Biological Properties of Angiotensin-Converting Enzyme Inhibitor Derived from Tuna Muscle

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A novel inhibitor of angiotensin-converting enzyme (ACE) derived from tuna muscle, Pro-Thr-His-Ile-Lys-Trp-Gly-Asp (tuna AI), was chemically synthesized, and its biological properties were investigated. Synthetic tuna AI was found to be chemically and biologically indistinguishable from the native one. Tuna AI inhibited rabbit lung ACE non-competitively with K_i values of 1.7 and 5.7 μM with substrates, hippuryl-L-histidyl-L-leucine and angiotensin I, respectively. This peptide (5.3 μM) also doubled the effect of bradykinin in the contraction of isolated guinea pig ileum. The peptide did not show zinc chelating activity and carboxypeptidase A inhibitory activity. Thus, tuna AI was found to be a unique ACE inhibitory peptide with non-competitive manner, differing from many naturally occurring peptide ACE-inhibitors.

Keywords — angiotensin-converting enzyme inhibitor; peptide; tuna muscle; enzyme inhibitor; bradykinin-potentiating activity; non-competitive inhibition

Introduction

We previously isolated an angiotensin-converting enzyme (ACE) inhibitor (tuna AI) from muscle extract of tuna (*Neothunnus macropodus*).¹⁾ This amino acid sequence was as follows; Pro-Thr-His-Ile-Lys-Trp-Gly-Asp. Since ACE is peptidyl dipeptide hydrolase, COOH-terminal tripeptide residues of the peptide inhibitors play a predominant role in competitively binding to the active site of ACE. The structure-activity relationship of snake venom peptides and their synthetic analogues indicated that ACE had little affinity for substrates or competitive inhibitors with COOH-terminal dicarboxylic acid residues and that penultimate branched-chain aliphatic amino acids were most effective for enhancing the binding to the enzyme.^{2,3)} Although tuna AI contains dicarboxylic acid, Asp, as its COOH terminus and Gly as its penultimate amino acid, it has almost the same activity as natural peptide inhibitors. Furthermore, it is noteworthy that this peptide derived from muscle glyceraldehyde 3-phosphate dehydrogenase (GAPDH).⁴⁾ In this paper, we prepared a synthetic tuna AI and in-

vestigated its biological properties.

Materials and Methods

Materials — Rabbit lung ACE, carboxypeptidase (Cpase) A from bovine pancreas and hippuryl-L-phenylalanine (Hip-Phe) were purchased from Sigma Chemical Co. (Mo., U.S.A.). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu), bradykinin (BK), bradykinin-potentiator B (BKP-B) and angiotensin I were obtained from Protein Research Foundation (Osaka, Japan). Bovine lung ACE was prepared as described previously.¹⁾ All other reagents and solvents were obtained from Nakarai Tesque Ltd. (Kyoto, Japan).

Synthesis of Tuna AI — Tuna AI was synthesized by solid phase method using an automated peptide synthesizer (Applied Biosystems Inc., model-430A), followed by treatment with hydrogen fluoride to cut off the support resins and to remove all of the protecting groups.^{5,6)} Crude products were purified by reverse phase high performance liquid chromatography (HPLC) on ODS columns. Amino acid analysis was carried out for the hydrolysates

with 6 N HCl containing 0.1% phenol or 4 N methanesulfonic acid containing 0.2% tryptamine with amino acid analyzer (Waters, PICO TAG®). Sequence analysis was performed by stepwise Edman degradation using a gas-phase automated sequencer (Applied Biosystems, model-470A), coupled with HPLC identification of the resulting phenylthiohydantoin (PTH)-amino acid.

Assays of ACE Activity — The activity of ACE was assayed both by spectrophotometric determination of the rate of formation of hippuric acid from Hip-His-Leu, and by fluorometric determination of the rate of formation of His-Leu from angiotensin I, according to the method of Cheung and Cushman.⁷⁾ For either assay method, incubations were carried out at 37 °C in a final volume of 0.25 ml, which contained 100 mM potassium phosphate buffer (pH 8.3), 300 mM NaCl, enzyme (6 mU) and 2.5 mM Hip-His-Leu, or 100 mM potassium phosphate buffer (pH 7.5), 30 mM NaCl, enzyme (2 mU) and 0.1 mM angiotensin I. The enzyme was rabbit or bovine lung ACE preparation. ACE inhibitory activity was assayed in the presence or absence of inhibitor. For kinetic stud-

ies, in which concentrations of substrate, as well as inhibitor, were varied, each activity measurement was based on incubations of 15 min for Hip-His-Leu and 20 min for angiotensin I.

