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1 **Caseinophosphopeptides released after tryptic hydrolysis *versus* simulated
2 gastrointestinal digestion of a casein-derived by-product**

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11

12 **Abstract**

13 The production of caseinophosphopeptides from a casein-derived by-product generated
14 during the manufacture of a functional ingredient based on antihypertensive peptides
15 was attempted. The casein by-product was submitted to tryptic hydrolysis during 30, 60,
16 and 120 min and further precipitated with calcium chloride and ethanol at pH 4.0, 6.0
17 and 8.0. The identification and semiquantification of the derived products by tandem
18 mass spectrometry allowed to assess some qualitative and quantitative changes in the
19 released caseinophophopeptides over time at the different precipitation pHs. The by-
20 product was also subjected to a simulated gastrointestinal digestion. Comparison of the
21 resulting peptides showed the large sequence homology in the phosphopeptides released
22 by tryptic hydrolysis and gastrointestinal digestion. Some regions, specifically α_{S1}-CN
23 43-59, α_{S1}-CN 60-74, β-CN 1-25, and β-CN 30-50 showed resistance to both tryptic
24 hydrolysis and simulated digestion. These results suggest that this casein-derived by-
25 product can be used as source of CPPs.

26

27

28 **Keywords:** Casein by-product, caseinophosphopeptides, simulated gastrointestinal
29 digestion, tandem mass spectrometry, tryptic hydrolysis

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1. Introduction

Caseinophosphopeptides (CPPs) are phosphorylated bioactive peptides that can be released from casein (CN) by *in vivo* or *in vitro* enzymatic digestion (Bouhallab & Bouglé, 2004). Some of them are characterized by the presence of three phosphoseryl groups followed by two residues of glutamic acid (SpSpSpEE). This motif is localized specifically in the sequences of α_{s1}-CN f(66-70), α_{s2}-CN f(8-12), α_{s2}-CN f(56-60), and β-CN f(17-21). Due to the phosphorylated serines, these regions are relatively resistant to be further hydrolysed and it has been proposed that they could prevent the precipitation of metal ions at alkaline pH in the distal small intestine (Fitzgerald, 1998; Zidane et al., 2012). This property implies that CPPs could be used as supplements for fortifying foods, with a view to improving mineral bioavailability. Different applications of CPPs have been reported such as prevention of osteoporosis, oligoelement supplementation and prevention of dental caries, the last one being the most used therapeutic application (Cross, Huq, O'Brien-Simpson, Perich, Attard, & Reynolds, 2007). In addition, it has been proposed that they may also display immunomodulatory, cytomodulatory and antioxidant activities (Meisel & Fitzgerald, 2003; Phelan, Aherne, Fitzgerald & O´Brien, 2009). Besides, CPPs can exert influence upon gastric secretion regulation (Guilloteau, Romé, Delaby, Mendy, Roger, & Chayvialle, 2009), and antimicrobial effects have been also reported (Arunachalam & Raja, 2010).

CPPs have been released from α_{s1}-, α_{s2}-, β-, and κ-CN subjected to hydrolysis with different enzymes such as trypsin pancreatin, alcalase, plasmin, or combinations of them, such as trypsin and chymotrypsin (Pinto, Caira, Cuollo, Lilla, Chianese & Addeo, 2012). The hydrolysis conditions were different taking into account the pH, time, enzyme:protein ratio and temperature, obtaining thus different sequences. In some

58 studies, the digests or hydrolysates are subjected to an enrichment step to isolate the
59 generated CPPs. Selective precipitation with $CaCl_2$ and ethanol is a usual method using
60 pHs in the range 3.5 to 8.5. It has been reported that at pH 3.5 only peptides containing
61 the phosphorylated cluster sequence SpSpSpEE selectively precipitate from a tryptic
62 digest of casein. However, at pH 4.6, the precipitate contained di- and tri-
63 phosphorylated peptides. At pH 8.0, an increase of the recoveries of the di- and
64 monophosphorylated peptides was observed (Aoki et al., 1998; Reynolds, Riley, &
65 Adamson, 1994; Zhao, Wang, & Zu, 2007). Whether the pH used during precipitation
66 could determine the characteristics of the products recovered remains to be evidenced
67 using a powerful identification technique such as tandem mass spectrometry (MS/MS).

68 Some animal studies have demonstrated the formation of CPPs after the ingestion of
69 CN or its fractions (Kasai, Honda, & Kiriyama, 1992; Meisel & Frister, 1989; Naito &
70 Suzuki, 1974). In humans, CPPs have been identified after the ingestion of milk, yogurt
71 and CN (Boutrou et al., 2013; Chabance et al., 1998). CPPs sharing some amino acid
72 sequences with them have been found using gastro-analogous digestion (pepsin) of β-
73 CN (Schmelzer, Schöps, Reynell, Ulbrich-Hofmann, Neubert, & Raith, 2007) or using
74 simulated gastrointestinal digestion of CN with pepsin and trypsin (Ono, Takagi, &
75 Kunishi, 1998) or pepsin and pancreatin (Miquel, Gómez, Alegría, Barberá, Farré, &
76 Recio, 2006).

77 The production of CPPs for commercial purposes is usually performed from the
78 whole casein fraction, CN or sodium caseinate. The aim of this work was to evaluate if
79 a by-product generated during the manufacture of an antihypertensive hydrolysate from
80 caseins (Contreras et al., 2011), could be used as substrate to obtain CPPs. From this
81 by-product, CPPs were released by tryptic hydrolysis, concentrated by selective
82 precipitation with $CaCl_2$ and ethanol at three pH values (4.0, 6.0 and 8.0), and identified

by HPLC-MS/MS. Moreover, the generation of CPPs was also evaluated after a hydrolysis process that simulates gastrointestinal digestion with the aim to investigate if CPPs could be also released from this CN-derived by-product during passage through the intestinal tract.

