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

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Abstract

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

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

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 phosphoserine 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

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

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

The production of CPPs for commercial purposes is usually performed from the whole casein fraction, CN or sodium caseinate. The aim of this work was to evaluate if a by-product generated during the manufacture of an antihypertensive hydrolysate from caseins (Contreras et al., 2011), could be used as substrate to obtain CPPs. From this by-product, CPPs were released by tryptic hydrolysis, concentrated by selective 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 100-105°C (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

duplicate on Precast Criterion XT 12% Bis-Tris gels and electrophoretic separations were carried out at 150 V using XT-MES as running buffer (Bio-Rad, Richmond, CA, USA), in the Criterion cell (Bio-Rad). Gels were stained with Bio-Safe Coomassie G-250 (Bio-Rad).

2.4 Preparation of CPPs

2.4.1 Hydrolysis with trypsin

Ten grams of CN-derived by-product were dissolved in Milli-Q water, and adjusted to pH 8.0 with 5 M NaOH. Food-grade trypsin (Biocatalysts, Nantgarw, UK) was added at final ratio of 2.0% w/w of substrate. Hydrolysis was performed at 50°C for 2 h with the pH maintained in the range 7.7-8.5 by the addition of 1 M NaOH. After hydrolysis, the pH was adjusted to pH 4.6 by the addition of 1 M HCl and insoluble material formed was removed by centrifugation ($12000 \times g$ for 10 min at 10°C).

2.4.2 Simulated gastrointestinal digestion

CN-derived by-product was subjected to a gastric and duodenal digestion according to Jiménez-Saiz, Martos, Carrillo, López-Fandiño R, and Molina (2011). The enzymes and reagents used in the simulated gastrointestinal digestion were purchased from Sigma-Aldrich (St. Louis, MO, USA). Briefly, the samples were dissolved in simulated gastric fluid, 0.35 M NaCl, mixed with vesicles containing phosphatidylcholine (9.58 mg/mL) and the pH was adjusted to 2.0 with 0.5 M HCl. The samples were pre-heated for 15 min at 37°C, digested with porcine pepsin (EC 3.4.23.1, 3640 U/mg protein) at 182 U/mg protein, and incubated for 60 min at 37 °C. Then gastric digests were adjusted to pH 7.0 by adding 0.5 M NaHCO₃.

Then, the supernatants of the gastric digests were re-adjusted to pH 6.5 by addition of 0.1 M NaHCO₃, 0.125 M bile salts equimolar mixture of sodium taurocholate and sodium glycodeoxycholate and 1 M CaCl₂ (7.6 mM final concentration). The samples were pre-heated at 37°C for 15 min. After, pancreatic porcine lipase (EC 232-619-9, type VI-S, 47900 U/mg protein) at 24.75 U/mg protein, pancreatic porcine colipase (EC 259-490-1) at 1:895 w/w protein, pancreatic bovine trypsin (EC 232-650-8, type I 10100 U/mg protein) at 34.5 U/mg protein, pancreatic bovine α -chymotrypsin (EC 232-671-2; type I-S; 55 U/mg protein) at 0.44 U/mg protein were added to the duodenal mixture, and incubated at 37 °C for 60 min. Then, the digestion was stopped by heating at 80°C for 5 min.

2.5 Isolation of CPPs by selective precipitation

CPPs in tryptic hydrolysate and gastrointestinal digest were precipitated according to Adamson and Reynolds (1995). The pH was adjusted to 4.0, 6.0 or 8.0 and CPPs were precipitated by the addition of 10% (w/v) CaCl₂ (20 mol/mol protein) and an equal volume of 99.8% (v/v) ethanol was added slowly with mixing. The suspension was centrifuged (12000 $\times$ g for 10 min at 10°C), and the precipitate was washed with 50% (v/v) ethanol, lyophilized and stored at -20°C until further analysis.

2.6 Analysis of CPPs by on-line RP-HPLC–ESI-MS/MS

The analysis of CPPs was carried out on-line by RP-HPLC MS/MS using an Esquire-LC quadrupole ion-trap mass spectrometer (Bruker Daltonics, GmbH, Bremen, Germany) (García-Nebot, Alegría, Barberá, Contreras, & Recio, 2010). The column used was (XBridgeTM BEH 300 C18 5 μ m, 4.6 $\times$ 250 mm, Waters), the injection volume was 50 μ L, and the flow was set at 0.8 mL/min. The flow was split post UV detector by

connecting a T-piece to give a flow of approx. 20 μ L/min to be directed to the mass spectrometer nebulizer.

