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Review

Renin Inhibitors in Foodstuffs: Structure-Function Relationship

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Renin, a highly specific aspartic proteinase originated from kidney, is a rate-limiting enzyme in renin-angiotensin system. Recently, we developed effective expression system for recombinant human (rh) renin using baculovirus-insect cell expression system, and simple and rapid assay method for rh-renin using internally quenched fluorogenic (IQF) substrate. Using the purified rh-renin and the IQF substrate, we constructed rapid and sensitive renin inhibitor screening system. Using the system, we found renin inhibitors in various foodstuffs. The isolation and characterization of renin inhibitors from soybean, rice, and wild vegetables and structure-function relationship of the inhibitors are also discussed.

Key words: renin, angiotensin, internally quenched fluorogenic substrate, soyasaponin I, oleic acid, linoleic acid, free fatty acids, kaurenic acid, pimaradienoic acid.

Introduction

Renin-angiotensin system (RAS) is one of the most important blood pressure control system in mammals. Renin catalyzes liberation of angiotensin I (AI) from plasma substrate angiotensinogen. AI is an inactive peptide and activated by angiotensin I converting enzyme (ACE). ACE cleaves C-terminal dipeptide from AI and produces active octapeptide

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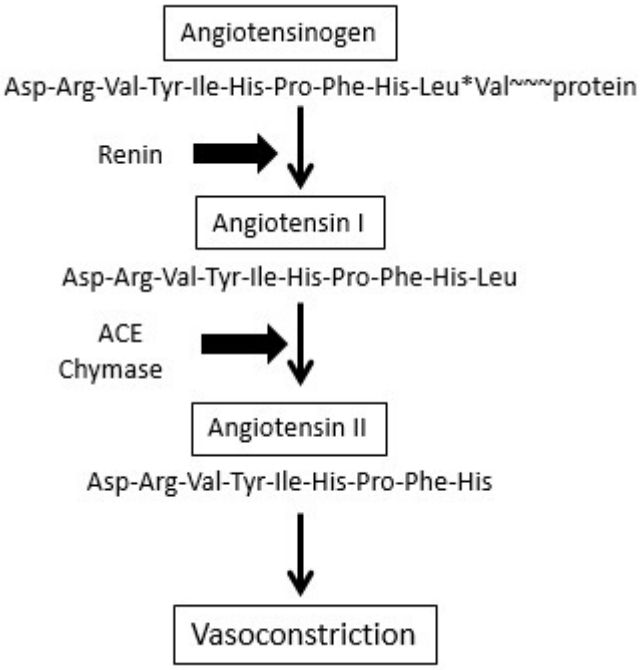

Fig. 1. Renin-angiotensin system (RAS).
Renin is synthesized mainly in the juxtaglomerular cells in the kidney cortex. The synthesized renin was released into the circulation by several stimuli. Renin catalyzes the liberation of angiotensin I from plasma substrate angiotensinogen. Thus, renin is the rate-limiting enzyme in RAS.

angiotensin II (AII) (Fig. 1). AII directly acts on arterial smooth muscle cells to maintain blood pressure and increases release of aldosterone from adrenal cortex to increase reabsorption of water from the kidney. This also causes high blood pressure [1]. ACE has been used to screen inhibitors from various foodstuffs because of its simple assay method. Although renin is the most important enzyme in RAS, screening for renin inhibitors among foodstuffs has not been well studied due to the complications of the renin assay. In this review we describe expression of recombinant human (rh) renin in *Spodoptera frugiperda* (Sf-9) insect cells, development of a simple and rapid assay method for human renin, occurrence of renin inhibitors in various foodstuffs, isolation of renin inhibitors, and structure-function relationship of them.

Expression of recombinant human renin in Sf-9 insect cells

The expression of rh-prorenin was first demonstrated in *Escherichia coli* cell [2]. The expressed rh-prorenin formed inclusion bodies and was difficult to refold. Recently, we expressed rh-prorenin in *E. coli* cells as a fusion protein with thioredoxin and the expressed rh-prorenin was refolded systematic dialysis and