2. Materials and methods

2.1 Samples

The CN-derived by-product obtained during the production of a hydrolysate with antihypertensive properties was provided by Innaves, S.A. (Porriño, Spain). The hydrolysate is prepared by pepsin digestion of commercial CN (Promilk 85®) from Arras Cedex (France) as described in Contreras et al. (2011).

2.2 Protein, moisture, and mineral content.

Moisture of commercial CN and CN-derived by-product was determined by drying for 3 h in an oven at 100-105ºC (International Dairy Federation, 1964). Protein amount was determined by the Kjeldahl method (International Dairy Federation, 1993). Mineral composition (Ca, P, K, Mg, and Na) was determined by inductively-coupled plasma emission spectrometry (ICP) using a Perkin Elmer Optima 4300 DV plasma emission spectrometer (Waltham, MA, USA). The analysis were performed in triplicate. Analysis of variance (ANOVA) was carried out using the test of confidence intervals at 95%.

2.3 SDS-PAGE

Samples were diluted to 1 mg/mL in sample buffer containing 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol and heated at 95°C for 4 min. Samples were analysed in

108 duplicate on Precast Criterion XT 12% Bis-Tris gels and electrophoretic separations
109 were carried out at 150 V using XT-MES as running buffer (Bio-Rad, Richmond, CA,
110 USA), in the Criterion cell (Bio-Rad). Gels were stained with Bio-Safe Coomassie G-
111 250 (Bio-Rad).
112
113 *2.4 Preparation of CPPs*
114 *2.4.1 Hydrolysis with trypsin*
115 Ten grams of CN-derived by-product were dissolved in Milli-Q water, and adjusted
116 to pH 8.0 with 5 M NaOH. Food-grade trypsin (Biocatalysts, Nantgarw, UK) was added
117 at final ratio of 2.0% w/w of substrate. Hydrolysis was performed at 50°C for 2 h with
118 the pH maintained in the range 7.7-8.5 by the addition of 1 M NaOH. After hydrolysis,
119 the pH was adjusted to pH 4.6 by the addition of 1 M HCl and insoluble material
120 formed was removed by centrifugation (12000 × *g* for 10 min at 10ºC).
121
122 *2.4.2 Simulated gastrointestinal digestion*
123 CN-derived by-product was subjected to a gastric and duodenal digestion according
124 to Jiménez-Saiz, Martos, Carrillo, López-Fandiño R, and Molina (2011). The enzymes
125 and reagents used in the simulated gastrointestinal digestion were purchased from
126 Sigma-Aldrich (St. Louis, MO, USA). Briefly, the samples were dissolved in simulated
127 gastric fluid, 0.35 M NaCl, mixed with vesicles containing phosphatidylcholine (9.58
128 mg/mL) and the pH was adjusted to 2.0 with 0.5 M HCl. The samples were pre-heated
129 for 15 min at 37ºC, digested with porcine pepsin (EC 3.4.23.1, 3640 U/mg protein) at
130 182 U/mg protein, and incubated for 60 min at 37 ºC. Then gastric digests were adjusted
131 to pH 7.0 by adding 0.5 M $NaHCO_3$.

132 Then, the supernatants of the gastric digests were re-adjusted to pH 6.5 by addition
133 of 0.1 M $NaHCO_3$, 0.125 M bile salts equimolar mixture of sodium taurocholate and
134 sodium glycodeoxycholate and 1 M $CaCl_2$ (7.6 mM final concentration). The samples
135 were pre-heated at 37ºC for 15 min. After, pancreatic porcine lipase (EC 232-619-9,
136 type VI-S, 47900 U/mg protein) at 24.75 U/mg protein, pancreatic porcine colipase (EC
137 259-490-1) at 1:895 w/w protein, pancreatic bovine trypsin (EC 232-650-8, type I
138 10100 U/mg protein) at 34.5 U/mg protein, pancreatic bovine α-chymotrypsin (EC 232-
139 671-2; type I–S; 55 U/mg protein) at 0.44 U/mg protein were added to the duodenal
140 mixture, and incubated at 37 °C for 60 min. Then, the digestion was stopped by heating
141 at 80°C for 5 min.
142
143 *2.5 Isolation of CPPs by selective precipitation*
144 CPPs in tryptic hydrolysate and gastrointestinal digest were precipitated according
145 to Adamson and Reynolds (1995). The pH was adjusted to 4.0, 6.0 or 8.0 and CPPs
146 were precipitated by the addition of 10% (w/v) $CaCl_2$ (20 mol/mol protein) and an equal
147 volume of 99.8% (v/v) ethanol was added slowly with mixing. The suspension was
148 centrifuged (12000 × *g* for 10 min at 10ºC), and the precipitate was washed with 50%
149 (v/v) ethanol, lyophilized and stored at -20ºC until further analysis.
150
151 *2.6 Analysis of CPPs by on-line RP-HPLC–ESI-MS/MS*
152 The analysis of CPPs was carried out on-line by RP-HPLC MS/MS using an
153 Esquire-LC quadrupole ion-trap mass spectrometer (Bruker Daltonics, GmbH, Bremen,
154 Germany) (García-Nebot, Alegría, Barberá, Contreras, & Recio, 2010). The column
155 used was (XBridge TM BEH 300 C18 5µm, 4.6 × 250 mm, Waters), the injection volume
156 was 50 µL, and the flow was set at 0.8 mL/min. The flow was split post UV detector by

157 connecting a T-piece to give a flow of approx. 20 μL/min to be directed to the mass
158 spectrometer nebulizer.
159 Freeze-dried samples (2.5 mg) were dissolved in 1 mL of 0.1% (v/v) trifluoroacetic acid
160 (TFA) (Sigma-Aldrich). Solvent A was a mixture of Milli-Q/TFA (1000:0.37, v/v), and
161 solvent B contained acetonitrile (HPLC grade)/TFA (1000:0.27, v/v). The gradient was
162 60% of solvent B in 90 min, after which the percentage of solvent B increased to 90%
163 in 5 min and remained constant at 90% for 5 min.

