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Note

Determination of Antihypertensive Peptides from an Izumi Shrimp Hydrolysate

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The izumi shrimp (*Plesionika izumiae* Omori, 1971) is an unused resource which can be caught off the southern coast of Tokushima Prefecture. We have previously found that an izumi shrimp hydrolysate significantly inhibited the age-associated spontaneous increase in blood pressure in stroke-prone spontaneously hypertensive rats. In this present study, two angiotensin I-converting enzyme inhibitory peptides were isolated from an izumi shrimp hydrolysate by using high-performance liquid chromatography, and their amino acid sequences were determined to be Val-Trp-Tyr-His-Thr and Val-Trp. A single oral administration of synthetic Val-Trp-Tyr-His-Thr or Val-Trp significantly decreased the blood pressure in stroke-prone spontaneously hypertensive rats. The antigenicity and allergenicity of the izumi shrimp hydrolysate against BALB/c mice were very low. These results demonstrate that the angiotensin I-converting enzyme inhibitory peptides isolated from the izumi shrimp hydrolysate had an anti-hypertensive effect on rats.

Key words: angiotensin; peptide; shrimp hydrolysate; anti-hypertension; allergenicity

The angiotensin I-converting enzyme (ACE; EC 3.4.15.1), which catalyzes the formation of the vasoconstrictor, angiotensin II, plays an important role in the regulation of blood pressure. The inhibition of this enzyme has been shown to have an anti-hypertensive effect, and hence there have been many studies on ACE inhibitory peptides derived from seafood.[1–4)]

The izumi shrimp (*Plesionika izumiae* Omori, 1971) is an unused resource which can be caught off the southern coast of Tokushima Prefecture. We have previously found that an izumi shrimp hydrolysate significantly inhibited the age-associated spontaneous increase in blood pressure in stroke-prone spontaneously hypertensive rats (SHRSP).[5)] In the present study, we attempted to isolate ACE inhibitory peptides derived from the izumi shrimp hydrolysate and examined the anti-hypertensive effect of these peptides on SHRSP. The antigenicity and allergenicity of the izumi shrimp hydrolysate were also examined against male BALB/c mice sensitized by the izumi shrimp hydrolysate.

Izumi shrimps were purchased from Yoshimi Kaisan Co. (Komatsushima, Japan). Whole izumi shrimps were thoroughly crushed in a blender (AP-12; Bibun Corp., Fukuyama, Japan) and then washed with two volumes of water at 98 °C for 1 h. After filtering through a stainless steel mesh (ϕ 150 μm), the resulting residue was suspended in two volumes of water and then digested with 0.1% (w/w) Protease S (Amano Enzyme, Nagoya, Japan) at 50 °C for 2 h (pH 6.5). The reaction was stopped by heating at 121 °C for 5 min. The digested solution was filtered through the stainless steel mesh (ϕ 150 μm) and then centrifuged at 11,000 g for 20 min. The supernatant was collected as the izumi shrimp hydrolysate.

The ACE inhibitory activity was assayed by the method of Cushman and Cheung[6)] with a slight modification. Hippuryl L-Histidyl L-Leucine (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in a 0.1 M sodium borate buffer (pH 8.3) containing 1 M sodium chloride. A 50 μl amount of ACE (Sigma Chemical Co.) dissolved in a 0.1 M sodium borate buffer at pH 8.3 (60 mUnit/ml) was mixed with 25 μl (2.5 μg) of the ACE inhibitory peptide derived from the izumi shrimp hydrolysate. The reaction was initiated by adding 50 μl of a 12.5 mM Hippuryl L-Histidyl L-Leucine solution, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 125 μl of 1 N hydrochloric acid. The hippuric acid liberated by ACE was extracted with 750 μl of ethyl acetate, and the ethyl acetate layer was evaporated to dryness by a

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Abbreviations: SHRSP, stroke-prone spontaneously hypertensive rat; ACE, angiotensin I-converting enzyme; HPLC, high-performance liquid chromatography

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centrifugal vaporizer (CVE-200D; Tokyo Rikakikai Co., Tokyo, Japan). The resulting residue was dissolved in 500 µl of distilled water, and hyppuric acid was spectrophotometrically measured at an optical density of 228 nm. The ACE inhibitory activity is expressed as the peptide concentration of a component that inhibited 50% of the ACE activity (IC_{50}).

