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Renin Inhibitor in Soybean

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1. Introduction

Renin-angiotensin-aldosterone system (RAS) is the most important blood pressure control system in animals (Fig. 1). Renin [EC 3. 4. 23. 15] is a highly specific aspartic proteinase that is mainly synthesized by juxtaglomerular (JG) cells in the kidney. The human renin gene encodes preprorenin consisting of 406 amino acids (1-23 signal sequence, 24-66 propeptide, and 67-406 mature renin) [Imai *et al.*, 1983]. The synthesized renin precursor is processed to mature renin by proteolysis and stored in renin granules in JG cells. The secretion of renin into the circulation is controlled by several stimuli. The enzyme catalyzes the release of angiotensin I from plasma substrate angiotensinogen. This conversion is the rate-limiting step in RAS. Angiotensin I is an inactive peptide activated by angiotensin converting enzyme (ACE) [EC 3. 4. 15. 11]. ACE cleaves C-terminus dipeptide from angiotensin I. The angiotensin II produced acts directly on arterial smooth muscle cells to maintain blood pressure and stimulate the synthesis and release of aldosterone. Hence, RAS is a major target in the treatment of hypertension. ACE inhibitor is commonly used in clinical treatment. In connection with the control of renin activity, renin-binding protein (RnBP), a cellular renin inhibitor, was first isolated from porcine kidney as a complex of renin, called high-molecular-weight renin [Takahashi *et al.*, 1983a, Takahashi *et al.*, 1983b]. The nucleotide sequences of porcine, human, and rat RnBP cDNAs were determined and the amino acid sequences consisted of 402, 417, and 419 amino acid residues, respectively [Inoue *et al.*, 1990, Inoue *et al.*, 1991, Takahashi *et al.*, 1994]. The co-expression of human renin and RnBP cDNAs in AtT-20 cells showed that RnBP regulates active renin secretion from the transfected cells [Inoue *et al.*, 1992].
ACE has been used to screen inhibitors from foodstuffs because of its simple assay method, but renin is a rate-limiting enzyme in RAS, so it was not used because of the complicated assay system. In this chapter we describe expression of recombinant human renin in *E. coli* and *Spodoptera frugiperda* (Sf-9) insect cells, development of a simple and rapid assay method for human renin, occurrence of renin inhibitor in fermented soybean, isolation of renin inhibitors from soybean, and structure-function relationship of saponins.

2. Expression of recombinant human renin in *E. coli* and Sf-9 insect cells

The isolation of human renin from the kidney was very difficult because of the starting materials and the extremely low concentration of renin in the kidney, although some groups have succeeded in purifying human kidney renin associated with juxtaglomerular cell

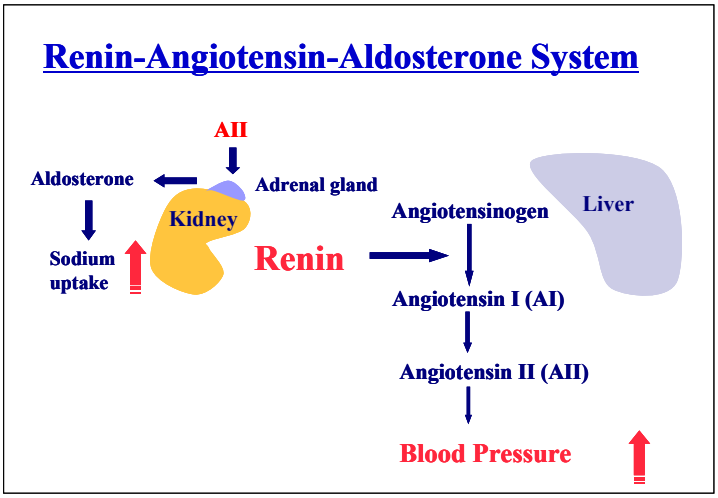

Fig. 1. Renin-angiotensin-aldosterone system

tumor [Galen *et al*., 1979] and using Haas's preparation [Yokosawa *et al*., 1980]. These human renins showed a heterogeneous electrophoretic pattern because of the variety of sugar chains and partial degradation. The expression of recombinant human prorenin in animal cells [Poorman *et al*., 1986, Weighous *et al*., 1986, Vlahos *et al*., 1990] or *Escherichia coli* cells [Imai *et al*., 1986] has also been reported. In the case of Chinese hamster ovary cells [Poorman *et al*., 1986], recombinant human prorenin was secreted into the medium. However, the expression level was very low. On the other hand, with the expression of human renin in *E. coli* cells, the expressed human prorenin formed inclusion bodies and did not properly refold into active renin [Imai *et al*., 1986].

