JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification of Casein Phosphopeptides Released after Simulated Digestion of Milk-Based Infant Formulas

ESTHER MIQUEL,† JOSÉ ÁNGEL GÓMEZ,‡ AMPARO ALEGRÍA,† REYES BARBERÁ,† ROSAURA FARRÉ,*,† AND ISIDRA RECIO‡

Nutrition and Food Chemistry, Faculty of Pharmacy, University of Valencia, Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain, and Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Adapted, follow-up, probiotic follow-up, toddler, and probiotic toddler infant formulas were subjected to an in vitro enzymatic procedure simulating physiological digestion. The formation and identification of casein phosphopeptides (CPPs) in the milk-based infant formulas were studied using reversed phase high-performance liquid chromatography coupled on line to an ion trap mass spectrometer. Most CPPs formed contained the cluster sequence SpSpSpEE, a mineral binding site. Phosphopeptide α_{s2}-CN(1−19)4P was present in all formulas analyzed. Probiotic formulas released CPPs not detected in nonprobiotic formulas and probably formed by bifidobacteria action. These observations suggest that physiological digestion of these products promotes the formation of bioactive peptides with mineral carrier properties in the gastrointestinal tract, which resist further proteolysis.

KEYWORDS: Casein phosphopeptides; simulated digestion; mineral bioavailability; milk-based infant formulas; reversed phase high-performance liquid chromatography; mass spectrometry

INTRODUCTION

The protein fraction of milk is an important source of bioactive peptides; the intrinsic bioactivities of these encrypted peptides remain latent until they are released and activated by enzymatic hydrolysis, gastrointestinal digestion, or food processing (*1*, *2*). Among these activated peptides, casein phosphopeptides (CPPs) may function as carriers for different minerals (*1*−*5*).

CPPs are phosphorylated casein-derived peptides that can be released through in vitro or in vivo enzymatic hydrolysis of α_{s1}-, α_{s2}-, and β-casein (CN). Many of these peptides contain a common structural motif: a sequence of three phosphoseryls followed by two glutamic acid residues. These highly polar acidic domains represent the binding sites for minerals such as calcium, iron, and zinc and play an important role in mineral bioavailability (*1*, *2*, *3*, *5*). These or related phosphorylated sequences occur in α_{s1}-CN(66−70), α_{s2}-CN(56−60), α_{s2}-CN(129−133), and β-CN(15−19) (*2*).

The amino acid sequence around the anionic hydrophilic domains seems to be significant in mineral binding (*6*). Moreover, dephosphorylated peptides do not bind minerals as efficiently as their equivalent phosphorylated derivatives (*2*).

The mineral chelating properties of CPPs coupled with their ability to form soluble complexes support their biological role as mineral carriers. Most minerals are dissociated from food at low pH in the stomach and may gradually become insoluble in the intestine as the pH increases. CPPs possess the ability to form soluble complexes with minerals, especially calcium—thereby preventing precipitation as calcium phosphate. Calcium weakly bound to CPPs can be progressively released in the intestinal lumen for absorption. These CPP effects are of nutritional relevance, since they occur at the intestinal pH suitable for calcium phosphate precipitation (*7*).

To date, several studies have investigated the effect of CPPs on mineral bioavailability. However, considerable controversy exists as to whether CPPs enhance calcium absorption. In general, in vitro animal studies have confirmed a positive effect of CPPs on calcium absorption (*7*−*9*), while most balance studies have failed to find an effect of CPPs in calcium absorption in rats (*10*, *11*) and piglets (*12*). Few studies have been published on the effect of CPPs upon human mineral bioavailability, and no effect on calcium absorption has been demonstrated (*13*−*15*). The discordance between the results of in vitro and in vivo studies can be attributed to differences in the methodology used to measure the intrinsic and extrinsic absorption of calcium, the CPP dosage involved, and the nature and presence of complex interactions with others food constituents. CPPs utilized in balance studies have usually been mixtures of different CPPs possessing different mineral binding capacities (*11*). CPPs also influence iron (*16*, *17*) and zinc (*13*, *14*) absorption, although studies of their effects on the bioavailability of these minerals are more scarce than studies on calcium.

Although bioactivity of phosphopeptides seemed more obvious in vitro and less clear in studies conducted under physi-

* To whom correspondence should be addressed. Tel: +34 96 3544950. Fax: +34 96 3544954. E-mail: rosaura.farre@uv.es.
† University of Valencia.
‡ Instituto de Fermentaciones Industriales (CSIC).

10.1021/jf0482111 CCC: $30.25
Published on Web 04/09/2005

ological conditions, many authors have shown that CPPs may improve the mineral balance in certain population groups at risk of intake below the recommended levels. A significant enhancement in calcium absorption was reported during the coingestion of CPPs with calcium by postmenopausal woman with a low basal absorptive capability (*18*). Iron bound to CN hydrolysates could offer a potential iron delivery source in programs for the treatment or supplementation of patients with iron deficiency anemia (*17*).

Several animal and human studies have reported the production of CPPs in vivo. Analyses of the intestinal fluid of minipigs fed a bovine CN-containing diet (*19*) and of the duodenal contents of adult humans after ingestion of milk and yogurt (*20*) showed that CPPs can survive intestinal transit after the ingestion of dairy foods. Other authors have reported better survival during intestinal passage by CPPs derived from the consumption of milk as opposed to the ingestion of CPPs per se (*21*).

Many studies have focused on the identification and characterization of CPPs in model systems of α_{s1}-CN (*22*), α_{s2}-CN (*23*), β-CN (*24*, *25*), and whole CN (*26*−*30*) subjected to tryptic hydrolysis. However, there have been no previously described investigations in which CPPs were identified after digestion procedures simulating human physiological conditions.

The protein fractions of a great number of infant formulas are derived from the milk used in their formulation. These products are supplemented with minerals and are formulated to fully or partially satisfy the mineral requirements of infants. Considering the effects of CPPs on mineral availability and the lack of studies designed to identify and characterize CPPs in infant formulas after simulated digestion, the present work was carried out to demonstrate the formation and resistance to gastrointestinal digestion of bioactive peptides with mineral carrier properties released after the digestion of milk-based infant formulas. The simultaneous separation and identification of CPPs in the samples studied was achieved by reversed phase high-performance liquid chromatography−electrospray ionization−tandem mass spectrometry (RP-HPLC-ESI-MS/MS) using an ion trap mass spectrometer. The potential mineral chelating properties of these peptides is discussed in relation to their structure.

