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Isolation and characterization of a bradykinin-potentiating peptide from a bovine peptic hemoglobin hydrolysate

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A bradykinin potentiating peptide was isolated from a peptic bovine hemoglobin hydrolysate, by the use of reversed-phase high-performance liquid chromatography (RP-HPLC). Its primary structure, determined by fast atom bombardment (FAB) and tandem mass spectrometry (MS/MS), was identical to fragment 129–134 of the α-chain of bovine hemoglobin. The bradykinin potency of this peptide, as exhibited by the guinea-pig ileum contraction, was significant and comparable with some others previously described.

Bovine hemoglobin; Bradykinin potentiation; Peptic hydrolysate; High-performance liquid chromatography; Mass spectrometry

1. INTRODUCTION

Bradykinin-potentiating activity is found in a large number of compounds from various chemical classes [1], among these compounds are a number of peptides from different sources, especially from protein enzymatic hydrolysates: tryptic hydrolysates of plasma proteins [1–3] and casein hydrolysates [4,5]. It was found that these peptides, which in themselves exhibited no bradykinin activity, enhanced the effect of subsequently added synthetic bradykinin on the guinea-pig ileum. Previous studies demonstrated that some active peptides able to potentiate kinin actions also showed their ability to inhibit more or less the angiotensin-converting enzyme (ACE) in vitro [3,6]. Therefore, although some peptides were able to inhibit ACE in vitro, this activity did not seem to be the only reason for the potentiation they cause [3]. Studies to confirm the possibility of a direct action of some potentiating peptides on the kinin receptor(s) as well as the specific mechanism of potentiation are still under way [7]. Their importance as naturally occurring ACE inhibitors are thus discussed. In addition, the question why molecules with quite different structures, i.e. peptides produced by trypsin digestion of serum albumin or casein, exert the same highly specific effect is not entirely solved [3,8].

Recently, a peptide called LVV-hemorphin-6, corresponding to the sequence at position 32–40 of the β-chain of the human hemoglobin, was reported to inhibit ACE activity [9]. This peptide was originally isolated from the human pituitary gland [10]. The normal concentrations of this peptide in plasma has still not been determined or similarly its possible importance in blood pressure regulation. Apparently, LVV-hemorphin-6 is a naturally occurring peptide which probably could be released from hemoglobin in the pituitary by the action of a trypsin-like and a chymotrypsin-like enzyme [10].

On the basis of this previous observation, we decided to investigate if a peptone preparation, obtained in our laboratory by peptic hydrolysis of bovine hemoglobin [11] could potentiate bradykinin activity towards isolated guinea-pig ileum. In the present paper the isolation of a bradykinin-potentiating peptide from this peptic hydrolysate is described. The active peptide was separated from the total hydrolysate, by HPLC and analysed by fast atom bombardment mass spectrometry (FAB-MS) [12].

Its structure revealed that it was a fragment of the α-chain of hemoglobin. This peptide was then synthetized in order to verify its activity.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

The synthetic peptide used in this study was synthetised by C. Guillon (Laboratoire de Technologie Enzymatique, University of Compiègne, Compiègne, France). Bradykinin, acetylcholine, angiotensin II, atropine sulfate and histamine were purchased from Sigma. All other chemicals and solvents were of analytical grade from commercial sources. The guinea-pig ileum contractions were recorded with a Washington isotonic transducer. A Waters system was used for the RP-HPLC separation. The instrument was equipped with a silica gel C-18 semi-preparative column (Waters Delta Pak, 19 × 300 mm, particle size 15 μm).

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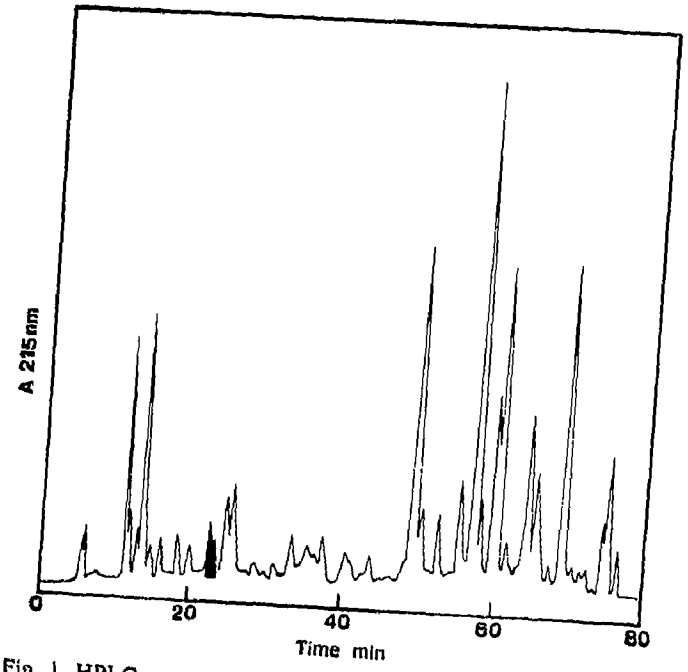

