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INHIBITION AND INHIBITION KINETICS OF ANGIOTENSIN CONVERTING ENZYME ACTIVITY BY HEMORPHINS, ISOLATED FROM A PEPTIC BOVINE HEMOGLOBIN HYDROLYSATE

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SUMMARY: The inhibitory effect on angiotensin converting enzyme (ACE) activity by hemorphins isolated from an enzymatic bovine hemoglobin hydrolysate was reported. Inhibitory kinetics proved that inhibition mechanism was non-competitive. The stability of hemorphins towards ACE was studied. © 1994 Academic Press, Inc.

The hemorphins, peptides with affinity for opioid receptor, were previously isolated from enzymatically treated bovine blood [1]. These peptides were found during the purification procedure of cytochrophins, opioid peptides derived from mitochondrial cytochrome b, originally obtained by treatment of bovine blood with a mixture of gastrointestinal enzymes. Two of these hemorphins (Tyr-Pro-Trp-Thr and Tyr-Pro-Trp-Thr-Gln) termed hemorphin-4 and hemorphin-5, respectively, were identified to the 34-37 and 34-38 fragments of the beta-chain of bovine hemoglobin and the 35-38 and 35-39 fragments of beta-chain of human hemoglobin. Recently, an opioid active fragment of hemoglobin was isolated from human pituitary gland[2,3]. This peptide (LVV-hemorphin-6) corresponds to the sequence at position 32-40 of the beta chain of human hemoglobin. This peptide is found in large amounts in the pituitary, so it is likely to occur in the circulation. The same group has also demonstrated that LVV-hemorphin-6 was able to inhibit angiotensin converting enzyme (ACE) activity [4]. ACE, an exopeptidase that cleaves dipeptide from the C-terminus of various oligopeptides, has an important physiological function in blood pressure regulation. ACE converts the inactive decapeptide angiotensin I to the potent vasopressor octapeptide, angiotensin II. The possible roles of the hemorphins as natural occuring hypotensive agents have been suggested [5] although the way of their occurence has not yet been established.

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We have previously reported the isolation of two opioid peptides, LVV-hemorphin-7 and VV-hemorphin-7, from a bovine hemoglobin peptic hydrolysate [6]. When we compared our isolated peptides with previously described hemorphins obtained either from the bovine or human beta-chain of hemoglobin, it was noticed that all these hemorphins whatever their source, originated from the same region of the beta-chain, termed as "strategic zone" [6]. In our work pepsin was solely used for hydrolysis of hemoglobin[7]. Such peptic hydrolysis was previously related by ZIOUDROU et al [8] for the production of exorphins from food protein.
In the present work, the inhibitory effect of our isolated peptic hemorphins towards ACE activity was tested . The influence of the hemorphins on ACE activity was examined by measuring their inhibitory effect on the hydrolysis of z-furanacryloyl-L-phenylalanylglycylglycine (FAPGG) [9], a specific substrate for ACE activity. In the same time, inhibition kinetics of ACE activity by the hemorphins was investigated. The stability of hemorphins towards ACE activity was also determined by incubation with ACE and then analysis of the peptides fragments.

MATERIALS AND METHODS

Materials and chemicals

All common chemicals and solvents were of analytical grade from commercial sources. Rabbit lung angiotensin converting enzyme, FAPGG and captopril were purchased from Sigma Chemicals. Hemorphin-7 was synthesized by C.Guillon, Laboratoire de Technologie Enzymatique, University of Compiègne, France

Methods

Hydrolysate preparation. Bovine hemoglobin hydrolysate was obtained at pilot- plant scale by peptic proteolysis in an ultrafiltration reactor as previously described in reference [7].