We constructed thioredoxin-human prorenin fusion protein expression vector. The constructed expression vector, pETHRN1, was transformed into *E. coli* BL21 (DE3) cells [Takahashi *et al*., 2006]. The addition of IPTG to the cells carrying pETHRN1 resulted in the highly efficient production of fusion protein. The SDS-PAGE analysis of whole cell extract showed the major protein in the *E. coli* cells to be the fusion protein. The expressed fusion prorenin formed inclusion bodies in *E. coli* cells. The inclusion bodies were purified by sonication and centrifugation. The purified inclusion bodies were solubilized with a 4 M guanidine hydrochloride solution. The gradual removal of guanidine hydrochloride by stepwise dialysis with the introduction of L-arginine and a non-ionic detergent Briji 35 resulted in efficient refolding of fusion prorenin. The refolded fusion prorenin was activated by trypsin, a model activator of prorenin. As shown in Fig. 2, the 58 kDa fusion prorenin disappeared with the emergence of 35-40 kDa mature renins. The 35-40 kDa mature enzymes are active species formed by limited proteolysis of trypsin. The active renin was used for the screening of renin inhibitor from various foodstuffs.

The expression of recombinant human prorenin and renin in mammalian cells has been reported. In these cases, the major secreted protein was inactive prorenin and trypsin treatment was essential for the activation of prorenin. We also expressed recombinant human renin in Sf-9 insect cells with recombinant baculovirus, vhpR, carrying human preprorenin cDNA in the polyhedrin locus of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) [Takahashi *et al*., 2007]. Sf-9 cells were infected with

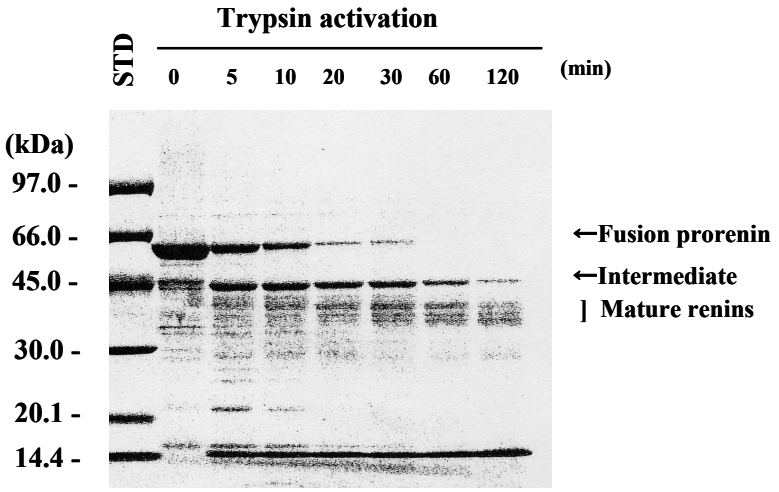

Fig. 2. Processing of fusion prorenin by trypsin.

recombinant baculovirus at a multiplicity of infection of 1.0 pfu/cell and cells were cultured in SF-900II serum-free medium using 250-ml shaker flasks on an orbital shaker at 100 rpm at 28°C. Cells grew continuously until day 3, but total cell numbers and viability decreased at days 4 and 5 of culture. Renin activity was not detected until day 3. A small amount of renin activity was detected at day 4 and increased dramatically at day 5. When the media were used for Western blotting, prorenin with a molecular weight of 43,000 was detected at days 3 and 4 of culture. On the other hand, only mature renin (molecular weight of 40,000) was detected in the day 5 culture. These results clearly show that the expressed prorenin was activated by proteinase appearing at the late stage of culture. This is the first demonstration of the accumulation of active renin in the baculovirus expression system [Takahashi *et al*., 2007]. Recently, we purified prorenin processing enzyme (PPE) from a medium of baculovirus-infected Sf-9 cells and revealed it to be a cysteine proteinase encoded by the AcNMPV gene [Gotoh *et al*., 2009, Gotoh *et al*., 2010a, Gotoh *et al*., 2010b].

