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Article

Isolation and Identification of Human Renin Inhibitor from *Aralia cordata* (Udo)

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We screened for human renin inhibitory activity in wild vegetables and found the activity in methanol extract of *Aralia cordata*. Physico-chemical data on the isolated inhibitors were identical to those of (–)-kaur-16-en-19-oic acid (kaurenic acid, KA) and pimaradienoic acids (PDA, 8:2 mixture of pimara-8(14), 15-dien-19-oic acid: pimara-9(11) 15-dien-19-oic acid). KA and PDA also inhibited porcine renin but had nearly no effect on other proteinases. This is the first demonstration of renin inhibitors in wild vegetables.

Key words: renin, inhibitor, *Aralia cordata*, kaurenic acid, pimaradienoic acid.

Introduction

Renin (EC 3. 4. 23. 15) is a highly specific aspartic proteinase mainly synthesized by juxtaglomerular cells in the kidney cortex. The enzyme catalyzes the liberation of decapeptide angiotensin I (AI) from plasma substrate angiotensinogen. This is the first step of the renin-angiotensin (RAS) system, a well-characterized physiological modulator of blood pressure and electrolyte balance [1]. The produced AI is an inactive peptide and activated by angiotensin converting enzyme (ACE, EC 3. 4. 15. 1). ACE removes a dipeptide His-Leu from the C-terminus of AI. The produced octapeptide giotensin II (AII) directly acts on arterial smooth muscle cells to maintain blood pressure. AII also accelerates the release of aldosterone from the adrenal cortex to induce sodium retention in the kidney. This also induces high blood pressure. Thus, control of RAS is the major target of cardiovascular disease therapies [2, 3].

ACE inhibitory peptides in various foodstuffs have been demonstrated to control RAS [4-6]. Renin is one of the most important enzymes in RAS. However, the screening for renin inhibitors among foodstuffs has not been well studied because of the need for preparation of human renin and the complication of the renin assay. Recently, we screened for renin inhibitory compounds in various foodstuffs using recombinant human (rh)-renin as a target enzyme [6-10] and found that fermented soybean paste miso contains a renin inhibitory compound,

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soyasaponin I, which is derived from soybean [10].

More recently, we found renin inhibitory activity in rice and cereals [11, 12]. The purified rice inhibitor preparation contained two unsaturated fatty acids, oleic acid and linoleic acid [11]. In the present study, we screened the extracts of 34 wild vegetables from Akita prefecture, Japan, for renin inhibitory activities. The screening showed that some wild-vegetable extracts had renin inhibitor activities. Methanol extract of *Aralia cordata* showed the highest renin inhibitory activity. We isolated active compounds from *A. cordata* and the chemical structures of the purified renin inhibitors were identified as kaurenic acid and pimaradienoic acids.

Materials and Methods

Materials Porcine pepsin (Lot SDK5232), bromeline (Lot DPG0653), bovine trypsin (Lot TSQ4876) were obtained from Wako Pure Chemical (Osaka, Japan). Subtilisin (Lot 20K1585), and rabbit lung ACE (Lot 056K74251) were from Sigma (St. Louis, MO, USA). Papain (Lot DL739221) was obtained from MP Biomedicals (Illkirch, France). Aminopeptidase M (Lot DL739221) was from Pierce (Rockford, IL, USA). Fluorogenic, internally quenched fluorogenic (IQF), and peptidyl-pNA substrates were obtained from Peptide Institute (Osaka, Japan). Porcine kidney renin was purified by a previously reported method [13]. Recombinant human (rh)-renin expressed in Sf-9 insect cells was prepared by the method of Takahashi *et al* [14].

Renin activity The rh-renin activity was measured using internally quenched fluorogenic (IQF) substrate: *N*-methylanthranyl-(Nma)-Ile-His-Pro-Phe-His-Leu*Val-Ile-Thr-His-N^{ε}-2, 4-dinitrophenyl-Lys (Lys(Dnp))-D-Arg(r)-r-HN_2 (*, scissile peptide bonds) [14]. The reaction mixture contained 40 µl of 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, 25 µM IQF substrate, 0.02% Tween 20, 5 µl of renin solution (5 µg/ml), and 5 µl of inhibitor solution in a total volume of 50 µl. The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by adding 200 µl of 0.1 M triethanolamine, pH 9.5. The increase of fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation at 340 nm. The sample concentration required to inhibit 50% of renin activity under the assay conditions was taken as the IC_{50} value.