164 The *m/z* spectral data were processed and transformed to spectra representing mass
165 values using Data Analysis version 4.0. Bio Tools version 3.2 (Bruker Daltoniks) was
166 used to process the MS(n) spectra and perform peptide sequencing. The semi-
167 quantification of the CPPs was performed by obtaining the extracted ion chromatograms
168 (EIC) and integration of the peak area thereof, after signal normalization to the total
169 peak area.

170

171 **3. Results and discussion**

172 *3.1 Characterization of the commercial casein, by-product and hydrolysed products*

173 Table 1 shows the protein, moisture and mineral content (Ca, P, K, Mg, Na, and Fe)
174 determined in the commercial CN, substrate of the hydrolysis process, and in the
175 corresponding CN-derived by-product. The contents in the commercial CN were similar
176 to those given by the manufacturer. The protein content found in the CN by-product
177 was lower than that of the commercial CN due to the industrial process (Contreras et al.,
178 2011). All mineral content values showed significant ($p < 0.05$) differences between
179 both samples. The K content was higher in the by-product, since food-grade KOH was
180 added during the hydrolysis process, whereas Ca, P, Mg, and Na contents were higher
181 in the commercial CN. The P content of the by-product represented more than 70% of

182 the content in the commercial CN on a protein basis. This led us to consider the by-
183 product a suitable source to obtain CPPs.

184 Figure 1 shows the SDS-PAGE electrophoretic separation of the commercial CN,
185 the CN-derived by-product, the product of its hydrolysis with trypsin during 120 min
186 and after its simulated gastrointestinal digestion. The bands corresponding to the CN
187 were intense in the commercial CN, although bands corresponding to residual serum
188 proteins as β-Lg and α-La could be observed too (Lane 2 in Fig.1). In the CN-derived
189 by-product bands corresponding to α_{s1}-, α_{s2}-, and β-CN were observed, as well as other
190 intense bands of lower molecular weight that corresponded to CN fragments, i.e., CN
191 partly hydrolysed generated by the action of pepsin during the production of the
192 antihypertensive ingredient (Lane 3 in the Fig.1).

193 When the CN-derived by-product was subjected to tryptic hydrolysis small
194 amounts of intact α_{s2}- and β-CN and partly hydrolysed CN were observed (Lane 4 in the
195 Fig. 1). However, other intact CN fractions were not observed, indicating that CN
196 fractions are mostly digested. Similar results were observed in the sample submitted to
197 gastrointestinal digestion, although even less intact CNs could be detected. These
198 results were confirmed when the CN-derived by-product was analyzed by (RP-HPLC)
199 (data not shown).

200

201 *3.2 Preparation of CPPs by hydrolysis with trypsin*

202 The tryptic hydrolysis (30, 60 and 120 min) of the CN-derived by-product was
203 followed by selective precipitation at pH values of 4.0, 6.0 and 8.0 in order to cover the
204 acidic-basic region, giving rise to nine different tryptic hydrolysates. A total of 44 CPPs
205 were identified, of which, 13 corresponded to α_{s1}-CN, 11 to α_{s2}-CN, 18 to β-CN, and
206 two to κ-CN (Table 2). It must be pointed out that 13 of the identified CPPs presented

207 the cluster sequence (SpSpSpEE). They corresponded to α_{s1}-CN f(61-79), α_{s2}-CN f(1-
208 20), α_{s2}-CN f(46-70) and β-CN f(1-25) or fragments thereof. All of the peptides were
209 found in every hydrolysis product, with the exception of α_{s1}-CN (61-79)5P, which was
210 only found in the precipitates at pH 8.0. Only two protein fragments corresponded to κ-
211 CN. This may be due to its low content in bovine milk and to scarcity of phosphoseryl
212 residues in this protein.

213 The identified peptides contained as C-terminal residue the amino acids Gln,
214 Lys, Arg, Asn, Glu, Ile, Ser, Asp, Leu, Tyr, Thr, and Phe. Some of these cleavage sites
215 cannot be explained on the basis of the trypsin specificity, thus being due the
216 chymotrypsin and carboxypeptidases present in the commercial food-grade trypsin and
217 to previous cleavages by the action of pepsin during the production of the
218 antihypertensive hydrolysate. This is in accordance to the non-trypsin-like activity
219 observed by Adamson and Reynolds (1995) in the Na caseinate hydrolysis with an
220 industrial-grade trypsin. Five peptides showed a lower number of phosphorylated
221 residues than maximal number allowed which could be due to incomplete Ser
222 phosphorylation or to the high temperature employed during the by-product drying
223 process. Dephosphorylation of the Ser 46 in α_{s1}-CN f(38-48)1P, Ser 64 and Ser 66 in
224 α_{s1}-CN f(59-71)2P and Ser 15 in β-CN f(1-25)3P or f(8-22)2P had previously been
225 shown in tryptic hydrolysates (Ellegard, Gammelgard-Larsen, Sörensen, & Fedosov,
226 1999; Zhu & Fitzgerald, 2010). Other potential phosphorylated sites that were not
227 phosphorylated in our samples corresponded to Ser 48 in α_{s1}-CN f(38-48)1P, Ser 56 in
228 SpSpSpEE and Ser 17 in f(8-22)2P, although dephosphorylation at these positions by
229 heat treatment had not previously reported.

230 In order to follow the release of CPPs during the tryptic hydrolysis, a relative
231 quantification of the identified sequences was conducted at the hydrolysis times studied.

