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Sensitivity of β-casein phosphopeptide-iron complex to digestive enzymes in ligated segment of rat duodenum

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Binding iron to the phosphorylated β(1-25) peptide derived from β-casein improves iron bioavailability in the rat. The aim of the present work was to learn how injected β(1-25) and iron-β(1-25) complex behave in the duodenum of rats using the technique of intestinal ligation in situ and reversed-phase (RP)-high performance liquid chromatography-electrospray mass spectrometry analysis of the lumen contents. The results demonstrate that β(1-25) is sensitive to digestive enzymes including proteases/peptidases and phosphatases during duodenal transit. The lumen contents of rats perfused with iron free β(1-25) contained all peptidic sequences derived from β(1-25). In contrast, the phosphorylated part of β(1-25) [i.e., β(15-24)] was not detected in lumen of rats perfused with iron-β(1-25) complex. (J. Nutr. Biochem. 10:723–727, 1999)

Keywords: iron; caseinophosphopeptide; enzymatic digestion; rat duodenum

Introduction

Phosphopeptides obtained by enzymatic digestion of α_s and β caseins with trypsin are known for their ability to form soluble complexes with different minerals such as calcium, zinc, and iron. PHOSPHOPEPTIDES can be formed in vivo as they have been isolated from the intestinal content of animals fed a high casein diet.[1,2] Several studies have reported that they might exert a positive influence on the intestinal absorption of calcium.[3]

In our previous work, we reported that one of these CCP, the β(1-25) derived from β-casein, can improve the bioavailability of iron in iron-deficient rats.[4] Rats fed iron bound to the β(1-25) [Fe/β(1-25)] complex showed a better improvement of blood parameters of iron status and higher liver storage than $FeSO_4$ group. The positive effect of β(1-25) as a chelator on iron absorption was also observed in situ using the intestinal ligation technique.[5] In the present work, the sensitivity of β(1-25) and of Fe/β(1-25) complex to digestive enzymes in situ was evidenced by RP-high performance liquid chromatography (HPLC)-electrospray mass spectrometry analyses of lumen contents of rats.

Methods and materials

Phosphopeptide β(1-25)

β(1-25) is the phosphorylated N-terminal fragment of β-casein of molecular mass 3124 containing four phosphoseryl residues (*Figure 1*). It chelates four iron atoms per molecule with high affinity.[5] β(1-25) and Fe/β(1-25) complex were prepared as previously reported.[6]

Duodenal ligation experiments

Adult Sprague-Dawley rats weighing 200 to 250 grams were obtained from the farm of the University of Caen. Rats had free access to a standard rat diet: proteins 20%, lipids 4.4%, carbohydrates 56.4%, and minerals and vitamins 5.5%. They were fasted overnight and anesthetized with 25 mg sodium pentobarbital per kilogram body weight. The abdomen was opened and the duodenum was ligated from the pylore to the treitz angle. The loop was then flushed with 3 mL of saline solution at room temperature and ligated on a cannula. Three groups of three rats were studied. The control group was perfused with 1 mL solution without β(1-25) peptide and containing 0.27 mg of iron as $FeSO_4$ salt. The other

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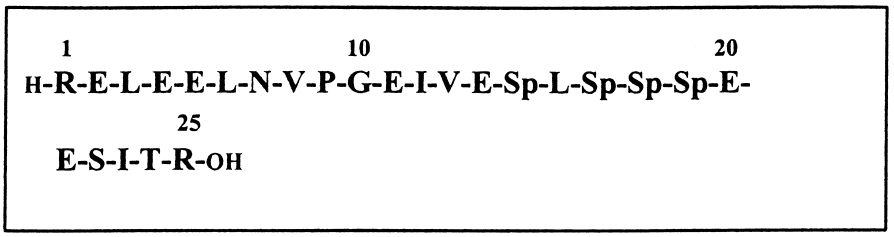

Figure 1 Amino acid sequence of β(1-25), the β-casein phosphopeptide. Sp, phosphoseryl residue.

two groups were perfused with 1 mL solution containing either 3 mg β(1-25) peptide alone or 3 mg β(1-25) complexed to iron (0.27 mg). A 200 μL sample was withdrawn at 15, 45, 60, and 90 minutes following perfusion without volume replacement. After 90 minutes, the loop was emptied and the lumen was flushed with cold saline solution at 4°C. Enzymatic activities were inactivated by heating the samples at 80°C for 10 minutes. The samples were stored at −20°C until analyzed. It was checked that the structure of β(1-25) phosphopeptide was not affected by this heating step.

