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# ISOLATION OF ANGIOTENSIN-CONVERTING ENZYME INHIBITOR FROM TUNA MUSCLE

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A novel inhibitor of angiotensin-converting enzyme (ACE) has been discovered and isolated in a pure form from acid extract of tuna muscle by successive column chromatographies and HPLC. The final preparation showed $IC_{50}$ values of 1 μM and 2 μM for ACEs from bovine and rabbit lungs, respectively. The amino acid sequence of the inhibitor has been established as Pro-Thr-His-Ile-Lys -Trp-Gly-Asp by the Edman procedure and carboxypeptidase digestion. 

The angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure. It cleaves angiotensin I to a powerful vasoconstrictor and salt-retaining octapeptide, angiotensin II, and inactivates the vasodilator and natriuretic nonapeptide, bradykinin (1, 2). Recently, great interest has developed in the role of the ACE inhibitor in the clinical treatment of hypertension (3). During an initial search for pharmacological activities of marine products, we found ACE inhibitory activity in muscle extract of tuna (Neothunnus macropterus). In this paper, we present the isolation and identification of an active component.

## MATERIALS AND METHODS

Enzyme assay; Rabbit lung ACE was purchased from Sigma Chemical Co. A crude preparation of ACE was obtained by extracting bovine lung acetone powder with 50 mM potassium phosphate buffer, pH 8.3, containing 1 μM $ZnCl_2$ at 4°C. The homogenate was centrifuged for 30 min at 37000 x g at 4°C and the clear

Abbreviation; ACE, angiotensin-converting enzyme; AI, ACE inhibitor; M. W., molecular weight.

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supernatant, containing the ACE, was dialyzed against the buffer at 4°C to remove low molecular weight inhibitors. The ACE activity was assayed by measuring the amount of liberated hippuric acid from hippuryl-His-Leu according to the method of Cushman et al. (4). The reaction mixture contained 0.1 M potassium phosphate buffer, pH 8.3, 0.3 M NaCl, 2.5 mM hippuryl-His-Leu and the ACE solution with or without the test sample in a final volume of 0.25 ml. After 30 min at 37°C, the reaction was stopped by adding 0.25 ml of 1 N HCl, and the hippuric acid was extracted with 1.5 ml of ethylacetate. A 1.0 ml aliquot of the extract was evaporated to dryness and the residue was dissolved in 1.0 ml of $H_2O$. The hippuric acid concentration was determined from the absorbance at 228 nm.

Sequence analysis; Amino acid analyses were carried out with an amino acid analyzer (Waters, PICO TAG TM ). The peptide (1 nmol) was hydrolyzed in 4 N methanesulfonic acid containing 0.2% tryptamine or 6 N HCl containing 0.1% phenol at 110°C for 20 hr. Sequence analysis of the peptide ( 2.5 nmol) was performed by stepwise Edman degradation using a gas-phase automated sequenator (Applied Biosystems, model 470 A), coupled with HPLC identification of resulting PTH-amino acid. Carboxy-terminal analysis of the peptide (5 nmol) was carried out by digesting with carboxypeptidase A and B (Sigma; 5 μg each) in 0.1 M $NaHCO_3$ at 37°C for 18 hr. Molecular weight was determined by HPLC on a Shodex OHpak Q-802/s column (0.8 x 25 cm) using 0.1 M sodium phosphate buffer, pH 7.0, as a solvent. Calibration peptides (Peptide Institute Inc.) were des-Arg$^9$-[Leu$^8$]-bradykinin (M.W. 870), bradykinin-potentiator B (mamushi, M.W. 1182) and parathyroid hormone (human, 69-84, M.W. 1717).

Isolation; The white muscle of tuna, approximately 1.0 kg, was diced and homogenized with 8 volumes of cold 1 M AcOH-20 mM HCl. The suspension was autoclaved at 120°C for 5 min, cooled and centrifuged at 12000 rpm for 20 min. Forty g of activated ODS (Waters, preparative grade, particle size; 55-105 μ) was added to the supernatant and stirred for 1 hr. The ODS recovered by filtration on a glass filter was washed with 400 ml of 4% AcOH and eluted with 500 ml of 15% $CH_3CN$. The 15% $CH_3CN$ fraction was evaporated under reduced pressure, and approximately 0.5 g of brown colored powder containing ACE inhibitory activity was obtained from 1 kg of white muscle. Subsequent purifications were performed by successive column chromatographies and HPLC. Column effluents were monitored by measuring absorbance at 210 nm, 280 nm and/or 750 nm due to the Folin-Lowry reaction (5). An aliquot of each fraction was subjected to the assay for ACE inhibitory activity. Through the purification steps bovine lung ACE was used as the monitoring enzyme. The 15% $CH_3CN$ fraction was dissolved in 0.2 M AcOH and absorbed on a SP-Sephadex C-25 column ($H^+$form, 1.8 x 50 cm). The column was washed with 330 ml of 0.2 M pyridine-acetate, pH 3.1, and then developed with a concave gradient made by 1 l of 0.2 M pyridine-acetate, pH 3.1, and 500 ml of 2.0 M pyridine-acetate, pH 5.0. ACE inhibitory activity emerged in 250-580 ml fractions. The active fraction was dried and redissolved in 0.2 M AcOH, followed by gel filtration on a Sephadex G-25 column (2.5 x 93 cm) with 0.2 M AcOH. The inhibitory activity was recovered in fractions 369-406 ml. The active fraction redissolved in 0.05% HCl was subjected to preparative HPLC on a Develosil ODS column. The activity was eluted in an unsymmetrical peak (Fig. 1). The dried active fraction was further fractionated by HPLC on an Asahipak GS-220 column. The activity was recovered in a symmetrical peak (Fig. 2). Finally, rechromatography by reverse-phase HPLC was necessary for desalting (Fig. 3).