232 The pH used during the selective precipitation can be a source of qualitative or
233 quantitative changes. Thus, when comparing pH 4.6 and 8.0, it has been reported that
234 relevant amounts of mono and diphosphorylated peptides were obtained predominantly
235 at pH 8.0 (Reynolds et al., 1994; Adamson & Reynolds, 1995). Figure 2A shows the
236 relative abundance of four related (10 common amino acid residues) diphosphorylated
237 peptides arisen from α_{s1}-CN at the three different precipitation pHs used. In agreement
238 with the mentioned studies, the pH 8.0 precipitates showed higher relative amounts than
239 the pH 4.0 and 6.0 precipitates. Conversely, CPPs with more than two phosphorylated
240 residues showed a different behaviour. Figure 2B shows the relative recovery of peptide
241 α_{s2}-CN f(56-68)3P, which presented a higher yield at the pH 6.0 precipitate at all the
242 studied times. When considering the effect of the hydrolysis time, it was observed that
243 this peptide was formed from 30 min and its relative amount decreased at 120 min of
244 tryptic hydrolysis. This reflects its susceptibility to degradation by trypsin in long-term
245 conditions.

246 To study the influence of the hydrolysis time on the amount of a CPP showing the
247 cluster sequence SpSpSpEE, we determined the relative amounts of the well-
248 characterized β-CN f(1-25)4P and four derived peptides with the same C-terminal end
249 in the pH 8.0 precipitates over time (Figure 3). A clear decrease was shown by β-CN
250 f(1-25)4P together with the increase in β-CN f(7-25)4P and f(12-25)4P, showing some
251 susceptibility of the parent peptide to the trypsin activity. In contrast, no relevant
252 changes were observed for β-CN f(2-25)4P and β-CN f(17-25)3P. The importance of
253 the structural conformation conferred by both, the acidic motif SpSpSpEE and the
254 preceding N-terminal portion, in relation to the biological activity is known (Ferraretto,
255 Gravaghi, Fiorilli, & Tettamanti, 2003). From the fragments whose amount is increased
256 in the pH 8.0 precipitates, only β-CN f(7-25)4P shows the N-terminal located amino

257 acids necessary to preserve the activity. This is the peptide showing a more steep
258 increase over time. It is then expected that, despite the decrease of the β-CN f(1-25)4P,
259 the increase in β-CN f(7-25)4P would help to keep the biological activity of the CPP
260 pool after 120 min of hydrolysis. The decrease in β-CN f(1-25)4P is in accordance with
261 the results of Su, Qi, He, Yuan, and Zhang (2007) who identified the same
262 phosphopeptide from 10 min to 1 hour of pancreatin hydrolysis of CN, but not at 2 h.
263 However, these authors could not identify the derived fragments above mentioned.
264

265 *3.3 CPPs released after simulated gastrointestinal digestion*

266 A total of 37 CPPs, of which 13 corresponded to α_{s1}-CN, 8 to α_{s2}-CN, 16 to β-CN
267 and two to κ-CN were identified after simulated gastrointestinal digestion of the CN-
268 derived by-product. Figure 4 shows the comparison of the tryptic and gastrointestinal
269 digestion products in the CN amino acid sequences. By using simulated gastrointestinal
270 digestion phosphorylated peptides belonging to the same domains that those generated
271 by tryptic hydrolysis were found. Furthermore, the pattern of resistant regions to trypsin
272 hydrolysis is very close to that of simulated digestion (90% of the amino acid residues).
273 Only small differences between sequences were found and make that the common CPPs
274 identified in the trypsin hydrolysates and in the simulated gastrointestinal digests
275 represented 73% of the total number of sequenced peptides. Well characterized CPPs
276 such as β-CN f(1-25)4P and, α_{s1}-CN f(43-58)2P and α_{s2}-CN f(56-68)3P and fragments
277 thereof were found using trypsin hydrolysis and simulated digestion.

278 In this regard, similar peptide profile had been shown between the CN and/or β-CN
279 tryptic hydrolysates (*in vitro* conditions) and the intestinal content of rats fed a CN-
280 and/or β-CN based diet (*in vivo* conditions), although Lys and Arg residues were lost in
281 the case of CN and β-CN based diet, respectively, compared to *in vitro* conditions

282 (Naito & Suzuki, 1974; Sato et al., 1991). In addition, the peptide α_{s1}-CN f(66-74)3P
283 was found in the duodenal content of minipigs fed a CN-based diet (Meisel & Frister,
284 1989), which presents the same cleavage than α_{s1}-CN f(61-74)4P and α_{s1}-CN f(62-
285 74)4P, and overlaps with α_{s1}-CN f(59-71)2P found in our digest (Figure 4). These
286 cleavages are also in agreement with those of peptides α_{s1}-CN f(62-69)4P and α_{s1}-CN
287 f(64-73)4P identified by Kasai et al. (1992) in feces of rats fed a CN-based diet. In this
288 context, β-CN f(33-44)4P found in our study was also identified in the human stomach
289 after milk intake after 20 min (Chabance et al., 1998).

290 In jejunal effluents of humans, after CN consumption, Boutrou et al. (2013) found
291 β-CN f(33-48)1P and α_{s1}-CN f(110-119)1P, two sequences found in our study, as well
292 as the fragment α_{s2}-CN f(138-146)1P that overlaps with the peptides α_{s2}-CN f(138-
293 147)1P and α_{s2}-CN f(138-149)1P (Figure 4). These results indicate that the simulated
294 gastrointestinal digestion can release some CPPs similar to those formed *in vivo*, in
295 agreement with the observations reported previously by García-Nebot et al. (2010).
296 These authors reported the resistance of CPPs pool when they were added to fruit
297 beverages and subjected to simulated gastrointestinal digestion. Most of the added CPPs
298 to the fruit beverage were recovered after digestion, although they lacked one or two
299 amino acid residues at the C- or N-terminal end.