Analyses

Lumen contents were incubated for 1 hour with 18 mM ethylenedaimine-tertraacetic acid (EDTA) at 25°C to dissociate iron-phosphopeptide complexes. This step was added to improve peptide separation and detection. After centrifugation (3,000 × g, 10 minutes), the supernatants were adjusted to pH 2 with 5% trifluoroacetic acid and analyzed by electrospray mass spectrometry (ESI-MS) working on-line with a HPLC system (Waters 625 LC System; Waters, Milford, MA USA). Chromatographic sepa-

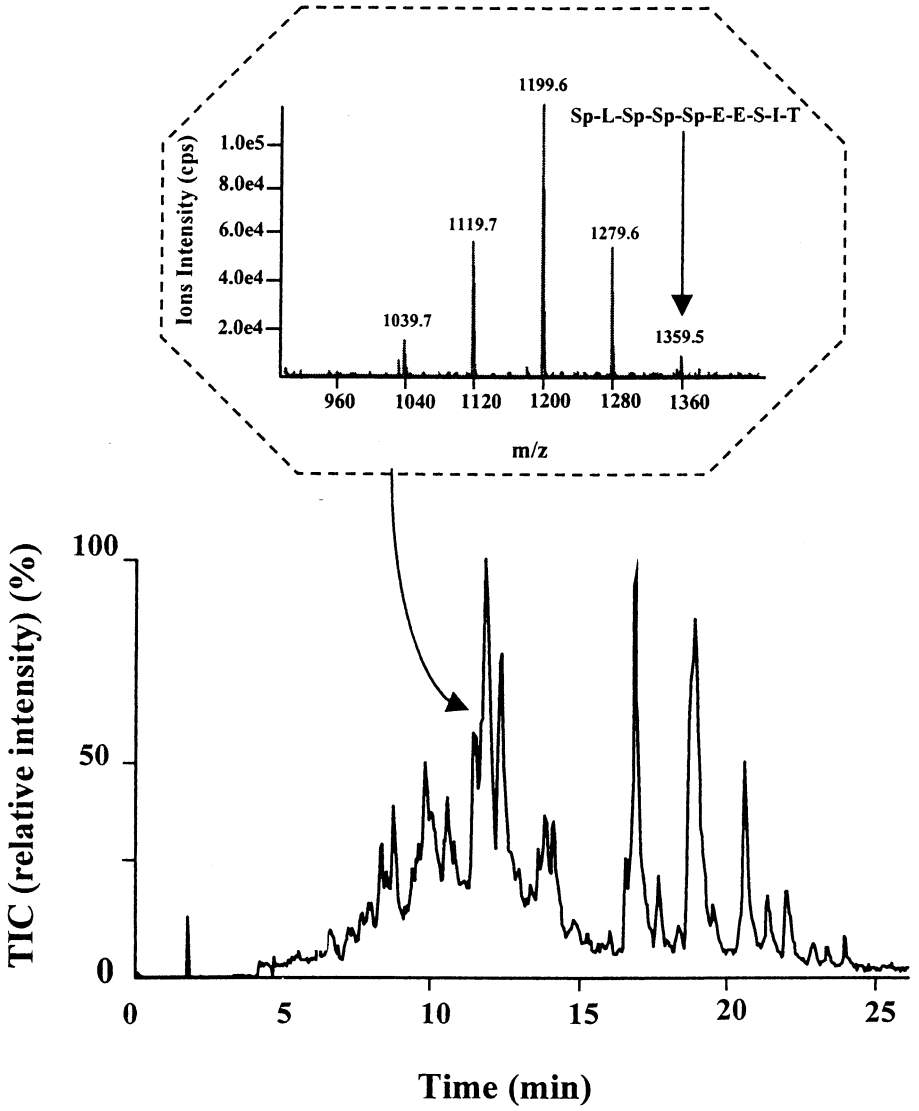

Figure 2 Total ion current (TIC, mass signal) of a high performance liquid chromatography run of lumen content withdrawn after 60 minutes from rat perfused with β(1-25) solution. TIC is expressed as a percent of the intensity of the highest peak. The insert represents the mass spectrum (ions intensity versus m/z) of selected ions of the peak eluted at 11.8 minutes. The observed ions were assigned to β(15-24) fragment m/z 1359.5 and its dephosphorylated forms corresponded to loss of one (−80 Da) to four HPO_3 groups (see text for attribution). cps, count per second.

Table 1 β(1-25)-derived peptides identified after 60 minutes in the lumen contents of rats perfused with either β(1-25) or Fe/β(1-25) solution

Observed molecular mass	Fragment	Corresponding sequence
Rats perfused with β(1-25) solution		
788.5	β(1-6)	RELEEL
632.3	β(2-6)	ELEEL
1626.8	β(1-4)	RELEELNVPGEIVE
856.6	β(7-14)	NVPGEIVE
546.3	β(10-14)	GEIVE
2967.8	β(1-24) or β(2-25)	(R)ELEELNVPGEIVESpLSpSpSpEESIT(R)
2354.2	β(7-25)	NVPGEIVESpLSpSpSpEESITR
1359.5, 1279.6, 1199.6, 1119.7, 1039.7	β(15-24) and dephosphorylated forms	SpLSpSpSpEESIT
Rats perfused with Fe/β(1-25) complex		
788.5	β(1-6)	RELEEL
856.6	β(7-14)	NVPGEIVE
546.3	β(10-14)	GEIVE

ration of sample components was performed at pH 2 using solvent A (0.1% trifluoroacetic acid, 5% acetonitrile in water) and solvent B (0.1% trifluoroacetic acid, 80% acetonitrile in water). Samples were introduced into the mass spectrometer (PE-Sciex API III; Sciex, Thornhill, Ontario, Canada) through a C18 symmetry column (2.1 × 150 mm, Waters) eluted at a flow rate of 0.25 mL/min, at 40°C, with a linear gradient of 5 to 60% of solvent B in solvent A over 30 minutes. In the ESI-MS technique, analytes are ionized at atmospheric pressure directly from the HPLC flowing liquid. Electrospray ion production requires two steps: dispersal of highly charged droplets at near atmospheric pressure, followed by conditions resulting in droplet evaporation.