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Anti-hypertensive substances in fermented soybean, natto

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Abstract. Natto is a traditional Japenese fermented food made by fermenting boiled soy beans with *Bacillus natto*. Its contents of inhibitors against the angiotensin converting enzyme (ACE, EC3.4.15.1) were investigated. Relatively strong inhibitory activity (IC_{50}: 0.4 mg/ml, 11.8 inhibition units/g natto) was detected in natto extracts and the inhibitory activity observed in the viscous fraction was more potent than in the bean extract. Two groups of inhibitors in the viscous material, high and low molecular weight inhibitors, were resolved by dialysis test. The inhibitor of high molecular weight was a protein with low IC_{50} value (0.12 mg/ml). The two types of low molecular weight inhibitors were detected in ethanol extracts (IC_{50}: 0.53 mg/ml and 0.95 mg/ml) and they were found to be stable over a wide range of pH and temperature up to 100 °C. They were different in the mode of ACE inhibition. One is competitive, and the other noncompetitive against the hydrolysis of Bz-Gly-His-Leu by ACE.

Introduction

Recently, new aspects of food functions other than nutrition or taste have been drawing considerable attention. Among them are the physiological functions of some food components in relation to certain ailments. One of the representatives is the epidemiological relationship between foods and hypertension [1, 2]. Some foods are known to be effective in suppressing the development of hypertension [3], which suggests the existence of some components having medicine-like action on the regulation systems of blood pressure.

The renin-angiotensin system is considered to be a blood pressure regulation system which is apt to be affected by food components [4]. The system starts by the conversion of angiotensinogen to a pre-hypertensive hormone angiotensin I (DRVYIHPFHL) by the action of renin which is secreted by the kidney. The angiotensin I is further converted to angiotensin II (DRVYIHPF), the active form of the hormone, by the action of angiotensin converting enzyme (ACE, EC3.4.15.1). Angiotensin II raises blood pressure by acting directly to blood vessels, sympathetic nerves and adrenal glands.

Therefore, food components which inhibit ACE have the possibility to suppress hypertension by decreasing the formation of angiotensin II. In fact, oral dose of synthetic ACE inhibitor like captopril to hypertensive patients becomes the first choice for the medical treatment.

From this point of view, we investigated natto, a Japanese traditional fermented food. Natto is produced by the fermentation of boiled soy beans with *Bacillus natto*, and is characterized by the viscous material covering the beans. The relationship between natto and blood pressure was studied by Hayashi et al. [5], who observed the suppression of blood pressure of rats when fed with natto. In the present study, we detected the ACE inhibitory activity in natto, and suggested that the inhibitor's hypotensive effect is due to the interception of the renin-angiotensin system. Some information about the ACE inhibitory substances contained in natto were also mentioned.

Materials and methods

Materials. Natto was obtained from Takano Foods Co., Ibaraki, Japan. It was principally made by the fermentation of boiled soy beans with *Bacillus natto* at 40 °C for 18 hours under the highly humid condition. Highly fermented natto is a natto with strong viscous intensity (The details of the manufacturing procedure are not given by Takano Foods Co.). All the chemicals used in this experiment were the analytical grade.

Preparation of ACE. ACE was prepared from porcine lung acetone powder by the following procedure. Fresh lung was cut into small pieces after removing the bronchus and tracheas, and then roughly homogenized with 5–10 volumes of cold acetone using Wahling blendor. The homogenate was filtered with suction and the resulting residue was used for the extraction of ACE after completely removing acetone. A total of 46.4 g of acetone powder was obtained from 259.2 g of the fresh lung. The prepared acetone powder was stable when stored at −20 °C, and more than 90% of the ACE activity remained after 1 year of storage under this condition.

