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Article

Renin Inhibitory Activity in Rice and Cereals

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Our recent study showed the occurrence of renin inhibitory activity in nonglutinous rice. In the present study we screened for renin inhibitory activity in glutinous, sake, and indica rice, as well as various cereals. Rice extracts tested in this study inhibited renin activity with IC_{50} values of 88-270 μg/ml. Glutinous rice extracts showed higher renin inhibitory activity than other rice extracts. On the other hand, extracts of cereals such as buckwheat (soba), rye, and barnyard millet (hie) had renin inhibitory activity, but other cereals had no effects on renin activity. The buckwheat had the highest renin inhibitory activity in this study. LC/MS analysis of rice and cereal extracts indicated that the major renin inhibitory compounds are oleic acid and linoleic acid.

Key words: renin, inhibitor, rice, cereals, oleic acid, linoleic acid.

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Introduction

Renin [EC 3.4.15.23] is a key enzyme of the renin-angiotensin system

(RAS), the most important blood pressure control system in animals [1]. The enzyme is a highly specific aspartic proteinase mainly synthesized in juxtaglomerular cells in the kidney [2]. Renin catalyzes the liberation of angiotensin I (AI) from plasma substrate angiotensinogen. Angiotensin converting enzyme (ACE) cleaves AI to form an active peptide, angiotensin II (AII). On the other hand, chymase, a chymotrypsin-like serine proteinase has been reported [3]. The enzyme is also known to form AII from AI in vascular tissues [4-6]. Chymase has no activity in normal tissues and its activity is only detected in mast cell-stimulated tissues. Thus, chymase inhibitors seem to be highly safety, because there is no chymase inhibitor target in normal tissues [3].

AII activates the type-1 angiotensin receptor to induce aldosterone synthesis, which increases water and salt absorption in kidney and increases blood pressure. Thus, control of RAS is the major target of cardiovascular disease therapies. ACE inhibitory peptides in various foodstuffs have been demonstrated to control RAS *in vitro* and *in vivo* [7-10]. Although renin is the most important enzyme in RAS, substantial screening for renin inhibitors among foodstuffs has not been undertaken because of the complexity of the renin assay [11, 12]. Recently, we developed a method of purification of recombinant human renin (rh) renin expressed in *E. coli* and Sf-9 insect cells [13, 14]. Using rh-renin as a target enzyme we screened various foodstuffs and found that soybean and minor legumes exhibits renin inhibitory activity [13, 15]. The renin inhibitory compound in soybean was identified as soyasaponin I [16]. Moreover, we studied the structure-function relationship of saponins on renin inhibition and revealed that the 3-O-β-D-glucopyranosiduronic moiety in saponins (glucuronide saponins) is essential for renin inhibition [17]. More recently, we found rh-renin inhibitory activity in non-glutinous rice and identified renin inhibitors as oleic acid and linoleic acid [18].

In the present study, we screened for renin inhibitory activity in various types of rice and cereal using rh-renin and renin-specific substrate, and found renin inhibitory activity in rice and some cereals.

Materials and Methods

Materials Oleic acid (Lot No. KWK2136), linoleic acid (Lot No. PEE3753), and rutin (Lot No. EPP5152) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Four important rice cultivars in Akita Prefecture, gluteinous rice strains (Tatsukokomochi and Kinunohada) and rice strains for sake brewing (Miyamanishiki and Akitasake-

komachi), grown in 2008 were obtained from Agricultural Experimental Station, Akita Prefectural Agriculture, Forestry, and Fisheries Research Center (Yuwa, Akita, Japan). Indica rice was obtained from Kitokushiryo Co. Ltd. (Tokyo, Japan). Rye, foxtail millet (awa), and Chinese millet (kibi) were obtained from Ikegaya Co. Ltd. (Shizuoka, Japan). Flour for bread, barnyard millet (hie), and amaranthus were obtained from Yamamoto Koujisyouten Co. Ltd. (Hyougo, Japan). Buckwheat (soba) was obtained from Totachiya Co. Ltd. (Tokyo, Japan). Weak flour was obtained from Nisshin Seifun Co. Ltd. (Tokyo, Japan).