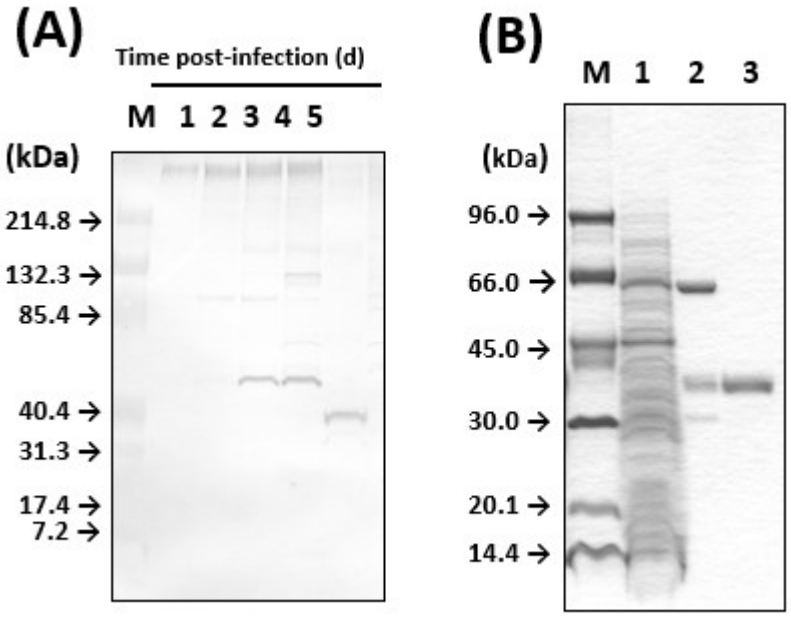

Fig. 2. Expression of rh-renin in Sf-9 insect cells (A) and SDS-PAGE of purified rh-renin.
A, Western blotting of culture medium using anti-human renin anti-serum. M, molecular weight marker (Kaleidoscope molecular weight marker, Bio-Rad), A 10 μl of culture mediums were used for the electrophoresis. B, SDS-PAGE of rh-renin. After the electrophoresis, proteins were stained with Coomassie brilliant blue R-250. M, molecular weight marker (Low molecular weight marker, GE-Health Care) ; lane 1, culture medium (10 μl); lane 2, pepstatin column eluate (1.3 μg of protein); lane 3, purified rh-renin (1.0 μg of protein).

activated by trypsin [3]. The active rh-renin was used for the screening of renin inhibitors and found that commercially available fermented soybean, miso contained renin inhibitory activity [3]. On the other hand, expression of rh-prorenin or rh-renin in mammalian and insect cells has been reported [4-9]. In these cases, major expressed protein was inactive prorenin. Trypsin treatment was essential for the activation of prorenin. We also used a recombinant baculovirus, vhpR, carrying human preprorenin cDNA in the polyhedrin locus of *Autorgapha californica* multiple nuclear polyhedrosis virus (AcMNPV) [10]. On an infection culture at a MOI of 1 pfu/cell using SF-900II serum-free medium on an orbital shaker at 100 rpm at 28°C. Cell grew continuously until day 3, but total cell numbers and viability decreased at day 4 and 5 of culture. Renin activity was not detected until day 3, but a small amount of renin activity was detected at day 4 and appeared dramatically at day 5. Western blotting using anti-human renin antiserum [11], 43 kDa prorenin was detected at days 3 and 4 cultures. On the other hand, only a

40 kDa mature renin was detected in the day 5 culture (Fig. 2A). These results clearly show that the expressed prorenin was activated by proteinase appearing at late stage of culture. Our recent study also showed that 32 kDa cysteine protease derived from AcMNPV gene is the prorenin processing enzyme [12]. Using the day 5 culture, we purified rh-renin using pepstatin affinity column chromatography and Mono Q FPLC. The purified rh-renin showed single protein band on SDS-PAGE with the molecular weight of 40 kDa [10] (Fig. 2 B).