MATERIALS AND METHODS

Samples. Adapted, follow-up, probiotic follow-up, toddler, and probiotic toddler milk-based infant formulas were provided by Hero España S. A. The protein content and the CN to whey protein ratio was as follows: adapted (0.112 g protein/g sample and 50:50), follow-up and probiotic follow-up (0.15 g protein/g sample and 50:50), toddler (0.026 g protein/mL sample and 65:35), and probiotic toddler (0.142 g protein/g sample and 65:35). Probiotic formulas contained *Bifidobacterium longum* and *Bifidobacterium bifidum*. An outline of the formulas manufacturing process is provided in **Figure 1**.

Simulated Digestion. *Enzymes and Bile Salts.* Pepsin A was from porcine gastric mucosa (EC 3.4.23.1) (Sigma Chemical Co., St. Louis, MO). Pepsin (1 g) was suspended in 25 mL of 0.1 N HCl. Pancreatin (0.04 g) was from porcine pancreas and bile extract (0.25 g) (Sigma Chemical Co.) and was dissolved in 10 mL of 0.1 N $NaHCO_3$.

The method of Jovaní et al. (*31*), with slight modifications, was applied, 10 g of adapted, follow-up, probiotic follow-up, probiotic toddler and 40 g of toddler were made up separately to 80 mL with distilled−deionized water. After homogenization, the pH was adjusted to 2 with 6 N HCl. After 15 min, the pH value was checked and if necessary readjusted to 2. Then, 0.5 g of freshly prepared pepsin solution was added. Samples were made up to 100 mL with distilled−deionized water and incubated in a shaking water bath at 37 °C for 2 h. The gastric digest was kept in ice for 10 min to stop pepsin digestion.

Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 5.2 by dropwise addition of 1 N $NaHCO_3$. Then, 1.25 g of the pancreatin−bile extract mixture was added and the incubation was continued for an additional 2 h. The sample was maintained for 10 min in an ice bath, and the pH was adjusted to 7.2 by dropwise addition of 0.5 N NaOH.

The gastrointestinal digest was centrifuged at 3500*g* for 1 h at 4 °C. The resulting supernatants (soluble fraction) were collected and centrifuged at 10000*g* for 10 min at 4 °C.

Isolation of CPPs by Selective Precipitation. According to the procedure of Reynolds et al. (*32*), a solution of 10% (w/v) of $CaCl_2$ was added to supernatants (20 mol $CaCl_2$/mol CN) adjusted to pH 8, and an equal volume of absolute ethanol 99.5% (v/v) was added slowly with constant mixing. After centrifugation at 12000*g* for 10 min at 10 °C, the precipitate was washed with 50% (v/v) ethanol and freeze-dried. The approximate yield of CPPs obtained after selective precipitation ranged from 2.5 to 7.2 mg CPP/g infant formula.

Analysis of CPPs by on Line RP-HPLC-ESI-MS/MS. RP-HPLC separation of the peptides was done on an Agilent HPLC system connected on line to an Esquire-LC quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The HPLC system was equipped with a quaternary pump, an in line degasser, an automatic injector, and a variable wavelength absorbance detector set at 214 nm (all 1100 Series, Agilent Technologies, Waldbronn, Germany). The column used in these analyses was a C_{18} Hi-Pore (250 mm × 4.6 mm; 5 μm particle size) (Bio-Rad Laboratories, Richmond, CA). The lyophilized precipitates resulting from selective precipitation of the infant formulas digests (2 mg) were dissolved in 1 mL of 0.1% trifluoroacetic acid, and the injection volume was 100 μL. Solvent A was a mixture of water/trifluoroacetic acid (1000:0.37 v/v) and solvent B contained water/acetonitrile/trifluoroacetic acid (200:800:0.27 v/v).

A linear gradient to 60% of B was applied in 90 min, the percentage of B was increased to 90% in 5 min and remained constant at 90% for 5 min more at a flow rate 0.8 mL/min. The flow rate was split up postdetector by placing a T-piece (Valco, Houston, TX) connected to a 75 μm i.d. polyether ether ketone outlet tube of a length adjusted to yield approximately 20 μL/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as the nebulizing and drying gas, and the helium collision gas pressure was approximately 5×10^{-3} bar. The capillary was held at 4 kV. Mass spectra were recorded over the mass/charge (*m*/*z*) range 100−2500. About 25 spectra were averaged in the MS analyses and about five spectra in the MS(*n*) analyses. The signal threshold to perform auto-MS(*n*) analyses was 5000, and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp from 0.39 to 2.6 V. The *m*/*z* spectral data were processed and transformed to spectra representing mass values using 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.

RESULTS AND DISCUSSION

The CPPs identified and the chromatographic profiles of the analyzed formulas are reported in **Tables 1**−**4** and **Figures 2** and **3**, respectively. CPPs released after simulated gastrointestinal digestion of milk-based infant formulas contained Arg, Lys at the C terminus—these results being consistent with the expected tryptic activity of pancreatin—in addition to Tyr (probably due to the expected chymotryptic activity of pancreatin). Furthermore, cleavage on the carboxyl side of Thr, Val, and Pro can be attributed to carboxypeptidase activity of pancreatin, because these residues are located penultimate to a tryptic or chymotryptic cleavage site (*33*). The formation of CPPs with Phe and Tyr at the C terminus can be due to the chymotryptic activity of pancreatin (*33*) or to the pepsin activity preferentially cleaving at the carboxyl side of such amino acids (*34*). Fragments at the C terminus, which contain Gln, could be formed after pancreatic hydrolysis, because pancreatin

