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Inhibitors of Angiotensin Converting Enzyme in Crude Drugs. I

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Hot 50% MeOH extracts of crude drugs considered to have a hypotensive effect were tested for inhibitory effects on hog kidney angiotensin converting enzyme. Of 65 samples tested, 14 showed reproducible inhibition in the preliminary screening. The extracts of 8 potent samples were fractionated by MCI gel and Sephadex LH-20 gel chromatographies to concentrate the inhibitors. The most potent fractions of Arecae Semen, Ephedrae Herba, Epimedii Herba, Polygoni avicularis Herba, Potentillae Herba and Rhei Rhizoma showed more than 90% inhibition at the concentration of 20 μg/ml. Those of Moutan Cortex and Cinnamomi Cortex showed 86 and 78% inhibitions, respectively, at the same concentration. All of these fractions consisted of tannin-type compounds.

Keywords——angiotensin converting enzyme (ACE); inhibitor; crude drug; hypotensive folk medicine; tannin

Angiotensin converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) is an exopeptidase which cleaves dipeptides from the carboxy-terminal end of various peptide substrates. ACE plays an important role in blood pressure regulation by catalyzing two important reactions: a) conversion of the inactive decapeptide angiotensin I to the potent vasoconstrictor and salt-retaining octapeptide angiotensin II,[1] and b) inactivation of the vasodilator and natriuretic nonapeptide bradykinin.[2]

Engel *et al.*[3] and other investigators[4] demonstrated that a nonapeptide (BPF_{9a} or SQ 20881) in snake venom potentiated the effect of bradykinin by blocking the conversion of angiotensin I and lowered the increased arterial blood pressure both in man and in experimental animals. Interest in ACE inhibitors has increased since Ondetti *et al.*[5] synthesized captopril (2-D-methyl-3-mercaptopropanoyl-L-proline, SQ 14225), which was designed to interact with maximal effectiveness at the active site of ACE on the basis of a hypothetical model that presumes homology with the active site of a similar exopeptidase, carboxypeptidase A. In the last few years, various compounds with modifications of the structure of captopril have been prepared and tested as ACE inhibitors.[6]

Yun *et al.*[7] have attempted to find other types of ACE inhibitors from plant sources. They investigated 27 medicinal plants traditionally used in Korea for the treatment of hypertension or related diseases, and found that several of them contain ACE inhibitors.

The purpose of the present work was also to investigate ACE inhibitors from plant sources. The crude drugs used in this work were selected from Japanese folk medicines used for the treatment of hypertension and related diseases, and from Chinese crude drugs referred to in the "Dictionary of Chinese Crude Drugs"[8] as being effective for lowering the blood pressure. Several of them were found to contain a new type of potent ACE inhibitor, tannins.

Experimental

Materials——Crude drugs were purchased from Uchida Pharmacy for Oriental Medicine, Tokyo, Japan. Crude

rhatannin was kindly supplied by Prof. I. Nishioka of Kyushu University. Bz–Gly–His–Leu, a substrate analog of ACE, and His–Leu, used as a standard, were obtained from the Protein Research Foundation, Minoh, Japan and *o*-phthaldialdehyde was from Sigma Chemical Co., U.S.A. The purified ACE (12.5 units/mg protein) was prepared from hog kidney as described previously.[9] MCI gel CHP20P (150—300 μ) was a product of Mitsubishi Chemical Industries Co., Tokyo, Japan.