Preparation of inhibitor solution and physico-chemical analysis Freeze-dried wild vegetables were extracted with methanol and evaporated to dryness. The evaporated sample was dissolved in methanol at a final concentration of 10 mg/ml. The sample was used for measuring renin inhibitory activity. Isolated compounds were analyzed by ESI-HR-MS, ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR, and $[\alpha]_D$ measurements.

Results and Discussion

Screening of renin inhibiton We tested renin inhibitory activity of methanol extracts from 34 wild vegetables (Fig. 1). Among them, *Pilea hamanoi* (Mizu), *Souchus asper* (Oninogeshi), *A. elata* (Taranoki), and *A. cordata* (Udo) extracts (final concentration of 0.1 mg/ml) inhibited renin activity by more than 20% compared with the control and *A. cordata* extract had the highest renin inhibitory activity. Thus, we tried to purify renin inhibitory compounds from *A. cordata*.

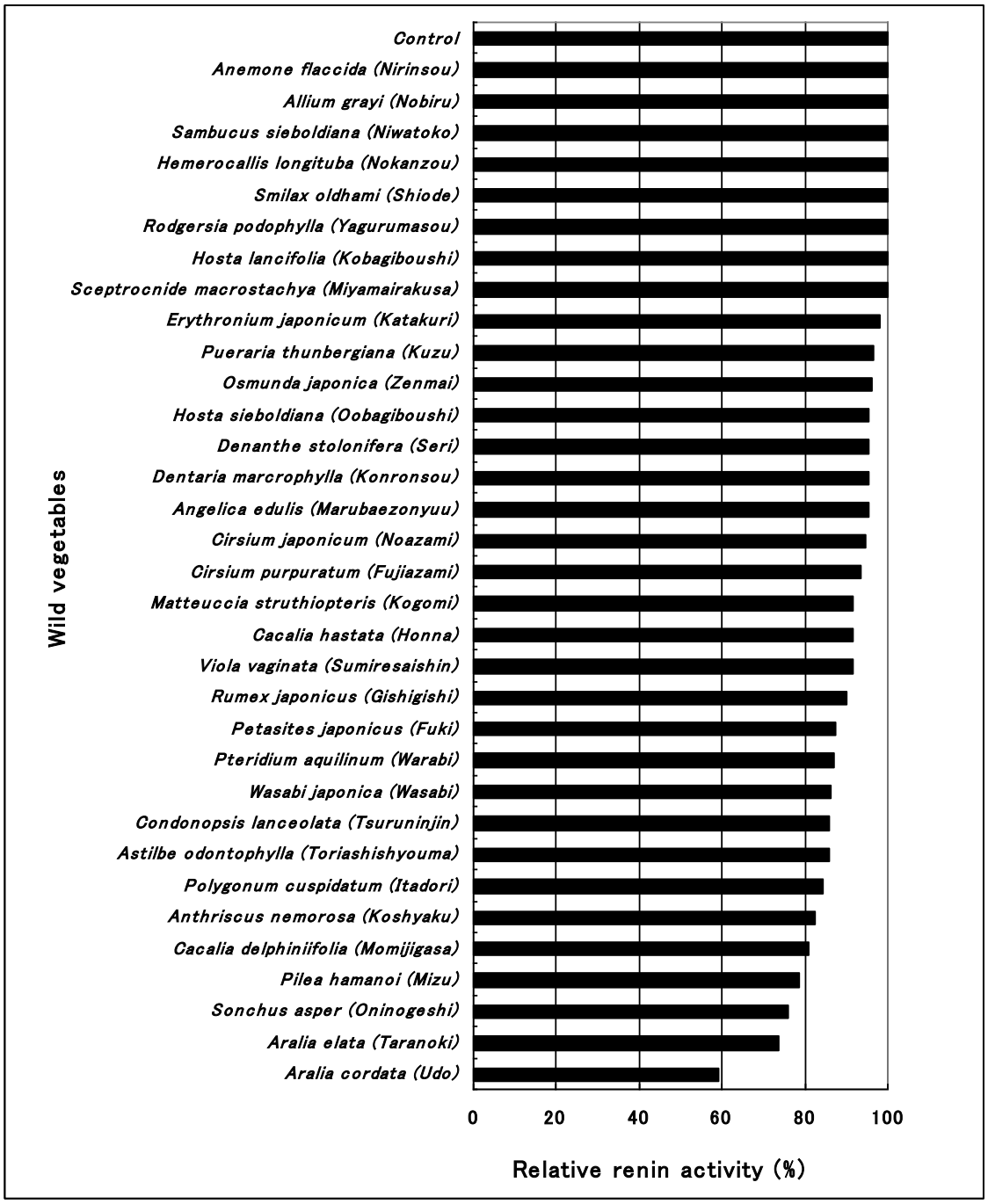

Fig. 1. Inhibition of rh-renin activity by wild-vegetable extracts.

Crude methanol extracts of wild vegetables were used for rh-renin inhibition assay. The final concentration of the extracts was 0.1 mg/ml. Each result is the mean value of triplicate determinations.

Purification and identification of renin inhibitors from *A. cordata* (Udo)

Freeze-dried *A. cordata* powder (75 g) was soaked in 1.2 liters of methanol at room temperature for 1 h then centrifuged at 10,000 x g for 30 min. The supernatant was evaporated to dryness, and the dry matter (8.1 g) was dissolved in 240 ml of 100% methanol. Insoluble materials were removed by centrifugation and the supernatant was evaporated to dryness (5.7 