE-inhibitory activity (AI; IC_{50}= 3.4 µmol L^{-1}) and several other
301 dipeptides (GLx, VP and DL) with higher IC_{50} values were isolated from fraction F1 (**Table 2**). The
302 dipeptide AI was previously isolated from soy sauce-like seasoning and pinto bean proteins after
303 gastro-intestinal digestion (Nakahara et al., 2010; Tagliazucchi et al., 2015).
304 In fraction F2 (**Table 3**), the most effective ACE-inhibitory peptide was VY (IC_{50}= 7.1 µmol L^{-1})
305 followed by TF (IC_{50}= 18 µmol L^{-1}). Three others peptides able to inhibit ACE activity were
306 detected in this fraction (VP, PLx and SF). In particular, the dipeptide VY, previously isolated from
307 brewed sake was effective also *in vivo* by decreasing systolic blood pressure of spontaneously
308 hypertensive rats of 31 mmHg (Saito, Wanezaki, Kawato & Imayasu, 1994). More interestingly,
309 VY was also found in human plasma after consumption of a peptide-enriched drink, indicating that
310 this peptide is bioavailable also in humans (Foltz et al., 2007). In addition, administration to mild
311 hypertensive subjects of hydrolysed sardine muscle proteins containing 6 mg of VY resulted in a
312 significant decrease of blood pressure (Kawasaki et al., 2000).
313 Four peptides in the fraction F4 showed very low IC_{50} values. The tripeptide LHP, previously
314 isolated from peptic hydrolysate of shrimp meat (Cao, Zhang, Hong, Ji & Hao, 2000) and the milk-

315 derived tripeptide IPP (Solieri et al., 2015) showed IC_{50} values of 1.6 and 5 μmol L^{-1}, respectively.
316 The dipeptides LY and IY, which were previously isolated from sardine muscle, demonstrated IC_{50}
317 values of 18 and 2.1 μmol L^{-1}, respectively (Matsufuji et al., 1994). Two additional dipeptides (PL
318 and IP) and a tripeptide (VPY) with higher IC_{50} values were found in fraction F4 as reported in
319 **Table 4.**
320 Some other peptides may have the potential to exert inhibitory activity towards ACE. Structure-
321 activity relationships study (Wu, Aluko & Nakai, 2006) reported that for dipeptides, amino acid
322 residues at the C-terminal position with large bulk chains or hydrophobic side chains such as
323 aromatic amino acids (phenylalanine, tyrosine and tryptophan) and branched aliphatic side amino
324 acids (leucine, isoleucine and valine) are preferred for ACE-inhibitory activity (Wu et al., 2006).
325 Based on these considerations, two dipeptides in fraction F1 (VV, SLx), one dipeptide in fraction
326 F4 (PF) as well as one dipeptide found in both F1 and F2 fractions (TLx) and one found in both F2
327 and F4 fractions (VLx) could be active against ACE.
328 Several of these dipeptides share significant structural attributes with previously identified ACE-
329 inhibitory peptides. The dipeptide SL matches the last two C-terminal residues, which play a main
330 role in binding to the ACE active site, with the peptide ASL isolated from the silkworm *Bombyx*
331 *mori* (Wu, Feng, Lan, Xu & Liao, 2015). Similarly, the dipeptides VL and PF correspond to the C-
332 terminal residues of the ACE-inhibitory peptides LVL, isolated from porcine plasma (Hazato &
333 Kase, 1986) and DKIHPF isolated from bovine β-casein (Gobetti, Ferranti, Smacchi, Goffredi &
334 Addeo, 2000).
335
336 *3.5. Quantification of IPP in fraction F4*
337 IPP was further quantified in the fraction F4. The amount of released IPP at the end of the digestion
338 procedure resulted in 2.56 ± 0.15 mg L^{-1} of milk.
339 In camel milk, the tripeptide IPP is present only in κ-casein in position 99-101. According to
340 Kappeler, Farah, and Puhan (2003), the κ-casein component in camel milk represents 3.47% of the

341 total caseins. As the casein concentration was 26.8 g L^{-1}, the estimated κ-casein concentration was
342 about 930 mg L^{-1} (approximately 50 µmol L^{-1}). Considering that, the IPP concentration found at the
343 end of the digestion was 7.87 µmol L^{-1} (IPP molecular weight 325.4182), the recovery yield of IPP
344 was approximately 15.7%.
345 The tripeptide IPP exhibited low susceptibility *in vitro* to the brush border peptidases activity
346 (Ohsawa et al., 2008), and was able to reach blood circulation un-degraded (Foltz et al., 2007).
347 Several clinical studies on hypertensive subjects showed that the administration of daily doses of
348 VPP/IPP in the range of 2-6 mg were associated with a decrease of the blood pressure between 1.5
349 and 10 mmHg (Cicero, Aubin, Azais-Braesco & Borghi, 2013).
350 In bovine milk, IPP is easily released from β- and κ-casein by the action of proteinases and
351 peptidases from several strains of Lactobacillus during milk fermentation and cheese ageing (Solieri
352 et al., 2015; Stuknite, Cattaneo, Masotti & De Noni, 2015). However, IPP was not released after
353 hydrolysis of bovine β-casein using an *in vitro* gastro-intestinal digestion system suggesting that
354 mammalian gastrointestinal proteolytic enzymes are not able to produce IPP itself (Ohsawa et al.,
355 2008).
356 The analysis of the theoretical cleavage sites of the IPP–containing sequence in camel milk κ-casein
357 (**Figure 5**), suggested that several digestive proteolytic enzymes might be involved in the release of
358 IPP. Camel milk κ-casein contains the tripeptide in position 100-102. The peptide bond A_{99}—I_{100}
359 can be easily hydrolyzed by pepsin, chymotrypsin, or elastase whereas the peptide bond K_{103}—K_{104}
360 is a cleavage site for trypsin. The action of these enzymes should result in the release of the
361 tetrapeptide IPPK (f100-103). The residue K_{103} can be removed by the action of the pancreatic
362 carboxypeptidase B (C-terminal exopeptidase) which cleave specifically C-terminal lysine and
363 arginine residues. As a confirmation of the suggested pathway of IPP formation, the tetrapeptide
364 IPPK was found as double-charged ion in the fraction F4 (**Table 4**).
365 **Figure 5** also reports the theoretical cleavage sites by gastro-intestinal proteases of the sequences of
366 β- and κ-caseins containing the tripeptide IPP. Bovine κ-casein contains IPP in position 108-110

