Short Paper

Identification of antihypertensive peptides from peptic digest of the short-necked clam Tapes philippinarum and the pearl oyster Pinctada fucata martensii

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KEY WORDS: angiotensin I-converting enzyme, antihypertensive effect, enzyme inhibitor, pearl oyster, peptide, short-necked clam, spontaneously hypertensive rat.

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) plays an important physiologic role in the regulation of blood pressure and electrolyte homeostasis. It has been known that some ACE-inhibitory peptides are produced by the enzymatic digests of various marine food proteins, including tuna muscle, sardine muscle, dried bonito, dried-salted fish, fish sauce, and fish watersoluble protein. Recently, we observed that the peptic digest of the seaweeds Porphyra yezoensis, Hizikia fusiformis, and Undaria pinnatifida lowered the blood pressure of spontaneously hypertensive rats (SHR), thus suggesting that appropriate peptides may be efficient for inducing potent antihypertensive effects that are comparable to therapeutic drugs. In addition, there have been a few reports on ACE-inhibitory peptides derived from shellfish, whereby Matsumoto et al. have purified Leu-Phe from oyster protein digest.

In the present study, we describe the purification of ACE-inhibitory peptides derived from the short-necked clam Tapes philippinarum and the pearl oyster Pinctada fucata martensii, their structures, and the antihypertensive action of orally administered peptides on SHR.

The short-necked clams were bought from the Shimonoseki fish market in June 1997, and the culture pearl oysters (3–5 years old, shell length 6–7 cm) were obtained from Uwa Bay, Ehime prefecture, in June 1997. After washing with deionized water, the edible portion of the short-necked clam and pearl oyster were homogenized and freeze-dried. Pepsin (porcine stomach mucosa; Wako Pure Chemicals, Osaka, Japan) was obtained from Peptide Institute (Osaka, Japan), and angiotensin I-converting enzyme from rabbit lung acetone powder was obtained from Sigma Chemical Co. (St Louis, MO, USA).

One hundred grams of the short-necked clam or pearl oyster powder was immersed in 200 mL of deionized water and adjusted to pH 2 with 1 N hydrochloric acid. Three grams of pepsin was added to the homogenate, and then peptic digestion was done for 5 h at 45°C. The peptic digest was adjusted to pH 7 with 1 N sodium hydroxide. The digest was boiled for 10 min to inactivate the enzyme, and then filtered. The filtrate of the peptic digest was dialyzed against 5 L of deionized water in seamless cellulose tubing (36 inches; Wako Pure Chemicals) for two days. The outer solution was applied to a Dowex 50W column (4.5 cm × 20 cm, 50–100 mesh, H+ form; Dow Chemical, Midland, MI, USA) equilibrated with deionized water. The column was washed thoroughly with deionized water to remove any impurities, and the peptides were then eluted with 500 mL of 3.7% ammonia solution. After being evaporated under vacuum, the concentrate was applied to a Sephadex G-25 column (2.3 cm × 140 cm, medium; Pharmacia, Uppsala, Sweden) equilibrated with deionized water and eluted with deionized water at a flow rate of 30 mL/h. Fractions of 8.6 mL were collected. Peptide content (mg/mL) was assayed according to the Lowry method. A major peak with ACE inhibitory activity was collected (molecular weight 300–5000) and evaporated under vacuum. The concentrate was applied to a SP-Sephadex C-25 column (1.5 cm × 47.2 cm, H+ form; Pharmacia) equilibrated with deionized water, and chromatographed using a linear gradient from deionized water to 1.5% sodium chloride solution at a flow rate of 90 mL/h. Fractions of 10 mL were col-
lected and monitored by the enzyme assay. The active fractions were then lyophilized (SP fraction). The extent of ACE inhibitory activity was assayed by using a modified method of Cheung and Cushman. Fifty microliters of a sample solution with 100 μL of 2.5 mU ACE solution was added to 100 μL of a 12.5 mM HHL solution in 1.0 M NaCl-borate buffer at pH 8.3. After incubation at 37°C for 1 h, the reaction was stopped by adding 250 μL of 0.5 N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate, and absorbance at 228 nm was determined.

