Note

Antihypertensive Effects of Natto, a Traditional Japanese Fermented Food, in Spontaneously Hypertensive Rats

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Natto is a popular traditional Japanese food made by fermenting steamed soybeans with *Bacillus subtilis* natto. It has long been thought that natto is effective in preventing hypertension. But there have not been any reports demonstrating in vivo antihypertensive effects of natto. In this study, we demonstrate for the first time the antihypertensive activity of natto in vivo. We determined optimal conditions for producing natto with the highest angiotensin I converting enzyme (ACE) inhibitory activity. An ACE inhibitor partially purified from natto was orally administered as a single dose (1 mg, 10 mg, and 100 mg/kg body weight) in spontaneously hypertensive rats, and then their blood pressure was measured five times every hour after administration. The administration of the inhibitor, even at the lowest dose, resulted in a significant decrease in blood pressure 4 h after administration. Thus, the ACE inhibitor from natto appears to moderately reduce blood pressure relative to increases in dosage.

Keywords: natto, soybean, angiotensin I converting enzyme, *Bacillus subtilis* natto, hypertension, spontaneously hypertensive rats

Introduction

Angiotensin I converting enzyme (EC 3.4.15.1; ACE), the key enzyme in the renin-angiotensin system, is associated with the regulation of blood pressure. ACE increases blood pressure by both converting the inactive angiotensin I to the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. ACE inhibitors from various foods have been studied in terms of their ability to prevent and alleviate hypertension.

It has long been thought that eating natto is useful for preventing an increase in blood pressure. Several reports on in vitro ACE inhibitory activity in natto has been presented (Okamoto *et al.*, 1995a, 1995b), but there have been no re-

ports demonstrating in vivo antihypertensive activity of natto. The objective of our study was to determine whether the ACE inhibitor in natto has antihypertensive activity in vivo.

We (Ibe *et al.*, 2006) previously demonstrated that ACE inhibitory activity of natto varied depending on the fermentation time and type of bacteria strain (*Bacillus subtilis* natto). In this study, we produced natto under the optimal conditions for producing the highest ACE inhibitory activity and partially purified the ACE inhibitor from natto. We fed spontaneously hypertensive rats (SHR) the crude ACE inhibitor in a single dose and examined whether the blood pressure decreased relative to the dosage.

Materials and Methods

Small-sized soybeans, cultivar Kosuzu, yielded in Iwate Prefecture were soaked at room temperature for 16 h, and

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then steamed at 126°C for 16 min with an autoclave. The steamed soybeans were inoculated with *B. subtilis* natto O9516 at approximately 10^4 cells/g. Our previous study on the ACE inhibitory activities of natto produced with various isolates of *B. subtilis* natto found that *B. subtilis* natto O9516 isolated from straw in Okinawa, Japan, was one of the most potential isolates (Ibe *et al.*, 2006). The inoculated soybeans were packed in polypropylene containers, and fermented in an incubator at 39°C for 20 h. After fermentation, the natto was left at 4°C for 24 h, then lyophilized, ground, and stored at -20°C prior to purification.

To determine an appropriate extracting solvent for partial purification of the ACE inhibitor of natto, the ground sample was suspended in 50 volumes each of 99.8% methanol, 80% methanol, 99.5% ethanol, 80% ethanol and distilled water (DW) and stirred for 1 h at room temperature. After each extract was centrifuged ($9100 \times g$, 10 min), the supernatant was evaporated and lyophilized. Based on the ACE inhibitory activity results of each extract, 80% methanol was used as the extracting solvent for partial purification of the ACE inhibitor of natto.

The sample extracted with 80% methanol was dissolved in water and applied to a column (ϕ 1.6 cm $\times$ 93 cm) for gel filtration chromatography (Sephadex G-15, 1,500 Da exclusion limit; GE Healthcare UK Ltd., England) at a flow rate of 20 mL/h. The column was eluted with DW, and each 2-mL eluted fraction was collected. The molecular weights of the inhibitors were estimated using the same Sephadex G-15 column with oxidized glutathione (MW 613), leucine enkephalin (MW 555) and reduced glutathione (MW 307) as standard molecular markers. The fraction with the highest ACE inhibitory activity was lyophilized for use for administration to SHR.

The ACE inhibitory activity was assayed according to the modified method of Lieberman (Yamamoto *et al.*, 1980). For each assay, 50 μ L of 0.1 M borate buffer (pH 8.4), 10 μ L of 4 M NaCl, 10 μ L of DW, 10 μ L of 50 mM Bz-Gly-His-Leu (Peptide Institute, Inc., Osaka) and 10 μ L sample solution were incubated with 10 μ L of 1.7 mU ACE (Sigma Chemical Co., USA) for 45 min at 37°C. The reaction was stopped

by adding 100 μ L of 0.5 M HCl. The liberated hippuric acid was extracted with 600 μ L ethyl acetate, and 400 μ L of the resulting extract was then dried with a centrifugal concentrator. The precipitate was dissolved in 1.2 mL of 1 M NaCl, and the absorbance at 228 nm was then measured. One unit of inhibitory activity is defined as the amount of inhibitor needed to inhibit 50% of the ACE activity (IC_{50} value).

