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ISOLATION OF ANGIOTENSIN-CONVERTING ENZYME INHIBITOR FROM PORCINE PLASMA

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An inhibitory peptide of angiotensin-converting enzyme was purified from porcine plasma. The final preparation showed IC_{50} values of 4.2, 0.6, and 0.9 μg/ml for angiotensin-converting enzymes from guinea pig serum, rabbit lung, and monkey brain, respectively. The amino acid sequence of the inhibitor was determined as leucyl-valyl-leucine by Edman procedure. This structural observation was supported by mass spectrometric analysis.

Angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) cleaves angiotensin I to the potent vasopressor angiotensin II, releasing C-terminal dipeptide. It also inactivates the vasodepressor bradykinin. The clinical importance of ACE inhibitors has been considered for the treatment of hypertension. Since the recent studies with captopril (1), much attention has been given to the synthesis of ACE inhibitors, based on structure-activity studies. However, the observation of natural occuring ACE inhibitors is rare; only snake venom is known as a natural source of ACE inhibitory peptides (2).

In the preliminary studies, we found the presence of ACE inhibitory factor in porcine plasma. The inhibitory activity was extracted by 80% methanol from plasma-treated charcoal powder. In this report, we describe the isolation and properties of ACE inhibitor from porcine plasma.

MATERIALS AND METHODS

Enzymes: Guinea pig serum ACE and monkey brain ACE were purified as described previously (3,4). Rabbit lung ACE was purchased from Sigma Chemical Co.

Enzyme assay: The ACE activity was assayed measuring the amount of the liberated hippuric acid from hippuryl-His-Leu by the modified method of Hayakari

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et al. (5). The reaction mixture contained 100 mM Tris-HCl buffer, 300 mM NaCl and 5.6 mM hippuryl-His-Leu, in a total volume of 250 µl. The pH of Tris-HCl buffer was 7.4 for guinea pig serum ACE and 8.2 for monkey brain and rabbit lung ACEs, respectively. Bestatin (100 µg/ml) was added to the reaction mixture of rabbit lung ACE for depression of aminopeptidase activity. After 3 min incubation, the reaction was initiated by addition of the enzyme and carried out at 37°C for 30 min. The reaction was terminated by the addition of 15 µl of 1N NaOH; after 30 min at room temperature, 1 ml each of 60 mM sodium phosphate buffer, pH 7.2, and 1% cyanuric chloride in ethylene glycol monomethyl ether (w/v) was added to the reaction mixture. After 15 min, the absorbance at 382 nm was determined.

Preparation of porcine plasma: Porcine blood (12 l) was obtained from the slaughterhouse, plasma (0.5% sodium citrate) was prepared by centrifugation at 600 x g. An equal volume (6 l) of water was added to the plasma, the pH was adjusted to 2 by conc HCl with test paper. The solution obtained was applied to a column (6 x 18 cm) of Amberlite XAD-4, the column was washed successively with 400 ml of 0.01N HCl, water, and 10% methanol; elution was carried out with 800 ml of 80% methanol. The 80% methanol fraction was evaporated under reduced pressure, 3-4 g of brown colored powder was obtained from 6 l of porcine plasma. The powder was stored at room temperature under vacuum. Subsequent purification procedures are described in "RESULTS AND DISCUSSION".

RESULTS AND DISCUSSION

The purification procedure is outlined in Table 1. Through the purification steps guinea pig serum ACE was used as the monitoring enzyme. Stage 1, pooled powder (50g) was dissolved by stirring for 20 h, then a large amount (18g) of precipitate was removed by filtration. Stage 5, 20 mg of the sample was applied to the reverse-phase HPLC column. On elution, two non-symmetrical peaks, overlapping each other, were appeared. The volume of these fractions was 56-80 ml. The ACE inhibitory activity of the late appearing peak (fractions 68-80 ml) was 5-fold higher than that of early peak (fractions 56-68 ml). The late appearing peak was further purified. Stage 6, rechromatography by reverse-phase HPLC was necessary for the final purification step, yielding 27 mg of a white amorphous powder.