367 and the amino acids close to IPP are the same as in camel κ-casein suggesting that the same
368 pathway can be involved in the release of IPP. In bovine β-casein, IPP is located at position 74-76.
369 As detailed in **Figure 5**, the peptide bonds N_{73}—I_{74} and L_{77}—T_{78} can be hydrolysed by
370 chymotrypsin, releasing the tetrapeptide IPPL. The C-terminal L_{77} residue can be subsequently
371 removed by pancreatic carboxypeptidase A releasing the tripeptide IPP.
372 On the other hand, as mentioned above, Ohsawa et al. (2008) failed to detect VPP and IPP after
373 gastro-intestinal hydrolysis of bovine β-casein. This discrepancy can be ascribed to different causes.
374 Firstly, the *in vitro* digestion system used in the Ohsawa study is different from that used in this
375 study. The biggest difference is the amount of digestive enzyme added to the mixture, which was
376 higher in our system. The digestion system used in this study has been validated for milk
377 macronutrient digestion and closely mimics the digestive process in humans, especially for protein
378 digestion, resulting in the formation of free amino acids and small peptides (2-6 amino acids)
379 (Kopf-Bolanz et al., 2012). Secondly, for the identification of peptides, we used a *de novo*
380 sequencing software, which is able to identify also shorter peptide such as di- or tri-peptides.
381 Instead, the software commonly used for proteomic study and adapted for peptide identification,
382 such as Mascot, have normally a minimum peptide length for identification of five residues
383 (Koskinen, Emery, Creasy & Cottrell, 2011).
384

385 **4. Conclusion**

386 This paper demonstrates for the first time the presence of ACE-inhibitory peptides in the low
387 molecular weight (< 3kDa) fraction of digested camel milk. In addition, some of the identified
388 peptides showed marked structural similarities with previously described ACE inhibitors.
389 This work also provides evidence, for the first time, of the fact that IPP may be released during the
390 gastrointestinal digestion of camel milk κ-casein. Basing on the protease specificity in the digestive
391 system and on the sequence of bovine β- and κ-caseins it can be predicted that the anti-hypertensive
392 tripeptide IPP (but also VPP) can be released during bovine milk digestion. Further experiment

393 using physiologically validated *in vitro* model of human digestion system and *de novo* sequencing
394 software could confirm this hypothesis. Our results highlight the ability of gastro-intestinal
395 proteases at physiological conditions to release ACE-inhibitory peptides. Likewise, based on the
396 structure-activity relationship studies, several putative new bioactive peptides could be selected for
397 further biological activities analysis. This research provides the basis to increase the exploitation of
398 the health benefits of camel milk proteins.

References

Acharya, K. R., Sturrock, E. D., Riordan, J. F., & Ehlers, M. R. W. (2003). ACE revisited a new target for structure-based drug design. *Nature Review*, 2, 891-902.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzensulfonic acid. *Journal of Agricultural and Food Chemistry*, 27, 1256-1262.

Bidasolo, I. B., Ramos, M., & Gomez-Ruiz, J. A. (2012). In vitro simulated gastrointestinal digestion of donkeys' milk. Peptide characterization by high performance liquid chromatographyetandem mass spectrometry. *International Dairy Journal*, 24, 146-152.

Cao, W., Zhang, C., Hong, P., Ji, H., & Hao, J. (2010). Purification and identification of an ACE inhibitory peptide from the peptic hydrolysate of Acetes chinensis and its antihypertensive effects in spontaneously hypertensive rats. *International Journal of Food Science and Technology*, 45, 959-965.

Cicero, A. F. G., Aubin, F., Azais-Braesco, V., & Borghi, C. (2013). Do the lactotripeptides isoleucine–proline–proline and valine–proline–proline reduce systolic blood pressure in European subjects? A meta-analysis of randomized controlled trials. *American Journal of Hypertension*, 26, 442-449.

Contreras, M. M., Carrón, R., Montero, M. J., Ramos, M., & Recio, I. (2009). Novel casein-derived peptides with antihypertensive activity. *International Dairy Journal*, 19, 566-573.

El-Agamy, E. I., Nawar, M., Shamsia, S. M., Awad, S., & Haenlein, G. F. W. (2009). Are camel milk proteins convenient to the nutrition of cow milk allergic children? *Small Ruminant Research*, 82, 1-6.

Dei Più, L., Tassoni, A., Serrazanetti, D. I., Ferri, M., Babini, E., Tagliazucchi, D., & Gianotti, A. (2014). Exploitation of starch industry liquid by-product to produce bioactive peptides from rice hydrolyzed proteins. *Food Chemistry*, 155, 199–206.

Foltz, M., Meynen, E. E., Bianco, V., van Platerink, C., Koning, T. M. M. G., & Kloek, J. (2007). Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *Journal of Nutrition*, 137, 953-958.

Gobetti, M., Ferranti, P., Smacchi, E., Goffredi, F., & Addeo, F. (2000). Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by Lactobacillus delbrueckii subsp. bulgaricus SS1 and Lactococcus lactis subsp. cremoris FT4. *Applied and Environmental Microbiology*, 66, 3898-3904.

Hazato, T., & Kase, R. (1986). Isolation of angiotensin-converting enzyme from porcine plasma. *Biochemical and Biophysical Research Communication*, 139, 52-55.

Helal, A., Tagliazucchi, D., Verzelloni, E., & Conte, A. (2014). Bioaccessibility of polyphenols and cinnamaldehyde in cinnamon beverages subjected to in vitro gastro-pancreatic digestion. *Journal of Functional Foods*, 7, 506-516.

Hernández-Ledesma, B., Recio, I., Ramos, M., & Amigo, L. (2002). Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. *International Dairy Journal*, 12, 805-812.

Hernández-Ledesma, B., Quirós, A., Amigo, L., & Recio, L. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal*, 17, 42-49.

Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I. (2014). Dairy protein hydrolysates: Peptides for health benefits. *International Dairy Journal*, 38, 82-100.

Kappeler, S., Farah, Z., & Puhan, Z. (1998). Sequence analysis of *Camelus Dromedarius* milk caseins. *Journal of Dairy Research*, 65, 209-222.

