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# Angiotensin I-Converting Enzyme Inhibitory Action of Fish Sauce

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**The angiotensin I-converting enzyme (ACE) inhibitory activity of fish sauce, the product with the strongest ACE inhibitory activity among liquid fermented foods, was fractionated into 2 fractions with ethanol treatment. The major part of the inhibitory substance was contained in the supernatant fraction (S-fraction) and its intraperitoneal administration had a hypotensive effect on spontaneously hypertensive rat (SHR). By octadecylsilane column chromatography and two successive kinds of gel filtration, the inhibitory activity of the S-fraction was further separated into several fractions indicating that the strong inhibitory activity of the fish sauce is caused by a combined action of various kinds of inhibitory substances present. From the fraction with the lowest 50% inhibition concentration $IC_{50}$ value, three kinds of dipeptides, glycyl-tryptophan, isoleucyl-tryptophan and valyl-tryptophan, were isolated as the ACE inhibitory compounds. Oral administration of these peptides to SHR showed a hypotensive effect.**

Keywords: Angiotensin I-converting enzyme, ACE, hypertension, fish sauce, glycyl-tryptophan, valyl-tryptophan, isoleucyl-tryptophan

Recently, the physiological function of food constituents has been attracting considerable attention as a new aspect of foods. Among them, the angiotensin I-converting enzyme (ACE, E.C. 3.4.15.1.) inhibitory activity of various kinds of food has been intensively studied in relation to the prevention of hypertension. To date, various kinds of substances such as small peptides and organic compounds have been isolated as ACE inhibitors (Suzuki *et al.*, 1983; Yokoyama *et al.*, 1992; Tsuzuki *et al.*, 1992; Saito *et al.*, 1992; Takahama *et al.*, 1992; Maruyama *et al.*, 1985; Okamoto *et al.*, 1995a). We have also investigated the ACE inhibitory action of various fermented foods and found that soy sauce and fish sauce had the strongest ACE inhibitory activity among the liquid fermented products tested. The 50% inhibition concentration ($IC_{50}$) values of both sauces were quite similar on both a liquid volume basis ($\mu$l/ml) and a dry weight basis (mg/ml) (Okamoto *et al.*, 1995b). The major part of the ACE inhibitory activity in soy sauce was reported to be attributable to a single compound with an $IC_{50}$ of 0.26 $\mu$M, nicotianamine (Kinoshita *et al.*, 1993), the most potent inhibitor that has so far been separated from foods. Therefore, it is interesting to survey the ACE inhibitory substances in fish sauce that showed almost the same $IC_{50}$ as soy sauce.

Recently, the development of ACE inhibitory peptides in the proteolytic hydrolyzates of various fish muscles was reported (Kohama *et al.*, 1988; Kawamura *et al.*, 1989; Ukeda *et al.*, 1991, 1992; Matsuda *et al.*, 1992; Yokoyama *et al.*, 1992; Seki *et al.*, 1993; Matsumoto *et al.* 1994). Fish sauce is a fermented fish food; therefore, it is assumed to contain many kinds of substances including peptides with ACE inhibitory activity that are produced during the fermentation of fish proteins. In this report, we describe the purification of ACE inhibitory peptides from a fish sauce, their structure, and the hypotensive action of the orally administrated peptides on spontaneously hypertensive rats.

## Materials and Methods

*Materials* Fish sauce made from salmon was obtained from Kaitakusha (Iwate, Japan). Hippuryl-L-histidyl-L-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan). ACE was prepared from porcine lung as previously described (Okamoto *et al.*, 1995a). YMC gel ODS-A60-230/70, Sephadex G-25 and Bio-gel P-2 were obtained from YMC Co. Ltd. (Kyoto, Japan), Pharmacia LKB Biotechnology Co., Inc. (U.S.A.) and Bio-rad Laboratories Co., Inc. (U.S.A.), respectively. TSK-gel ODS 80 TM was a product of Tosoh Co., Inc. (Tokyo, Japan). The synthetic glycyl-tryptophan, valyl-tryptophan and isoleucyl-tryptophan were purchased from Kokusan Chemical Works Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade.

