Isolation of inhibitory substance acting on angiotensin converting enzyme from the leaf of *Quercus stenophylla* MAKINO

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Abstract

The leaf of *Quercus stenophylla* MAKINO (*Fagaceae*) has been traditionally used for treatments of urolithic and hypertensive diseases in Japan, especially in Tokushima Prefecture. We studied the inhibitory effects of the various fractions extracted from the leaf of *Q. stenophylla* on angiotensin converting enzyme activity. Since the methanol extract was shown to exhibit an inhibitory effect on angiotensin converting enzyme, the active substance was further fractionated by silica gel column chromatography after treatment with chloroform. The crystallized active principle was identified as (+)-catechin. Not only authentic (+)-catechin but also its stero-isomer, (−)-epicatechin were shown to be the strong inhibitor of angiotensin converting enzyme and they act as competitive inhibitors for the enzyme.

Key words ACE activity, (+)-catechin, *Quercus stenophylla* MAKINO
Abbreviation ACE, angiotensin converting enzyme

Introduction

The leaf of *Quercus stenophylla* MAKINO (*Fagaceae*) (Japanese name : Urajirogashi) has been used for treatment of urolithic and hypertensive diseases.

Recently, Nishioka *et al.* isolated various tannins such as ellagitannins containing a salidroside (*p*-hydroxyphenethyl alcohol 1-*O*-*β*-D-glucoside) and proto-quercitol gallates *etc.* from the bark of *Q. stenophylla* MAKINO.[1-7] However, biological activities of these tannins are remained to be elucidated. The present experiments were designed to clarify biological activities of the tannins and their related compounds, especially focusing on hypertension.

Renin is well known to be released in blood stream from kidney and converted angiotensinogen to angiotensin I, which is then hydrolyzed to angiotensin II by angiotensin converting enzyme (ACE).[8] It is well known that angiotensin II has hypertensive activity. Recently, some inhibitors of *in vitro* ACE activity such as captopril (SQ 14,225) and nonapeptide (SQ 20,881) have been widely used for the treatment of hypertension.[9,10]

In this study, we attempted the isolation of substances inhibiting ACE activity from the leaf of *Q. stenophylla*.

Materials and Methods

Materials : A Hippuryl-L-Histidyl-L-Leucine (Hip-His-Leu) was obtained from Sigma Co. and used as a substrate for angiotensin converting enzyme (ACE). 2.5 mM Hip-His-Leu was dissolved in 100 mM phosphate buffer containing 300

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mM NaCl (PBS, pH 8.3). Test compounds were dissolved in PBS (pH 8.3). Column chromtography was carried out using silica gel 60 (Wako Chemical Co.) as the absorbent. Other chemicals were reagent grade.

Preparations of angiotensin converting enzyme (ACE) : ACE were isolated from the rat lung by the methods of Takada *et al.*[11] ACE were dissolved in PBS (pH 8.3).

Measurement of inhibitory effects on ACE activity : A mixture of 2.5 mM Hip-His-Leu (0.15 ml) and ACE solution (0.1 ml ; ACE activity : 2.64 units/mg protein, 1 unit : 1 μmoles/hippuric acid/min/ml reaction mixture) were incubated with or without indicated amounts of test compounds at 37℃ for 30 min in a final volume of 0.35 ml. The reaction was stopped by adding 1 N HCl (0.25 ml) and the mixture was extracted with ethyl acetate (2.0 ml). The ethyl acetate phase (1.0 ml) was evaporated, and the residue was dissolved in water (2.0 ml). And then, the free hippuric acid was determined by ultraviolet (UV) absorption at 280 nm for detection.

Isolation of the inhibitory substances from the leaf of Q. stenophylla on ACE activity : The leaf (500 g) of *Q. stenophylla* MAKINO collected in Tokushima Prefecture of Japan was extracted with methanol (1 l×2) at 37℃ for 3 hrs, and then the methanol solutions were concentrated *in vacuo* to give the methanol extracts. The methanol extracts were suspended in water, the suspensions were extracted with chloroform and divided into two fractions, chloroform-insoluble and chloroform-soluble fractions, respectively. The chloroform-insoluble fraction was chromatographed on a silica gel column with acetone-methanol-water (6 : 2 : 1, v/v) and further rechromatographed on a silica gel column to fractionate an inhibiotry substance.