Freeze-dried samples (2.5 mg) were dissolved in 1 mL of 0.1% (v/v) trifluoroacetic acid (TFA) (Sigma-Aldrich). Solvent A was a mixture of Milli-Q/TFA (1000:0.37, v/v), and solvent B contained acetonitrile (HPLC grade)/TFA (1000:0.27, v/v). The gradient was 60% of solvent B in 90 min, after which the percentage of solvent B increased to 90% in 5 min and remained constant at 90% for 5 min.

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

3. Results and discussion

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

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

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

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

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

3.2 Preparation of CPPs by hydrolysis with trypsin

The tryptic hydrolysis (30, 60 and 120 min) of the CN-derived by-product was followed by selective precipitation at pH values of 4.0, 6.0 and 8.0 in order to cover the acidic-basic region, giving rise to nine different tryptic hydrolysates. A total of 44 CPPs were identified, of which, 13 corresponded to α_{s1} -CN, 11 to α_{s2} -CN, 18 to β -CN, and 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, Sørensen, & 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.

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

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

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

3.3 CPPs released after simulated gastrointestinal digestion

A total of 37 CPPs, of which 13 corresponded to α_{s1} -CN, 8 to α_{s2} -CN, 16 to β -CN and two to κ -CN were identified after simulated gastrointestinal digestion of the CN-derived by-product. Figure 4 shows the comparison of the tryptic and gastrointestinal digestion products in the CN amino acid sequences. By using simulated gastrointestinal digestion phosphorylated peptides belonging to the same domains that those generated by tryptic hydrolysis were found. Furthermore, the pattern of resistant regions to trypsin hydrolysis is very close to that of simulated digestion (90% of the amino acid residues). Only small differences between sequences were found and make that the common CPPs identified in the trypsin hydrolysates and in the simulated gastrointestinal digests represented 73% of the total number of sequenced peptides. Well characterized CPPs 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 thereof were found using trypsin hydrolysis and simulated digestion.

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

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

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

Picariello et al. (2010) studied the formation of peptides after simulated digestion with pepsin and a mixture of trypsin, chymotrypsin, carboxypeptidase and elastase followed by incubation with extracts of human intestinal brush border membranes. The CPPs enrichment was performed by selective isolation on TiO₂ micro-column. In this study, the α_{s1} -CN region 104-119 was covered by six CPPs, in contrast with the unique peptide α_{s1} -CN f(110-119) found in our samples. However, they reported no peptide in the α_{s1} -CN region 43-59 where numerous CPPs appeared in our samples. In the β -CN

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

In animal studies, Bouhallab et al. (1999) evaluated the sensitivity of β -CN f(1-25)4P to digestive enzymes using a duodenal loop system in rats, and the fragments β -CN f(2-25)4P and β -CN f(7-25)4P were found. Both cleavage sites (Arg₁-Glu₂ and Asn₆-Val₇) have been also found in our study. Likewise, α_{s1} -CN f(59-71)2P, α_{s1} -CN f(61-74)4P and α_{s1} -CN f(62-74)4P identified in our digested sample overlap with the main peptide, α_{s1} -CN f(59-79)5P, found in the ileum of rats after administration by stomach gavage of a CPPs pool (Brommage, Juillerat, & Jost, 1991). In the ileum extract of rats after ingestion of a CPPs preparation, α_{s1} -CN f(61-74)4P and β -CN f(7-24)4P were identified. The first sequence is present in our digest while the second overlaps with β -CN f(7-25)4P (Hirayama, Toyota, Hidaka, & Naito, 1992).

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

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

4. Conclusions

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

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Figure captions

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

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

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

Figure 4. Caseinophosphopeptides sequences identified from casein derived by-product subjected to hydrolysis with trypsin (solid line) and after simulated gastrointestinal digestion (dotted line). **S_p** Indicates Phosphoserine; **S_pS_pS_pEE** Indicates cluster sequence; * Indicates Serine residue without phosphate.

