Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Bonito Bowels Autolysate

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Six angiotensin I-converting enzyme inhibitory peptides were isolated from a bonito bowels autolysate. Their amino acids were sequenced as Tyr-Arg-Pro-Tyr, Gly-His-Phe, Val-Ang-Pro, Ile-Lys-Pro, Leu-Ang-Pro, and Ile-Ang-Pro. Peptides having corresponding amino acid sequences were synthesized by a solid-phase method and their inhibition of the activity measured. IC_{50} of these peptides were estimated to be 320, 1100, 2.2, 2.5, 1.0, and 1.8 \mu M, respectively. The role of carboxyl terminal proline residues on the inhibition is discussed.

Angiotensin I-converting enzyme [EC 3.4.15.1] (ACE) is important in the maintenance of blood pressure. The enzyme removes histidyl-leucine from angiotensin I to form the blood-vessel-constricting octapeptide, angiotensin II. ACE also removes two amino acids from the vasodilating nonapeptide, bradykinin. An orally effective ACE inhibitor, captorpril (D-3-mercapto-2-methylpropyl-L-proline), is established itself in the therapy of hypertension and congestive heart failure, but attempts have been made to find new ACE inhibitory peptides from food proteins, i.e. tuna muscle, casein, zein, and gelatin.

About 300,000 tons of bonitos are landed every year in Japan and the content of bowels is 5 to 5.5% of the bonito body. Only a small amount of bonito bowels are processed into the Japanese traditional fermented food called shiokara, however, the rest can be used for feeds and fertilizers unless they are scrapped. Although fish bowels are rarely used in these ways, it might be possible to find a new use for them. In this paper, isolation and sequencing of the amino acids of ACE inhibitory peptides are described.

Materials and Methods

Materials. Bonito bowels, which contain stomach, intestine, and liver, were collected from a katsuo bushi (Japanese traditional seasoning made from dried bonito muscle) factory in Yamagawa, Kagoshima Prefecture in Japan. Rabbit lung acetyl powder and hippuryl-L-histidyl-L-leucine were purchased from Sigma Chemical Co. (U.S.A.). HPLC solvents were purchased from Kanto Kagaku Co., Ltd.

Amino acid sequencing. Amino acids were sequenced by automated Edman degradation using an Applied Biosystem Model 470A protein sequencer.

Estimation of ACE inhibition. The method of Cushman and Cheung for the estimation of ACE inhibitory activity was used with some modifications described by Marayama and Suzuki. Each assay was done in 76 \mu l of reaction mixture containing 5 mM hippuryl-L-histidyl-L-leucine, 1.6 milliliters of ACE, 300 mM NaCl, and 100 mM borate buffer (pH 8.3). The IC_{50} of the peptides were estimated for synthetic peptides. Preparations of synthetic peptides were requested from Fujiya Co., Ltd. They were synthesized by a solid method. Since many peptide preparations were diluents, precise concentration of the peptide was confirmed by the amino acid analyzer after hydrochloric acid hydrolysis.

Isolation of ACE inhibitory peptides. One kilogram of bonito bowels were crushed and a half weight of water added, then this was autolyzed at 60°C for 3h with gentle stirring. Autolysis was stopped by heating at 90°C; the mixture was passed through a 200-mesh screen, and then filtered again by ultrafilter Sip-1013 (Asahi Chemical Industry Co., Ltd., 6000 dalton cut off). Eight ml of the filtrate was put into a Sep-Pak Plus C_{18} cartridge (Waters) and washed with the same volume of water. Peptides were eluted with 8 ml of 15% [v/v] acetonitrile solution. A series of another six of the same Sep-Pak treatments were done and the eluents were mixed together. After being concentrated by a rotary evaporator, the concentrate was put on a Toyopak ISC-SP M cartridge that had been pretreated with 10 mM phosphate buffer (pH 6.0). The cartridge was washed with 4 ml of the same buffer (pH 6.0) and peptides were eluted with 4 ml of 10 mM Na_{2}HPO_{4} solution (crude peptide solution). Peptides were further purified by high performance liquid chromatography. For the first reverse phase chromatography, the crude peptide preparation was put on an RP-18(e) column (10 mm i.d. x 250 mml, Merck) equilibrated with 0.05% trifluoroacetic acid, eluted with a linear gradient of acetonitrile (0 to 30%) in 0.05% trifluoroacetic acid at a flow rate of 4 ml/min. An RP-18(e) column (4 mm i.d. x 250 mml) was used as a second stage of purification. The conditions of chromatography were the same as above except the flow rate was 1 ml/min. For gel permeation chromatographies, Asahipak GS-220 (7.6 mm i.d. x 500 mml, Asahi Chemical Industry Co., Ltd.) and GS-320 (7.6 mm i.d. x 500 mml, Asahi Chemical Industry Co., Ltd.) were used. Peptides were eluted with 50 mM of ammonium acetate solution at a flow rate of 1 ml/min. An ion exchange chromatography was done using an SP-25W column (4.6 mm i.d. x 250 mml, Tosoh Co., Ltd.), and peptides were eluted with a linear gradient of NaCl (0 to 0.5 M) in 20 mM sodium phosphate buffer (pH 6.0). Peptides eluted from the SP-25W column were desalted by isocratic HPLC on RP-18(e) column (4 mm i.d. x 250 mml) using 0.05% trifluoroacetic acid plus 7% (v/v) acetonitrile as a mobile phase. Each chromatography was monitored by the absorbance at 210 nm ultraviolet light, and the peptide contents of fractions were assayed occasionally with Micro BCA Kit purchased from Pierce (Lockford, IL, U.S.A.).