Preparation of LVV-hemorphin-7 and VV-hemorphin-7. LVV-hemorphin-7 and VV-hemorphin-7 were prepared by GP and RP HPLC using a Waters 600 E system.
GP-HPLC. Separation were performed on a 60 cm x 21.5 mm i.d. TSK G2000 SWG column eluting with 10 mM ammonium acetate buffer (prepared daily from analytical ammonium acetate and adjusted to pH 6.0 with acetic acid). Hydrolysate powder samples of 50 mg were dissolved in 500µl of the same buffer and filtered through 0.2 µm filters before being applied to the column. The flow rate was 6 ml/min. Fraction were collected and freeze dried.
RP-HPLC. The active fraction eluted from TSK G2000 SWG was analyzed by RP-HPLC on a 30 cm x 19 mm i.d. Delta Pak C-18 column. The mobile phase comprised 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. The flow rate was 12 ml/min. Samples were dissolved in buffer A (200 mg/ml) filtered through 0.20 µm filters and 500µl were injected. The gradient applied was 0-40% B in 40 min. Fraction were collected and freeze dried.

Amino acid analysis. Amino acids were analyzed using a Waters Picotag Work Station. Peptide hydrolysis was achieved with constant-boiling HCl containing 1% phenol, for 24 h. at 100 °C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column(150 mm x 3.9 mm i.d.) were performed according to Bidlingmeyer et al. [10]. The detection wavelength was 254 nm and the flow rate 1 ml/min.

Mass spectrometry analysis. Mass spectra, generated from Fast Atom Bombardment (FAB) mass spectrometry 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 KeV. The peptide was dissolved in water (250µg/µl) and 1µl 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. Caesium iodide was the standard for mass calibration.

Inhibition studies. ACE activities were routinely measured with 5 X 10^{-5} M FAPGG as substrate in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.3 M NaCl by the method of HOLMQUIST [9]. In a typical run, 1.2 ml of substrate in the above buffer was placed in the spectrophotometer and allowed to reach thermal equilibrium at 25°C. ACE 7.5 mU, was added to initiate hydrolysis.The absorbance decrease at 328 nm in the first 5 min. was monitored with a PERKIN ELMER model Lambda 16 UV/VIS spectrophotometer. The comparison of enzymatic reaction slopes obtained with different hemorphins concentration allowed us to determine their inhibitory effect on ACE activity and Michaelis constants.

Verification of ACE purity. In order to verify the absence of unexpected enzyme and particularly carboxypeptidase, hydrolysis kinetics of FAPGG (2.5 10^{-5} M) by ACE(15mU) followed in the presence or absence of captopril (0.1 µM) added to the substrate prior to the incubation.

Stability studies. 0.1 mg of either VV-hemorphin-7 or LVV-hemorphin-7 were dissolved in 1 ml above Tris - HCl buffer and incubated at 37 °C with ACE. (7.5 mU) during 7 h. The reaction mixture was then analyzed by reversed phase high performance liquid chromatography (RP-HPLC). The degradation products of the hemorphins were resolved on a Nova-Pak C-18 column (3.9 mm i. d. X 150 mm). The mobile phase comprised: 10 mM ammonium acetate buffer pH 6.0 as eluent A and acetonitrile as eluent B. A linear gradient (15-30% B in 15 minute) was applied.

RESULTS AND DISCUSSION

The peptic hydrolysate of bovine hemoglobin was first fractionated in 9 fractions by TSK column, as previously described [6]. One fraction (FVII) exhibited an opioid activity determined by use of electrically stimulated myenteric plexus/longitudinal muscle preparation of the guinea-pig ileum (GPI). This active fraction was collected for further separation by RP-HPLC. The figure 1 shows that this active fraction was resolved into two peaks, which were identified as VV-hemorphin-7 (P1) and LVV-hemorphin-7 (P2) respectively by positive FAB-MS and amino acid analysis.

