JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 7955–7961 7955
DOI:10.1021/jf100568r

Phosphorylation and Coordination Bond of Mineral Inhibit the Hydrolysis of the β-Casein (1−25) Peptide by Intestinal Brush-Border Membrane Enzymes

Rachel Boutrou,* Elodie Coirre, Julien Jardin, and Joëlle Léonil

INRA and Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Oeuf, 65 rue de Saint Brieuc, F-35042 Rennes, France

Caseinophosphopeptides (CPP) are food mineral-rich components that may resist intestinal enzyme hydrolysis. We wondered whether phosphorylation and/or mineral binding induces resistance of CPP to intestinal hydrolysis. We used intestinal brush-border membrane vesicles to digest different forms of the β-casein (1−25) peptide: unphosphorylated and phosphorylated carrier of varied cations. The results showed that the activity of alkaline phosphatase seems not to be specific to either the phosphorylation degree or the phosphorylation sites whereas phosphorylations limited the action of peptidases. Studying the mechanism and the kinetics of hydrolysis of the different peptides allows understanding how some cations prevent more CPP from hydrolysis than others. The action of both exo- and endopeptidases was limited for the β-CN (1−25) peptide bound to zinc or copper. Actually the peptide bound to copper was almost not hydrolyzed during the digestion, suggesting that coordination bond of copper to CPP inhibits the action of both phosphatase and peptidases.

KEYWORDS: Caseinophosphopeptides; intestinal hydrolysis; brush-border membrane enzymes; phosphorylation; minerals

INTRODUCTION

Caseinophosphopeptides (CPPs) are encrypted in caseins that represent 80% of milk proteins. Casein derived CPPs correspond to various phosphorylated regions of α_{s1}-, α_{s2}- and β-casein. Most CPPs contain clusters of three phosphorylated serine residues followed by two glutamic acid residues named the "phosphoserine cluster". As these peptides have a high content of negative charges, they efficiently bind divalent cations with the formation of soluble complexes. CPPs then function as carriers of minerals. Complexes of peptides and minerals of calcium, zinc, iron, magnesium, manganese, copper and selenium have been reported (*1*). Natural mineral-rich components such as CPPs have important implications in the treatment and/or prevention of diseases. They have already found applications in the food industry that have developed soft drinks ingredients or fortifiers to aid mineral absorption (*2*). CPP are also used in the pharmaceutical industry included in nonfood matrices for the treatment and/or prevention of dental disease (*3*).

The significance of the interaction between CPPs and mineral, in particular for enhancing mineral absorption at the intestinal level, is a controversial issue due to the diversity of the experimental approaches used as well as variations in the methodologies used to assess mineral bioavailability (*4*). For example *in vitro* and *in vivo* animal studies have confirmed a positive effect of CPP on calcium absorption (*5*−*7*) while other studies failed to find an effect in rats (*8*) and piglets (*9*). The difficulty to control all the factors affecting mineral absorption (food matrix, mineral dose, CPP preparation, CPP dose, and CPP/mineral ratio, physicochemical environment) justifies the controversial results from the literature. In addition, the CPP-promoting effect on calcium concentration in intestinal cells was proven to depend on the structural conformation conferred both by the "phosphoserine cluster" and the preceding N-terminal portion (*10*).

To ensure mineral bioavailability CPPs have to be close to the epithelial cells that absorb minerals. CPPs have been found in the intestinal lumen after *in vitro* and *in vivo* digestion (*11*). They were detected in a number of *in vivo* animal studies after ingestion of casein (*8*, *12*−*14*) and crude CPP preparations (*15*) thus demonstrating that CPPs are produced and found naturally following ingestion of casein-containing diets. Analysis of the duodenal content of adult humans after ingestion of milk and fermented products showed that CPP, at least partly, are produced and survive intestinal transit after ingestion of dairy food (*16*). Furthermore, part of the CPPs generated during the digestion in the small intestine of rats fed casein was not hydrolyzed in the digestive tract but was excreted into the feces (*12*). All these results justify that it is generally assumed that CPP from caseins are rather resistant to further hydrolysis by digestive enzymes. However further investigation of the hydrolysis of mineral carrier CPPs is needed to define what makes CPPs resistant to enzymatic digestion at the intestine level. Controversial results exist in the literature with respect to the absorption mainly due to (i) change in CPP conformation according to the amino acids sequence and (ii) the involvement of other residues, non-phosphorylated, in the mineral binding (*10*). In the present investigation we used the well-characterized phosphorylated N-terminal fragment of β-casein, namely, the β-CN (1−25) peptide, carrying various minerals to address the question whether phosphorylation and/or

*Corresponding author. Tel: +33 2 23 48 53 46. Fax: +33 2 23 48 53 50. E-mail address: Rachel.Boutrou@rennes.inra.fr.

Published on Web 06/02/2010

mineral binding induces resistance of CPPs to intestinal hydrolysis. An important step in the intestinal processing of peptides derived from food protein takes place at the brush-border membrane (BBM) level. This membrane which covers the entire surface of the small intestine contains many hydrolytic enzymes and transport systems involved in the final digestion and nutrient absorption. The brush border enzymes face outward from the membrane into the intestinal lumen, to hydrolyze peptides that come into contact with the epithelial cell surface. We used intestinal brush-border membrane vesicles (BBMV) that possess the intestinal enzymatic cocktail, especially peptidases and alkaline phosphatase, to digest different forms of the β-casein (1−25) peptide: unphosphorylated, phosphorylated carrier of sodium, calcium, zinc or copper. The kinetics of digestion were assessed and the products of digestion were identified using tandem mass spectrometry.