g). A part of the sample (1 g) was dissolved in 10% methanol and applied to Sep-Pak Vac C_{18} 35cc (Waters, Bedford, USA) that had been equilibrated with distilled water. The column was washed with distilled water and 50% methanol. The adsorbed materials were eluted with 100% methanol. The eluate was evaporated to dryness, and the dry matter (0.116 g) was dissolved in 10 ml of 100% methanol. The sample was put on a Sephadex LH-20 column (2.0 x 60 cm) equilibrated with 100% methanol. The column was eluted with the same solvent and fractions containing rh-renin inhibitory activity were pooled. The active fractions were evaporated to dryness (21.6 mg). The dry matter was purified by silica gel chromatography (hexane–EtOAc) to afford a 4:4:1 mixture of three compounds (31.1 mg), which were separated by PTLC (Merck RP-18, 100% acetonitrile) into a polar mixture of two isomers and a less polar compound. These compounds were identified as an 8:2 mixture of (–)-pimara-8(14), 15-dien-19-oic and pimara-9(11), 15-dien-19-oic acids (PDAs, Fig. 2 B and C), and (–)-kaur-16-en-19-oic acid (KA, Fig. 2A), on the basis of HR-MS, NMR, and $[\alpha]_D$ data. The ^{1}H and ^{13}C NMR shifts were identical to those reported in the literature [15, 16] (Fig. 2).

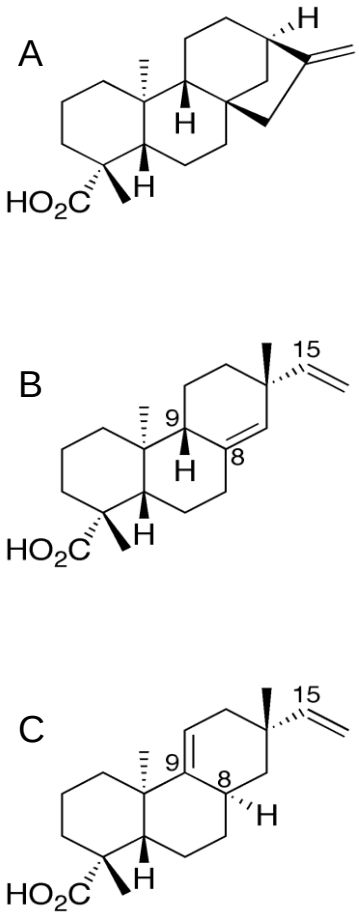

Fig. 2. Chemical structures of (–)-kaur-16-en-19-oic acid (KA) (A), pimara-8(14), 15-dien-19-oic acid (B), and pimara-9(11), 15-dien-19-oic acid (C).

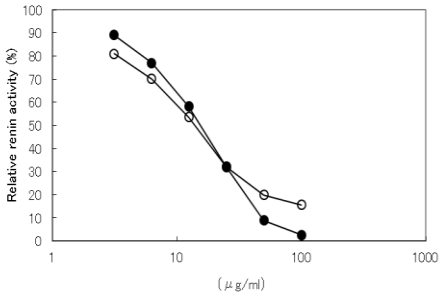

Fig. 3. Effects of KA and PDA on rh-renin activity. The rh-renin was incubated with the indicated amount of KA (-○-) and PDA (-●-). Each result is the mean value for triplicate determinations.