We purified recombinant human renin in day 5 culture. Table 1 shows a summary of purification. Approximately 0.6 mg of purified preparation was obtained from 200 ml of culture with a yield of 35%. The quantity of renin production in the medium was estimated to be 8.7 mg/l from the yield. Previously, the amounts of recombinant prorenin produced by mammalian and insect cells were 2-15 mg/l of medium [Poorman *et al*., 1986, Weighous *et al*., 1986, Fritz et al., 1986]. However, the production of active renin was very low even in insect cells. Thus, our result is the highest production of active human renin in conventional reports.

Steps	Total protein (mg)	Total activity (U/mg)	Specific activity (%)	Yield
1. Medium	875	470	0.55	100
2. Pepsatin column	1.69	171	101	36.4
3 Mono Q	0.613	166	270	35.3

Table 1. Purification of recombinant human renin from Sf-9 medium.

The purified renin preparation showed a single protein band on SDS-PAGE with an apparent molecular weight of 40,000. The N-terminal amino acid sequence of the purified preparation was determined to be NH_2-Leu-Gly-(X)-Thr-Thr-Ser-Ser-Val-Ile-Leu-. The sequence agreed with the N-terminal sequence from +3 to +12 of mature human renin, except for a unidentified residue, which appeared to be a glycosylated Asn residue, as reported previously [Imai *et al.*, 1983]. The processing site of the renin expressed in Sf-9 cells was different from that of authentic renin because of the substrate specificity of PPE in Sf-9 cells [Gotoh *et al.*, 2010b].

3. Development of internally quenched fluorogenic substrate for human renin

The internally quenched fluorogenic (IQF) substrate for human renin, *N*-methylanthranyl (Nma)-Ile-His-Pro-Phe-His-Leu*Val-Ile-Thr-His-N^{ε}-2, 4,-dinitrophenyl (Dnp)-Lys-D-Arg-D-Arg-NH_2 (*, scissile peptide bond) was custom-synthesized at Peptide Institute (Osaka, Japan). Hydrolysis of IQF substrate at the Leu-Val bond was spectrophotometrically determined. The reaction mixture contained 1 µl of 1 mM IQF substrate solution in DMSO, 44 µl of sodium phosphate buffer, pH 6.5, 0.1 M NaCl, 0.02% Tween 20, 0.02 % NaN_3, and 5 µl of 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 9.0. The increase in fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation at 340 nm. The k_{cat} and K_m values of recombinant renin for the IQF substrate at pH 6.5 and at 37℃ were 833 s^{-1} and 35.7 μM^{-1}, respectively (Fig. 3) [Takahashi *et al.*, 2007].

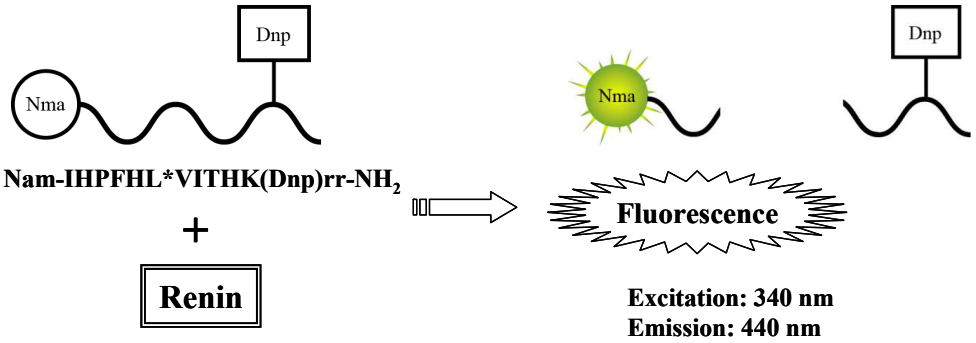

Fig. 3. Measurement of human renin activity using IQF substrate.

