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Inhibitors of Angiotensin-Converting Enzyme in Crude Drugs. II[1]

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The effects of rhatannin, Chinese gallotannin and tannin fractions isolated from Arecae Semen, Cinnamomi Cortex, Ephedrae Herba, Epimedii Herba, Moutan Cortex, Polygoni avicularis Herba, Potentillae Herba and Rhei Rhizoma on the activities of angiotensin-converting enzyme (ACE) and other six proteases were investigated.

These tannin samples had no significant effect on the activity of kallikrein, but all of them inhibited the activities of carboxypeptidase B, leucine aminopeptidase, trypsin and chymotrypsin although to lesser extents as compared to the effects on ACE. On the other hand, all the samples except for Chinese gallotannin and the fraction from Moutan Cortex, enhanced the activity of carboxypeptidase A. All the samples showed non-competitive inhibition patterns with ACE. Rhatannin and the tannin samples obtained from Arecae Semen, Ephedrae Herba, Epimedii Herba, Polygoni avicularis Herba, and Rhei Rhizoma showed more potent inhibitory effects on ACE than the other samples, and their I_{50} values were 3.7, 1.0, 1.9, 2.9, 1.1, and 1.2 μg/ml, respectively. The apparent K_i value of rhatannin for ACE was 5×10^{-7} M with Bz–Gly–His–Leu as the substrate, and this value is 5 times and 300 times higher than those of SQ 20881 ($K_i = 1 \times 10^{-7}$ M) and SQ 14225 ($K_i = 1.7 \times 10^{-9}$ M), respectively. Thus, it is suggested that rhatannin and some of the tannin samples obtained from the above crude drugs (which are believed to have hypotensive effects) show high specificity as ACE inhibitors.

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

In the preceding paper,[1] we described the results of screening of 65 crude drugs for inhibitory effects on angiotensin-converting enzyme (ACE). All of the potent fractions of the eight inhibitory crude drugs have been proved to consist of higher molecular weight condensed-type tannins based on the results of Sephadex LH-20 column chromatography[2] and vanillin-HCl color reaction,[3] although the homogeneities, molecular weights and structures were not established.

Recently, several investigations on the biological properties of tannins have been reported using structure-determined and homogeneous tannins.[4] The most interesting finding was that by Oura *et al.*,[4c] who showed that rhatannin, the major tannin in Rhei Rhizoma, causes a remarkable decrease of the blood urea nitrogen (BUN) concentration in rat serum upon intraperitoneal administration, and it stimulates the activity of glutamine synthetase without affecting the activities of five enzymes of the urea cycle, or glutamate oxaloacetate transaminase, glutamate dehydrogenase and glutaminase. These results are contrary to the generally accepted view that tannins nonspecifically inhibit the activities of enzymes, and strongly imply the presence of distinct interactions between tannins and enzymes.

Inhibition of the ACE activity by our tannin samples is a noteworthy addition to the

increasing list of biological activities of tannins, but the specificity of our samples as ACE inhibitors should be further investigated to establish whether these tannins are candidate for hypotensive drugs. We deal in this paper with the effects on the activities of other proteases, carboxypeptidases A and B (Cpases A and B), leucine aminopeptidase (LAP), trypsin, chymotrypsin and kallikrein, employing rhatannin and Chinese gallotannin (tannic acid, a mixture of galloylglucoses[5]) as representative tannin samples of condensed-type and hydrolyzable-type, respectively. Some information on the inhibition mechanisms is presented.

Experimental

Materials——Bz–Gly–His–Leu, His–Leu, Bz–Gly–Lys, Bz–Arg–OEt, Ac–Tyr–OEt, L-leucine 4-methylcoumaryl-7-amide (LMC) were purchased from the Protein Research Foundation, Minoh, Japan. Bz–Gly–Phe was synthesized in this laboratory and was homogeneous on high-performance liquid chromatography (HPLC). Trypsin and α-chymotrypsin were purchased from Miles Biochemicals, Cpase A (porcine pancreas, type I), Cpase B (porcine pancreas, type I), LAP (porcine kidney, type V) and kallikrein (porcine pancreas) were from Sigma Chemical Co. Hog kidney ACE was prepared as described previously.[6] Various tannin samples listed in Table I were isolated as described in the preceding paper.[1] All other chemicals were of analytical grade.

