Note

**Effects of Angiotensin I-Converting Enzyme Inhibitor from
Ashitaba (*Angelica keiskei*) on Blood Pressure
of Spontaneously Hypertensive Rats**

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Summary The inhibitory activity of angiotensin I-converting enzyme (ACE) was extracted with 80% ethanol from the leaves of Ashitaba (*Angelica keiskei*). The present ACE inhibitor was fractionated and separated with various chromatographies. The antihypertensive effects of the sample (G fraction) from Ashitaba on spontaneously hypertensive rats (SHR) were observed by long-term administration for 10 wk. Another sample (S fraction) from Ashitaba also had antihypertensive effects after a single intravenous administration to SHR. The sample was further purified by using several chromatographies. The ACE inhibitor fraction was characterized as follows: no significant absorbance, a zwitterion, a water-soluble substance and a positive ninhydrin reaction. According to a mass spectrum analysis, the molecular weight of the ACE inhibitor was determined to be 303 and Na-salt ions of carboxyl groups were detected. The ACE inhibitor from Ashitaba contained in the anti-hypertensive fraction was speculated to be very similar to authentic nicotianamine based on a comparative study of inhibitory activity, mass spectrum analysis and thin-layer chromatographies.

Key Words ACE inhibitor, Ashitaba (*Angelica keiskei*), SHR, blood pressure, antihypertensive factor

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Hypertension may induce cerebral apoplexy and cardiovascular disease. Over 90% of the hypertension cases are diagnosed as essential hypertension. The renin-angiotensin (R-A) system plays an important role in essential hypertension. Angiotensinogen is cleaved by an aspartyl proteinase renin (EC 3.4.23.15) to release decapeptide angiotensin I (AI). Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) cleaves dipeptides from the free COOH-terminal of AI and produces angiotensin II (AII), which has some physiological actions, vasoconstrictive and aldosterone secretory (1). Therefore, the inhibition of ACE can reduce blood pressure. ACE inhibitors such as captopril and enalapril have been used as antihypertensive drugs (2).

In recent years, many ACE inhibitory peptides from food protein sources have been isolated and reported, such as casein (3), tuna (4) and bonito (5). There have been a few reports about non-peptidyl ACE inhibitors, polyphenols (6) and nicotianamine (7). Ashitaba (*Angelica keiskei*) is a well-known vegetable and a wild plant that grows mainly on the Izu Islands, Japan (8). It contains highly nutritional components and is speculated to have various physiological effects on health (9, 10).

In this paper, the effects of the ACE inhibitor from Ashitaba on the blood pressure of SHR was studied, and the ACE inhibitor was purified and characterized.

Materials and methods

Materials. Ashitaba was obtained from Saitama Prefecture and Hachijyoujima Island Agricultural Corp. ACE from rabbit lung acetone powder was purchased from Sigma Chemical Co. (U.S.A.). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was from the Peptide Institute (Osaka, Japan). TLC (silica gel 60 F₂₅₄) was from Merck Co. Dowex 50W-X8 and Dowex 1-X4 were obtained from Dow Chemical Co. (U.S.A.). Sephadex G-10 and G-25 were from Pharmacia Fine Chemicals. The Asahipak ODP-50 column was from Asahi Chemical Industry (Tokyo, Japan). CE-2 was purchased from CLEA Japan, Inc. Other chemicals were from Wako Co. (Japan). Authentic nicotianamine was kindly supplied by Kikkoman Co. (Japan).

Assay method of ACE inhibitory activity. The activity of the ACE inhibitor was assayed by the modified method of Cushman and Cheung (11). The reaction mixture was as follows: 500 μ L of 7 mM Hip-His-Leu in 200 mM borate buffer (pH 8.3), 400 μ L of 2.0 M NaCl, 40 μ L of distilled water and 30 μ L of inhibitor. ACE activity was evaluated with 153.6 mU/mL. After incubation at 37°C for 30 min, the reaction was stopped by adding 500 μ L of 1 M HCl. The hippuric acid released by the action of ACE was extracted with 3 mL of ethyl acetate. After centrifugation for 15 min at 3,000 rpm, 2 mL of the upper layer was dried for 3 h at 60°C in vacuo by a speed back concentrator (TAITEC, VC-960). The residue was dissolved in 1 mL of distilled water, and the absorbance was measured at 228 nm. The inhibitory activity was calculated by the following formula: Residual activity (%) = (Sample - Blank)/(Control - Blank) $\times$ 100; Inhibitory activity (%) = 100 - Residual activity. One unit of ACE activity is defined as the amount catalyzing the formation of 1 μ mol

of hippuric acid from Hip-His-Leu in 1 min at 37°C. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value under those conditions.