4. The occurrence of renin inhibitor in fermented soybean (miso)

Using recombinant human renin, we screened the inhibitory activity of desalted miso extract. Miso is a very common seasoning in Japan. The water extracts of miso were not suitable for the renin inhibition assay because of the high salt concentration. A high concentration of NaCl interrupted the renin activity. Thus, the water extracts of miso were desalted using a Sep-Pak C18 cartridge (Millipore). We tested commercially available miso and found that some miso exhibited renin inhibitory activity [Takahashi *et al.*, 2006].
To understand the origin of the renin inhibitory activity in the miso samples, we studied the renin inhibitory activity during fermentation of miso. As shown in Table 2, young miso

showed high renin inhibitory activity. Seven-day fermented miso was more potent than 30-day fermented miso. These results suggest that the renin inhibitory activity in miso decreased during fermentation, so that soybean or koji may exhibit renin inhibitory activity. Thus, we prepared extracts of soybean, steamed soybean, and koji. Soybean and steamed soybean extracts showed high renin inhibitory activity (Table 2). On the other hand, koji had nearly no renin inhibitory activity. These results clearly show that miso exhibited the renin inhibitory activity derived from soybeans.

Samples	n	Renin activity (%)	
		Mean	Standard deviation
Control	9	100.23	4.68
7-day miso	5	67.10	13.28
30-day miso	5	83.52	4.87
Koji	5	90.01	3.70
Soybean	5	49.42	3.16
Steamed soybean	5	37.38	3.78

Table 2. Effects of miso, koji, and soybean extracts on renin activity.

5. Isolation of renin inhibitor from soybean

Before isolation of renin inhibitor from soybean, we investigated the localization of renin inhibitor in soybean. Soybean was separated into two parts, embryo and cotyledon, and then extracted and evaluated for renin inhibitory activity. Embryo extract contained about 3-fold-higher renin inhibitory activity than cotyledon extract. Hence, we used soybean embryo for isolation of renin inhibitor. The scheme for the purification of soybean renin inhibitor is shown in Fig. 4. Approximately 70 mg of purified inhibitor was obtained from 750 g of soybean embryo. Isolated soybean renin inhibitor (SRI) gave soyasapogenol moiety and sugar chain unit as rhamnopyranosyl (1→2) galactopyranosyl (1→2) glucopyranosiduronic acid for ^{1}H and ^{13}C NMR spectra [Kitagawa *et al*., 1982, Kitagawa *et al*., 1988, Tsunoda *et al*., 2008]. Finally, the soybean renin inhibitor was identified as soyasaponin I (Fig. 5) by direct comparison with standard compounds for $[\alpha]_D$, mixed melting point, ^{1}H NMR, and IR spectra [Takahashi *et al*., 2008]. Soyasaponin I is one of the major saponins in soybean [Gu *et al*., 2002].

The purified SRI inhibited renin activity in a dose-dependent manner. An IC_{50} value of 30 μg/ml was obtained. Kinetic studies with SRI indicated partial noncompetitive inhibition with a *K*i value of 37.5 μM. The inhibitory spectra of SRI were studied using various proteinases. SRI also inhibited porcine kidney renin activity with an IC_{50} value of 30 μg/ml. SRI had very little effect on porcine pepsin or cysteine proteinases (papain and bromeline), and had no effect on serine proteinases (bovine pancreatic trypsin and human urinary kallikrein) or metalloproteinases (rabbit lung ACE and porcine kidney aminopeptidase) [Takahashi *et al*., 2008]. Moreover, a significant decrease in systolic blood pressure of spontaneously hypertensive rats was observed when commercially available soyasaponin was orally administered at 80 mg/kg/day for 8 weeks [Hiwatashi *et al*., 2010].