HPLC Method——The HPLC system (Waters Assoc., Milford, MA, U.S.A.) consisted of a model 6000A pump, coupled with a U6K injector and an absorbance detector (model 440) set at 254 nm. Chromatography on a reverse phase column (μ Bondapak C_{18}, 4 mm × 30 cm, Waters Assoc.) was performed at room temperature and at a flow rate of 1 ml/min with the following buffer systems: (I) 20% methanol–water (v/v) in 1% AcOH, (II) 1 mM $K_2HPO_4 + H_3PO_4$, pH 3.0/methanol (75 : 25, v/v) or (III) 40% methanol–water (v/v) with PIC-A reagent (Waters Assoc.). System I was used for the separation of Bz–Arg–OEt and Bz–Arg, which showed retention times of 8.2 and 4.7 min, respectively. System I was also used for the separation of Ac–Tyr–OEt and Ac–Tyr, which gave retention times of 7.8 and 4.1 min. System II was used for the separation of Bz–Gly–Lys and Bz–Gly (retention times of 4.3 and 3.8 min) and System III was used for the separation of Bz–Gly–Phe and Bz–Gly (retention times of 3.9 and 3.3 min). Under these HPLC conditions, all of the tannin samples used in this study were retained on the column.

Enzyme Assays——In all experiments, the volume of the reaction mixture was 0.5 ml, and incubation (15—30 min) was carried out at 37 °C in 13 × 100 mm siliconized glass tubes. Tannin samples were preincubated for 10 min with each enzyme. Fluorometric assay of the hydrolysis of Bz–Gly–His–Leu by ACE was performed as described in the preceding paper.[1] LAP activity was determined using LMC as a substrate according to the method of Saifuku *et al.*[7]

Bz–Gly–Phe, Bz–Gly–Lys, Bz–Arg–OEt and Ac–Tyr–OEt were employed as substrates for the estimation of Cpase A, Cpase B, trypsin (or kallikrein) and chymotrypsin activities, respectively, and the enzymic reaction was stopped by the addition of 0.5 ml of absolute ethanol. The mixture was then centrifuged and the clear supernatant was subjected to HPLC analysis. The analytical system was calibrated with Bz–Gly when Bz–Gly–Lys or Bz–Gly–Phe was the substrate, with Bz–Arg when Bz–Arg–OEt was the substrate, and with Ac–Tyr when Ac–Tyr–OEt was the substrate. Details of assay conditions for these enzymes are given in Table II.

TABLE I. Origins and Fractions of Tannin Samples

Sample name	Origin	Fraction[a]
Areca	Arecae Semen	Fr. II-5-c
Cinnamomum	Cinnamomi Cortex	Fr. III-5-c
Ephedra-I	Ephedrae Herba	Fr. II-5-a
Ephedra-II	Ephedrae Herba	Fr. II-5-c
Epimedium	Epimedii Herba	Fr. II-5-c
Moutan	Moutan Cortex	Fr. II-5-c
Polygonum	Polygoni avicularis Herba	Fr. II-5-c
Potentilla	Potentillae Herba[b]	Fr. II-5-c
Rheum	Rhei Rhizoma	Fr. II-5-c

a) The fractionation procedure was described in the preceding paper.[1]
b) The herb of *Potentilla chinensis* SERINGE or *P. discolor* BUNGE. The original plant was not specified.