In other experiments, the inhibitor was preincubated with various concentrations of ZnCl_2 for 5 min at 37 °C before the enzyme reaction was initiated. Controls were run containing enzyme, buffer and Hip-His-Leu with or without ZnCl_2 , as the divalent cation may react allosterically to alter ACE activity.

Assay of Cpease A — The activity of Cpease A was determined according to the method of Folk and Schirmer.⁸⁾ The assay mixture (total volume, 3 ml) was as follows: 1 mM Hip-Phe, 25 mM tris-HCl containing 0.5 M NaCl (pH 7.5) and 0.1 mU enzyme, with or without inhibitor. The reaction velocity was determined by measuring the increase in absorbency at 254 nm.

Augmentation of BK-Induced Contraction of Guinea Pig Ileum — BK activity was determined in a 5 ml organ bath by measuring contraction of guinea pig ileum in aerated tyrode solution at 30 °C. Test samples were added just before addition of 0.05 ml of the standard BK solution.

Fig. 1. HPLC of Synthetic and Native Tuna AI

The peptides were chromatographed on a Develosil ODS-7 column (0.46 × 25 cm) eluted with a gradient of acetonitrile in 0.05% hydrochloric acid (A). They were run on an Asahipak GS-220 column (0.76 × 50 cm) eluted with 50 mM sodium phosphate buffer, pH 7.0 (B). a) Synthetic and b) native tuna AI.

TABLE I. ACE Inhibitory Activity of Tuna AI

Inhibitor	IC ₅₀ (μ M)	
	Bovine lung ACE	Rabbit lung ACE
Native tuna AI	0.9	2
Synthetic tuna AI	0.9	2
BKP-B	5	3

The data represent the mean value in two independent assays.

Results

Comparison of Synthetic Tuna AI with Native One

The final synthetic product was homogeneous by HPLC using condition different from that of the final purification steps, and showed identical retention time with native tuna AI in HPLC (Fig. 1). The results of amino acid analysis and sequence analysis agreed well with expected values. Synthetic and native peptides were found to be almost equipotent in regard to their ACE inhibitory activities (Table I). Its potency was a little stronger than that of BKP-B,⁹⁾ in the present assay system. Thus, synthetic tuna AI was found to be chemically and biologically indistinguishable from the native one.

Kinetics of ACE Inhibition by Tuna AI

Figure 2 shows Lineweaver-Burk plots for inhibition of ACE hydrolysis of Hip-His-Leu or angiotensin I by tuna AI.

Table II summarizes the IC₅₀ values, K_i values and type of inhibition against two different substrates. Tuna AI inhibited non-competitively both substrates as indicated from Lineweaver-Burk plots. IC₅₀ values of tuna AI were 2.0 and 4.9 μ M for Hip-His-Leu and angiotensin I, respectively and the K_i values obtained from Dixon plots (data not shown) were 1.7 and 5.7 μ M, respectively.

Effect of ZnCl₂ on ACE Inhibition by Tuna AI

When tuna AI (2 μ M) was preincubated without ZnCl₂, its ACE inhibitory activity was 53%. When 2, 10 and 20 μ M of ZnCl₂ were added, respective inhibitory activities were 50, 48 and 45%. On the contrary, the inhibitory activity of ethylenediaminetetraacetic acid (EDTA, 3 μ M) was reversed completely by 20 μ M of ZnCl₂ (Table III). These data indicated that tuna AI had not the chelating effect of ACE active site zinc at all.

Effect of Tuna AI on Cpase A

ACE was completely inhibited by 100 μ M of tuna AI, while Cpase A was not inhibited at all (Table IV).

BK-Potentiating Activity of Tuna AI

Tuna AI showed clearly BK-potentiating activity (Fig. 3). The final concentration of tuna

Fig. 2. Lineweaver-Burk Plots of Inhibition of Rabbit Lung ACE by Tuna AI

Enzyme concentrations are as follows: (A) 6 mU/assay, (B) 2 mU/assay. For each assay, 1 unit of ACE activity is defined as the amount catalyzing the formation of 1 μ mol hippuric acid from Hip-His-Leu in 1 min at 37 °C. Tuna AI concentration: (A) ●, 0 μ M; ▲, 3 μ M; ■, 6 μ M; (B) △, 0 μ M; □, 2 μ M.

TABLE II. Kinetics of Inhibition of Rabbit Lung ACE by Tuna AI

Substrate	IC ₅₀ (μ M)	K _i (μ M) ^{a)}	K _m (mM) ^{b)}	Type of inhibition
Hip-His-Leu	2.0	1.7	1.45	Non-competitive
Angiotensin I	4.9	5.7	0.47	Non-competitive

a) K_i values were determined from Dixon plots.b) K_m values were determined from Lineweaver-Burk plots.TABLE III. Effect of ZnCl₂ on Inhibition of ACE by Tuna AI

Sample	ZnCl ₂ added (μ M)	ACE inhibition (%)
Tuna AI	0	53
	2	50
	10	48
	20	45
EDTA	0	61
	3	9
	20	0

Tuna AI (2 μ M) or EDTA (3 μ M) was preincubated with the indicated concentrations of ZnCl₂ at 37 °C for 5 min before the enzyme reaction was initiated. Substrate: Hip-His-Leu.