Assay of Inhibitory Effects——Inhibitory effects of the samples on ACE activity were determined by a modification of the method described by Friedland and Silverstein.[10] The substrate solution was prepared by dissolving 43 mg of Bz–Gly–His–Leu in 10 ml of phosphate-saline buffer (final concentrations in the assay mixture: 100 mM potassium phosphate, pH 8.3, 300 mM NaCl; 5 mM Bz–Gly–His–Leu). Enzyme solution was prepared by dissolving purified ACE in the same buffer. Two per cent *o*-phthaldialdehyde (200 mg/10 ml MeOH) was freshly prepared every day prior to use. The assay mixture, consisting of 240 μl of enzyme solution (0.8 munits ACE), 10 μl of sample solution and 250 μl of substrate solution, was incubated at 37 °C. As a control, assay mixture containing water or MeOH instead of the sample solution was run in parallel. The enzymic reaction was initiated by the addition of the substrate. After 1 h of incubation, 1.45 ml of 0.3 M NaOH was added to the assay mixture to stop the enzymic reaction, then 100 μl of *o*-phthaldialdehyde reagent was added to form the fluorescent adduct of the aldehyde and the histidyl moiety of His–Leu released from the substrate. Exactly 10 min later, the reaction was terminated by the addition of 200 μl of 3 M HCl. The solution was diluted 100 times with water and the fluorescence was read in a 1 cm cuvette on a Hitachi 650-10S fluorometer between 30 and 90 min after addition of HCl, during which time it was stable. The excitation wavelength was 340 nm and the emission fluorescence wavelength was 455 nm. Except for the preliminary screening test, samples were dissolved in 50 or 80% MeOH and added to the assay mixture. The MeOH concentration in the assay mixture was held constant at 2%. All assays were performed in duplicate. The rate of inhibition was calculated by comparison with the amount of His–Leu obtained from the control.

Preliminary Screening of Inhibitory Effects of Crude Drug Extracts on ACE——A sample of each crude drug (2 g) was extracted with 50% MeOH (20 ml) at 90—100 °C for 1 h, and 10 ml of the filtrate was concentrated to dryness *in vacuo*. Water (2 ml) was added to the dried residue and the mixture was sonicated and centrifuged. Ten μl of the supernatant was tested for inhibitory effect on ACE.

Crude drugs used for the preliminary screening were as follows. Among them, the crude drugs asterisked showed more than 50% inhibition: Achyranthis Radix, *Actinostemma lobatum* MAXIM. (herb and seed*), *Ajuga decumbens* THUNB. (herb), Akebiae Caulis, Alismatis Rhizoma, Angelicae Radix, Angelicae pubescentis Radix, *Apocynum venetum* L. (herb), Arctii Fructus, Arecae Semen*, Artemisiae capillaris Herba, Asari Herba, Astragali Radix, Belamcandae Rhizoma, *Capsella bursa-pastoris* MEDICUS (herb), Cassiae Semen, *Chenopodium album* L. var.