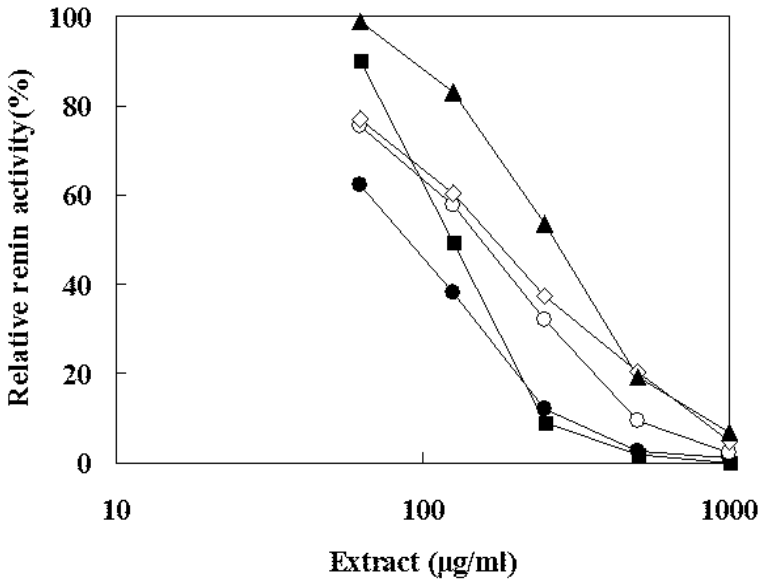

Fig. 1 Effects of Rice Extracts on rh-Renin Activity

rh-Renin was incubated with the indicated amounts of Tatsukomochi (●), Kinunohada (◆), Miyamanishiki (○), Akitasakekomachi (◇), and Indica rice (▲) extracts. Each result is the mean value for triple determinations.

Preparation of Rice and Cereal Extracts Sample powder (25 g) was soaked in 200 ml 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 was dissolved in 100% methanol for renin inhibition assay.

Renin Activity The rh-renin expressed in Sf-9 cells was prepared by the method of Takahashi *et al*. [14]. The internally quenched fluorogenic (IQF) substrate for human renin, *N*-methylanthranyl (Nma)-Ile-His-Pro-Phe-His-Leu*Val-Ile-Thr-His-Lys-2,4 -dinitrophenyl(Dnp)-D-Arg-D-Arg-NH_2 (*, scissile peptide bond) and a reference compound were custom-synthesized at the Peptide Institute (Osaka, Japan). Hydrolysis of the IQF substrate at the Leu-Val bond was spectrophotometrically determined. The reaction mixture contained 39 µl of 50 mM sodium phosphate buffer, pH 6.5, 0.1 M NaCl, 0.02% NaN_3, 0.02% Tween 20, 1 µl of 1 mM IQF substrate solution in DMSO, 5 µl of inhibitor solution, and 5 µl of rh-renin solution (5 µg/ml) 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 0.2 ml of 0.1 M triethanolamine, pH 9.5. The increase in 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 condition was taken as the IC_{50} value. One unit of renin inhibitory activity results in 50% inhibition of 25 ng of rh-renin.

Identification of Oleic Acid and Linolenic Acid by LC-ESI-MS LC-ESI-MS analysis was performed with an Agilent 1100 Series HPLC system and API-2000 mass spectrometer (Applied Biosystems) in the negative mode. HPLC was carried out on a TSKgel ODS-100V (Tosoh, 3 μm, ϕ2.0 x 50 mm) column used at 40 °C with acetonitrile–H_2O containing 0.1% formic acid as eluent (0.4 ml/min, a linear gradient from 70% to 80% acetonitrile for 10 min). Five microliters of a 10 ppm solution of fatty acid samples in MeOH was injected. Linoleic acid (*m*/*z* 279) and oleic acid (*m*/*z* 281) were detected at t_R 3.4 and t_R 5.1 min, respectively.

