



**HAL**  
open science

## Sensitivity of $\beta$ -casein phosphopeptide-iron complex to digestive enzymes in ligated segment of rat duodenum

Said Bouhallab, Nabil Aït Oukhatar, Daniel Mollé, Gwenaele Henry,  
Jean-Louis Maubois, Pierre Arhan, Dominique L Bouglé

### ► To cite this version:

Said Bouhallab, Nabil Aït Oukhatar, Daniel Mollé, Gwenaele Henry, Jean-Louis Maubois, et al.. Sensitivity of  $\beta$ -casein phosphopeptide-iron complex to digestive enzymes in ligated segment of rat duodenum. *Journal of Nutritional Biochemistry*, 1999, 10 (12), pp.723-727. 10.1016/S0955-2863(99)00062-5. hal-01568137

**HAL Id: hal-01568137**

**<https://hal.science/hal-01568137v1>**

Submitted on 24 Jul 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - ShareAlike 4.0 International License

# Sensitivity of $\beta$ -casein phosphopeptide-iron complex to digestive enzymes in ligated segment of rat duodenum

Saïd Bouhallab,\* Nabil Aït Oukhatar,<sup>†</sup> Daniel Mollé,\* Gwénaële Henry,\*  
Jean-Louis Maubois,\* Pierre Arhan,<sup>†</sup> and Dominique L. Bouglé<sup>†</sup>

\*Laboratoire de Recherches de Technologie Laitière, INRA, Rennes, France; and <sup>†</sup>Laboratoire de Physiologie Digestive et Nutritionnelle, CHU de Caen, Caen, France

*Binding iron to the phosphorylated  $\beta(1-25)$  peptide derived from  $\beta$ -casein improves iron bioavailability in the rat. The aim of the present work was to learn how injected  $\beta(1-25)$  and iron- $\beta(1-25)$  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  $\beta(1-25)$  is sensitive to digestive enzymes including proteases/peptidases and phosphatases during duodenal transit. The lumen contents of rats perfused with iron free  $\beta(1-25)$  contained all peptidic sequences derived from  $\beta(1-25)$ . In contrast, the phosphorylated part of  $\beta(1-25)$  [i.e.,  $\beta(15-24)$ ] was not detected in lumen of rats perfused with iron- $\beta(1-25)$  complex. (J. Nutr. Biochem. 10:723–727, 1999) © Elsevier Science Inc. 1999. All rights reserved.*

**Keywords:** iron; caseinophosphopeptide; enzymatic digestion; rat duodenum

## Introduction

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

In our previous work, we reported that one of these CCP, the  $\beta(1-25)$  derived from  $\beta$ -casein, can improve the bioavailability of iron in iron-deficient rats.<sup>4</sup> Rats fed iron bound to the  $\beta(1-25)$  [Fe/ $\beta(1-25)$ ] complex showed a better improvement of blood parameters of iron status and higher liver storage than FeSO<sub>4</sub> group. The positive effect of  $\beta(1-25)$  as a chelator on iron absorption was also observed in situ using the intestinal ligation technique.<sup>5</sup> In the present work, the sensitivity of  $\beta(1-25)$  and of Fe/ $\beta(1-25)$  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 $\beta(1-25)$

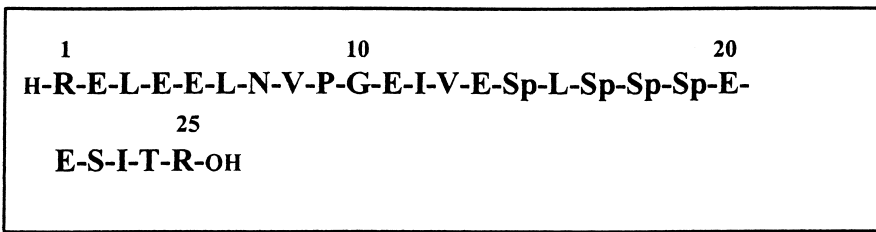
$\beta(1-25)$  is the phosphorylated N-terminal fragment of  $\beta$ -casein of molecular mass 3124 containing four phosphoserine residues (Figure 1). It chelates four iron atoms per molecule with high affinity.<sup>5</sup>  $\beta(1-25)$  and Fe/ $\beta(1-25)$  complex were prepared as previously reported.<sup>6</sup>

### 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  $\beta(1-25)$  peptide and containing 0.27 mg of iron as FeSO<sub>4</sub> salt. The other

Address correspondence and reprint requests to Dr. S. Bouhallab, Laboratoire de Recherches de Technologie Laitière, INRA, 65 rue de Saint Brieu, 35042 Rennes cedex, France.