Development of internally quenched fluorogenic substrate for human renin

In 1969 Haber *et al.* developed sensitive renin assay method using angiotensin I radioimmunoassay [13]. This method is highly sensitive, but we have to prepare various reagents including ^{125}I-labeled AI, angiotensinogen, and anti-AI anti-serum. In 1980, Murakami *et al.* developed fluorogenic substrate for porcine renin, succinyl (Suc)-Arg-Pro-Phe-His-Leu*Leu-Val-Tyr-4-methylcoumaryl-7-amide (MCA) [14]. Porcine renin cleaves the substrate at Leu-Leu bonds, releasing Leu-Val-Tyr-MCA. This peptide was then cleaved by aminopeptidase to release free 7-amino-4-methyl- coumarin. This method is relatively simple, but the sensitivity for human renin is very low. Recently, we developed novel internally quenched fluorogenic (IQF) substrates for porcine [15] and human renin [10, 16]. The structures of both substrates are as follows: 2-(methylamino) benzoyl (Nma)-His-Pro-Phe-His-Leu*Leu-Val-Tyr-[N^{ξ}-(2,4- dinitro phenyl)-Lys] [Lys(Dnp)]-D-Arg (r)-r-NH_2 and Nma-Ile-His-Pro-Phe-His-Leu*Val-Ile- His-Thr-Lys(Dnp)-r-r-NH_2 (*, scissile peptide bond). Hydrolysis of Leu-Leu bond of porcine renin substrate or Leu-Val bond of human renin substrate was spectrophotometrically determined. In the case of human renin assay, the reaction mixture contained 1 μl of 1 mM IQF substrate in DMSO, 44 μl of sodium phosphate buffer, pH 6.5, containing 0.1 M NaCl, 0.02% Tween 20, and 0.02% NaN_3, and 5μl of rh-renin solution in a total volume of 50 μl. The reaction mixture was incubated at 37℃ for 30 min and the reaction was terminated by adding 0.1 M triethanolamine, pH 10.5. The increase in the fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation wavelength at 340 nm. The K_m and k_{cat} values of rh-renin for the synthetic substrate at the above conditions were $35.7\mu M^{-1}$ and $833s^{-1}$ respectively. The assay system is suitable for the measurement of rh-renin activity and the screening of rh-renin inhibitors from various foodstuffs and natural products.

Renin inhibitor in soybean

Using the rh-renin as target enzyme,

we screened the inhibitory activity of various foodstuffs and found that miso extract contains rh-renin inhibitory activity originated from soybean. Thus, we try to isolate renin inhibitor from soybean.

Before isolation of rh-renin inhibitor from soybean, we investigated the localization of renin inhibitor in soybean. Soybean was separated into two parts, embryo and cotyledon. Both parts were extracted with hot water and the extracts were concentrated by Sep-Pak ODS column (Millipore, USA) and evaluated for renin inhibitory activity. Embryo extract contained about 3-fold higher inhibitory activity than cotyledon extract, hence we used soybean embryo for isolation of renin inhibitor. Approximately 70 mg of soybean renin inhibitor (SRI) was obtained from 750 g of soybean embryo. The isolated SRI gave soyasapogenol B moiety and sugar chain unit as rhamnopyranosyl (1 → 2) galactopyranosyl (1 → 2) glucopyranosiduronic acid for ^{1}H and ^{13}C NMR spectra [17-19]. Finally, the SRI was identified as soyasaponin I by direct comparison with standard compound for $[\alpha]_D$, mixed melting point, ^{1}H NMR, and IR spectra (Fig. 3), [16].

Soyasaponin I inhibited rh-renin activity in a dose dependent manner with IC_{50} value of 30 μg/ml. Kinetic studies with soyasaponin I indicated partial noncompetitive inhibition with K_i value of

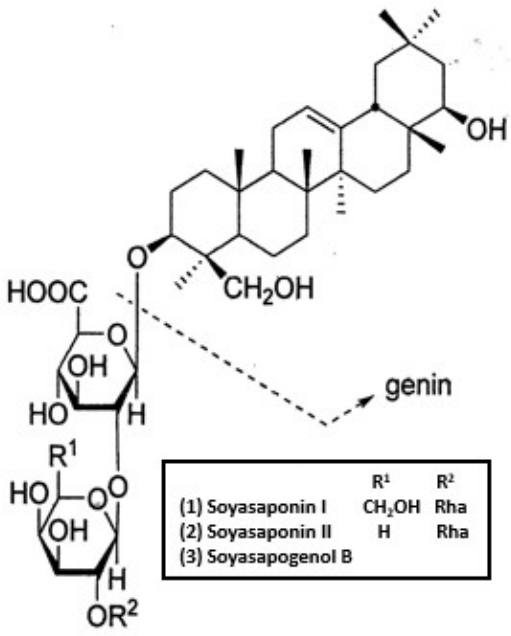

Fig. 3. Structures of soyasaponins.
Soyasaponin I and II inhibited rh-renin activity. On the other hand, soyasapogenol B had no effect on rh-renin.

