In vitro digestion of caseinophosphopeptide–iron complex

By NABIL AÎT-OUKHATAR*, SAÏD BOUHALLAB†, FRANÇOIS BUREAU‡, PIERRE ARHAN*, JEAN-LOUIS MAUBOIS† and DOMINIQUE L. BOUGLÉ*

**Laboratoire de Physiologie Digestive et Nutritionnelle, Service de Pédiatrie A, Centre Hospitalier Universitaire de Caen, F-14033 Caen Cedex, France*

†INRA Laboratoire de Recherches de Technologie Laitière, 65 rue de Saint-Brieuc, F-35042 Rennes Cedex, France

‡Laboratoire de Biochimie A, Service de Pédiatrie A, Centre Hospitalier Universitaire de Caen, F-14033 Caen Cedex, France

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Caseins bind strongly to Fe by their phosphoseryl residues (Hegenauer *et al*. 1979; Brulé & Fauquant, 1982; Bouhallab *et al*. 1991; Emery, 1992) and keep it soluble at the alkaline pH of the duodenum (Manson & Annan, 1971; Bouhallab *et al*. 1991). It has been suggested that this strong binding prevents the release of free Fe during digestion and impairs its absorption (West, 1986; Hurrell, 1997), but in human studies hydrolysis by digestive enzymes to give low molecular mass peptides improves Fe absorption (Hurrell *et al*. 1988, 1989).

β-Casein peptide 1–25 (β-CN(1–25)) is the phosphorylated N-terminal fragment of β-CN. Its molecular mass is 3124 Da and it contains four phosphoseryl residues that bind four Fe atoms and keep them soluble (Bouhallab *et al*. 1991).

Preliminary results showed that binding Fe to β-CN(1–25) enhances its bioavailability in the rat (Aît-Oukhatar *et al*. 1997) and its absorption by the duodenal rat loop model (Pérès *et al*. 1997). We recently showed that the β-CN(1–25)–Fe complex is hydrolysed to a lesser extent than free β-CN(1–25) during duodenal digestion, and that the phosphorylated region of the peptide to which Fe is bound, β-CN(15–24), cannot be detected in the digestive lumen (our unpublished results).

These previous studies did not examine the effect of luminal digestion that could release free inorganic Fe. We considered the possibility that Fe bound to β-CN(1–25) is not released in the digestive lumen, and that it reaches the absorbing surface of the small intestine brush border membrane as a complex. To test this hypothesis we examined *in vitro* the influence of pH and digestive enzymes on the dialysability of the β-CN(1–25)–Fe complex.

MATERIALS AND METHODS

Reagents and materials

Double distilled and deionized water was used. All glassware and the polyethylene tubes used for the samples were washed and rinsed in distilled water.

Porcin pepsin (EC 3.4.23.1), bovine α-chymotrypsin (EC 3.4.21.1) and TPCK-treated bovine trypsin (EC 3.4.21.4) were purchased from Sigma Chemicals (St Louis,

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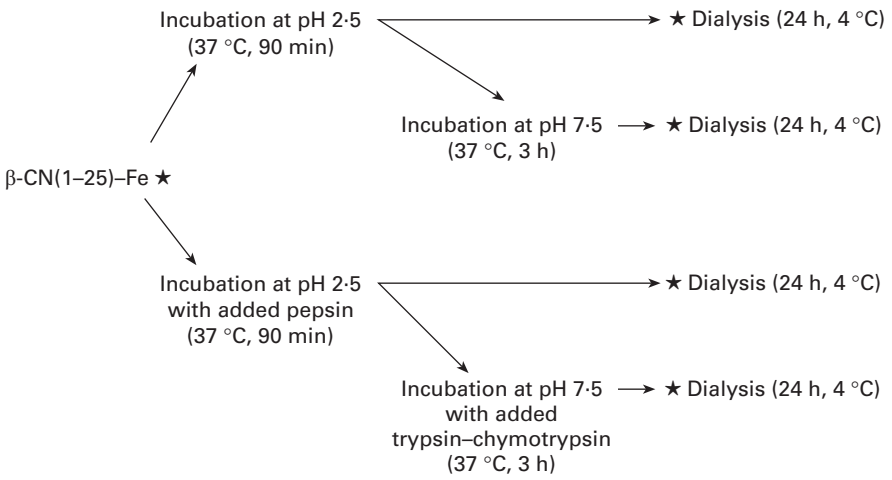


