EFFECT OF DIGESTIVE ENZYMES ON THE BIOACTIVE PROPERTIES OF GOAT MILK PROTEIN HYDROLYSATES

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ABSTRACT

The aim of this research was to study the influence of the gastrointestinal digestion on the bioactivity of goat milk protein hydrolysates prepared with subtilisin, trypsin and a combination of these two enzymes. All hydrolysates had excellent ACE inhibitory, antioxidant and bile acid-binding capacity. Peptide profiles and bioactivities were mainly altered during the intestinal digestion, whereas the effect of the gastric digestion was negligible. The influence of the intestinal digestion varied depending on the hydrolysate and the bioactivity studied. In the case of ACE inhibitory activity, it exclusively decreased when peptides were produced with trypsin. Contrarily, antioxidant activity and bile acid-binding capacity improved after the gastrointestinal digestion, regardless the enzymatic treatment conducted. Hydrolysis employing mixtures of subtilisin and trypsin is considered a good approach to produce peptides that maintain, or even enhance, their bioactivity after digestion.

1. INTRODUCTION

Food proteins, and specially milk proteins, have been described as an important source of bioactive peptides (Korhonen & Pihlanto, 2006). Apart from their nutritional value, these compounds present a physiological activity which might have beneficial effects on health. To be considered as bioactive, the compounds should generate a measurable biological effect and have the potential of generate health benefits without presenting toxicity, allergenicity or mutagenicity (Möller, Scholz-Ahrens, Roos, & Schrezenmeir, 2008). Bioactive peptides are encrypted in the sequence of the proteins and should be released to exert their activity. Enzymatic hydrolysis is the main procedure for liberating active peptides. Enzymes such as subtilisin and trypsin generate peptides with hydrophobic or positively-charged amino acids at the C-terminus, respectively. These features have been shown to be favourable for exerting bioactivities (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011; Power, Jakeman, & FitzGerald, 2013). Regarding the substrates, most of the studies dealing with the production of bioactive peptides from milk proteins employ cow milk as a raw material (Nagpal et al., 2011). However, more recently, other types of milk, such as goat milk have also shown its potential as a source of bioactive peptides (Ahmed, El-Bassiony, Elmalt, & Ibrahim, 2015; Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013). Compared to cow milk, goat milk has a slightly higher casein fraction, which is mainly composed by β-casein (50% of total caseins) while, in bovine casein fraction, the α-caseins represent almost 50% of the total (Bernaka, 2011). Moreover, goat milk proteins have better digestibility and hypoallergenic properties (Park, 2009). The bioactivities observed in milk protein derived peptides cover a wide range, including antihypertensive, antioxidant and cholesterol lowering (Korhonen & Pihlanto, 2006; Muro Urista, Álvarez Fernández, Riera Rodriguez, Arana Cuenca, & Téllez Jurado, 2011).

Antihypertensive activity is one of the most relevant bioactivities since hypertension is the main risk factor for cardiovascular diseases. The antihypertensive effect of hydrolysates is usually studied *in vitro* by the capacity of inhibiting angiotensin converting enzyme (ACE). In the organism, this enzyme is a key component of the blood pressure regulation process (Johnston, 1992) and its inhibition would help to control high blood pressure. Contrary to commercial ACE inhibitory drugs, food derived peptides have not shown any side effects, even at high doses

(Ishida et al., 2011). The ACE inhibitory potential of goat milk protein hydrolysates has been reported in previous studies employing subtilisin or trypsin as enzymes. (Espejo-Carpio et al., 2013; Manso & López-Fandiño, 2003). Additionally, some of the peptides released from goat protein have shown *in vivo* effects. For example, a goat milk hydrolysate originated a significant decreased in systolic blood pressure in spontaneously hypertensive rats. Moreover it reduced the ACE activity in aorta, left ventricle, and kidney in a similar way that a commercial drug (Geerlings et al., 2006).

Antioxidants compounds, apart from inhibiting or retard lipid oxidation in food, might also prevent oxidation at physiological level (Hajieva & Behl, 2006). Because of the potentially hazardous effects of synthetic antioxidants (Ito, Fukushima, & Tsuda, 1985), there is an increasing interest in finding natural antioxidants with reduced side effects. In this context, goat milk proteins have shown its potential as raw material for the production of hydrolysates exhibiting antioxidant activity. Recently, De Gobba, Espejo-Carpio, Skibsted and Otte (2014) produced antioxidant peptides by enzymatic hydrolysis of goat milk microfiltration fractions using subtilisin and trypsin. Likewise, Li et al. (2013) identified antioxidant peptides in goat milk casein hydrolysates produced by a combination of neutral and alkaline proteases. Bezerra et al. (2013) also reported antioxidant activity for the permeate of goat casein hydrolysates produced by using papain.