[7] An electrospray is generally produced by application of a high electric field and a potential difference of 3 to 6 kV. The voltage on the sprayer in this study was set between 5 and 5.5 kV. The relative abundance (relative intensity) of each peak to "total ion current" (TIC) was recorded as function of elution time. Each peak contained several ions corresponding to endogenous components of the lumen. Mass spectrum of selected ions present in selected peaks is given as ion intensity (counts per second) versus mass to charge ratio value (m/z).[8] Only ions attributed to β(1-25)-derived peptides are reported.

Complete characterization of peptides was performed by the determination of amino acid sequence of selected peptides using tandem mass spectrometry (MS/MS). The MS/MS procedure was carried out in three stages: (1) Ions were introduced to the first quadrupole from the ion source and the ion of interest was allowed to pass through while all others were rejected. (2) The second quadrupole contained argon as an inert gas, with which the selected ion collided. Multiple collisions with the gas caused the ion to fragment. (3) The resulting fragments of ions were passed to the third quadrupole, which separated them by mass. The resulting spectra were used to sequence peptides because fragmentation occurs principally at the amide bonds between residues.[7] Series of ions that differ in mass by one amino acid residue were used to reconstruct the sequence of the peptide, taking into account that one series of ions may all contain the C terminus (y ions) and another all contain the N terminus (b ions). The nomenclature of Roepstorff and Fohlman,[9] which was proposed to describe the cleavage of backbone bonds in linear peptides, was used. MS/MS experiments were performed on collected peptides at an infusion rate of 3 μL/min. The collision energy, chosen as a function of m/z of the parent ion, was in the range of 25 to 50 eV. Mass data were collected on an Apple Macintosh Quadra 900 computer and were processed using the software package Mac Spec 3.2 Sciex (Sciex, Thornhill, Ontario, Canada).