Results and Discussion

Tannins are known to have the ability to combine with proteins, and the albumin precipitation reaction is the first choice of test reaction to investigate the properties of tannins. Therefore, we first checked the inhibitory effects of the tannin samples on ACE in the presence of a large amount of bovine serum albumin (BSA). In the standard assay method, the sample and ACE were mixed first and then the substrate was added to start the enzymic reaction. In the experiment in the presence of BSA, the sample was added to the mixture of BSA (25 μg/ml) and ACE (0.3 μg/ml) prior to the addition of the substrate. In the case of Ephedra-I, 30% of the ACE activity was protected by the addition of BSA, whereas all the other samples showed almost the same degrees of inhibition both in the presence and absence

TABLE II. Effects of Various Tannin Samples on the Activities of ACE, Cpase A, Cpase B, LAP, Trypsin, Chymotrypsin (Chymo.) and Kallikrein (KL)

Sample	Activity (%)													
	ACE[a]		Cpase A[b]		Cpase B[c]		LAP[d]		Trypsin[e]		Chymo.[f]		KL[g]	
Amount added (μg/ml)	20	4	20	4	20	4	20	4	20	4	20	4	20	4
Control	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Areca	3	25	131	115	63	92	32	80	94	100	60	100	97	100
Cinnamomum	22	59	200	187	52	100	60	90	90	100	47	90	93	100
Ephedra-I	2	61	210	180	66	97	10	82	40	75	47	95	88	100
Ephedra-II	2	15	127	96	61	89	13	71	42	82	37	85	88	100
Epimedium	2	41	170	120	41	73	32	88	85	100	98	100	95	100
Moutan	15	64	82	95	61	84	40	85	40	85	74	100	96	100
Polygonum	2	21	145	113	55	76	12	71	23	80	58	90	93	100
Potentilla	8	70	135	127	61	78	61	96	88	100	100	100	100	100
Rheum	2	18	111	93	54	75	9	83	23	78	46	85	94	100
Rhatannin	2	40	127	95	48	97	18	88	25	75	65	95	95	100
Chinese gallotannin	23	65	77	109	54	89	70	100	61	96	88	100	93	100

Assay conditions: *a*) 5 mM Bz–Gly–His–Leu, 0.15 μg of ACE and 100 mM potassium phosphate buffer, pH 8.3, containing 300 mM NaCl; *b*) 5 mM Bz–Gly–Phe, 0.45 μg of Cpase A and 100 mM potassium phosphate buffer, pH 7.5; *c*) 5 mM Bz–Gly–Lys, 0.34 μg of Cpase B and 100 mM potassium phosphate buffer, pH 7.5; *d*) 0.11 mM LMC, 0.15 μg of LAP and 100 mM potassium phosphate buffer, pH 8.0; *e*) 1 mM Bz–Arg–OEt, 0.10 μg of trypsin and 100 mM potassium phosphate buffer, pH 8.0; *f*) 1 mM Ac–Tyr–OEt, 0.10 μg of chymotrypsin and 100 mM potassium phosphate buffer, pH 7.0; *g*) 1 mM Bz–Arg–OEt, 0.10 μg of kallikrein and 100 mM potassium phosphate buffer, pH 8.0, containing 100 mM NaCl.

TABLE III. Inhibition of ACE by Various Tannin Samples

Sample	I_{50} (μg/ml)
Areca	1.0
Cinnamomum	4.7
Ephedra-I	4.5
Ephedra-II	1.9
Epimedium	2.9
Moutan	7.0
Polygonum	1.1
Potentilla	8.5
Rheum	1.2
Rhatannin	3.7
Chinese gallotannin	7.0

of BSA. These results imply that the samples other than Ephedra-I inhibit the activity of ACE even in the presence of other proteins.