Received November 4, 1998; accepted August 9, 1999.

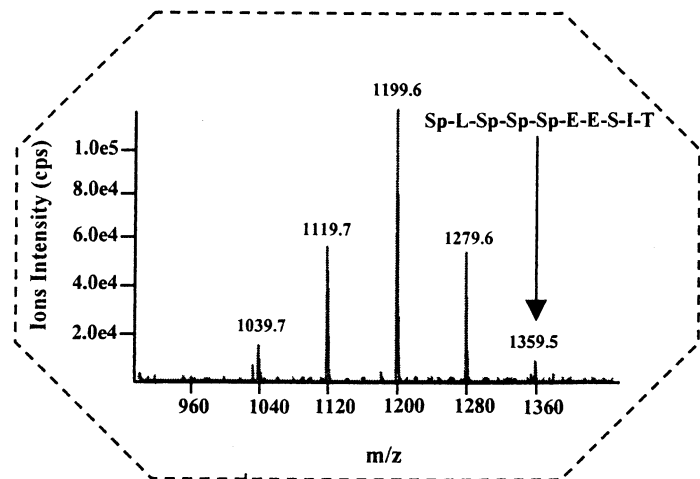


**Figure 1** Amino acid sequence of  $\beta(1-25)$ , the  $\beta$ -casein phosphopeptide. Sp, phosphoserine residue.

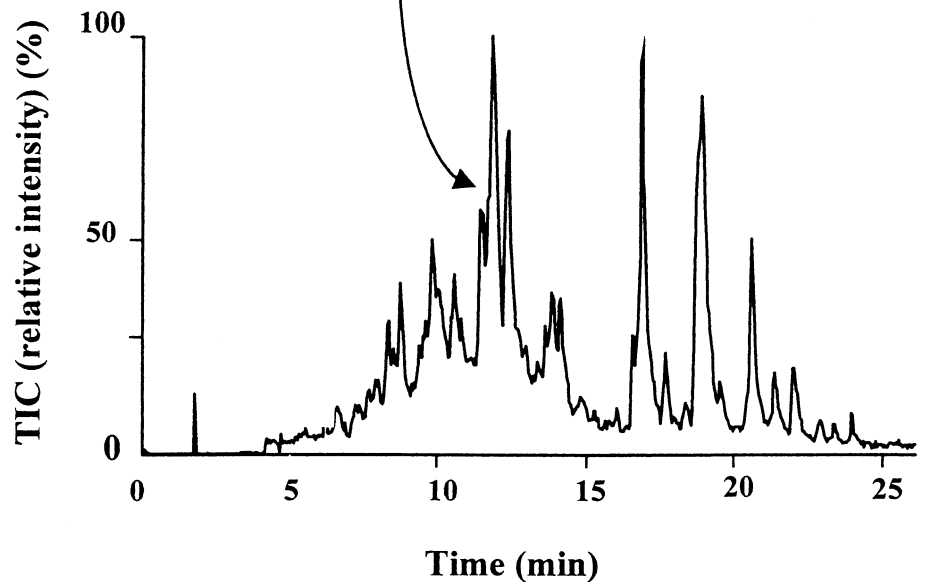
two groups were perfused with 1 mL solution containing either 3 mg  $\beta(1-25)$  peptide alone or 3 mg  $\beta(1-25)$  complexed to iron (0.27 mg). A 200  $\mu$ L sample was withdrawn at 15, 45, 60, and 90 minutes following perfusion without volume replacement. After 90 minutes, the loop was emptied 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  $\beta(1-25)$  phosphopeptide was not affected by this heating step.

*Analyses*

Lumen contents were incubated for 1 hour with 18 mM ethylenediamine-tetraacetic acid (EDTA) at 25°C to dissociate iron-phosphopeptide complexes. This step was added to improve peptide separation and detection. After centrifugation (3,000  $\times$  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 separa-



**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  $\beta(1-25)$  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  $\beta(15-24)$  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**  $\beta$ (1-25)-derived peptides identified after 60 minutes in the lumen contents of rats perfused with either  $\beta$ (1-25) or Fe/ $\beta$ (1-25) solution

