Human prorenin was expressed in *Escherichia coli* as a fusion protein of thioredoxin. The chimeric protein, which accumulated insoluble inclusion bodies, was solubilized in 4M guanidine–HCl and refolded by an arginine-detergent buffer system and by systematic dialysis. The refolded fusion prorenin was activated by trypsin. The antiserum against human kidney renin specifically inhibited the recombinant human renin activity. Using the recombinant human renin, we screened its inhibitory activity in fermented soybean paste (miso) and demonstrated that miso contained renin inhibitory activity derived from soybean. The IC\textsubscript{50} values for soybean and steamed soybean extracts were determined to be 1.9 and 1.6 mg/ml, respectively. This is the first demonstration of renin inhibitory activity in miso and soybean.

**Key words:** renin; inhibitor; prorenin; miso; soy bean

Renin is a key enzyme for blood pressure control in the renin–angiotensin–aldosterone system (RAS). The enzyme is a highly specific aspartic proteinase mainly synthesized in the juxtaglomerular (JG) cells in the kidney cortex.\textsuperscript{1} The secretion of renin into the circulation is controlled by several stimuli. The renin activity is also regulated by the renin-binding protein (RnBP), an endogenous renin inhibitor.\textsuperscript{2–6} RnBP inhibits renin activity by forming a complex of renin, so-called high-molecular-weight renin.

The isolation of human renin from the kidney was very difficult because of the starting materials, although some groups have succeeded in purifying human kidney renin including JG cell tumor and Haas’s preparation.\textsuperscript{7–12} These human renins showed a heterogeneous electrophoretic pattern because of the variety of sugar chains and partial degradation. The expression of human renin in animal cells or *E. coli* cells has also been reported. In the case of animal cells, recombinant human (rh)-renin was secreted into the medium.\textsuperscript{13–19} On the other hand, with the expression of human renin in *E. coli* cells, the expressed human prorenin made inclusion bodies and did not properly refold into active renin.\textsuperscript{20–22} The angiotensin I-converting enzyme (ACE) has been used as a target enzyme in RAS for screening inhibitors because of its simple assay method. However, renin is a rate-limiting enzyme in RAS, so it was not used because the measurement was so complicated.

In the present study, we expressed human prorenin in *E. coli* cells as a fusion protein with thioredoxin. The expressed rh-prorenin was refolded by systematic dialysis, and then activated by trypsin as a model activator. Using the rh-renin as a target enzyme, we screened the renin inhibitory activity in fermented soybean paste (miso) and found that soybean, the major material in miso, contained renin inhibitory activity.

**Materials and Methods**

**Materials.** Restriction enzymes, the DNA ligation kit Ver. 2.0, and Taq DNA polymerase were obtained from Takara Bio (Tokyo, Japan). Expression vector pET32a and *E. coli* BL21 (DE3) competent cells were from Novagen (Merck, Darmstad, Germany). \textsuperscript{125}I-Angiotensin I (5 L-isoleucine) was from Amersham Biosciences (UK), and rabbit anti-human renin antisera was prepared by the method of Takahashi et al.\textsuperscript{6,23}

**Construction of the expression plasmid for human prorenin.** To delete the pre-sequence (20 amino acid residues) of human preprorenin and to make a fusion protein with thioredoxin, 660 bps of the human preprorenin cDNA, pSVL-HR4,\textsuperscript{23} was amplified with the sense primer containing the additional Bam HI site upstream from the pro-sequence, 5’-GGATCCACCTT-
TGTTCTCCCCAGACGA-3', and the antisense primer containing the Eco RI site of the cDNA clone, 5'-AGCGATTTGGAATTCTCG GA-3'. The PCR reaction was performed for 30 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 3 min). The amplified fragment was subcloned into the T-vector (Promega, WI, USA). The resulting plasmid is named pGHRPRC-1. To delete the 3' untranslated region of human preprorenin cDNA, 672 bps of the 3' region of human preprorenin cDNA was amplified with the sense primer about 100 bps upstream from Eco RI site, 5'-CAGGTCAACCTATCTCCG-3', and an antisense primer containing Xba I site downstream of the stop codon. PCR was performed under the same conditions as those just described. The amplified fragment was subcloned into the T-vector. The right-oriented plasmid is named pGHRPRC-2. The Bam HI-Eco RI fragment of pGHRPRC-1 was subcloned into the same site of pGHRPRC-2. The resulting plasmid, pGHREN1, was digested with Bam HI and Sph I (multiple cloning site of the T-vector), and then subcloned into the Bam HI-Sph I sites of pET32a. The resulting expression plasmid, designated pETHRN1, was used to transform E. coli BL21 (DE3) cells. The construction of the plasmid was confirmed by a sequence analysis.