Protein content was measured with a micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL, USA), using bovine serum albumin as a standard.

The izumi shrimp hydrolysate was passed through an ultra-filter (Molcut L; Nihon Millipore, Yonezawa, Japan) to remove molecules larger than 5,000 Da. The filtrate was applied to a reverse-phase column (TSK-GEL ODS-80TM, 250 × 4.6 mm i.d.; Tosoh Corp., Tokyo, Japan) and eluted with a linear gradient of acetonitrile (0.05–80% in 40 min) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The elution was monitored at 210 nm. The fraction with the highest ACE inhibitory activity was concentrated with the centrifugal vaporizer and lyophilized. The active fractions obtained from the first step of high-performance liquid chromatography (HPLC) were then applied to a reverse-phase column (J'sphere ODS-H80, 150 × 4.6 mm i.d.; YMC Co., Kyoto, Japan) and eluted with a linear gradient of acetonitrile (12–24% in 15 min) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The fraction with the highest ACE inhibitory activity was concentrated with the centrifugal vaporizer and lyophilized. The amino acid sequences of the purified peptides were analyzed with an LF-3000 protein sequencer (Beckman Coulter, Fullerton, CA, USA). The peptides were synthesized with an ACT348 peptide synthesizer (Applied Biosystems, Foster City, CA, USA).

The ACE inhibitory peptides were digested by pepsin and trypsin that constitute the gastro-intestinal proteases. After the ACE inhibitory peptides had been adjusted to pH 2.0 with 1 N hydrochloric acid, they were digested at 37 °C for 4 h in the presence of 1 mg of a pepsin solution dissolved in 0.075 N hydrochloric acid. The digesting solution was then adjusted to pH 8.0 with 1 N sodium hydroxide, and digestion was continued at 37 °C for 4 h in the presence of 1 mg of trypsin dissolved in a 0.05 N potassium phosphate buffer (pH 8.0). The reaction was stopped by heating at 98 °C for 5 min. The amino acid sequences of the HPLC peak were determined after the treatment with pepsin and trypsin of the ACE inhibitory peptides.

Male stroke-prone spontaneously hypertensive rats (SHRSP/Izm) at 4 weeks of age were purchased from Disease Model Cooperative Research Association (Kyoto, Japan). The rats were given a stock diet (SP; Funabashi Farms Co., Funabashi, Japan) and distilled water *ad libitum* for 4 or 8 weeks. All the rats were housed in individual stainless steel cages in a room with a controlled temperature (24 °C) and a 12-h light-dark cycle. The rats were divided into 3 groups of 5 rats each. Two milligrams of synthesized peptides were dissolved in 2 ml of distilled water and administered by intubation to rats that had been fasted overnight. Control rats were given captopril (1 mg) dissolved in 2 ml of distilled water or distilled water alone. After a single oral administration of the sample, the systolic blood pressure was measured by the tail-cuff method with a programmed electrosphygmomanometer (PS-100; Riken Kaihatsu Co., Tokyo, Japan). At least five readings were recorded and averaged for each rat. Data are expressed as the mean and standard deviation. Student's t test was conducted after a one-way analysis of variance to examine the difference which was considered statistically significant at $p < 0.05$.

Male BALB/c mice at 8 wk of age (Japan SLC Co., Hamamatsu, Japan) were maintained on a stock diet (MF; Oriental Yeast Co., Tokyo, Japan). One mg of the izumi shrimp hydrolysate or distilled water was intraperitoneally administered with a complete adjuvant. After 3 wk, the sample was intraperitoneally administered three times with an incomplete adjuvant. As a positive control, 1 mg of tropomyosin purified from giant tiger shrimp (*Penaeus monodon*) was intraperitoneally administered to mice with the adjuvant. The mice were then anesthetized and killed, and serum was collected.