Since hemorphins might be formed in plasma during physiological or pathological degradation of hemoglobin [4], it is interesting to determine their action on ACE activity. Figure 2 exhibits the percentage of inhibition of ACE activity by the hemorphins. The IC_{50} (the final concentration of peptide which inhibited 50% ACE activity) of hemorphin-7, VV-hemorphin-7 and LVV-hemorphin-7 were 16, 10.2 and 1.7µM respectively. A remarkable difference of inhibition capacity of ACE activity between LVV-hemorphin-7 and VV-hemorphin-7 appeared although these two peptides only differ from one another by the presence of leucine in N-terminus (LVV-hemorphin-7). This phenomena was already described in fragments of human beta casein [11]. It indicated that the prolongation of N-terminus in some peptides can increase

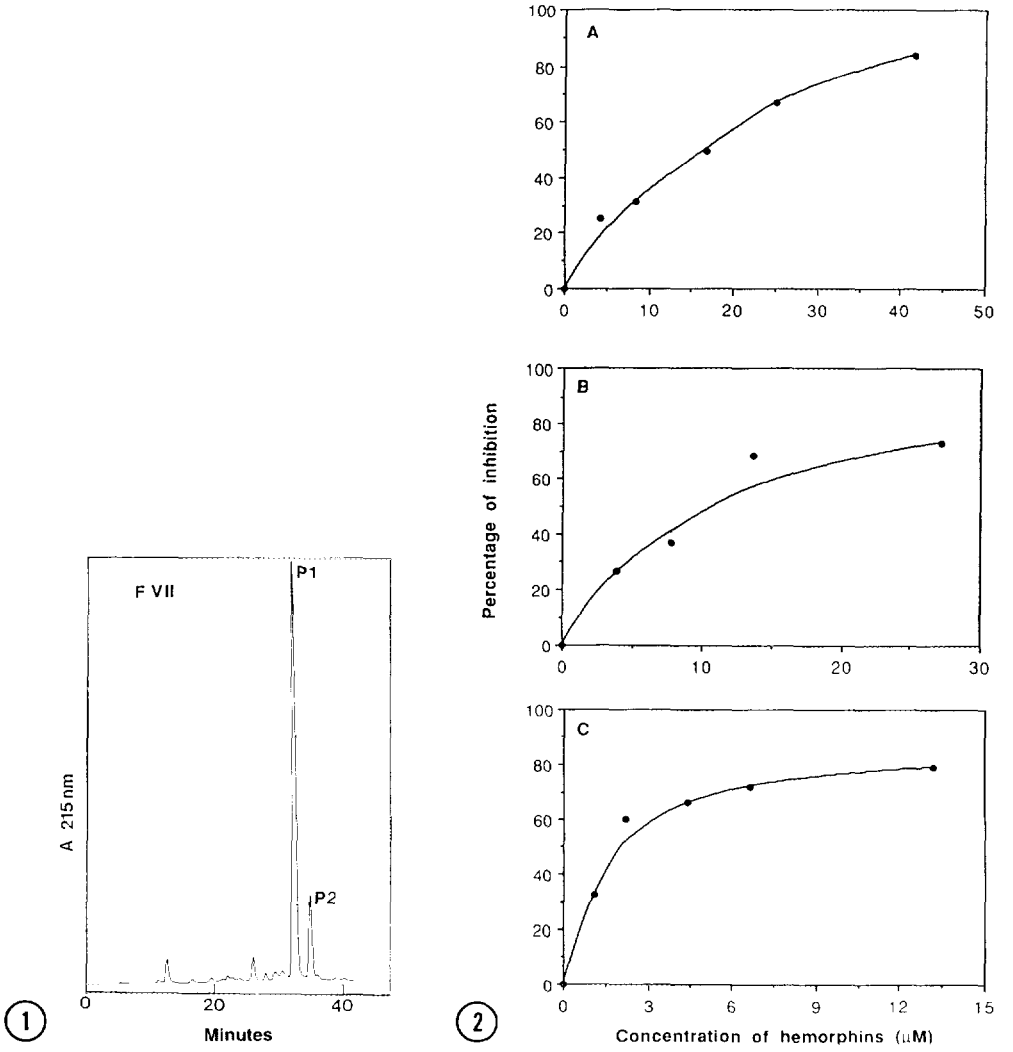

Figure 1. Purification of VV-hemorphin-7 and LVV-hemorphin-7 on a reverse-phase HPLC column. An active fraction from the TSK G2000 SWG column was applied to a Delta Pak C18 column and eluted with a mobile phase consisting of 10 mM ammonium acetate buffer,pH 6.0, as eluent A and acetonitrile as eluent B. The gradient was 0 to 40% B for 40 min with a flow rate of 12 ml/min.

Figure 2. Percentage of inhibition of ACE activity in the presence of various concentrations of hemorphin-7(A), VV-hemorphin-7(B) and LVV-hemorphin-7(C). The constant concentrations of FAPGG and ACE were 10^{-4} M and 7.5 mU/1.2 ml. Temperature was maintained at 25 °C.

the potency to inhibit ACE activity. The relative inhibitory potencies of the three hemorphins in the present study are consistent with this hypothesis. The most active hemorphins for inhibiting ACE activity was LVV-hemorphin-7. Its potency was comparable to both Bothrops and Agkistrodon venom peptides [12, 13, 14], which were competitive with respect to ACE substrate. The structure - activity relationships study suggested that the snake venom peptide inhibitors bind to ACE via their C-terminal sequence and terminal carboxyl group [15,16, 17], the proline