Observed molecular mass	Fragment	Corresponding sequence
<i>Rats perfused with <math>\beta</math>(1-25) solution</i>		
788.5	$\beta$ (1-6)	RELEEL
632.3	$\beta$ (2-6)	ELEEL
1626.8	$\beta$ (1-4)	RELEELNVPGEIVE
856.6	$\beta$ (7-14)	NVPGEIVE
546.3	$\beta$ (10-14)	GEIVE
2967.8	$\beta$ (1-24) or $\beta$ (2-25)	(R)ELEELNVPGEIVESpLSpSpSpEESIT(R)
2354.2	$\beta$ (7-25)	NVPGEIVESpLSpSpSpEESITR
1359.5, 1279.6, 1199.6, 1119.7, 1039.7	$\beta$ (15-24) and dephosphorylated forms	SpLSpSpSpEESIT
<i>Rats perfused with Fe/<math>\beta</math>(1-25) complex</i>		
788.5	$\beta$ (1-6)	RELEEL
856.6	$\beta$ (7-14)	NVPGEIVE
546.3	$\beta$ (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  $\times$  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.<sup>7</sup> 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).<sup>8</sup> Only ions attributed to  $\beta$ (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.<sup>7</sup> 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,<sup>9</sup> 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  $\mu$ L/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).