In addition, some protein fractions present in milk have also been reported to exert cholesterol lowering activity. The lowering activity of a given compound depends on its ability to inhibit the intestinal absorption of dietary cholesterol or to sequester bile acids, which are not reabsorbed but excreted. In response to a lower rate of bile recycling, the liver increases bile acid synthesis, which is done at the expense of removing cholesterol from bloodstream. Several studies have reported the cholesterol lowering ability of cow milk proteins but none of goat milk proteins. For instance, Lanzini, Fitzpatrick, Pigozzi, and Northfield (1987) conducted *in vivo* studies on ileal resection patients demonstrating the bile binding capacity of caseins. Furthermore, Nagaoka et al. (2001) identified an active peptide in tryptic β-lactoglobulin hydrolysates able to inhibit the absorption of dietary cholesterol through the intestine wall.

78 In any case, to exert their biological effect, bioactive peptides should reach their target organs

79 and tissues in an active form. In this regard, gastrointestinal digestion is a key process

80 determining the bioavailability of the peptides. *In vitro* digestion processes, which employ

81 gastrointestinal proteases, could be employed for evaluating the effect of digestive enzymes

82 over bioactive hydrolysates and peptides. To the best of the authors' knowledge, there are no

83 published studies dealing with the effect of gastrointestinal digestion on antioxidant or

84 cholesterol lowering activity of milk protein-derived peptides. With respect to ACE inhibitory

85 activity, the few studies carried out are focused on the changes originated on isolated peptides

86 instead of on the whole hydrolysate (Contreras, Sanchez, Sevilla, Recio, & Amigo, 2013;

87 Quirós, Contreras, Ramos, Amigo, & Recio, 2009; Tavares et al., 2011). Although the

88 evaluation of the effect of digestive enzymes over isolated peptides could be interesting, as far

89 as nutraceuticals are concern, large scale purification or synthesis of individual bioactive

90 peptides would be very costly. Furthermore, bioactivities such as antihypertensive could be

91 influenced by apparent inactive peptides which could interact with subsites different from the

92 active center of ACE (López-Fandiño, Otte, & van Camp, 2006; Meisel, 1997). Additionally,

93 because of the different conformational requirements of the catalytic sites of ACE, a mixture of

94 peptides with different conformational features would be necessary for inhibiting ACE (Gobbetti,

95 Stepaniak, De Angelis, Corsetti, & Di Cagno, 2002). Therefore, studying the effect of the

96 digestion over the complete hydrolysate seems a better approach to evaluate the global

97 bioactive capacity.The aim of this work was to evaluate the effect of gastrointestinal enzymes in

98 the ACE inhibitory activity, antioxidant activity and cholesterol lowering activity of hydrolysates

99 of goat milk caseins.

100 2. MATERIALS AND METHODS

101 2.1. ENZYME HYDROLYSIS

102 Goat milk caseins were hydrolysed according to the procedure described in Espejo-Carpio et al.

103 (2013). Firstly, commercial UHT goat milk was microfiltered through a 0.14 μm ceramic

104 membrane, where the casein fraction was obtained in the retentate side. Subtilisin

105 (EC.3.4.21.62) and trypsin (EC.3.4.21.4), both from Novozymes A/S (Bagsværd, Denmark),

106 were used as enzymes for producing three different hydrolysates: subtilisin hydrolysate (SH),

107   trypsin hydrolysate (TH) and a hydrolysate produced with a mixture of both enzymes (STH). In

108   all the cases, the reaction was carried out in a 0.2 L stirrer tank reactor at 50 ºC and pH 8. The

109   degree of hydrolysis (DH) was monitored throughout the reaction by the pH-stat method (Adler-

110   Nissen, 1986). After 3 h of reaction, enzyme was thermally deactivated in boiling water for 15

111   min. The final hydrolysates were freeze dried for further analysis.

112   ## 2.2. PROTEIN DETERMINATION

113   The protein content of both the hydrolysates and the digested hydrolysates were determined by

114   the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, St. Louis, MO, USA).

115   The dried samples were re-dissolved in Milli-Q water and analysed in triplicate according to the

116   kit instructions. The absorbance was measured at 650 nm.

117   ## 2.3. GASTROINTESTINAL DIGESTION

118   The method proposed by Lo and Li Chan (2005) with some modifications was used for

119   evaluating the susceptibility of active peptides to digestive enzymes. In this work, the effects of

120   both the gastric digestion alone and the gastrointestinal digestion over the bioactivities of

121   hydrolysates were evaluated. To this end, each hydrolysate sample was subjected, in triplicate,

122   to two digestion procedures: gastric and gastrointestinal digestion.

123   The digestion process was carried out in a jacketed stirred reactor at 37 ºC. Firstly, the freeze

124   dried samples were reconstituted in water until obtaining a protein concentration of 10 mg mL^{-1}.

125   Then, the pH was decreased to pH 2 by adding 1 M HCl. Subsequently, the gastric digestion

126   was initiated by adding pepsin (EC 3.4.23.1, Merck, Darmstadt, Germany) at enzyme-substrate

127   ratio of 4 % (w/w). The reaction was stopped after 1 h raising the pH to 5.3 with 0.9 M NaHCO$_3$.

128   For gastric digested samples, the digestion process was finished at this point by inactivating

129   thermally the enzyme (100 ºC for 15 min). However, the digestion process continued for the

130   gastrointestinal digested samples by the incorporation of pancreatin to the reaction media.