Three mg of the izumi shrimp hydrolysate (150 µl) was added to the same volume of an SDS-sample buffer (20% glycerol, 4.6% SDS and 125 mM Tris-hydrochloric acid containing 0.002% bromophenol blue). After adding 2-mercaptoethanol, each sample was boiled at 100 °C for 5 min. These samples were electrophoresed in 12.5% acrylamide gel according to Laemmli's method,[7] and the proteins that had been electrically separated were transferred to a nitrocellulose membrane according to the method of Towbin *et al.*[8]

In order to detect IgG in the serum from those mice sensitized with the izumi shrimp hydrolysate, the transfer membrane was reacted in turn with the serum from these sensitized mice and the biotin labeled anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA). Likewise, in order to detect IgE in the serum from those mice sensitized with the izumi shrimp hydrolysate, the transfer membrane was reacted in turn with the serum from these sensitized mice and the biotin labeled anti-mouse IgE, rabbit IgG antibody (Vector Laboratories), and the biotin labeled anti-rabbit IgG antibody (Vector Laboratories). The color reaction was performed by using an ABC kit (Vector Laboratories). The captured image of each band on the transfer membrane was scanned at 600 dpi resolution.

We have already reported that the oral administration of an izumi shrimp hydrolysate decreased the blood pressure in SHRSP.[5] In the present study, we attempted to isolate the ACE inhibitory peptides derived from the izumi shrimp hydrolysate and also examined the anti-hypertensive effects on SHRSP of these peptides.

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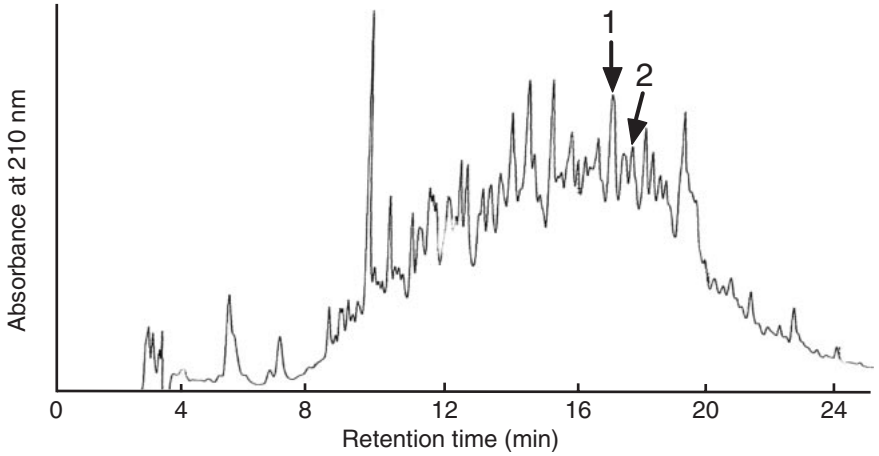


Fig. 1. First HPLC Step for the Izumi Shrimp Hydrolysate in a TSK-GEL ODS-80TM Column.
The elution was performed as described in the Materials and Methods section. Peaks 1 and 2 with arrows show the ACE inhibitory activity.

As a first step, we isolated the ACE inhibitory peptides derived from the izumi shrimp hydrolysate. Figure 1 shows the elution pattern of the izumi shrimp hydrolysate in the first step of the HPLC analysis in the TSK-GEL ODS-80TM column. The fractions with ACE inhibitory activity were eluted from 10 to 18 min after injection. These fractions were used in the further purification step in the J'sphere ODS-H80 column. Two kinds of ACE inhibitory peptides were obtained from fractions 1 and 2, the amino acid sequences of these peptides being Val-Trp-Tyr-His-Thr and Val-Trp, respectively. The IC_{50} value for the former was 28.3 μM, and for the latter was 6.6 μM. Although the isolation of Val-Trp-Tyr-His-Thr had not previously been reported, Val-Trp had already been isolated from a hydrolysate of wakame,[9] pelagic thresher muscle hydrolysate,[10] hot water extract of wakame,[11] and salmon muscle hydrolysate.[12] Furthermore, Cheung *et al.*[13] have reported that Val-Trp was the most potent peptide, while Sato *et al.*[9] have reported that Val-Trp showed competitive inhibition. Val-Trp showed high ACE inhibitory activity in this study as well as in the other reports.