ACE was extracted from the acetone powder as follows: the acetone powder was suspended in 10 volumes of 125 mM Tris-HCl buffer (pH 8.3) containing 1M NaCl and homogenized with polytron type homogenizer (Kinematica mbH, Switzerland). The homogenate was stirred overnight at 4 °C, and then centrifuged (30,000 × g, 20 min). The resulting supernatant contained the ACE activity which could be completely inhibited by ethylenediamine tetraacetic acid (EDTA), but not by diisopropyl fluorophosphate (DFP), N-ethylmaleimide (NEM) and pepstatin. The cleavage products from Bz-Gly-His-Leu were only Bz-Gly and His-Leu, indicating that this activity is attributed to ACE. Therefore, the extract was used in the subsequent experiments as a ACE solution. One unit of ACE was defined as the amount of the enzyme that produce 1 μmol of Bz-Gly per minute from Bz-Gly-His-Leu under the assay condition.

Assay procedure of ACE inhibitory activity. The ACE inhibitory activity was measured principally according to the method of Cushmen et al. [6]. ACE solution (50 μl, 4 mU) was preincubated with each sample (50 μl) for 5 min at 37 °C, then the enzyme reaction was started by adding 150 μl of 8.33 mM of the substrate, Bz-Gly-His-Leu (Peptide Institute Co., Osaka, Japan) in 125 mM Tris-HCl buffer (pH 8.3), to the mixture. After incubation for 30 min at 37 °C, the reaction was stopped by adding 250 μl of 1N HCl. Bz-Gly produced by this reaction was extracted with 1.5 ml ethyl acetate, and then the mixture was centrifuged to separate the ethyl acetate layer. Exactly 1 ml of the ethyl acetate layer was transferred to another test tube and evaporated. The extracted hippuric acid was redissolved in distilled water, and its amount was measured from the absorbance at 228 nm. The ACE inhibitory activities was expressed in two ways: (1) IC_{50}; the sample concentration that inhibits 50% of ACE activity, and (2) inhibition unit; 1 inhibition unit is defined as the activity of a inhibitor which inhibits 1 unit of ACE activity under the assay condition.

Gel filtration and molecular weight estimation. Bio-Gel P-2 (Bio-Rad Laboratories Co., USA) chromatography was carried out in a column (Ø 1.0 cm × 70 cm) at a flow rate of 5.6 ml/hour. The column was eluted with distilled water, and 1 ml of the eluate was collected. The molecular weights of the inhibitors were estimated using this Bio-gel P-2 column with bacitracin (MW 1,400), oxidized glutathione (MW 600) and reduced glutathione (MW 300) as standard molecular markers.

Kinetic analysis of ACE inhibitor. The mode of inhibition by partially purified inhibitors and their Ki values were analyzed using Lineweaver-Burk plots [7]. The activity of ACE (4 mU)) on the substrate at various concentration (0.5, 1.0, 2.5, 5.0, 10.0 mM) with and without inhibitors was measured. Inhibitor concentrations used were 0, 0.3 and 0.8 mg/ml in case of Fraction 2 and 0, 0.4 and 0.8 mg/ml in case of Fraction 3.

Results and discussion

ACE inhibitory activity of natto. Ten gram of natto was homogenized with 10 volumes of distilled water and then mixed for 30 min followed by centrifugation (12,000 × g, 15 min). The supernatant (2.6 g dry weight) showed the IC_{50} value of 0.4 mg/ml (total inhibitory unit: 118 units), a relatively strong ACE inhibitory activity while the washed precipitate suspended in water showed little activity. This indicates that natto contains soluble materials that inhibit ACE. To determine which part of natto has the ACE inhibitory activity, natto (25 g) was washed with 10 volumes of distilled water with stirring for 1 hour at 4 °C and then the viscous material was separated from the beans. Two kinds of natto, namely, normally fermented and highly fermented natto, were used in this experiment. The residual beans were homogenized with 10 volumes of

distilled water and stirred for 1 hour at 4 °C followed by centrifugation (40,000 × g, 15 min). The lyophilized separated viscous material fraction (4.0 g for normally fermented and 4.5 g for highly fermented natto) and bean extract fraction (0.9 g for normally fermented and 1.5 g for highly fermented natto) were subjected to the ACE inhibition assay. Table 1 shows that both viscous material and bean extract possessed the ACE inhibitory activities. Although both parts had considerably strong IC_{50}s in both kinds of natto, the viscous material showed higher IC_{50} value and stronger total inhibition activity than the bean extract in the both kinds of natto. Therefore, the ACE inhibitory substances present in the viscous material were investigated in the subsequent experiments.

