Abstract—Effects of condensed tannins isolated from Rhei Rhizoma on the activities of angiotensin converting enzyme (ACE) and various proteases were examined in vitro. Among the various condensed tannins tested, procyanidin B-5 3,3'-di-O-gallate and procyanidin C-1 3,3',3''-tri-O-gallate strongly inhibited the activity of ACE. The concentration of procyanidin B-5 3,3'-di-O-gallate required for 50% inhibition of ACE was $1.3 \times 10^{-6}$ M. The inhibition of ACE by condensed tannins was reversible and non-competitive, according to dialysis and to Dixon plots. However, over one hundred times the concentration was required to inhibit activities of other proteases such as trypsin, chymotrypsin, leucine aminopeptidase, carboxypeptidase A and urinary kallikrein. These results suggest that the inhibitory effects of condensed tannins on the activities of ACE are specific.

Angiotensin converting enzyme (ACE, kininase II, EC 3.4.15.1) is a dipeptidyl carboxypeptidase which removes a dipeptide from the biologically inactive decapeptide angiotensin I and produces the potent vasopressor octapeptide angiotensin II (1). The vasodepressor peptide bradykinin is inactivated by the enzymatic removal of a dipeptide by kininase II (2). Thus, ACE plays a pivotal role in blood pressure regulation.

Captopril (D-2-methyl-3-mercaptopropanoyl-L-proline, SO 14,225) (3), a synthetic orally active inhibitor of ACE, reduces blood pressure in essential and renal hypertension in man (4) and in many animal models of experimental hypertension (5, 6). Since captopril was synthesized by Ondetti et al. (3), different inhibitors of ACE have been investigated.

Inokuchi et al. (7, 8) investigated ACE inhibitors extracted from Chinese crude drugs which are clinically prescribed to treat hypertensives, and they demonstrated that fractions containing tannins showed a high specificity as ACE inhibitors.

We examined the inhibitory effects of pure condensed tannins isolated from Rhei Rhizoma on the activities of ACE and various proteases.

The rates of hydrolysis of hippuryl-L-histidyl-L-leucine by ACE were determined, by quantitating spectrofluorimetrically, the liberated L-histidyl-L-leucine (9). Rabbit lung ACE (10) and Bz-Gly-His-Leu were each dissolved in 100 mM potassium phosphate buffer, pH 8.3, containing 0.3 M NaCl. The assay mixture containing 240 $\mu$l of enzyme solution (0.8 x 10^{-3} units ACE) (10) and 10 $\mu$l of inhibitor solution (in methanol) was preincubated at 37°C for 5 min in siliconized tubes. The reaction was initiated by the addition of 250 $\mu$l of substrate solution, the final concentration of the substrate being 5 mM. After 1 hr of incubation, the reaction was halted by the addition of 1.45 ml of 0.3 M NaOH; then 100 $\mu$l of 2% o-phthalaldehyde

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reagent (200 mg/10 ml methanol) was added to the mixture. Exactly 10 min later, the reaction was terminated by the addition of 200 μl of 3 M HCl. The relative fluorescence intensity was measured in an Aminco-Bowman spectrophotofluorometer, between 30 and 90 min after addition of HCl, during which time it was stable. Excitation and emission wavelengths of 364 and 464 nm, respectively, were used. As a control, the activity of ACE in the absence of tannins was measured. The control activity was taken as 100%, and the percent activity of enzyme with tannins was calculated. Kᵢ value of procyanidin B-5 3,3'-di-O-gallate for ACE was determined using Dixon plots (11).

The inhibitory effects of procyanidin B-5 3,3'-di-O-gallate on various proteases were next examined. Bovine trypsin (type I), bovine α-chymotrypsin (type II) and porcine kidney leucine aminopeptidase (type III-CP) were each dissolved in 25 mM Tris-HCl buffer, pH 7.5. Urinary kallikrein and Bz-Gly-Phe were dissolved in 20 mM Tris-HCl buffer, pH 8.0, and 100 mM potassium phosphate buffer, pH 8.3, containing 0.3 M NaCl, respectively. The activities of trypsin, chymotrypsin, leucine aminopeptidase and urinary kallikrein were determined by spectrofluorimetric quantitations, using Bz-Arg-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Leu-MCA and Pro-Phe-Arg-MCA (Protein Research Foundation) and o-phthalaldehyde (Nakarai Chemicals). Urinary kallikrein was purified by the method of Geiger and Fritz (12). Condensed tannins were isolated from Rhei Rhizoma by the methods described in previous papers (13, 14). The formulae of these compounds are shown in Fig. 1A.

The effects of the condensed tannins on the hydrolysis of ACE were examined, and the concentration required for 50% inhibition is shown in Table 1. The inhibitory activities on ACE by the monomers (−)-epicatechin 3-O-gallate and (−)-epigallocatechin 3-O-gallate were much the same. However, a comparison of monomers with the procyanidin B-2 3,3'-di-O-gallate (dimer) and procyanidin C-1 3,3',3''-tri-O-gallate (trimer) suggested that the inhibition of ACE by these tannins showed a tendency to increase when they were polymerized. The inhibition of ACE by tannins proportional to their degree of polymerization was similar to that reported by Inokuchi et al. (15). Among the various condensed tannins tested, procyanidin B-5 3,3'-di-O-gallate was most effective. The concentration of this inhibitor required for 50% inhibition of ACE was 1.3×10⁻⁶ M. The concentration for 50% inhibition was 60 times more effective than that of (−)-epicatechin 3-O-gallate. However, the inhibitory effect of procyanidin B-5 3,3'-di-O-gallate on ACE was less potent than captopril (IC₅₀ value was 2.3×10⁻⁸ M) (3).

The effect of dialysis was next examined. A mixture of ACE and procyanidin B-5 3,3'-di-O-gallate (10⁻⁴ M) and 20 mM Tris-HCl buffer containing 0.15 M NaCl, pH 8.3, was dialyzed overnight at 4°C. Since 50% activity of ACE was recovered after the dialysis, the inhibition of ACE by procyanidin B-5 3,3'-di-O-gallate was reversible. ACE is a Zn-containing enzyme and tannins have metal-chelating character (8); however, it is assumed that the inhibition of ACE by
Fig. 1. Structural formulae of tannins (A) and Dixon plot of inhibition of ACE at two different concentrations of substrate (5 × 10^{-3} M (●), 1 × 10^{-3} M (○)) (B).
procyanidin B-5 3,3'-di-O-gallate was not only due to the metal chelation. The $K_i$ value was determined from a Dixon plot (Fig. 1B). The $K_i$ value of procyanidin B-5 3,3'-di-O-gallate for ACE was $1 \times 10^{-6}$ M, a result indicating that the inhibition was non-competitive.

When the inhibitory effects of procyanidin B-5 3,3'-di-O-gallate on various proteases were examined, the concentrations required for 50% inhibition of trypsin, chymotrypsin, leucine aminopeptidase, carboxypeptidase A and urinary kallikrein were found to be $1 \times 10^{-4}$ M, $>10^{-4}$ M, $1 \times 10^{-4}$ M, $>10^{-4}$ M and $4 \times 10^{-4}$ M, respectively. Procyanidin B-5 3,3'-di-O-gallate inhibited ACE, but did not inhibit the enzyme activities of trypsin, chymotrypsin, leucine aminopeptidase, carboxypeptidase A and urinary kallikrein. While condensed tannins possess the ability to combine with proteins, they apparently do not combine with protein non-specifically.

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