MATERIALS AND METHODS

Preparation of the Different Forms of β-CN (1−25) Peptide. Phosphorylated β-casein (1−25)−calcium complex, subsequently called β-CN (1−25)P−Ca, was prepared by limited hydrolysis of β-casein as previously described by Léonil et al. (*17*) through the action of trypsin novo (Gist-brocades, The Netherlands). Briefly β-casein was isolated from sodium caseinate (Armor Proteins, Saint-Brice en Coglès, France) as previously described (*18*). β-Casein solution was hydrolyzed for 3 h at pH 7.5 and 40 °C at an enzyme/substrate ratio of 1:1000 (w:v). The reaction was stopped by adding 1 M HCl until reaching pH 4.6. At this pH the nonhydrolyzed casein fraction precipitates (1 h at room temperature). After centrifugation (12000*g*, 20 min, 20 °C), the supernatant was filtered through Whatman 41 (Laboratoires Humeau, la Chapelle sur Erdre, France). Phosphopeptides were precipitated with 10% $CaCl_2$ (w/v) equivalent to 20 mol of calcium/mol of β-casein and 50% ethanol, under magnetic agitation during 1 h at 30 °C. The pellet (centrifugation 12000*g*, 20 min, 20 °C) was solubilized in distilled water and dialyzed through 1000 Da cutoff membrane tubing (Medicell International Ltd., London, U.K.) for 72 h at 4 °C against distilled water at pH 7.0.

β-CN (1−25)P−Na, used as control peptide, was prepared by dialysis of the β-CN (1−25)P−Ca solution through cutoff 500 Da membrane tubing (Spectra/Por, Medicell International Ltd., London, U.K.) for 48 h at 4 °C against distilled water at pH 3.5 to remove calcium. The pH was adjusted to 7.0 by addition of 1 M NaOH during 1 h incubation under agitation.

β-CN (1−25)P−Ca was solubilized at 10 $g \cdot L^{-1}$ to prepare β-CN (1−25)P−Zn and β-CN (1−25)P−Cu complexes. The solution was temperate at 30 °C before addition of $ZnCl_2$ or $CuCl_2$ solution whose pH was previously adjusted to 6.5 by addition of 1 M NaOH; 4 mol of cation was added per mol of β-CN (1−25)P. After 1 h incubation under agitation, the solution was diafiltered (spiral cellulose 3 kDa cutoff membrane (Amicon, Lexington, MA) under water; 1 bar at 40 °C) to remove unbound cation. The amount of Zn and Cu complexed with β-CN (1−25) was determined by atomic absorption spectrometry (model AA 1275; Varian, F-91941 Les Ulis, France).

Non-phosphorylated β-CN (1−25), subsequently called β-CN (1−25)-unP, was prepared from β-CN (1−25)P−Ca through the action of acid phosphatase from potatoes (EC 3.1.8.2) (2 U/mg; Sigma, St Louis, MO) used at an enzyme/substrate ratio of 1:10 (w:w) in 0.1 M sodium acetate buffer pH 5.8, at 37 °C during 3 h. The reaction was stopped by addition of 3% trifluoroacetic acid (TFA, trifluoroacetic acid, Pierce, Touzart et Matignon, Vitry sur seine, France).

All the obtained solutions were freeze-dried. A blue powder was obtained in the case of the β-CN (1−25)P−Cu.

Preparation of Brush-Border Membrane Vesicles (BBMV). BBMV from the ileum of a freshly killed pig were prepared as described by Boutrou et al. (*19*). Purification and enrichment of the BBMV was checked by determination of the marker enzymes alkaline phosphatase (EC 3.1.3.1) and dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5). Samples were diluted 1:100 in 0.1 M sodium carbonate buffer pH 9.4 and mixed to an equal volume of *p*-nitrophenyl phosphate. The absorbance at 405 nm was measured each min during 10 min to determine the activity. To measure the activity of DPP IV, samples were diluted 1:80 in 0.02 M Tris-HCl buffer pH 7.5. Fifty microliters was incubated with 50 μL of 0.66 mM Phe-Pro β-naphthylamide at 37 °C. The reaction was stopped by adding 50 μL of a mixture containing 1 $mg \cdot mL^{-1}$ Fast Garnet, 10% (v:v) Triton X-100 and 1 M sodium acetate pH 4.0 after 0, 5, 10, 15, and 20 min, and the absorbance at 550 nm was measured. Protein concentration was determined by using the Bradford reagent (Sigma) with bovine serum albumin as standard. The specific alkaline phosphatase and DPP IV activities were 19.6- and 17.0-fold enriched, respectively, in the final BBMV fraction.

Hydrolysis of β-CN (1−25) Peptides by BBMV Enzymes. Digestion of the different forms of β-CN (1−25) peptide was performed at 37 °C in 35 mM Hepes-Tris buffer, 0.15 M KCl pH 7.0. Digestion was started by mixing equal volume of substrate solution (5 g/L) and BBMV preparation diluted 1:10 (v:v) in Hepes-Tris buffer. At selected times 0.3 mL samples were withdrawn and the reaction was stopped by removing the BBMV (centrifugation at 2000*g* for 1 min). The supernatants were stored at −20 °C until analysis. A blank sample was obtained by replacing the substrate with buffer. A control was obtained by replacing BBMV preparation with buffer.

Measure of Decrease of Substrate Amount Using RP-HPLC. To monitor the decrease of the amount of substrate throughout hydrolysis, the samples were analyzed by reversed-phase HPLC on a Waters HPLC system fitted with Waters 2695 separation module and UV Waters 2487 dual absorbance detector (214 and 280 nm). One hundred microliters of digested substrate was diluted 1:3 (v:v) in solvent A (0.106% TFA (v:v) in Milli-Q water), and 100 μL was injected onto a C18 Vydac column (250 × 4.6 mm i.d., Touzart et Matignon, Vitry sur seine, France). The elution was performed at a flow rate of 1 $mL \cdot min^{-1}$ at 40 °C, with a linear gradient from 5% to 80% of solvent B (0.1% TFA (v:v) and 80% acetonitrile (v:v) in Milli-Q water) for 24 min. The peak height of each substrate was determined.

Assessment of Peptide Hydrolysis. Global digestion was determined by measuring free amino groups with trinitrobenzenesulfonic acid. After 1:2 dilution in distilled water, 10 μL of the digested substrate was added to 100 μL of 1 M potassium borate, pH 9.2, and 40 μL of 1.2 g/L of trinitrobenzenesulfonic acid and the sample was incubated for 1 h at 37 °C. The absorbance at 405 nm was measured using a microplate spectrophotometer (SpectraMax M2, Molecular Devices, Paris, France), and free amino groups were quantified with glycine as the standard.