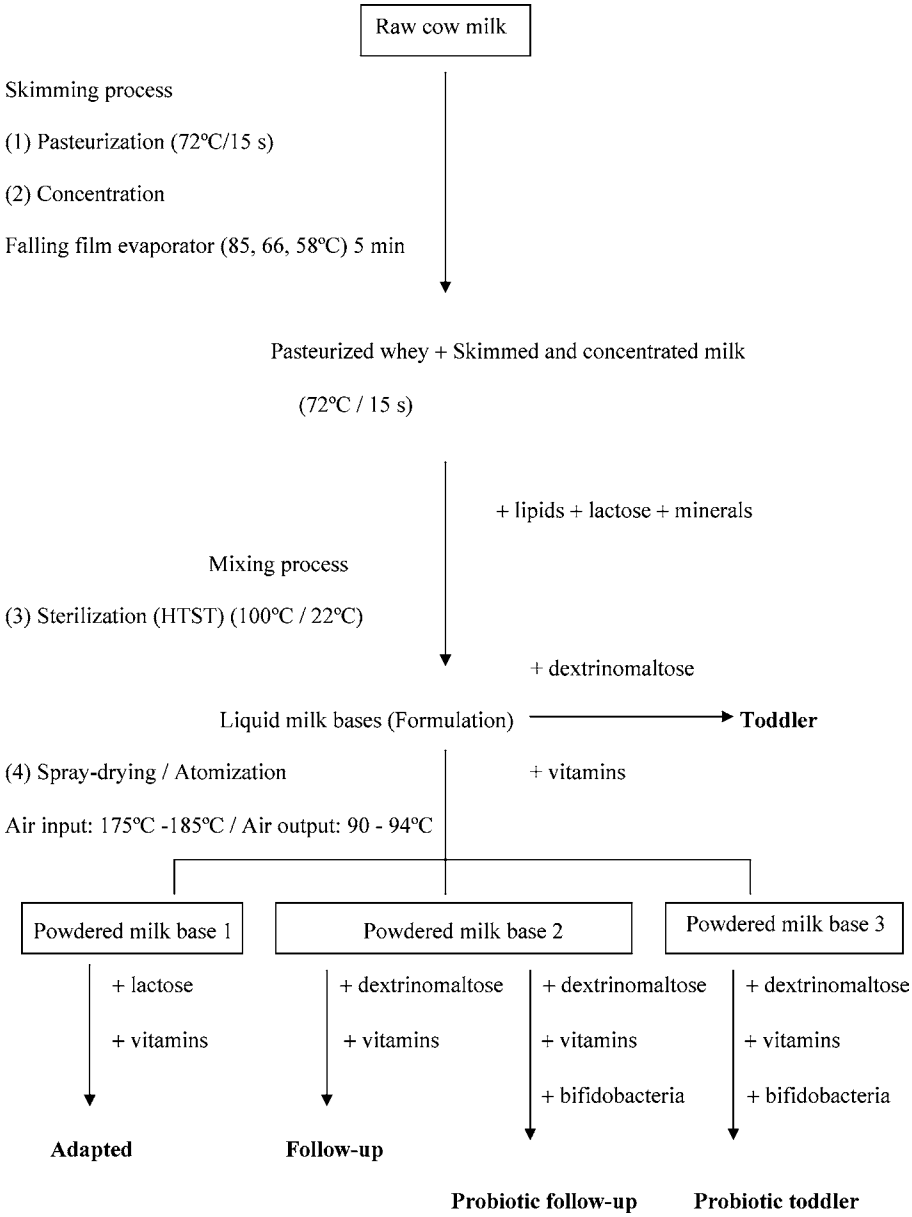

Figure 1. Outline of the formulas manufacturing process.

exhibits broad specificity in cleaving bonds on the carboxyl side of Arg, Lys, Tyr, Leu, Met, Gln, Ser, Thr, Glu, Val, and Pro (*35*).

Given that it has been suggested that enterocytes are able to uptake large peptides up to 30kDa (*36*) and plasma detection of CPPs has also been consistently reported by Chabance et al. (*20*), CPPs released after simulated digestion of milk-based infant formulas, whose molecular masses are ~1125−6512 Da (**Tables 1**−**4**), are good candidates for intestinal absorption and for playing a possible physiological role in mineral bioavailability. Although the mechanism by which CPPs interact with intestinal cells has not been elucidated, the structural conformation conferred by the phosphorylated cluster and specific amino acids located around them is presumably implicated (*6*).

Identification of CPPs after Simulated Digestion. The method applied to identify these components involved HPLC-ESI-MS measurement of the masses of peptides and a search for the amino acid sequences of α_{s1}-, α_{s2}-, and β-CN fitting the experimentally observed mass. **Figure 4A** shows the ESI-MS spectrum of peptide α_{s2}-CN(1−19)4P (*m*/*z* 2455.8). Three losses of 49 Da with respect to the precursor ion (*m*/*z* 1228.5), corresponding to phosphate groups (H_3PO_4), were observed in the ESI-MS spectrum. **Figure 4B** shows the ESI-MS/MS spectrum of the doubly charged ion (*m*/*z* 1228.5). The amino acid sequence and major fragment ions are also indicated. In the fragmentation profile of the peptide, an ion at *m*/*z* 1179.5 corresponding to $(M + 2H)^{2+}$-Pi was the most noticeable fragment ion in the spectrum, although a large series of y and b type ions were also observed.

Phosphopeptides are susceptible to dephosphorylation during exposure to alkaline and heat treatment. These conditions result in β-elimination of phosphoserine and the generation of dehydroalanine (*37*). Meisel et al. (*38*) indicated significant losses in peptide-bound phosphorus in sodium caseinate after heating at 170 °C during 45, 60, and 75 min. However, the spray drying time during the infant formulas manufacturing process is shorter than those indicated by Meisel et al. (*38*), so less dephosphorylation may occur. The identification of different CPPs carried out in this study, including those containing the SpSpSpEE cluster, which could affect mineral availability, could be an indicative signal showing that several CPPs may survive the processing conditions in infant formulas.

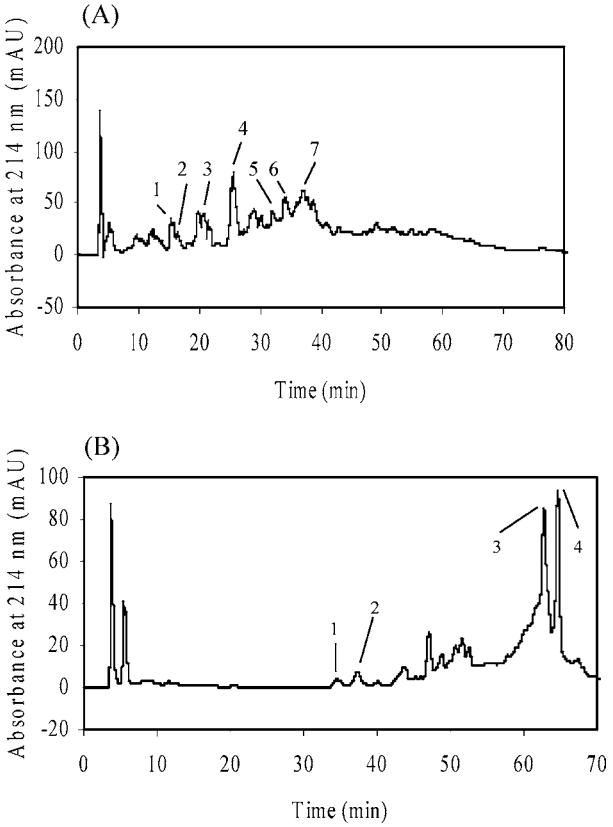

Figure 2. Separation of CPPs by RP-HPLC in adapted (**A**) and follow-up (**B**) infant formulas.