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. Caseinophosphopeptides 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 SpK	1828.9	1829.8	1829.9(+1)
α_{s1} -CN f(38-48)1P	NEL SpK DIGSES	1258.5	1257.5	1259.5(+1)
α_{s1} -CN f(43-52)2P	DIG SpESp TEDQ	1239.4	1239.3	1240.4(+1)
α_{s1} -CN f(43-55)2P	DIG SpESp TEDQAME	1570.6	1570.4	1571.6(+1)
α_{s1} -CN f(43-56)2P	DIG SpESp TEDQAMED	1685.6	1685.5	1686.6(+1)
α_{s1} -CN f(43-58)2P	DIG SpESp TEDQAMEDIK	1926.7	1926.6	1927.7(+1)
α_{s1} -CN f(43-59)2P	DIG SpESp TEDQAMEDIKQ	2054.8	2054.7	2055.8(+1)
α_{s1} -CN f(44-59)2P	IG SpESp TEDQAMEDIKQ	1940.8	1939.7	1941.8(+1)
α_{s1} -CN f(59-71)2P	QMEAESISS SpSpEEI	1598.6	1598.5	1599.6(+1)
α_{s1} -CN f(61-74)4P	EAES SpISpSpSpEEI VPN	1808.9	1809.5	1809.9(+1)
α_{s1} -CN f(61-79)5P*	EAES SpISpSpSpEEI VPNS SpVEQK	2462.0	2460.8	1232.0(+2)
α_{s1} -CN f(62-74)4P	AE SpISpSpSpEEI VPN	1680.8	1680.5	1681.8(+1)
α_{s1} -CN f(110-119)1P	EIVPN SpAEER	1222.6	1222.5	1223.6(+1)
α_{s2} -CN f(1-20)4P	KNTMEHV SpSpSpEESIISpQETY	2618.0	2617.8	1310.0(+2)
α_{s2} -CN f(37-58)3P	STFCKEVVRNANEEEEYSIG SpSpSp	2678.2	2677.0	1340.1(+2)
α_{s2} -CN f(46-70)4P	NANEEEEYSIG 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 SpSpEESp AEVATEE	1592.5	1593.4	1593.5(+1)
α_{s2} -CN f(117-132)2P	VPITPTLNREQL SpTSpe	1942.7	1943.8	1943.7(+1)
α_{s2} -CN f(126-136)2P	EQL SpTSpe ENSK	1410.5	1410.4	1411.5(+1)
α_{s2} -CN f(126-137)2P	EQL SpTSpe ENSKK	1538.7	1538.5	1539.7(+1)
α_{s2} -CN f(138-147)1P	TVDMES SpTEVF	1236.5	1236.4	1237.5(+1)
α_{s2} -CN f(138-149)1P	TVDMES SpTEVFTK	1465.6	1465.6	1466.6(+1)
α_{s2} -CN f(138-150)1P	TVDMES SpTEVFTKK	1593.7	1593.7	1594.7(+1)
β -CN f(1-25)4P	RELEELNVPGEIVES SpLSpSpSpEESITR	3122.4	3121.2	1562.2(+2)
β -CN f(1-25)3P	RELEELNVPGEIVES SpLSpSpSpEESITR	3042.4	3041.2	1522.2(+2)
β -CN f(2-25)4P	RELEELNVPGEIVES SpLSpSpSpEESITR	2966.4	2965.1	1484.2(+2)
β -CN f(2-18)3P	ELEELNVPGEIVES SpLSpSp	2082.8	2082.8	2083.8(+1)
β -CN f(4-25)4P	EELNVPGEIVES SpLSpSpSpEESITR	2724.2	2723.0	1363.1(+2)
β -CN f(7-25)4P	NVPGEIVES SpLSpSpSpEESITR	2352.0	2351.8	2353.0(+1)
β -CN f(8-22)2P	VPGEIVESL SpSpEES	1707.5	1707.6	1708.5(+1)
β -CN f(12-25)4P	IVES SpLSpSpSpEESITR	1855.7	1855.6	1856.7(+1)
β -CN f(17-25)3P	SpSpSpEESITR	1234.4	1234.3	1235.4(+1)
β -CN f(30-44)1P	IEKFQ SpEEQQQTEDE	1946.9	1946.7	1947.9(+1)
β -CN f(30-48)1P	IEKFQ SpEEQQQTEDELQDK	2432.2	2431.0	1217.1(+2)
β -CN f(31-46)1P	EKFQ SpEEQQQTEDELQ	2074.7	2074.8	2075.7(+1)
β -CN f(33-44)1P	FQ SpEEQQQTEDE	1576.7	1576.5	1577.7(+1)
β -CN f(33-45)1P	FQ SpEEQQQTEDEL	1689.7	1689.6	1690.7(+1)
β -CN f(33-46)1P	FQ SpEEQQQTEDELQ	1817.8	1817.6	1818.8(+1)
β -CN f(33-47)1P	FQ SpEEQQQTEDELQD	1932.8	1932.7	1933.8(+1)
β -CN f(33-48)1P	FQ SpEEQQQTEDELQDK	2060.9	2060.8	2061.9(+1)
β -CN f(33-52)1P	FQ SpEEQQQTEDELQDKIHFP	2556.2	2555.0	1279.1(+2)
k-CN f(147-161)1P	ED SpPEVIESPPEINT	1734.8	1734.7	1735.8(+1)
k-CN f(146-161)1P	LED SpPEVIESPPEINT	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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