Free amino acids (AAs) produced throughout digestion were determined after precipitation of peptides with 3% sulfosalicylic acid and centrifugation at 3000*g* for 10 min. The supernatant was filtered through a 0.45 μm filter and injected on a Pharmacia LKB-Alpha Plus series 2 amino acid analyzer (Pharmacia Biotech., Saclay, France). The concentration of each of the 20 free AAs analyzed was summed to estimate the total amount of free AAs.

Identification of Peptides by Nano LC/MS−MS. All mass spectra were performed using a hybrid quadrupole time-of-flight (Q/TOF) mass spectrometer QStar XL (MDS Sciex, Toronto, Canada). The instrument was calibrated with a multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein, β-CN (193−209) (NeoMPS S.A., Strasbourg, France). After 1:20 dilution in 0.1% TFA, the peptide fraction (10 μL) was trapped onto a micro-pre-column cartridge C_{18} PepMap 100 (300 μm i.d. × 5 mm, Dionex) before separation of peptides onto a column C_{18} PepMap 100 (75 μm i.d. × 150 mm, Dionex). The separation started with 10% solvent B for 5 min, and a linear gradient from 10 to 60% solvent B for 55 min was performed at a flow rate of 200 nL/min. Solvent A contained 2% acetonitrile, 0.08% formic acid and 0.01% TFA in LC grade water; and solvent B contained 95% acetonitrile, 0.08% formic acid and 0.01% TFA in LC grade water.

The online separated peptides were analyzed by ESI Q/TOF in positive ion mode. An optimized voltage of 3.5 kV was applied to the nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). MS and MS/MS data were acquired in continuum mode. Data-direct analysis was employed to perform MS/MS analysis on 1+ to 4+ charged precursor ions. Precursor selection was based upon ion intensity, charge state and if the precursors had been previously selected for fragmentation they were excluded for the rest of the analysis. Spectra were collected in the selected mass range 350−2000 m/z for MS spectra and 60−2000 m/z for MS/MS.

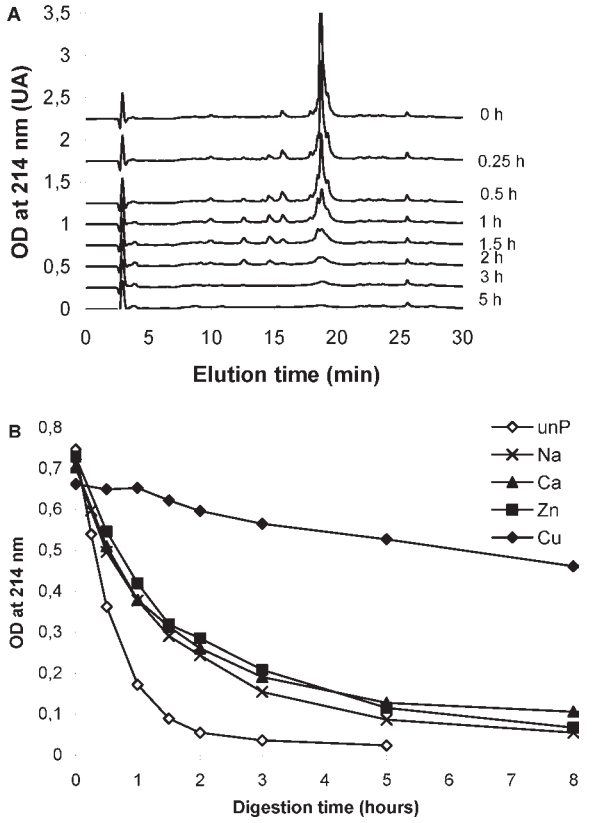

Figure 1. RP-HPLC profiles of non-phosphorylated β-CN (1−25) (**A**). Decrease of peak height was used to determine the half-life ($t_{1/2}$) of each substrate (**B**).

The mass spectrometer was operated in data-dependent mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software (Applied Biosystems, Framingham, MA) when the intensity of the ions was above 5 cps. To identify peptides, all data (MS and MS/MS) were submitted to MASCOT (v.2.2). The search was performed against a homemade database dealing with major milk proteins which represents a portion of the Swissprot database (http://www.expasy.org). No specific enzyme cleavage was used, and the peptide mass tolerance was set to 0.2 Da for MS and 0.1 Da for MS/MS. Phosphorylation on serine and threonine residues were selected as a variable modification. For each peptide identified, a minimum MASCOT score corresponding to a p value < 0.05 was considered as a prerequisite for peptide validation with a high degree of confidence. The attribution of the phosphorylation site by the software Mascot after tandem mass spectrometry analysis can be complicated if incomplete ion series are obtained, thus sometimes giving multiple phosphoserine candidates.

RESULTS

Before presentation of the results, it is noteworthy that at neutral pH the phosphoserine cluster of CPP is negatively charged and thus cations naturally bind to this cluster. Consequently, "phosphorylation" and "mineral binding" cannot be considered as independent events and the phosphorylated β-CN (1−25) peptide without cation does not exist per se. The mineral binding is lower for monovalent cation than for divalent ones. This is the reason why we used the β-CN (1−25)P−Na as a control.

The RP-HPLC profiles allowed visualizing the substrate breakdown throughout the digestion time. The non-phosphorylated β-CN (1−25) peptide (peak eluted between 18.5 and 19.5 min) totally disappeared after three hours of digestion; it was hydrolyzed into numerous peaks (**Figure 1A**). All peaks eluted at the time below that of β-CN (1−25)-unP peptide increased in intensity up to 2 h of digestion, with the appearance of new peaks in the medium part of the chromatogram (eluted in the range 8−16 min). These peaks themselves disappeared and no peak was visible anymore after 5 h of digestion. The change of RP-HPLC profiles was similar for the phosphorylated substrates; however the peak of the initial substrate decreased slower and the neoformed peaks differed (results not shown).