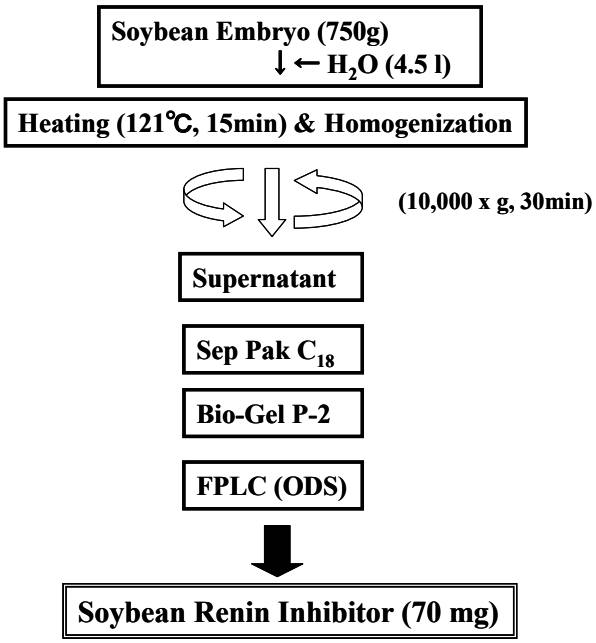

Fig. 4. Isolation of renin inhibitor from soybean embryo.

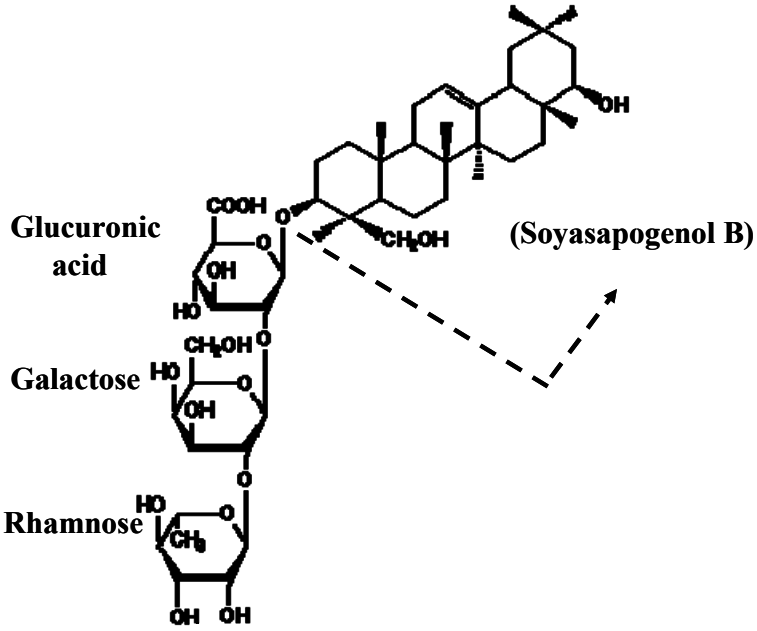

Fig. 5. Chemical structure of soyasaponin I.

