Angiotensin I-converting enzyme (ACE) inhibitory activity was observed in a tofuyo (fermented soybean food) extract with an IC50 value of 1.77 mg/ml. Two ACE inhibitors were isolated to homogeneity from the extract by adsorption and gel filtration column chromatography, and by reverse-phase high-performance liquid chromatography (HPLC). The purified substances reacted with 2,4,6-trinitrobenzensulfonic acid sodium salt. The amino acid sequences of these inhibitors determined by Edman degradation were Ile-Phe-Leu (IC50, 44.8 μM) and Trp-Leu (IC50, 29.9 μM). The Ile-Phe-Leu sequence is found in the α- and β-subunits of β-conglycinin, while the Trp-Leu sequence is in the B-, B1A- and BX-subunits of glycinin from soybean. Both of the peptides are non-competitive inhibitors. The inhibitory activity of Trp-Leu was completely preserved after a treatment with pepsin, chymotrypsin or trypsin. Even after successive digestion by these gastrointestinal proteases, the activity remained at 29% of the original value.

Key words: angiotensin I-converting enzyme inhibitor; bioactive peptide; fermented soybean food; tofuyo

The angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxy peptidase associated with the regulation of blood pressure. It converts angiotensin I to the potent pressor peptide, angiotensin II, and also degrades depressor peptide bradykinin.1-12) ACE inhibitors from various foods have recently been studied in terms of their ability to prevent and alleviate hypertension. Physiologically functional foods enriched with ACE inhibitors such as tripeptide (Val-Pro-Pro, Ile-Pro-Pro) from sour milk,3) dipeptide (Val-Tyr) from a sardine muscle hydrolysate6) and dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) from a casein hydrolysate9) are used as supplements to improve hypertension. In respect of soybean, Kawamura6) has purified ACE inhibitory peptides from a soybean protein hydrolysate and observed that the peptide fragments existed in the primary structures of β-conglycinin and glycinin. Wu and Ding7,8) have also confirmed the hypotensive effect of ACE inhibitory peptides derived from a soy protein alkaline hydrolysate on spontaneously hypertensive rats; they isolated and characterized two ACE inhibitory peptides. ACE inhibitors have also been isolated from fermented soybean foods, and were identified to be nicotiamine from soy sauce,9) as well as phytic acid10) and peptide (Ser-Tyr) from miso paste.11) Although the ACE inhibitory activity in natto has already been studied, the chemical structure of the inhibitor has not yet been reported.12) More information on the ACE inhibitors in soybean foods is necessary for the development of healthy foods.

Tofuyo is a traditional fermented tofu from Okinawa in Japan. It has a mild flavor and fine taste similar to cream cheese. It also has high nutritional value because it is from soybean. We have been studying the production of tofuyo and have characterized it.13-16) However, the physiological functions of tofuyo remain to be clarified.

We report here the isolation and identification of ACE inhibitors from tofuyo. Moreover, we investigated the mechanism underlying the inhibition at the kinetic level and the stability of the purified ACE inhibitors against digestion.

Materials and Methods

Materials and reagents. Tofuyo prepared from red and yellow koji is supplied by Benihama Co., Ltd. The enzymes used throughout this study were the angiotensin I-converting enzyme (rabbit lung, 2.5 units), pepsin (porcine gastric mucous membrane, 3,900 units/mg of solid), chymotrypsin (bovine pan-
creas, 40–60 units/mg of protein), and trypsin (bovine pancreas, 8,600 units/mg of solid) from Sigma Chemical Co. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and isoleucyl-phenylalanyl-leucine were obtained from Peptide Institute Inc., and tryptophanyl-leucine was obtained from Nikka Techno Service Co. SEPABEADS SP825 and Sephadex G-25 resin were from Mitsubishi Chemical Co. and American Bioscience, respectively. All other chemicals used were of analytical grade.

Assay for ACE inhibitory activity. The ACE inhibitory activity was assayed by using the modified method of Lieberman.\(^{(17)}\) For each assay, 65 \(\mu l\) of a sample solution and 50 \(\mu l\) of 12.5 mM Hip-His-Leu as a substrate in a borate buffer (pH 8.3) containing 1 M NaCl were incubated with 10 \(\mu l\) of ACE (2 \(\mu l\)) for 1 h at 37°C. The reaction was stopped by adding 125 \(\mu l\) of 0.5 M HCl. The liberated hippuric acid was extracted with 750 \(\mu l\) of ethyl acetate, and 200 \(\mu l\) of the resulting extract was then evaporated to dryness with a centrifugal concentrator (Tomy CC-105). The precipitate was dissolved in 1.2 ml of 1 M NaCl, and the absorbance at 228 nm was then measured. The sample concentration required to inhibit 50% of the ACE activity under the assay conditions is taken as the IC\(_{50}\) value.

Protein assay. The protein concentration in each sample was determined with a micro BCA protein assay reagent kit (Pierce Chemical), with bovine serum albumin used as the standard.