Kappeler, S., Farah, Z., & Puhan, Z. (2003). 5′-Flanking regions of camel milk genes are highly similar to homologue regions of other species and can be divided into two distinct groups. *Journal of Dairy Science*, 86, 498-508.

Kawasaki, T., Seki, E., Osajima, K., Yoshida, M., Asada, K., Matsui, T., & Osajjima, Y. (2000). Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *Journal of Human Hypertension*, 14, 519–523.

Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an in vitro digestive system for studying macronutrient decomposition in humans. *Journal of Nutrition*, 142, 245–250.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: production and functionality. *International Dairy Journal*, 16, 945-960.

Koskinen, V. R., Emery, P. A., Creasy, D. M., & Cottrell, J. S. (2011). Hierarchical clustering of shotgun proteomics data. *Molecular and Cellular Proteomics*, 10, M110.003822.

Ling, E.R. (1963). A text book of Dairy Chemistry, Vol 2, 3rd Ed., Chapman & Hall, London.

Matsufuji, H., Matsui, T., Seki, E., Osajima, K., Nakashima, M., & Osajima Y. (1994). Angiotensin I-converting enzyme inhibitory peptides in an alkaline proteinase hydrolysate derived from sardine muscle. *Bioscience Biotechnology and Biochemistry*, 58, 2244-2245.

Marier, J. R., & Baulet, M. (1959). Direct analysis of lactose in milk and serum. *Journal of Dairy Science*, 42, 1390 – 1394.

Moslehishad, M., Eshani, M. R., Salami, M., Mirdamadi, S., Ezzatpanah, H., Niasari-Niaslaji, A., & Moosavi-Movahedi, A. A. (2013) The comparative assessment of ACE-inhibitory and antioxidant activities of peptide fractions obtained from fermented camel and bovine milk by *Lactobacilus rhamnosus* PTCC 1637. *International Dairy Journal*, 29, 82-87.

Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., & Uchida R. (2010). Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its Angiotensin I-Converting Enzyme inhibitory substances. *Journal of Agricultural and Food Chemistry*, 58, 821-827.

Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Morotta, F., Jain, S., & Yadav, H. (2011). Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food and Function*, 2, 18–27.

Ohsawa, K., Satsu, H., Ohki, K., Enjoh, M., Takano, T., & Shimizu, M. (2008). Producibility and digestibility of antihypertensive β-casein tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in the gastro-intestinal tract: Analyses using an in vitro model of mammalian gastro-intestinal digestion. *Journal of Agricultural and Food Chemistry*, 56, 854-858.

Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S., & Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins: biological and toxicological implications. Journal of Chromatography B, 878, 295-308.

Picariello, G., Iacomino, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., Di Luccia, A., & Addeo, F. (2013). Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins. Food Chemistry, 139, 203-212.

Pihlanto, A. (2006). Antioxidative peptides derived from milk protein. *International Dairy Journal*, 16, 1306-1314.

Phelan, M., & Kerins, D. (2011). The potential role of milk-derived peptides in cardiovascular disease. *Food and Function*, 2, 153-167.

Ronca-Testoni, S. (1983). Direct spectrophotometric assay for angiotensin-converting enzyme in serum. *Clinical Chemistry*, 29, 1093–1096.

Rowland, S. J. (1938). The determination of the nitrogen distribution in milk. *Journal of Dairy Research*, 9, 42 – 46.

Saito, Y., Wanezaki, K., Kawato, A., & Imayasu S. (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Bioscience Biotechnology and Biochemistry*, 58, 1767-1771.

Salami, M., Yousefi, R., Eshani, M. R., Dalgalarrondo, M. Chobert, J. M., Haertlé, T., Razavi, S. H., Saboury, A. A., Niasari-Niaslaji, A., & Moosavi-Movahedi, A. A. (2008). Kinetic characterization

of hydrolysis of camel and bovine milk proteins by pancreatic enzymes. *International Dairy Journal*, 18, 1097-1102.

Salami, M., Moosavi-Movahedi, A. A., Moosavi-Movahedi, F., Eshani, M. R., Yousefi, R., Farhadi, M., Niasari-Niaslaji, A., Saboury, A. A., Chobert, J. M., Haertlé, T. (2011). Biological activity of camel milk casein following enzymatic digestion. *Journal of Dairy Research*, 78, 471-478.

Solieri, L., Rutella, G. S., Tagliazucchi D. (2015). Impact of non-starter lactobacilli on release of peptides with angiotensin-converting enzyme inhibitory and antioxidant activities during bovine milk fermentation. *Food Microbiology*, 51, 108-116.

Stuknite, M., Cattaneo, S., Masotti, F., & De Noni, I. (2015). Occurrence and fate of ACE-inhibitory peptides in cheeses and in their digestates following *in vitro* static gastrointestinal digestion. *Food Chemistry*, 168, 27-33.

Tagliazucchi, D., Martini, S., Bellesia, A., Conte, A. (2015). Identification of ACE-inhibitory peptides from *Phaseolus vulgaris* after *in vitro* gastro-intestinal digestion. *International Journal of Food Sciences and Nutrition*, 66, 774-782.

Wu J., Aluko R. E., & Nakai S. (2006). Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-activity relationship study of di- and tri-peptides. *Journal of Agricultural and Food Chemistry*, 54, 732-738.

Wu S., Feng X., Lan X., Xu Y., & Liao D. (2015). A novel angiotensin-I converting enzyme (ACE) inhibitory peptide from gastrointestinal protease hydrolysate of silkworm pupa (Bombyx mori) protein: Biochemical characterization and molecular docking study. *Peptides*, 68, 17-24.

Figure captions

Figure 1. Changes in hydrolysis degree (DH) of camel milk proteins during *in vitro* gastric and pancreatic digestion. Values represent means ± SD of triplicate digestions. Different letters indicate that the values are significantly different ($P < 0.05$).

Figure 2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of camel milk proteins. (**A**) Un-digested camel milk is shown in lane 1. Sample after salivary digestion is shown in lane 2. Sample in lane 3 is the camel milk protein profile after addition of simulated gastric fluid without pepsin. Samples after gastric digestion are in lanes 4 (5 minutes of gastric digestion), 5 (30 minutes), 6 (60 minutes), 7 (90 minutes) and 8 (120 minutes of gastric digestion). Samples after pancreatic digestion are reported in (**B**). Lane 1 is un-digested camel milk. Sample in lane 2 is the camel milk protein profile after addition of simulated intestinal fluid without pancreatin. Samples after pancreatic digestion are in lanes 3 (5 minutes of intestinal digestion), 4 (30 minutes), 5 (60 minutes), 6 (90 minutes) and 7 (120 minutes of intestinal digestion). SDS-PAGE was carried in triplicate for each digestion. The showed electropherogram is representative of three independent experiments.

