Does casomorphin have a functional role?

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Received 25 November 1983; revised version received 27 January 1984

Degradation of buffalo β-casein by various physiological enzymes was studied. Digestion with gastric and pancreatic proteases plus leucine aminopeptidase did not release casomorphins but a putative precursor (procasomorphin) which was further digested by brush border peptidases into peptides differing from casomorphins.

Casomorphin *Brush border protease* *Casein* *High-performance liquid chromatography*

1. INTRODUCTION

Authors in [1] have shown the presence of opioid materials in bovine milk and milk products. Authors in [2] isolated from casein peptone (an undefined enzymatic digest of bovine whole casein) an opioid peptide resistant to pronase. The sequence of this peptide, β-casomorphin 7, corresponds to the sequence 60–66 of bovine β-casein and seems to be of general occurrence in all the β-caseins so far investigated, i.e., bovine [3], ovine [4] and buffalo [5].

However, it is not clear whether β-casomorphin could be released in the gastrointestinal tract and reach concentrations of physiological significance (β-casomorphin is 250-times less potent than normorphin [2]). Thus, whether this peptide plays a physiological or toxicological role is an open question.

We have undertaken a study to answer this question. We report here preliminary results obtained by studying in vitro proteolysis of buffalo β-casein by gastric, pancreatic and brush border proteases plus leucine aminopeptidase.

Abbreviation: GPE, gastric and pancreatic enzymes plus leucine aminopeptidase

2. MATERIALS AND METHODS

2.1. *Materials*

Buffalo β-casein was isolated from whole casein as in [6]. Rabbit small intestine brush border was obtained as in [7]. Peptidase activities were assayed as in [8]; working solutions were prepared with a total activity of 1.5 units/ml aminopeptidase N, 2.0 units/ml aminopeptidase A and 0.5 units/ml dipeptidyl aminopeptidase IV. β-Casomorphin 7 and proteases were purchased from Sigma.

2.2. *Enzyme digestions and separation of peptides*

Buffalo β-casein (500 mg), in 50 ml aqueous solution of 30% acetic acid, was first hydrolysed with pepsin (5 mg). A long incubation time (5 h) was required because of the poor solubility of the protein at acidic pH. The pH of the digest was then adjusted to 8.5 with ammonium hydroxide and at intervals of 1 h trypsin (4 mg), chymotrypsin (4 mg), elastase (4 mg), carboxypeptidase A (1 mg), carboxypeptidase B (1 mg) and leucine aminopeptidase (1 mg) were added. One h after the last addition the digest (GPE digest) was directly analysed by HPLC.

Purified peptides (150 nmol) were incubated in 0.1 M ammonium bicarbonate (pH 8) with 75 μl of

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

brush border preparation. Aliquots of this mixture, at the times indicated, were acidified with glacial acetic acid and analysed by HPLC. Peptides were purified either on a Waters liquid chromatograph equipped with two pumps (model 6600 A), a solvent programmer (model 660) and a variable wavelength UV detector (model 450) or on a Beckman liquid chromatograph equipped with two pumps (model 110), a solvent programmer (model 420) and a variable wavelength UV detector (model 100-40 Hitachi). A μ-Bondapak C-18 reverse-phase column (5 μm, 4.6 × 250 mm) was used. Peptides were eluted at a flow rate of 1 ml/min with a gradient of CH_3CN in 0.1% trifluoroacetic acid as indicated in fig.1. Details for amino acid analysis were as reported in [5].

3. RESULTS AND DISCUSSION

3.1. *GPE digest*

Fig.1. shows the HPLC pattern of the GPE digest of buffalo β-casein in comparison with that obtained by running the same sample spiked with β-casomorphin 7. It is evident that the gastric and pancreatic proteases used here are unable to release β-casomorphin 7 from β-casein.

Furthermore from the GPE digest a pure peptide was isolated (peak P in fig.1a). This peptide, whose amino acid composition is reported in table 1, corresponds to the sequence positions 59–68 of buffalo β-casein [5] and contains β-casomorphin. We shall henceforth refer to this peptide as procasomorphin. It should be noted that Lys 68 in buffalo β-casein [5] replaces Asn 68 in cow β-casein [3].