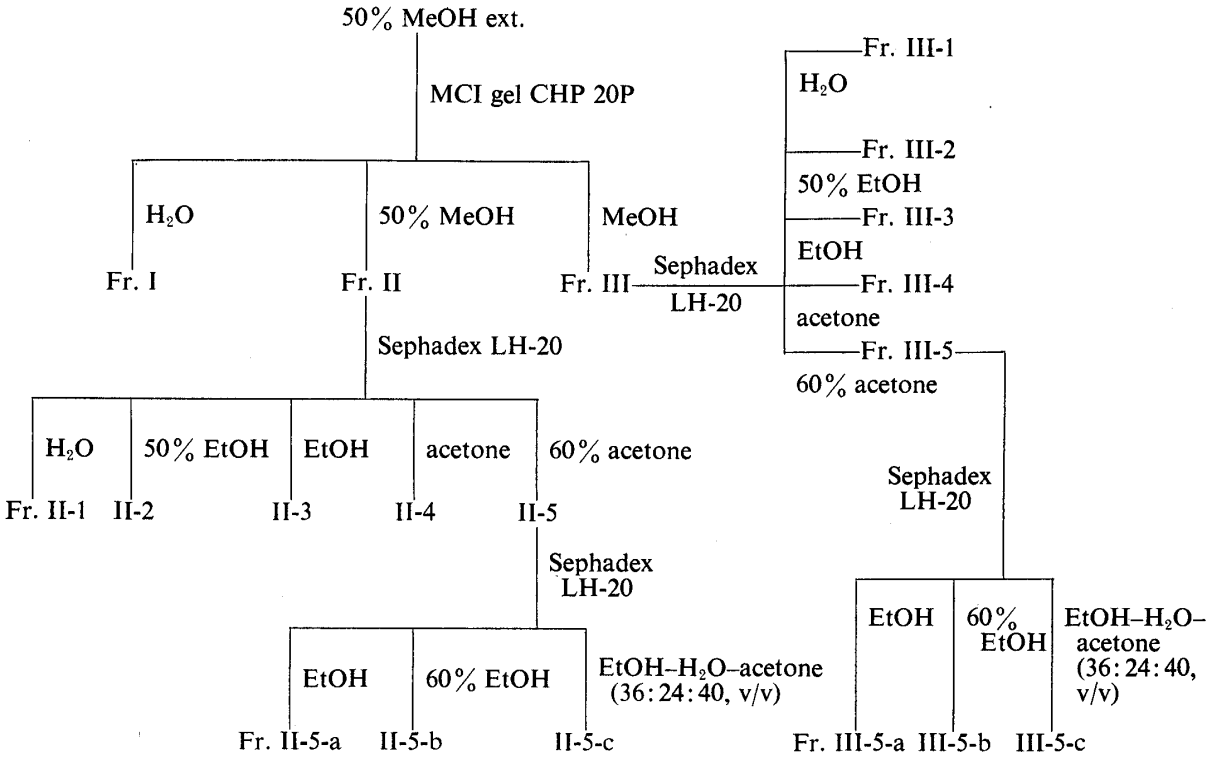

Chart 1. Fractionation of 50% MeOH Extracts of Crude Drugs

centrorubrum MAKINO (herb), Cimicifugae Rhizoma, Cinnamomi Cortex*, Clematidis Radix, Codonopsis pilosulae Radix, Corni Fructus, Crataegi Fructus*, Dendrobii Herba, *Dianthus superbus* L. (seed), *Diospyros kaki* L. *fil.* (leaf), Ephedrae Herba*, Epimedii Herba*, Eucommiae Cortex, Fritillariae Bulbus*, Gardeniae Fructus, Gentianae macrophyllae Radix, Ginseng Radix, Houttuyniae Herba, Leonuri Herba, Cnidii Rhizoma, *Luffa cylindrica* ROEM. (seed), Lycii Folium, Lycii Radicis Cortex, Lycii Fructus, Magnoliae Cortex, Magnoliae Flos, Momordicae Semen, Mori Folium*, Mori Cortex, Moutan Cortex*, Plantaginis Folium, Plantaginis Semen, Polygonati sibiricae Rhizoma, Polygoni avicularis Herba*, Potentillae herba*, Prunellae Spica, Puerariae Radix, Rauwolfiae Radix, Rhei Rhizoma*, Salviae militiorrhizae Radix, Sanguisorbae Radix*, Saussureae Radix, Scrophulariae Radix, Scutellariae Radix, Sinomeni Caulis *et* Rhizoma, *Trachycarpus excelsa* WINDL. (leaf), Uncariae Ramulus *et* Uncis*, Xanthii Fructus and Zizyphi spinosi Semen.

Extraction and Fractionation (Chart 1)——Each crude drug (100 g) which showed a potent inhibitory effect on ACE activity in the screening test was suspended in 50% MeOH (500—700 ml) and refluxed for 1 h. After cooling to room temperature, the solution was filtered and the residue was extracted again in the same way. The filtrates were combined and concentrated to dryness *in vacuo* at below 60 °C. The yields are listed in Table I. The 50% MeOH extract (3 g) was dissolved in hot water (3 ml) and the insoluble material was removed by centrifugation. The supernatant was applied to the MCI gel (30 ml) column and eluted with water (200 ml). The water-insoluble material was dissolved in hot 50% MeOH (3 ml) and centrifuged. The supernatant was applied to the same column and eluted with 50% MeOH (200 ml). The 50% MeOH-insoluble material was dissolved in hot MeOH (3 ml), applied to the column and eluted with MeOH (200 ml). Each effluent was concentrated to dryness *in vacuo*. The yields are listed in Table I. Fraction III of Cinnamomi Cortex and Fr. II of the other crude drugs were further fractionated by Sephadex LH-20 gel chromatography. Each fraction (1 g) was extracted with 3 ml each of water, 50% EtOH, EtOH, acetone and 60% aqueous acetone successively and each fraction was chromatographed on a Sephadex LH-20 (100 ml) column using the same solvents (200 ml) in the same manner as described for the MCI gel chromatography. The yields are listed in Table II. The fractions containing most of the ACE inhibitor (Fr. III-5 of Cinnamomi Cortex and Fr. II-5 of the others, 100 mg each) were rechromatographed on a Sephadex LH-20 (20 ml) column according to the method described by Nishizawa *et al.*[11] for fractionation of Paeonia gallotannins. Elution was carried out with 50 ml each of EtOH, 60% EtOH and a mixture of EtOH, water and acetone (36 : 24 : 40, v/v). The results are shown in Table III.