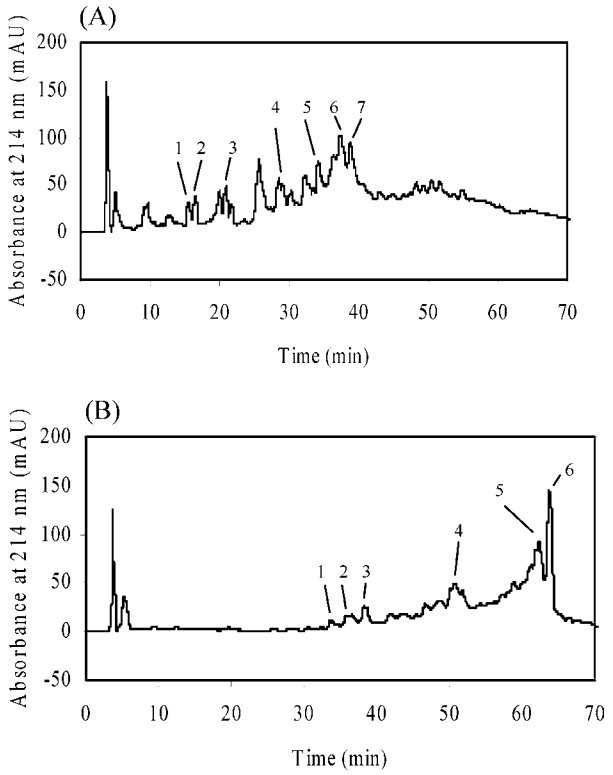

Figure 3. Separation of CPPs by RP-HPLC in toddler (**A**) and probiotic follow-up (**B**) infant formulas.

Adapted Formula. The chromatographic profile of peptides released after simulated digestion of the adapted infant formula is reported in **Figure 2A**. A total of seven CPPs, two of them with the cluster sequence SpSpSpEE and with 2−5 phosphate groups, are identified (**Table 1**).

These CPPs derived from α_{s1}- and α_{s2}-CN, and none of them derived from β-CN. This observation is interesting in terms of mineral bioavailability, because some authors have reported that CPPs derived from α_s-CN exhibit greater calcium binding affinity (measured by a calcium selective electrode) than β-CN-derived CPPs (*39*).

Among the CPPs identified, two of them, α_{s1}-CN(43−52)2P and α_{s2}-CN(1−19)4P, coincide with those obtained after hydrolysis of sodium caseinate with pancreatin (*35*)—the enzyme used in this study during gastrointestinal digestion of the formulas. CPPs derived from α_{s1}-CN had singly charged precursor ions, and these CPPs contained 9−11 amino acids and two phosphate groups; only α_{s1}-CN(56−90)5P with 34 amino acids had a doubly charged precursor ion. CPPs derived from α_{s2}-CN had doubly charged precursor ion, and these CPPs contained 19−23 amino acids and 3−4 phosphate groups.

Follow-up Formula. Figure 2B shows the chromatographic separation of the CPPs released after simulated human digestion of the follow-up infant formula. A total of four CPPs, all of them presenting the cluster sequence SpSpSpEE with four or five phosphate groups, were identified (**Table 2**).

Follow-up formula contained larger CPPs than adapted formula, with 19−52 amino acids and molecular masses of ∼2400−6500 Da. In contrast to adapted formula, these CPPs derived from α_{s2}- and β-CN and none of them derived from α_{s1}-CN.

Fragment α_{s2}-CN(1−19)4P was common to adapted formulas, and fragment α_{s2}-CN(1−24)4P coincides with those identified by other authors after tryptic hydrolysis of CN (**Table 2**).

The pentaphosphorylated peptide β-CN(1−52)5P included in its amino acid sequence two previously described tryptic peptides, β-CN(1−25)4P and β-CN(33−48)1P (**Table 2**), with mineral chelating properties and calcium binding constants at pH 7 that are 0.35−0.39 and 0.30−0.33 mM^{-1}, respectively (*40*). The beneficial effects of β-CN(1−25)4P and β-CN(33−48)1P stimulating calcium absorption have been previously demonstrated (*6*). Furthermore, β-CN(1−25)4P improves iron (*16*, *17*) and zinc (*41*) bioavailability. Although the physiological effect of a particular mineral carrier tryptic peptide may not always be extrapolated to longer peptides or peptides with common amino acid sequences, the structural similarity may allow a similar mineral binding activity to be anticipated.

The protein fragment β-CN(1−32)4P has been identified in the human stomach after the ingestion of milk or yogurt (*20*). This suggests that cleavage of the Lys_{32}−Phe_{33} bond may have been due to pepsin activity—the latter being the enzyme used in this study during the gastric step.

Toddler Formula. The chromatographic separation of CPPs released after simulated digestion of the toddler formula is reported in **Figure 3A**. A total of 11 CPPs were identified in the toddler formula representing the greatest number of CPPs of all of the studied formulas (**Table 3**). This may suggest that the liquid form could facilitate accessibility of the enzymes to the cleavage sites. Among these CPPs, six derived from α_{s1}-CN, three from α_{s2}-CN, and two from β-CN. Only two CPPs [α_{s1}-CN(1−19)4P and α_{s1}-CN(57−90)5P] contained the aforementioned cluster sequence. Peaks 1−3 contained different protein fragments located between residues 40 and 52 in the primary structure of α_{s1}-CN. Peak 7 contained the fragment β-CN(33−52)1P, which in its amino acid sequence includes phosphopeptide β-CN(33−48)4P, which has been described to