3.2. *Brush border digest*

Procasomorphin was digested by brush border enzymes for 0.5, 1 and 4 h and the digest analysed by HPLC (fig.2). A blank run without peptide shows that peaks X and Y arise from the brush border preparation. It is evident that digestion of procasomorphin by brush border proteases gives rise to peptides A–C. Table 1 lists the composi-

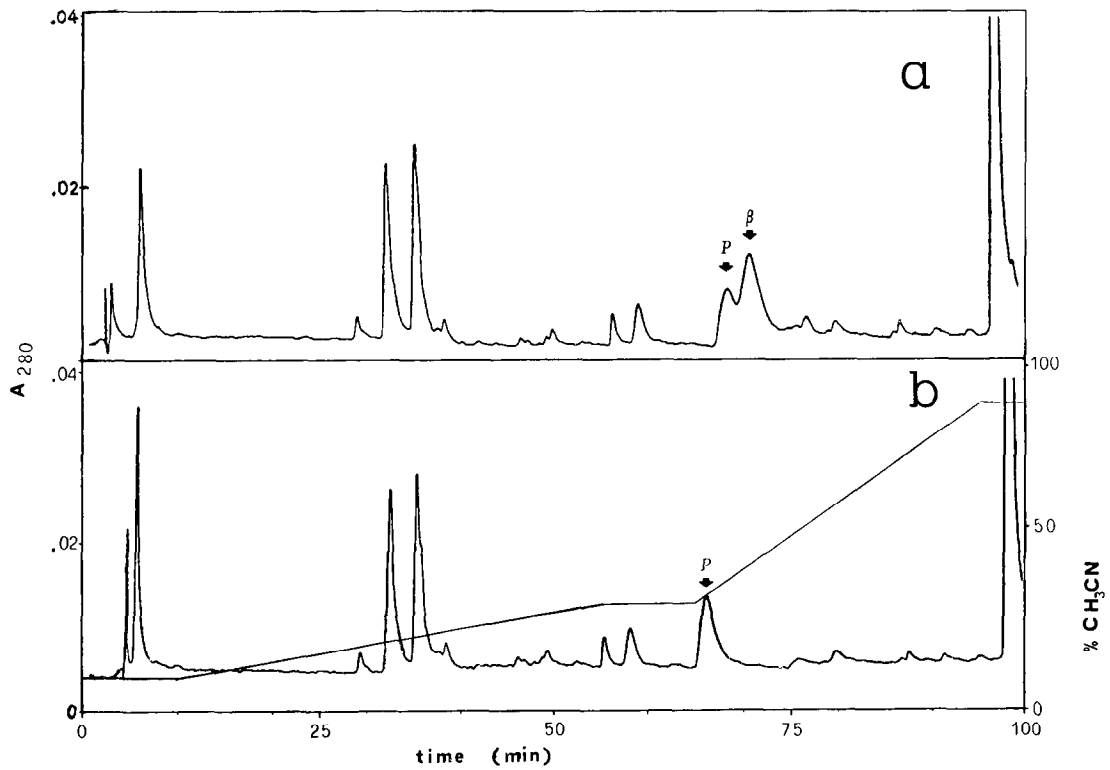

Fig.1. HPLC chromatograms of GPE digest (for details see section 2) with (b) and without (a) β-casomorphin. β, β-casomorphin 7; P, procasomorphin.

Table 1

Amino acid compositions of peptides purified from GPE and brush border digest

Peptide	Amino acid composition
Procasomorphin (fig.1)	Pro(3.7),Gly(1.0),Val(0.9), Ile(1.0),Tyr(0.8),Phe(1.0), Lys(0.8)
Peptide A (fig.2a–c)	Pro(1.2),Tyr(0.9),Phe(1.0)
Peptide B (fig.2a,b)	Pro(3.9),Gly(1.0),Ile(0.8), Tyr(0.6),Phe(0.9)
Peptide C (fig.2a)	Pro(4.3),Gly(1.2),Val(1.0), Ile(1.0),Tyr(0.8),Phe(0.9)