Fig. 1. HPLC separation of peptic hemoglobin hydrolysate on C_{18} Delta Pak column. Bradykinin-potentiating activity co-eluted with the absorbance peak marked in black.

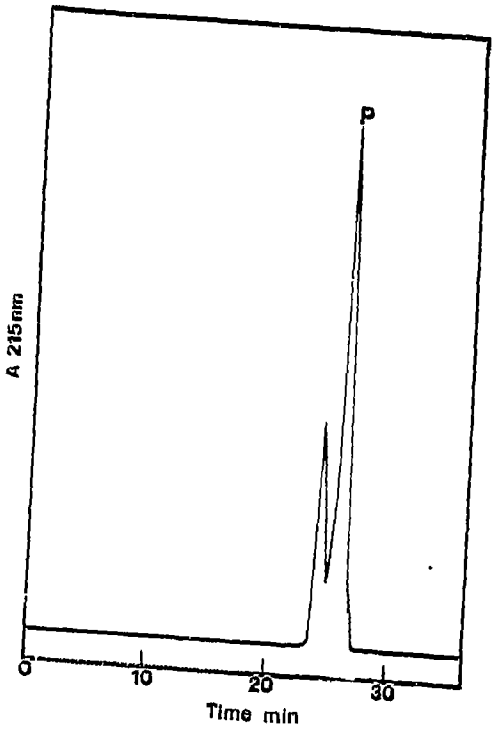

Fig. 2. HPLC elution of the bradykinin-potentiating peptide (P) from the active peak collected in Fig. 1. The column was a C_{18} Delta Pak.

2.2. *Hydrolysate preparation*

Bovine hemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor as previously described in [11]. Hemoglobin obtained by hemolysing erythrocytes was added to water and 4 M hydrochloric acid to obtain 300 liters of 5% denatured hemoglobin at pH 2. For peptic digestion, 80 liters of hemoglobin were heated to 40°C in a reactor and added to porcine pepsin (240 Anson units). Hydrolysis lasted 8 h and pH 2 was maintained by a pH stat.

After 8 h, the reactor was connected to ultrafiltration membranes (mineral membranes, with nominal molecular weight cut-off lower than 20,000 Da) with an area of 0.32 m^2. Ultrafiltration was conducted at 40°C for 22 h. Operating pressures were adjusted in order to keep a constant ultrafiltration flow (10 l/h). Incubation volume was maintained constant in the ultrafiltration reactor by adding hemolysate. The ultrafiltrate was desalted by electrodialysis and then atomized. The nitrogen content of the peptidic powder was determined by the Kjeldahl method and allowed us to evaluate amounts of peptides in the hydrolysate greater than 90% ($N \times 6.25$). The percentage of free amino acids was estimated under 1%.