131   Pancreatin (Sigma-Aldrich) is a mixture of porcine pancreas enzymes containing proteases

132   such as trypsin, chymotrypsin, elastase, carboxy-peptidase A and carboxypeptidase B. The

133   enzyme mixture was prepared at 2 mg mL^{-1} in NaHCO$_3$ 0.1 M and added to the reactor to obtain

134   an enzyme-substrate ratio of 4% (w/w). Immediately, the pH was raised to pH 7.5 with 1 M

135   NaOH. The intestinal digestion was performed for 2 h at 37 ºC, then, the enzymes were

136 thermally deactivated (100 ºC for 15 min). Once deactivated, both gastric samples and

137 gastrointestinal samples were freeze-dried until further analysis.

2.4. SIZE EXCLUSION CHROMATOGRAPHY

139 The molecular size distribution of the hydrolysates and their digests was determined by size

140 exclusion chromatography (SEC). A fast protein liquid chromatography system (Pharmacia LKB

141 Biotechnology AB, Uppsala, Sweden) mounted with Superdex Peptide 10/300GL column (GE

142 Healthcare, Uppsala, Sweden) was used. The column was operated at 25 ºC with a mobile

143 phase composed of 70:30 water/acetonitrile with 0.1 % trifluoroacetic acid (TFA). The

144 absorbance was measured at 280 nm.

145 Hydrolysates and digested hydrolysates were diluted in distilled water (5 mg protein mL^{-1}) and

146 filtered through a 0.2 μm filter. An aliquot of 100 μL was injected and eluted at 0.5 mL min^{-1}.

147 Glycine (75 Da), alanine (89 Da), Phe-Gly-Gly (279 Da), (Gly)$_6$ (360 Da), vitamin B12 (1355

148 Da), insulin (5733 Da), aprotinin (6511 Da) and ribonuclease (13700 Da) were used as

149 standards.

2.5. ACE-INHIBITORY ACTIVITY

151 A spectrophotometric assay (Shalaby, Zakora, & Otte, 2006) was selected to measure the

152 capacity of the hydrolysates and their digested samples to inhibit ACE. The method is based on

153 the decrease in absorbance observed when the synthetic tripeptide N-[3-(2-furyl)acryloyl]-L-

154 phenylalanyl-glycyl-glycine (FAPGG) is hydrolysed by ACE (EC 3.4.15.1). Both reagents were

155 purchased from Sigma-Aldrich. The assay was carried out in a 96-well microplate at 37 ºC

156 measuring the absorbance at 340 nm for 30 min in a Multiskan FC microplate photometer

157 (Thermo Scientific, Vantaa, Finland). Inhibition percentage was determined according to Eq. 1.

158 Samples were analysed in triplicate.

159
$$ACE\ inhibition\ (\%) = \left(1 - \frac{p_i}{p_0}\right) \times 100 \qquad [1]$$

160 Where p_i was the slope in the presence of inhibitor and p_0 the slope in the absence of inhibitor

161 (pure water). These slopes were calculated in the interval from 10 to 25 min, when a better

162 linearity was registered. The IC$_{50}$ value, defined as the concentration of sample which reduces

163 the ACE activity to 50%, was calculated for each sample in triplicate using five different protein

164 concentrations.

2.6. ANTIOXIDANT ACTIVITY

166 The antioxidant activity of the hydrolysates produced and their digested samples was

167 determined *in vitro* by testing their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH)

168 radical. The method described by Picot et al. (2010) was used. In short, a volume of 1 mL of

169 each sample having different protein concentrations (0.5 - 6 mg mL^{-1}) was added to 1 mL of 0.1

170 mM DPPH in methanol. The mixture was shaken and left for 30 min at room temperature in the

171 dark. Then, the absorbance of the reaction mixture was measured at 517 nm. A blank was run

172 in the same way by using distilled water instead of sample, and sample control was also made

173 for each sample by adding methanol instead of DPPH solution. Triplicate measurements were

174 carried out for each sample and DPPH scavenging activity was calculated by Eq. 2:

175
$$DPPH\ inhibition\ (\%) = \left(1 - \frac{(A_{sample} - A_{sample\_control})}{A_{blank}}\right) \times 100 \qquad [2]$$

176 The IC$_{50}$ value, which defines the concentration of hydrolysate (mg protein mL^{-1}) needed to

177 inhibit DPPH activity by half, was also calculated.

2.7. BILE ACID BINDING CAPACITY

179 Both the hydrolysates and their digested samples were tested for their *in vitro* bile acid-binding

180 capacity. A bile preparation was formulated containing 75 % of glycocholic bile acids and 25 %

181 of taurine bile acids, simulating human bile (Lin, Tsai, Hung, & Pan, 2010). The hydrolysate

182 powder (100 - 110 mg) was mixed with 4 mL of 0.72 mM bile acid solution. This suspension was

183 incubated under agitation at 1 h and 37 ºC. It was then centrifuged at 60,000 g for 30 min, and

184 the supernatant was recovered. Bile acid binding was related to the amount of bile retained by

185 the hydrolysate (i.e. present in the sludges after centrifugation). The unbound bile acids in the

186 supernatant were determined using a total bile acids determination kit (Diazyme, Poway, CA,