Fractionation of ACE inhibitors in viscous material. The viscous material was then extracted by both distilled water and ethanol as described in the legend of Table 2, and subjected to dialysis with a membrane with molecular cut-off of 3,500. Table 2 shows the comparison of IC_{50} of both extracts before and after dialysis. For both normally and highly fermented natto, IC_{50}s of water extracts of the viscous material decreased after dialysis, while those of ethanol extracts increased. This indicates that the ACE inhibitory substance in water extracts is of high molecular weight, and that in ethanol extracts is of low molecular weight. ACE inhibitory activity of the water extracts was higher than that of

Table 1. ACE inhibitory activities of two kinds of natto

Kind of natto	IC_{50}(mg/ml)		Total inhibition activity (unit)	
	Viscous material	Beans	Viscous material	Beans
Normally fermented	0.32	0.33	250	54.5
Highly fermented	0.27	0.51	333	60.1

Table 2. Effect of dialysis on ACE inhibition by the viscous substance

Sample	IC_{50}(mg/ml)	
	Before dialysis	After dialysis
Normally fermented		
water extract	0.32	0.15
ethanol extract	0.33	0.74
Highly fermented		
water extract	0.27	0.14
ethanol extract	0.52	1.82

Water extract and ethanol extract were prepared as follows: The viscous material was extracted with 10 volumes of both water and 80% ethanol by stirring the mixture for 1 hour at 4 °C, and then the extracts were dried up by lyophilization and evaporation, respectively. Both extracts were redissolved in 2 volumes of distilled water and dialysed against distilled water followed lyophilization.

the ethanol extracts especially in highly fermented natto both before and after dialysis.

To obtain the high molecular weight substances in the viscous material, water extracts of the material were further fractionated by precipitating with 90% saturated ammonium sulfate. To the viscous material (5200 ml) prepared from 500 g of natto with 10 volumes of water was added 3442 g of solid ammonium sulfate and the mixture was stirred for 1 hour after dissolving the ammonium sulfate completely, and then stood overnight. The resulting precipitate was collected by centrifugation (35,000 × g, 15 min) followed by dialysis (MW cut-off 10,000) against distilled water. All procedures were carried out at 4 °C. The dialysate thus obtained was lyophilized and tentatively named as Fraction 1 (33.5 g). To obtaining the low molecular weight substances, ethanol extraction of the viscous material was carried out after centrifugation (40,000 × g, 15 min) of the material (4860 ml, prepared from 500 g of natto with 10 volumes of water) because the material was turbid. The resulting supernatant and precipitate were then lyophilized (62.5 g and 11.8 g, respectively) and refluxed for 1 hour with 10 volumes of 80% ethanol. Extracts of the supernatant and the precipitate separated from the residue by filtration were dried up by rotary evaporator and named as Fraction 2 and Fraction 3, respectively. The yield of Fraction 2 was 26.5 g and that of Fraction 3 was 6.3 g. The IC_{50} of Fraction 1, 2 and 3 was 0.12, 0.53 and 0.95 mg/ml, respectively and the total inhibitory activities of them were 5561, 1020.5 and 168 units, respectively, showing the highest inhibition in Fraction 1. Although the total activities of Fraction 2 and 3 were relatively small compared with that of Fraction 1, the characterization of both fractions were carried out in the subsequent experiment since their IC_{50}s were low enough to inhibit ACE and their low molecular weight nature is favorable for practical use by oral intake.

Characterization of Fraction 1

Effect of temperature. To determine the temperature stability, Fraction 1 was incubated at various temperatures (0–100 °C) for 1 hour. The inhibitory activity of Fraction 1 was stable up to 40 °C. It almost disappeared, however, when incubated above 50 °C, indicating that this substance is not resistant to heating. This fact and its precipitability by concentrated ammonium sulfate suggest that the inhibitory substance in Fraction 1 is a proteinaceous one.