Expression of the fusion protein. E. coli BL21 (DE3) cells transformed with pETHRN1 were cultured in 3 ml of a 2 × YT medium (1.6% (w/v) polypeptone, 1.0% (w/v) yeast extract, and 0.3% (w/v) NaCl at pH 7.0) containing 0.1 mg/ml of ampicillin at 30 °C for 20 h. The culture (2.5 ml) was inoculated with 125 ml of 2 × YT containing 0.1 mg/ml of ampicillin and incubated at 30 °C for 5 h, before expression of the fusion protein was induced with 1 mM isopropyl-β-d-thiogalactopyranoside (IPTG) at 30 °C for 3 h. After this incubation, the cells were harvested by centrifugation.

Purification of the inclusion body. The cells (20.5 g wet weight) were suspended in 400 ml of a 20 mM sodium phosphate buffer at pH 7.0, 0.15 M NaCl and 1 mM EDTA, and then sonicated for 3 min with Sonifier (Branson, Danbury, CT, USA). The sonicated sample was centrifuged at 20,000 × g for 30 min and resuspended in 400 ml of the same buffer. Sonication and centrifugation were conducted four times, and the resulting precipitate was used as the inclusion body preparation.

Solubilization of the inclusion body. One gram of the inclusion body preparation containing 100 mg of protein was solubilized in 5 ml of a 20 mM sodium phosphate buffer at pH 6.5, 0.1 M NaCl, 1 mM EDTA, and 4 M guanidine–HCl while gently stirring for 4 h at 25 °C. The solution was centrifuged at 20,000 × g for 30 min, and the supernatant was used for refolding.

Refolding of the fusion prorenin. The solubilized fusion protein (2.5 ml of a 10 mg/ml solution) was mixed with 80 ml of 50 mM Tris–HCl at pH 8.2, 0.24 M NaCl, 10 mM KCl, 0.05% (w/v) PEG 3350, 0.5 M guanidine–HCl, 2 mM MgCl₂, 2 mM CaCl₂, 0.4 M sucrose, 0.5 M l-arginine, 0.3 mM lauryl maltoside, 0.1 mM oxidized glutathione, and 1 mM reduced glutathione, and then stirred at 4 °C for 4 h. The solution was dialyzed against 51 of 30 mM Tris–HCl at pH 7.0, 0.15 M NaCl, 5% (w/v) sucrose, 0.5 M l-arginine, and 0.5% (w/v) Brij 35, and then dialyzed against same buffer except for Brij 35. The concentration of l-arginine was then reduced to 0.25, 0.1, and 0 M in a stepwise manner by dialysis (51 each). The solution was finally dialyzed against 51 of 20 mM Tris–HCl at pH 7.0, 0.15 M NaCl, 5% (w/v) sucrose, and 0.02% (w/v) NaN₃, and the aggregate formed was removed by centrifugation. The clear supernatant (100 ml) was concentrated 5-fold by ultrafiltration (PM-10, Amicon).

Activation of rh-prorenin. The refolded fusion protein (5 mg) was incubated with trypsin (20 μg) at 25 °C for 2 h, and the reaction was terminated by the addition of leupeptin (2 mg). The reaction mixture was dialyzed against a 20 mM sodium phosphate buffer at pH 7.0, 0.02% (w/v) Tween 20, and 10 μM leupeptin, and the aggregate formed was removed by centrifugation. The dialyzed sample was then applied to FPLC equipped with a Mono-Q RH 5/5 column (0.5 × 5 cm, Amersham Bioscience) that had been equilibrated with the same buffer. The renin activity was eluted with 30 ml of a 0–0.2 M NaCl linear gradient in the same buffer at a flow rate of 1 ml/min. The fractions containing renin activity (250 U/ml, 2 ml) were pooled and stored at −80 °C until needed.

Renin activity. The renin activity was determined on the basis of the generation of angiotensin I by using porcine plasma angiotensinogen as a substrate. A sample (10 μl) was incubated for 60 min at 37 °C with the porcine plasma substrate (2 mg) in a total volume 50 μl of a 0.1 M sodium phosphate buffer at pH 6.5 containing 2 mM EDTA and 1 mM PMSF. The generated angiotensin I was quantified by a radioimmunoassay. One unit of enzymatic activity is defined as the amount of the enzyme required to release one microgram of angiotensin I from angiotensinogen per h at 37 °C.

