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Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding

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Abstract

Adapted and follow-up milk-based infant formulas were subjected to gastrointestinal digestion simulating physiological conditions. The naturally occurring casein phosphopeptides (CPPs) generated were fractionated by anion exchange high-performance liquid chromatography and sequenced by tandem mass spectrometry. In both infant formula digests, a total of 19 CCPs from bovine casein were identified, of which 7 corresponded to α_{s1}-casein and 12 to α_{s2}-casein. Most CPPs had the cluster sequence SpSpSpEE, representing the binding sites for minerals. The distribution of calcium, iron and zinc content in CPP fractions eluted from the anion exchange column was also studied. The results obtained suggest that calcium could be preferably bound to CPPs with the cluster sequence SpSpSpEE, whereas iron and zinc could be bound to CPPs containing the phosphorylated cluster and phosphoserine residues.

Keywords: Casein phosphopeptides; Infant formulas; Mass spectrometry; Mineral bioavailability; Simulated gastrointestinal digestion

1. Introduction

The protein fraction of milk-based infant formulae is derived from the milk used in their formulation. These products are supplemented with minerals, mainly calcium, iron and zinc. Human milk is the reference standard for the manufacture of infant formulae, though in the case of minerals, amounts higher than the human milk content are added because their bioavailability is lower. Therefore, from a nutritional point of view, it is important to know the mineral contents of infant formulae and also their bioavailability to fully or partially satisfy the mineral requirement of infants. Bioavailability is defined as the fraction of the ingested nutrient that is absorbed and subsequently used for normal physiological functions (Barberá & Farré, 1992).

Among the biologically active peptides derived from the digestion of milk proteins, casein phosphopeptides (CPPs) may function as carriers for different minerals, playing an important role in their bioavailability (Clare & Swaisgood, 2000; Fitzgerald, 1998; Korhonen & Pihlanto-Leppälä, 2001; Meisel & Fitzgerald, 2003). These peptides contain highly polar acidic sequences of three phosphoseryl groups followed by two glutamic acid residues, SpSpSpEE, representing the binding sites for minerals.

Most studies on the role of CPPs focus on calcium bioavailability, while iron and zinc have been less extensively investigated. In vitro assays have demonstrated an enhancing effect of CPPs on calcium absorption (Ferraretto, Gravaghi, Fiorilli, & Tettamanti, 2003; Mykkänen & Wasserman, 1980; Sato, Noguchi, & Naito, 1986). However, in vivo studies in animals (Kopra, Scholz-Ahrens, & Barth, 1992; Pointillart & Guéguen, 1989) and humans (Hansen, Sandström, Jensen, & Sorensen, 1997a; Narva, Kärkkäinen, Poussa, Lamberg-Allardt, & Korpela, 2003) have failed to find an effect of CPPs on indicators of calcium utilization. The bioavailability of iron in rats is improved by CPPs (Aït-Oukhatar et al., 1999; Bouhallab et al., 2002), and iron bound to β-CN(1-25)4P displayed better iron absorption and uptake than inorganic salts (Aït-Oukhatar, et al., 2002). In the same way, CPPs were reported to stimulate zinc absorption from infant foods in humans (Hansen, Sandström, Jensen, & Sorensen, 1997b)

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and to decrease the inhibitory effect of phytate on zinc absorption from high phytate infant diets in rats (Hansen, Sandström, & Lönnerdal, 1996).

Considering the effects of CPPs on mineral bioavailability, and the lack of studies designed to identify and characterize CPPs released from infant formulas through gastrointestinal digestion, the present work was designed to investigate the formation and gastrointestinal survival of naturally occurring mineral carrier peptides released by simulated gastrointestinal digestion of infant formulas. The peptides generated were fractionated by anion exchange high-performance liquid chromatography (AE-HPLC) and sequenced by tandem mass spectrometry. In addition, the distribution of minerals in CPP fractions eluted from the anion exchange column was evaluated in order to study their potential mineral binding behaviour.

2. Materials and methods

2.1. Samples

Adapted and follow-up milk-based infant formulas (IF) were provided by Hero España S.A. (Murcia, Spain). The protein content (g protein g^{-1} sample) was 0.11 and 0.15, respectively, and the casein to whey protein ratio was 50:50 for both IF.