187 USA), employing cholic acid (Sigma Aldrich) as standard. This method is based on the

188 enzymatic conversion of bile acids to 3-ketosteroids, which can be followed

189 spectrophotometrically at 405 nm. The bile acid-binding capacity is commonly expressed as the

190 μmol of bile acids bound by 100 mg of dry matter (i.e. retained in the sludge after

191 centrifugation). A sample of 24 - 26 mg of cholestyramine (Sigma-Aldrich) was employed as

192 positive control. In our case, 100 mg of cholestyramine sample bound on average 9.42 ± 0.02

193 μmol of the bile acids, which represents 90 % of bile acid binding capacity. Considering that

194 proteins were the major compounds responsible for the sequestration of bile acids, the bile acid-

195 binding capacity of the hydrolysates was related to cholestyramine on equal protein basis.

196 ## 2.8. STATISTICAL ANALYSIS

197 Statgraphics Centurion XV (Statistical Graphics Corp., Rockville, MD, USA) was used for data

198 analysis. Data were expressed as mean ± standard deviation (SD) with three replications. Firstly

199 a one-way analysis of variance (ANOVA) was performed to identify significant differences

200 between original and digested samples. Secondly, mean values were compared by using the

201 Fisher's least significant difference (LSD) test. Differences between means were considered

202 significant at p values ≤ 0.05.

203 # 3. RESULTS AND DISCUSSION

204 ## 3.1. PRODUCTION OF HYDROLYSATES

205 The variation of the DH along the reaction was similar for the three treatments. As observed in

206 Fig. 1, an initial period of constant reaction rate was followed by a period of decreasing rate

207 where the curve became asymptotic. The lowest DH was achieved for TH, which might be due

208 to the higher specificity of this enzyme and the relatively high temperature employed in the

209 hydrolysis. Nevertheless, in all the treatments, the final DH obtained was above 20 %, which

210 assures hydrolysates containing small peptides. Indeed, the average peptide chain length

211 (PCL), calculated as the inverse of DH (Adler-Nissen, 1986), would be 5 residues. Short length

212 chain peptides are highly desirable since they usually exhibit better antihypertensive and

213 antioxidant activities than larger peptides (Contreras, Carrón, Montero, Ramos, & Recio, 2009;

214 Di Pierro, O'Keeffe, Poyarkov, Lomolino, & FitzGerald, 2014; Pihlanto-Leppälä, Rokka, &

215 Korhonen, 1998) and also have better resistance to degradation by digestive enzymes (Cinq-

216 Mars, Hu, Kitts, & Li-Chan, 2007; Roberts, Burney, Black, & Zaloga, 1999). The final DH

217 depends mainly on the enzyme employed, its concentration and the reaction time. The high

218   enzyme-substrate ratio employed in this work allowed to reach high DH level in a relatively short

219   reaction time. Similar final DH values were obtained in the hydrolysis of goat caseins by papain

220   when employing lower E:S ratios (0.5-1.5 %) but longer reactions times (5 h) (Bezerra et al.,

221   2013).

222   3.2. MOLECULAR SIZE DISTRIBUTION

223   Fig. 2 shows the SEC chromatograms of the original hydrolysates and their digests. The original

224   hydrolysates and their gastric digests presented similar molecular size distributions in all cases.

225   This would indicate that pepsin did not modify the original hydrolysates. In contrast,

226   gastrointestinal digests presented important differences compared to the original and gastric

227   digested hydrolysates. Particularly, the fraction of long chain peptides (29 - 0.9 kDa) which

228   eluted before 32 min, showed an increase. This raise could be attributed to large protein

229   fragments released from the previously added enzymes by the action of pancreatin. As a

230   consequence, this initial fraction was not considered for studying the variation between the

231   original hydrolysates and their digested samples. Then, three fractions were selected from each

232   chromatogram for comparison. These fractions, which are delimited by vertical lines in Fig. 2,

233   were noted as A, B and C, and had a molecular size ranging between 900 – 330 Da, 330 – 160

234   and <160 Da, respectively. According to the values of area percentage of each fraction shown

235   in Table 1, in almost all the cases there were significant differences between the original

236   hydrolysate and their gastric and/or gastrointestinal digested samples.

237   As shown Table 1, the original SH and STH presented a very similar molecular size distribution.

238   In contrast, TH presented a higher proportion of large peptides (fraction A) and lower of smaller

239   fractions (B and C). These differences are consistent with the fact that TH had a considerably

240   lower degree of hydrolysis than SH and STH (Fig. 1), and then the proportion of long chain

241   peptides should be higher.

242   Gastric digestion produced small changes in the proportion of fractions A and B in the

243   hydrolysates produced with subtilisin (SH and STH), while no significant changes were

244   observed for TH. On the other hand, the smallest peptides (fraction C) seemed to remain

245   unmodified by gastric digestion in any case. With respect to gastrointestinal digestion, it

246   generated changes in the fractions of peptides larger than 160 Da (A and B fractions) for all the

247 hydrolysates. Contrary to TH, fraction A (900 – 330 Da) increased whereas fraction B (330 –

248 160 Da) decreased after the gastrointestinal digestion of SH and STH. In the case of the

249 hydrolysates with the highest DHs (SH and STH), fraction C (< 160 Da) did not change after

250 gastrointestinal digestion. Nevertheless, fraction C of TH increased after the complete digestion

251 (Table 1).