Results and Discussion

The 50% MeOH extracts of 65 crude drugs were tested for inhibitory effects on hog kidney ACE. Among them, 14 samples showed more than 50% inhibition in the preliminary screening test. The extracts of 8 potent samples among them were fractionated into water-soluble and MeOH-soluble fractions in order to investigate the solubilities of the inhibitors. The water-soluble fraction of the 50% MeOH extract of Arecae Semen showed high inhibitory activity, but generally, the MeOH-soluble fractions showed higher activity than the corresponding water-soluble fractions (data not shown), suggesting that the inhibitors were less soluble in water. Therefore, the 50% MeOH extracts were fractionated by MCI gel chromatography into three fractions. As expected, none of the water-eluted fractions (Fr. I) had inhibitory activity, and the inhibitors were concentrated in the 50% MeOH-eluted fractions (Fr. II) except for the inhibitors in Cinnamomi Cortex. The inhibitors in Cinnamomi Cortex were mostly eluted in Fr. III by MeOH indicating that they are less polar than those in the other crude drugs (Table I). Fraction III of Cinnamomi Cortex and Fr. II of the others were further fractionated by Sephadex LH-20 gel chromatography to concentrate the inhibitors. As shown in Table II, almost all the inhibitors were concentrated in the fraction eluted by 60% aqueous acetone (Fr. II-5 and III-5). These fractions were obtained as amorphous light brown or reddish-brown powders. When the inhibitory effects of these fractions of ACE were measured in borate-saline buffer instead of phosphate-saline buffer, none of the samples showed inhibition. Further, prolonged storage of the methanolic solution of these fractions at room temperature decreased the inhibitory effect (data not shown). These observations suggested that the inhibitors in these fractions may be the same or similar compound(s). The gel chromatographic behavior of the fractions, their astringent taste, their ultraviolet spectra (λ_{max}^{MeOH} 280 nm) and colorations with ferric chloride and vanillin-HCl reagents strongly suggested that these fractions consisted mainly of tannin-type compounds. Therefore, these

TABLE I. Inhibitory Effects of 50% MeOH Extracts and Their Fractions Obtained by MCI Gel Chromatography on Angiotensin Converting Enzyme

Crude drugs	Inhibition %			
	50% MeOH ext. 200 μg/ml	Fr. I 20 μg/ml	Fr. II 20 μg/ml	Fr. III 20 μg/ml
Arecae Semen	98 (14.3)[a]	0 (5.4)	95 (7.4)	94 (0.6)
Cinnamomi Cortex[b]	87 (10.3)	0 (2.6)	76 (2.2)	75 (5.5)
Ephedrae Herba	98 (33.8)	0 (7.0)	95 (21.3)	19 (2.5)
Epimedii Herba	84 (27.2)	0 (14.3)	60 (5.2)	0 (4.3)
Moutan Cortex	61 (29.8)	0 (25.2)	58 (4.3)	6 (1.3)
Polygoni avicularis Herba	94 (20.2)	0 (14.1)	98 (3.8)	88 (1.3)
Potentillae Herba	54 (16.1)	0 (9.6)	52 (4.7)	10 (1.3)
Rhei Rhizoma	95 (34.4)	0 (7.5)	55 (18.9)	7 (4.9)

a) The values in parentheses are the yields (%) on the basis of the weight of crude drug.
b) Fractions of Cinnamomi Cortex were dissolved in 80% MeOH and added to the assay mixture. The fractions of the other crude drugs were dissolved in 50% MeOH.

