Purification and Identification of an Angiotensin I-converting Enzyme Inhibitor from Soy Sauce

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The inhibitory activity of an angiotensin I-converting enzyme (ACE) detected in soy sauce was fractionated into two major fractions of high molecular weight (Hw) and low molecular weight (Lw) by gel filtration chromatography on Bio-gel P-2 after treating with ethanol. The Hw fraction reduced the blood pressure in hypertensive rats after orally administering, while the Lw fraction did not. The ACE inhibitor in the Hw fraction was further purified by Dowex 50W ion-exchange chromatography and four subsequent steps of HPLC. On the basis of the SIMS-mass spectrum, NMR spectrum and other characteristics, the purified ACE inhibitor was identified as nicotianamine (N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid). The IC50 value for this ACE was 0.26 μM.

The angiotensin I-converting enzyme (EC 3.4.15.1, ACE) catalyzes the hydrolysis of angiotensin I to generate a potent vasoconstrictor, angiotensin II.1) This enzyme plays an important role in the renin-angiotensin system to regulate both the arterial blood pressure, and salt and water balance.2) Since snake venom peptide inhibitors played a role in establishing the clinical value of ACE inhibitors,3) many peptide derivatives of ACE inhibitor have been developed.4) Many natural products have also been examined for ACE inhibitory activity, and teprotide from Bothrops Jaraaca,5) aconvenin from Streptomyces,6) and others7,8) have been identified.

The ACE inhibitory abilities of foods have recently been studied,9) ACE inhibitors being isolated and six peptides and phytin identified from sake and its by-products.10) We found an ACE inhibitor in Japanese-style fermented soy sauce which was already known to have many kinds of peptides produced from raw protein by proteolysis.11,12) The most potent active fraction from gel filtration of this ACE inhibitor lowered the blood pressure in hypertensive rats by oral administration. This report describes the purification and identification of an ACE inhibitor having antihypertensive effect from soy sauce.

Materials and Methods

Materials. Hippuryl-l-histidyl-l-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan). Captopril, angiotensin I and II, and ACE from rabbit lung acetonide powder were obtained from Sigma Chemical Co. (U.S.A.). The partially purified ACE preparation was obtained from pig epididymis according to Cushman and Cheung.13) TSKgel ODS-120T and TSKgel Amide 80 columns were products of Tosoh (Tokyo, Japan), and the poly-hydroxyethyl aspartamide column was from Poly LC (U.S.A.). Bio-gel P-2 was obtained from Bio-Rad laboratories (U.S.A.), and Dowex 50W X-2 was obtained from Dow Chemical Co. (U.S.A.). Authentic nicotianamine was kindly supplied by Dr. Noma of Japan Tobacco.

Assay of ACE inhibitory activity. The activity of ACE inhibition was assayed by a modification of the method of Cushman and Cheung.14) A mixture (300 μl) containing a 100 mM sodium borate buffer (pH 8.3), 300 mM NaCl, 8 μM ACE from rabbit lung, and an appropriate amount of the inhibitor solution was preincubated for 10 min at 37°C. The reaction was initiated by adding 0.15 ml of HHL at a final concentration of 5 mM, and terminated by adding 0.5 ml of 1M HCl after 30 min of incubation. The hippuric acid liberated was extracted with 1.5 ml of ethyl acetate, 1.0 ml of the extract was evaporated to dryness by a Speed back concentrator (Savant, U.S.A.), and the residue was dissolved in 1.0 ml of deionized water. The absorbance at 228 nm was measured to evaluate the degree of inhibition of ACE activity. One unit of inhibitory activity is defined as the amount of inhibitor needed to inhibit 50% of the ACE activity (IC50 value).