252 3.3. ACE-INHIBITORY ACTIVITY

253 The ACE-inhibitory activity obtained for the original hydrolysates, with IC_{50} values ranging from

254 230.7 to 269.0 μg mL^{-1} (Table 2). Higher IC_{50} values (760 – 1143 µg mL^{-1}) were found in

255 subtilisin hydrolysates of bovine casein (S.-J. Jiang, Qian, Shen, & Mu, 2013) and yak casein

256 (Jiang, Chen, Ren, Luo, & Zeng, 2007). The hydrolysates produced with subtilisin (SH and

257 STH) exhibited similar ACE inhibitory activity (Table 2). This is in line with the similarities

258 observed in their molecular size distribution (Table 1). Differently, TH presented a lower

259 inhibition capacity, which could be explained by the lower degree of hydrolysis.

260 The ACE inhibitory activity of all the hydrolysates after gastric digestion presented a slight

261 improvement. Only SH showed a significant reduction in the IC_{50} value (Table 2). However, after

262 gastric digestion, there were no differences in the inhibitory capacity of SH and STH. In the case

263 of TH, the absence of changes in the peptide distribution (Fig 2) was reflected in the similar

264 ACE inhibitory of the original hydrolysate and the gastric digest thereof (Table 2).

265 Contrary to the slight influence of gastric digestion, gastrointestinal digestion caused important

266 changes in ACE inhibitory activity, particularly to the hydrolysates produced using trypsin (TH

267 and STH). A reduction by almost 50 % of the initial value of ACE inhibition was obtained after

268 the gastrointestinal digestion of TH. This decrease could be explained because the original

269 peptides generated by trypsin in TH are not modified by pepsin (Table 1 and 2). Therefore,

270 before intestinal digestion, active peptides would have arginine and lysine in the C-terminal,

271 which could be released by the action of the carboxy-peptidase B during intestinal digestion.

272 This modification of the C-terminal sequence would substantially reduce its inhibitory capacity

273 (López-Fandiño et al., 2006). Furthermore, active peptides could also be degraded by other

274 intestinal enzymes reducing the global ACE inhibitory activity. Both mechanisms would be

275 related to the decrease in fraction A (900 – 330 Da) and the increase in fraction B (330 – 160

276   Da) observed after the gastrointestinal digestion of TH. In the case of SH, it maintained the ACE

277   inhibitory activity of the original hydrolysate after the gastrointestinal digestion. Subtilisin seems

278   to produce bioactive hydrolysates with gastrointestinal digestion resistance independently of the

279   substrate employed, since similar results were obtained for the hydrolysates of pumpkins

280   proteins (Vaštag, Popović, Popović, Peričin-Starčević, & Krimer-Malešević, 2013) and tuna

281   cooking juice (Hwang, 2010). The final ACE inhibitory activity of these hydrolysates after

282   gastrointestinal digestion varied from IC_{50} = 404 to 250 μg mL^{-1}, which is in the same range as

283   to those obtained in this study for goat milk protein hydrolysates. According to the studies

284   dealing with the modifications by gastrointestinal digestion of isolated casein peptides, the fate

285   of the peptides is very variable. Some of the peptides could resist digestion process without

286   degradation (Contreras et al., 2013), while others would be hydrolysed (Quirós et al., 2009) with

287   or without modification of its ACE inhibitory activity. Therefore, in the case of SH, the loss of

288   some active peptides during gastrointestinal digestion would be balanced with the release of

289   active fragments. This effect would also occur, to some extent, in the hydrolysate produced with

290   the combination of enzymes (STH). The ACE inhibitory activity of the gastrointestinal digested

291   of STH only reduced 15 % and it was not significant different from digested SH.

292   3.4. ANTIOXIDANT ACTIVITY

293   The DPPH radical scavenging of the hydrolysates and their digests were measured as a

294   function of the protein concentration in order to determine their IC_{50} values (Table 2). TH

295   exhibited the highest antioxidant activity (IC_{50}=3.78±0.07 mg mL^{-1}), followed by SH and STH

296   (IC_{50}=4.62±0.15 mg mL^{-1} and IC_{50}=5.51±0.08 mg mL^{-1}, respectively) (Table 2). The differences

297   found in the antioxidant activity between the two hydrolysates produced using subtilisin (SH and

298   STH) were not statistically significant. This may be explained by the similarities observed

299   between the molecular size distributions of both hydrolysates (Table 1). Furthermore, statistical

300   significance was not observed for TH when compared to SH (Table 2). This finding is in

301   agreement with the results reported by Mao, Cheng, Wang, and Wu (2011) These authors

302   obtained yak casein hydrolysates with the highest DPPH scavenging activity when using trypsin

303   or subtilisin as the enzyme (without significant differences between both enzymes). In this

304   study, the highest antioxidant activity exhibited by TH can be attributed to its lower DH value

305   (Fig. 1), which results in a higher percentage of peptides in fraction A (900 – 330 Da) (Table 2).