The final preparation showed positive ninhydrin and Rydon-Smith reactions on silica-gel plate. A single spot (Rf=0.36) was observed on silica-gel thin layer chromatography with the solvent system of ethyl acetate : n-butanol : acetic acid : water = 4 : 4 : 1 : 1 (v/v). High voltage paper electrophoresis at 3.5 kv for 15 min was carried out at pH 1.8; formic acid : acetic acid : water = 75 : 225 : 1700 (v/v), a spot (Rm=0.60) was observed,

Table 1. Separation of angiotensin-converting enzyme inhibitor from porcine plasma

1.	The pooled powder (50g) was dissolved in 1 l of 0.05 M pyridine-acetate pH 2.7. Filtered (filter paper) supernatant was applied to the column (4.5 x 14 cm) of Dowex 50W-X8 (H^+type). Elution was with a 2 l linear gradient of 0.05 M pyridine-acetate pH 2.7 to 1 M pyridine-acetate pH 4.7. Inhibitory activity emerged in fractions 900-1170 ml.
2.	The dried sample was dissolved in water, absorbed to the column (4.5 x 14 cm) of Dowex 50W-X8 (H^+type). Elution was with a 2 l linear gradient of water of 4N NH_4OH. Inhibitory activity was found in fractions 540-790 ml.
3.	The dried sample was dissolved in 1 M acetic acid. Gel chromatography was carried out on Bio-Gel P-2 column (2.8 x 95 cm) with 1 M acetic acid. Inhibitory activity was recovered in fractions 320-400 ml.
4.	The dried sample was dissolved in water, absorbed to the column (1.2 x 4.5 cm) of Porapak Q*. Elution was with ethanol.
5.	The dried sample was dissolved in the solvent; 0.1% acetic acid : acetonitrile = 90 : 10 (v/v), applied to the column (0.78 x 30 cm) of μ-Bondapak C-18. Isocratic elution (2ml/min) was with the column development solvent as described above; absorption at 215 nm was measured. The inhibitory activity appeared mainly in fractions 68-80 ml.
6.	The dried sample was dissolved and rechromatographed on a μ-Bondapak C-18 column by the same conditions as described in stage 5. The inhibitory activity was recovered in fractions 56-60 ml.

* Waters Associates, INC.

taking the migration of alanine as 1.0. These results indicated that the final preparation contained a single peptide or amino acid.

Amino acid analysis (Hitachi 835 analyzer) after 6N HCl hydrolysis at 120°C for 24h revealed the presence of valine and leucine or isoleucine. An estimation of molar ratios indicates 1.0 valine and 1.6 leucine or isoleucine. The amino acid sequence was analyzed by Edman procedure (Applied Biosystems 470-A). The sequence of Leu-Val-Leu was indicated; the calculated molecular weight is 343. The Leu-Val-Leu structure of the ACE inhibitor was supported by the results of fast atom bonbardment (positive) mass spectrometric analysis. Two peaks of m/z 366 and m/z 344 appeared which were interpreted as arising from $[M + Na]^+$ and $[M + H]^+$ of Leu-Val-Leu, respectively. Peaks of m/z 231 and m/z 213 were also observed. They were inter-

preted as arising from Val-Leu and Leu-Val(CO) which caused by the elimination of N-terminal Leu and C-terminal Leu from Leu-Val-Leu, respectively.

The final preparation showed IC_{50} values of 4.2, 0.6, and 0.9 μg/ml for guinea pig serum ACE, rabbit lung ACE, and monkey brain ACE, respectively. Its inhibition mode with rabbit lung ACE was competitive. The snake venom (Bothrop jararaca) nonapeptide, <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, showed IC_{50} value of 1.0 μg/ml on rabbit lung ACE (6), our final preparation showed almost same potency. The structure of three aliphatic amino acid residues, Leu-Val-Leu, may be suitable for binding to ACE. Previously, Cheung et al. reported inhibitory effects on ACE with various dipeptides (7). They concluded that the most favorable C-terminal amino acids of the dipeptides were the aromatic acids and the imino acids and N-terminal amino acids were branched-chain aliphatic amino acid, valine and isoleucine.

On reverse-phase HPLC analysis, hydrolysis of the final preparation by rabbit lung ACE was not observed, but evidence for hydrolysis by guinea pig serum aminopeptidase (8) was obtained. Aminopeptidase is present in tissues and body fluids and the protection of the N-terminal amino acid of Leu-Val-Leu will be important to prolong the in vivo potency of the inhibitor.

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