Effects of the antiserum on the renin activity. An 80-μl amount of the rh-renin solution (0.1 U/ml of 50 mM Tris–HCl at pH 7.5, containing 1 mM EDTA, 10 μM leupeptin, and 0.1% (w/v) BSA) was incubated with several concentrations of the diluted anti-human renin antiserum (20 μl) for 16 h at 4 °C. After this incubation, the residual renin activity in each sample was measured.

Inhibition of the renin activity by the miso, soybean,
and koji extracts. A 10-μl amount of the renin solution (75 mU/ml of 20 mM sodium phosphate buffer at pH 6.5, 2 mM EDTA, 0.2 mM PMSF, 10 μM leupeptin, and 0.02% (w/v) NaNO₃ and 10 μl of the extract or distilled water were incubated for 120 min at 37 °C with porcine plasma angiotensinogen (2 mg) in a total volume of 100 μl in a 0.1 M sodium phosphate buffer at pH 6.5, containing 2 mM EDTA and 1 mM PMSF. The generated angiotensin I was measured by a radioimmunoassay. The range of the standard curve for the radioimmunoassay was 25–1600 pg of angiotensin I. The sample concentration required to inhibit 50% of the renin activity under the assay conditions is taken as the IC₅₀ value.

Preparation of miso. Miso was prepared from soybean (Tachiyutaka) that had been soaked overnight (16 h) and steamed for 15 min. Ten kilogram of the steamed soybeans was mixed with 10 kg of koji and salt (16 h) and steamed for 15 min. Ten kilogram of the soybean (Tachiyutaka) that had been soaked overnight and koji extracts. A 10-μl amount of the renin solution (75 mU/ml of 20 mM sodium phosphate buffer at pH 6.5, 2 mM EDTA, 0.2 mM PMSF, 10 μM leupeptin, and 0.02% (w/v) NaNO₃ and 10 μl of the extract or distilled water were incubated for 120 min at 37 °C with porcine plasma angiotensinogen (2 mg) in a total volume of 100 μl in a 0.1 M sodium phosphate buffer at pH 6.5, containing 2 mM EDTA and 1 mM PMSF. The generated angiotensin I was measured by a radioimmunoassay. The range of the standard curve for the radioimmunoassay was 25–1600 pg of angiotensin I. The sample concentration required to inhibit 50% of the renin activity under the assay conditions is taken as the IC₅₀ value.

Preparation of miso. Miso was prepared from soybean (Tachiyutaka) that had been soaked overnight (16 h) and steamed for 15 min. Ten kilogram of the steamed soybeans was mixed with 10 kg of koji and salt (4 kg). The mixture was incubated with Zygosaccharomyces rouxii as a starter at 30 °C.

Extraction of miso, soybean and reacted rice (koji). Twenty five grams of miso, soybean, and koji were homogenized in 100 ml of distilled water with a Polytron homogenizer (Kinematica, Switzerland). The homogenate was heated in boiling water for 10 min and then cooled on ice. The sample was centrifuged at 10,000 × g for 30 min to remove the insoluble materials. The supernatant was applied to a Sep-Pak C18 cartridge (Millipore, Massachusetts, USA) that had been equilibrated with distilled water. The column was washed extensively with distilled water, and then the adsorbed materials were eluted with methanol. The methanol was evaporated to dryness, and the dry matter was dissolved in distilled water to evaluate the renin inhibitory activity.

SDS–PAGE and western blotting. SDS–PAGE was performed by the method of Laemmli, using 10–20% polyacrylamide gel (E-T1020L, Atto, Tokyo, Japan). After the electrophoresis, the proteins were transferred on to a nitrocellulose membrane for western blotting by using the anti-human renin antiserum.

Results and Discussion

Expression rh-prorenin in E. coli cells

The constructed expression vector, pETHRN1, was transformed into E. coli BL21(DE3) cells. The addition of IPTG to the cells carrying pETHRN1 resulted in the highly efficient production of a fusion protein. An analysis of the whole cell extract by SDS–PAGE and subsequent Coomassie brilliant blue staining and western blotting with the rabbit anti-human renin antiserum revealed the major protein in the E. coli cells to be the fusion protein (data not shown). The estimated molecular weight of the fusion protein (58 kDa) is in good agreement with its theoretical molecular weight.