300 Picariello et al. (2010) studied the formation of peptides after simulated digestion
301 with pepsin and a mixture of trypsin, chymotrypsin, carboxypeptidase and elastase
302 followed by incubation with extracts of human intestinal brush border membranes. The
303 CPPs enrichment was performed by selective isolation on TiO_2 micro-column. In this
304 study, the α_{s1}-CN region 104-119 was covered by six CPPs, in contrast with the unique
305 peptide α_{s1}-CN f(110-119) found in our samples. However, they reported no peptide in
306 the α_{s1}-CN region 43-59 where numerous CPPs appeared in our samples. In the β-CN

307 sequence, numerous peptides were identified around the β-CN region 30-52 in
308 agreement with our results. However, the N-terminal end was only represented in two
309 β-CN f(1-25) peptides while none of the five derived fragments present in our digested
310 samples were found. This had been also the case in the study of Su et al. (2007), where
311 pancreatin was used and no CPP isolation procedure was applied. In a study on the
312 intestinal brush-border enzymes action on β-CN f(1-25) it has been reported that, in
313 addition to phosphorylation, binding cations might prevent CPP from hydrolysis
314 (Boutrou et al., 2010). Therefore, some of the differences found between studies using
315 *in vitro* hydrolysis could be attributed to the different mineral content of the substrate
316 and the hydrolysis or gastrointestinal digestion conditions applied.

317 In animal studies, Bouhallab et al. (1999) evaluated the sensitivity of β-CN f(1-
318 25)4P to digestive enzymes using a duodenal loop system in rats, and the fragments β-
319 CN f(2-25)4P and β-CN f(7-25)4P were found. Both cleavage sites (Arg_1-Glu_2 and
320 Asn_6-Val_7) have been also found in our study. Likewise, α_{s1}-CN f(59-71)2P, α_{s1}-CN
321 f(61-74)4P and α_{s1}-CN f(62-74)4P identified in our digested sample overlap with the
322 main peptide, α_{s1}-CN f(59-79)5P, found in the ileum of rats after administration by
323 stomach gavage of a CPPs pool (Brommage, Juillerat, & Jost, 1991). In the ileum
324 extract of rats after ingestion of a CPPs preparation, α_{s1}-CN f(61-74)4P and β-CN f(7-
325 24)4P were identified. The first sequence is present in our digest while the second
326 overlaps with β-CN f(7-25)4P (Hirayama, Toyota, Hidaka, & Naito, 1992).

327 The CN regions where a larger amount of peptides both in tryptic hydrolysates and
328 simulated digests has been identified are α_{s1}-CN 43-59, β-CN 1-25 and β-CN 30-43.
329 These regions are characterized by a high hydrophobicity at pH 3.0 (gastric conditions)
330 and/or the presence of post-translational modifications, parameters that have already
331 been identified as playing a key role in the resistance of CN to *in vitro* digestion

332 (Dupont et al., 2010). These facts suggest the resistance of these peptides in the case of
333 the CN-derived by-product being ingested.
334

335 **4. Conclusions**

336 The results in this study show that the partially hydrolysed CN fraction obtained as a
337 by-product during the production of an antihypertensive hydrolysate can be further
338 exploited as source of CPPs. The identification and semiquantitative determination of
339 CPPs has shown that the selection of the tryptic hydrolysis time and precipitation pH
340 can modulate the recovery of specific phosphorylated sequences. This can be important
341 in view of selecting the conditions where the length of the N-terminal portion preceding
342 the phosphoserine cluster SpSpSpEE in the CPP is enough to keep the biological
343 activity. Identification of CPPs in the samples of CN-derived by-product after being
344 subjected to simulated gastrointestinal digestion (aiming to mimic what would occur in
345 an *in vivo* digestion) has shown that this process generates CPP sequences very similar
346 to those produced by tryptic hydrolysis. These results suggest that this CN-derived by-
347 product can be used as a source for CPPs after its passage through the gastrointestinal
348 tract opening the door for its use as functional ingredient to improve mineral
349 bioavailability. Because the phosphorylated regions of CN are resistant to hydrolysis, it
350 is likely that many of the by-products or insoluble fractions generated during milk
351 protein hydrolysis, for instance, in the manufacture of active hydrolysates or hydrolysed
352 infant formulas, could be further exploited as CPPs sources.
353

354 **Acknowledgments**

355 This work has received financial support from projects AGL2011-24643, FEDER-
356 INNTERCONECTA-GALICIA (ENVELLEFUN), and FP7-SME-2012-315349

357 (FOFIND). The authors are participants in the FA1005 COST Action INFOGEST on
358 food digestion. E. C-H. thanks the University of Veracruz, México, scholarship
359 PROMEP/103.5/13/6408 for the support for PhD studies abroad. The authors thank B.
360 Hernández-Ledesma for reviewing the article.

361

362 **References**

363 Adamson, N. J. & Reynolds, E. C. (1995). Characterization of tryptic casein
364 phosphopeptides prepared under industrially relevant conditions. *Biotechnology and*
365 *Bioengineering, 45,* 196-204.

366 Aoki, T., Nakano, T., Iwashita, T., Sugimoto, Y., Ibrahim, H. R., Toba, Y., Aoe, S., &
367 Nakajima, I. (1998). Preparation and characterization of micellar calcium phosphate-
368 casein phosphopeptide complex. *Journal of Nutritional Science and Vitaminology,*
369 *44,* 447-456.

370 Arunachalam, K., & Raja, R. B. (2010). Isolation and characterization of CPP (casein
371 phosphopeptides) from fermented milk. *African Journal of Food Science, 4*, 167-
372 175.

373 Bouhallab, S., Aït-Oukhatar, N. A., Mollé, D., Henry, G., Maubois, J. L., Arhan, P., &
374 Bouglé, D. L. (1999). Sensitivity of beta-casein phosphopeptide-iron complex to
375 digestive enzymes in ligated segment of rat duodenum. *The Journal of Nutritional*
376 *Biochemistry, 10,* 723-727.