The activity of the ACE inhibitor during the purification steps was assayed by the HPLC method.15) using angiotensin I as a substrate. After a solution (100 μl) containing 200 mM Tris-HCl (pH 8.3), 600 mM NaCl, and 10 μM ACE from epididymis had been mixed with 100 μl of the inhibitor solution, pre-incubation was performed for 10 min at 37°C. The enzyme reaction was initiated by adding 10 μl of an angiotensin I solution (20 μg). After incubating for 10 min at 37°C, the reaction was stopped by adding 10 μl of 6 M HCl. Twenty μl of the resulting solution was analyzed by HPLC to determine the concentration of the product, angiotensin II.

Experimental animals and measurement of blood pressure. Male spontaneously hypertensive 11-week-old rats (SHR) were obtained from Charles River Japan, the body weight of each being about 300 g, and the mean blood pressure about 182-197 mmHg. To prepare the two-kidney Goldblatt hypertensive (2KGH) rats, a silver clip was applied to the left renal artery of Wistar rats to produce an internal diameter of 0.2 mm according to the method of Yamakoshi et al.16) A single oral administration of the test substances dissolved in distilled water was given, and control rats were given the same volume of distilled water. The tail systolic blood pressure was measured by the tail cuff method,17) using a programmed MK-1000 electrophygomonanometer (Muromachi Kikai Co.).

Identification and characterization of the purified inhibitor. The free amino group was analyzed by the method of Sanger and Thompson,18) and sugar(s) were detected by the method of Dubois et al.19) Melting points were measured by Yanagimoto micro-melting point apparatus, and specific rotation was measured by a polarimeter with a 10-cm cell at room temperature and at a 1% concentration. The IR spectrum was obtained for KBr discs with a JASCO FT/IR-7300 instrument. 1H-NMR and 13C-NMR spectra were obtained in D2O, using TSP or DMSO as an internal standard, with a JEOL JNM-FX200 NMR spectrometer. The SIMS-mass spectrum was taken on a Hitachi M-80B mass spectrometer, and the elemental analysis was performed at Toray Research Center.

Results

Fractionation of soy sauce with ethanol and subsequent gel filtration

Five liters of soy sauce was mixed with 18 liters of eth-
Table I. Relationship between the Oral Administration of the Hw or Lw Fraction and Blood Pressure Reduction in SHR and 2KGH Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Blood pressure before administration</th>
<th>Blood pressure reduction after administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
<td>4h</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Captopril, 10 mg/kg</td>
<td>4</td>
<td>197.0±20.2</td>
<td>39.3±16.7</td>
</tr>
<tr>
<td>Hw, 3.6g/kg</td>
<td>5</td>
<td>187.7±8.2</td>
<td>22.3±6.6</td>
</tr>
<tr>
<td>Lw, 6.0g/kg</td>
<td>5</td>
<td>181.9±11.0</td>
<td>1.1±2.4</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4</td>
<td>195.4±13.4</td>
<td>1.8±1.9</td>
</tr>
<tr>
<td>2KGH rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Captopril, 10 mg/kg</td>
<td>4</td>
<td>204.0±31.5</td>
<td>54.8±15.8</td>
</tr>
<tr>
<td>Hw, 3.6g/kg</td>
<td>3</td>
<td>183.0±14.9</td>
<td>22.1±16.8</td>
</tr>
<tr>
<td>Lw, 6.0g/kg</td>
<td>4</td>
<td>187.3±9.5</td>
<td>0.3±0.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>4</td>
<td>187.3±19.7</td>
<td>0.8±0.9</td>
</tr>
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</table>

The blood pressure reduction (mmHg) in each group is given by (blood pressure before administration) - (blood pressure after administration). Values are shown as mean±S.D.