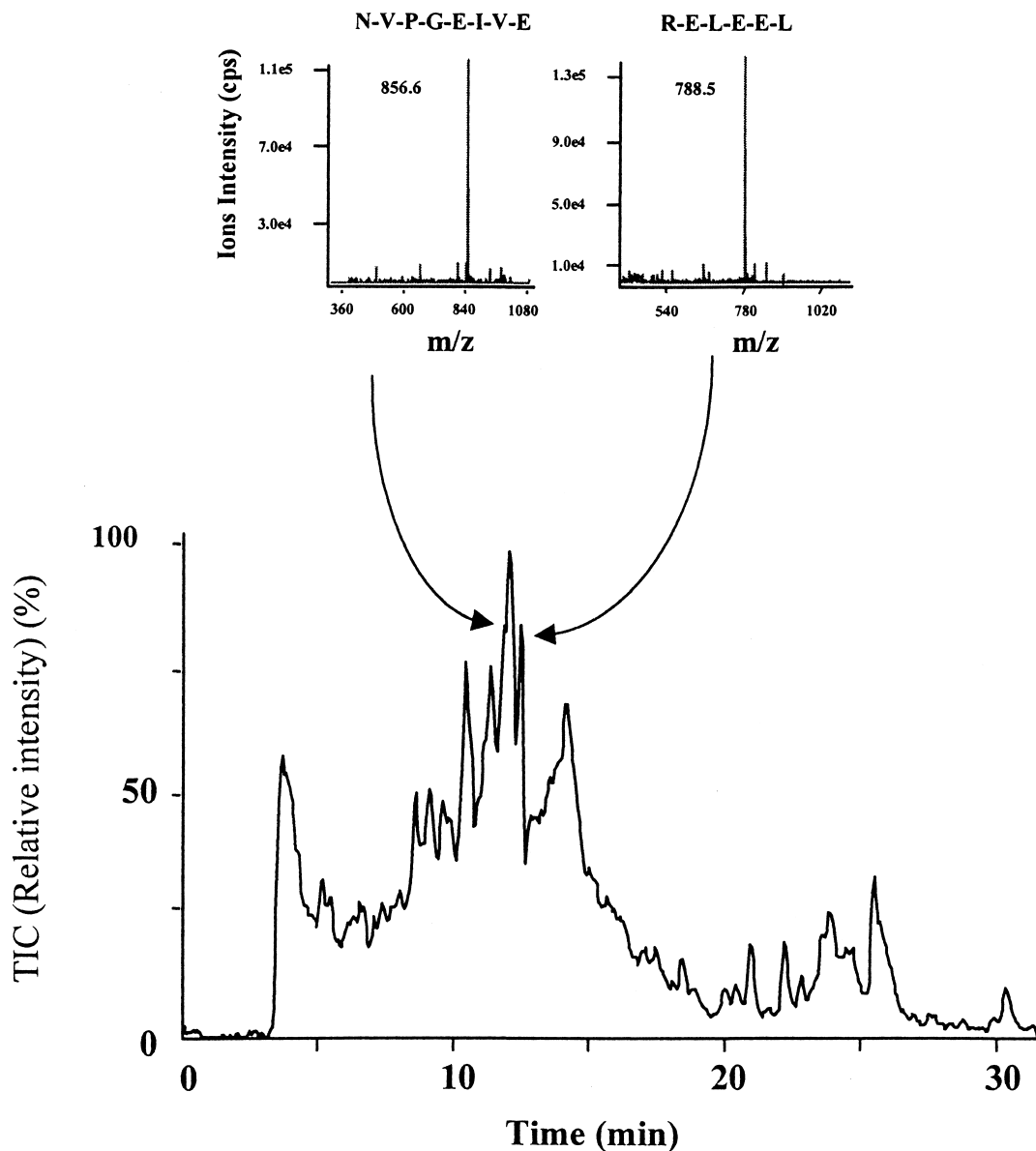
## Results and discussion

$\beta$ (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  $\beta$ (1-25) fragments, (2) absence of such masses in control lumen from rats perfused without  $\beta$ (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  $\beta$ (1-25) on-line to MS. A similar profile was found for control rats perfused with FeSO<sub>4</sub> solution. However, by comparing the data between the luminal content of  $\beta$ (1-25)-injected rats and that of the control (FeSO<sub>4</sub>) rats, several masses not found in the control were detected. With the help of the known sequence of  $\beta$ (1-25), the observed masses can be unambiguously assigned as  $\beta$ (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  $\beta$ (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<sub>3</sub> groups. Such results underline the sensitivity of  $\beta$ (1-25) and/or derived  $\beta$ (15-24) to phosphatases during duodenal transit.

It is generally assumed that phosphopeptides from caseins are resistant to further hydrolysis by digestive enzymes.<sup>10</sup>  $\beta$ (7-24) is the smallest phosphopeptide from  $\beta$ -casein that has been identified in the contents of small intestine of rats fed a casein diet.<sup>11</sup> The approach described here, which is based on the analysis of luminal content by ESI-MS without further purification steps, evidenced the sensitivity of  $\beta$ (1-25) to both proteases/peptidases and phosphatases in situ. At least five peptidic bonds of  $\beta$ (1-25) (Figure 1; i.e., R<sub>1</sub>-E<sub>2</sub>, L<sub>6</sub>-N<sub>7</sub>, P<sub>9</sub>-G<sub>10</sub>, E<sub>14</sub>-S<sub>15</sub>, and T<sub>24</sub>-R<sub>25</sub>) were cleaved. In addition, the action of phosphatases was shown by successive dephosphorylation of the  $\beta$ (15-24) fragment.

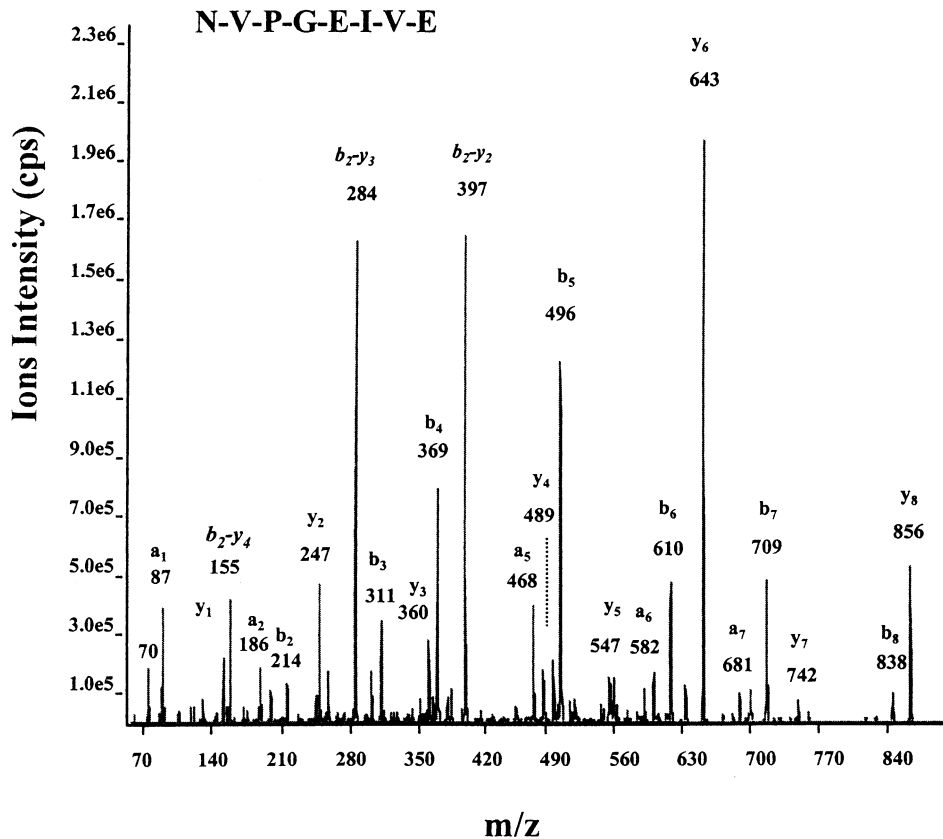
Figure 3 shows the TIC of a HPLC run of luminal