377 Bouhallab, S., & Bouglé, D. (2004). Biopeptides of milk: Caseinophosphopeptides and
378 mineral bioavailability. *Reproduction Nutrition Development, 44,* 493-498.

379 Boutrou, R., Coirre, E., Jardin, J., & Léonil, J. (2010). Phosphorylation and
380 coordination bond of mineral inhibit the hydrolysis of the β-casein (1-25) peptide by

381 intestinal brush-border membrane enzymes. J*ournal of Agricultural and Food*
382 *Chemistry, 58*, 7955-7961.
383 Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A.,
384 Nenamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein-
385 derived bioactive peptides in the jejunum in healthy human. *American Journal of*
386 *Clinical Nutrition, 97,* 1314-1323.
387 Brommage, R., Juillerat, M. A., & Jost, R. (1991). Influence of casein phosphopeptides
388 and lactulose on intestinal calcium-absorption in adult female rats. *Lait, 71,* 173-180.
389 Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M.,
390 Perrotin, P., Guillet, R., Jolles, P., & Fiat, A. M. (1998). Casein peptide release and
391 passage to the blood in humans during digestion of milk or yogurt. *Biochimie, 80,*
392 155-65.
393 Contreras, M. d. M., Sevilla, M. A., Monroy-Ruiz, J., Amigo, L., Gómez-Sala, B.,
394 Molina, E., Ramos, M., & Recio, I. (2011). Food-grade production of an
395 antihypertensive casein hydrolysate and resistance of active peptides to drying and
396 storage. *International Dairy Journal*, *21*(7), 470-476.
397 Cross, K. J., Huq, N. L., O'Brien-Simpson, N. M., Perich, J. W., Attard, T. J., &
398 Reynolds, E. C. (2007). The Role of Multiphosphorylated Peptides in Mineralized
399 Tissue Regeneration. *International Journal of Peptide Research and Therapeutics*,
400 *13*(4), 479-495.
401 Dupont, D., Mandalari, G., Molle, D., Jardin, J., Léonil, J., Faulks, R. M., Wickham, M.
402 S. J., Clare Mills, E. N., & Mackie, A. R. (2010). Comparative resistance of food
403 proteins to adult and infant in vitro digestion models. *Molecular Nutrition & Food*
404 *Research*, *54*(6), 767-780.

405 Ellegard, K. H., Gammelgard-Larsen, C., Sörensen, E. S., & Fedosov, S. (1999).
406 Process scale chromatographic isolation, characterization and identification of tryptic
407 bioactive casein phosphopeptides. *International Dairy Journal*, *9*, 639-652.
408 Ferraretto, A., Gravaghi, C., Fiorilli, A., & Tettamanti, G. (2003). Casein-derived
409 bioactive phosphopeptides: role of phosphorylation and primary structure in
410 promoting calcium uptake by HT-29 tumor cells. *FEBS Letters*, *551*(1-3) 92-98.
411 Fitzgerald, R. J. (1998). Potential uses of caseinophosphopeptides. *International Dairy*
412 *Journal, 8,* 451-457.
413 García-Nebot, M. J., Alegría, A., Barberá, R., Contreras, M. M., & Recio, I. (2010).
414 Milk versus caseinophosphopeptides added to fruit beverage: Resistance and release
415 from simulated gastrointestinal digestion. *Peptides*, *31,* 555-561
416 Guilloteau P., Romé V., Delaby L., Mendy F., Roger L., & Chayvialle J. A. (2009).
417 New role of phosphopeptides as bioactive peptides released during milk casein
418 digestion in the young mammal: Regulation of gastric secretion. *Peptides, 30*, 2221 -
419 2227.
420 Hirayama, M., Toyota, K., Hidaka, H., & Naito, H. (1992). Phosphopeptides in rat
421 intestinal digests after ingesting casein phosphopeptides. *Bioscience, Biotechnology*
422 *and Biochemistry, 56,* 1128-9.
423 International Dairy Federation Determination of moisture content, Method 26 (1964).
424 Joint IDF/ISO/AOAC International Standard IDF, Brussels, Belgium.
425 International Dairy Federation Determination of nitrogen content, Kjeldalh method
426 20B, (1993). Joint IDF/ISO/AOAC International Standard IDF, Brussels, Belgium.
427 Jiménez-Saiz R., Martos, G., Carrillo W., López-Fandiño R., & Molina E. (2011).
428 Susceptibility of lysozyme to in-vitro digestion and immunoreactivity of its digests.
429 *Food Chemistry, 127*, 1719-1726.

430 Kasai T., Honda T., & Kiriyama S. (1992). Caseinphosphopeptides (CPP) in feces of
431 rats fed casein diet. *Bioscience, Biotechnology and Biochemistry, 56*, 1150 -1151.
432 Meisel, H., & Fitzgerald, R. J. (2003). Biofunctional peptides from milk proteins:
433 Mineral binding and cytomodulatory effects. *Current Pharmaceutical Design, 9,*
434 1289-1295.
435 Meisel, H., & Frister, H. (1989). Chemical characterization of bioactive peptides from
436 *in vivo* digests of casein. *Journal of Dairy Research, 56,* 343-349.
437 Miquel, E., Gómez, J. A., Alegría, A., Barberá, R., Farré, R., & Recio, I. (2006).
438 Identification of casein phosphopeptides after simulated gastrointestinal digestion by
439 tandem mass spectrometry. *European Food Research & Technology, 222,* 48-53.
440 Naito, H. & Suzuki, H. (1974). Further evidence for formation *in vivo* of
441 phosphopeptide in intestinal lumen from dietary beta-casein. *Agricultural and*
442 *Biological Chemistry, 38,* 1543-1545.
443 Ono, T., Takagi, Y., & Kunishi, I. (1998). Casein phosphopeptides from casein micelles
444 by successive digestion with pepsin and trypsin. *Bioscience, Biotechnology and*
445 *Biochemistry, 62,* 16-21.
446 Phelan, M., Aherne, A., Fitzgerald, R., & O´Brien, N. (2009). Casein-derived bioactive
447 peptides: Biological effects, industrial uses, safety aspects and regulatory status.
448 *International Dairy Journal, 19,* 643-654.
449 Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S.,
450 & Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of
451 milk proteins: Biological and toxicological implications. *Journal Chromatography*
452 *B, 878*, 295-308.