*Measurement of ACE inhibitory activity* ACE inhibition was assayed principally according to the method of Cushman and Cheung (1971). ACE solution (50 $\mu$l) was preincubated with each sample (50 $\mu$l) for 5 min at 37°C, followed by the addition of 150 $\mu$l of 8.33 mM HHL (in 125 mM Tris-HCl buffer, pH 8.3 containing 1 M NaCl). The mixture was incubated for 30 min at 37°C, and then the

reaction was stopped by addition of 250 $\mu$l of 1 N HCl. The hippuric acid produced by this reaction was extracted with 1.5 ml of ethyl acetate, and 1.0 ml of the extract was dried by a heat block with gentle aeration. The extracted hippuric acid was redissolved in distilled water, and its amount was measured from the absorbance at 228 nm. The ACE inhibition ratio (%) was obtained from the following equation:

$$\text{ACE Inhibition (\%)} = \{1-(A_S-A_{B2})/(A_c-A_{B1})\}\times 100$$

$A_S$ : the absorbance value with the sample

$A_c$ : the absorbance value with water instead of the sample

$A_{B1}$: the absorbance value with inactivated enzyme and water

$A_{B2}$: the absorbance value with inactivated enzyme and the sample.

The ACE inhibitory activity was expressed as $IC_{50}$, the sample concentration that inhibits 50% of ACE activity. One ACE inhibition unit is defined as the amount of inhibitor needed to inhibit 50% of ACE activity.

*Effect of pH and temperature on ACE inhibition* The stability of ACE inhibitory activity at various pHs was measured as follows: samples (500 $\mu$l) were incubated with 0.1 M McIlvane buffer with various pHs (200 $\mu$l) for 1 h at 4°C. After adjusting the pH of the mixture to 8.3 by adding 800 $\mu$l of assay buffer, the ACE inhibition was measured as described above. The temperature stability of the ACE inhibitory activity was determined by measuring the residual activity of the samples after incubating them for 1 h at various temperatures.

*Experimental animals and measurement of blood pressure* Spontaneously hypertensive rats (SHR) were purchased from Charles River Japan, Co., Inc. The rats were bred in a room with a 12 h light-dark cycle. Temperature and humidity were controlled at 22±1°C and 60±5% RH, respectively. The diet (CRF-1, Charles River Japan, Co., Inc.) and tap water were available *ad libitum*. Systolic blood pressure (SBP) was measured by the tail-cuff method using a programmable sphygnomanometer (type PS-100, Riken Kaihatsu Co., Inc.) after warming the rat in a chamber maintained at 38°C for 15 min. For one measurement, SBP was measured 5 times successively. The significance of the differences in SBP before and after administration of the sample was analyzed using the Student's *t*-test.

*1) Intraperitoneal injection* Thirteen week old male SHR (about 300 g B.W.) were used for this experiment. The sample solution (500 $\mu$l) was intraperitoneally injected. The control rats were injected with the same volume of 3.6% NaCl in the same manner, because the sample contained 3.6% of NaCl. Six rats were used for one group.

*2) Oral administration* Forty-five week old male SHRs (about 500 g B.W.) were used in this experiment. The samples were suspended in 3 ml of 1% carboxymethylcellulose (CMC) and orally administered to rats at a dose of 200 mg/kg B.W. with a flexible sonde. The control group was given the same volume of 1% CMC solution. Five rats were used for one group.

*Chromatography*

*1) Octadecylsilane (ODS) column chromatography* The column ($\phi$5.0×40 cm) was equilibrated with water and eluted with 70% methanol after washing unadsorbed substances with water. The flow rate was set at 52 ml/h and every 15 ml of the eluate was collected.

*2) Sephadex G-25 column chromatography* The column ($\phi$2.6×96 cm) was equilibrated with 0.05 M acetic acid and eluted with the same solution. The flow rate was set at 30 ml/h and every 5 ml of the eluate was collected.

*3) Bio-gel P-2 column chromatography* The column ($\phi$1.6×90 cm) was equilibrated with 0.05 M acetic acid and eluted with the same solution. The flow rate was set at 23 ml/h and every 2 ml of the eluate was collected.

*4) High performance liquid chromatography (HPLC)* A TSK-gel ODS 80TM ($\phi$4.6×250 mm) column was equilibrated with 10% acetonitrile containing 0.1% trifluoroacetic acid, and the elution was done by increasing the acetonitrile concentration linearly from 10% to 30% within 30 min. The flow rate was set at 0.8 ml/min and every peak fraction was collected.

*Amino acid analysis and amino acid sequencing* The amino acid composition was determined with an Hitachi amino acid analyzer 8500, after hydrolyzing the sample in 6 N HCl for 24 h at 110°C in vacuo. The amino acid sequence was determined using a protein sequencer (ABI 473A).