Effect of a protease inhibitor. Natto is very rich in a serine protease, subtilisin. Fraction 1 was then investigated its resistance against phenylmethanesulfonyl fluoride (PMSF), a serine protease inhibitor. PMSF solution (100 mM in ethanol) was added to Fraction 1 to give final concentrations of 0.5 and 1.0 mM, and the mixtures were incubated at 4 °C for 1 hour. The ACE inhibitory activities before and after the addition of PMSF were measured and compared. The addition of 0.5 and 1.0 mM PMSF resulted in the decrease of ACE inhibitory activity by 61.3 and 63.5 percent, respectively (these concentrations

of PMSF and ethanol did not affect ACE), however, 38.7 and 36.5 percent of the inhibitory activity still remained, respectively. These results suggest that about two thirds of ACE inhibition by this fraction may be due to the direct action of coexisting serine protease, and one third due to the inhibitors, or PMSF may have reacted with and changed the efficiency of the inhibitors.

Characterization of Fraction 2 and 3

Molecular size. Fraction 2 and 3 were subjected to gel filtration using Bio-Gel P-2 under the same condition (Fig. 1A and B). As shown in these figures, the ACE inhibitory activities of these two fractions were eluted at the different elution volumes, indicating that the ACE inhibitors in Fraction 2 and Fraction 3 were different substances. Based on the elution volumes, the molecular weights of the substances in Fraction 2 and Fraction 3 were estimated roughly to be 780 and 200 daltons, respectively (Fig. 2).

Effect of pH and temperature. The pH stability of the substances in Fraction 2 and 3 were investigated by incubating them with buffers between pH 2 to pH 10 for 1 hour at 4 °C, after which the remaining inhibitory activities were measured at the standard assay pH. The ACE inhibitory activities of these two

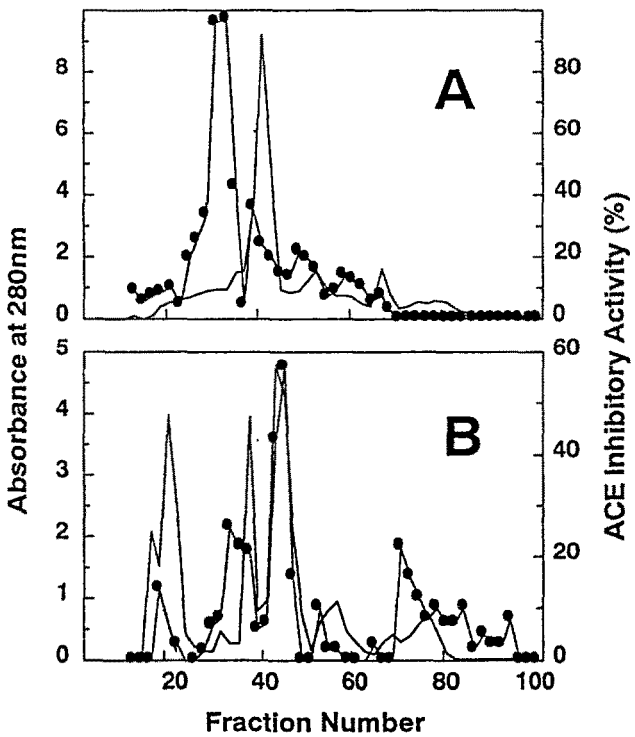

Fig. 1. Bio-Gel P-2 column chromatography of Fraction 2(A) and Fraction 3(B) —, absorbance at 280 nm; ●—●, relative ACE inhibitory activity (%)