Table II. Purification of the ACE Inhibitor from Soy Sauce

<table>
<thead>
<tr>
<th>Step</th>
<th>ACE inhibitory activity (×10⁶ units)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-gel P-2 of Hw fraction</td>
<td>1.27</td>
<td>100</td>
</tr>
<tr>
<td>Dowex 50W X-2</td>
<td>1.24</td>
<td>98</td>
</tr>
<tr>
<td>TSKgel ODS-120T</td>
<td>0.98</td>
<td>77</td>
</tr>
<tr>
<td>TSKgel Amide 80</td>
<td>0.68</td>
<td>54</td>
</tr>
<tr>
<td>Poly-hydroxyethyl A</td>
<td>0.40</td>
<td>31</td>
</tr>
<tr>
<td>TSKgel Amide 80</td>
<td>0.23</td>
<td>18</td>
</tr>
</tbody>
</table>

2KGH rats. The change in blood pressure was measured 1, 4, 8, and 24 h after administering the test sample. Blood pressure was significantly decreased 4-8 h after administering the Hw fraction, and returned to the control level within 24 h after the administration. These results indicate that only the Hw fraction decreased the blood pressure in SHR and 2KGH rats, while the Lw fraction had no effect on the blood pressure in either type rat.

Purification of the ACE inhibitor from soy sauce

The Hw fraction was dissolved in distilled water, and the pH value was adjusted to 1.5 with HCl. After sample solution had been applied to a column (60×200 mm) of Dowex 50W X-2 (H⁺ form), the column was washed with 1 liter of 50 mM acetic acid, and then eluted with 5 mM acetic acid containing 1 M NaCl (pH 6.0). Twenty ml of each fraction was obtained and assayed for ACE inhibitory activity. The active fractions were pooled and concentrated to approx. 20 ml, and desalted by filtration through Bio-gel P-2. The ACE inhibitor was further purified by HPLC, and Table II summarizes the result of purifying the ACE inhibitor from the Hw fraction. Five liters of soy sauce initially had 1.15×10⁶ units of ACE inhibitory activity. However, after filtration through Bio-gel P-2, the Hw fraction contained 1.27×10⁶ units, and the Lw fraction contained 0.78×10⁶ units. This result indicates that the total inhibitory activity (2.05×10⁶ units) was increased by 177% compared with that of the original soy sauce. As it was observed that only the Hw fraction was effective for decreasing the blood pressure by orally administering to SHR and 2KGH rats, further purification of the ACE in-

Fig. 1. Bio-gel P-2 Column Chromatogram of Soy Sauce.
The experimental details are described in the text. (A) the precipitate obtained after treating soy sauce with ethanol, (B) the supernatant. The fractionated volume was 19 ml. Closed circles show ACE inhibitory activity, and dotted lines show absorbance at 400 nm. The active fractions collected are shown by arrows.

Effects on blood pressure of a single administration of the Hw or Lw fraction

Table I shows the effects on blood pressure of an oral administration of the Hw or Lw fraction in both SHR and
Fig. 2. HPLC Purification of the ACE Inhibitor from the Hw Fraction Obtained by Bio-gel P-2.
(A) Reverse-phase HPLC in a TSKgel ODS-120T column (21.5 × 300 mm) of the active fraction eluted from Dowex 50W X-2 with 5 mM acetic acid containing 1 M NaCl (pH 6.0). The active fractions indicated by bar 1 were collected and further purified by TSKgel Amide 80. (B) First normal-phase HPLC in a TSKgel Amide 80 column (4.6 × 200 mm) of 1. The eluent was 65% acetonitrile at a flow rate of 1 ml/min; detection by RI. Two active fractions were collected and further purified by Poly-hydroxyethyl A. (C) Hydrophilic interaction HPLC in a Poly-hydroxyethyl A column (4.6 × 200 mm) of 2. The solvent system was a linear gradient from 80% acetonitrile containing 10 mM triethylamine phosphate (TEAP) at pH 2.8 to 25% acetonitrile containing 10 mM TEAP at a flow rate of 1 ml/min. Absorbance was monitored at 220 nm of the active fractions eluted at about a 40 min retention time. 3 were collected and further purified by TSKgel Amide 80. (D) Second normal-phase HPLC in a TSKgel Amide 80 column (4.6 × 200 mm) under the same HPLC conditions as those in (A). The indicated fraction 4 was collected, concentrated and lyophilized.