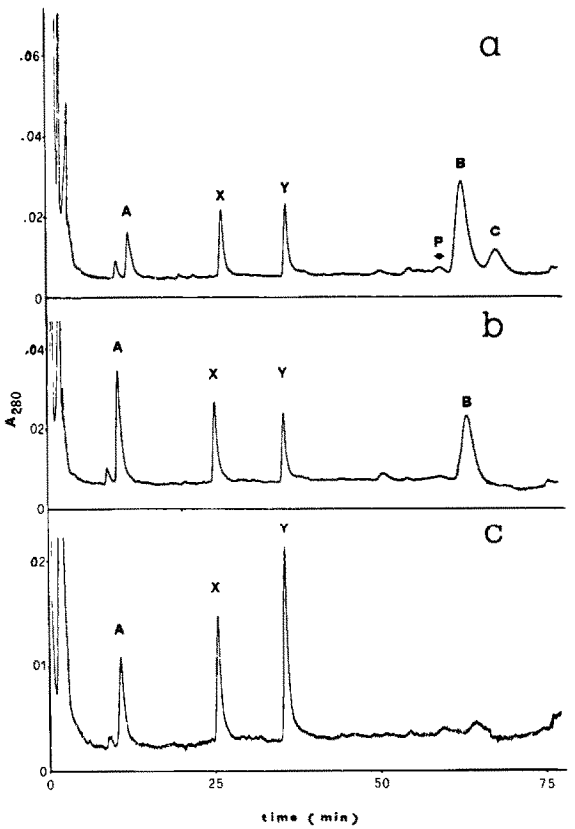

Fig.2. HPLC pattern of brush border digest of procasomorphin at 0.5 (a), 1 (b) and 4 h (c). Conditions were as in fig.1. Peaks X and Y arise from the brush border preparation; for the identification of peptides A–C refer to table 1 and fig.3. The arrow indicates the retention time of procasomorphin.

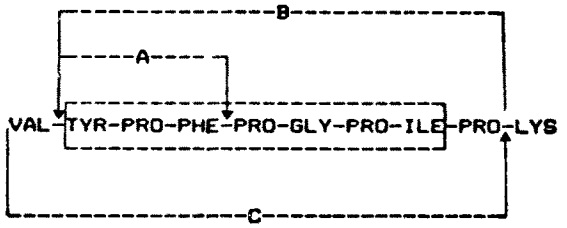

Fig.3. Amino acid sequence of procasomorphin. The sites of brush border protease hydrolysis are indicated by arrows. The sequence of β-casomorphin is boxed.

tions of purified peptides from which the sequences shown in fig.3 have been inferred. Under our experimental conditions we were not able to detect β-casomorphin 7 which could be putatively split from procasomorphin by the action of brush border amino- and carboxypeptidase activities.

In fig.3 the amino acid sequence of procasomorphin and the putative sites of hydrolysis by brush border proteases are reported. It is worth noting that peptide B purified from brush border digest at 0.5 and 1 h of incubation (fig.2a,b) differs from casomorphin 7 in having an additional proline residue at the C-terminal. The brush border carboxypeptidase activity which removes the C-terminal lysine from procasomorphin seems to be unable to remove the following proline residue. Furthermore, the presence of the peptide A (Tyr-Pro-Phe) at 0.5 h would indicate an endopeptidase as the major cause of the complete degradation of procasomorphin at 4 h of incubation.

The results can be summarized as follows:

(i) Proteolytic enzymes of gastrointestinal tract plus leucine aminopeptidase, under controlled conditions, do not release β-casomorphin 7 from β-casein but a putative precursor, i.e., procasomorphin;

(ii) Procasomorphin is further digested by brush border peptidases but not to casomorphin;

(iii) The peptide Tyr-Pro-Phe still resistant to proteolytic activity of the brush border is devoid of any opioid activity [9].

Apparently our results seem then to exclude a physiological role for β-casomorphin. However, only in vivo studies would unequivocally clarify whether β-casomorphin is of any physiological significance.

ACKNOWLEDGEMENTS

This work was supported by grants of Ministero della Pubblica Istruzione (to F.A. and S.A.: 'Peptidi Bioattivi da Fonti Alimentari'; and to P.P.) and of Progetto Finalizzato Chimica Fine e Secondaria CNR (to G.M.). The technical assistance of Miss G. Travaglione is acknowledged.

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