37.5μM. The inhibitory spectra of soyasaponin I are shown in Table I. Soyasaponin I also inhibited porcine renin activity with an IC_{50} value of 30 μg/ml. Soyasaponin I weakly inhibited porcine pepsin or cysteine proteases, papain and bromeline, and had no effect on serine proteases or metallo proteinases. These results clearly indicate that soyasaponin I is a renin-specific inhibitor in soybean.

The effects of soyasaponin on blood pressure of spontaneously hypertensive rats (SHR) were also investigated [20]. Oral administration of commercially available soybean saponin (Wako Pure Chemical Industries, Osaka) at 80 mg/kg of body weight per day to SHR/Izm strain for eight weeks significantly decreased the

Table 1 Effects of soyasaponin I on proteinase activity [16]

Enzyme	Inhibition* (%)	IC_{50} (μg/ml)
[Aspartic proteinase]		
Human renin	74.0	30
Porcine renin	85.0	30
Porcine pepsin	15.0	N. D.
[Serine proteinase]		
Bovine trypsin	0	N. D.
Human urinary kallikrein	0	N. D.
[Cysteine proteinase]		
Papain	25.5	N. D.
Bromeline	22.0	N. D.
[Metallo proteinase]		
Rabbit ACE	0	N. D.
Aminopeptidase M	0	N. D.

*, The ratios of enzyme inhibition in the presence of 0.1 mg/ml of soyasaponin I are indicated.
N. D., not determined.

systolic blood pressure. These results show that soybean saponin inhibited human and porcine renin activity *in vitro*, and improved hypertension in SHR.

The structure and function relationship of saponins on renin inhibition was also investigated using several saponins and sapogenols [21]. Soyasaponin I and II (from soybean) (Fig. 3), chikusetusaponin IV (from *Panax japonicas* Rhizome), glycyrrhizin (form licorice root), monoglucuronyl glycyeehetic acid (form licorice root), and *Kichia scoparia* fruit saponins (momordins) inhibited rh-renin activity. On the other hand, sapogenols (soyasapogenol B from soybean (Fig. 3) and glycyrrhetic acid from licorice root), saikosaponins b2 and c (from bupleurum root), and ginsenoside Rb_1 (from ginseng root) had no effects on rh-renin activity. These results clearly indicate that the 3-*O*-β-D-glucopyranosiduronic acid moiety in saponins is essential for renin inhibition.

Renin inhibitor from rice

We found rh-renin inhibitory activity in cooked rice after screening

Table 2 Effects of rice extract on renin activity and the concentration of fatty acids in rice extracts [22]

Rice cultivars	IC_{50} (μg/ml)	Linoleic acid (μmole/ml)	Oleic acid (μmole/ml)
Akitakomachi	280	9.96	2.76
Dewahikari	250	11.79	3.99
Haenuki	170	12.96	4.11
Hitomebore	280	12.38	3.04
Menkoina	150	13.11	3.94
Sasanishiki	160	14.41	4.21
Takaneminori	180	14.20	4.88
Yumeobako	270	8.81	3.43

Rice powder (25g) was soaked in 200 ml of methanol at room temperature for 1 h and then centrifuged at 10,000 x g for 30 min. The supernatant was evaporated to dryness, and the dry matter was dissolved in 10% ethanol at a final concentration of 40 mg/ml for renin inhibition assay and determination of the fatty acid concentration. Each results is the mean value of triplicate determination.

several foodstuffs. Therefore, we tested the effects of local cultivar rice extracts on rh-renin activity. Table II shows the effects of methanol extracts of various nonglutinous rice strains cultivated in Akita Prefecture on rh-renin activity [22]. All of the rice extracts tested inhibited rh-renin activity with IC_{50} values of 150-280μg/ml. Next we tried to isolate rice renin inhibitor (RRI) from Akitakomachi because it is the most important rice strain in Akita Prefecture. Akitakomachi powder (300 g) was soaked in methanol (2.4 litters) at room temperature for 1 h and then centrifuged at 10,000 x g for 30 min. The supernatant was evaporated to dryness. The dry matter was used for further purification. After several column chromatography steps, we isolate RRI preparation (59.6 mg). The preparation was used for the identification of RRI. The ^{1}H NMR spectrum of the isolated RRI showed signals arising from a mixture of unsaturated fatty acids, and the negative ESI-MS gave *m/z* 279 and 281 peaks. These data indicate that the RRI was a 2.5:1 mixture of linoleic acid and oleic acid. The isolated RRI preparation inhibited rh-renin activity with an IC_{50} value of 9.9μg/ml. To clarify the direct effects of oleic acid and linoleic acid on rh-renin activity, we used commercially available extra pure reagents for rh-renin inhibitory activity. Oleic acid and linoleic acid inhibited rh-renin activity in a dose-dependent manner with IC_{50} values 8.0 and 10.5μg/ml, respectively.