AI required to double the effect of BK was 5.3 ± 0.3 μ M (means $\pm$ S.E., $n = 3$). BKP-B, on the other hand, showed a little stronger sen-

TABLE IV. Effect of Tuna AI on Cpsae A Activity

Protease	Tuna AI (μ M)	Inhibition (%)
Cpsae A	100	0
ACE	100	100

Enzyme concentrations are as follows: 0.1 U/assay for Cpsae A, 6 mU/assay for ACE.

sitizing action than tuna AI.

Discussion

Tuna AI inhibited ACE non-competitively. It has not been well known that there are natural peptide inhibitors which show non-competitive inhibition for ACE except for ACTH fragments.¹⁰⁾ On the other hand, there are many competitive inhibitors, such as SQ-20881,⁷⁾ SQ-20858,⁷⁾ β -lipotropin,¹¹⁾ β -endorphin,¹²⁾

Fig. 3. BK-Potentiating Activity of Tuna AI on Guinea Pig Ileum

BK activity was assayed by contraction of isolated guinea pig ileum in aerated Tyrode solution at 30 °C. Tuna AI or BKP-B was applied at the indicated concentration just before the addition of BK (1 nM).

enkephalin¹²⁾ and BK-potentiator C.⁹⁾ The structure-activity relationship of the venom peptides and their synthetic analogues indicated that ACE had little affinity for substrates or competitive inhibitors with COOH-terminal dicarboxylic acid, or with penultimate proline residues. But aromatic amino acid and imino acid as COOH-terminus were most effective for enhancing binding to ACE, and penultimate branched-chain aliphatic amino acid and antepenultimate aromatic amino acid residue also appeared to enhance binding for ACE active site.^{2,3)} Tuna AI contains dicarboxylic acid, Asp, as its COOH terminus and penultimate Gly, and the amino acid sequence was different from those of the venom peptide family, except that the antepenultimate amino acid, Trp, was aromatic. These facts may support non-competitive inhibition of ACE by tuna AI.

N^α-Phosphonyl dipeptides,¹³⁾ *N*^α-phosphonyl-L-Ala-L-Pro and *N*^α-phosphonyl-L-Val-L-Trp, which were synthesized by consulting a schematic model of the active site of ACE,¹⁴⁾ showed the different models of inhibition between not only substrates (Hip-His-Leu and angiotensin I) but also the other assay conditions, such as pH and buffer. Cheung *et al.*⁷⁾ suggested that Cl⁻ appeared to serve as an allosteric modifier, that subtly changes the shape of the active site of ACE. Such a change could make the active site more suitable for binding some substrates (Hip-His-Leu and angiotensin I). Tuna AI also may have other types of inhibition in other assay conditions.

ACE contains zinc ion which plays an important role in the active site.^{15,16)} Some inhibitors,^{17,18)} such as EDTA, show powerful inhibitory activity by chelating the ACE active site zinc. Because EDTA lost its inhibitory activity by chelating with exogenous zinc, its main inhibitory mechanism was shown to be a chelating effect. However, tuna AI did not show any zinc chelating effect. It is certain that its inhibitory mechanism is non-competitive in our assay condition with Hip-His-Leu.

ACE was often compared with C₁ase A,¹⁵⁾ because their active sites resemble each other. C₁ase A also contains zinc ion in the active site, and not only its amino acid sequence but also its

structure is similar to that of ACE.¹⁹⁾ Inhibition of tuna AI against C₁ase A was investigated to study the specificity of tuna AI. Tuna AI had no inhibitory activity for C₁ase A even at a concentration of 100 μM. Tuna AI was a specific inhibitor of ACE.

ACE cleaves angiotensin I to angiotensin II, and inactivates BK.^{20,21)} Tuna AI really showed BK potentiating-activity on the isolated guinea pig ileum like BKP-B. The differences were found among the effects on bovine lung, rabbit lung and guinea pig ileum ACE's of both tuna AI and BKP-B. This fact may be partly explained by the differences in species and tissue specificities of ACE or kininase II.^{22,23)} Although tuna AI is only a moderately strong inhibitor of ACE, compared with known ACE inhibitors, it is noteworthy that this peptide derives from tuna muscle GAPDH and inhibits rabbit lung ACE non-competitively.

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