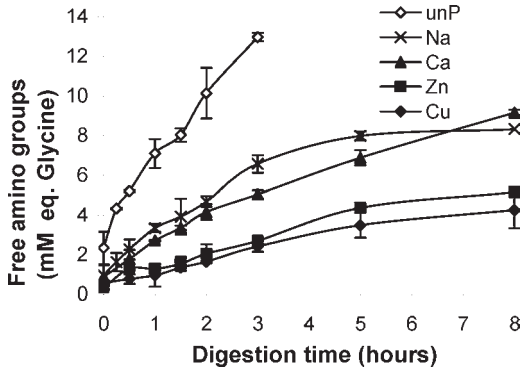

Figure 2. Determination of free amino groups in the hydrolysate from the non-phosphorylated β-CN (1−25) peptide (unP) and the phosphorylated peptide carrying different cations: β-CN (1−25)P−Na (Na), β-CN (1−25)P−Ca (Ca), β-CN (1−25)P−Zn (Zn) and β-CN (1−25)P−Cu (Cu) throughout the hydrolysis by enzymes of BBMV.

The disappearance of each substrate was estimated using peak height as determined by RP-HPLC. The peak height decreased exponentially, except for β-CN (1−25)P−Cu peptide (**Figure 1B**). The peak height of β-CN (1−25)-unP decreased faster than the one of all the phosphorylated substrates. Decrease was similar for β-CN (1−25)P−Na, β-CN (1−25)P−Ca and β-CN (1−25)P−Zn. The half-life $t_{1/2}$, i.e. the time required for the disappearance of one-half of the substrate during digestion, was determined from the curves whereas the equation of the linear decrease ($y = -0.026x + 0.06605$) was used to calculate the half-life of β-CN (1−25)P−Cu peptide. Half-life values of 0.42, 1.16, 1.23, 1.26, and 12.7 h were determined for β-CN (1−25)-unP, β-CN (1−25)P−Na, β-CN (1−25)P−Ca, β-CN (1−25)P−Zn and β-CN (1−25)P−Cu peptides, respectively.

Measure of free amino groups, representative of the number of peptide bonds cleaved, evaluated the degree of hydrolysis. For all the substrates, the quantity of free amino groups increased throughout digestion time (**Figure 2**). However after 2 h of digestion, the quantity was twice higher for the non-phosphorylated substrate than for β-CN (1−25)P−Na and β-CN (1−25)P−Ca peptides themselves twice higher than for β-CN (1−25)P−Zn and β-CN (1−25)P−Cu peptides. In addition, the kinetics of hydrolysis of the non-phosphorylated peptide was 2.5 to 20 times faster than the one of β-CN (1−25)P−Na and P−Cu peptides, respectively. It was similar for β-CN (1−25)P−Na and β-CN (1−25)P−Ca, both being faster than the β-CN (1−25)P−Zn and β-CN (1−25)P−Cu ones. Free AAs were also continuously released during the 8 h of digestion (**Figure 3**). As for the free amino groups, kinetics of the free AAs release was the fastest for β-CN (1−25)-unP and similar for β-CN (1−25)P−Na and β-CN (1−25)P−Ca. During the digestion, the concentration of free AAs was lower for β-CN (1−25)P−Zn than for β-CN (1−25)P−Ca and β-CN (1−25)P−Na, and it was the lowest for β-CN (1−25)P−Cu.

The peptides released throughout the hydrolysis of the β-CN (1−25)P−Ca peptide were identified (**Figure 4**). The β-CN (1−25)P−Ca peptide was concomitantly dephosphorylated and proteolyzed throughout the digestion. The action of the alkaline phosphatase was visible through dephosphorylation of the peptide; there seem to be no preferred phosphorylated sites. Indeed

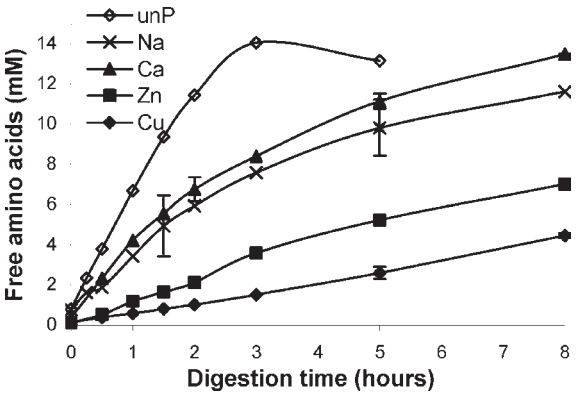

Figure 3. Determination of free amino acids in the hydrolysate from the non-phosphorylated β-CN (1−25) peptide (unP) and the phosphorylated peptide carrying different cations: β-CN (1−25)P−Na (Na), β-CN (1−25)P−Ca (Ca), β-CN (1−25)P−Zn (Zn) and β-CN (1−25)P−Cu (Cu) peptides throughout the hydrolysis by enzymes of BBMV.