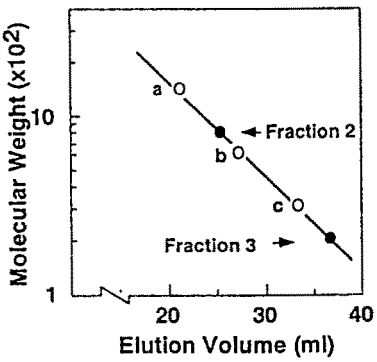

Fig. 2. Molecular weights of inhibitors in Fraction 2 and Fraction 3 (○), molecular weight standards: a, bacitracin (MW 1400); b, oxidized glutathione (MW 600); c, reduced glutathione (MW 300)

fractions were almost the same at any given pH. This indicates that the inhibitory substances in Fraction 2 and 3 were stable over a wide range of pH. The fractions were incubated at various temperatures for 1 hour, and the remaining inhibitory activities were assayed at the standard temperature. The inhibitory activities of the fractions were found to be stable between 0–100 °C.

Mode of ACE inhibition by Fraction 2 and 3. The modes of inhibition of the partially purified inhibitors were analyzed using the Lineweaver-Burk plot as shown in Fig. 3. In the case of Fraction 2, the K_m value was not altered by the change in inhibitor concentration, while the V_{max} value varied from 10 nmol/min (no inhibitor) to 2.7 nmol/min (inhibitor concentration: 0.3 mg/ml) and 1.2 nmol/min (inhibitor concentration: 0.8 mg/ml), indicating that the inhibitor in Fraction 2 is a noncompetitive inhibitor. The Ki value of this fraction was calculated from this result and the estimated molecular weight as 0.14 mM. On the other hand, the inhibitor in Fraction 3 showed a constant

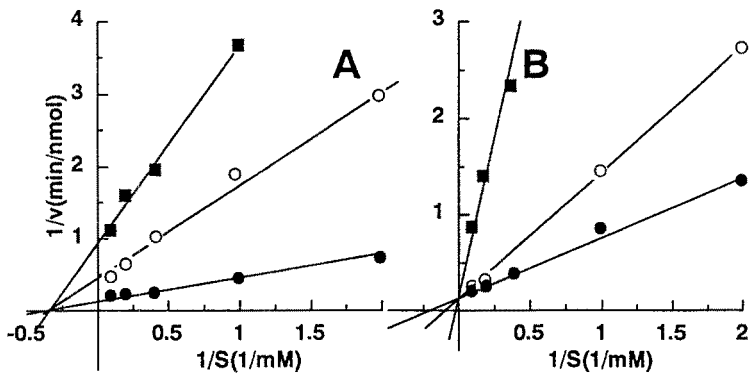

Fig. 3. Kinetic profile of ACE inhibition by the inhibitors in Fraction 2(A) and Fraction 3(B) ●—●, without the inhibitor; ○—○, with the inhibitor (0.3 mg/ml in case of Fraction 2, 0.4 mg/ml in case of Fraction 3); ■—■, with the inhibitor (0.8 mg/ml of both Fractions)

V_{max} and K_ms changed from 4.2 mM (no inhibitor) to 8.3 mM (inhibitor concentration: 0.4 mg/ml) and 50 mM (inhibitor concentration: 0.8 mg/ml). This result indicates that the inhibitor in Fraction 3 causes competitive inhibition. The Ki value of this fraction was 1.4 mM. Thus, inhibitors in Fraction 2 and Fraction 3 were shown to differ in the mode of inhibition.

Discussion

Natto is a popular traditional Japanese food made by fermentation of boiled soybean with *Bacillus natto* [8]. It has been said to be effective in preventing the so-called adult diseases such as hypertension, hypercholesteromia and arteriosclerosis.

Hayashi et al. [5] showed that the blood pressure of spontaneous hypertension rat (SHR) decreased when it was fed with natto, but did not when fed with boiled soybean. This fact suggests that a certain hypotensive material is produced in natto preparation during fermentation.

In this study, we have found the presence of the inhibitors in natto against angiotensin-converting enzyme (ACE), a key enzyme that catalyzes the production of a hypertensive peptide hormone, angiotensin II, in renin- angiotensin system, a major blood pressure regulating system. The renin-angiotensin system is closely related to food components absorbed from digestive ducts to blood circulation since this system functions in the blood stream of both lung and endothelium blood vessels. Accordingly, ACE inhibitors in natto detected in the present study may explain, at least partly, the hypotensive effects of natto feeding to SHR as reported by Hayashi.