Results and discussion

β(1-25)-derived peptides were identified in the luminal content of rats according to the following parameters: (1) correspondence of detected molecular masses (ions) with well-known molecular masses of β(1-25) fragments, (2) absence of such masses in control lumen from rats perfused without β(1-25) phosphopeptide, and (3) confirmation of attributed amino acid sequence of detected ion by MS/MS procedure.

Figure 2 shows the TIC of an HPLC run of lumen content from rats perfused with β(1-25) on-line to MS. A similar profile was found for control rats perfused with $FeSO_4$ solution. However, by comparing the data between the luminal content of β(1-25)-injected rats and that of the control ($FeSO_4$) rats, several masses not found in the control were detected. With the help of the known sequence of β(1-25), the observed masses can be unambiguously assigned as β(1-25)-derived peptides. The recovered masses and corresponding sequences are listed in *Table 1*. The mass spectrum of peak eluted at 11.8 minutes is shown in the *Figure 2* insert. The peak contains several ions that correspond to a mixture of native β(15-24) peptide (m/z 1359.5) and its dephosphorylated forms; that is, minus one (−80 Da, m/z 1279.6), two (−160 Da, m/z 1199.6), three (−240 Da, m/z 1119.7), and four (−320 Da, m/z 1039.7) HPO_3 groups. Such results underline the sensitivity of β(1-25) and/or derived β(15-24) to phosphatases during duodenal transit.

It is generally assumed that phosphopeptides from caseins are resistant to further hydrolysis by digestive enzymes.[10] β(7-24) is the smallest phosphopeptide from β-casein that has been identified in the contents of small intestine of rats fed a casein diet.[11] The approach described here, which is based on the analysis of luminal content by ESI-MS without further purification steps, evidenced the sensitivity of β(1-25) to both proteases/peptidases and phosphatases in situ. At least five peptidic bonds of β(1-25) (*Figure 1*; i.e., R_1–E_2, L_6–N_7, P_9–G_{10}, E_{14}–Sp_{15}, and T_{24}–R_{25}) were cleaved. In addition, the action of phosphatases was shown by successive dephosphorylation of the β(15-24) fragment.