2.3. *Reversed-phase high-performance liquid chromatography (RP-HPLC)*

RP-HPLC separations were performed on a silica gel C-18 column

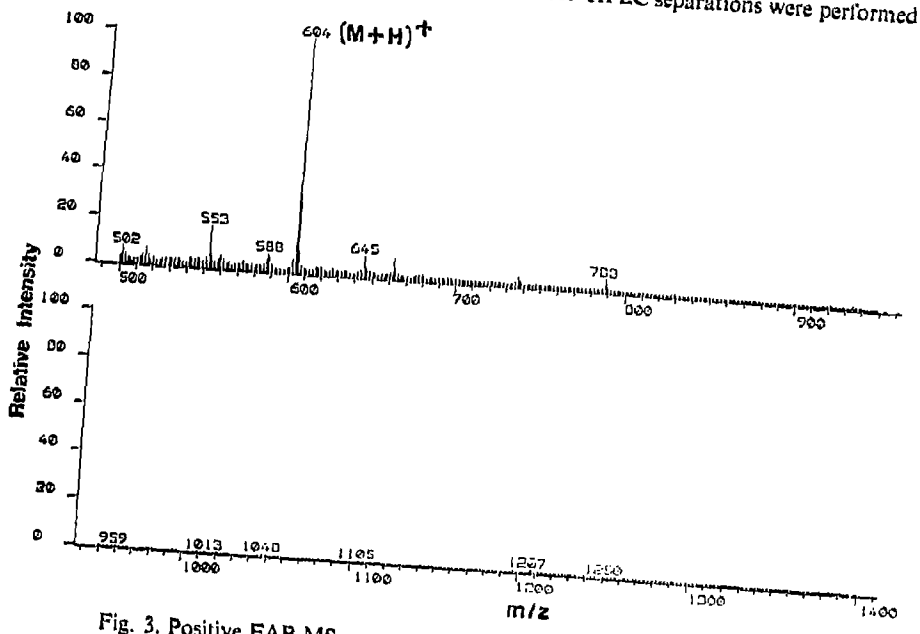

Fig. 3. Positive FAB-MS spectrum of the bradykinin-potentiating peptide.

with a mobile phase comprising 10 mM ammonium acetate buffer, pH 6.0, as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples of hemoglobin hydrolysate were dissolved in buffer A (200 mg/ml) filtered through 0.20 μm filters and 500 μl were injected. The linear gradient applied was 0–40% B in 80 min. Re-equilibration at 0% B for 10 min was performed between each analysis. Eluted fractions were collected and freeze-dried prior to mass spectrometry analysis and bradykinin-potentiating activity studies.

2.4. *Mass spectrometry analysis*

Mass spectra, generated from FAB-MS and tandem mass spectrometry (MS/MS) of the active peptide, were recorded on a four-sector 'Concept II' tandem mass spectrometer (Kratos, Manchester, UK). Ions were produced in a standard FAB source by bombarding the sample with xenon atoms having a kinetic energy of 8 keV and the instrument was operated at an accelerating voltage of 8 kV.

The peptide was dissolved in water (250 μg of 50 μl) and 1 μl of the solution was loaded on the stainless steel tip with thioglycerol as matrix. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Cesium iodide was the standard for mass calibration. In the MS/MS mode, only the ^{12}C component of the $(M+H)^+$ ion is selected by the first two sectors and transmitted into the collision region. The collision cell was filled with helium and the collision energy was 6 keV. Thus, the peptide sequence was determined from the spectrum produced by the collision-induced decomposition (CID) of the protonated molecule, $(M+H)^+$.

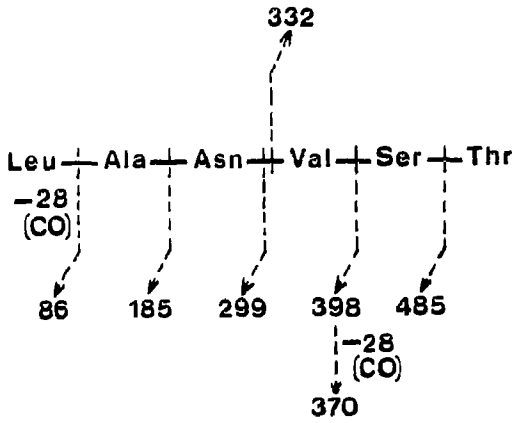

Table 1

Specificity of the potentiation of bradykinin and other agonists on the guinea-pig ileum. Our values are given by means from 3 separate experiments

Agonist (ng/ml bath fluid)	Contraction (mm)		Potentiating factor ($P f$)
	− Peptide	+ Peptide (3.5 μg/ml)	
Bradykinin (5)	30	79	2.63
Angiotensin II (0.75)	79	80	1.01
Histamine (3)	48	53	1.1
Acetylcholine (1.5)	128	131	1.02

2.5. *Bioassays*

This was done routinely on guinea-pig ileum. The preparation previously described [3] was used: a 3–4 cm length of ileum obtained from female animals (250 g) was suspended in a 10 ml, 37°C bath with Tyrode solution containing 1.0 μg of atropine sulfate/ml. Peptides to be assayed for bradykinin-potentiating activity were at first dissolved in Tyrode. A certain dose of bradykinin was added either alone or 1 min after a peptide, the potentiating effect of which was to be determined. The effect obtained from addition of the combination was compared with the dose–effect curve of bradykinin alone and a 'bradykinin equivalent' of the combination was determined [1]. A potentiating factor was calculated as:

