Note

Purification and Identification of Angiotensin I-Converting Enzyme Inhibitor from Morokheiya (*Corchorus olitorius*)

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In order to investigate another function such as food in Morokheiya, the angiotensin-converting enzyme (ACE) inhibitor was extracted with 80% ethanol from the leaves of Morokheiya (*Corchorus olitorius*). The extract was dried, dissolved in water, defatted with ether and decolorized with activated carbon. The ACE inhibitor was purified by successive ion-exchange chromatography, Amberlite IR-120B, Dowex 50W-X8 and Dowex 1-X4, respectively. The ACE inhibitor was further purified by silica gel column chromatography and finally purified and isolated by high performance liquid chromatography (HPLC) on Asahipak NH2P-50. The ACE inhibitor showed a positive ninhydrin reaction and no significant absorbance. The present ACE inhibitor was identified as Nicotianamine based on the comparative study using amino acid analyzer, TLC and capillary electrophoresis.

Keywords: ACE inhibitor, amino acid, nikotianamine

Life-style related diseases, such as obesity, diabetes mellitus, hypertension and cardiovascular disease, have been recently increasing. In each population, deaths due to heart disease or vascular disease have been the number 2 and 3 killers. However, their total is greater than that of the number 1 cause of death, cancer. Especially, hypertension is a most important disease because it may induce cerebral apoplexy and cardiovascular disease, moreover, obesity can cause hypertension. Over 90% of the hypertension cases is called essential hypertension. About 2/3 of the essential hypertension is related to renin-angiotensin systems (RA system). This system is well understood (Dzau, 1988) as follows: renin from the kidney hydrolyzes angiotensinogen, the prehormone of the hypertensive peptide, from the liver. Angiotensin I is produced and converted to angiotensin II, a hypertensive peptide, by the angiotensin-converting enzyme (ACE). ACE participates in the control of blood pressure, and its inhibitors such as captopril and enalapril have been used as antihypertensive drugs. In recent years, a number of ACE inhibitors have been isolated from various food protein sources (Murayama & Suzuki, 1982; Kohama *et al.*, 1988; Ukeda *et al.*, 1991), and some of them have an effect on the decrease in blood pressure of spontaneously hypertensive rats (SHR). Morokheiya is a well-known vegetable which contains high nutritional components and is speculated to have good effects on health. We found ACE inhibitory activity in the extract from Morokheiya. In this paper, we present the isolation and identification of the ACE inhibitor from Morokheiya leaves.

Materials Morokheiya was obtained from the Hanyuu Agricultural Corp., Saitama Prefecture. ACE was purchased from the Sigma Chemical Co. (St. Louis, MO). Hip-his-leu

was from the Peptide Institute (Osaka). The TLC plate (silica gel 60F₂₅₄) was obtained from the Merck Co. (Frankfurt, Germany). Other chemicals were purchased from Wako Pure Chemicals (Osaka).

Assay for ACE inhibitory activity ACE inhibitory activity was assayed by a modification of the method of Cushman and Cheung (1971). The reaction mixture contained 500 μ l of 7 mM hip-his-leu in 200 mM of borate buffer, pH 8.3, 400 μ l of 2 M NaCl, 40 μ l of distilled water and 30 μ l of inhibitor sample or H₂O. After 30 min at 37°C, the reaction was stopped by adding 500 μ l of 1 N HCl, and the hippuric acid was extracted with ethylacetate. A 2.0 ml aliquot of the extract was evaporated to dryness and the residue was dissolved in 1.0 ml of H₂O. The hippuric acid concentration was determined from the absorbance at 228 nm. Inhibitory activity was calculated as follows: Residual activity=(sample-blank)/(control-blank) $\times$ 100, Inhibitory activity=100-residual activity.