Figure 3 shows the TIC of a HPLC run of luminal

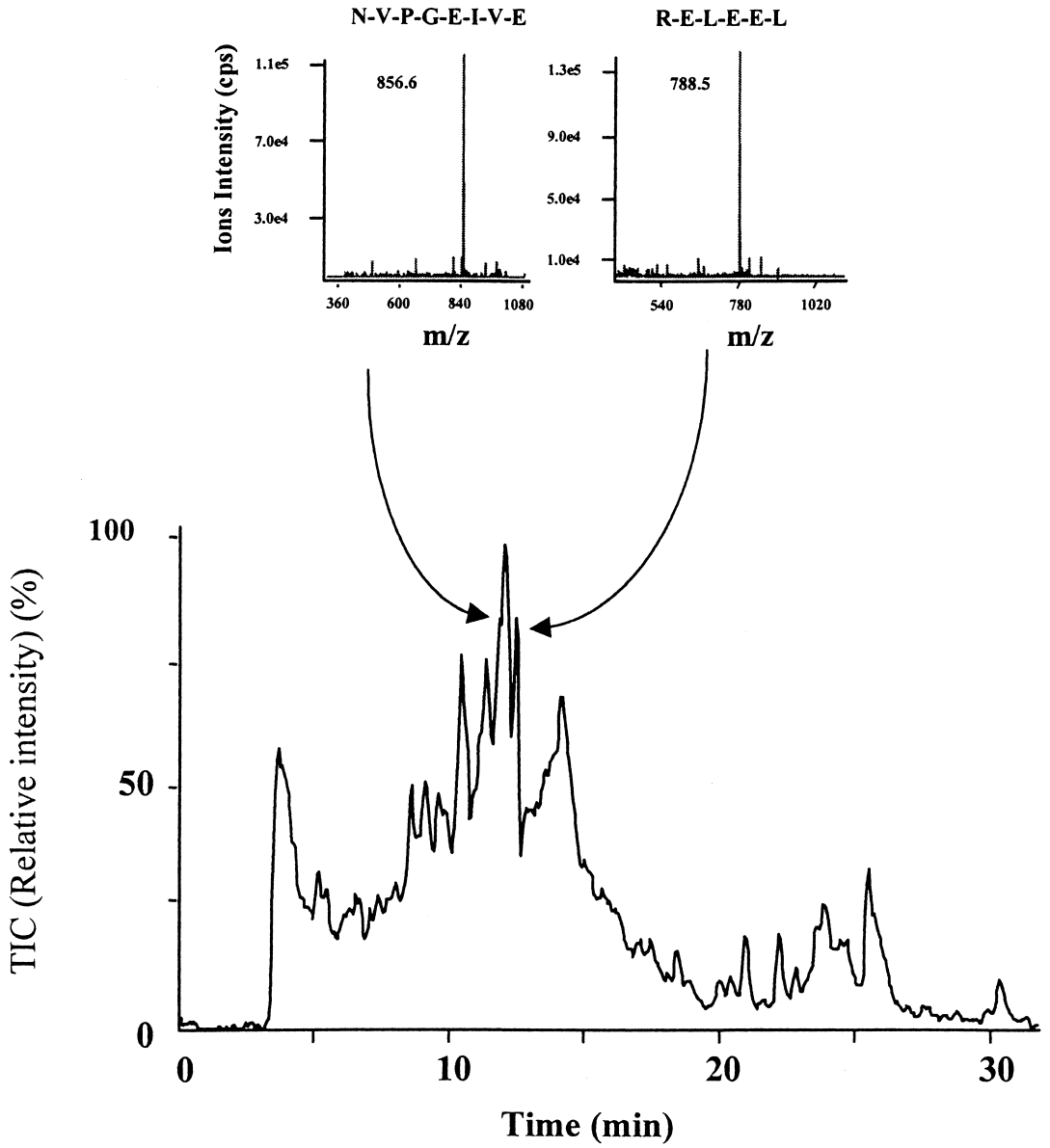

Figure 3 Total ion current (TIC, mass signal) of a high performance liquid chromatography run of lumen content withdrawn after 60 minutes from rat perfused with Fe/β(1-25) complex solution. TIC is expressed as a percent of the intensity of the highest peak. The extracted mass spectra attributed to β(1-25)-derived fragments [i.e., β(1-6) (m/z 788.5) and β(7-14) (m/z 856.6)] are shown. cps, count per second

content from rats perfused with Fe/β(1-25) complex. Again, masses corresponding to peptides derived from β(1-25) were detected (*Table 1*). The mass spectrum of detected peptides [i.e., β(1-6) and β(7-14)] are shown (*Figure 3*, insert). To confirm the structure of assigned peptides, the corresponding HPLC peaks were collected, and components were further identified by MS/MS. As an example, the product ion spectrum for the singly charged ion $[M + H]^{+1}$ with a m/z value of 856.6 (shown in *Figure 4*) confirmed the sequence NVPGEIVE attributed to β(7-14) peptide. However, in contrast to samples from rats perfused with β(1-25), the phosphorylated region [i.e., β(15-25)] was not detected in the luminal contents of rats perfused with Fe/β(1-25) complex. Indeed, the hydrolysis rate of the complex seems to be slower because β(1-25)-derived peptides were only detected after 60 minutes following perfusion instead of 15 minutes in rats group perfused with iron-free β(1-25). The absence of the phosphorylated β(15-24) in the luminal content could be linked to the reported positive effect of β(1-25) on iron absorption in vivo.[4] Because of its lower molecular weight, soluble Fe/β(15-24) complex may be better able to reach the brush border than the whole Fe/β(1-25) complex and could be the effective enhancer of iron absorption. Although such an assumption cannot be excluded, more studies must be performed about the relationship between the positive role of β(1-25) in iron availability and its susceptibility to digestive enzymes.