## Results

*Fractionation of fish sauce with ethanol* Four liters of a salmon fish sauce ($IC_{50}$ of 1.6 mg/ml, $1.9\times10^7$ inhibition units) was added with 20 *l* of ethanol and stirred for 1 h at the room temperature followed by centrifugation (18,000 rpm× 20 min). The resulting precipitate and supernatant were then dried by lyophilization and evaporation, respectively. Their $IC_{50}$ was measured after dissolving them in an appropriate volume of distilled water. The supernatant fraction (S-fraction) showed an $IC_{50}$ of 1.8 mg/ml and $1.1\times10^7$ inhibition units while the precipitated fraction (P-fraction) gave an $IC_{50}$ of 3.2 mg/ml and $3.75\times10^6$ inhibition units, indicating that the S-fraction had a stronger ACE inhibitory activity than the P-fraction. The S-fraction was therefore subjected to further investigation.

*Temperature and pH stability of ACE inhibitory activities of the S-fraction* As shown in Fig. 1, the remaining ACE inhibitory activity was almost the same over a wide

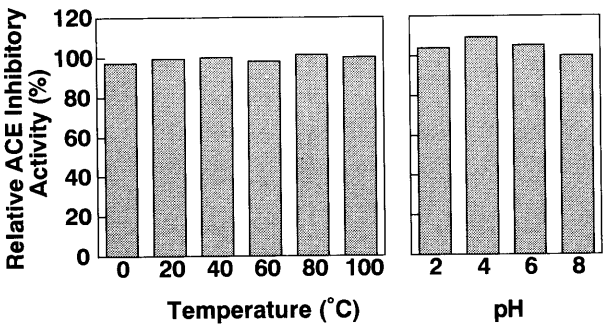


**Fig. 1.** Effect of pH and temperature on ACE inhibitory activity of S-fraction. The ordinate shows the remaining ACE inhibitory activity vs. the initial inhibitory activity as 100%. The experiment was done with the sample concentration which expressed 70% ACE inhibition after the dilution as described in Materials and Methods 3.

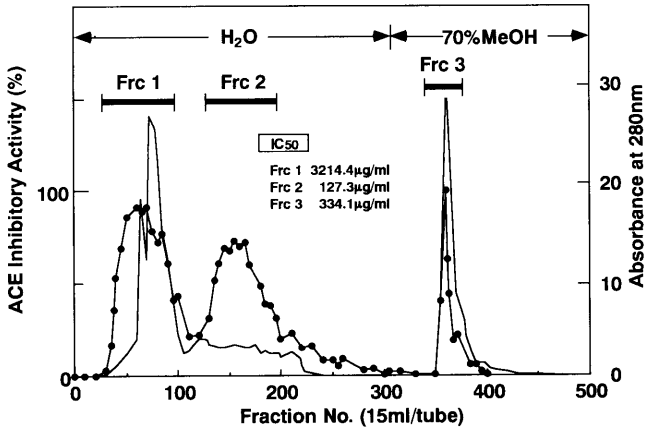


**Fig. 2.** ODS column chromatography of S-fraction. The details are described in Materials and Methods. Fifty $\mu$l of each fraction was subjected to the assay. ●——●, ACE inhibitory activity (%); ——, absorbance at 280 nm.