306 According to previous studies about the influence of DH and molecular mass of peptides on

307 antioxidant capacity, short peptides (<1500 Da) show higher antioxidant activity than higher

308 molecular mass peptides (Peña-Ramos & Xiong, 2002; Wu, Chen, & Shiau, 2003). However, an

309 excessive hydrolysis would reduce the DPPH inhibition of the hydrolysates (García-Moreno et

310 al., 2014; Jao & Ko, 2002; Mao et al., 2011). Moreover, the presence of cationic peptides,

311 containing arginine and lysine released in the hydrolysis with trypsin, may be also responsible

312 for the enhanced antioxidant activity of this hydrolysate (García-Moreno et al., 2013).

313 Similar to for ACE-inhibitory activity, the gastric digestion slightly modified the DPPH radical

314 scavenging activity of all the hydrolysates, with IC_{50} values ranging from 3.04 to 4.33 mg mL^{-1}

315 (Table 2). Vaštag et al. (2013) also indicated high stability in the antioxidant activity of pumpkin

316 oil cake protein hydrolysate produced with subtilisin after incubation with pepsin. A significant

317 improvement was observed in the IC_{50} value for STH (Table 2), which may be attributable to an

318 increase in the percentage of fraction A (900 – 330 Da) of this hydrolysate after gastric digestion

319 (Table 1). In contrast, the gastrointestinal digestion improved the DPPH scavenging activity with

320 statistical significance for all the hydrolysates when compared with their originals. This is in

321 agreement with previous studies which also reported higher antioxidant activity for pumpkin oil

322 cake and fish protein hydrolysates after digestion with pancreatin (Samaranayaka, Kitts, & Li-

323 Chan, 2010; Vaštag et al., 2013). Similar and considerably lower IC_{50} values were found for the

324 gastrointestinal digest of goat casein hydrolysates, varying from 1.30 to 1.57 mg mL^{-1} (Table 2).

325 In the case of STH, the antioxidant capacity improvement can be explained due to an increase

326 in the percentage of fraction A. For SH, similar molecular size distributions were obtained,

327 whereas, for TH, fraction A was reduced resulting in an increase of fraction B (Table 1).

328 Therefore, the improved antioxidant activity of the gastrointestinal digest should be mainly

329 attributable to the specificity of the different enzymes which are present in pancreatin. These

330 enzymes favour the release of hydrophobic and cationic peptides which have been reported to

331 be responsible for the antioxidant capacity of protein hydrolysates (Rajapakse, Mendis, Byun, &

332 Kim, 2005).

333 3.5. BILE ACID-BINDING CAPACITY

334 The mean values of the bile acid-binding capacities of hydrolysates and their digests are

335 presented in Table 2, relative to cholestyramine on an equal protein basis. The original

336 hydrolysates SH, TH and STH presented similar levels of bile acid binding capacity, showing no

337 significant differences among the enzymatic treatments. Indeed, they sequestrated on average

338 2.08 ± 0.28 µmol of bile acids per 100 mg of protein (23.37 ± 1.79 % relative to cholestyramine).

339 The gastric digests from TH and STH exhibited an improved ability to bind bile acids ($41.08 \pm$

340 6.39 %), while no significant differences were found between SH and their gastric digests.

341 Intestinal digestion reduced in 23 % the bile acid-binding capacity of TH gastric digests, while it

342 improved that of SH and STH gastric digests. Indeed, STH gastrointestinal digests presented

343 the highest value of bile acid-binding capacity among the samples (52.74 ± 6.35 %).

344 Most of the studies on hypocholesterolemic activity deal with dietary fibers (Kahlon & Smith,

345 2007), while the references to protein compounds are less common. Standard procedures to

346 evaluate *in vitro* sequestration of bile acids include simulated gastrointestinal digestion of the

347 samples. Therefore, no previous references on the intrinsic bile acid-binding capacity of protein

348 hydrolysates (i.e. without being submitted to digestion) are available. Nagaoka et al. (2001)

349 studied the *in vitro* bile acid-binding of casein and β-lactoglobulin hydrolysates produced with

350 trypsin at the same operating conditions (pH=8, 50ºC, 3-6 h) and lower enzyme-substrate ratio

351 (0.4 % w/w). After purification, the gastrointestinal digests presented taurocholate binding

352 capacities of 35% for the casein hydrolysates and 47 % for the β-lactoglobulin hydrolysates,

353 related to cholestyramine. These levels are comparable to those obtained in this work for TH

354 (31.47 ± 1.16 %).

355 The influence of the enzymatic treatment and digestion upon the hypocholesterol activity is

356 related with the nature of the peptidic fractions herein released. Sugano et al. (1990) suggested

357 mean molecular weights between 1 and 10 kDa for hypocholesterolemic peptides. Both the

358 original and the digests obtained in this work present a molecular size distribution within this

359 range. Moreover, Iwami et al. (Iwami, Sakakibara, & Ibuki, 1986) related bile acid sequestration

360 to the presence of hydrophobic peptides. To this regard, it should be noted that subtilisin and

361 pancreatine enzymes release prefereable peptides containing hydrophobic residues. This fact

362 could explain the improved bile sequestration shown by SH and STH hydrolysates, compared to

363 that of TH. Furthermore, both SH and STH bile acid-binding capacities increased after the

364 intestinal digestion with pancreatine.