453 Pinto, G., Caira, S., Cuollo, M., Lilla, S., Chianese, L, & Addeo, F. (2012). Bioactive
454 casein phospopeptides in dairy products as nutraceuticals for functional foods. In
455 W.L. Hurley, *Milk Protein*. Chapter 1 (pp. 3-44). Croatia: InTech Open Science.
456 Reynolds, E. C., Riley, P. F., & Adamson, N. J. (1994). A selective precipitation
457 purification procedure for multiple phosphoseryl-containing peptides and methods
458 for their identification. *Analytical Biochemistry, 217,* 277-284.
459 Sato, R., Shindo, M., Gunshin, H., Noguchi, T., & Naito, H. (1991). Characterization of
460 phosphopeptide derived from bovine beta-casein - an inhibitor to intra-intestinal
461 precipitation of calcium-phosphate. *Biochimica et Biophysica Acta, 1077,* 413-415.
462 Schmelzer, C. E. H., Schöps, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R. H. H.,
463 & Raith, K. (2007). Peptic digestion of beta-casein time course and fate of possible
464 bioactive peptides. *Journal of Chromatography A, 1166,* 108-115.
465 Su, R., Qi, W., He, Z., Yuan, S., & Zhang, Y. (2007). Pancreatic hydrolysis of bovine
466 casein: Identification and release kinetics of phosphopeptides. *Food Chemistry, 104,*
467 276-286.
468 Zhao, L., Wang, Z., & Xu, S. Y. (2007). Preparation of casein phosphorylated peptides
469 and casein non-phosphorylated peptides using alcalase. *European Food Research*
470 *and. Technology*, *225,* 579-584.
471 Zhu, Y. S., & Fitzgerald, R. J. (2010). Direct nanoHPLC-ESI-QTOF MS/MS analysis
472 of tryptic caseinophosphopeptides. *Food Chemistry, 123*, 753-759.
473 Zidane, F., Aurélie, M., Cakir-Kiefer, C., Miclo, L., Rahuel-Clermont, S., Girardet J., &
474 Corbier C. (2012). Binding of divalent metal ions to 1-25 β-caseinophosphopeptide:
475 An isothermal titration calorimetry study. *Food Chemistry, 132*, 391-398.

476 **Figure captions**

477 Figure 1. SDS-PAGE analysis of commercial casein, casein-derived by-product and the
478 product of its hydrolysis with trypsin or simulated gastrointestinal digestion under
479 reducing conditions. Lane 1: Molecular marker; Lane 2: Commercial casein; Lane 3:
480 Casein derived by-product; Lane 4: Casein-derived by product hydrolyzed with trypsin
481 for 120 min; Lane 5: Casein derived by-product subjected to simulated gastrointestinal
482 digestion.

483

484 Figure 2. Relative amount of A) Peptides f(43-52), f(43-55,) f(43-56) and f(43-59) from
485 α_{s1}-CN at 120 min hydrolysis time and at the precipitation pHs 4.0, 6.0 and 8.0 and B)
486 α_{s2}-CN f(56-68)3P hydrolysis times 30, 60, and 120 min at the precipitation pHs 4.0,
487 6.0 and 8.0.

488

489 Figure 3. Relative amount of β-CN f(1-25)4P, f(7-25)4P, f(12-25)4P, f(17-25)3P and
490 f(2-25)4P after 30, 60 and 120 min of tryptic hydrolysis with selective precipitation at
491 pH 8.0.

492

493 Figure 4. Caseinophosphopeptides sequences identified from casein derived by-product
494 subjected to hydrolysis with trypsin (solid line) and after simulated gastrointestinal
495 digestion (dotted line). $\mathbf{S_p}$ Indicates Phosphoserine; $\mathbf{S_PS_PS_PEE}$ Indicates cluster
496 sequence; * Indicates Serine residue without phosphate.

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498

499

500 Table 1. Moisture, protein and mineral content of casein and casein-derived by-product

501 (expressed as %, w/w)

Component	Casein	Casein-derived by-product
Moisture	5.022 ± 0.010	4.999 ± 0.020
Protein	79.960 ± 0.152	71.052 ± 0.339*
Ca	2.506 ± 0.437	0.418 ± 0.027*
P	1.144 ± 0.030	0.788 ± 0.040*
K	0.362 ± 0.014	0.478 ± 0.034*
Mg	0.092 ± 0.003	0.016 ± 0.001*
Na	0.066 ± 0.004	0.022 ± 0.001*

*indicates significant differences ($p<0.05$). Data are expressed as mean ± standard deviation ($n = 3$)

Table 2. Caseinophopshopeptides identified in the CN-derived by product subjected to hydrolysis with trypsin. Observed mass, calculated mass and ion (m/z) selected for MS/MS analysis, where the charge of the ion is given between brackets.