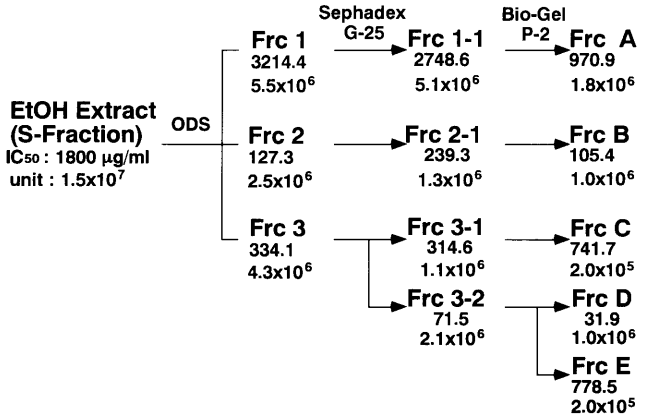


**Fig. 3.** Fractionation of ACE inhibitory substances in fish sauce.

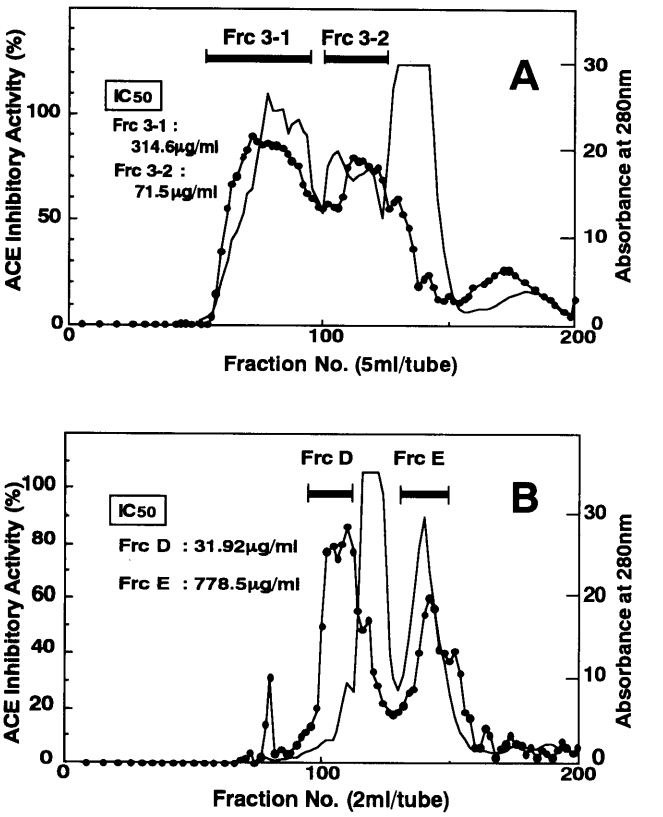


**Fig. 4.** A. Sephadex G-25 column chromatography of fraction 3. B. Bio-gel P-2 column chromatography of fraction 3-2. The symbols are the same as in Fig. 2. The details are described in Materials and Methods. Fifty $\mu$l of each fraction was subjected to the assay.

range of temperature and pH treatment, indicating that the inhibitory substances in the fish sauce S-fraction are stable under various temperature and pH conditions.

*Effect of intraperitoneal administration of S-fraction on blood pressure of SHR* The S-fraction was then administered to SHRs at a dose of 166 mg/kg B.W. Prior to administration, the SBP of SHRs was 182±4 mmHg, which decreased significantly ($p<0.01$) to 166±3 mmHg 24 h after administration. On the other hand, the SBP of the control group, to which 3.4% NaCl alone was administered, was 184±4 mmHg while that before administration was 186±5 mmHg. This difference was found statistically insignificant. These results have shown that the S-fraction of the fish sauce has a hypotensive effect *in vivo*, and in this connection, the participation of ACE inhibitory substances in causing such an effect was considered.

*Fractionation of ACE inhibitory substance of S-fraction* The S-fraction was then subjected to various chromatographies to identify the substance responsible for the hypotensive effect with ACE inhibition. The S-fraction was dissolved in distilled water and applied to an ODS column. The elution profile is shown in Fig. 2. Two major peaks with inhibitory activity were obtained from the unadsorbed fraction (Frc 1 and Frc 2), and one in the 70% methanol-eluted fraction (Frc 3). These peak fractions were pooled and lyophilized or evaporated to dryness and then subjected to sequential gel filtration on Sephadex G-25 and Bio-gel P-2. The $IC_{50}$s and units of each fraction obtained in the series of the procedures are summarized in Fig. 3. The results suggests that the strong ACE inhibitory activity of fish sauce is not caused mainly by a single compound like that in soy sauce but by the combined action of many substances. Among the fractions obtained above, fraction D showed the smallest $IC_{50}$ (31.9 $\mu$g/ml, $1.0\times10^6$ inhibitory units) and was further purified in the subsequent investigation. The elution profiles of the column chromatographies to obtain fraction D from fraction 3 of the initial ODS chromatography are shown in Fig. 4.

*Isolation of ACE inhibitory substances in fraction D* Fraction D, the former peak on Bio-gel P-2 gel filtration, was then lyophilized and subjected to HPLC with TSK-gel ODS 80 TM (Fig. 5). Three peaks with inhibitory activity were collected and were tentatively named AI-1, AI-2 and AI-3 in the order of the elution on HPLC. All of them showed a single peak on rechromatography using the same HPLC column. Their yields were 98.3 mg, 1.7 mg and 1.3 mg, respectively.