## RESULTS AND DISCUSSION

An active component for ACE inhibition in the extract of tuna muscle was purified by successive chromatographies on SP-Sephadex C-25, Sephadex G-25, Develosil ODS-7 and Asahipak GS-220 columns. The homogeneity was confirmed by another HPLC of the final preparation on Develosil ODS-7 and Asahipak GS-220

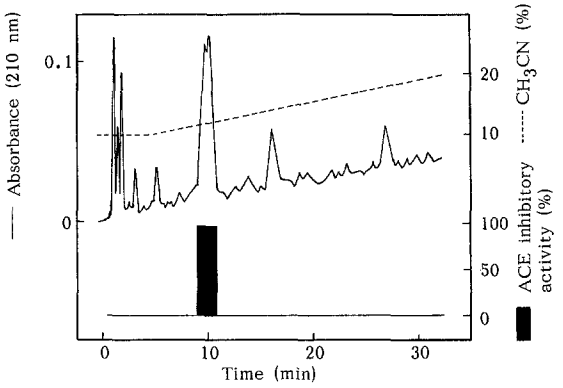
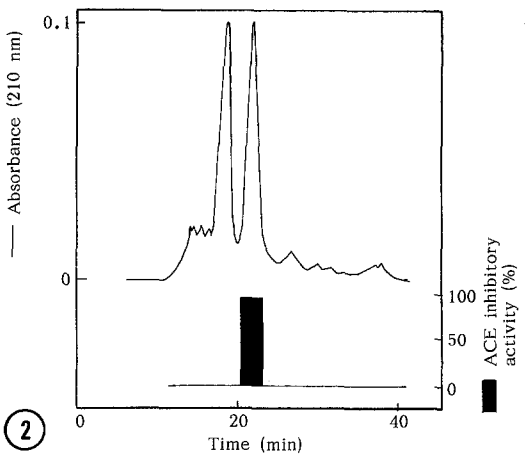



Fig.1. Reverse phase HPLC of active fraction obtained after Sephadex G-25. Column: Develosil ODS-7 (0.8 x 25 cm). Solvent system: (a) 10% $CH_3CN$ in 0.05% HCl, (b) 20% $CH_3CN$ in 0.05% HCl. Linear gradient from (a) to (b) (30 min). Flow rate: 5 ml/min. One peak of a potent ACE inhibitory activity was fractionated.

under the same conditions as shown in Figs. 2 and 3. The final preparation was designated as tuna ACE inhibitor (AI). The yield was approximately 100 μg from 1 kg of muscle. Tuna AI showed $IC_{50}$ values of 1 μM and 2 μM for ACEs from bovine and rabbit lungs, respectively (Table I). Tuna AI was positive to the Folin-Lowry reaction and its hydrolysate with 6 N HCl at 110°C for 20 hr was positive to the ninhydrin reaction. The UV spectrum (Fig. 4) revealed the presence of



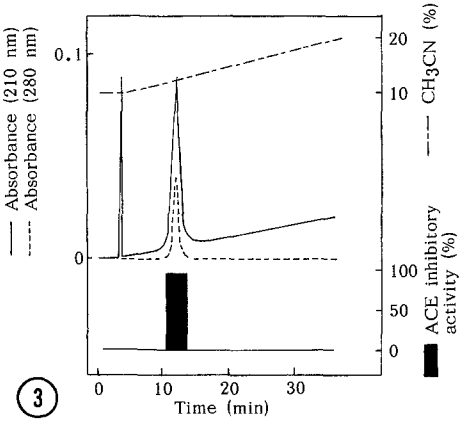




Fig.2. Gel filtration HPLC of active fraction after reverse phase HPLC. Column: Asahipak GS-220 (0.76 x 50 cm). Solvent: 50 mM sodium phosphate buffer, pH 7.0. Flow rate: 1 ml/min. One peak of a potent ACE inhibitory activity was seperated.