5. CONCLUSIONS

366 Hydrolysates of goat milk caseins produced with subtilisin (S) and the combination subtilisin and

367 trypsin (ST) showed similar characteristics in both molecular size distribution and bioactivities.

368 In contrast, the lower DH reached in trypsin hydrolysates (T) led to hydrolysates with lower ACE

369 inhibitory activity but with better DPPH radical scavenging activity and bile acid-binding

370 capacity. Gastrointestinal digestion modified considerably the original hydrolysates. Particularly,

371 the digestion process reduced drastically the ACE inhibitory activity of the trypsin hydrolysate

372 while ACE inhibition of subtilisin hydrolysate remained constant. On the other hand, both gastric

373 and gastrointestinal digestion enhanced notably the antioxidant activity and bile acid-binding

374 capacity of all the hydrolysates assayed. Taking into account all the information provided by

375 simulated digestions, the hydrolysis with the combination of enzymes would be the best option

376 for obtaining potent bioactive hydrolysates from goat milk resistant to digestive enzymes.

377 However, *in vivo* studies would be necessary for evaluating the capacity of these hydrolysates

378 of exerting bioactive effect.

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539

FIGURE CAPTIONS

Figure 1. Evolution of degree of hydrolysis (DH) during the hydrolysis of caseins with subtilisin (dashed line), trypsin (dotted line) and the combination of subtilisin plus trypsin (solid line).

Figure 2. Molecular size distribution of the hydrolysates produced with subtilisin (a), trypsin (b) and the combination of subtilisin plus trypsin (c). Each chart shown the original hydrolysate (solid line), the gastric digested hydrolysate (gray solid line) and the gastrointestinal digested hydrolysate (dotted line)
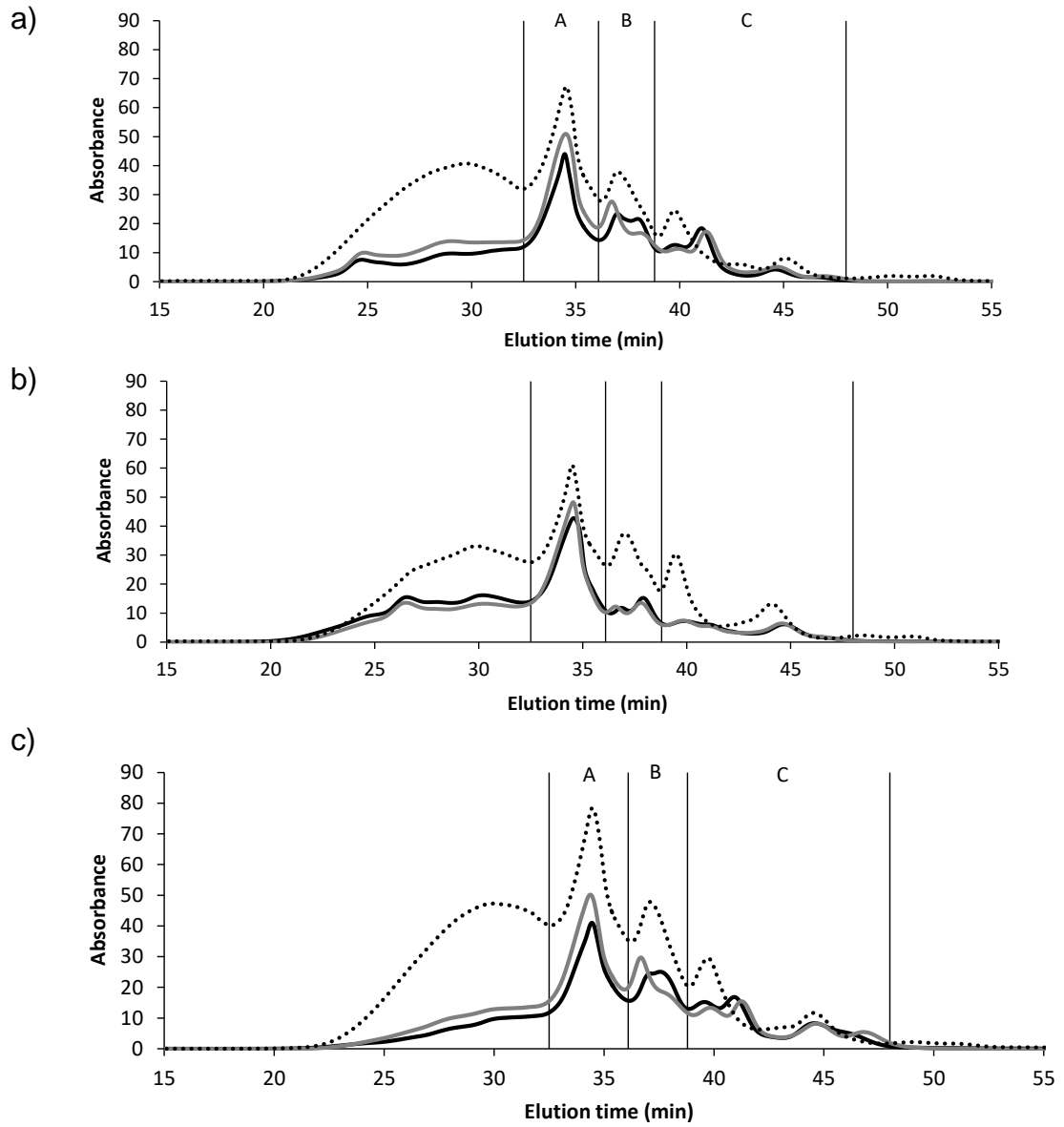
549 FIGURE 1
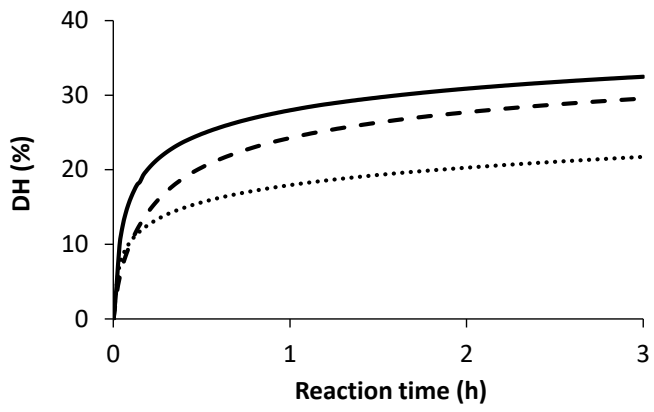
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553　FIGURE 2