Results and Discussion

Inhibition of rh-Renin by Rice Extracts Nonglutinous rice is the most important agricultural product in Japan, especially in rice-producing counties in the Tohoku area. Recently, we found rh-renin inhibitory activity in nonglutinous rice extracts and identified the active compounds from Akitakomachi, the most important rice cultivar in Akita Prefecture, as oleic acid and linoleic acid. Moreover, the structure-function relationship of fatty ac-

Table 1 Effect of Rice Extracts on rh-Renin Activity

Sample	Total extract (mg/g powder)	IC_{50} (μg/ml)	IU*1 (U/mg powder)	Concentration (μmol/ml)*2 LA*3	OA*4
[Nonglutinous rice]*5 Akitakomachi	5.7	280	4.04	2.49	0.69
[Glutinous rice]*6 Tatsukomochi	10.0	88	22.73	4.13	1.83
Kinunohada	10.1	125	16.03	7.52	3.22
[Sake rice]*6 Miyamanishiki	6.1	155	7.82	3.72	1.47
Akitasakekomachi	5.8	170	6.82	3.08	1.21
Indica Rice*6	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.
*3, LA, linoleic acid
*4, OA, oleic acid
*5, Data from published results [18].
*6, This study

ids on rh-renin activity was also investigated [18]. In the present study, we tested the effects of glutinous, sake, and Indica rice extracts on rh-renin activity to understand the existence of renin inhibitors in rice universally. As shown in Fig. 1, all of the rice extracts tested in this study inhibited rh-renin activity in a dose-dependent manner. Among them, Tatsukomochi extract, the most important glutinous rice in Akita Prefecture, had the highest renin inhibitory activity with an IC_{50} value of 88.0 μg/ml. Another glutinous rice extract, Kinunohada, also showed high renin inhibitory activity with an IC_{50} value of 125.0 μg/ml. On the other hand, sake and Indica rice extracts showed nearly the same inhibitory activity as nonglutinous rice.

The amount of rice extracts, IC_{50} values, inhibitor units, and oleic acid and linoleic acid contents are summarized in Table 1. The glutinous rice, Tatsukomochi and Kimunohada, gave large amounts of extract with 10.0 and 10.1 mg/g powder, respectively. The yields of sake rice and nonglutinous rice extract were 5.7 to 8.8 mg/g powder. Indica rice extract showed the lowest yield of 3.7 mg/mg powder. Our previous study showed that oleic acid and linoleic acid are the major rice rh-renin inhibitors [18]. Thus, we measured the concentration of these free fatty acids in rice extracts. The IC_{50} values of rice extracts are correlated with the contents of oleic acid and linoleic acid. To evaluate the rh-renin inhibitory activity per gram weight, we calculated renin inhibitor unit (IU) per gram powder. Nonglutinous rice had 4.04 to 9.47 U/mg of powder. Glutinous rice, Tatsukomochi and Kinunohada, showed 22.73 and 16.03 IU, respectively. Glutinous rice had two- to three-fold higher renin inhibitory activity than the other strains. Indica rice showed the lowest value of 2.74 IU. These results showed that glutinous rice extracts had higher renin