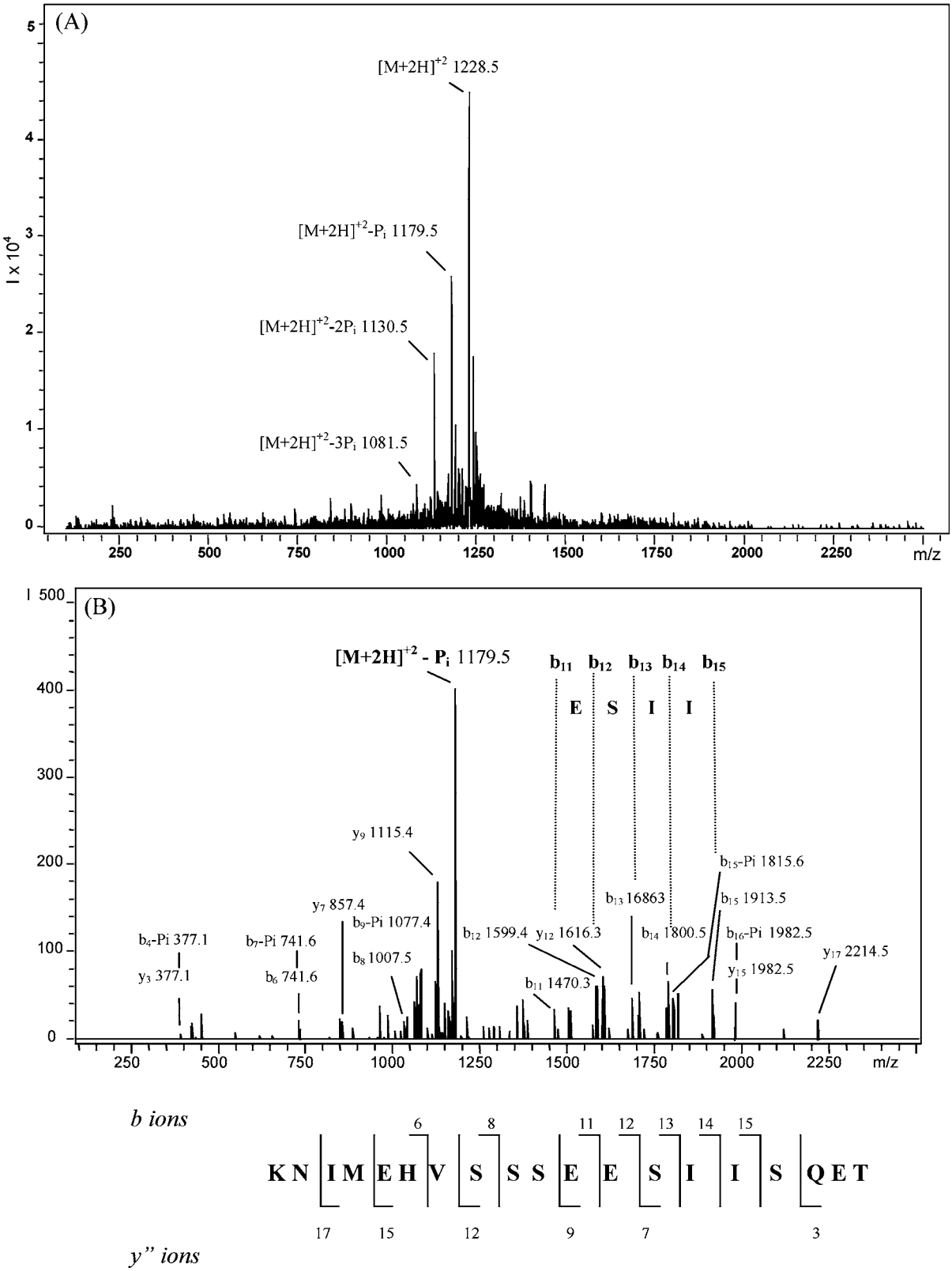

Figure 4. ESI-MS (**A**) and ESI-MS/MS (**B**) spectra of the peptide at *m*/*z* 2455.8 from α_{s2}-CN. The peptide was identified by RP-HPLC-ESI-MS/MS. The doubly charged ion *m*/*z* 1228.5 was subjected to collision-induced dissociation. Following sequence interpretation and database searching, the ESI-MS/MS spectrum was matched to α_{s2}-CN(1–19)4P. The sequence of this peptide is displayed with the fragment ions observed in the MS/MS spectrum. Fragment ions are labeled according to the nomenclature proposed by Papayannopoulus (*47*).

act as a mineral carrier (**Table 3**). Fragment α_{s1}-CN(43−52)2P was common to adapted formula, and α_{s2}-CN(1−19)4P was to adapted and follow-up formulas.

Probiotic Formulas. Figure 3B shows the chromatographic profile of CPPs identified in the probiotic follow-up formula. A total of six CPPs, three of them with the aforementioned cluster sequence and 1, 4, and 5 phosphate groups, were identified in both probiotic formulas (**Table 4**).

Probiotic formulas released a greater number of CPPs than follow-up infant formula. These CPPs derived from α_{s1}-, α_{s2}-,

Table 1. CPPs Formed and Identified after Simulated Digestion of Adapted Infant Formula[a]

peak[b]	protein fragment	amino acid sequence	observed *m/z*	calcd *m/z*[c]	ion (*m/z*) selected for MS/MS (charge)	mineral carrier peptide[d]	refs
1	α_{s1}-CN(43–52)2P	DIG**Sp**E**Sp**TEDQ	1240.4	1240.4	1240.4 (1)	*DIG**Sp**E**Sp**TEDQ*	*35*
2	α_{s1}-CN(44–52)2P	IG**Sp**E**Sp**TEDQ	1125.5	1125.3	1125.5 (1)	DIG**Sp**E**Sp**TEDQ	
3	α_{s1}-CN(41–52)2P	SKDIG**Sp**E**Sp**TEDQ	1455.5	1455.5	1455.5 (1)	as above	
4	α_{s1}-CN(40–52)2P	LSKDIG**Sp**E**Sp**TEDQ	1568.5	1568.6	1568.5 (1)	as above	
5	α_{s2}-CN(124–146)3P	NREQL**Sp**T**Sp**EENSKKTVDME**Sp**TEV	2881.2	2881.1	1441.1 (2)	EQL**Sp**T**Sp**EENSK	*23, 35*
6	α_{s2}-CN(1–19)4P	KNIMEHV**SpSpSpEE**SII**Sp**QET	2455.8	2455.8	1228.5 (2)	*KNTMEHV**SpSpSp**EESII**Sp**QET*	*35*
7	α_{s1}-CN(56–90)5P	DIKQMEA**Sp**I**SpSpSpEE**IVPN**Sp**VEQ-KHIQKEDVPSER	4398.4	4397.8	1100.7(2)	QMEA**Sp**I**SpSpSpEE**-IVPN**Sp**VEQK	*24, 27–29*

[a] **Sp** phosphoserine. **SpSpSpEE** cluster sequence. [b] Chromatographic peaks were reported in **Figure 2A**. [c] Calculated monoisotopic mass. [d] Sequences in italics indicate CPPs identified previously that are identical to those obtained in the present study.

Table 2. CPPs Formed and Identified after Simulated Digestion of Follow-up Infant Formula[a]

peak[b]	protein fragment	amino acid sequence	observed *m/z*	calcd *m/z*[c]	ion (*m/z*) selected for MS/MS (charge)	mineral carrier peptide[d]	refs
1	α_{s2}-CN(1–19)4P	KNTMEHV**SpSpSpEE**SII**Sp**QET	2455.8	2455.8	1228.4 (2)	*KNTMEHV**SpSpSp**EESII**Sp**QET*	*35*
2	α_{s2}-CN(1–24)4P	KNTMEHV**SpSpSpEE**SII**Sp**QETYKQEK	3132.4	3132.2	1567.5 (2)	*KNTMEHV**SpSpSpEE**SII**Sp**QETYKQEK*	*23, 26,30*
3	β-CN(1–32)4P	RELEELNVPGEIVE**Sp**L**SpSp**-**SpEE**SITRINKKIEK	3976.3	3975.8	1326.2 (3)	*RELEELNVPGEIVE**Sp**L**SpSp**-**SpEE**SITRINKKIEK*	*20*
4	β-CN(1–52)5P	RELEELNVPGEIVE**Sp**L**SpSpSpEE**SITRINKK-IEKFQ**Sp**EEQQQTEDELQDKIHPF	6512.2	6512.9	1629.6 (4)	RELEELNVPGEIVE**Sp**L**SpSpSpEE**SITR	*24, 25,28, 29*
						FQ**Sp**EEQQQTEDELQDKF	*24, 25,29, 30*

[a] **Sp** phosphoserine. **SpSpSpEE** cluster sequence. [b] Chromatographic peaks were reported in **Figure 2B**. [c] Calculated monoisotopic mass. [d] Sequences in italics indicate CPPs identified previously that are identical to those obtained in the present study.