Fig.3. Reverse phase HPLC of active fraction obtained after Asahipak GS-220. Column: Develosil ODS-7 (0.46 x 25 cm). Solvent system: (a) 10% $CH_3CN$ in 0.05% HCl, (b) 20% $CH_3CN$ in 0.05% HCl. Linear gradient from (a) to (b) (30 min). Flow rate: 2 ml/min. One peak of a potent ACE inhibitory activity was obtained as a final preparation.

Table 1. ACE inhibitory activity of tuna AI

| | $IC_{50}$ (μM) | |
|---|---|---|
| | Bovine lung ACE | Rabbit lung ACE |
| Tuna AI | 1 | 2 |
| Bradykinin-potentiator B | 5 | 3 |

The data represent the mean value in two independent assays.

tryptophanyl residue in the molecule. This evidence indicated that tuna AI consisted of a peptide.

The apparent molecular weight of tuna AI was estimated to be 920 by gel filtration on a Shodex OHpak Q-802/s calibrated with peptides of known molecular weight. Amino acid analyses revealed that this peptide contained Asp, Gly, His Thr, Pro, Ile, Trp and Lys per mol of the molecule (Table II). By stepwise Edman degradatioin of the peptide, liberated PTH-amino acids were successfully identified up to the 8th step, as shown in Fig. 5, although the 8th PTH-Asp was recovered in a very poor yield. The first release of Asp took place by carboxypeptidase A and B digestion (Fig. 5). It was known that these exopeptidases require a free COOH-group at the terminus of the peptide to be hydroryzed (6, 7). Consequently, the amino acid sequence was established as Pro-Thr-His-Ile-Lys-Trp-Gly-Asp. The molecular weight of the peptide calculated from its amino acid sequence was 952, which was in agreement with the apparent molecular weight determined by gel filtration as mentioned above.

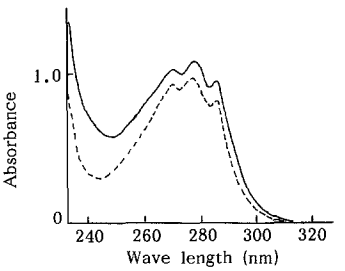



Fig.4. UV spectrum of tuna AI.
—— Tuna AI (165 μg/ml of $H_2O$).
----- Trp (160 μM in $H_2O$).

Table 2. Amino acid composition of tuna AI

| Amino acid | Residues in 20 hr hydrolysate at 110°C with | |
|---|---|---|
| | 6 N HCl | 4 N methanesulfonic acid |
| Asp | 1.1 (1) | 1.1 (1) |
| Gly | 1.1 (1) | 1.2 (1) |
| His | 1.0 (1) | 1.0 (1) |
| Thr | 1.0 (1) | 0.9 (1) |
| Pro | 1.0 (1) | 1.1 (1) |
| Ile | 1.0 (1) | 1.0 (1) |
| Trp | ——— | 0.8 (1) |
| Lys | 0.9 (1) | 0.9 (1) |

The figures in parentheses are rounded values.

Thus the ACE inhibitor in tuna muscle was identified to be the octapeptide. So far as we know, this finding is the first report with respect to the ACE inhibitory peptide in marine products. The homology examination of the amino acid sequence using a data base "PRF/SEQDB" revealed that tuna AI was a novel peptide and that its sequence was not contained within any other proteins. Among the naturally occurring peptides with ACE inhibitory activity, the most potent and specific inhibitors are several structually similar peptides that have been isolated from the venoms of the South American pit viper Bothrops jararaca and the Japanese pit viper Agkistrodon halys blomhoffii. These venom peptides are Pyr-Lys-Trp-Ala-Pro (8), Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (9) and Pyr-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro (10). The structure-activity relationship of the venom peptides and their synthetic analogues indicated that the ACE had little affinity for substrates or competitive inhibitors with COOH-terminal dicarboxylic acids, but an antepenultimate aromatic amino acid residue appeared to enhance binding (11, 12). Tuna AI contains dicarboxylic acid, Asp, as its COOH-terminus and the amino acid sequence was different from those of the

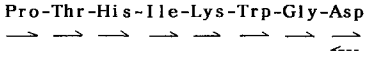



Fig.5. Amino acid sequence determination of tuna AI.
—→ by Edman degradation.
←--- by carboxypeptidase A and B digestion.

venom peptide family, except that the antepenultimate amino acid, Trp, was aromatic. However, Tuna AI showed a more potent ACE inhibitory activity than bradykinin-potentiator B (Table II), especially for bovine lung ACE. These findings, which require confirmation, suggest that this octapeptide may interact with the ACE via another mechanism.

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