We further investigate the structure-function relationship of fatty acids on rh-renin activity; we tested the effects of several saturated and unsaturated fatty acids on it. Figure 4 shows the structures and IC_{50} values of various free fatty acids on rh-renin activity. In the case of C18 unsaturated fatty acids, mono- to tri-unsaturated fatty acids, *cis*-vaccenic acid, oleic acid, linoleic acid, and linolenic acid inhibited rh-renin activity. On the other hand, saturated fatty, stearic acid had no effect on activity. A C16 saturated fatty acid, palmitic acid, also had no effect on the activity. Other polyunsaturated fatty acids, arachidonic acid, eicosapentanoic acid (EPA), and docosahexanoic acid (DHA), also inhibited rh-renin activity. These results indicate that free unsaturated fatty acids inhibited rh-renin activity.

In connection with these results, the effects of circulating natural lipids on human plasma renin activity have been reported [23, 24]. Kotchen *et al*. found that acetone soluble lipid extracts from human plasma inhibit the *in vitro* renin activity [23]. Furthermore, they isolated a renin inhibitor from human plasma by affinity chromatography using mouse submaxillary grand renin as a ligand and concluded that circulating linoleic acid inhibits renin activity [24]. The effects of n-3 polyunsaturated fatty acids (PUFAs) on a model of human renin hypertension had also been reported [25]. Fischer *et al*.

compared the effects of PUFAs with direct renin inhibition in electrophysiological remodeling. They treated double-transgenic rats expressing the human renin and angiotensinogen genes weeks 4 to 7 with n-3 PUFAs ethyl-esters. Sprague-Dawley rats were used as control. Dietary n-3 PUFAs increased the cardiac contents of EPA and DHA. At week 7, mortality in the hypertension model rats was 31%, whereas none of the n-3 PUFAs or aliskiren-treated hypertension model rats died. Aliskiren is the first orally active inhibitor of human renin to be approved for clinical use as an antihypertensive agent [26]. Aliskiren-treated hypertension model rats and Sprague-Dawley rats were normotensive. These results indicate that n-3 PUFAs and aliskiren improved high blood pressure and the development of cardiac hypertrophy.

Renin inhibitor from cereals

We further screened for renin inhibitory activity in various types of rice and cereals and found renin inhibitory activity in rice and some cereals [27]. At the beginning, we tested the effects of

Table 3 Effect of Rice Extracts on rh-Renin Activity [22, 27]

Sample	Total extract (mg/g powder)	IC_{50} (μg/ml)	IU*1 (U/mg powder)	Linoleic acid (μmole/ml)*2	Oleic acid (μmole/ml)*2
[Nonglutinous rice]					
Akitakomachi	5.7	280	4.04	2.49	0.69
Dewahikari	6.3	250	5.04	2.95	1.00
Haenuki	6.3	170	7.41	3.24	1.03
Hitomebore	6.3	280	4.50	3.10	0.76
Menkoina	7.1	150	9.47	3.28	0.99
Sasanishiki	6.8	160	8.50	3.60	1.05
Takaneminori	7.4	180	8.22	3.55	1.22
Yumeobako	8.9	270	6.59	2.20	0.86
[Glutinous rice]					
Tatsukomochi	10.0	88	22.73	4.13	1.83
Kinunohada	10.1	125	16.03	7.52	3.22
[Sake rice]					
Miyamanishiki	6.1	155	7.82	3.72	1.47
Akitasakekomachi	5.8	170	6.82	3.08	1.21
Indica Rice	3.7	270	2.74	3.49	1.27

*1, IU, inhibitor unit
*2, Rice extracts (10 mg/ml) were used for determination of fatty acid concentration.