residue at C-terminus position being essential for eliciting the inhibitory activity [18, 19]. An aromatic amino acid such as tryptophan or phenylalanine in the antepenultimate position was required for maximal inhibitory activity [19]. In these regards, LVV-hemorphin-7 has not any relationships in its structure of C-terminus with that of snake venom peptide inhibitors, but their inhibitory activities were very similar. The group of KOHMURA [11] has synthesized 69 peptides mimicking human beta-casein fragment. All of these synthetic peptides were considered to satisfy the requirements for binding to active site model of ACE and expected to possess the inhibitory activity. However, compounds with very varying potency were obtained. Our results showed that the inhibition degree of ACE activity by the hemorphins remained stable around 80%. According to the above results it was necessary to investigate the inhibition mechanism of ACE activity by the hemorphins.

In order to ascertain ACE purity, the verification of the absence of unexpected enzyme and particularly carboxypeptidase was carried out. Hydrolysis kinetics of FAPGG ($2.5\ 10^{-5}$ M) by ACE(15mU) followed in the presence or absence of captopril (0.1 µM) added to the substrate prior to the incubation were performed. The results showed in figure 3 clearly evidenced the absence of unexpected enzyme , particularly carboxypeptidases, in the commercial purchased ACE.

As mentioned by HOLMQUIST et al [9], at low concentration of FAPGG,([S] < Km), the reaction kinetics was first order and hydrolysis were allowed to proceed to more than 95%

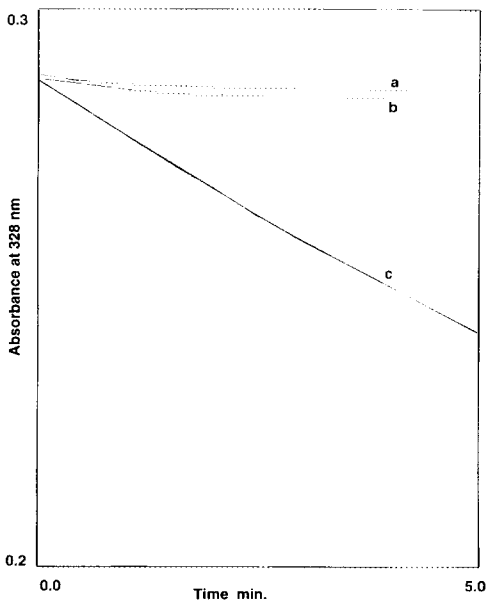

Figure 3. Hydrolysis kinetics of FAPGG by ACE in presence or absence of captopril with the condition described in experimental section. a: Substrate FAPGG (2.5×10^{-5}M); b: FAPGG (2.5×10^{-5}M) + Captopril (0.1 µM) + ACE (15 mU); c: FAPGG (2.5×10^{-5}M) + ACE (15 mU).