Mineral contents (mg g^{-1}), as stated on the label, were: calcium (3.92 and 6), iron (0.06 and 0.09) and zinc (0.04 and 0.04) for adapted and follow-up milk-based IF, respectively.

2.2. Simulated gastrointestinal digestion

Milk-based IF were digested according to the method described by Jovaní, Barberá, Farré, and Martín de Aguilera (2001). Briefly, 10 g of each sample were digested with pepsin (pH 2) (E.C. 3.4.23.1; 1: 60,000, 3400 U mg^{-1}) (Sigma, St. Louis, MO, USA) at 37 °C for 2 h, followed by exposure to pancreatin and bile extract (pH 5) (Sigma) at 37 °C for 2 h. The pH was adjusted to 7.2, the gastrointestinal digests were centrifuged (3500 × *g* for 1 h, 4 °C), and the supernatants (soluble fractions) were stored at −20 °C until use.

2.3. Fractionation of CPPs by AE-HPLC

The analytical HPLC system consisted of a 600E quaternary pump and 2487 absorbance detector set at 210 nm (Waters, Milford, MA, USA). The soluble fractions resulting from IF gastrointestinal digests were injected into a Mono Q® column (50mm × 5 mm; 10 μm particle size) (Amersham Biosciences, Uppsala, Sweden) and eluted with a linear gradient as has been reported elsewhere (Miquel, Alegría, Barberá, & Farré, 2005). Data were analysed using the Millennium^{32} Chromatography Manager Simple System software package (Waters).

The fractions from AE-HPLC obtained from 10 repetitive runs were pooled and lyophilized prior to the separation and identification of CPPs by reversed phase HPLC electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS/MS).

2.4. Analysis of CPPs by online RP-HPLC-ESI-MS/MS

Separation of the peptides eluted from the anion exchange column was performed on an Agilent HPLC system connected online to an Esquire-LC quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The HPLC system was equipped with a quaternary pump, an inline degasser, an automatic injector and a variable wavelength absorbance detector set at 214 nm (1100 Series, Agilent Technologies, Waldbronn, Germany). A C_{18} Hi-Pore® column (250mm × 4.6 mm; 5 μm particle size) (Bio-Rad Laboratories, Hercules, CA, USA) was used in this analysis.

Peptide samples collected from AE-HPLC fractionation were prepared and analysed using RP-HPLC-ESI-MS/MS as described elsewhere (Miquel et al., 2005). The *m/z* spectral data were processed and transformed to spectra representing mass values by the program Data Analysis version 3 (Bruker Daltonics). BioTools version 2.1 (Bruker Daltonics) was used to process the MS(*n*) spectra and to perform peptide sequencing.

2.5. Calcium, iron and zinc analysis

Calcium, iron and zinc were determined by flame atomic absorption spectroscopy on a Perkin-Elmer atomic absorption spectrophotometer, model 2380 (Perkin-Elmer, Norwalk, USA), equipped with a deuterium lamp as background correction system. Previously reported instrumental conditions were applied (García, Alegría, Barberá, Farré, & Lagarda, 1998; Roig, Alegría, Barberá, Farré, & Lagarda, 1999).

Prior to determination of the mineral contents, the organic matter of the soluble fraction from simulated gastrointestinal digests of both IF (3 mg) and the pool of fractions eluted after 6 consecutive runs from AE-HPLC was ashed at 450 °C for 24 h.

3. Results and discussion

3.1. Identification of CPPs in IF digests

The gastrointestinal digests of both IF were initially fractionated using AE-HPLC. Fractionation of the adapted and follow-up IF digest-using AE-HPLC is shown in Fig. 1a and c. The UV chromatograms corresponding to fraction 4 from AE-HPLC separation of adapted and follow-up IF digest are reported in Fig. 1b and d.

It is accepted that CPPs elute from Mono Q® column in the range between >0.3 and <0.6 M of the linear NaCl gradient (Juillerat et al., 1989) in which fractions 2–4 were