Results

Isolation of ACE inhibitory substances from the leaf of Quercus stenophylla

The leaf of *Q. stenophylla* was extracted with methanol and the resulting extract was concentrated *in vacuo*. The residue was then suspended in water and extracted with chloroform, divided into two fractions, chloroform-insoluble and chloroform-soluble fractions. The preparation procedure was outlined in Fig. 1.

As shown in Fig. 2, both the methanol extracts and chloroform-insoluble fractions were found to inhibit the ACE activity.

Furthermore, the chloroform insoluble fraction was chromatographed on a silica gel column with acetone-methanol-water (6 : 2 : 1, v/v) as the eluants. The active fraction was rechromatographed on a slica gel column to give an inhibitory substance on ACE activity (Fig. 3). The inhibitory substance was obtained as a colorless crystalline powder having mp. 171—174 and $(\alpha)D^{20}+17.1$ (c=1.01, ethanol) by recrystallization from a mixture of methanol and chloroform. The infrared (IR) and proton nuclear magnetic resonance (^{1}H-NMR) spectra of the inhibitory sub-

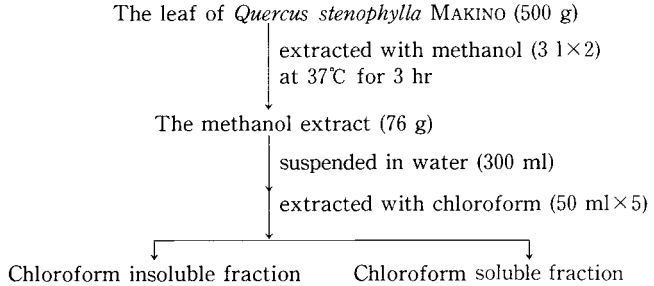

Fig. 1 The preparations of various fractions of the leaf of *Q. stenophylla.*

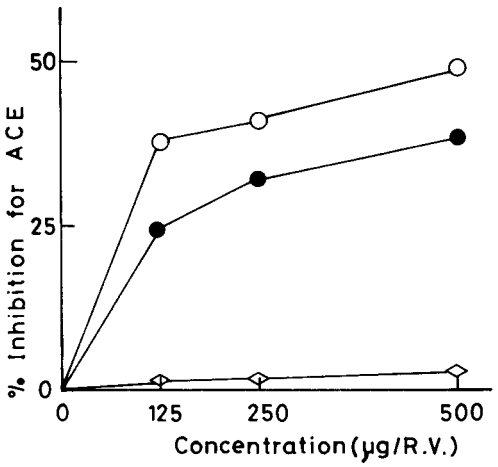

Fig. 2 Inhibitory effects of the various fractions of the leaf of *Q. stenophylla* on ACE activity. Values are means for 2 experiments. •, methanol extracts ; ○, chloroform insoluble fraction ; ◇, chloroform soluble fraction ; R.V., reaction volume.