Protein fragment	Amino acid sequences	Observed mass	Calculated mass	Ion m/z (charge)
α_{s1}-CN f(28-42)1P	FPEVFGKEKVNEL**Sp**K	1828.9	1829.8	1829.9(+1)
α_{s1}-CN f(38-48)1P	NEL**Sp**KDIGSES	1258.5	1257.5	1259.5(+1)
α_{s1}-CN f(43-52)2P	DIG**Sp**E**Sp**TEDQ	1239.4	1239.3	1240.4(+1)
α_{s1}-CN f(43-55)2P	DIG**Sp**E**Sp**TEDQAME	1570.6	1570.4	1571.6(+1)
α_{s1}-CN f(43-56)2P	DIG**Sp**E**Sp**TEDQAMED	1685.6	1685.5	1686.6(+1)
α_{s1}-CN f(43-58)2P	DIG**Sp**E**Sp**TEDQAMEDIK	1926.7	1926.6	1927.7(+1)
α_{s1}-CN f(43-59)2P	DIG**Sp**E**Sp**TEDQAMEDIKQ	2054.8	2054.7	2055.8(+1)
α_{s1}-CN f(44-59)2P	IG**Sp**E**Sp**TEDQAMEDIKQ	1940.8	1939.7	1941.8(+1)
α_{s1}-CN f(59-71)2P	QMEAESIS**SpSp**EEI	1598.6	1598.5	1599.6(+1)
α_{s1}-CN f(61-74)4P	EAE**SpISpSpSpEE**IVPN	1808.9	1809.5	1809.9(+1)
α_{s1}-CN f(61-79)5P*	EAE**SpISpSpSpEE**IVPN**Sp**VEQK	2462.0	2460.8	1232.0(+2)
α_{s1}-CN f(62-74)4P	AE**SpISpSpSpEE**IVPN	1680.8	1680.5	1681.8(+1)
α_{s1}-CN f(110-119)1P	EIVPN**Sp**AEER	1222.6	1222.5	1223.6(+1)
α_{s2}-CN f(1-20)4P	KNTMEHV**SpSpSpEE**SII**Sp**QETY	2618.0	2617.8	1310.0(+2)
α_{s2}-CN f(37-58)3P	STFCKEVVRNANEEEYSIG**SpSpSp**	2678.2	2677.0	1340.1(+2)
α_{s2}-CN f(46-70)4P	NANEEEYSIG**SpSpSpEESp**AEVATEEVK	3008.2	3007.0	1505.1(+2)
α_{s2}-CN f(53-70)4P	SIG**SpSpSpEESp**AEVATEEVK	2157.7	2157.7	2158.7(+1)
α_{s2}-CN f(56-68)3P	S**SpSp**EE**Sp**AEVATEE	1592.5	1593.4	1593.5(+1)
α_{s2}-CN f(117-132)2P	VPITPTLNREQL**Sp**T**Sp**E	1942.7	1943.8	1943.7(+1)
α_{s2}-CN f(126-136)2P	EQL**Sp**T**Sp**EENSK	1410.5	1410.4	1411.5(+1)
α_{s2}-CN f(126-137)2P	EQL**Sp**T**Sp**EENSKK	1538.7	1538.5	1539.7(+1)
α_{s2}-CN f(138-147)1P	TVDME**Sp**TEVF	1236.5	1236.4	1237.5(+1)
α_{s2}-CN f(138-149)1P	TVDME**Sp**TEVFTK	1465.6	1465.6	1466.6(+1)
α_{s2}-CN f(138-150)1P	TVDME**Sp**TEVFTKK	1593.7	1593.7	1594.7(+1)
β-CN f(1-25)4P	RELEELNVPGEIVE**Sp**L**SpSpSpEE**SITR	3122.4	3121.2	1562.2(+2)
β-CN f(1-25)3P	RELEELNVPGEIVESL**SpSpSpEE**SITR	3042.4	3041.2	1522.2(+2)
β-CN f(2-25)4P	RELEELNVPGEIVE**Sp**L**SpSpSpEE**SITR	2966.4	2965.1	1484.2(+2)
β-CN f(2-18)3P	ELEELNVPGEIVE**Sp**L**SpSp**	2082.8	2082.8	2083.8(+1)
β-CN f(4-25)4P	EELNVPGEIVE**Sp**L**SpSpSpEE**SITR	2724.2	2723.0	1363.1(+2)
β-CN f(7-25)4P	NVPGEIVE**Sp**L**SpSpSpEE**SITR	2352.0	2351.8	2353.0(+1)
β-CN f(8-22)2P	VPGEIVESLS**SpSp**EES	1707.5	1707.6	1708.5(+1)
β-CN f(12-25)4P	IVE**Sp**L**SpSpSpEE**SITR	1855.7	1855.6	1856.7(+1)
β-CN f(17-25)3P	**SpSpSpEE**SITR	1234.4	1234.3	1235.4(+1)
β-CN f(30-44)1P	IEKFQ**Sp**EEQQQTEDE	1946.9	1946.7	1947.9(+1)
β-CN f(30-48)1P	IEKFQ**Sp**EEQQQTEDELQDK	2432.2	2431.0	1217.1(+2)
β-CN f(31-46)1P	EKFQ**Sp**EEQQQTEDELQ	2074.7	2074.8	2075.7(+1)
β-CN f(33-44)1P	FQ**Sp**EEQQQTEDE	1576.7	1576.5	1577.7(+1)
β-CN f(33-45)1P	FQ**Sp**EEQQQTEDEL	1689.7	1689.6	1690.7(+1)
β-CN f(33-46)1P	FQ**Sp**EEQQQTEDELQ	1817.8	1817.6	1818.8(+1)
β-CN f(33-47)1P	FQ**Sp**EEQQQTEDELQD	1932.8	1932.7	1933.8(+1)
β-CN f(33-48)1P	FQ**Sp**EEQQQTEDELQDK	2060.9	2060.8	2061.9(+1)
β-CN f(33-52)1P	FQ**Sp**EEQQQTEDELQDKIHPF	2556.2	2555.0	1279.1(+2)
k-CN f(147-161)1P	ED**Sp**PEVIESPPEINT	1734.8	1734.7	1735.8(+1)
k-CN f(146-161)1P	LED**Sp**PEVIESPPEINT	1847.9	1847.8	1848.9(+1)

Sp: phosphoserine residue; **SpSpSpEE:** Cluster Sequence. *indicates that this phosphopeptide only has been identified in the pH 8 precipitate.

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