**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/ $\beta(1-25)$  complex solution. TIC is expressed as a percent of the intensity of the highest peak. The extracted mass spectra attributed to  $\beta(1-25)$ -derived fragments [i.e.,  $\beta(1-6)$  ( $m/z$  788.5) and  $\beta(7-14)$  ( $m/z$  856.6)] are shown. cps, count per second

content from rats perfused with Fe/ $\beta(1-25)$  complex. Again, masses corresponding to peptides derived from  $\beta(1-25)$  were detected (Table 1). The mass spectrum of detected peptides [i.e.,  $\beta(1-6)$  and  $\beta(7-14)$ ] 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  $[M + H]^+$  with a  $m/z$  value of 856.6 (shown in Figure 4) confirmed the sequence NVPGEIVE attributed to  $\beta(7-14)$  peptide. However, in contrast to samples from rats perfused with  $\beta(1-25)$ , the phosphorylated region [i.e.,  $\beta(15-25)$ ] was not detected in the luminal contents of rats perfused with Fe/ $\beta(1-25)$  complex. Indeed, the hydrolysis rate of the complex seems

to be slower because  $\beta(1-25)$ -derived peptides were only detected after 60 minutes following perfusion instead of 15 minutes in rats group perfused with iron-free  $\beta(1-25)$ . The absence of the phosphorylated  $\beta(15-24)$  in the luminal content could be linked to the reported positive effect of  $\beta(1-25)$  on iron absorption in vivo.<sup>4</sup> Because of its lower molecular weight, soluble Fe/ $\beta(15-24)$  complex may be better able to reach the brush border than the whole Fe/ $\beta(1-25)$  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  $\beta(1-25)$  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  $\beta(7-14)$  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.<sup>8</sup>

## Conclusion

The data presented here demonstrate that the  $\beta$ -casein phosphopeptide  $\beta(1-25)$ , 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.

## References

- Meisel, H. and Frister, H. (1988). Chemical characterization of a caseino-phosphopeptide isolated from in vivo digest of a casein diet. *Biol. Chem. H.-S.* **369**, 1275–1279
- Kasai, T., Iwasaki, R., Tanaka, M., and Kiriya, S. (1995). Casein-phosphopeptides (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. *Biosci. Biotech. Bioch.* **59**, 26–30
- Kitts, D.D. and Yuan, Y.V. (1992). Caseinophosphopeptides and calcium bioavailability. *Trends Food Sci. Tech.* **3**, 31–35
- Ait-oukhatar, N., Bouhallab, S., Bureau, F., Arhan, P., Maubois, J.L., Drosdowsky, M.A., and Bouglé, D.L. (1997). Bioavailability of caseinophosphopeptide bound iron in the young rat. *J. Nutr. Biochem.* **8**, 190–194
- Bouhallab, S., Léonil, J., and Maubois, J.L. (1991). Complexation du fer par le phosphopeptide (1-25) de la caséine  $\beta$ : Action de l'alcalase et de la phosphatase acide. *Lait* **71**, 435–443
- Peres, J.M., Bouhallab, S., Bureau, F., Maubois, J.L., Arhan, P., and Bouglé, D. (1997). Absorption du fer lié au caseinophosphopeptide 1-25 de la  $\beta$ -caséine. *Lait* **77**, 433–440
- Carr, S.A., Hemling, M.E., Bean, M.F., and Roberts, G.D. (1991). Integration of mass spectrometry in analytical biotechnology. *Anal. Chem.* **63**, 2802–2824
- Busman, M., Schey, K.L., Oatis, J.E., and Knapp, D.R. (1996). Identification of phosphorylation sites in phosphopeptides by positive and negative mode electrospray ionisation—tandem mass spectrometry. *J. Am. Soc. Mass Spectr.* **7**, 243–249
- Roepstorff, P. and Fohlman, J. (1984). Proposal for common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **11**, 601
- Naito, H. and Suzuki, H. (1974). Further evidence for the formation of phosphopeptide in the intestinal lumen from dietary  $\beta$  casein. *Agric. Biol. Chem.* **38**, 1543–1545
- Hirayama, M., Toyota, K., Hidaka, H., and Naito, H. (1992). Phosphopeptides in rat intestinal digests after ingesting casein phosphopeptides. *Biosci. Biotech. Bioch.* **56**, 1128–1129