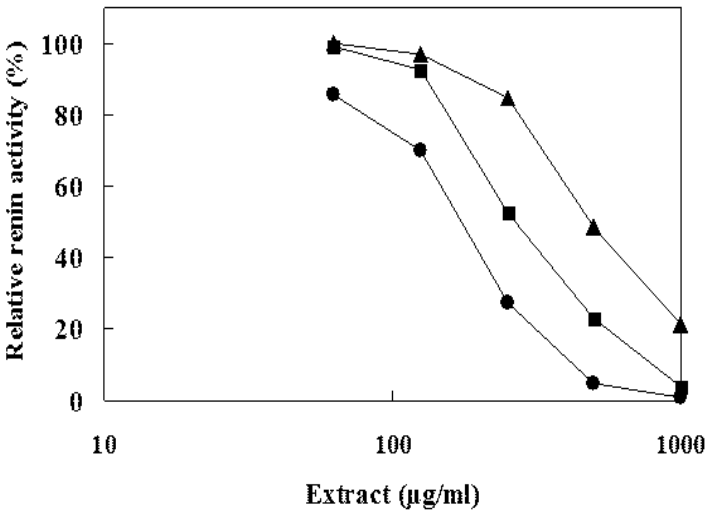

Fig. 2 Effects of Cereal Extracts on rh-Renin Activity

rh-Renin was incubated with the indicated amounts of buckwheat (●), rye (▲), and barnyard millet (■) extracts. Each result is the mean value for triple determinations.

Table 2 Effect of Cereal Extracts on rh-Renin Activity

Sample	Total extract (mg/g powder)	IC_{50} (μg/ml)	IU*1 (U/mg powder)	Concentration (μmole/ml)*2	
				LA*3	OA*4
Buckwheat (Soba)	39.8	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
Amaranthus	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.
*3, LA, linoleic acid
*4, OA, oleic acid

inhibitory activity than the other rice extracts.

Inhibition of rh-Renin Activity by Cereal Extracts For cereals, growth is possible in sterile land. Therefore, cold regions and highlands can be used for indispensable crops. Moreover, cereals are a staple food in many countries around the world, including Japan. In this study, we used buckwheat (soba), rye, foxtail millet (awa), barnyard millet (hie), Chinese millet (kibi), and amaranthus as cereals. Flour for bread and weak flour were also used as a control for rye. Among methanol extracts of the different cereals studied, buckwheat extract had the highest rh-renin inhibitory activity followed by barnyard millet and rye extracts, as shown in Fig. 2. The IC_{50} values of buckwheat, barnyard millet, and rye extracts were determined to be 173, 265, and 490 μg/ml, respectively. No other cereal extracts had rh-renin inhibitory activity. This is the first demonstration of renin inhibitory activity in cereals. Table 2 shows a summary of the total extracts, IC_{50} values, IU, and concentrations of oleic acid and linoleic acid. Buckwheat showed the highest renin inhibitory units among the renin inhibitory cereals. Renin inhibitory cereals contain relatively high concentrations of oleic acid and linoleic acid. On the other hand, concentrations of these unsaturated fatty acids in non-renin inhibitory cereals are lower. The concentrations of oleic acid and linoleic acid seem to be correlated with rh-renin inhibitory activity. Buckwheat contains several functional

compounds such as hyperin, quercetin, isoquercetin, or rutin [19]. Among these, rutin is a well-known functional compound in buckwheat. This compound inhibits alpha-glucosidase activity [20]. Rutin-containing foodstuffs are expected to be a good source for the development of powerful anti-diabetes functional foods [20]. Thus, we tested the effects of rutin on rh-renin activity. Rutin had no effects of rh-renin activity up to 1.0 mg/ml (data not shown). As shown in Table 2, oleic acid and linoleic acid are major renin inhibitory compounds in buckwheat. However, the existence of other unknown rh-renin inhibitory compound(s) in buckwheat extract cannot be ruled out. More detailed studies are necessary for the identification of renin inhibitory compound(s) in cereals.

Conclusion

In the present study, we investigated the effects of rice and cereal extracts on rh-renin activity and found that glutinous rice exhibits higher renin inhibitory activity than other rice extracts. Moreover, buckwheat contains the highest renin inhibitory activity among the tested cereals. This is the first demonstration of renin inhibitory activity in cereals.

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