Natto was characterized by its viscous materials covering the surface of the fermented beans. These viscous materials were secondary products of fermentation and have been reported to be complex materials mainly composed of polymer of D-glutamate and fractan [9, 10]. In this study, the viscous materials were separated from the bean part and were analyzed for ACE inhibition. Fraction 1 from the viscous materials was of high molecular weight and is supposed to be a proteinaceous one. About 60–65% of the inhibition by Fraction 1 was abolished by the addition of PMSF, a serine protease inhibitor, suggesting the possibility that this ACE inhibition was largely due to the action of a protease, subtilisin, produced by *Bacillus natto.* However, the remaining 35–40% of the inhibitory activity was insensitive to further PMSF treatment. The fact that the inhibition ratio changed only slightly with the change of the concentration of PMSF suggests that the effect of the protease is completely removed by the addition of PMSF and that the remaining ACE inhibitory activity is due to ACE inhibitor other than subtilisin. Therefore, the real inhibition unit of Fraction 1 is calculated to be about 2200 units. Taking these facts into consideration, the contributions of Fraction 1, 2 and 3 to the whole ACE inhibitory activity in natto are about 64.9%, 30.1% and 5.0%, respectively, indicating that Fraction 1 possesses the highest inhibitory activity. To date, there are very few reports about high

molecular weight ACE inhibitor. The detailed study of this substance is currently under way.

The inhibitory substances in Fraction 2 and 3 detected in the ethanol extracts of the viscous material were of low molecular weight. They are judged to be different from one another from their estimated molecular weights and their modes of inhibition. To express any hypotensive effect in the living body, ACE inhibitory substances must be absorbed into the blood circulation system from the digestive organs after being eaten. Their stability over wide range of pH and temperature and their low molecular weight nature are favorable in this point, therefore, not only Fraction 2 but also Fraction 3 can not be ignored although the proportions of them to the whole ACE inhibitory activity in natto were relatively low especially in Fraction 3. In addition, the fact that natto contains as little amount of sodium chloride as does Tofu, another traditional soybean food, makes it favorable to hypertensive patient, too. Further purification of the ACE inhibitors necessary for the structural analysis and the clinical test is now in progress.

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References

1. Smith-Vaniz GT (1970) Diet-induced hypertension and cardiovascular lesions in mice. Yale J Bio Med 43: 61–70.
2. Wynn A (1987) Inequalities in nutrition. Nutr Health 5: 79–94.
3. Nestle PJ (1989) Current strategies for atherosclerosis and lowering cholesterol. Clin Exp Hypertens [A]11: 915–925.
4. Kim S, Yamamoto K (1992) The in vivo role of renin-angiotensin system. Cell Science 8: 146–151.
5. Hayashi U, Nagao K, Tosa Y, Yoshioka Y (1977) Natto no Eiyoka ni Kansuru Jikkenteki Kenkyu. Natto Kagaku Kenkyu Kaishi 1: 85–93.
6. Cushman DW, Cheng HS (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochemical Pharmacol 20: 1637–1648.
7. Lineweaver H, Burk D (1934) Determination of enzyme dissociation constants. J Am Chem Soc 56: 658–666.
8. Ohkubo K (1992) Natto. In: Yamauchi F, Ohkubo K, eds. Daizu no Kagaku. Tokyo Asakurashoten, pp 117–123.
9. Fujii H (1963) On the formation of mucilage by *Bacillus natto*, Part III: Chemical constituents of mucilage in natto (1). Nippon Nogei Kagaku Kaishi 37: 407–411.
10. Fujii H (1963) On the formation of mucilage by *Bacillus natto*, Part IV: Chemical constituents of mucilage in natto (2). Nippon Nogei Kagaku Kaishi 37: 474–477.