We examined whether there was a change in Val-Trp or Val-Trp-Tyr-His-Thr after treating with gastro-intestinal proteases. Although Val-Trp was not changed after being treated with gastro-intestinal proteases, Val-Trp-Tyr-His-Thr was hydrolyzed to Val-Trp and Val-Trp-Tyr by pepsin and trypsin digestion. It was found that Val-Trp was also produced by gastro-intestinal proteases. These results suggest that these peptides having ACE inhibitory activity were absorbed in their intact form from the intestinal tract, and showed an anti-hypertensive effect.

Val-Trp or Val-Trp-Tyr-His-Thr was administered in this study to SHRSP, and the change in blood pressure was observed. The blood pressure 0.5 and 1 h after a single oral administration of Val-Trp-Tyr-His-Thr or Val-Trp was significantly lower than that in the control group of 8-week-old SHRSP (Fig. 2A). Similarly, the

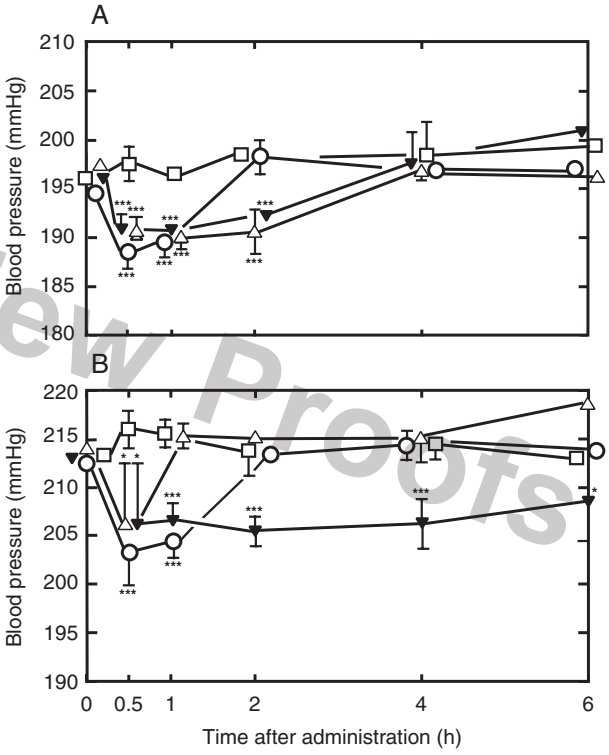


Fig. 2. Change in Blood Pressure after a Single Oral Administration of Val-Trp-Tyr-His-Thr (unfilled circles), Val-Trp (unfilled triangles), Captopril (filled triangles) and Water (unfilled squares) in 8- (A) and 12- (B) Week-Old SHRSP.
Each value is the mean ± SD, $n = 5$ per group. Values are considered significantly different at $^{*}p < 0.05$ or $^{***}p < 0.001$ compared to the control groups.

blood pressure 0.5 h after a single oral administration of these two peptides was significantly lower in 12-week-old SHRSP (Fig. 2B). Although a single oral administration of Val-Trp (1 mg/kg of body weight) to spontaneously hypertensive rats by Sato *et al.*[9] did not clearly show any anti-hypertensive effect, both Val-Trp-Tyr-His-Thr and Val-Trp (about 10 mg/kg of body

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weight) showed a significant anti-hypertensive effect on SHRSP in this study. It is considered that this result depended on the difference in dose level of Val-Trp.

We examined the antigenicity and allergenicity of the izumi shrimp hydrolysate in mice. No IgG or IgE against the izumi shrimp hydrolysate could be detected in the serum from mice sensitized with this hydrolysate. On the other hand, the production of IgG and IgE against tropomyosin was detected in the serum from mice that had been sensitized to tropomyosin purified from the giant tiger shrimp. These results indicate that the antigenicity and allergenicity of the izumi shrimp hydrolysate toward mice were very low. Similarly, the likelihood of any antigenicity or allergenicity due to the intake of the izumi shrimp hydrolysate in humans seems to be low.

These results demonstrate that angiotensin I-converting enzyme inhibitory peptides isolated from the izumi shrimp hydrolysate had an anti-hypertensive effect on rats.

Acknowledgments

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