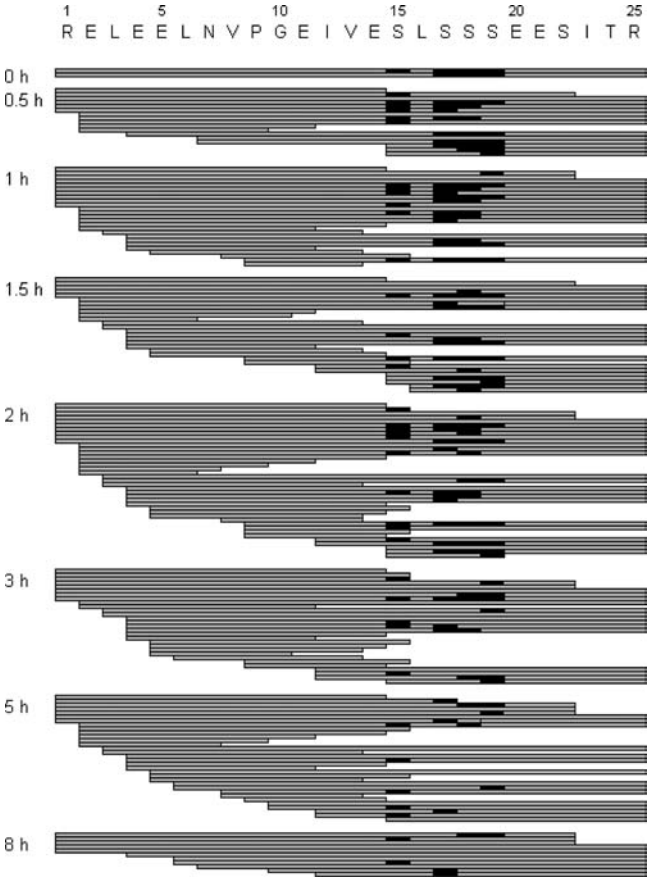

Figure 4. Amino acid sequence of the β-CN (1−25) peptide and the peptides identified from the hydrolysis of the β-CN (1−25)P−Ca peptide at 0, 0.5, 1, 1.5, 2, 3, 5, and 8 h of digestion using tandem mass spectrometry. The black boxes correspond to the phosphoserine residues.

the phosphorylated residue Sp_{15} was not preferentially dephosphorylated compared to the three Sp within the triad, and none of the Sp within the triad was first dephosphorylated. The action of the alkaline phosphatase was not complete after 8 h of digestion, and peptides with one or two phosphorylated residues were still identified. It is of note that automatic identification via tandem mass spectrometry and MASCOT database search cannot be performed on peptides smaller than 5 amino acids which are thus "invisible" with this approach. With respect to the activity of

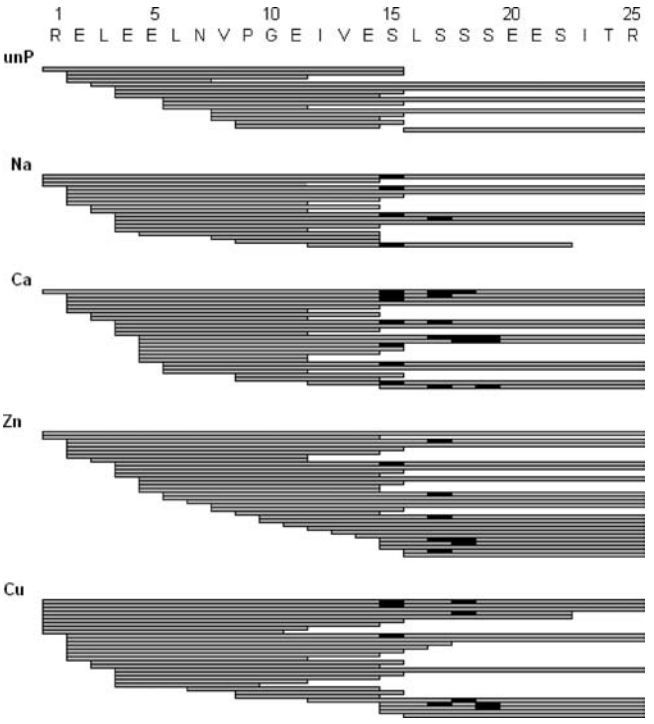

Figure 5. Amino acid sequence of the β-CN (1−25) peptide and the peptides identified after two hours digestion of the non-phosphorylated β-CN (1−25) peptide (unP) and the phosphorylated peptide carrying different cations: β-CN (1−25)P−Na (Na), β-CN (1−25)P−Ca (Ca), β-CN (1−25)P−Zn (Zn) and β-CN (1−25)P−Cu (Cu) peptides using tandem mass spectrometry. The black boxes correspond to the phosphoserine residues.

the peptidases, the sequence alignment of the hydrolyzed peptides showed the action of both endo- and exopeptidases. The N-terminal end of the peptide was first cleaved through the action of aminopeptidases that released one or two amino acids while an endopeptidase cleaved the peptidic bonds $E_{11}-I_{12}$ and $E_{14}-S_{15}$. Consecutively to the action of the endopeptidase, the amino- and carboxypeptidases went on to hydrolyze the β-CN (1−14)P−Ca peptide, and rather all the peptidic bonds within this fragment were cleaved after 5 h of digestion. In contrast, both types of exopeptidases were almost inactive within the β-CN (15−25)P−Ca fragment. Only the $S_{22}-I_{23}$ bond was cleaved at the C-terminal end of the β-CN (1−25)P−Ca peptide throughout the digestion, thus generating the β-CN (1−22) fragment. The activity of peptidases seems to be influenced by the phosphorylation degree because exopeptidases hydrolyzed preferentially the non-phosphorylated β-CN (1−15) region of the peptide and almost none of the β-CN (12−25) region, regardless the sites and number of phosphorylation.

The peptides released throughout the hydrolysis of the diverse forms of the β-CN (1−25) peptide at two hours digestion were identified (**Figure 5**). These samples have been analyzed in an other set of experiments to compare the pattern of peptides generated from the diverse substrates; the hydrolysis patterns from the β-CN (1−25)P−Ca (**Figures 4** and **5**) cannot be compared because the conditions of analyses were not quite identical. Similar peptides were identified from the different forms of the peptide. The peptidic bonds $E_{11}-I_{12}$ and $E_{14}-S_{15}$ were cleaved in the non-phosphorylated peptide and in all the phosphorylated peptides regardless of the mineral bound. In addition, the peptidic bond $S_{15}-L_{16}$ was noticeably cleaved only when the residue S_{15} was no more phosphorylated. This result showed that the activity of the endopeptidase depends on phosphorylation.

It is noteworthy that the β-CN (15−25) fragment was not identified from the digestion of either the unphosphorylated peptide or the β-CN (1−25)P−Na peptide. Also remarkable is that the C-terminal end of the peptide was hydrolyzed only in the β-CN (1−25)P−Cu peptide where the peptidic bonds $L_{16}-S_{17}$ and $S_{17}-S_{18}$ were cleaved.