Male SHR were purchased from the Saitama Animal Facility Center (Saitama, Japan) and fed laboratory chow (CE-2; Clea Japan, Tokyo, Japan). The systolic blood pressure (SBP) of 15-week-old SHR (280–330 g bodyweight) was measured. Six SHR were given peptidic fraction powder (200 mg/kg) and captopril (10 mg/kg) dissolved in 0.9% saline by gastric intubation, and kept at 40°C for 10 min, and their SBP was measured by the tailcuff using a UR-5000 programmed electrosphygmomanometer (Ueda Co. Ltd, Tokyo, Japan). At least five readings were recorded, the maximum and minimum values were discarded, and an averaged SBP value was calculated from the remaining three values. The significance of differences of SBP before and after administration was analyzed using Student’s t-test.

The antihypertensive activity of each peptidic fraction obtained from the short-necked clams and the pearl oysters was evaluated by measuring the change in SBP at 1 h, 2 h, 3 h, 4 h, and 6 h after oral administration. Systolic blood pressure did not change in the control rats during the experimental period (6 h). Captopril (10 mg/kg) lowered SBP significantly from 1 h to 4 h after administration of the drug. A single dose of the peptidic fraction from the short-necked clam reduced SBP significantly by 42.2 mmHg at 2 h, and this antihypertensive effect continued for 4 h after administration (Fig. 1a). When the peptidic fraction from the pearl oyster was orally administered to the SHR, the fraction reduced SBP significantly by 45.9 mmHg at 3 h, and the antihypertensive effect continued for 4 h (Fig. 1b).

Results of the present study confirm that the peptidic fraction from the peptic digests of the short-necked clam and pearl oyster show antihypertensive activities in SHR after oral administration. Oral administration of the peptidic fraction (200 mg/kg) showed blood pressure-reducing activity that was qualitatively similar to that of captopril. The ACE inhibitory peptide (200 mg/kg) was weaker than captopril (10 mg/kg) used medical treatment in dose. Although a number of ACE inhibitory peptides have been isolated from various proteinous food materials, some peptides with potent ACE inhibitory activity in vitro or in-
Antihypertensive peptides

Table 1  Analytical data and angiotensin I-converting enzyme (ACE) inhibitory activity of synthetic peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid ratio in acid hydrolysate*</th>
<th>IC₅₀ ‡</th>
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<tbody>
<tr>
<td>Ile-Ala-Glu</td>
<td>Ile 1.11, Ala 0.89, Glu 0.95</td>
<td>34.7</td>
</tr>
<tr>
<td>Ile-Val-Glu</td>
<td>Ile 1.07, Val 1.02, Glu 0.91</td>
<td>95.6</td>
</tr>
<tr>
<td>Phe-Glu</td>
<td>Phe 1.03, Glu 0.99</td>
<td>51.8</td>
</tr>
<tr>
<td>Ala-Leu-Ala-Phe-Glu</td>
<td>Ala 1.97, Leu 1.07, Glu 0.87</td>
<td>167.5</td>
</tr>
<tr>
<td>Val-Glu-Val</td>
<td>Val 2.04, Glu 0.93</td>
<td>8.7</td>
</tr>
<tr>
<td>Ala-Glu-Leu</td>
<td>Ala 0.87, Glu 0.99, Leu 1.04</td>
<td>57.1</td>
</tr>
<tr>
<td>Leu-Val-Glu</td>
<td>Val 1.01, Val 0.98, Glu 0.96</td>
<td>14.2</td>
</tr>
<tr>
<td>Ile-Glu-Leu-Pro-Leu-Gly</td>
<td>Ile 1.03, Glu 0.95, Leu 2.03</td>
<td>72.1</td>
</tr>
</tbody>
</table>

*Each peptide was hydrolysed with 6 N HCl at 110°C for 24 h. Concentration of ACE inhibitory peptide required to inhibit 50% of the ACE activity.

The peptidic fraction that had been dissolved in hydrochloric acid containing 0.1% phenol at 110°C for 24 h, and the hydrolysates were analyzed using a PICO-TAG™ amino acid analyzer (Waters, Milford, MA, USA). Sequence analyses were done according to the method of stepwise Edman degradation using a 477A gas-phase automated sequencer (Applied Biosystems, Foster City, CA, USA) coupled to a HPLC. Peptides were synthesized by the solid-phase method using a 430A automated peptide synthesizer (Applied Biosystems). The results of amino acid and sequence analyses agreed well with the expected values.

Regarding the relationship between structure and activity of ACE inhibitory peptides, Cheung et al.15 have reported that those peptides with highly potent inhibitory activity had Pro, Phe or Tyr at the C-terminus, and Val and Ile at the N-terminus. In the present study, all eight ACE inhibitory peptides obtained contained glutamic acid. It is considered that glutamic acid might play an important role in ACE inhibition.

REFERENCES