completion. Figure 4 displays the 1/ rate versus 1/[S] with different amounts of hemorphins. The Michaelis constant (Km) for FAPGG in these conditions was found to be around 1.25 X 10^{-4} M which is very similar to that obtained by HOLMQUIST et al [9], (Km=3 X 10^{-4} M).The unaltered Km with increasing concentration of inhibitor i.e., VV-hemorphin-7 and LVV-hemorphin-7 suggested a noncompetitive mechanism. It is quite different with peptides inhibitors of ACE often described in the literature, for which the inhibition is frequently competitive [20]. It indicates that the hemorphins may interact with ACE in different way from that of the substrate, and produce an inhibitory effect.

In our study, the stability of the hemorphins in presence of ACE was also examined. When VV-hemorphin-7 was incubated with ACE the Gln-Arg bond was cleaved and VV-hemorphin-5 appeared (Table 1, Figure 5, a,c). The later was then relatively stable and remained even after 7 h. of incubation. LVV-hemorphin-7 was incubated in the same conditions. After 7 h. of incubation, Arg-Phe was released and gave no further cleavage of LVV-hemorphin-5 (Table 1, Fig. 5, b,d). Similar results have been obtained by Glämsta[4]. It indicated that the C-terminus of VV- hemorphin-7 and LVV-hemorphin-7 could interact with the active site of ACE and be cleaved. The N-terminus of the hemorphins was not hydrolysed by

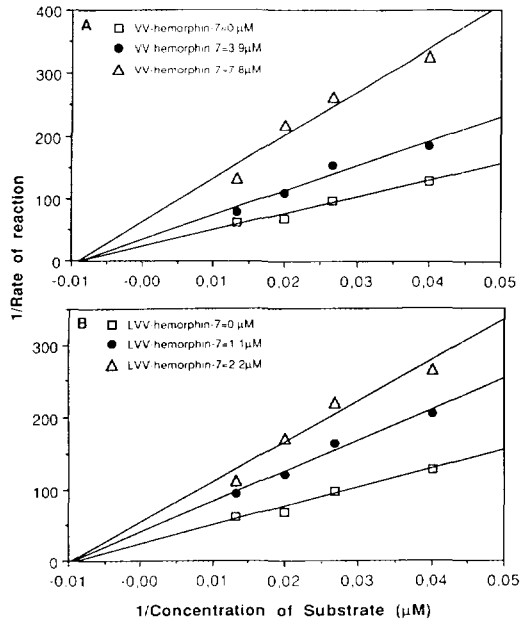

Figure 4. Lineweaver-Burk plot of FAPGG concentration effect on the activity of ACE in absence or presence of VV-hemorphin-7 (A) and LVV-hemorphin-7 (B). The concentration of ACE was 7.5 mU/1.2 ml. Temperature was maintained at 25 °C.

Table 1. Hemorphins hydrolyzed in vitro by ACE. 0.1 mg of VV-hemorphin-7 or LVV-hemorphin-7 was incubated at 37°C in 1 ml Tris -HCl buffer in the presence of 7.5 mU ACE. The incubation time was up to 7 h, then the reaction mixture was analyzed by reversed phase high performance liquid chromatography (RP-HPLC).

Peptide	Structure and cleavage point
VV-hemorphin-7	Val - Val - Tyr - Pro - Trp - Thr - Gln ↓ Arg - Phe
LVV-hemorphin-7	Leu - Val - Val - Tyr - Pro - Trp - Thr - Gln ↓ Arg - Phe

ACE. This pointed out that this part of molecule has probably no direct interaction with the active site of ACE, in spite of an inhibitory effect on ACE.