Table 3. CPPs Formed and Identified after Simulated Digestion of Toddler Infant Formula[a]

peak [b]	protein fragment	amino acid sequence	observed *m/z*	calcd *m/z*[c]	ion (*m/z*) selected for MS/MS (charge)	mineral carrier peptide[d]	refs
1	α_{s1}-CN(43–52)2P	DIG**Sp**E**Sp**TEDQ	1240.4	1240.4	1240.4 (1)	*DIG**Sp**E**Sp**TEDQ*	*35*
2	α_{s1}-CN(44–52)2P	IG**Sp**E**Sp**TEDQ	1125.5	1125.3	1125.5 (1)	DIG**Sp**E**Sp**TEDQ	
3	α_{s1}-CN(41–52)2P	SKDIG**Sp**E**Sp**TEDQ	1455.5	1455.5	1455.5 (1)	as above	
	α_{s1}-CN(42–52)2P	KDIG**Sp**E**Sp**TEDQ	1368.5	1368.5	1368.5 (1)	as above	
	α_{s1}-CN(40–52)2P	LSKDIG**Sp**E**Sp**TEDQ	1568.5	1568.6	1568.5 (1)	as above	
4	α_{s2}-CN(137–146)1P	KTVDME**Sp**TEV	1218.5	1218.5	1218.5 (1)	VDME**Sp**TEVFTK	*35*
5	α_{s2}-CN(124–146)3P	NREQL**Sp**T**Sp**EENSKKTVDME**Sp**TEV	2881.2	2881.1	1441.5 (2)	VDME**Sp**TEVFTK	
	α_{s2}-CN(1–19)4P	KNTMEHV**SpSpSpEE**SII**Sp**QET	2456.6	2455.8	1228.4 (2)	*KNTMEHV**SpSpSp**EESII**Sp**QET*	*35*
6	β-CN(30–46)1P	IEKFQ**Sp**EEQQQTEDELQ	2189.2	2188.9	1095.9 (2)	FQ**Sp**EEQQQTEDELQDK	*24, 25,29, 30*
7	α_{s1}-CN(57–90)5P	IKQMEA**Sp**I**SpSpSpEE**IV-PN**S**VEQKHIQKEDVPSER	4281.4	4282.2	1427.8 (3)	QMEA**Sp**I**SpSpSpEE**-IVPN**Sp**VEQK	*24,27–29*
	β-CN(33–52)1P	FQ**Sp**EEQQQTEDELQDKIHPF	2557.2	2556.1	1279.1 (2)	FQ**Sp**EEQQQTEDELQDK	*24, 25,29, 30*

[a] **Sp** phosphoserine. **SpSpSpEE** cluster sequence. [b] Chromatographic peaks were reported in **Figure 3A**. [c] Calculated monoisotopic mass. [d] Sequences in italics indicate CPPs identified previously that are identical to those obtained in the present study.

Table 4. CPPs Formed and Identified after Simulated Digestion of Probiotic Formulas[a]

peak[b]	protein fragment	amino acid sequence	observed *m/z*	calcd *m/z*[c]	ion (*m/z*) selected for MS/MS (charge)	mineral carrier peptide[d]	refs
1	α_{s2}-CN(1–19)4P	KNTMEHV**SpSpSpEE**SII**Sp**QET	2455.8	2455.8	1228.4 (2)	*KNTMEHV**SpSpSp**EESII**Sp**QET*	*35*
2	α_{s1}-CN(73–90)1P	PN**Sp**VEQKEDVPSER	2200	2200.0	1100.5 (2)	QMEA**Sp**I**SpSpSpEE**IVPN**S**VEQK	*24,27–29*
3	α_{s1}-CN(73–91)1P	PN**Sp**VEQKEDVPSERY	2363	2363.1	1182.6 (2)	as above	
4	β-CN(1–32)4P	RELEELNVPGEIVE**Sp**L**SpSp**-**SpEE**SITRINKKIEK	3976.3	3975.8	1326.7 (3)	*RELEELNVPGEIVE**Sp**L**SpSp**-**SpEE**SITRINKKIEK*	*20*
5	α_{s1}-CN(111–147)1P	IVPN**Sp**AEERLHSMKEGIHA-QQKEPMIGVNQELAYFY	4333.6	4334.0	1445.6 (3)	VLEIVPN**Sp**AEER	*30*
6	β-CN (1–52)5P	RELEELNVPGEIVE**Sp**L**SpSpSp**-**EE**SITRINKKIEKFQ**Sp**E-EQQQTEDELQDKIHPF	6512.2	6512.8	1629.6 (4)	*RELEELNVPGEIVE**Sp**L**Sp**-**SpSpEE**SITR*	*24, 25,28, 29*
						*FQ**Sp**EEQQQTEDELQDK*	*24, 25,29, 30*

[a] **Sp** phosphoserine. **SpSpSpEE** cluster sequence. [b] Chromatographic peaks were reported in **Figure 3B**. [c] Calculated monoisotopic mass. [d] Sequences in italics indicate CPPs identified previously that are identical to those obtained in the present study.

and β-CN, three of them [α_{s2}-CN(1−19)4P, β-CN(1−32)4P, and (β-CN(1−52)5P] were common to follow-up infant formula (**Table 4**).

Little work has been carried out on peptidase activities of bifidobacterium species in milk (*42*−*44*). Aminopeptidase, carboxypeptidase, iminopeptidase, dipeptidase, and tripeptidase activities of *B. longum* were observed in synthetic substrates (*45*). According to these studies, monophosphorylated peptides α_{s1}-CN(73−90)1P, α_{s1}-CN(73−91)1P, and α_{s1}-CN(111−147)-1P) could be formed by bifidobacteria action, because these CPPs were only detected in probiotic formulas and no references support their formation as a result of tryptic or pancreatic digestion.