In order to check the possible specificity of the samples as inhibitors of ACE, we examined the effects on other proteases, three Zn-containing exopeptidases (Cpases A and B, and LAP), two digestive enzymes (trypsin and α-chymotrypsin) and kallikrein, the kinin-releasing enzyme. The results are summarized in Table II. The samples did not have any significant effect on the activity of kallikrein, but all of them showed inhibitory effects on the activities of Cpase B, LAP, trypsin and chymotrypsin, although to lesser extents as compared to the effect on ACE. Among nine samples and the two defined tannin samples, Areca, Ephedra-II, Epimedium, Polygonum, Rheum and rhatannin showed more potent inhibitory effects on ACE than the other samples, and their I_{50} values were 1.0, 1.9, 2.9, 1.1, 1.2 and 3.7 μg/ml, respectively (Table III). On the other hand, their inhibitory effects on Cpase B, LAP, trypsin and chymotrypsin did not exceed 30% even at the concentration of 4 μg/ml, so that there were marked differences in the degrees of inhibitory effects towards ACE and the other proteases. The effects of the samples on Cpase A were quite different from those on the other enzymes. All the samples, except for Moutan and Chinese gallotannin, enhanced the activity of Cpase A and the stimulatory effects were in the order of Ephedra-I > Cinnamomum > Epimedium > Polygonum > Potentilla > Areca > Ephedra-II > rhatannin > Rheum at the concentration of 20 μg/ml. In contrast, Moutan and Chinese gallotannin showed 18 and 23% inhibitions, respectively, at the same concentration. These results indicate that each tannin sample has a distinct interaction with each enzyme. Our tannin samples showed higher inhibitory effect on ACE than on the other enzymes and thus seem to be rather specific inhibitors of ACE.

As mentioned in the introduction, Oura *et al.*[4c] reported a stimulatory effect of rhatannin on glutamine synthetase. Elevation of the Cpase A activity is the second example of the stimulatory effect of tannins on enzyme activity.

In Fig. 1, the kinetics of inhibition of ACE by each sample are plotted according to Lineweaver and Burk. All samples produced non-competitive inhibition patterns. The calculated enzyme-inhibitor dissociation constant (K_i value) of ACE with rhatannin (proposed average molecular weight: *ca.* 4500) is shown in Fig. 2. Its apparent K_i value was 5×10^{-7} M with Bz–Gly–His–Leu as the substrate.

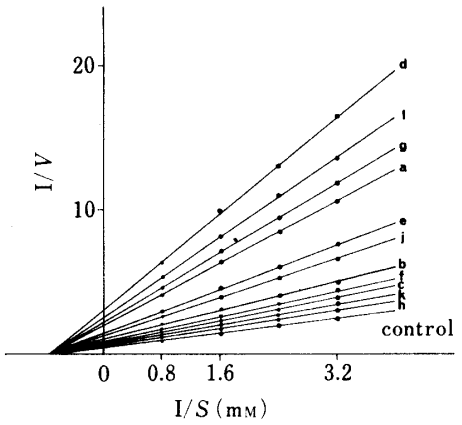

Fig. 1. Lineweaver–Burk Plots of ACE Activity in the Presence of Various Tannin Samples

The concentration of each tannin sample in the assay mixture was 4 μg/ml. Control: no tannin sample was added. a, Areca; b, Cinnamomum; c, Ephedra-I; d, Ephedra-II; e, Epimedium; f, Moutan; g, Polygonum; h, Potentilla; i, Rheum; j, Rhatannin; k, Chinese gallotannin.

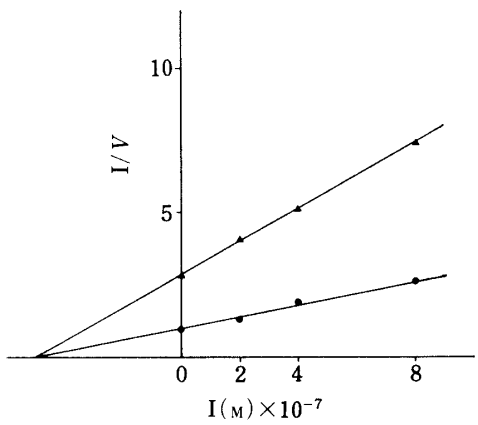

Fig. 2. Dixon Plot of the Inhibition of ACE by Rhatannin

Solid circles, 1.25×10^{-3} M Bz–Gly–His–Leu; solid triangles, 3.1×10^{-4} M Bz–Gly–His–Leu; $K_i = 5 \times 10^{-7}$ M.