Male SHR (26 weeks old; approx. 350 g; Sankyo Labo Service Co., Tokyo), were housed in a temperature-controlled room (22°C) under a 12-h light-dark cycle and had free access to tap water and a commercial diet (Oriental Yeast Co., Ltd., Tokyo). This study was conducted under the animal care guidelines established by the animal study committee of Tokyo University of Agriculture. A single oral administration of the test sample dissolved in DW was fed to rats (6 rats/group) at concentrations of 1 mg/kg, 10 mg/kg and 100 mg/kg body weight (bw), respectively. Control rats received an equal volume of DW. The tail systolic blood pressure was measured for 5 h after administration by the tail cuff method, using a blood pressure monitor (MK-2000; Muromachi Kikai Co., Ltd., Tokyo). The means of data were compared by the Student's t-test.

Results and Discussion

Table 1 shows extract yield, IC_{50} , and total inhibitory activity available from each extraction of 1 g lyophilized natto ($n = 2$). Because of its high level of both IC_{50} and total inhibitory activity, 80% methanol was adopted as the extracting solvent in this experiment.

Figure 1 shows the elution curve of gel filtration chromatography. There were two peaks of ACE inhibitory activity (peaks 1 and 2) and the molecular weights of two substances corresponding to the peaks were estimated to be about 592 and 507, respectively. As the peak 1 fraction showed remarkable ACE inhibitory activity, it was collected, lyophilized and administered to SHR.

The values of IC_{50} and total inhibitory activity in lyophilized natto (100 g) were 0.27 mg/mL and 3.7×10^5 units, respectively; in 80% methanol extract, they were 0.078 mg/mL and 2.5×10^5 units. After gel filtration, the active fraction

Table 1. ACE inhibitory activities of natto extract.

Solvent	IC_{50} (mg/mL)	Extract from 1g natto (mg)	Total activity (unit)
99.8% Methanol	0.235	330	1404
80.0% Methanol	0.07	210	3000
99.5% Ethanol	not detected	260	—
80.0% Ethanol	0.08	190	2375
DW	0.15	520	3470

$n = 2$

Fig. 1. Sephadex G-15 column chromatography of natto extract.

The sample (2 mL) containing 0.2 g of 80% methanol extract was applied on the column (1.6 × 93 cm) that had been equilibrated and eluted with distilled water. The flow rate was 20 ml/h, and 2-ml fractions were collected. The standard markers were oxidized glutathione (MW 613), leucine enkephalin (MW 555) and reduced glutathione (MW 307). ----, absorbance at 254 nm; ●, ACE inhibitory activity (%).

Fig. 2. Effect of partially purified ACE inhibitor of natto on changes in the blood pressure of SHR. A single oral administration of the test sample dissolved in distilled water was given to rats at concentrations of 1 mg/kg (A), 10 mg/kg (B) and 100 mg/kg (C) body weight. Control rats received an equal volume of distilled water. The change of systolic blood pressure was measured for 5 h. Data are expressed as the mean ± SE ($n = 6$).

*Significant difference from the initial blood pressure ($p < 0.05$). ●, control; ○, ACE inhibitor from natto.

contained 0.023 mg/mL IC_{50} and 0.8×10^5 units.

Figure 2 shows changes in the blood pressure of SHR for 5 h after a single oral administration of the inhibitor (peak 1 fraction). Blood pressure was significantly decreased within 4 h after administration of the inhibitor at a dosage of 1 mg/kg bw and from 3 h to 5 h at a dosage of 10 mg/kg bw, indicating that oral administration of the ACE inhibitor of the natto extract moderately reduced the blood pressure of SHR relative to increases in dosage.

Kinoshita *et al.* (1993) showed that the ACE inhibitor of soy sauce was nicotianamine derived from soy beans. In addition, it was recently shown that the strength of ACE inhibition of beans is correlated to the amount of nicotianamine content (Izawa *et al.*, 2008). Kuba *et al.* (2003) isolated two ACE inhibitors from the fermented soybean food tofuyo, and

Shin *et al.* isolated an ACE inhibitor derived from Korean soybean paste that significantly decreased ACE activity in the aorta, leading to lowered systolic blood pressure in SHR (Shin *et al.*, 1995 and 2001). Like these previous reports on ACE inhibitors isolated and identified in fermented soybean foods, the ACE inhibitor reported in this study also has a low molecular weight.

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