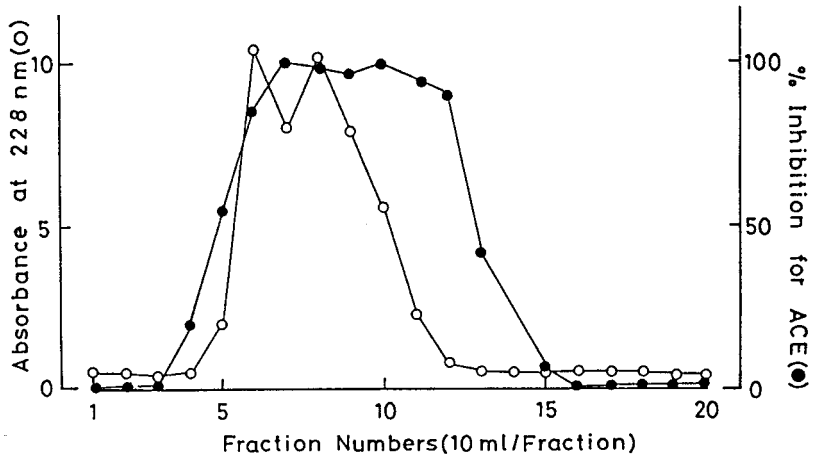

Fig. 3 Isolation of inhibitory substances from the leaf of *Q. stenophylla*. The chloroform insoluble extracts (2 g) were chromatographed on a silica gel column (ϕ 1.5 cm×80 cm) with acetone-methanol-water (6 : 2 : 1) as the eluant.

stance are identical with those of authentic sample of (+)-catechin. Furthermore, the melting point was not depressed on admixture with the authentic sample of (+)-catechin. And the inhibitory substance was identified as (+)-catechin. (+)-Catechin showed of the dose-dependent inhibition for ACE activity (Fig. 4).

Effects of (+)-catechin and (−)-epicatechin on ACE activity

As the inhibitory substance isolated from the leaf of *Q. stenophylla* on ACE activity was identical with (+)-catechin, (−)-epicatechin, a steroisomer of (+)-catechin was subjected to examination of an inhibitory action on ACE activity. It

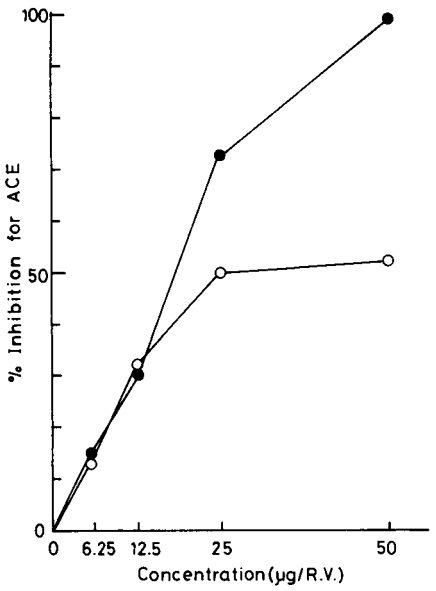

Fig. 4 Inhibitory effects of (+)-catechin and (−)-epicatechin on ACE activity.
Values are means for 2 experiments.
○, (+)-catechin ; ●, (−)-epicatechin ; R.V., reaction volume.

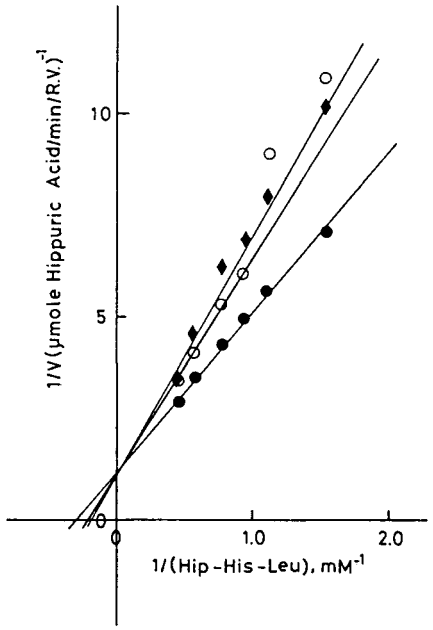

Fig. 5 Lineweaver-Burk plot of ACE with Hip-His-Leu as substrate in the presence or absence of (+)-catechin and (−)-epicatechin.
●, Hip-His-Leu alone ; ○, (+)-catechin (15 μg/reaction volume) ; ◆, (−)-epicatechin (15 μg/reaction volume).

was found that (−)-epicatechin also strongly inhibited the ACE activity. For characterization of the mechanism of inhibition of ACE by (+)-catechin and (−)-epicatechin, the enzyme activity was assayed at various concentrations of Hippuryl-L-Histidyl-L-Leucine (His-His-Leu) as a substrate in the presence and absence of (+)-catechin or (−)-epicatechin. A Lineweaver-Burk plot of the data in Fig. 5 shows that (+)-catechin and (−)- epicatechin inhibited ACE activity competitively. The V_{max} value of the ACE for Hip-His-Leu (substrate) was 8.1×10^{-4}M. The 50% inhibitory concentration (IC_{50}) values of (+)-catechin and (-)-epicatechin for ACE activity were 1.55×10^{-4}M and 2.16×10^{-4}M, respectively.