Figure 3. UV-chromatogram of the low molecular weight peptidic fraction (<3 kDa) obtained from camel milk subjected to consecutive gastro-pancreatic digestion. 1-8 represent the collected fractions used for the identification of the angiotensin converting enzyme-inhibitory peptides. The black line represents the trace at 214 nm whereas the grey line represents the trace at 280 nm. The showed chromatogram is representative of three independent experiments.

Figure 4. Mass spectrum of peptide fractions F1 (**A**), F2 (**B**) and F4 (**C**) from nanoflow LC-ESI-QTOF MS/MS analysis. Identified peptides are reported in tables 2, 3 and 4. The showed mass spectra are representative of three independent experiments.

Figure 5. Theoretical cleavage sites in the Ile-Pro-Pro (IPP)-containing sequences of camel and bovine κ-casein and bovine β-casein. CA: carboxypeptidase A; CB: carboxypeptidase B; Ch: chymotrypsin; E: elastase; P: pepsin; T: trypsin.

Table 1. Peptides concentration, yield, angiotensin-I converting enzyme (ACE)-inhibitory activity (IC_{50} values) of the post-pancreatic < 3 kDa permeate and peptide fractions obtained through reversed phase-high performance liquid chromatography purification of the post-pancreatic fraction. Values represent means ± standard deviation of triplicate determination.

	Peptides concentration (mg mL⁻¹)	*Estimated yield[a] (%)*	*IC_{50}[b] (µg peptides mL⁻¹)*
< 3 kDa permeate	21.74 ± 0.49[a]	100	1171.4 ± 3.1[a]
HPLC F1	11.10 ± 0.33[b]	51.1	38.6 ± 0.4[b]
HPLC F2	2.50 ± 0.11[c]	11.5	107.9 ± 1.2[c]
HPLC F3	1.07 ± 0.07[d]	4.9	720.3 ± 5.9[d]
HPLC F4	1.08 ± 0.09[d]	5.0	37.2 ± 0.3[b]
HPLC F5	0.44 ± 0.02[e]	2.0	> 1000
HPLC F6	0.79 ± 0.04[f]	3.6	927.4 ± 8.8[e]
HPLC F7	0.71 ± 0.02[f]	3.3	> 1000
HPLC F8	1.00 ± 0.02[d]	4.6	> 1000

[a] yield was calculated as follows: (peptides concentration in the fraction)*100/ (peptides concentration in the post-pancreatic < 3 kDa permeate).

[b]IC_{50} is defined as the concentration of peptides needed to inhibit of 50% ACE activity.

Different letters within the same column indicate that the values are significantly different ($P < 0.05$).

Table 2. Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F1 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion.

Observed mass (m/z)	*Calculated mass*[a]	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]
120.0807	122.0855	T	Various proteins	/
132.1014	132.1019	Lx	Various proteins	/
166.0861	166.0863	F	Various proteins	/
182.0805	182.0812	Y	Various proteins	Antioxidant
189.1246	189.1234	GLx	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (GL/GI IC_{50}= >1000 µmol L^{-1}) DPP IV inhibitor (GL/GI)
203.1427	203.1390	ALx	β-, αS1-,αS2-, κ-casein	ACE-inhibitor (AI IC_{50}= 3.4 µmol L^{-1}) DPP IV inhibitor (AL)
215.1413	215.1390	VP	β-,αS2-, κ-casein	ACE-inhibitor (IC_{50}= 420 µmol L^{-1}) DPP IV inhibitor
217.1561	217.1547	VV	β-, αS1, κ-casein, and α-lactalbumin	DPP IV inhibitor
219.1330	219.1339	SLx	β-, αS1-,αS2-casein	DPP IV inhibitor (SL)
233.1496	233.1496	TLx	β-,αS2-casein	DPP IV inhibitor
233.1573	233.1496	LxT	β-, αS2, κ-casein, and α-lactalbumin	DPP IV inhibitor (LT)
246.1450	246.1448	GGI	α-lactalbumin f(19-21)	/
247.1295	247.1288	DLx	β-, αS1-,αS2-casein, and α-lactalbumin	ACE-inhibitor (DL IC_{50}= >1000 µmol L^{-1})
253.1177	253.1183	FS	κ-casein f(96-97)	/
267.1349	267.1339	FT	β-,αS2-casein	/
269.1712	269.1608	LH	β-, αS1-,αS2-, κ-casein	Inhibitor of lipid peroxidation DPP IV inhibitor
276.1567	276.1554	SLG	Lactoferrin and lactotrasferrin	/
302.1971	302.2074	LxLxG	Cytochrome b, peptidoglycan recognition protein 1, milk fat globule EGF factor 8	ACE-inhibitor (LLG IC_{50}= >1000 µmol L^{-1})

316.2101	316.2067	VPT	αS2-casein f(116-118)		/
326.2049	326.2074	PPL	β-casein f(78-80)	DPP IV inhibitor	
371.2290	371.1962	GS(phospho)Q	Various proteins		/

[a]Monoisotopic mass

[b]Lx indicates leucine or isoleucine

[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity

Table 3. Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F2 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion