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Table 1. Area percentages of the fractions from the goat caseins hydrolysates and their digests.

Hydrolysate	Sample	Percentage of Area		
		Fraction A 900-330 Da	*Fraction B 330-160 Da*	*Fraction C < 160 Da*
SH (subtilisin)	Original	44.9 ± 0.5[ab]	26.7 ± 0.1[a]	28.4 ± 0.6[ab]
	Gastric Digested	50.0 ± 0.2[cd]	23.9 ± 0.1[b]	26.1 ± 0.3[ac]
	Gastrointestinal Digested	50.4 ± 1.1[cd]	25.1 ± 0.5[cd]	26.9 ± 4.0[ac]
TH (trypsin)	Original	56.3 ± 0.6[e]	19.8 ± 0.5[e]	23.9 ± 0.2[ac]
	Gastric Digested	58.2 ± 0.4[e]	18.8 ± 0.7[e]	23.2 ± 1.0[c]
	Gastrointestinal Digested	46.9 ± 3.9[bc]	26.8 ± 0.6[a]	26.3 ± 4.1[ac]
STH (subtilisin +trypsin)	Original	40.9 ± 0.1[a]	26.0 ± 0.4[ad]	33.1 ± 0.3[b]
	Gastric Digested	45.6 ± 2.1[b]	22.8 ± 0.6[f]	32.6 ± 2.0[b]
	Gastrointestinal Digested	51.0 ± 2.2[d]	24.8 ± 0.6[c]	24.2 ± 2.1[ac]

559 Data are means of triplicate determinations ± standard deviation. Equal super index in the same column

560 indicates no statistical differences between the area percentages ($p < 0.05$).

561

562

563   Table 2. ACE inhibitory, antioxidant and bile acid-binding capacities of the original goat caseins

564   hydrolysates and their digests.

Hydrolysate	Sample	ACE inhibitory activity	Antioxidant activity	Bile acid-binding capacity
		IC$_{50}$ (µg mL^{-1})	IC$_{50}$ (mg mL^{-1})	(%)
SH (subtilisin)	Original	243.1 ± 12.6[ab]	4.62 ± 0.15[ab]	22.03 ± 1.76[a]
	Gastric Digested	215.2 ± 10.7[c]	3.26 ± 0.16[ac]	27.18 ± 4.37[ab]
	Gastrointestinal Digested	249.3 ± 13.7[abd]	1.57 ± 0.62[d]	41.74 ± 4.18[c]
TH (trypsin)	Original	269.0 ± 3.2[d]	3.78 ± 0.07[ac]	26.23 ± 5.21[ab]
	Gastric Digested	259.1 ± 17.9[bd]	3.04 ± 0.14[c]	40.73 ± 1.16[c]
	Gastrointestinal Digested	399.8 ± 15.3[e]	1.39 ± 0.56[d]	31.47 ± 1.16[b]
STH (subtilisin +trypsin)	Original	230.7 ± 19.2[ac]	5.51 ± 0.08[b]	21.86 ± 4.36[a]
	Gastric Digested	212.0 ± 12.7[c]	4.33 ± 0.24[ac]	41.43 ± 10.20[c]
	Gastrointestinal Digested	266.9 ± 5.7[d]	1.30 ± 0.22[d]	52.74 ± 6.35[d]

565   Data are means of triplicate determinations ± standard deviation. Equal super index in the same column

566   indicates no statistical differences (p<0.05)

567