DISCUSSION

The N-terminal CPP issued from the hydrolysis of β-casein is one of the main CPPs produced *in vivo* during digestion of casein and milk products (*8*, *12*, *14*). As all CPPs arisen from casein hydrolysis, the β-CN (1−25) peptide has the ability to bind and keep soluble diverse minerals, thus enhancing their absorption (*20*−*23*). We hypothesize that phosphorylation and/or mineral bound to phosphoserine clusters hinders CPP hydrolysis by intestinal enzymes, thus enhancing the bioavailability of minerals. The aim of the present study was to define the mechanisms by which CPPs carrying mineral are hydrolyzed through the action of intestinal proteases and alkaline phosphatase. The hydrolysis of casein yields several CPPs which differ in amino acid composition, charge and hydrophobicity (*24*). To avoid conflicting results previously obtained using a mixture of CPP produced from the hydrolysis of whole casein (*25*) we used in the present study a pure CPP from β-casein. Using different forms of the pure β-CN (1−25) peptide allowed access to the effect of phosphorylation and mineral binding on peptide hydrolysis. In the present study, we evaluated the intestinal hydrolysis of the β-CN (1−25) peptide in its non-phosphorylated form and phosphorylated forms bound to Na, Ca, Zn or Cu cations.

The results showed that phosphorylations prevent CPP from intestinal enzymatic hydrolysis. Although all the substrates were hydrolyzed by the enzymes from intestinal brush-border membrane, the kinetics of hydrolysis of the β-CN (1−25) peptide was much faster for the non-phosphorylated form than for the phosphorylated forms, regardless of the type of mineral bound. Indeed, the calculated half-life was at least three times higher for the phosphorylated peptides than for the non-phosphorylated one. The quantity of the different substrates gradually decreased (**Figure 1B**) while the quantity of free amino groups and free AAs increased (**Figures 2** and **3**). The quantities as well as the rates of release of both free amino groups and free AAs were the highest for the non-phosphorylated peptide while those of the β-CN (1−25)P−Na peptide considered as control sample were twice lower. This result suggests that phosphorylations hinder the action of peptidases, probably by steric hindrance because the major phosphorylation sites in bovine caseins are hydrophilic and correspond to a loop structure which could include only β-turn as regular secondary structure (*26*). This result also fit to the accepted idea that phosphorylations hinder CPP from hydrolysis through the action of peptidases until it reaches epithelial cells where phosphatase releases mineral which is subsequently absorbed. For example the enzymatic dephosphorylation of the β-CN (1−25)P−Fe complex, which was slower than that of the β-CN (1−25)P−Ca, rendered the iron initially bound to the phosphopeptide entirely dialyzable (*18*). Thus it appeared that the release of insoluble iron by alkaline phosphatase was prevented by providing iron in the β-CN (1−25)P−Fe complex form (*21*).

Among the digestive enzymes identified from human and mouse intestinal BBM (*27*, *28*), alkaline phosphatase is the enzyme that hydrolyzes phosphate linkages. From the patterns of hydrolysis of the β-CN (1−25)P−Ca peptide we deduced that the activity of alkaline phosphatase seems to be not specific to the phosphorylation degree and the phosphorylation sites. We observed that the β-CN (1−25)P−Ca peptide was not totally dephosphorylated after 8 h of digestion. This may be due to the BBMV/peptide ratio used in this study that was evaluated in a preliminary study to be able to elucidate the mechanisms of CPP hydrolysis by brush-border membrane enzymes. Hence the implied enzymes are actually the ones that digest peptides *in vivo*, but the excess of peptide over enzymes certainly prevents the complete digestion of the β-CN (1−25) peptide. In addition, alkaline phosphatase is present at the surface of the intestinal BBMV, but it hardly cleaves phosphate groups bound to peptide. On one hand an explanation could be that alkaline phosphatase is anchored in the apical microvilli of brush-border membrane of the epithelial cells and thus hardly accessible to the substrates (*29*). On the other hand the *in vivo* neutral pH is lower than the pH required for optimum activity of the alkaline phosphatase. Thus, the lack of activity of intestinal phosphatase which is an alkaline phosphatase prevents CPP hydrolysis thereby favoring mineral carriage and absorption at the intestinal level.

Studying the mechanism of hydrolysis of the different forms of the β-CN (1−25) peptide by intestinal peptidases allows understanding of the observed differences in the kinetics of hydrolysis. The mechanism of CPP hydrolysis can be deduced from the peptides identified throughout the digestion (**Figure 4**) and give information on the potential enzymes involved. BBM expresses at least 15 peptidases that are involved in peptide hydrolysis (*30*), and among them various classes of peptidases have been detected in human intestinal BBM (*31*): endopeptidases, aminopeptidases and carboxypeptidases, and the dipeptidyl peptidase IV. For β-CN (1−25)P−Ca, aminopeptidases early cleaved the N-terminal AAs while the C-terminal end of the peptide remained quite noncleaved. An endopeptidase, certainly the EC 3.4.24.11 one (*30*), cleaved the bond $E_{14}-S_{15}$, and consecutively carboxypeptidases cleaved the fragment β-CN (1−14). It is noteworthy that the region β-CN (15−25), i.e. the phosphorylated part of the peptide, was scarcely hydrolyzed; it was slowly dephosphorylated throughout the digestion. An important point is that the mechanism of hydrolysis that is described in details in the Results section matches for the different forms of the β-CN (1−25) peptide used in the present study. Indeed the identification of the peptides generated from all the different forms of the β-CN (1−25) peptide at 2 h digestion indicated that the mechanism of hydrolysis was qualitatively similar. This result is not surprising since the different forms of the β-CN (1−25) peptide differed in terms of the nature of the cation bound but possess the same residue sequence which was hydrolyzed through the action of the same enzymes from BBMV. In addition the mechanism defined in the present study is in agreement with the results of Bouhallab et al. (*32*) who have shown that β-CN (1−25)P−Ca is sensitive to digestive enzymes including proteases/peptidases and phosphatases during *in situ* duodenal transit in rats. It is of note that peptidases are external components of the brush-border membrane of the epithelial cells and thus are more prone to hydrolyze peptides than alkaline phosphatase (*29*). Our results obtained using different forms of the β-CN (1−25) peptide reflect this differential accessibility of the peptide to the enzymes of the brush-border membrane. They also reinforced the idea that partial hydrolysis of the peptide bonds exposes Sp residues to attack by intestinal alkaline phosphatase (*33*).