Observed mass (m/z)	*Calculated mass[a]*	*Peptide sequence[b]*	*Protein precursor*	*Bioactivity[c]*
120.0792	122.0855	T	Various proteins	/
166.0854	166.0863	F	Various proteins	/
215.1333	215.1390	VP	β-, αS2-, κ-casein	ACE-inhibitor (IC_{50} 420 µmol L^{-1}) DPP IV inhibitor
229.1554	229.1547	PLx	β-, αS1-, κ-casein and α-lactalbumin	ACE-inhibitor (PL IC_{50}= 330 µmol L^{-1}) DPP IV inhibitor (PL/PI)
231.1706	231.1703	VLx	β-, αS1-, κ-casein	DPP IV inhibitor (VL/VI) Glucose uptake stimulating peptide (VL)
231.1764	231.1703	LxV	β-, αS2-casein	Glucose uptake stimulating peptide (LV/IV) DPP IV inhibitor (LV)
233.1453	233.1496	TLx	β-casein and α-lactalbumin	DPP IV inhibitor (TL/TI)
249.1273	249.1267	VM	β-, αS1-, αS2-casein	DPP IV inhibitor
253.1238	253.1183	SF	κ-casein f(96-97)	ACE-inhibitor (IC_{50} 130 µmol L^{-1}) DPP IV inhibitor
267.1293	267.1339	TF	β-, αS2-casein	ACE-inhibitor (IC_{50} 18 µmol L^{-1}) DPP IV inhibitor
281.1478	281.1496	VY	β-, αS2-casein	ACE-inhibitor (IC_{50} 7.1 µmol L^{-1}) Antioxidant DPP IV inhibitor
187.1329	373.2445	LLQ	β-, αS1-casein	/
272.1599	272.1605	PGV	Pancreatic lipase	/
290.1769	290.1710	TVA	κ-casein f(116-118)	/
316.2188	316.2231	IAI	κ-casein f(98-100)	/
318.1940	318.2023	VVT	αS1-casein f(151-53)	/
320.1868	320.1816	SLxT	β-casein f(20-22) f(125-127)	/
331.1643	331.1612	NPT	Cytochrome b f(207-209)	/
344.1734	344.1565	SHT	β-casein	/

			f(62-64)	
345.2180	345.2132	VLN	αS1-casein f(22-24)	/
348.1860	348.1765	DLT	α-lactalbumin f(84-86)	/
357.2130	35.2132	PLxQ	β-, αS1-, κ-casein	/
361.1988	361.2082	QIT	κ-casein f(156-158)	/
368.1748	368.1816	VYS	β-casein f(60-62)	/
400.2561	400.2554	GVPK	Various proteins	/
414.2329	414.2711	KPVA	κ-casein f(63-66)	/
457.2305	457.2194	TVTH	Various proteins	/
485.3254	485.3082	LQPK	β-casein f(89-92)	/

[a]Monoisotopic mass

[b]Lx indicates leucine or isoleucine

[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme ; DPP IV: Dipeptidyl peptidase IV; IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity

Table 4. Peptides and amino acids identified in the reversed phase-high performance liquid chromatography fraction F4 of < 3 kDa permeate obtained from camel milk after simulated gastro-intestinal digestion

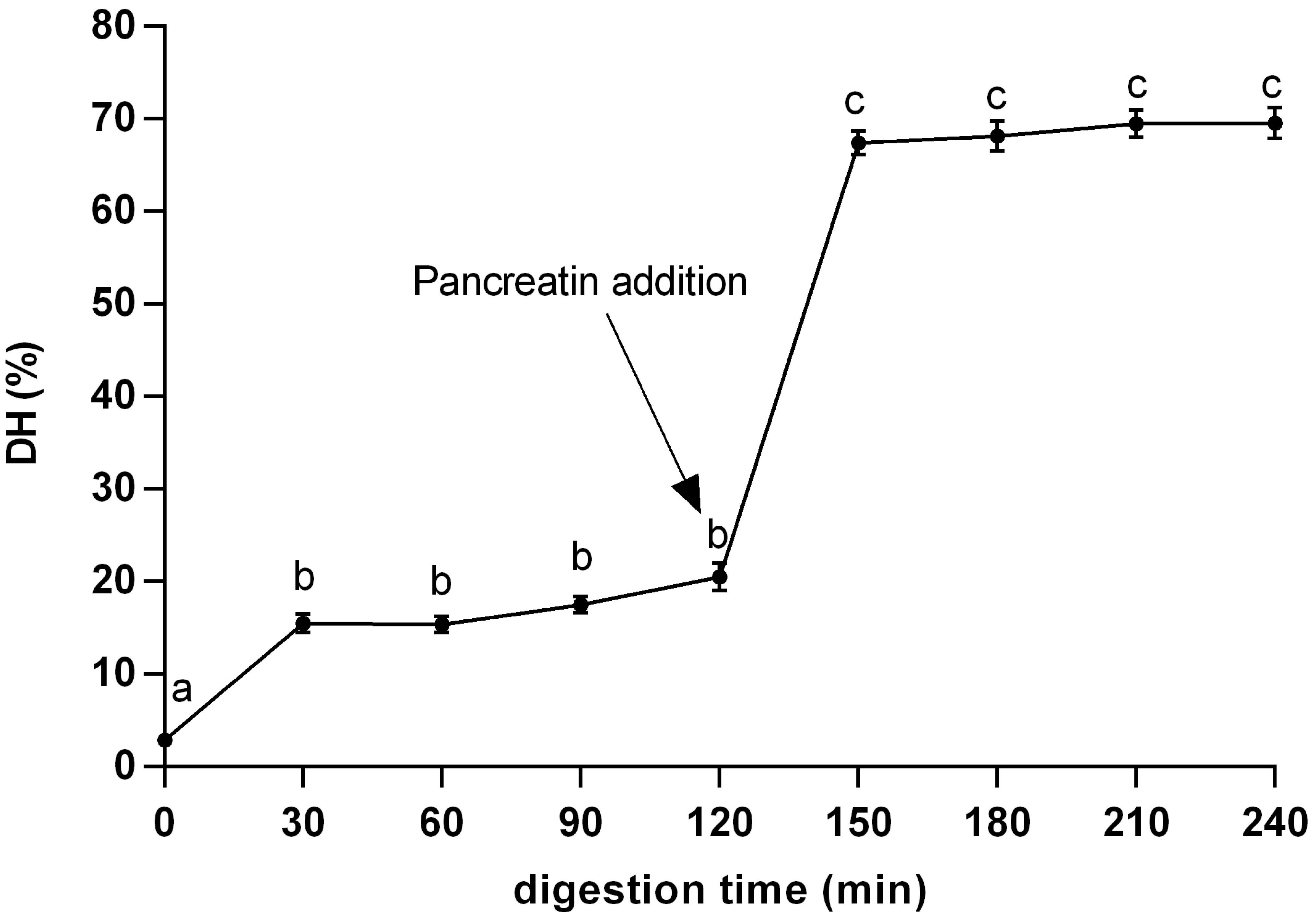
Observed mass (m/z)	*Calculated mass*[a]	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]
205.0942	205.0972	W	Various proteins	Antioxidant
229.1504	229.1547	PLx	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (PL IC_{50}= 330 µmol L^{-1}) DPP IV inhibitor (PL/PI)
229.1504	229.1547	LxP	β-, αS1, αS2-, κ-casein	ACE-inhibitor (IP IC_{50}= 130 µmol L^{-1}) DPP IV inhibitor (LP/IP)
231.1675	231.1703	VLx	β-, αS1, κ-casein, and α-lactalbumin	DPP IV inhibitor (VL/VI) Glucose uptake stimulating peptide (VL)
245.1903	245.1860	LxLx	β-, αS1, κ-casein, and α-lactalbumin	ACE-inhibitor (LL IC_{50}= n.r.) DPP IV inhibitor (LL/LI/IL/II) Glucose uptake stimulating peptide (LL/LI/IL/II)
263.1351	263.1390	PF	β-, αS1, αS2-casein	DPP IV inhibitor
295.1626	295.1652	LxY	β-, αS1, αS2-casein	ACE-inhibitor (LY IC_{50}= 18 µmol L^{-1} ; IY IC_{50}= 2.1 µmol/L) Antioxidant (LY/IY)
196.1704	391.1976	FPQ	β-, αS1-casein	/
288.1856	288.1918	GLxV	Lactoferrin, lactotransferrin, cytochrome b, porcine pepsin A, pancreatic lipase	/
314.2002	314.2074	PVV	β-casein f(116-118)	/
326.2048	326.2074	IPP	κ-casein f(99-101)	ACE-inhibitor (IC_{50}= 5.0 µmol L^{-1})
350.1644	350.1710	AYP	αS2-casein f(95-97)	/
360.2050	360.2129	ILD	α-lactalbumin f(95-97)	/
366.2057	366.2136	LxHP	β-, αS1-casein	ACE-inhibitor (LHP IC_{50}= 1.6 µmol L^{-1})
374.2358	374.2286	ILE	αS1-casein f(28-30)	/
378.1953	378.2023	VPY	β-casein f(178-180)	ACE-inhibitor (IC_{50}= 288 µmol L^{-1})
400.1937	400.2013	HIM	αS1-casein f(58-60)	/
219.1259	437.2507	IAHP	αS1-casein f(188-191)	/