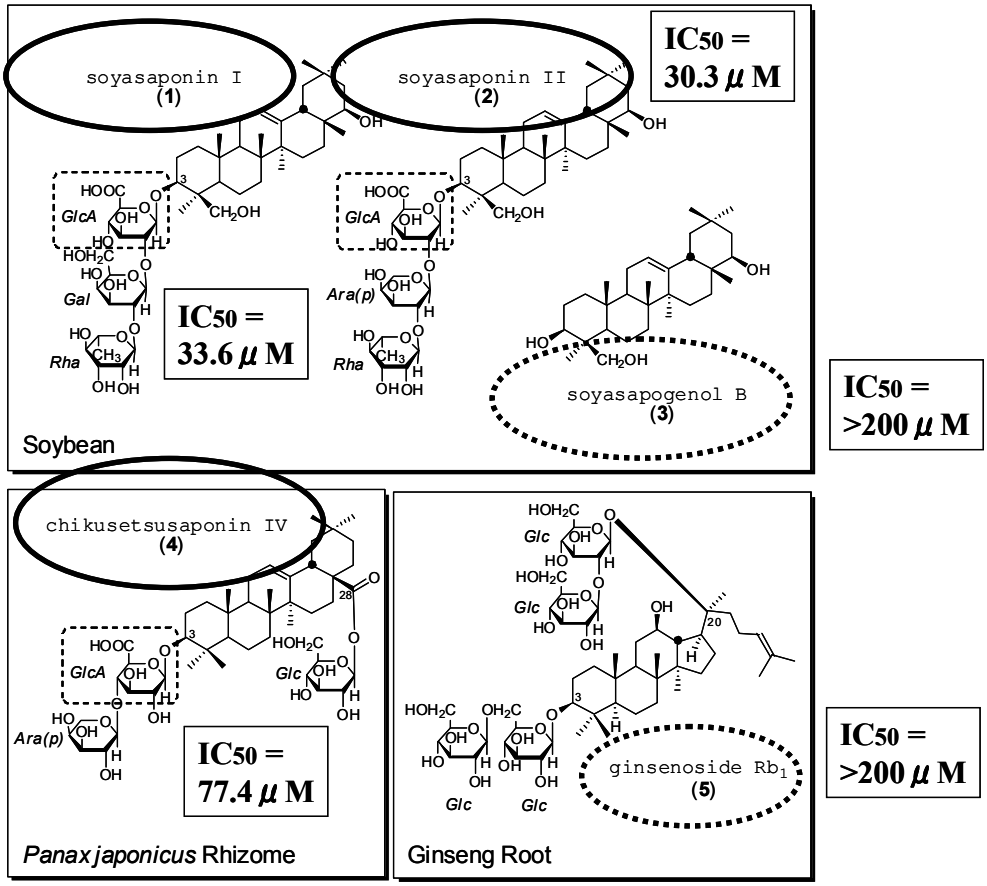

Fig. 6. Chemical structures and IC_{50} values of soyasaponin I (1), soyasaponin II (2), soyasapogenol B (3), chikusetsusaponin IV (4), and ginsenoside Rb_1 (5). Compounds 1, 2, and 4 had renin inhibitory activity. Compounds 3 and 5 had no effects on renin activity. *Ara(p)*, arabinose; *Glc*, glucose; *GlcA*, glucuronic acid; *Rha*, rhamnose.