The cleavage of Val_{72}−Pro_{73} and Arg_{90}−Tyr_{91} is involved in the formation of α_{s1}-CN(73−90)1P, Val_{72}−Pro_{73}, and Tyr_{91}−Leu_{92} in the origin of α_{s1}-CN(73−91)1P and Glu_{110}−Ile_{111} and

Pro_{147}−Glu_{148} in the formation of α_{s1}-CN(111−147)1P. Hydrolysis of all of these bonds is most probably the result of aminopeptidase and carboxypeptidase activities.

B. longum hydrolyzed CN and seems to possess carboxypeptidase activity, since it hydrolyzed the monosubstituted substrate hippuryl Arg (*45*). Minagawa et al. (*46*) found *B. longum and B. bifidum* to contain selectively hydrolyzing X-Pro type aminopeptidases—These results are consistent with those found in α_{s1}-CN(73−90)1P and α_{s1}-CN(73−91)1P and aminopeptidases with broad substrate specificity.

The application of simulated digestion to milk-based infant formulas revealed formation and possible persistence in the gastrointestinal tract of CPPs having a high degree of phosphorylation, including the SpSpSpEE cluster that could affect mineral availability. It should be noted that the tetraphosphorylated peptide with the cluster sequence SpSpSpEE α_{s2}-CN(1−19)4P was common to all formulas analyzed and that the probiotic formulas contained unique CPPs probably formed by bifidobacteria action. The effect on mineral bioavailability of other CPPs, including those of the aforementioned cluster identified in this study, is not known. It therefore could be of interest to examine this aspect more in depth. Although CPPs hold promise for use as food ingredients with the ability to carry minerals in infant formulas, further studies on the availability of CPPs derived from physiological digestion and their interrelations with other components in complex matrixes such as infant formulas should be carried out.

LITERATURE CITED

(1) Korhonen, H.; Pihlanto-Leppälä, A. Milk protein-derived bioactive peptides novel opportunities for health promotion. *Bull. IDF* **2001**, *363*, 17−26.

(2) Meisel, H.; Fitzgerald, R. J. Biofunctional peptides from milk proteins: Mineral binding and cytomodulatory effects. *Curr. Pharm. Des.* **2003**, *9*, 1289−1295.

(3) Clare, D. A.; Swaisgood, H. E. Bioactive milk peptides: a prospectus. *J. Dairy Sci.* **2000**, *83*, 1187−1195.

(4) Shah, N. P. Effects of milk-derived bioactives: An overview. *Br. J. Nutr.* **2000**, *84*, 3−10.

(5) Fitzgerald, R. J. Potential uses of caseinophosphopeptides. *Int. Dairy J.* **1998**, *8*, 451−457.

(6) Ferraretto, A.; Gravaghi, C.; Fiorilli, A.; Tettamanti, G. Casein-derived bioactive phosphopeptides: Role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells. *FEBS Lett.* **2003**, *551*, 92−98.

(7) Erba, D.; Ciappellano, S.; Testolin, G. Effect of caseinphosphopeptides on inhibition of calcium intestinal absorption due to phosphate. *Nutr. Res.* **2001**, *21*, 649−656.

(8) Mykkänen, H. M.; Wasserman, R. H. Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *J. Nutr.* **1980**, *110*, 2141−2148.

(9) Sato, R.; Noguchi, T.; Naito, H. Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. *J. Nutr. Sci. Vitaminol.* **1986**, *32*, 67−76.

(10) Brommage, R.; Juillerat, M. A.; Jost, R. Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. *Lait* **1991**, *71*, 173−180.

(11) Kopra, N.; Scholz-Ahrens, E.; Barth, C. A. Effect of casein phosphopeptides on utilization of calcium in vitamin D-replete and vitamin D-deficient rats. *Milchwissenschaft* **1992**, *47*, 488−490.

(12) Pointillart, A.; Gueguen, L. Absence of effect of the incorporation of a milk phosphopeptide on the utilization of calcium and phosphorus in the young pig. *Reprod., Nutr., Dev.* **1989**, *29*, 477−486.

(13) Hansen, M.; Sandström, B.; Jensen, M.; Sorensen, S. S. Casein phosphopeptides improve zinc and calcium absorption from rice-based but not from whole-grain infant cereal. *J. Pediatr. Gastroenterol. Nutr.* **1997**, *24*, 56−62.

(14) Hansen, M.; Sandström, B.; Jensen, M.; Sorensen, S. S. Effect of casein phosphopeptides on zinc and calcium absorption from bread meals. *J. Trace Elem. Med. Biol.* **1997**, *11*, 143−149.

(15) Narva, M.; Karkkainen, M.; Poussa, T.; Lamberg-Allardt, C.; Korpela, R. Casein phosphopeptides in milk and fermented milk do not affect calcium metabolism acutely in postmenopausal women. *J. Am. Coll. Nutr.* **2003**, *22*, 88−93.

(16) Bouhallab, S.; Cinga, V.; Aït-Oukhatar, N.; Bureau, F.; Neuville, D.; Arhan, P.; Maubois, J. L.; Bouglé, D. Influence of various phosphopeptides of caseins on iron absorption. *J. Agric. Food Chem.* **2002**, *50*, 7127−7130.

(17) Chaud, M. V.; Izumi, C.; Nahaal, Z.; Shuhama, T.; Bianchi, M. P.; Freitas, O. Iron derivatives from casein hydrolysates as a potential source in treatment of iron deficiency. *J. Agric. Food Chem.* **2002**, *50*, 871−877.

(18) Heaney, R. P.; Saito, Y.; Orimo, H. Effect of caseinophosphopeptides on absorbability of co-ingested calcium in normal postmenopausal women. *J. Bone Miner. Metab.* **1994**, *12*, 77−81.

(19) Meisel, H.; Frister, H. Chemical characterization of a casein-phosphopeptide isolated from in vivo digest of a casein diet. *Biol. Chem. Hoppe-Seyler* **1988**, *369*, 1275−1279.

(20) Chabance, B.; Marteau, P.; Rambaud, J. C.; Migliore-Samour, D.; Boynard, M.; Perrotin, P.; Guillet, R.; Jolls P.; Fiat, A. M. Casein peptide release and passage to the blood in humans during digestion of milk or yoghurt. *Biochimie* **1998**, *80*, 155−165.

(21) Meisel, H.; Bernard, H.; Fairweather-Tait, S.; Fitzgerald, R. J.; Hartmann, R.; Lane, C. N.; McDonagh, D.; Teucher, B.; Wal, J. M. Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. *Br. J. Nutr.* **2003**, *89*, 351−358.

(22) Carr, S. A.; Huddleston, M. J.; Annan, R. S. Selective detection and sequencing phosphopeptides at the femtomole level by mass spectrometry. *Anal. Chem.* **1996**, *68*, 180−192.