Fig. 1. Schematic outline of procedures for *in vitro* digestion of the β-casein (1−25)−iron complex. At the points indicated by ★ a 1 ml sample was taken for iron analysis.

MO 63178, USA). Enzymes were suspended in 0·1 M-HCl. The natural cellulose dialysis tubing (Spectra/Por, molecular mass cut-off 1000 Da, diam. 11·5 mm; Spectrum, Bioblock Scientific, F-67403 Illkirch, France) was washed three times with double distilled and deionized water and soaked overnight in $NaHCO_3$ (0·5 g/l).

Protein and peptide source

β-Casein was isolated from sodium caseinate as described previously (Bouhallab *et al.* 1991). The β-CN(1–25) was purified from a tryptic hydrolysate of bovine β-casein by the method of Gaucheron *et al.* (1995)

Binding of iron to β-casein peptide 1–25

Pure β-CN(1–25) was incubated at 25 °C for 30 min with a 10-fold excess of $FeCl_2$ solution. After incubation, the solution was subjected to diafiltration on an ultrafiltration membrane with a molecular mass cut-off of 3 kDa. It was then extensively dialysed against water at 4 °C for 24 h to remove unbound Fe. Four to five moles of Fe were bound for each mole of β-CN(1–25). The amount of Fe complexed with β-CN(1–25) was determined by atomic absorption spectrometry (Model AA 1275; Varian, F-91941 Les Ulis, France). The dialysed sample was freeze dried.

In vitro *digestion*

The dialysis model has been thoroughly used in *in vitro* studies of Fe solubility (Demott & Dincer, 1976; Bouhallab *et al.* 1991; Emery, 1992) and bioavailability (Miller *et al.* 1981; Hurrell *et al.* 1988, 1989; Pérez-Llamas *et al.* 1996). The pH values used, 2·5 and 7·5, are found in the stomach and the duodenum (Miller & Berner, 1989), and are close to the optimal value of enzyme activity (pepsin, pH 2; trypsin–chymotrypsin, pH 7–9).

The protocol given in Fig. 1 was adapted from Miller *et al.* (1981). Dialysis was performed after rather than during digestion, and Fe was bound to the peptide while in Miller's method it was only in presence of the proteins. Peptide (30 mg) containing

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Table 1. *Dialysability of iron after in vitro digestion of a β-casein (1–25)–iron complex*

(Values are expressed as percentages of the initial amount of iron present, means ± SD for five experiments)

	Incubation conditions			
	pH 7†	pH 2·5	pH 2·5, then pH 7·5	Significance‡
Control (without enzymes)	ND	3·4 ± 2·8	3·5 ± 2·2	$P = 0{\cdot}93$ NS
Incubation with enzymes	ND	−1·4 ± 3·5	−0·2 ± 2·9	$P = 0{\cdot}30$ NS
Significance‡		$P = 0{\cdot}13$ NS	$P = 0{\cdot}12$ NS	

ND, not detected; NS, not significant.
† Dialysis during the production process.
‡ By Student's *t* test.

270 μg Fe was placed in 10 ml 0·01 M-HCl, from which two samples of 4 ml were used for studying the effects of pH and enzymes or of pH alone.