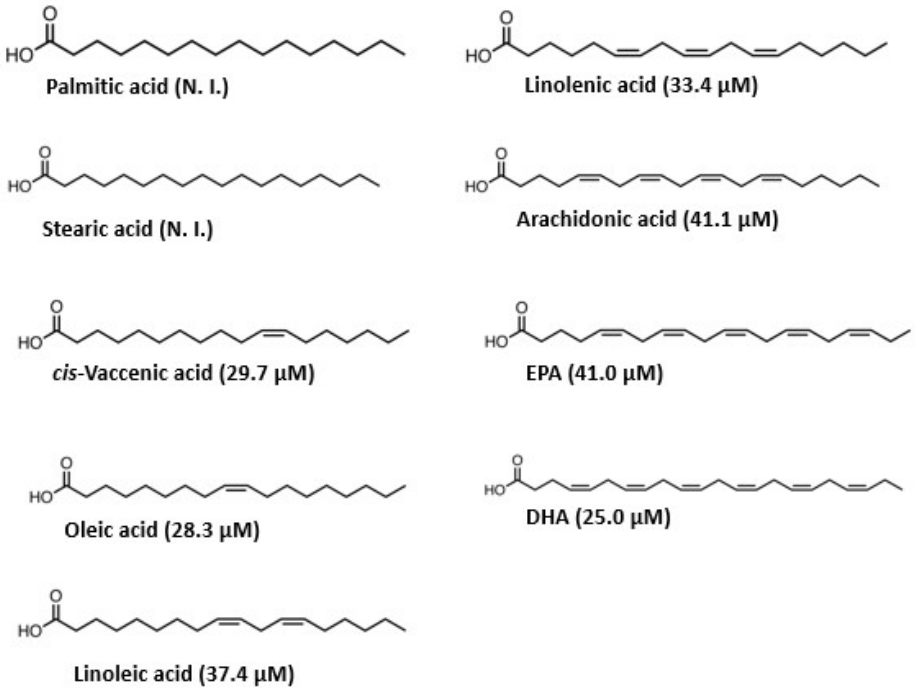

Fig. 4. Structures of free fatty acids and IC_{50} values.
Saturated fatty acids, palmitic acid and stearic acids, had no effect on rh-renin activity. Unsaturated fatty acids inhibited rh-renin activity in a dose-dependent manner. The IC_{50} values are indicated in the parenthesis. N. I., no inhibition.

glutinous, sake, and Indica rice extracts on rh-renin activity to understand the existence of renin inhibitor in rice universally. Table 3 shows the summary of the amount of rice extracts, IC_{50} values, inhibitor units (IU), and oleic acid and linoleic acid contents of various rice extracts. Rice extracts tested in this study inhibited rh-renin activity with IC_{50} values of 88-270 µg/ml. In the previous study, we found that oleic acid and linoleic acid are the major rice rh-renin inhibitors [24]. The IC_{50} values of rice extracts are correlated with the contents of oleic acid and linoleic acid. To evaluate the renin inhibitory activity per gram weight, we calculated the renin inhibitor per gram extracts. Nonglutinous rice, Akitakomachi and other strain had 4.04 to 9.47 IU of extract (Table 3) [22]. Glutinous rice, Tatsukomochi and Kimunohada showed 22.73 and 16.03 IU, respectively. Indica rice showed the lowest value of 2.74 IU. These results clearly showed that glutinous rice extracts had higher renin inhibitory activity than other rice extracts.

Cereals are staple food in many countries around the world including Japan. Table 4 shows effects of methanol extracts of various cereals on rh-renin activity. Flour for bread and weak flour were also used as a control. Among

Table 4 Effect of Cereal Extracts on rh-Renin Activity

Sample	Total extract (mg/g powder)	IC_{50} (μg/ml)	IU*1 (U/mg powder)	Linoleic acid (μmole/ml)*2	Oleic acid (μmole/ml)*2
Buckwheat (Soba)	9.8	173	44.7	2.61	2.66
Flour for Bread	11.9	>1000	Not determined	0.76	0.12
Weak Flour	14.2	>1000	Not determined	1.95	0.30
Rye	20.1	490	8.20	5.52	1.60
Foxtail millet (Awa)	36.3	>1000	Not determined	0.62	0.09
Barnyard millet (Hie)	28.7	265	21.6	7.15	2.39
Chinese millet (Kibi)	21.9	>1000	Not determined	0.41	0.11
Amaranth	40.9	>1000	Not determined	0.54	0.23

*1, IU, inhibitor unit
*2, Rice extracts (10 mg/ml) were used for determination of fatty acid concentration.

different cereal extracts, buckwheat extract showed highest rh-renin inhibitory activity followed by barnyard millet and rye extracts. On the other hand, control flours, foxtail millet, Chinese millet, and amaranth extracts had no effects on rh-renin activity. The concentration of oleic acid and linoleic acids are also shown in Table 4. Renin inhibitory cereals contain relatively high concentration of these unsaturated free fatty acids. The concentration of oleic acid and linoleic acid seem to be correlated with rh-renin inhibitory activity. However the existence of other unknown renin inhibitory compound in cereals cannot be ruled out.