Considering the release time course of free amino groups and free AAs throughout the digestion also informs about the hydrolysis mechanism of the different forms of the peptide through the activity of endo- and exopeptidases. Both exopeptidases concomitantly hydrolyzed the non-phosphorylated peptide that was consequently the fastest degraded in free AAs. In contrast the action of both endo- and exopeptidases on phosphorylated peptides was quantitatively limited. Furthermore, regarding the different phosphorylated forms, the kinetics of

release of free AAs was exponential for the β-CN (1−25) peptide bound to sodium or calcium whereas it was linear for β-CN (1−25) peptide bound to zinc or copper. This result shows that the action of both exo- and endopeptidases was hindered for the latter peptides. The inhibition of activity for endo- and/or exopeptidases observed using the phosphorylated β-CN(1−25) peptide bound to different cations was in accordance with the affinity order Ca < Zn < Cu estimated by Baumy and Brulé (*34*) when they have studied the binding of bivalents cations to β-casein.

Our results showed that, in addition to phosphorylation, binding cation also prevents CPP from hydrolysis. However, some cations better prevent CPP from hydrolysis than others, as suggested by the higher half-life of β-CN (1−25)P−Cu ($t_{1/2}$ 12.7 h) compared to the one of β-CN (1−25)P−Ca and β-CN (1−25)P−Zn ($t_{1/2}$ close to 1.2 h). Among all the phosphorylated β-CN (1−25) peptides used in the present study, the β-CN (1−25)P−Cu peptide was rather not hydrolyzed during the eight hours of digestion. This result demonstrates that the type of bound cations deeply modifies the intestinal enzyme action. From our result we can extrapolate that in particular binding cations such as copper limited the action of both phosphatase and peptidases. Calcium and zinc are bound to CPP through ionic bonds whereas copper is bound through ionic bonds with phosphoserine plus coordination bonds to NH_2, CO_2H and CONH (*34*). From our data, coordination bond of mineral to CPP is able to inhibit the action of both phosphatase and peptidases. The very low hydrolysis observed for the β-CN (1−25)P−Cu peptide may be true for other coordinated cations such as iron. Indeed a MS analysis of the products of intraluminal digestion of Fe−β-CN (1−25) peptide suggested that the phosphorylated 15−25 fragment of β-CN-(1−25) which contains the four Fe bound phosphoserine is resistant to proteinases (*35*). It is now interesting to note that the lumen contents of rats perfused with iron free β-CN (1−25) contained all peptidic sequences derived from β-CN (1−25) peptide while the phosphorylated part of β-casein (1−25), i.e. β-casein (15−25) was not detected in lumen of rats perfused with iron−β-casein (1−25) complex as suggested by the results of Bouhallab et al. (*32*). These results allow increasing about the knowledge that some minerals can cause changes in conformation, and thus alter (inhibit/accelerate) the enzymatic attack on the protein and on the peptide and to specify that in particular the minerals coordinated to CPP inhibited the proteolysis and the dephosphorylation of peptide.

In conclusion, by studying the hydrolysis of different forms of the β-casein (1−25) peptide, we demonstrated that first phosphorylations and second coordination link of mineral induced the CPP resistance to intestinal enzymes. Post-translational modifications in general could represent a protective mechanism for food-derived bioactive peptides. Indeed, as phosphorylations do, glycosylations inhibit the enzymatic action on peptides. Indeed digestion product yields of glycosylated peptides were lower than those observed for the unglycated substrate (*19*). The resistance of the post-translational modified proteins and the derived peptides to hydrolysis by intestinal enzymes raises questions about their further processing and their potential resistance to colonic enzymes.

ABBREVIATIONS USED

AA, amino acids; BBM, brush-border membrane; BBMV, brush-border membrane vesicles; Ca, calcium; Cu, copper; CPP, caseinophosphopeptide; CN, casein; ESI, electrospray ionization; HPLC, high performance liquid chromatography; NA, sodium; MS, mass spectrometry; TFA, trifluoroacetic acid; Zn, zinc.

ACKNOWLEDGMENT

Saïd Bouhallab and Fréderic Gaucheron (INRA, UMR1253 STLO, Rennes, France) are acknowledged for interesting and helpful discussions about complexes formed by phosphopeptides and minerals.