*Structural analysis of isolated ACE inhibitory substances* The characteristics of the isolated substances are summarized in Table 1. The three peaks were all ninhydrin-

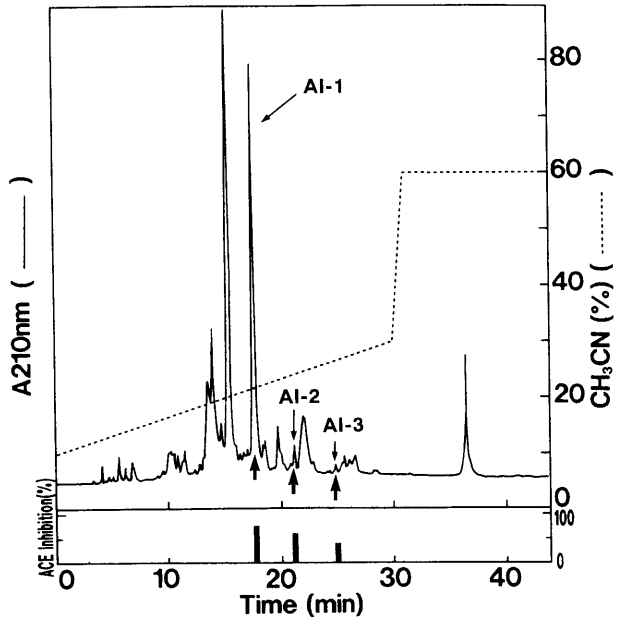


**Fig. 5.** HPLC profile of fraction D on TSK-gel ODS 80 TM. The details are described in Materials and Methods. Fifty $\mu$l of each peak fraction (about 4% of AI-1, 15% of AI-2 and 3) was subjected to the assay. The arrows show the peaks with ACE inhibitory activity.

positive and their UV absorption spectra were characteristic of tryptophan with the maximal absorption at 217 and 277 nm. They were, therefore, assumed to be peptides. Amino acid analysis gave only glycine for AI-1, glycine and valine for AI-2, and glycine and isoleucine for AI-3. From the amino acid sequence analysis, the structures of AI-1, 2, 3 were estimated to be glycyl-tryptophan (GW), valyl-tryptophan (VW) and isoleucyl-tryptophan (IW), respectively. The retention times of these substances on TSK-gel ODS 80 TM showed good agreement with the authentic peptides. Based on these data, the structures of AI-1, 2, 3 were determined to be GW, VW and IW, respectively, and their $IC_{50}$ values were determined to be 35.0 $\mu$M, 2.0 $\mu$M and 1.8 $\mu$M, respectively.

*Effect of oral administration of isolated peptides on blood pressure of SHR* The three ACE inhibitory peptides isolated from the fish sauce were orally administered to SHR to investigate their hypotensive action (Table 2). With a single dose of the peptides at 200 mg/kg B.W., the blood pressure of the treated SHR decreased with time after administration of the peptides, while the blood pressure of the control SHRs which were given only 1% CMC solution hardly decreased. The maximum depression of blood pressure occurred at 6 h, 3 h and 1 h after the administration of GW, VW, IW, respectively. After the maximum depression was attained, the blood pressure of the treated animals was restored within 24 h, indicating that the decrease was only a temporary effect of the peptides.

**Table 1.** Characteristics of the isolated peptides.

| | AI-1 | AI-2 | AI-3 |
|---|---|---|---|
| Ninhydrin reaction | positive | positive | positive |
| $\lambda_{max}$ (nm) | 273, 217 | 274, 217 | 277, 217 |
| Amino acids composition[a] | G | G, V | G, I |
| Sequential analysis 1st | G | V | I |
| 2nd | W | W | W |
| 3rd | — | — | — |

[a] This indicates the amino acid composition of the sample after hydrolysis in 6 N HCl at 110°C for 24 h in vacuo.

## Discussion

In relation to the physiological function of foods, development of peptides with anti-hypertensive action has been observed in proteolytic hydrolyzates of food proteins such as fish proteins, milk (Maruyama *et al.*, 1987) and plant proteins (Miyoshi *et al.*, 1990; Samoto *et al.*, 1990). Many kinds of fish sauces are commercially available and popular in Asian countries including Japan. Because they are made by fermentation of various kinds of fish, they are assumed to be rich in the degradation products such as small peptides and amino acids. In fact, fish sauces showed a relatively strong ACE inhibition, the degree of which was similar to that of soy sauce which is known to contain a strong ACE inhibitor, nicotianamine. In the present experiment, salmon-fish sauce was used because it showed the smallest $IC_{50}$ among the fish sauces tested.