Discussion

The renin-angiotensin system is one of the major homeostatic mechanisms regulating arterial pressure and salt and water balance.[12,13] The renal enzyme renin, reacting with a substrate present in blood, forms firstly an inactive decapeptide, angiotensin I ; angiotentin I is then converted to the active octapeptide, angiotensin II by angiotensin converting enzyme (ACE).[8] It is well known that angiotensin II has a hypertensive activity. It has been reported that the nonapeptide SQ 20,881, Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro is shown to be a specific and potent inhibitor of angiotensin converting enzyme *in vitro* and *in vivo*.[10] Ondetti *et al.*[10] found that angiotensin converting enzyme, like carboxypeptidase A, was a zinc-containing metalloprotein. Moreover, they suggested that the active site of angiotensin converting enzyme would be similar to that carboxypeptidase A. And they hypothesized that the active site of this enzyme might have a group

capable of interacting with the COOH-terminal amide bond of the substrate, probably through hydrogen bonding, and the zinc ion must be suitably located at the active site of this enzyme to polarize the carbonyl groups of the scissile amide bonds making it more sussceptible to hydrolytic cleavage. For the mechanisms of an angiotensin converting enzyme inhibitor, SQ 14,225 (captopril), Ondetti *et al*.[10] suggested that the interaction of the carboxyl group with the zinc atom of this enzyme plays an important role in determining the inhibitory potency.

Okuda *et al*.[14] reported that three tannins such as geraniin, punicalin and punicalagin, and a related compound (−)-epigallocatechin gallate interacted with the heavy metal ions such as Fe^{3+}, Cu^{2+}, Pb^{2+}, Cr^{6+} and Hg^{2+}. The present studies were found that (+)-catechin isolated from the leaf of *Q. stenophylla* and (−)-epicatechin were competitive inhibitor for angiotensin converting enzyme activity. These results suggest that (+)-catechin and (−)-epicatechin might be interacted with the zinc atom in angiotensin converting enzyme, and they might cause the competitive inhibitions for angiotensin converting enzyme in cosequence. Furthermore, it is found that the inhibitory effects of (+)-catechin on angiotensin converting enzyme activity are stronger than those of (−)-epicatechin. These results suggest that the difference of inhibitory actions of (+)-catechin and (−)-epicatechin on angiotensin converting enzyme may be due to stero-configuration of these compounds.

It was thus of great interest that (+)-catechin was firstly isolated from the leaf of *Q. stenophylla* or medicinal plants as the inhibitory substances on angiotensin converting enzyme, and flavan-3-ol such as (+)-catechin and (−)-epicatechin were structurally different from the well-known angiotensin converting enzyme inhibitor, such as, nonapeptide SQ 20,881 and captopril SQ 14,225.

Experiments are further needed to examine their clinical significances in the treatment of hypertension.

和文抄録

ウラジロガシ葉（*Quercus stenophylla*）は日本，特に徳島県下で尿路結石および高血圧の治療薬として民間的に用いられている。著者らはウロジロガシ葉の抽出物を分画し，その各フラクションのアンギオテンシン変換酵素に対する阻害効果を検討した。その結果，メタノール抽出エキスがアンギオテンシン変換酵素活性を阻害することが判明したので，その活性成分はメタノール抽出エキスをクロロホルムで処理した後，シリカゲルカラムクロマトグラフィーによって分離した。その阻害物質は(+)-catechin として同定した。(+)-catechin ばかりでなく立体異性体である（−)-epicatechin もまた強い阻害効果を示し，そして，両者はアンギオテンシン変換酵素に対して拮抗阻害剤として作用した。

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