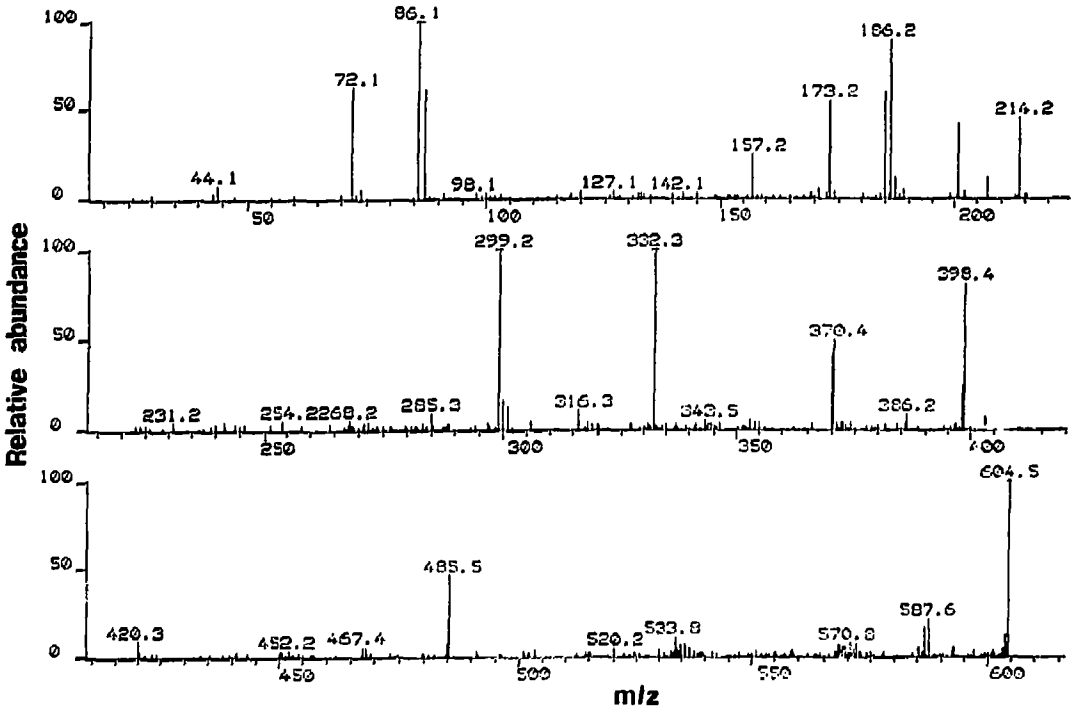

Fig. 4. CID spectrum of $(M+H)^+$ ion of the bradykinin-potentiating peptide, and notation of fragment ions.

$$Pf = \frac{\text{bradykinin equivalent of bradykinin + peptide}}{\text{administered dose of bradykinin}}$$

3. RESULTS AND DISCUSSION

The UV-profile from the fractionation of a total peptic hydrolysate of hemoglobin on the C_{18} reverse-phase column is shown in Fig. 1. As can be seen in Fig. 1, the hydrolysate resolved into a large number of peaks corresponding to peptides of different molecular size. One active fraction, found among several other inactive peptides, was collected for further purification by RP-HPLC under the same conditions. By this technique, the active fraction was resolved into two peaks (Fig. 2). The major bradykinin-potentiating component eluted at 13% of acetonitrile. This component was subjected to an additional separation on RP-HPLC in order to check its purity (not shown). Based on this data, the active peptide obtained in significant amounts from the semi-preparative RP-HPLC was used throughout the present work for mass spectrometry analysis and bradykinin-potentiating activity studies.

Fig. 3 shows the positive FAB-MS spectrum of this selected peptide. Accurate relative molecular mass, deduced from the *m/z* value of $(M+H)^+$ by substraction of one mass unit for the attached proton is 603. In order to determine the precise amino acid sequence of the peptide, tandem MS was performed. In the collision cell of the mass spectrometer, the precursor ion fragments by CID into structurally significant product ions. Fig. 4 shows the CID spectrum of the $(M+H)^+$ ion and displays notation of fragment ions of the peptide according to this CID spectrum. Interpretation along the fragmentation process results in the recognition of a series of ions indicating the following sequence: Leu-Ala-Asn-Val-Ser-Thr. Owing to this amino acid sequence, this peptide effectively represents fragment 129–134 of the α-chain of bovine hemoglobin.