Effect of pH. One sample was adjusted to pH 2·5 with 4 M-HCl and incubated at 37 °C for 90 min in a shaking water bath, maintaining the pH at 2·5 throughout by adding 4 M-HCl when necessary. At the end of the incubation, 1 ml was transferred to a dialysis bag and 1 ml was drawn for analysis by atomic absorption spectrometry (model 1100B; Perkin Elmer, F-91400 Villebon-sur-Yvette, France). The other 2 ml was incubated at pH 2·5 as above, then the pH was adjusted to 7·5 with 0·5 M-NaOH and it was incubated at 37 °C for 3 h, checking the pH regularly. At the end of the incubation 1 ml was transferred for dialysis and the remaining 1 ml used for analysis.

Dialysis experiment. Dialysis bags containing 1 ml of either mixture were dialysed at 4 °C for 24 h against 500 ml solution: 0·001 M-HCl after incubation at pH 2·5, and 50 mM-Tris HCl after incubation at pH 7·5. The bags were then removed and Fe levels measured.

Dialysability, expressed as a percentage of the total amount of Fe that disappeared from the samples, was calculated as $(I - F/I) \times 100$, where F is the final amount of Fe present in the bag at the end of the dialysis and I the initial amount of Fe present at the end of incubation but before dialysis.

Pepsin digestion. Pepsin (0·12 mg) suspension was added to 4 ml of the 10 ml solution containing the β-CN(1–25)–Fe complex at pH 2·5. The pH was readjusted to 2·5 using 4 M-HCl and the mixture incubated at 37 °C for 90 min. The reaction was stopped by heating at 90 °C for 10 min. The mixture was cooled to room temperature and 1 ml was taken for analysis and 1 ml dialysed in the same way as after incubation at pH 2·5.

Trypsin–chymotrypsin digestion. To simulate physiological digestion, the pH of the remaining 2 ml of the peptic digest was adjusted to 7·5 with 0·5 M-NaOH and the digest further incubated at 37 °C for 3 h with a mixture of trypsin and chymotrypsin and an enzyme: substrate ratio of 1:100. The reaction was stopped and 1 ml was dialysed as described above.

RESULTS

Results are given in Table 1. Changes in pH did not increase Fe dialysability (release of free Fe or Fe chelated by peptides of < 1000 Da). Moreover, mass spectrometry showed that neither pepsin nor pancreatic proteinases significantly increased Fe dialysability, although they partly hydrolysed the non-phosphorylated moiety of the peptide (results not shown).

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DISCUSSION

This study confirms that Fe binding to β-CN(1–25) is resistant to changes in pH. Previous work has shown that Fe is not released from β-CN(1–25) at neutral pH during the production process (Bouhallab *et al.* 1991), and that bound Fe is also resistant to the low gastric pH (Hegenauer *et al.* 1979; Galdi & Valencia, 1988; Emery, 1992).

Phosphatase activity, which could release free Fe, is located in the brush border of the apical membrane of enterocytes, and not in the digestive lumen. Furthermore, the partial denaturation of the phosphate residues that occurs during binding of Fe to phosphoserine residues (Emery, 1992) could explain why phosphatases are less active towards Fe bound to β-CN(1–25) (Mellander & Fölsch, 1972; Bouhallab *et al.* 1991).

Fe was not released as a low molecular mass peptide complex by pepsin or trypsin–chymotrypsin, and the high concentration of negatively charged phosphates in β-CN(1–25) seems to protect it from the action of pepsin and of pancreatic endopeptidases (Mellander & Fölsch, 1972; West, 1986). This explains why they are detected in the faeces (Kasai *et al.* 1995). A mass spectrometric analysis of the products of intraluminal digestion suggests that the phosphorylated 15–24 fragment of β-CN(1–25), which contains the four Fe bound phosphoserines, is resistant to proteinases (our unpublished results).

In conclusion, we have shown that *in vitro* digestion was unable to release dialysable Fe when it was bound to β-CN(1–25). This makes it possible for Fe to reach the apical membrane of the enterocyte in a soluble, bound form. These findings support the results of our previous study, which showed that Fe bound to β-CN(1–25) can be absorbed by a separate pathway from inorganic Fe (Pérès *et al.* 1999).

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