In the present work, we demonstrated an inhibitory effect on ACE activity by hemorphins isolated from a peptic hydrolysate of bovine hemoglobin. The N-terminus of the hemorphins played a very important role for their inhibitory activity, and the most active

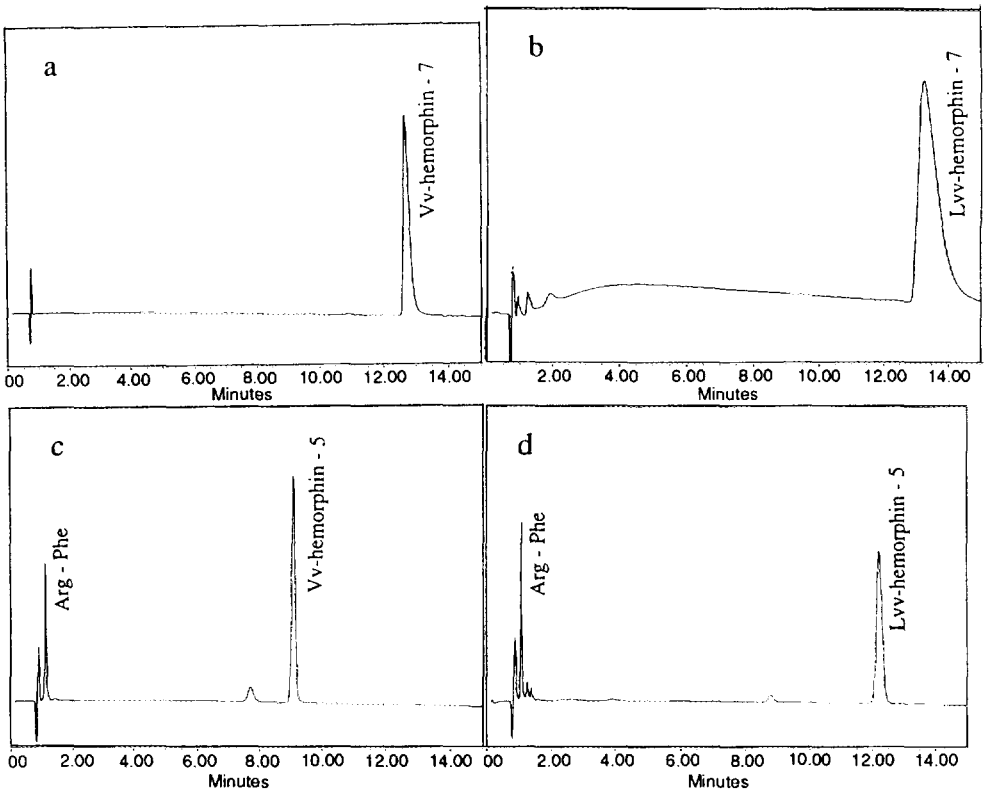

Figure 5. HPLC separation of VV-hemorphin-7 and LVV-hemorphin-7 and their degradation products after 7 h of incubation. The hemorphins and their degradation products by ACE were resolved on a Nova-Pak C-18 3.9 X 150 mm column with a linear gradient of acetonitrile. The mobile phase comprised 10 mM ammonium acetate buffer,pH 6.0,as eluent A and acetonitrile as eluent B. The gradient applied was 15-30% B in 15 minutes. The identity of the peptide fragments was confirmed by FAB mass spectrometry.

hemorphin was LVV-hemorphin-7. Inhibitory kinetics demonstrated that the inhibitory mechanism was non competitive, the stability of our hemorphins towards ACE was similar to that of LVV-hemorphin-6 [4]. Further investigations are needed to find how these blood - born peptides may occur as degradation products in vivo and then can exert a physiological activity in blood pressure regulation.

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