444.2396	444.2453	LQSP	β-casein f(113-116)	/
227.6459	454.3024	IPPK	κ-casein f(99-102)	/
459.2536	459.2449	TEPI	β-casein f(64-67)	/
235.6654	470.3337	IIPK	β-casein f(103-106)	/
504.2348	504.2453	YPPQ	αS1-casein f(180-183)	/
312.1753	623.3511	VAHIPS	αS2-casein f(27-32)	/
328.6844	656.3726	TPVSPR	Serum albumin f(313-318)	/
342.1603	683.3359	SHTEPI	β-casein f(62-66)	/
344.1814	687.3762	SLNEPK	Lactophorin f(1-6)	/

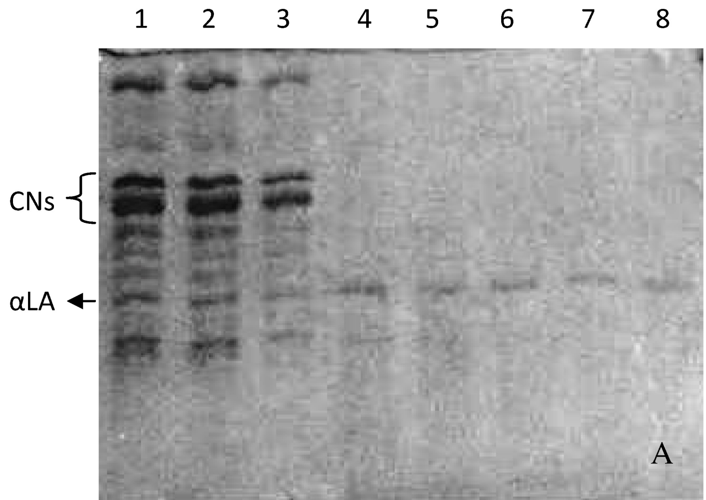
[a]Monoisotopic mass