The specificity of the active peptide for bradykinin potentiation is indicated by the results obtained in a set of experiments, which showed firstly (Table I) that this peptide did not change significantly the effect of several smooth muscle agonists such as acetylcholine, angiotensin II and histamine, whereas the potentiating factor is 2.63 in the presence of bradykinin. Then, the data presented in Fig. 5 exhibit the additive effect of the peptide, when increased amounts of bradykinin were added to the guinea-pig ileum in the presence or in the absence of this peptide. The points of the curves represent the mean ± SD of data obtained with three different pieces of smooth muscle. Almost parallel curves were obtained in this bradykinin concentration range (2–12 ng/ml bath fluid). It must be pointed out that the peptide added by itself involved no contraction of the muscle. Furthermore when increased amounts of peptides were added with a constant concentration of bradykinin (3.75 ng/ml), the potentiation increased as a function of peptide amount (Fig. 6). All these experiments were performed with the identical synthetic peptide and gave very similar results (not shown).

Thus, as far as the specificity is concerned, the peptide isolated from bovine hemoglobin gave marked potentiations in the range 2–12 ng/ml of bradykinin, which is not very far from the results previously described for kinin-potentiating peptides generated from human serum protein [3] or casein hydrolysates [4,5]. Thus our peptide could be classified along with such natural peptides and more precisely with hemoglobin-derived biologically active peptides such as hemorphins. Such correlations have already been noted between ACE inhibitors from bovine casein hydrolysate and casomorphin [13].

This could be further extrapolated for hemoglobin-

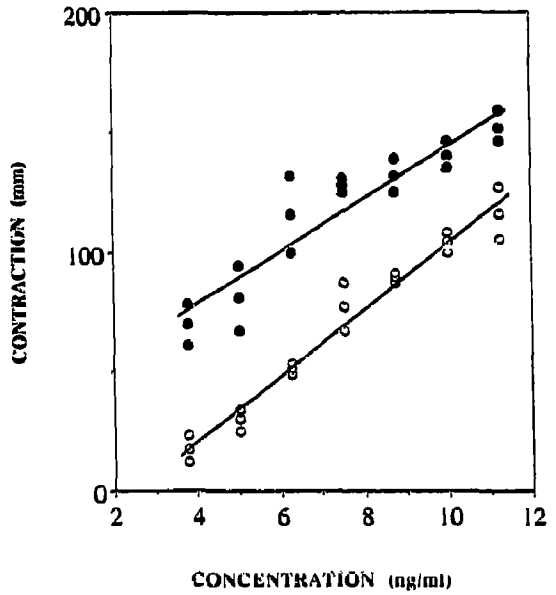

Fig. 5. Dose-response curves of bradykinin in the absence (○) and in the presence (●) of bradykinin-potentiating peptide (3.5 μg/ml bath fluid) with the guinea-pig ileum.

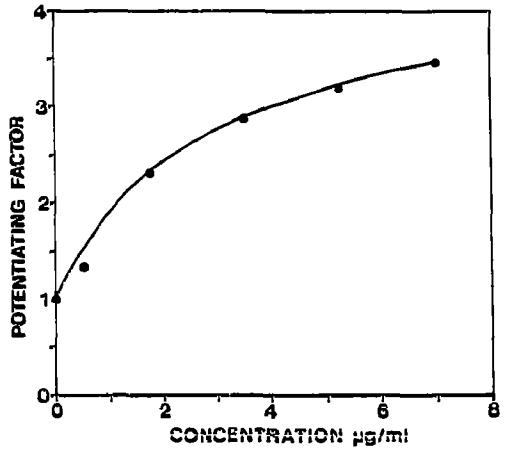

Fig. 6. Dose-dependence of the potentiating activity of bradykinin-potentiating peptide in the guinea-pig ileum. Bradykinin was fixed at 3.75 ng/ml bath fluid.

derived peptides since one of them, the opioid active fragment LVV-hemorphin-6 [10] was also recently described as an ACE inhibitor [9].

A comparison between the α-chains of bovine and human hemoglobin revealed that the hexapeptides located in position 129–134 differ only in one amino acid: Leu-Ala-Asn-Val-Ser-Thr (bovine) versus Leu-Ala-Ser-Val-Ser-Thr (human). Thus, it would probably be of interest to test the specificity of this human hemoglobin-derived peptide for bradykinin potentiation. In the present work, the bradykinin potentiating hexapeptide was found in a peptic hemoglobin hydrolysate, so that it could be produced plausibly during digestion in the stomach of foods containing blood [14]. Thus, one may speculate, about the formation of this kind of biologically active peptide in the gastrointestinal tract, and if it would be able to exert a bradykinin potentiation activity in vivo. There are now indications that fragments of proteins, in the form of small peptides, can cross the small intestine to a certain degree and thus gain access to peripheral tissue via the circulation [15,16].

Further investigations need to be done with our peptide to examine its influence on ACE activity, in order to determine if it exerts its potentiating effect by an ACE-dependant or -independant way.

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