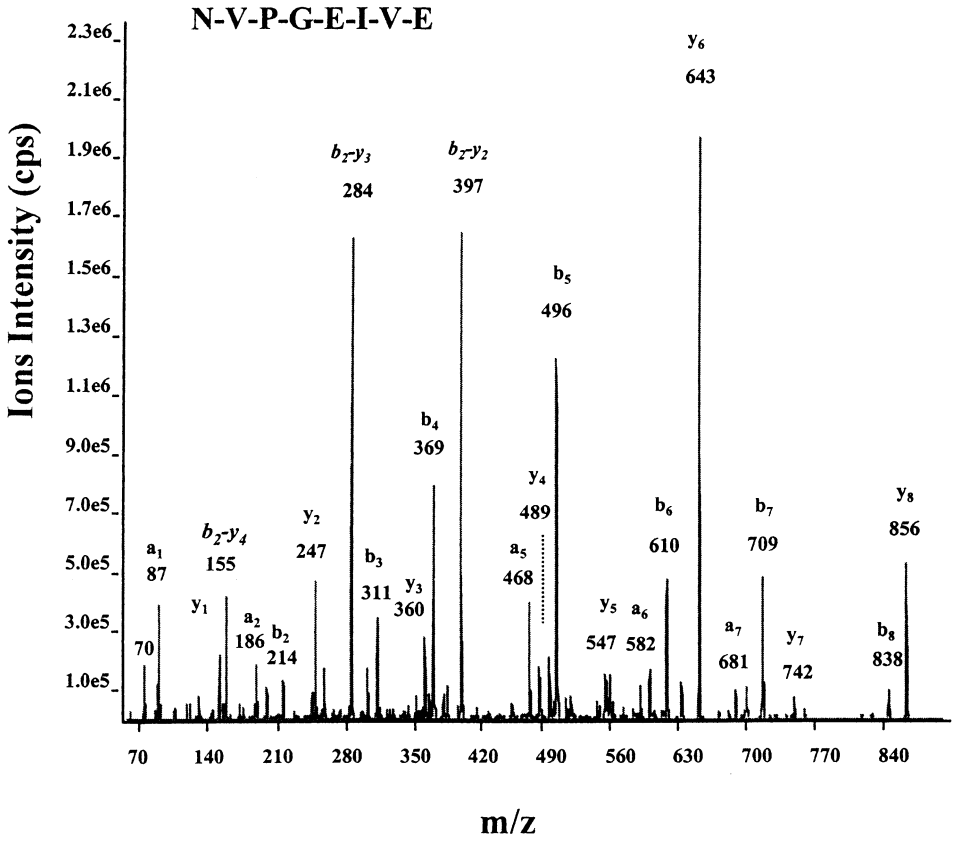

Figure 4 Product ion spectrum for the $[M + H]^{+1}$ (m/z 856.6) ion, which confirmed the indicated amino acid sequence of β(7-14) peptide. $[M + H]^{+1}$, singly charged ion (molecular mass + one proton); cps, count per second. *y* and *b* correspond to the C terminus and N terminus parts of fragmented peptide (ion), respectively.[8]

Conclusion

The data presented here demonstrate that the β-casein phosphopeptide β(1-25), whether to iron complexed or not, is hydrolyzed by the digestive enzymes in situ. Generated peptides were detected by direct analysis of duodenal content of rats by HPLC-ESI-MS/MS. Such an approach could be of more general use in studies on the nutritional and biological values of food-derived peptides.

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