Results

Preparation of crude peptide solution

Bonito bowels were crushed, autolyzed, and filtered through a 200-mesh screen. The autolysate (1500 ml) contained 85 g of peptides measured by the Micro BCA method. The autolysate was treated with an ultrafilter Sip-1013 to remove high molecular weight proteins, colloids, and solid substances. About one half the amount (40 g) of peptides were recovered as filtrate. The ACE

Abbreviations: ACE, angiotensin I-converting enzyme; IC_{50}, the concentration of an ACE inhibitor required to inhibit 50% of the ACE activity; HPLC, high performance liquid chromatography.
inhibitory activities were assayed in 76 μl of reaction mixture that contained 20 μg of peptide. Relatively high inhibitory activity was appeared after the unfiltration (Table I).

The ultrafiltrate of the autolysate containing about 30 mg of peptides/ml was put into a Sep-Pak C18 cartridge and washed with water, and peptides eluted with 15% [v/v] of acetonitrile solution were collected. After seven Sep-Pak treatments, a peptide preparation containing 600 mg of peptides was recovered from 55 ml of the ultrafiltrate. The Sep-Pak-treated peptides were concentrated and assayed for the ACE inhibitory activity (Table I). The Sep-Pak treatment slightly enhanced the ACE inhibitory activity of the peptide.

The peptides were further purified by Toyopak IC-SP treatments. A large amount of peptides which had low ACE inhibitory activity were washed out with the buffer (pH 6.0), while a peptide preparation having high ACE inhibitory activity was obtained by the elution with the alkaline buffer (pH 9.0). The crude peptide preparation thus obtained was used for the next step of purification by HPLC.

**Purification of ACE inhibitory peptides by HPLC**

The crude peptide preparation was treated by reverse phase HPLC. Figure 1 shows the chromatographic pattern.

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**Table I. ACE Inhibitory Activities of Peptide Preparations**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of peptide (mg)</th>
<th>ACE inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autolysate</td>
<td>85,000</td>
<td>22</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>40,000</td>
<td>35</td>
</tr>
<tr>
<td>Ultrafiltrate</td>
<td>1,680</td>
<td>35</td>
</tr>
<tr>
<td>Sep-Pak C18</td>
<td>600</td>
<td>45</td>
</tr>
<tr>
<td>IC-SP (pH 6)</td>
<td>504</td>
<td>41</td>
</tr>
<tr>
<td>IC-SP (pH 9)</td>
<td>38</td>
<td>78</td>
</tr>
</tbody>
</table>

Clear filtrate (1.3 liters) obtained by the ultrafiltration of autolysate (1.5 liters) was assayed its peptide content and the ACE inhibitory activity. Fifty-five ml of the ultrafiltrate was subjected to the Sep-Pak C18 and Toyopak IC-SP treatments.

*a* The amount of peptide was measured by the Micro BCA method.

*b* The ACE inhibitory activity were assayed in a 76-μl reaction mixture containing 1.6 milliunits of ACE and 20 μg of peptide to be assayed.