Purification of ACE inhibitor For the first step, 80% ethanol was used as the extraction solvent because an aqueous solvent was jellied by the Morokheiya contents. The extract was treated by activated carbon for decoloration and ether for defatting. ACE inhibitory activity was separated with Amberlite IR-120B. The ACE inhibitor was applied to the Dowex 1-X4 column (32 ϕ $\times$ 835 mm) previously activated as the acetate type. The active fractions were collected, evaporated to dryness and dissolved in a pyridine-acetate mixture, pH 3.1. The ACE inhibitor was applied to a Dowex 50W-X8 column (32 ϕ $\times$ 840 mm) previously equilibrated with a 0.2 M pyridine-acetate mixture, pH 3.1, and eluted by a linear gradient of pH 3.1 to pH 5.0 with the pyridine-acetate buffer.

Active fractions were collected, evaporated to dryness and dissolved in acetate. The ACE inhibitor was applied to the second Dowex 1-X4 column ($32\phi \times 840$ mm) and eluted with a linear gradient of 0.01 to 2 N acetate. However, the second Dowex 1-X4 column was not as efficient as expected. The ACE inhibitor was further purified on a silica gel column ($32\phi \times 460$ mm), previously equilibrated with a mixture of propanol and ammonia (7.5: 2.5). Active fractions were separated and retarded after high ninhydrin positive fractions. The ACE inhibitor was rechromatographed on the silica gel column. The active fractions were obtained as before. The ACE inhibitor was then gel-filtrated on the Sephadex G-25 column ($22\phi \times 980$ mm), previously equilibrated with deionized water to separate by the differences in molecular weights. These results are not shown, but the molecular weight of the ACE inhibitor was estimated to be about 300, judging from the authentic substance, alanine, glutathione, and oxidized glutathione. The ACE inhibitor was finally purified by high performance liquid chromatography (HPLC) on an Asahi-pak NH2P column as shown in Fig. 1. The ACE inhibitory activity was eluted with a linear gradient of 62% acetonitrile in water to 50% acetonitrile in 0.2 N NH_4OH . The active fraction did not have a strong absorbance at 220 nm. This phenomenon suggested that the molecular weight of the present ACE inhibitor has no characteristic chemical group with absorbance. The final ACE inhibitor from Morokheiya was attempted to be chemically identified. In the present study, the authors tried various purification methods, and some volume of the ACE inhibitor was lost during each purification step. Therefore, a table of the purification steps is not shown.

Identification of ACE inhibitor from Morokheiya

According to the mass spectrum (Voyager MALDI-TOF/MS, Japan Perceptive Co., Tokyo) of the ACE inhibitor after gel filtration on Sephadex G-25, two components with molecular weights of 303 and 202 were identified. Nicotianamine from tobacco leaves (Noma *et al.*, 1971) was reported to have a molecular weight of 303, and nicotianamine from Japanese soy sauces (Kinoshita *et al.*, 1993) was also reported to have ACE inhibitory activity. Moreover, on the preliminary TLC, the present ACE inhibitory activity was detected on the same migration as nicotianamine. Therefore, the final preparation, obtained after HPLC, was applied to the follow-

ing identification procedures with nicotianamine.

Final preparation of the ACE inhibitor after normal phase HPLC was applied to an amino acid analyzer (Yanagimoto L-7, Yanagimoto Co., Kyoto). As shown in Fig. 2, the elution profile of the ACE inhibitor was similar to that of nicotianamine. It was determined that the ACE inhibitor from Morokheiya was nicotianamine, but this elution time is also similar to cystine. Therefore, the following identification was made with nicotianamine and cystine.

The present ACE inhibitor was identified by TLC (silica gel 60F₂₅₄, Merck Co.) with three kinds of developing solvents. As shown in Fig. 3, in any solvent, the migration of the ACE inhibitor was coincident with nicotianamine and different from cystine. The ACE inhibitor from Morokheiya was definitely nicotianamine.

The ACE inhibitor was applied to capillary electropho-

Fig. 2. Chromatography of ACE inhibitor on amino acids analyzer. ACE inhibitor was applied to an amino acid analyzer. Elution profiles of ACE inhibitor (A) and nicotianamine (B) are shown at absorbances of 440 nm and 570 nm.