Identification of the purified ACE inhibitor from soy sauce

The purified ACE inhibitor as a white powder was soluble in water, and insoluble in methanol, chloroform, acetone, pyridine, and DMSO. The inhibitor gave a positive ninhydrin reaction and a DNP-derivative result characteristic of an ε-amino group. A negative color reaction by the phenol-sulfuric acid method indicated that the inhibitor had no sugar moiety, and the ultra-violet absorption spectrum in water exhibited no characteristic peak.

The inhibitor had a melting point of 220–223°C with decomposition, and [α]D of –48.8° (c 1.0, H2O). The 1H-NMR spectrum showed no signal assignable to a methyl group, and the 13C-NMR spectrum proved the presence of 12 carbon atoms. The 13C-NMR chemical shifts and the off-resonance proton decoupled spectrum specified their character as 6CH2, 3CH and 3C=O. The molecular weight of the inhibitor was determined to be 303 from the SIMS-mass spectrum. Elemental analysis gave the following results. Found: C, 40.4; H, 7.3; N, 11.5; O, 36.5%; P and S, not detected. Calcd. for C12H21N3O6·3H2O: C, 40.3; H, 7.56; N, 11.76; O, 40.3%.

These data indicate that the purified ACE inhibitor from soy sauce was identical with nicotianamine (N-[N-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid). A comparison of the physical constants, IR spectrum, HPLC and TLC behavior with the data for authentic nicotianamine, as well as a comparison of the 13C- and 1H-NMR spectra of our material with the literature values for nicotianamine 20,21 proved the identity of the ACE inhibitor to be nicotianamine.

Characterization of the purified ACE inhibitor from soy sauce

The IC50 value obtained by using rabbit lung enzyme with HHL as the substrate was 0.26 μM. A signal oral administration to SHR of 20 mg/kg of the purified inhibitor decreased the blood pressure. This decrease in blood pressure measured 1 h, 4 h, and 8 h after administration was 24, 20, and 19 mmHg (average values, N=2), respectively.
Discussion

Japanese-style soy sauce contained two major ACE inhibitors (the Hw and Lw fractions) that were fractionated with ethanol and subsequently by gel filtration. We purified the ACE inhibitor in soy sauce from the Hw fraction because the specific activity for ACE inhibition of the Hw fraction was about 10 times higher than that of the Lw fraction. In addition, a single oral administration of the Hw or Lw fraction to SHR and 2K-GH rats decreased the blood pressure only in the case of the Hw fraction. It might be considered that the ACE inhibitory substance present in the Hw fraction comprised mainly one kind of inhibitor because of its high yield (18%) and single chromatographic distribution of ACE inhibitory activity during purification. On the other hand, the Lw fraction contained many kinds of inhibitor that were detected by HPLC with an ODS column (data not shown).

From the results of an instrumental analysis, the purified inhibitor was identified as nicotianamine, which has been reported in some higher plants.\(^22,23\) Although nicotianamine has an optimal molecular structure for chelating iron and is considered to be a possible phytosiderophore with an essential function in cellular iron transport and/or metabolism,\(^24\) the ACE inhibitory activity of nicotianamine has not previously been reported, except for one patent application claiming its production by a microorganism (Medicago sp.). However, it is very likely that a powerful chelating agent might be a representative inhibitor of ACE.\(^25\) In addition, a proline analogue, azetidic acid 2-carboxylic acid, present in the structure of nicotianamine must be rendered for ACE inhibition.

Buděnský et al. have reported the presence of nicotianamine in Leguminosae, and isolated it from the aerial parts of alfalfa (Medicago sativa L.). In the soy sauce fermentation process, one of the raw materials is soy beans, which belong to Leguminosae.\(^26\) Therefore, the origin of the nicotianamine in soy sauce might be considered to be soy bean. We have detected a large quantity of nicotianamine in soy bean (unpublished data), but more experiments will be needed to prove the origin of the nicotianamine in soy sauce.

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References