Preparation of the tofuyo extract. Tofuyo was suspended in 3 volumes of distilled water, homogenized with a labo-stirrer (Yamato L-35) and then shaken in an incubator (Yamato BT-21) at room temperature for 1 h. The mixture was then centrifuged at 12,000 \(\times\) g for 15 min and the resulting precipitate was removed. The turbid supernatant was filtered, and the filtrate was boiled for 20 min. The filtered solution was used as the tofuyo extract.

Purification of the ACE inhibitors. The extract obtained from 600 g of tofuyo was applied to an adsorptive column of SEPABEADS SP825 (65 cm \(\times\) 21.4 cm) that had been pre-equilibrated with distilled water. After washing with the same distilled water, the inhibitors were eluted with a step-wise gradient of an ethanol solution from zero to 70%. Each fraction was evaporated in a rotary evaporator (Yamato RE-46), and the resulting residue was dissolved in distilled water.

The active fraction was subjected to reverse-phase HPLC in a column of Cosmosil SCX-AR-300, elution being carried out with a linear gradient of acetonitrile from zero to 40% in 0.05% trifluoroacetic acid for 80 min at a flow rate of 0.5 ml/min with monitoring at 220 nm. The fraction corresponding to an active peak was rechromatographed in the same column. Purification of the other potent inhibitor was done by gel filtration (Sephadex G-25, 1 cm \(\times\) 5 cm) before reverse-phase HPLC.

Amino acid sequence analysis. The amino acid sequences of the ACE inhibitors were determined by automated Edman degradation with a gas/liquid phase protein sequencer (Applied Biosystems 473A).

Digestion test. The stability of each purified ACE inhibitory peptide against gastrointestinal proteases in vitro was assessed. An inhibitor solution (1.5 mM, 0.2 ml) was incubated with 0.2 ml of a 0.05% pepsin, chymotrypsin or trypsin solution (0.1 M HCl at pH 2.0 or 0.1 M potassium phosphate buffer at pH 8.0) and 0.2 ml of each buffer for 6 h at 37°C. The inhibitor solution was then subjected to successive digestion tests. The pepsin solution was evaporated in a centrifugal concentrator and then redissolved in 0.6 ml of a solution containing both 0.025% (w/v) chymotrypsin and 0.025% (w/v) trypsin. The reaction mixture was incubated for 6 h at 37°C and then boiled for 5 min to stop the digestion. After the enzymatic treatment, each sample was centrifuged, and the supernatant was adjusted to pH 8.3 for measuring the ACE inhibition.

Results and Discussion

ACE inhibitory activity in tofuyo

The ACE inhibitory activity of the tofuyo extract compared with that of other fermented soybean foods is shown in Table 1. The miso paste and natto extracts were obtained by using distilled water with the same method as that used for the tofuyo extract, while soy sauce was used directly. Before the assay, each extract and the soy sauce were adjusted to pH 8.3 with a 0.1 M sodium hydroxide solution. The natto extract showed the highest inhibitory activity, and the ACE inhibitory activity in the tofuyo extract was almost as strong as that in the miso paste extract.

| Table 1. IC\(_{50}\) Values for Typical Fermented Soybean Foods |
|---------------------------------|-----------------|
| Fermented soybean food* | IC\(_{50}\) (mg/ml) |
| Soy sauce | 3.44 |
| Miso paste | 1.27 |
| Natto | 0.16 |
| Tofuyo | 1.77 |

* Miso paste and natto were extracted by following the same method as that for tofuyo described in the text. Soy sauce was used directly for the assay.

Purification and identification of the ACE inhibitory peptides

The crude tofuyo extract was loaded into a column...
Table 2. Separation of ACE Inhibitors from the Tofuyo Extract by Chromatography in a SEPABEADS SP825 Column

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (g)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% ethanol</td>
<td>498.91</td>
<td>5.1</td>
</tr>
<tr>
<td>20% ethanol</td>
<td>6.83</td>
<td>25.7</td>
</tr>
<tr>
<td>40% ethanol</td>
<td>0.59</td>
<td>10.1</td>
</tr>
<tr>
<td>70% ethanol</td>
<td>0.04</td>
<td>28.6</td>
</tr>
</tbody>
</table>

* The final concentration of protein in each fraction was 0.1 mg/ml in the reaction mixture.

of SEPABEADS SP825 and eluted with a step-wise gradient of ethanol (0, 20, 40 and 70%). The ACE inhibitory activity in each of these fractions is shown in Table 2. Potent activity and a large sample amount were found in the 20% ethanol fraction, so this fraction was concentrated. The concentrate was subjected to reverse-phase HPLC (Cosmosil 5C$_18$-AR-300 column), elution being performed with a linear gradient of an acetonitrile solution as shown in Fig. 1(A). ACE inhibitory activity was detected in several peaks. Peak I, which had the highest activity, was used for the next step of purification. After peak I had been concentrated, it was rechromatographed in the same column with a moderate acetonitrile gradient. As shown in Fig. 1(B), a homogeneous preparation (compound I) was obtained.