Table 1. Effects of KA and PDA on Proteinase Activities.

Enzyme[*1]	Substrate[*2]	Reaction buffer	Inhibition (%)[*3] KA	PDA
Aspartic protease				
Human renin (0.5 μg/ml)	Nma-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dnp)-r-r-NH_2 (20 μM)	Buffer A*4	79.0	90.8
Porcine renin (0.5 μg/ml)	Nma-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Lys(Dnp)-r-r-NH_2 (20 μM)	Buffer A[*4]	74.4	88.1
Porcine pepsin (0.5 μg/ml)	MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-r-NH_2 (20 μM)	Buffer B[*5]	N. I.	N. I.
Serine protease				
Bovine trypsin (1.0 μg/ml)	Pro-Phe-Arg-MCA (0.2 mM)	Buffer C[*6]	N. I.	N. I.
Subtilisin (10 ng/ml)	Suc-Ala-Ala-Ala-pNA (0.2 mM)	Buffer D[*7]	N. I.	N. I
Cysteine protease				
Papain (10 ng/ml)	Pro-Phe-Arg-MCA (0.2 mM)	Buffer E[*8]	N. I.	N. I.
Bromeline (2.0 μg/ml)	Pro-Phe-Arg-MCA (0.2 mM)	Buffer E[*8]	18.4	9.9
Metallo protease				
Rabbit ACE (1 mU/ml)	Hip-His-Leu (2.0 mM)	Buffer F[*9]	N. I.	N. I.
Aminopeptidase M (0.2 μg/ml)	Leu-MCA (0.2 mM)	Buffer G[*10]	N. I.	N. I

[*1] Values in parentheses indicate final enzyme concentrations; [*2] Values in parentheses indicate final substrate concentrations; [*3] The ratios of enzyme inhibition in the presence of 0.15 mM of KA or PDA are indicated; [*4] 50 mM Na-phospahte, pH 6.5, 0.1 M NaCl, and 0.02% Tween 20; [*5] 0.1 M Na-citrate, pH 3.0, 0.1 M NaCl, and 0.02% Tween 20; [*6] 50 mM Tris-HCl, pH 7.5, and 0.02% Tween 20; [*7] 50 mM Tris-HCl, pH 8.0, and 0.02% Tween 20; [*8] 20 mM Na-phosphate, pH 6.0, and 0.02% Tween 20; [*9] 0.1 M HEPES, pH7.5, 0.3 M NaCl, and 0.01% Triton X-100; [*10] 50 mM Tris-HCl, pH 7.5, and 0.02% Tween 20.

N.I., no inhibition.

Effects of KA and PDA on renin and other proteinases

The crude methanol extract of *A. cordata* inhibited rh-renin activity in a dose-dependent manner with IC_{50} value of 130 μg/ml. The isolated KA and PDA inhibited rh-renin activity strongly with IC_{50} values of 14.0 and 15.6 μg/ml (46.3 and 51.2 μM), respectively (Fig. 3).

Table 1 shows the inhibitory spectra of KA and PDA. KA and PDA strongly inhibited rh-renin and porcine renin activities. KA and PDA also weakly inhibited cysteine proteinase bromeline but they had no effects on pepsin, bovine trypsin, subtilisin, papain, rabbit ACE, or aminopeptidase M. These results indicate that KA and PDA are renin-specific inhibitors from *A. cordata*.

Some physiological functions of KA and PDA have been reported. These compounds exhibit pharmacological activities such as analgesic, sedative, and anti-inflammatory effects [15, 17]. Moreover, the effects of PDA and its related compounds on large-conductance K^+ channels were examined using human embryonic kidney 293 cells. The measurement of BKαβ1 opening under whole-cell voltage clamp showed that these compounds activated BKαβ1 in a similar manner [18]. On the other hand, effects of these compounds on proteinases have not been reported.

In conclusion, this is the first demonstration of renin inhibitory activities of KA and PDA. To understand the anti-hypertensive effects of KA and PDA, animal experiments using spontaneously hypertensive rats or Dahl salt-sensitive rats are necessary.

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