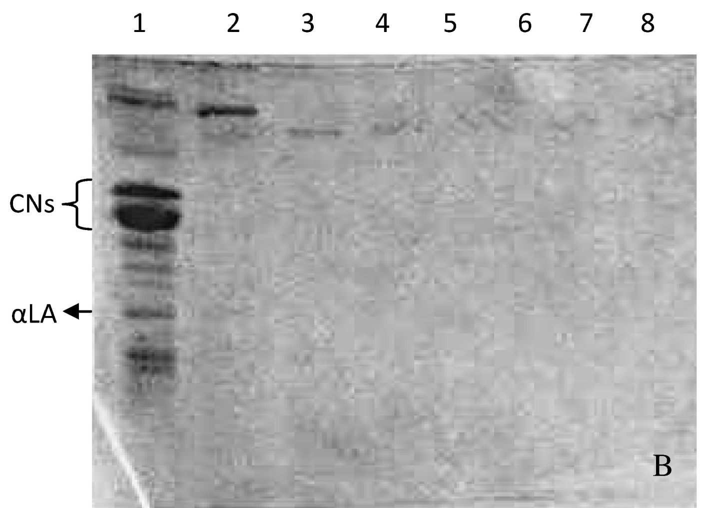
[b]Lx indicates leucine or isoleucine

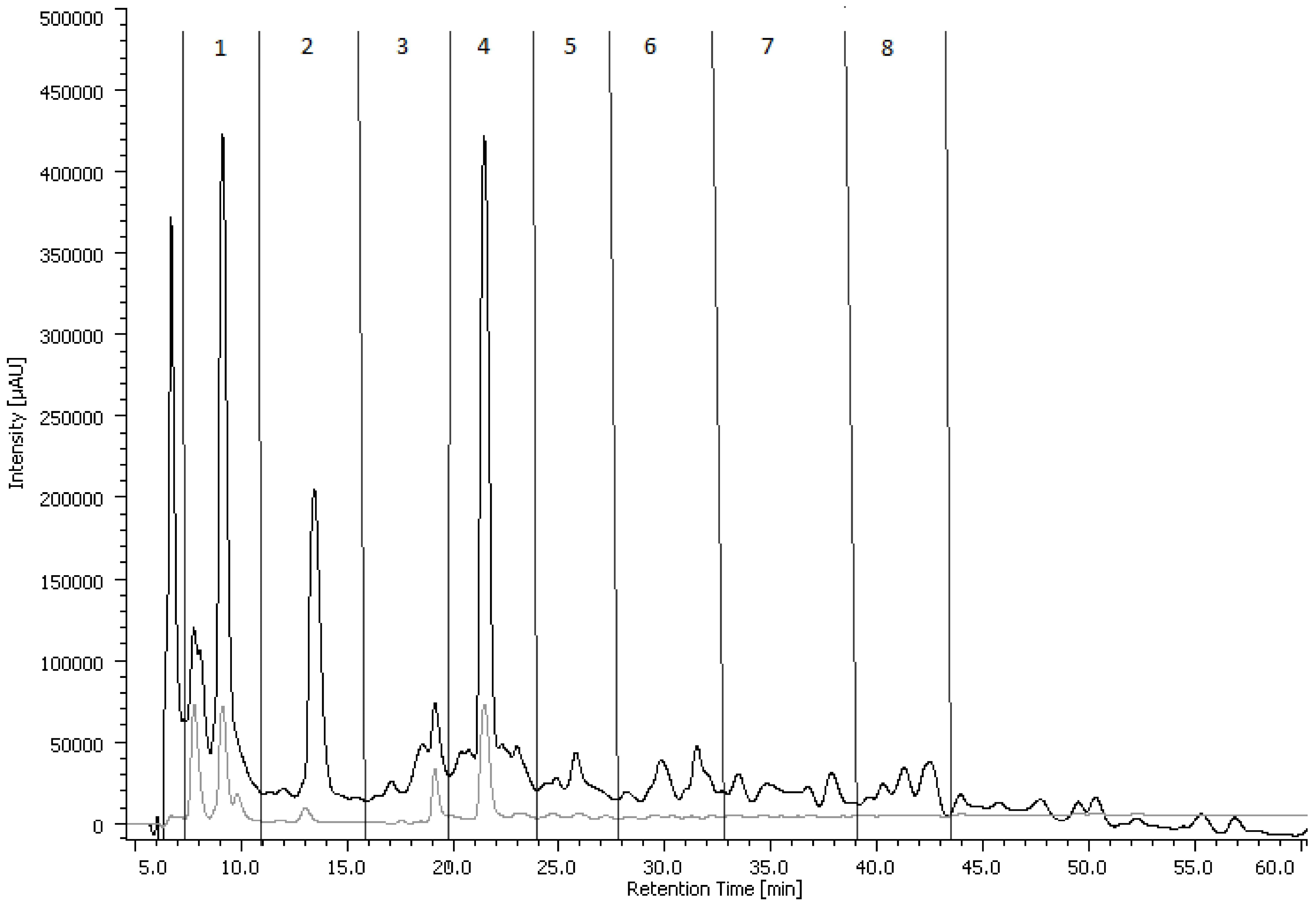
[c]Potential bioactivities were achieved from the BIOPEP database; ACE: Angiotensin Converting Enzyme; DPP IV: Dipeptidyl peptidase IV; IC_{50} is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity



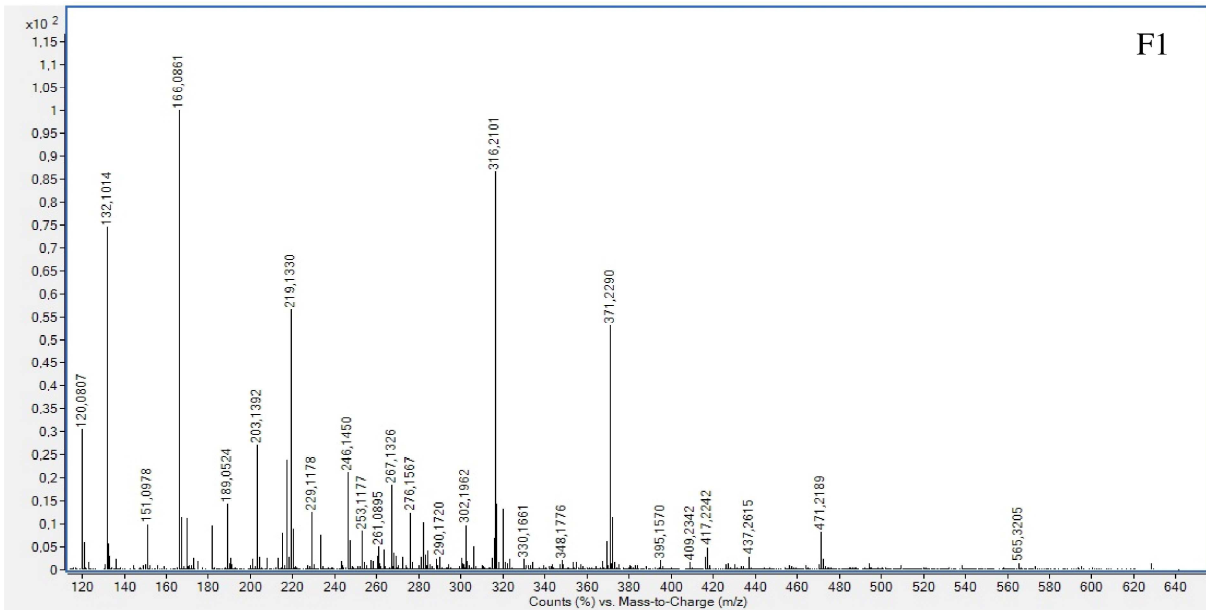
DH (%)
80
70
60
50
40
30
20
10
0
0
30
60
90
120
150
180
210
240
digestion time (min)
Pancreatin addition
a
b
b
b
b
c
c
c
c