TABLE II. Inhibitory Effects of Fractions Obtained by the 1st Sephadex LH-20 Gel Chromatography

Crude drugs	Inhibition % (20 μg/ml)				
	Fr. II-1	Fr. II-2	Fr. II-3	Fr. II-4	Fr. II-5
Arecae Semen	0 (0.5)[a]	24 (1.9)	50 (0.7)	66 (1.2)	98 (3.5)
Ephedrae Herba	0 (8.3)	6 (3.6)	0 (0.8)	24 (0.4)	98 (5.8)
Epimedii Herba	0 (1.9)	0 (1.3)	11 (0.4)	33 (0.2)	98 (1.4)
Moutan Cortex	0 (1.5)	0 (0.8)	2 (0.3)	20 (0.4)	75 (1.3)
Polygoni avicularis Herba	11 (1.7)	10 (0.7)	37 (0.2)	85 (0.2)	98 (1.0)
Potentillae Herba	3 (1.3)	3 (1.2)	2 (0.5)	15 (0.5)	89 (1.4)
Rhei Rhizoma	0 (4.6)	0 (4.0)	7 (2.0)	96 (1.8)	99 (6.0)
	Fr. III-1	Fr. III-2	Fr. III-3	Fr. III-4	Fr. III-5
Cinnamomi Cortex	0 (0.1)	0 (1.5)	17 (0.5)	50 (0.4)	80 (2.4)

a) The values in parentheses are the yields (%) of the fractions based on the weight of crude drug.

fractions were further purified by fractionation based on molecular weight differences.[11] The last fractions eluted by the mixture of EtOH, water and acetone (36 : 24 : 40, v/v) seemed to consist of higher molecular weight tannins, and showed the highest inhibitory effect of the three fractions at the concentration of 20 μg/ml (Table III). Crude rhatannin, which is the major tannin of Rhei Rhizoma, and a mixture of (−)-epicatechin 3-*O*-gallate polymers[12] (probable average molecular weight: *ca.* 4500) showed 67% inhibition at the concentration of 5 μg/ml (data not shown). These experimental data suggest that the ACE-inhibiting activity might be dependent on the molecular weight of tannin, that is, the degree of polymerization, except for the inhibitors in Ephedrae Herba. In the case of Ephedrae Herba, Fr. II-5-a, which appears to consist of the lower molecular weight tannins, also showed a potent inhibitory effect. As shown in Table III, the extents of inhibitory effects of the most potent fractions vary from 11 to 73% at the concentration of 2 μg/ml. These differences might be attributed to differences of the component tannins. Thus, each fraction may have different effects on other enzymes. The effects of each fraction have so far been examined on three Zn-containing exopeptidase (carboxypeptidases A and B, and leucine aminopeptidase), two digestive

TABLE III. Inhibitory Effects of Fractions Obtained by the 2nd Sephadex LH-20 Gel Chromatography

Crude drugs	Yields[a] (%)	Inhibition %		Yields[a] (%)	Inhibition %		Yields[a] (%)	Inhibition %	
		20 μg/ml	2 μg/ml		20 μg/ml	2 μg/ml		20 μg/ml	2 μg/ml
		Fr. II-5-a			Fr. II-5-b			Fr. II-5-c	
Arecae Semen	0.04	0		0.17	79	2	3.22	95	73
Ephedrae Herba	1.10	99	30	0.23	95	19	4.39	98	52
Epimedii Herba	0.16	32		0.26	85	2	0.99	98	46
Moutan Cortex	0.03	0		0.09	68	5	1.08	86	11
Polygoni avicularis Herba	0.02	0		0.05	93	5	0.93	99	61
Potentillae Herba	0.07	0		0.23	66	0	1.11	92	20
Rhei Rhizoma	0.18	0		0.24	68	0	5.78	98	64
		Fr. III-5-a			Fr. III-5-b			Fr. III-5-c	
Cinnamomi Cortex	0.87	66	10	0.33	72	14	1.11	78	28

a) The yields (%) are on the basis of the weight of the crude drug.

enzymes (trypsin and chymotrypsin) and kallikrein, the kinin-releasing enzyme. None of the fractions had any significant effect on the activity of kallikrein. However, they showed inhibitory effects on the activities of carboxypeptidase B, leucine aminopeptidase, trypsin and chymotrypsin although to lesser extents than on ACE. On the other hand, the effects on carboxypeptidase A were quite different from those on the other enzymes. All samples, except for the fraction from Moutan Cortex, increased the activity of the enzyme. These results are contrary to the common belief that tannins nonspecifically inhibit the activities of enzymes, and support Nishioka's prediction that there should be a specific interaction between individual tannins and enzymes.[12] Details of the experiments on the effects of these fractions on the above-mentioned enzymes will be presented in the next paper.

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