Food Chemistry 168 (2015) 648–655

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

E. Cruz-Huerta, M.J. García-Nebot, B. Miralles, I. Recio, L. Amigo *

Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI-UAM+CSIC), Nicolás Cabrera 9, 28049 Madrid, Spain

ARTICLE INFO

Article history:
Received 14 February 2014
Received in revised form 24 June 2014
Accepted 16 July 2014
Available online 24 July 2014

Keywords:
Casein by-product
Caseinophosphopeptides
Simulated gastrointestinal digestion
Tandem mass spectrometry
Tryptic hydrolysis

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 for 30, 60 and 120 min and further precipitated with calcium chloride and ethanol at pH 4.0, 6.0 and 8.0. Identification and semi quantification of the derived products by tandem mass spectrometry revealed some qualitative and quantitative changes in the released caseinophosphopeptides over time at the different precipitation pHs. The by-product was also subjected to simulated gastrointestinal digestion. Comparison of the resulting peptides showed large sequence homology in the phosphopeptides released by tryptic hydrolysis and simulated 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. The results of the present study suggest that this casein-derived by-product can be used as a source of CPPs.

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 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 further hydrolysis 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 latter being the most used therapeutic application (Cross et al., 2007; Nongonierma & Fitzgerald, 2012). In addition, it has been proposed that they may also display immunomodulatory, cytomodulatory and antioxidant activities (Meisel & Fitzgerald, 2003; Phelan, Aherne, Fitzgerald, & ÓBrien, 2009). Besides, CPPs can influence gastric secretion regulation (Guilloteau et al., 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 these, such as trypsin and chymotrypsin (Pinto et al., 2012). The hydrolysis conditions, including pH, time, enzyme:protein ratio and temperature, were all different, thereby producing different sequences. In some studies, the digests or hydrolysates are subjected to an enrichment step to isolate the generated CPPs. Selective precipitation of CPPs with $CaCl_2$ and ethanol is a usual method using pHs in the range 3.5–8.5 (Aoki et al., 1998; Reynolds, Riley, & Adamson, 1994; Zhao, Wang, & Xu, 2007), but immobilized metal affinity chromatography (IMAC) involving different metal ions and titanium dioxide chromatography (Picariello et al., 2010) have also been used. Combination of selective precipitation at pH 7 with gallium IMAC has been recently described (Zhu & Fitzgerald, 2010). 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 contains di- and tri-phosphorylated peptides as well as the cluster peptides. At pH 8.0, all of the cluster peptides and the diphosphorylated peptides as well as the monophosphorylated peptides containing the sequence Ser(P)-Glu-Glu- precipitate, but the other monophosphorylated peptides containing the sequence Ser(P)-Ala-Glu-Glu- and Glu-Ser(P)-Thr-Glu- do not (Reynolds et al., 1994). 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).

* Corresponding author. Tel.: +34 910017939; fax: +34 910017905.
E-mail address: lourdes.amigo@csic.es (L. Amigo).

http://dx.doi.org/10.1016/j.foodchem.2014.07.090
0308-8146/

Some animal studies have demonstrated the formation of CPPs after the ingestion of CN or its fractions (Kasai, Honda, & Kiriyama, 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 CPPs found in human samples have been released using gastro-analogous digestion (pepsin) of β-CN (Schmelzer et al., 2007) or using simulated gastrointestinal digestion of CN with pepsin and trypsin (Ono, Takagi, & Kunishi, 1998) or pepsin and pancreatin (Miquel et al., 2006).

The production of CPPs for commercial purposes is usually performed from the whole CN fraction, or sodium caseinate. The aim of this work was to evaluate if a by-product generated during the manufacture of an antihypertensive CN hydrolysate (Contreras et al., 2011), could be used as a 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 antihypertensive hydrolysate was prepared at industrial scale by pepsin digestion of commercial CN (Promilk 85® 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 analyses were performed in triplicate. Analysis of variance (ANOVA) was carried out using the test of confidence intervals at 95%.

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (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 analyzed in duplicate on Precast Criterion XT 12% Bis-Tris gels (Bio-Rad, Richmond, CA, USA) and electrophoretic separations were carried out at 150 V using XT-MES as running buffer (Bio-Rad), 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 grammes 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 a 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 (12,000×*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 the method by Jiménez-Saiz, Martos, Carrillo, López-Fandiño, 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 $NaHCO_3$ for enzyme inactivation.

For the duodenal phase, gastric digests were re-adjusted to pH 6.5 by addition of 0.1 M $NaHCO_3$, 0.125 M bile salts (equimolar mixture of sodium taurocholate and sodium glycodeoxycholate) and 1 M $CaCl_2$ (7.6 mM final concentration). The samples were pre-heated at 37 °C for 15 min. Pancreatic porcine lipase (EC 232-619-9, type VI-S, 47,900 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 10,100 U/mg protein) at 34.5 U/mg protein and pancreatic bovine α-chymotrypsin (EC 232-671-2; type I–S; 55 U/mg protein) at 0.44 U/mg protein were then added to the duodenal mixture, and incubated at 37 °C for 60 min. The digestion was then 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) by the slow addition of 10% (w/v) $CaCl_2$ (20 mol/mol protein) and an equal volume of 99.8% (v/v) ethanol with mixing. In order to evaluate the possible different composition of the precipitate with the pH employed, it was adjusted to 4.0, 6.0 or 8.0 The suspension was centrifuged (12,000×*g* for 10 min at 10 °C), the precipitate 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 an XBridge™ BEH 300 C18 (5 μm, 4.6 × 250 mm, Waters, Wexford, Ireland), with an injection volume of 50 μl, and a flow rate of 0.8 ml/min. The flow was split post UV detector by connecting a T-piece to give a flow of approx. 20 μl/min which was sent to the mass spectrometer.

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% solvent B for 90 min, after which the percentage of solvent B increased to 90% over 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