LITERATURE CITED

(1) Meisel, H. Overview on milk protein-derived peptides. *Int. Dairy J.* **1998**, *8*, 363–373.
(2) Phelan, M.; Aherne, A.; Fitzgerald, R. J.; O'Brien, N. M. Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *Int. Dairy J.* **2009**, *19*, 643–654.
(3) Cross, K. J.; Huq, N. L.; Reynolds, E. C. Casein phosphopeptides in oral health - Chemistry and clinical applications. *Curr. Pharm. Des.* **2007**, *13*, 793–800.
(4) Korhonen, H.; Pihlanto, A. Bioactive peptides: Production and functionality. *Int. Dairy J.* **2006**, *16*, 945–960.
(5) Erba, D.; Ciappellano, S.; Testolin, G. Effect of caseinphosphopeptides on inhibition of calcium intestinal absorption due to phosphate. *Nutr. Res.* (*N.Y.*) **2001**, *21*, 649–656.
(6) Mykkanen, H. M.; Wasserman, R. H. Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *J. Nutr.* **1980**, *110*, 2141–2148.
(7) Sato, R.; Noguchi, T.; Naito, H. The necessity for the phosphate portion of casein molecules to enhance Ca absorption from the small intestine. *Agric. Biol. Chem.* **1983**, *47*, 2415–2417.
(8) 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.
(9) Pointillart, A.; Gueguen, L. Absence d'effet de l'incorporation d'un phosphopeptide du lait sur l'utilisation du calcium et du phosphore chez le jeune porc. *Reprod. Nutr. Dev.* **1989**, *29*, 477–486.
(10) 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.
(11) Silva, S. V.; Malcata, F. X. Caseins as source of bioactive peptides. *Int. Dairy J.* **2005**, *15*, 1–15.
(12) Kasai, T.; Iwasaki, R.; Tanaka, M.; Kiriyama, S. Caseinphosphopeptide (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. *Biosci,. Biotechnol., Biochem.* **1995**, *59*, 26–30.
(13) Lee, Y. S.; Noguchi, T.; Naito, H. Intestinal absorption of calcium in rats given diets containing casein or amino acid mixture: the role of casein phosphopeptides. *Br. J. Nutr.* **1983**, *49*, 67–76.
(14) Hirayama, M.; Toyota, K.; Hidaka, H.; Naito, H. Phosphopeptides in rat intestinal digests after ingestion casein phosphopeptides. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1128–1129.
(15) 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*, 351–359.
(16) Chabance, B.; Marteau, P.; Rambaud, J. C.; Miglioresamour, D.; Boynard, M.; Perrotin, P.; Guillet, R.; Jolles, P.; Fiat, A. M. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **1998**, *80* (2), 155–165.
(17) Léonil, J., Nau, F., Mollé, D., Maubois, J. L., INRA. Procédé d'obtention, à partir de la caséine beta, de fractions enrichies en peptides à activité biologique et les fractions peptidiques obtenues. Patent number 2650955, 1991, pp 1−35.
(18) Bouhallab, S.; Léonil, J.; Maubois, J. L. Complexation du fer par le phosphopeptide (1−25) de la caseine beta: action de l'alcalase et de la phosphatase acide. *Lait* **1991**, *71*, 435–443.
(19) Boutrou, R.; Jardin, J.; Blais, A.; Tomé, D.; Léonil, J. Glycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not exointestinal brush border membrane peptidases. *J. Agric. Food Chem.* **2008**, *56*, 8166–8173.
(20) Ait-Oukhatar, N.; Peres, J. M.; Bouhallab, S.; Neuville, D.; Bureau, F.; Bouvard, G.; Arhan, P.; Bougle, D. Bioavailability of caseinophosphopeptide-bound iron. *J. Lab. Clin. Med.* **2002**, *140*, 290–294.

(21) Ani-Kibangou, B.; Bouhallab, S.; Mollé, D.; Henry, G.; Bureau, F.; Neuville, D.; Arhan, P.; Bouglé, D. Improved absorption of caseinophosphopeptide-bound iron: role of alkaline phosphatase. *J. Nutr. Biochem.* **2005**, *16*, 398–401.
(22) Peres, J. M.; Bouhallab, S.; Petit, C.; Bureau, F.; Maubois, J. L.; Arhan, P.; Bougle, D. Improvement of zinc intestinal absorption and reduction of zinc-iron interaction using metal bound to the caseinophosphopeptide 1−25 of beta-casein. *Reprod. Nutr. Dev.* **1998**, *38*, 465–472.
(23) Gravaghi, C.; Del Favero, E.; Cantu', L.; Donetti, E.; Bedoni, M.; Fiorilli, A.; Tettamanti, G.; Ferraretto, A. Casein phosphopeptide promotion of calcium uptake in HT-29 cells - relationship between biological activity and supramolecular structure. *FEBS J.* **2007**, *274*, 4999–5011.
(24) Bouhallab, S.; Cinga, V.; Ait-Oukhatar, N.; Bureau, F.; Neuville, D.; Arhan, P.; Maubois, J. L.; Bougle, D. Influence of various phosphopeptides of caseins on iron absorption. *J. Agric. Food Chem.* **2002**, *50*, 7127–7130.
(25) Yeung, A. C.; Glahn, R. P.; Miller, D. D. Effects of iron source on iron availability from casein and casein phosphopeptides. *J. Food Sci.* **2002**, *67*, 1271–1275.
(26) Holt, C.; Sawyer, L. Primary and predicted secondary structures of the caseins in relation to their biological functions. *Protein Eng.* **1988**, *2*, 251–259.
(27) Maestracci, D.; Preiser, H.; Hedges, T.; Schmitz, J.; Crane, K. Enzymes of the human intestinal brush border membrane identification after gel electrophoretic separation. *Biochim. Biophys. Acta* **1975**, *382*, 147–156.
(28) Babusiak, M.; Man, P.; Petrak, J.; Vyoral, D. Native proteomic analysis of protein complexes in murine intestinal brush border membranes. *Proteomics* **2007**, *7*, 121–129.
(29) Maestracci, D. Enzymatic solubilization of the human intestinal brush border membrane enzymes. *Biochim. Biophys. Acta* **1976**, *433*, 469–481.
(30) Woodley, J. F. Enzymatic barriers for GI peptide and protein delivery. *Crit. Rev. Ther. Drug Carrier Syst.* **1994**, *11*, 61–95.
(31) Tobey, N.; Heizer, W.; Yeh, R.; Huang, T.-I.; Hoffner, C. Human intestinal brush border peptidases. *Gastroenterology* **1985**, *88*, 913–926.
(32) Bouhallab, S.; Oukhatar, N. A.; Mollé, D.; Henry, G.; Maubois, J. L.; Arhan, P.; Bougle, D. L. Sensitivity of beta-casein phosphopeptide-iron complex to digestive enzymes in ligated segment of rat duodenum. *J. Nutr. Biochem.* **1999**, *10*, 723–727.
(33) Yeung, A. C.; Glahn, R. P.; Miller, D. D. Dephosphorylation of sodium caseinate, enzymatically hydrolyzed casein and casein phosphopeptides by intestinal alkaline phosphatase: implications for iron availability. *J. Nutr. Biochem.* **2001**, *12*, 292–299.
(34) Baumy, J. J.; Brulé, G. Effect of pH and ionic strength on the binding of bivalent cations to beta-casein. *Lait* **1988**, *68*, 409–418.
(35) Ait-Oukhatar, N.; Bouhallab, S.; Bureau, F.; Arhan, P.; Maubois, J. L.; Bougle, D. L. *In vitro* digestion of caseinophosphopeptide-iron complex. *J. Dairy Res.* **2000**, *67*, 125–129.

Received for review February 10, 2010. Revised manuscript received May 19, 2010. Accepted May 20, 2010.