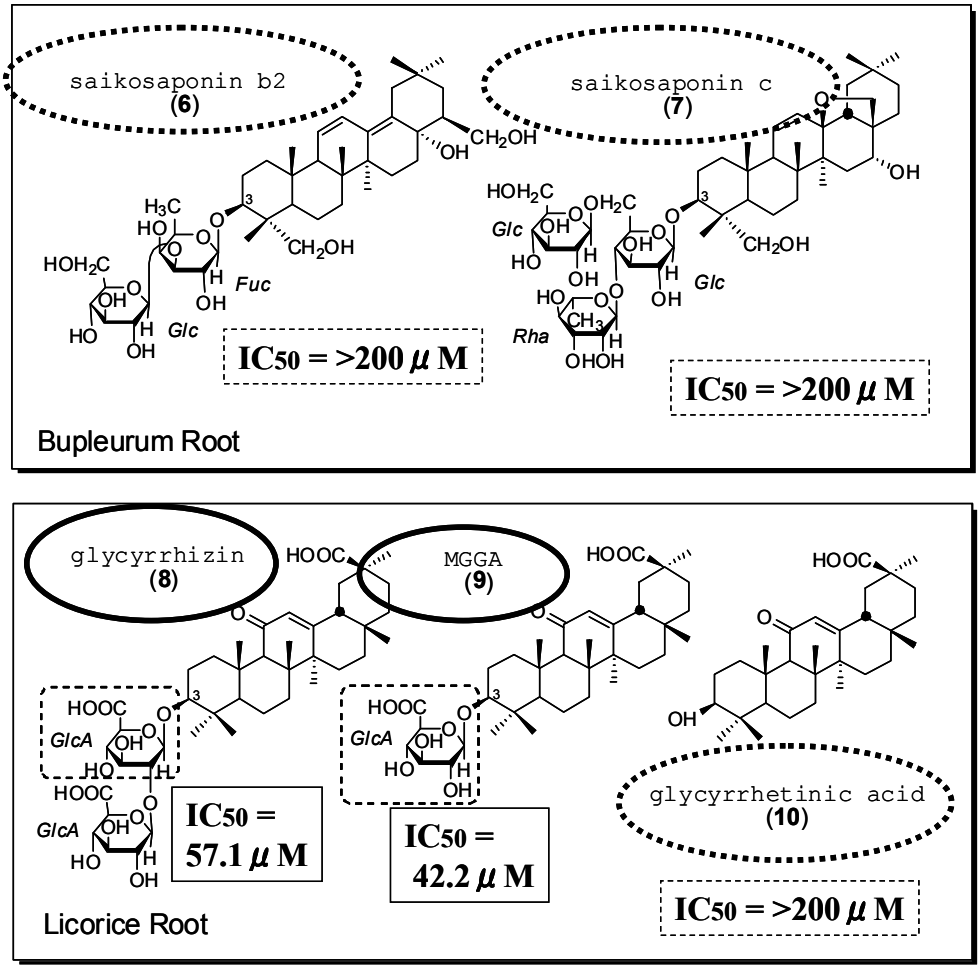

Fig. 7. Chemical structures and IC_{50} values of saikosaponin b2 (6), saikosaponin c (7), glycyrrhizin (8), monoglucuronyl glycyrrhetinic acid (MGGA) (9), and glycyrrhetinic acid (10). Compounds 8 and 9 inhibited renin activity. Compounds 6, 7, and 10 had no effect on renin activity. Fuc, fructose; *Glc*, glucose; *GlcA*, glucuronic acid; *Rha*, rhamnose.