Purification was carried out on the other potent inhibitor in the 20% ethanol fraction from SEPABEADS SP825 already described. The active solution was loaded into a column of Sephadex G-25 and then eluted with distilled water. Seven active fractions were obtained as shown in Fig. 2. The major active fraction, No. 6, was then obtained. The active solution was subjected to reverse-phase HPLC (Cosmosil 5C$_18$-AR-300 column), elution being performed with a linear gradient of an acetonitrile solution as shown in Fig. 3. Strong inhibitory activity was found in peak II that was eluted at 35 min.
(Fig. 3(A)). A homogeneous preparation of compound II was finally obtained (Fig. 3(B)).

Since both substances (compounds I and II) reacted with 2,4,6-trinitrobenzensulfonic acid sodium salt, which binds only to primary amines and the sulfhydryl group, they both seemed to be peptide-like substances.

The chemical structures of compounds I and II were identified to be Ile-Phe-Leu and Trp-Leu, respectively, by an amino acid sequence analysis. Their IC₅₀ values were determined to be 44.8 and 29.9 μM, respectively. The contents of Ile-Phe-Leu and Trp-Leu were estimated to be 23.9 and 0.3 mg in 100 g of the tofuyo extract. This is the first report of ACE inhibitory peptides derived from tofuyo. A computer search of the SWISS-PROT protein sequence database showed that the amino acid sequence Ile-Phe-Leu exists in the primary structures of the α- and β-subunits of β-conglycinin (Ile (476)-Phe (477)-Leu (478) in the α-subunit, and Ile (247)-Phe (248)-Leu (249) in the β-subunit), whereas Trp-Leu exists in the primary structures of the B1A- and BX-subunits of glycmin (Trp (44)-Leu (45) in the B- and BX-subunits, and Trp (43)-Leu (44) in the B1A-subunit). Since the α'-, α-, and β-subunits in β-conglycin and the acidic subunit in glycmin are degraded to low-molecular-weight elements during tofuyo fermentation for 3 months, it is likely that Ile-Phe-Leu was liberated from β-conglycin by proteases that were produced by Monascus purpureus and/or Aspergillus oryzae used in the fermentation process. Although the basic subunit in glycmin cannot be easily degraded by these enzymes, as compared with each subunit in β-conglycin or the acidic subunit in glycmin, Trp-Leu might have been liberated from the subunit during long-term fermentation (3 months).

**Clarification of the ACE inhibition mechanism**

To clarify the mechanism underlying the inhibition at the kinetic level, Lineweaver-Burk plots were determined for Ile-Phe-Leu and Trp-Leu. As shown in Fig. 4, these plots with an intersection on the 1/S axis indicate that both peptides are non-competitive inhibitors. Moreover, Dixon plots displaying the 1/v versus inhibitor concentration with an intersection on the 1 axis were also characteristic of non-competitive inhibition (data not shown). Competitive ACE inhibitors have been most frequently reported, and some non-competitive inhibitors have also been found in such foods as natto, sake, tuna, sardine, and chickpea. However, the inhibition site on ACE of these non-competitive inhibitors has not previously been investigated. Interestingly, captopril, enalaprilat and ramiprilat, which are competitive inhibitors of ACE, have also shown a non-competitive mode on the Dixon plots. One of the reasons for such an inhibition mode, i.e., the mixed type, has been consi-

dered to be the slow-tight binding of these inhibitors at the ACE active site. Further investigation is required to determine the mechanism for non-competitive inhibition in foods containing the Ile-Phe-Leu and Trp-Leu peptides.

**Digestion test on the inhibitors**

In order to understand the resistance of both the Ile-Phe-Leu and Trp-Leu peptides in tofuyo to digestion in vivo, changes in their IC₅₀ values before and after treatment with gastrointestinal proteases in vitro were examined. As shown in Table 3, the ACE inhibitory activities of both peptides were completely preserved after the pepsin treatment. Although the inhibitory activity of Trp-Leu was also completely preserved after the chymotrypsin or trypsin treatment, that of Ile-Phe-Leu had decreased to 62% and 75% of the original value following the chymotrypsin and trypsin treatment, respectively. In spite of the successive digestion with pepsin, chymotrypsin and trypsin, the inhibitory activity of Ile-Phe-Leu was found to be 38%, and that of Trp-Leu was 29% of the original value. It has been reported that the IC₅₀
values of "Ile-Phe" and "Phe-Leu", which are parts of Ile-Phe-Leu, were 930 and 16 μM, respectively. Thus, Ile-Phe-Leu is likely to preserve its activity until it is degraded to its individual amino acids.

It is well known that di- and triptides are more rapidly absorbed and reach a higher concentration in the blood than single amino acids. Chun et al. have recently demonstrated that short peptides (average residue length of 3.2) in a soybean hydrolysate were more rapidly absorbed than the long ones (average residue length of 5.2) when using a rat intestinal sac system. Therefore, Ile-Phe-Leu and Trp-Leu isolated from tofuyo are expected to be easily absorbed and contribute to the antihypertensive effect via an in vivo transport system.

Further studies are necessary and will be performed to confirm the antihypertensive effect of tofuyo and its ACE inhibitors in vivo.

References

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