(23) Tauzin, J.; Miclo, L.; Roth, S.; Mollé, D.; Gaillard, J. L. Tryptic hydrolysis of bovine α_{s2}-casein: Identification and release kinetics of peptides. *Int. Dairy J.* **2003**, *13*, 15−27.

(24) Gaucheron, F.; Mollé, D.; Léonil, J.; Maubois, J. L. Selective determination of phosphopeptide β-CN(1−25) in a β-casein digest by adding iron: characterization by liquid chromatography with on-line electrospray-ionization mass spectrometric detection. *J. Chomatogr. B* **1995**, *664*, 193−200.

(25) Visser, S.; Slangen, C. J.; Lagerwerf, F. M.; Van Dongen, W. D.; Haverkamp, J. Identification of a new genetic variant of bovine β-casein using reversed-phase high-performance liquid chromatography and mass spectrometric analysis. *J. Chromatogr. A* **1995**, *711*, 141−150.

(26) Gagnaire, V.; Pierre, A.; Mollé, D.; Léonil, J. Phosphopeptide interacting with colloidal calcium phosphate isolated by tryptic hydrolysis of bovine casein micelles. *J. Dairy Res.* **1996**, *63*, 405−422.

(27) Berrocal, R.; Chanton, S.; Juillerat, M. A.; Pavillard, B.; Scherz, J. C.; Jost, R. Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium. *J. Dairy Res.* **1989**, *56*, 335−341.

(28) Juillerat, M. A.; Baechler, R.; Berrocal, R.; Chanton, S.; Scherz, J. C.; Jost, R. Tryptic phosphopeptides from whole casein. I. Preparation and analysis by fast protein liquid chromatography. *J. Dairy Res.* **1989**, *56*, 603−611.

(29) Goepfert, A.; Meisel, H. Semipreparative isolation of phosphopeptides derived from bovine casein and dephosphorylation of casein phosphopeptides. *Nahrung* **1996**, *40*, 245−248.

(30) Ellegard, K. H.; Gammelgard-Larsen, C.; Sorensen, E.; Fedosov, S. Process scale chromatographic isolation, characterization and identification of tryptic bioactive casein phosphopeptides. *Int. Dairy J.* **1999**, *9*, 639−652.

(31) Jovaní, M.; Barberá, R.; Farré, R.; Martín de Aguilera, E. Calcium, iron, and zinc uptake from digests of infant formulas by Caco-2 cells. *J. Agric. Food Chem.* **2001**, *49*, 3480−3485.
(32) Reynolds, E. C.; Riley, P. F.; Adamson, N. J. A selective precipitation purification procedure for multiple phosphoseryl-containing peptides and methods for their identification. *Anal. Biochem.* **1994**, *217*, 277−284.
(33) Allen, G. Sequencing of proteins and peptides. In *Laboratory Techniques in Biochemistry and Molecular Biology*; Burdon, R. H., van Knippenberg, P. H., Eds.; Elsevier Science Publishing: Amsterdam, 1989; Vol. 9, p 73.
(34) Sweeney, P. J.; Walker, J. M. In *Enzymes of Molecular Biology*; Burrell, M. M., Eds.; Humana Press: Totowa, NJ, 1993; p 290.
(35) Adamson, N. J.; Reynolds, E. C. Characterization of multiply phosphorylated peptides selectively precipitated from a pancreatic casein digest. *J. Dairy Sci.* **1995**, *78*, 2653−2659.
(36) Pantzar, N.; Westrm, B. R.; Luts, A.; Lundin, S. Regional small-intestinal permeability in vitro to different-sized dextrans and proteins in the rat. *Scand. J. Gastroenterol.* **1993**, *28*, 205−211.
(37) Léonil, J.; Gagnaire, V.; Mollé, D.; Pezennec, S.; Bouhallab, S. Application of chromatography and mass spectrometry to the characterization of food proteins and derived peptides. *J. Chromatogr. A* **2000**, *881*, 1−21.
(38) Meisel, H.; Andersson, H. B.; Buhl, K.; Erbersdobler, H. F.; Schlimme, E. Heat-induced changes in casein-derived phosphopeptides. *Z. Ernahrungswiss.* **1991**, *30*, 227−232.
(39) Park, O.; Swaisgood, E.; Allen, J. C. Calcium binding of phosphopeptides derived from hydrolysis of α_s-casein or β-casein using immobilized trypsin. *J. Dairy Sci.* **1998**, *81*, 2850−2857.
(40) Meisel, H.; Olieman, C. Estimation of calcium-binding constants of casein phosphopeptides by capillary zone electrophoresis. *Anal. Chim. Acta* **1998**, *372*, 291−297.
(41) Pérès, J. M.; Bouhallab, S.; Petit, C.; Bureau, F.; Maubois, J. L.; Arhan, P.; Bouglé, D. Improvement of zinc intestinal absorption and reduction of zinc/iron interaction using metal bound to the caseinophosphopeptide 1−25 of β-casein. *Reprod., Nutr., Dev.* **1998**, *38*, 465−472.
(42) Abu-Taraboush, H. M.; Al-Dagal, M. M.; Al-Royli, M. A. Growth, viability, and proteolytic activity of bifidobacteria in whole camel milk. *J. Dairy Sci.* **1997**, *81*, 354−361.
(43) Desjardins, M.; Roy, D.; Goulet, J. Growth of bifidobacteria and their enzyme profiles. *J. Dairy Sci*. **1990**, *73*, 299−307.
(44) Desjardins, M.; Roy, D.; Goulet, J. β-galactosidase and proteolytic activities of bifidobacteria in milk: A preliminary study. *Milchwissenchaft* **1991**, *46*, 11−13.
(45) El-Soda, M.; Macedo, A.; Olson, N. F. The peptide hydrolase system of Bifidobacterium species. *Milchwissenschaft* **1992**, *47*, 87−90.
(46) Minagawa, E.; Kaminogawa, S.; Tsukasaki, F.; Motoshima, H.; Yamauchi, K. Exopeptidase profiles of bifidobacteria. *J. Nutr. Sci. Vitaminol.* **1985**, *31*, 599−606.
(47) Papayannopoulos, I. A. The interpretation of collision-induced dissociation tandem mass spectra of peptides. *Mass Spectrom. Rev.* **1995**, *14*, 49−73.

Received for review October 26, 2004. Revised manuscript received February 3, 2005. Accepted March 15, 2005. This study is part of project AGL 2000-1483 financially supported by the FEDER/CICYT (Spain). Thanks to Hero S.A. (Spain) for providing the samples and for financial support for this study. Thanks to Generalitat Valenciana for the financial support given to the Bionutest group. E.M. is the holder of a grant from the Ministerio de Ciencia y Tecnología, Spain.

JF0482111