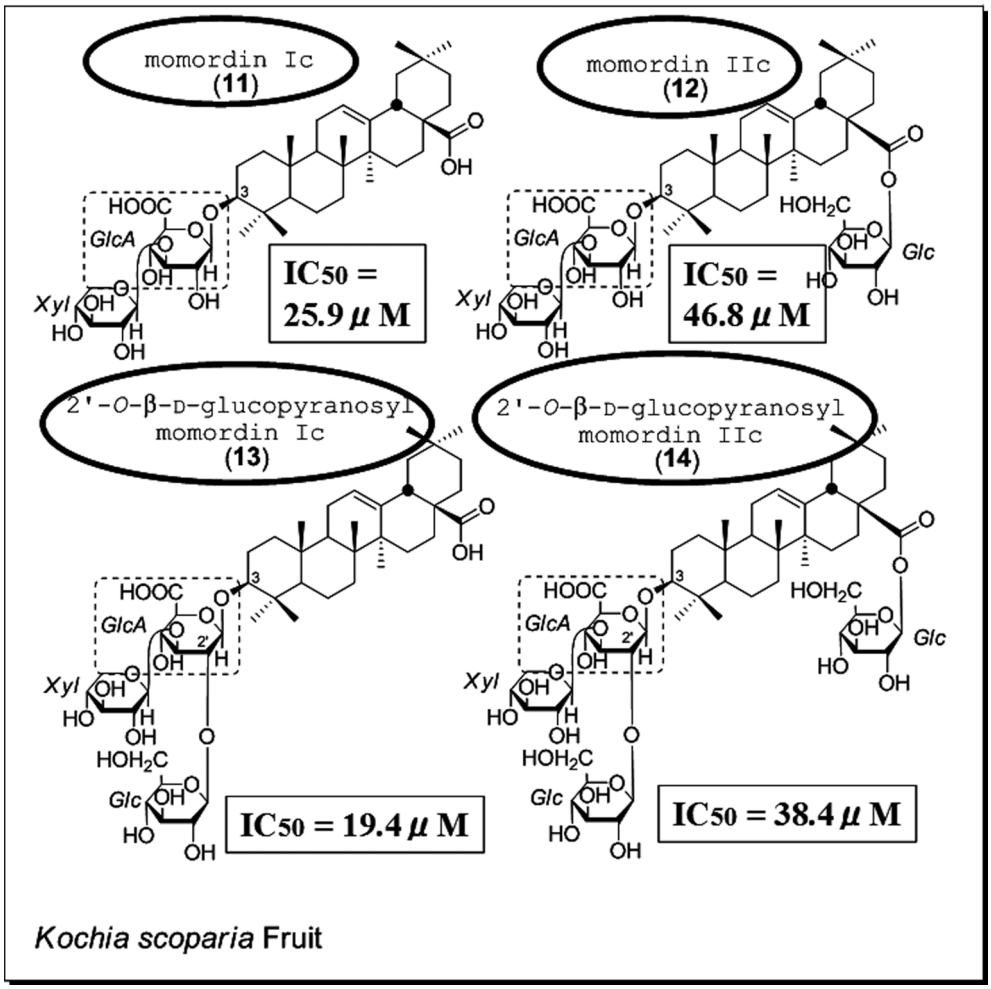

Fig. 8. Chemical structures and IC_{50} values of momordin Ic (11), momordin IIc (12), 2′-O-β-D-glucopyranosyl momordin Ic (13), and 2′-O-β-D-glucopyranosyl momordin IIc (14). Compounds 11, 12, 13, and 14 inhibited renin activity. *Glc*, glucose; *GlcA*, glucuronic acid; *Xyl*, xylose.

6. Renin inhibition by saponins

We investigated the effects of various saponins and sapogenols on human renin activity to elucidate the structure-function relationship of saponins [Takahashi et al., 2010]. Figures 6 to 8 show the chemical structure of saponins and sapogenols tested. Among them, soyasaponin I (**1**), soyasaponin II (**2**), saikosaponin c (**7**), 2′-*O*-β-D-glucopyranosyl momordin Ic (**13**), and 2′-*O*-β-D-glucopyranosyl momordin IIc **(14)** contain three sugar units attached at the 3β-hydroxyl position. Chikusetsusaponin IV (**4**), ginsenoside Rb_1 (**5**), saikosaponin b_2 (**6**), glycyrrhizin (**8**), momordin Ic (**11**), and momordin IIc (**12**) contain two sugar units at the same position. Compound **9** (monoglucuronyl glycyrrhetic acid: MGGA) contains one sugar unit at the same position. Soyasapogenol B (**3**) and glycyrrhetinic acid (**10**) are sapogenols.
Soyasaponin I (**1**), soyasaponin II (**2**), chikusetsusaponin IV (**4**), glycyrrhizin (**8**), MGGA (**9**), and saponins from *Kochia scoparia* fruit (**11-14**) inhibited human renin activity in a dose-dependent manner with IC_{50} values of 19.4-77.4 μM. These saponins have a glucuronide residue at the 3β-hydroxy position. On the other hand, ginsenoside Rb_1 (**5**), saikosaponin b_2 (**6**), saikosaponin c (**7**), and sapogenol compounds [soyasapogenol B (**3**) and glycyrrhetinic acid (**10**)] had no effect on renin activity. Compounds **5**, **6**, and **7** have glucose or fructose residues at the 3β-hydroxy sugar chain's first inner position. These results clearly indicate that glucuronic acid residues at the 3β-hydroxyl sugar chain's first inner position are essential for renin inhibition.

7. Conclusion

We developed efficient production of recombinant human renin in *E. coli* and *Spodoptera frugiperda* (Sf-9) insect cells. Using recombinant human renin and newly developed IQF substrate, we screened for renin inhibitor from several foodstuffs and found renin inhibitory activity in miso originated from soybean. The purified renin inhibitor from soybean was identified as soyasaponin I. Moreover, we investigated the effects of various saponins and sapogenols on human renin activity and showed that glucuronide saponins, glucuronic acid residues at the 3β-hydroxyl sugar chain's first inner position are essential for renin inhibition.

8. Acknowledgements

This research was supported in part by a Grant-in-Aid for Scientific Research (no. 20380081) from the Japan Society for the Promotion of Science and by a Grant for City Area Program from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan. The authors thank Dr. Yukiyoshi Tamura (Maruzen Pharmaceutical Co. Ltd., Fukuyama, Japan) for providing MGGA. The authors also thank Ms. Nao Suzuki and Ms. Mika Hokari for technical assistance.

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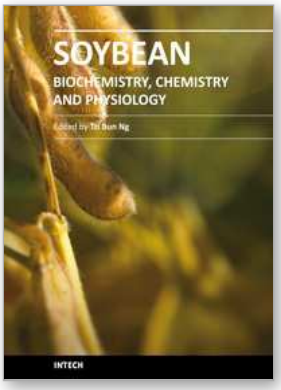

Soybean - Biochemistry, Chemistry and Physiology
Edited by Prof. Tzi-Bun Ng

ISBN 978-953-307-219-7
Hard cover, 642 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

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