The sample preparation for long-term and single administration. Inhibitory activity was observed in the leaves but not the stems of Ashitaba, and the ACE inhibitor could be extracted with 80% ethanol but not with 100% ethanol. Approximately 7 kg of lyophilized Ashitaba leaves was homogenized and extracted twice with 10 volumes of 80% ethanol. After treatment with activated carbon and ether, ACE inhibitory activity was purified by ion exchange chromatographies on Amberlite IR120-B (column was previously prepared as H^+ type and the ACE inhibitor was eluted with 2 N NH_4OH), Dowex 50W-X8 (column was previously prepared as H^+ type and the ACE inhibitor was eluted with 0.5 M NH_4OH), Dowex 1-X4 (column was previously prepared as CH_3COO^- type and the ACE inhibitor was eluted with 0.05 M CH_3COOH), and gel filtrations on Sephadex G-10 and G-25. This ACE inhibitor fraction was termed the G fraction (IC_{50} value = 4.1 $\mu g/mL$) for long-term administration.

The G fraction was further purified by silica gel chromatography with a mixture of *n*-propanol–ammonia solution (3:1, v/v). The ACE inhibitory fraction (IC_{50} value = 2.7 $\mu g/mL$) was obtained and called the S fraction for single administration to SHR.

Experimental animals and measurement of blood pressure.

1. *Long-term administration:* Male spontaneously hypertensive rats (SHR) were purchased from Charles River Japan, Inc. Nineteen male SHR were divided into three groups at 5 wk of age. The G fraction (IC_{50} = 4.1 $\mu g/mL$) was mixed with drinking water at 21.8 mg/kg/d, and captopril was used at 0.3 mg/kg/d. The diet (CE-2) was given ad libitum for 10 wk. Rats were bred in a room with the temperature set at $21 \pm 2^\circ C$, humidity at $55 \pm 10\%$ and lighting for 12 h daily (light from 06:00 to 18:00). During the experimental period, blood pressure was measured once a week, and body weight, food intake and drinking volume were measured every day. Systolic blood pressure was measured without anesthesia by the tail-cuff method (PB98A, Softron).

2. *Single administration:* A male SHR, weighing 293 g, was anesthetized by an intraperitoneal injection of nembutal. Polyethylene cannulas were inserted into the femoral artery to monitor blood pressure and into the jugular vein to inject the test agents. The arterial blood pressure was measured by a pressure transducer connected to the arterial cannula. The S fraction (IC_{50} = 2.7 $\mu g/mL$, 5.1 and 13.1 mg/kg) and captopril (44.4 $\mu g/kg$) were dissolved in physiological saline and injected into the carotid vein.

Statistical analysis. The experimental data are shown as the mean $\pm$ standard deviation. After the variance of values to be compared was assayed, the results were analyzed by Student's *t*-test or Welch's *t*-test to determine the significance of difference. $p < 0.05$ was defined as being significant.

Fig. 1. Effects of long-term administration of the G fraction and captopril on the blood pressure (A) and body weight (B) of SHR. ■, control group; ●, G fraction-treated group ($IC_{50}=4.1 \mu\text{g/mL}$, 21.8 mg/kg/d); ○, captopril-treated group ($IC_{50}=0.1 \mu\text{g/mL}$, 0.3 mg/kg/d). The values are expressed as the mean $\pm$ SD. The vertical bars indicate the SEM. *1, $p < 0.05$ for differences between groups' control and the G fraction; *2, $p < 0.05$ for differences between groups' control and captopril.

Results and discussion

Effects of long-term administration of the G fraction from Ashitaba and captopril on SHR. The G fraction (IC_{50} value = $4.1 \mu\text{g/mL}$), which was used for long-term experiments, was prepared as described in Materials and Methods and contained high ACE inhibitory activity from Ashitaba. Figure 1 shows the effects of the G fraction (21.8 mg/kg/d) and captopril (0.3 mg/d/kg) on the blood pressure and body weight of SHR. The blood pressure of the G fraction-treated group was $200 \pm 7.3 \text{ mmHg}$ ($n=7$) at the end of 10 wk of administration. The blood pressure of the control group was $211 \pm 3.7 \text{ mmHg}$ ($n=7$), and that of the captopril-treated group was $198 \pm 8.1 \text{ mmHg}$ ($n=5$). The blood pressure of the G fraction-treated group was significantly lower than that of the control group from the eleventh to fifteenth weeks, and that of the captopril treated-group was also significantly lower than that of the control group ($p < 0.05$) from the twelfth to fifteenth weeks.

Averages of body weight did not show any difference in these groups. Averages of food intake and water consumed, ratios of organ weights, and serum lipid levels

Fig. 2. Effects of a single administration of the S fraction on the blood pressure of SHR. The S fraction ($IC_{50}=2.7 \mu\text{g/mL}$) and captopril were given intravenously. (A) S fraction (5.1 mg/kg); (B) S fraction (13.1 mg/kg); (C) captopril (44.4 $\mu\text{g/kg}$).

of SHR also indicated no difference among them (data are not shown).

Effects of single administration of the S fraction from Ashitaba and captopril on SHR. The S fraction (IC_{50} value = $2.7 \mu\text{g/mL}$) was used for single administration. It was obtained as described in Materials and Methods, and contained a high concentration of ACE inhibitor from Ashitaba. Figure 2 shows the time course of the effects of a single intravenous injection of S fraction on the blood pressure of SHR, 11 wk of age. The blood pressure was monitored from the artery with a blood pressure transducer connected to a recorder. This figure showed that the S fraction decreased blood pressure and dose-dependent activity (A, 5.1 mg/kg; and B, 13.1 mg/kg).