Fig. 1. Normal phase HPLC of ACE inhibitor on Asahi pack NH2P-50. Active fraction obtained after Sephadex G-25 was applied to a HPLC column previously equilibrated with 62% acetonitrile. The column was eluted with a linear gradient of aqueous 62% acetonitrile soln. to 50% acetonitrile-0.2 N-ammonia mixture. The fraction was 5 ml each.

Fig. 3. Thin layer chromatography of ACE inhibitor on silica gel plates. Cystine (1), ACE inhibitors before (2) and after (3) HPLC and nicotianamine (4) were applied to silica gel G TLC plates and developed with three kinds of solvent, (A) *n*-propanol and ammonia (7:3), (B) *n*-propanol: pyridine: acetic acid and water (15:10:3:12) and (C) *n*-butanol: methanol: water (2:1:1). After developing the plates, the substances were detected by ninhydrin reaction.

resis (Quanta 4000E, Millipore Co., Milford, MA) at 25 mM sodium phosphate buffer, pH 2.5. As shown in Fig. 4, the electropherogram of the ACE inhibitor from Morokheiya was similar to that of nicotianamine. The present ACE inhibitor was then mixed with nicotianamine and the mixture was applied to capillary electrophoresis. The electropherogram indicated only one peak. These results suggested that the present ACE inhibitor was the same substance as nicotianamine.

As previously mentioned above, the ACE inhibitor from Morokheiya was identified to be nicotianamine. The nicotianamine from tobacco leaves had been first reported by Noma *et al.* (1971). They (Noma & Noguchi, 1976) reported that nicotianamine had an optimal molecular structure, chelating irons, and was considered to be a possible phytosiderophore with an essential function in plant cellular iron transport and metabolism. Budesinsky *et al.* (1980) reported the presence of nicotianamine in Leguminosae and isolated it from the aerial parts of alfalfa (*Medicago sativa* L.). They suggested that nicotianamine might cause the present ACE inhibitory activity because of a powerful chelating action. Nicotianamine has been reported in some higher plants and a kind of microorganism, based on a patent application claim (Ouchi *et al.*, 1995) in Japan. Kinoshita *et al.* (1993) reported the physiological and biochemical natures of nicotianamine from soy-source. Their report showed that nicotianamine had ACE inhibitory activity and depressed the blood pressure of SHR (spontaneously hypertensive rat) after a single oral injection. Many reports about ACE inhibitors have recently been published (Maruyama *et al.*, 1987; Miyoshi *et al.*, 1991; Matsufuji *et al.*, 1994; Murakami *et al.*, 1995). Most of their peptides have a COOH-terminal dicarboxylic acid and an antepenult aromatic amino acid residue, chelating and enhancing the binding of the ACE. Nicotianamine has

Fig. 4. Electropherograms of ACE inhibitor on capillary electrophoresis. ACE inhibitor was applied to a capillary column previously equilibrated with phosphate buffer, pH 2.5, and electroded under the same conditions. The electropherogram of ACE inhibitor (A) was compared to nicotianamine (B), cystine (C) and a mixture (D) of ACE inhibitor and nicotianamine.

carboxylic acids and an antipenultimate amino acid with a ring structure.

Many studies on ACE inhibitors from food stuffs have been focused on peptide products from the hydrolysates of various protein sources. The present ACE inhibitor from Morokheiya was very characteristic due to its naturally occurring substance in Morokheiya and very different from peptide, secondary products from protein sources. We may not expect to obtain a greater volume of nicotianamine from Morokheiya than from any other protein sources. However, Morokheiya is very famous for being abundant in some nutrient components, calcium etc. Especially, dietary fiber participates in controlling cholesterol and cardiovascular disease and is speculated to have a good effect on hypertensive patients. Therefore, nicotianamine in Morokheiya may have additional effects with these authentic nutritional components for human health. The authors plan to clarify the biological and physiological systems for hypertension *in vivo*. There must be further investigations on the metabolism of nicotianamine after long-term administration.

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