With the progress of fractionation, the ACE inhibiting activity was recovered not just in one fraction but in many different fractions (Fig. 3), indicating the possible occurrence of various substances with the activity including peptides. Strong ACE inhibition by fish sauce may be attributable to the combined action of these products. This kind of dispersed fractionation is very common to peptide isolation from protein hydrolyzates.

Thus, the isolation of ACE inhibitor was conducted from a fraction with the lowest $IC_{50}$. Three kinds of peptides isolated were designated GW, VW and IW, all dipeptides containing tryptophan at the C-terminal position. In a detailed study of the relationship between ACE inhibition and the sequence of synthetic dipeptides, Cheung *et al.*

**Table 2.** Changes in blood pressure after administration of isolated peptides.

| | B.W. (g) | Blood pressure (mmHg) | | | | |
|---|---|---|---|---|---|---|
| | | before | after | | | |
| | | | 1 h | 3 h | 6 h | 9 h |
| Control | 473±12 | 178±5 | 190±8 | 176±4 | 174±8 | not tested |
| VW | 461±9 | 207±3 | 199±7 | 169±13* | 194±5* | not tested |
| IW | 448±5 | 193±5 | 177±4* | 179±5 | 189±10 | not tested |
| GW | 459±5 | 206±4 | 191±10 | 185±2** | 177±5* | 191±11 |

Systolic blood pressure is shown as mean±SD, $n$=5.
Significant differences from "before administration": *$p<0.05$; **$p<0.01$.

reported that tryptophan at the COOH-terminal and valine and isoleucine at the $NH_2$-terminal of dipeptide are the most favorable sequences for ACE inhibitory activity (Cheung *et al.*, 1980). Among the 3 peptides obtained in this study, two of them, VW and IW, were found to be the same peptides as described above. VW has also been isolated from sake lees, and its hypotensive action on SHR has been confirmed (Saito *et al.*, 1994a). The other dipeptide, GW, which was shown to be a considerably strong ACE inhibitor ($IC_{50}$ of 35 $\mu$M), was already isolated from sardine muscle hydrolyzate by Seki *et al.* (1993). They reported it as having a good taste, rather sweet, not bitter in spite of having tryptophan in its structure, in addition to the ACE inhibitory activity. Having both anti-hypertensive and taste-improving action, this peptide is therefore considered to be suitable as a functional food material. In any case, it is interesting to note that, among various kinds of dipeptides, characteristic peptides, VW and IW, which have the strongest ACE inhibitory action, and GW, which has a good taste in addition to its inhibitory activity, were contained in the fish sauce that is commonly used as a food. This fact is suggestive for designing foods having anti-hypertensive action.

To our knowledge, the hypotensive action of orally administrated IW and GW on SHR was first observed in our study, although a similar action of VW on SHR has already been reported by Saito *et al.* (1994a). Our results as shown in Table 2 and as reported by other laboratories (Saito *et al.*, 1994a, b) suggest a possibility that the ACE inhibitory dipeptides are absorbed from the intestine to express the hypotensive action. This consideration is also supported by the fact that dipeptides are small enough to pass through the intestine and are relatively resistant to digestive proteases.

One of the practical questions is how much of these peptides are in the fish sauces. We can estimate this very roughly to be 25 mg/*l* original sauce (sum of the 3 kinds of peptides isolated), which is much more than that of nicotianamine, a strong ACE inhibitor in soy sauce (1.3 mg/*l*) (Kinoshita *et al.*, 1993). This quantification and duration of the hypotensive effect (Table 2) suggest the probable contribution of the peptides to the hypotensive action but not parallel to the hypotensive activity assumed from the result of intraperitoneal injection of the S-fraction. There are other active fractions remaining unpurified, and among them, other functional substances must be present. Judged from the stability of the ACE inhibitory activity of the S-fraction (Fig. 1), the existence of other ACE inhibitory substances which can possibly be absorbed into a living body, other than the peptides from fraction D, is considered. The concerted action of these substances together with the peptides in fraction D may express the anti-hypertensive effect of the S-fraction. Therefore, further investigation of the active substances in the other fraction (Fig. 3) will be desirable for the elucidation of the hypotensive action of fish sauce through ACE inhibition. This study is now in the progress.

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