Cushman *et al.*[8] have reported the K_i values with the same substrate for two well-known competitive ACE inhibitors, SQ 20881 (a nonapeptide obtained from the venom of *Bothrops jararaca*) and SQ 14225 (D-3-mercapto-2-methylpropanoyl-L-proline) as 1×10^{-7} M and 1.7×10^{-9} M, respectively. Thus, the K_i value for rhatannin is 5 times and 300 times higher than those for SQ 20881 and SQ 14225, respectively. From these results, rhatannin and some of our tannin samples can be regarded a new group of relatively potent ACE inhibitors from natural sources.

Since ACE, like Cpase B and LAP, is a Zn-containing exopeptidase, we next checked the effect of the addition of $ZnCl_2$ on the inhibitory effects of these samples in order to cast light on the inhibition mechanisms. The results are shown in Table IV. The addition of $ZnCl_2$ did not alter the inhibitory effects of Areca, Cinnamomum, Moutan and Potentilla on ACE activity, and only slightly decreased the inhibitory effects of Ephedra-I, -II, Rheum and rhatannin. All samples except for Epimedium inhibited the Cpase B activity to the same degree both in the presence and absence of $ZnCl_2$, *i.e.*, $ZnCl_2$ had practically no influence on the inhibition of Cpase B activity by our tannin samples. However, the inhibitory effects of all the samples on LAP were considerably reduced by the addition of $ZnCl_2$. In the case of Chinese gallotannin, almost all the activities of the three enzymes were restored by the addition of $ZnCl_2$. From these results, the inhibition of enzyme activities by Chinese gallotannin may be attributed, as is generally accepted, to metal-chelating character. The inhibitory effects of our tannin samples on ACE and Cpase B are due to uncharacterized mechanisms other than metal chelation. Combination with the tannins might change the steric conformations of the enzymes in such a way that the substrates have less easy access to the active sites of the enzymes. The recovery of the LAP activity after the addition of $ZnCl_2$ is rather perplexing, but might partly reflect chelation by the tannins of some of the zinc (4—6) in LAP. These results suggest that inhibition of the LAP activity by the tannin samples is not caused by a single mechanism but by complex mechanisms.

Cushman *et al.*[8] have developed many competitive inhibitors of ACE modeled on the bradykinin potentiating nonapeptide SQ 20881. One of them, SQ 14225 (captopril), showed a remarkable specificity for ACE inhibition, and it has been widely used for the treatment of a

TABLE IV. Effect of $ZnCl_2$ on the Inhibitory Activities of Various Tannin Samples on ACE, Cpase B and LAP

Sample	Activity (%)		
	ACE	Cpase B	LAP
	20 μg/ml + 500 μM $ZnCl_2$		
Control	100 (100)	100 (100)	100 (100)
Areca	3 (3)	58 (63)	58 (32)
Cinnamomum	22 (22)	55 (52)	76 (60)
Ephedra-I	20 (2)	65 (66)	69 (10)
Ephedra-II	5 (2)	54 (61)	53 (13)
Epimedium	13 (2)	64 (41)	65 (32)
Moutan	14 (15)	57 (61)	82 (40)
Polygonum	19 (2)	51 (55)	63 (12)
Potentilla	9 (8)	60 (61)	77 (61)
Rheum	10 (2)	47 (54)	58 (9)
Rhatannin	21 (2)	52 (48)	76 (18)
Chinese gallotannin	86 (23)	88 (54)	98 (70)

Values in parentheses represent the activities in the absence of $ZnCl_2$.

variety of hypertensive diseases. Our tannin samples, which were obtained from the crude drugs generally considered to have hypotensive effects, also show high specificity as ACE inhibitors, and do not have any significant effect on kallikrein, which plays an important hypotensive role by releasing kinin.

The tannin samples examined in this study were unfortunately, much less potent as inhibitors of ACE activity than SQ 14225, but a more potent and specific ACE inhibitor might well be isolated by further chemical investigation from these tannin fractions, or from among the huge variety of tannin resources in the plant kingdom.

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