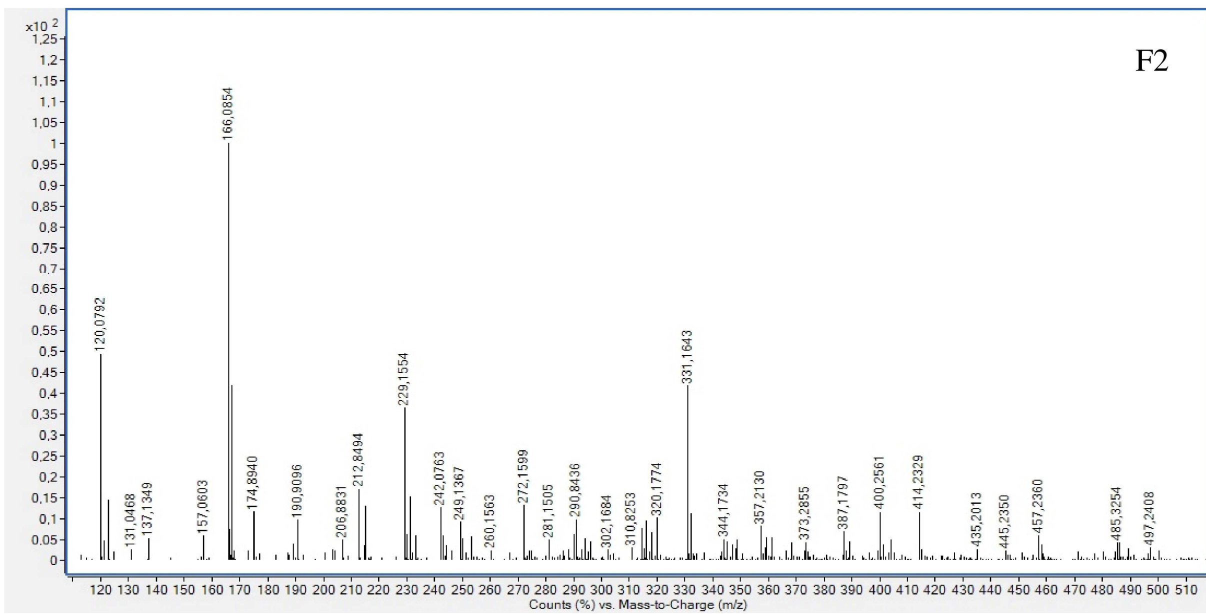

1 2 3 4 5 6 7 8
CNs
αLA
A

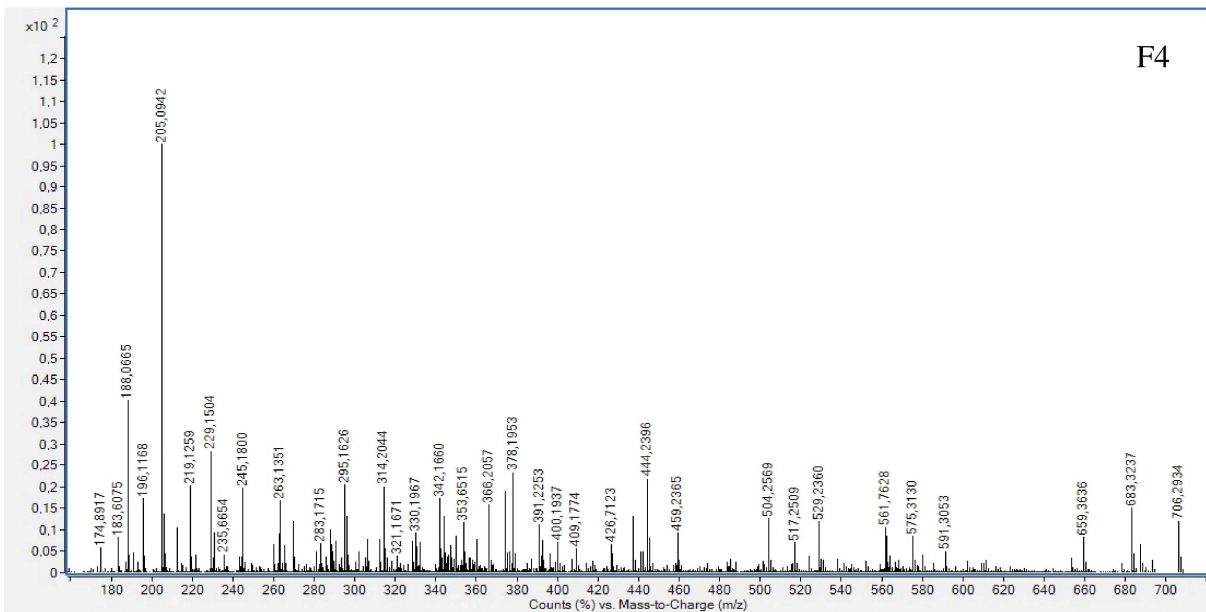

1 2 3 4 5 6 7 8
CNs
αLA
B



1
2
3
4
5
6
7
8
Intensity [µAU]
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
60.0
Retention Time [min]


F1
x10 2
Counts (%) vs. Mass-to-Charge (m/z)
120,0807
132,1014
151,0978
166,0861
189,0524
203,1392
219,1330
229,1178
246,1450
253,1177
261,0895
267,1326
276,1567
290,1720
302,1962
316,2101
330,1661
348,1776
371,2290
395,1570
409,2342
417,2242
437,2615
471,2189
565,3205


F2
x10 2
Counts (%) vs. Mass-to-Charge (m/z)
120,0792
131,0468
137,1349
157,0603
166,0854
174,8940
190,9096
206,8831
212,8494
229,1554
242,0763
249,1367
260,1563
272,1599
281,1505
290,8436
302,1684
310,8253
320,1774
331,1643
344,1734
357,2130
373,2855
387,1797
400,2561
414,2329
435,2013
445,2350
457,2360
485,3254
497,2408


F4
x10 2
Counts (%) vs. Mass-to-Charge (m/z)
174,8917
183,6075
188,0665
196,1168
205,0942
219,1259
229,1504
235,6654
245,1800
263,1351
283,1715
295,1626
314,2044
321,1671
330,1967
342,1660
353,6515
366,2057
378,1953
391,2253
400,1937
409,1774
426,7123
444,2396
459,2365
504,2569
517,2509
529,2360
561,7628
575,3130
591,3053
659,3636
683,3237
706,2934

P;Ch;E CB T

Camel κ-casein SFIAIPPKKTQ f(96-106)

Ch CA Ch

Bovine β-casein LPQNIPPLTQT f(70-80)

P;Ch;E CB T

Bovine κ-casein SFMAIPPKKNQ f(104-114)