Purification and characterization of the ACE inhibitor from Ashitaba leaves. The S fraction was further purified by thin-layer chromatography (TLC) and reverse-phase high-performance liquid chromatography (Hitachi HPLC system). After development by TLC with a mixture of *n*-propanol–29% ammonia solution (7:3, v/v), some fractions were carefully scraped loose with a narrow metal spatula and then extracted with 65% ethanol–1% ammonia solution. ACE inhibitory activity was detected in only one fraction under cystine (data are not shown). The ACE inhibitor was then applied to an ODP-50 column ($0.6 \times 25 \text{ cm}$). It was eluted with a linear gradient from 0 to 95% acetonitrile in 0.05% trifluoroacetic acid (TFA). ACE inhibitory activity passed through the column. The final ACE inhibitor from Ashitaba was purified about 300-fold.

The mass spectrum of the final preparation was measured on a Kompact MALDI-4 (Shimadzu Co., Ltd.). 2,5-Dihydroxybenzoic acid (DHBA) was used as the matrix. The mass spectrum indicated ion peaks $[M+H]^+$ (m/z 304), $[M+Na]^+$ (m/z 326) and $[M+2Na]^+$ (m/z 348). Nicotianamine from tobacco leaves (12) reported to be a molecular weight of 303, and nicotianamine from Japanese soy

saucos (7) was also reported to have ACE inhibitory activity. Nicotianamine has carboxylic groups. The present mass spectrum shows those two Na-salt forms. It was suggested that the ACE inhibitory fraction from *Ashitaba* contained nicotianamine.

The final ACE inhibitor was investigated by TLC with solvents of *n*-propanol-pyridine-acetic acid-water (2:1:1:1, v/v) and *n*-propanol-29% ammonia solution (7:3, v/v). Both locations of the ACE inhibitor and nicotianamine coincided as shown in Fig. 3, I and II. Another TLC with a solvent of butanol-methanol-water (2:1:1, v/v) was done and ACE inhibitory activity assayed (Fig. 3III). The spots of the ACE inhibitor and nicotianamine indicated the same location (Fig. 3III, left) and had ACE inhibitory activity (Fig. 3III, right). These results suggest that the ACE inhibitor from *Ashitaba* was very similar to nicotianamine. The IC_{50} value of the final purified ACE inhibitor was estimated to be about 1.1 $\mu\text{g/mL}$, which was the same value as that of nicotianamine presented by Kinoshita et al (7).

Kinoshita et al (Kikkoman Co., Ltd.) have reported, the ACE inhibitory activity of nicotianamine and the effect of a single oral administration in SHR (7). We also investigated the effects of the ACE inhibitory fraction from *Ashitaba* on hypertensive rats over long-term administration. These effects further confirmed the experiment of a single oral administration to SHR. Nicotianamine was reported by Noma and Noguchi (12) from tobacco leaves and by Kristensen and Larsen (13) from seeds. Budesinsky et al (14, 15) have reported the presence of nicotianamine in Leguminosae and isolated it from the aerial parts of alfalfa (*Medicago sativa* L). Nicotianamine has an optimal molecular structure for chelating irons, and was considered to be a possible phytosiderophore with an essential function in cellular iron transport and metabolism. Nicotianamine might be a representative inhibitor of ACE because of a powerful chelating action. In addition, the 2-carboxylic acid present in the structure of nicotianamine must be rendered for ACE inhibition. There is one patent application claiming production by a microorganism (*Basidiobolus* sp) (16, 17).

In recent years, several ACE inhibitory peptides were isolated and identified from food materials (2-4), and some inhibitors of this enzyme showed antihypertensive effects in vivo (18-23). However, most of them were peptides of enzymatic hydrolysates from food protein sources. Non-peptidyl ACE inhibitory substances have been reported (9, 24, 25). The present ACE inhibitor from *Ashitaba* may also be a non-peptidyl molecule.

Ashitaba has been said to have a good effect on human health. It contains dietary fiber, potassium, etc., and must have great efficacy in blood pressure in a living body. In this study, we found that the ACE inhibitor from *Ashitaba* contributed to the control of blood pressure. We need to conduct more experiments to clarify the detailed mechanism of the effects in vivo.

Fig. 3. Thin-layer chromatograms of the ACE inhibitor on silica-gel. Three kinds of developing solvents were used as follows: I, *n*-propanol-ammonia solution (7:3, v/v); II, *n*-propanol-pyridine-acetic acid-water (2:1:1:1, v/v); and III, butanol-methanol-water (2:1:1, v/v). In III, ACE inhibitory activity is shown on the right side. The ACE inhibitors from Ashitaba, nicotianamine and cystine were detected by spraying 0.2% ninhydrin-butanol. C, cystine; N, nicotianamine; A, ACE inhibitor from Ashitaba.

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