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**Table II. Purification of ACE Inhibitory Peptides**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount of peptide (μg)</th>
<th>ACE inhibitory activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>640</td>
<td>73</td>
</tr>
<tr>
<td>B</td>
<td>620</td>
<td>69</td>
</tr>
<tr>
<td>C</td>
<td>460</td>
<td>67</td>
</tr>
<tr>
<td>D</td>
<td>900</td>
<td>86</td>
</tr>
</tbody>
</table>

*a* The ACE inhibitory activity were assayed in a 76-μl reaction mixture containing 1.6 milliunits of ACE and 5 μg of peptide to be assayed.

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**Fig. 1. Purification of ACE inhibitory Peptides by HPLC.**

The crude peptide preparation was put on an RP-18(e) column (10 mm i.d. × 250 mm) and eluted with a linear gradient of 0 to 30% of acetonitrile. A, B, C, and D indicate the fractions with high ACE inhibitory activities.

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**Fig. 2. Purification of ACE Inhibitory Peptides by HPLC.**

The fraction C of Fig. 1 was once purified on the second reverse phase HPLC, then put on a GS-220 column. Two peptide peaks (C-1 and C-2) with high ACE inhibitory activities are indicated.

Peptides were fractionated appropriately, concentrated, and assayed for their ACE inhibitory activities. Four fractions indicated as A, B, C, and D in Fig. 1 had high ACE inhibitory activities (Table II).

Peptides in fraction A were further purified by the second RP-18(e), GS-220, and GS-320 chromatography, then a final peptide preparation (A-1) containing about 1 μg of peptide was obtained. One-fifth microgram of the peptide inhibited ACE about 60% in the same assay system. The amino acid sequence of the peptide was Val-Arg-Pro.

Peptides in fraction B of the first RP-18(e) chromatography were treated by the second RP-18(e), GS-220, and GS-320 chromatographies. A fraction containing 35 μg of peptide which strongly inhibited ACE (52% inhibition by 1 μg of the peptide) was obtained, however, the amino acid sequencing failed. Ion exchange HPLC on SP-25W column was used for the next stage of the purification. Active fraction was collected, desalted on RP-18(e) HPLC, and the final purification designated as B-1 was obtained. The peptide B-1 also strongly inhibited ACE (53% inhibition by one-fifth μg of peptide). The amino acid sequence was Ile-Lys-Pro.

Two active peptides (C-1 and C-2, see Fig. 2) were isolated from fraction C after the purification on gel permeation HPLC that followed the second RP-18(e) chromatography. These fractions contained about 4 and 2 μg of peptides and inhibited 27 and 35% of the ACE activities per μg of peptide, respectively. The amino acid sequences of C-1 and C-2 were Tyr-Arg-Pro-Tyr and Gly-His-Phe, respectively.

The peptide fraction D was purified by the same treatment as that used for fraction C, then a single active peak was obtained, however, the amino acids could not be sequenced. Since the second gel permeation chromatography on the
### Discussion

We have isolated six ACE inhibitory peptides from bonito bowels. Four of six peptides which had strong ACE inhibitory activities were tripeptides, composed of a hydrophobic amino acid at the amino terminal, a basic amino acid residue at the center, and a carboxyl terminal proline. Cheung et al.\(^6\) studied the ACE inhibitory activities of a series of dipeptides. They indicated that tryptophan, tyrosine, proline, or phenylalanine at the carboxyl terminal and branched-chain aliphatic amino acids at the amino terminal were suitable for a peptide binding to ACE as a competitive inhibitor. Although the peptides obtained in this study were tripeptides, these results almost coincided with that for dipeptides proposed by Cheung et al.\(^5\).

Two peptides which have the same amino acid sequence as the peptide A-1 (Val-Arg-Pro) and D-1 (Leu-Arg-Pro) had already been reported by Kohmura et al.\(^7\) and Miyoshi et al.\(^8\), respectively, suggesting that many proteins might contain the sequence Val-Arg-Pro and Leu-Arg-Pro.

Small peptides that have biological activities such as ACE inhibition might have some advantages for clinical use as follows; 1) specific amino acid sequence corresponding to the peptide of interest would exist abundantly in natural proteins; 2) the amount of peptide to be administered is less than that of larger peptides having equivalent molar activities; 3) smaller peptides would be less susceptible to proteolytic enzymes; 4) smaller peptides could easily be absorbed after oral administration since the size of the peptide taken up by the absorptive cells in the small intestine is limited to tri- or tetrapeptides.\(^9\) However, there is very little information about peptides that had been absorbed appearing intact in blood or lymph. Development of the effective preparation of these peptides, the investigation of anti-hypertensive effect after oral administration, and the use as health-care foods await further study.

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### References