Renin inhibitor from wild vegetables

We tested renin inhibitory activity of methanol extracts from 34 wild vegetables grew in Akita prefecture [28]. Among them, *Aralia cordata* (Udo) extract showed highest renin inhibitory activity. Thus, we tried to identify renin

Table 5 Effects of KA and PDA on proteinase activities [27]

Enzyme	Inhibition (%)* KA	PDA
[Aspartic protease]		
Human renin	79.0	90.8
Porcine renin	74.4	88.1
Porcine pepsin	N. I.	N. I.
[Serine protease]		
Bovine trypsin	N. I.	N. I.
Subtilisin	N. I.	N. I
[Cysteine protease]		
Papain	N. I.	N. I.
Bromeline	18.4	9.9
[Metallo protease]		
Rabbit ACE	N. I.	N. I.
Aminopeptidase M	N. I.	N. I

* The ratio of enzyme inhibition in the presence of 0.15 mM of KA or PDA is indicated; N.I., no inhibition.

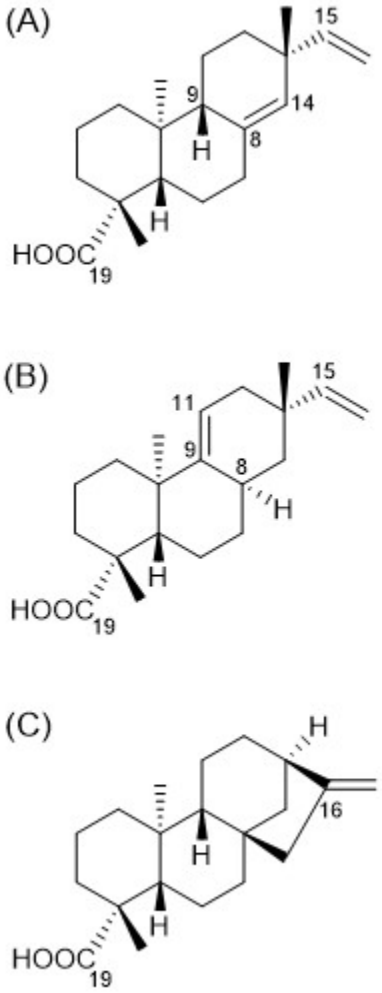

Fig. 5. Structures of (-)-pimera-8(14), 15-dien-19-oic acid (A) and pimera-9(11), 15-dien-19-oic acid (B), and (-)-kaur-16-en-19-oic acid (C).

inhibitor from *A.* cordata. From lyophilized powder, we finally obtained a polar mixture of two isomers and a less polar compound by preparative TLC. These compound were identified as an 8:2 mixture of (-)-pimera-8(14),15-dien-19-oic acid and pimera-9(11),15-dien-19-oic acid (PDAs, Fig 5 A and B), and (-)-kaur-16-en-19-oic acid (KA, Fig 5C). KA and PDA inhibited rh-renin activity dose-dependent manner with IC_{50} values of 46.3 and 51.2 μM, respectively. The effects of KA and PDA on several proteases were also investigated. Table 5 shows summary of inhibition of proteases by KA and PDA. KA and PDA strongly inhibited rh-renin and porcine renin activities. Both compounds also weakly inhibited bromeline. But they had no effect on other proteases. These results indicate that KA and PDA are renin-specific inhibitor from *A. cordata*.

Conclusion

In this study we isolated several renin inhibitory compounds from foodstuffs. The isolated compounds are soyasaponin I from soybean, oleic acid and linoleic acid from rice, and KA and PDA from *A. cordata*. As for renin inhibitors from foodstuffs, safety is guaranteed. These compounds will be beneficial ingredients for a therapeutic treatment against hypertension and related diseases.

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