Solubilization and refolding of the fusion rh-prorenin

The expressed fusion rh-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–HCl solution. The gradual removal of guanidine–HCl by stepwise dialysis with the introduction of L-arginine and a non-ionic detergent resulted in efficient refolding of rh-prorenin. Approximately 5.5 mg of soluble rh-prorenin was obtained from 25 mg of inclusion bodies.

Activation of refolded rh-prorenin

The human plasma and porcine kidney prorenins are inactive precursors of renin. The prorenin has been activated by trypsin and other endopeptidases. In the present study, we used trypsin as a model activator of rh-prorenin. The refolded rh-prorenin (100 μl) was incubated with trypsin (0.2 μg) at 25 °C for the indicated times. The reaction was stopped by the addition of leupeptin (20 μg), and the renin activity of the samples was then measured. The rh-prorenin showed hardly any renin activity before trypsin activation. During the incubation of rh-prorenin with trypsin, the renin activity increased in a time-dependent manner (Fig. 1). The activation pattern was very similar to that of porcine kidney prorenin by trypsin.

Processing of rh-prorenin by trypsin

SDS–PAGE and western blotting of the activated rh-prorenin by trypsin are also shown in Fig. 2. The 58-kDa protein disappeared with emerging 35–40 kDa mature enzymes. The 35–40 kDa mature enzymes might have been active species formed by the limited proteolysis of trypsin. Trypsin is not a physiological processing enzyme of prorenin. The prohormone processing enzymes, PC1 and PC5, seemed to be endogenous prorenin processing enzymes. Thus, the use of prohormone processing enzymes for rh-prorenin activation may give a clear activation pattern.

Incubation time (min)

Fig. 1. Activation of Recombinant Human Prorenin by Trypsin.

Refolded rh-prorenin (1 μg) was incubated by trypsin (1 ng) for the indicated times. After the incubation, the emerging renin activity was measured by using porcine angiotensinogen as a substrate.
Inhibition of rh-renin activity by the anti-human renin antiserum

The rh-renin was evaluated by the anti-human renin antiserum. When the rh-renin was incubated with the anti-human renin antiserum prepared against pure human kidney renin,\textsuperscript{11,35)} the renin activity was inactivated by the antiserum in a dose-dependent manner (data not shown). These results clearly indicate that the fusion rh-prorenin was activated by trypsin and that the enzyme activity that appeared was true renin activity.

Inhibition of human renin activity by the miso extracts

Using the rh-renin, we screened the inhibitory activity of desalted miso extracts. The water extracts of miso were not suitable for the renin inhibition assay because of the high salt concentration. Sodium chloride interrupted the renin activity or radioimmunoassay for angiotensin I. Thus, the water extracts of miso were desalted by a Sep-Pak C18 cartridge. Table 1 shows the effects of the desalted miso extracts on the renin activity. Interestingly, some miso used in this study contained renin inhibitory activity. The miso 2 and 4 extracts significantly inhibited the renin activity. Nearly no renin inhibitory activity could be detected in the miso 1, 3, 5, and 6 extracts. This is the first report on the renin inhibitory activity in miso, a very common seasoning in Japan.

**Origin of the renin inhibitory activity in miso**

To understand the origin of the inhibitory activity in the miso samples, we studied the renin inhibitory activity in miso during the 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 cultivated miso. These results suggest that the renin inhibitory activity in miso decreased during fermentation, so that soybean or koji may contain renin inhibitory activity. We therefore prepared the extracts of soybean, steamed soybean, and koji. High inhibitory activity was detected in the soybean and steamed soybean extracts (Table 2). The IC\textsubscript{50} values for the soybean and steamed soybean extracts were estimated to be 1.9 and 1.6 mg/ml, respectively. No renin inhibitory activity was detected in the koji extract (Table 2). These results suggest that miso contains the renin inhibitory activity derived from soybeans. Oligo-peptide(s) in the soybean extract may inhibit recombinant human renin activity. The ACE inhibitory activities in a soy protein hydrolysate and in fermented soybeans have already been reported.\textsuperscript{37–40)} To our knowledge, this is the first report of renin inhibitory activity in miso and soybean. The newly discovered renin inhibitory activity in the miso and soybean extracts will provide the potential to develop antihypertensive functional foods. The identification of the active compound(s) is underway. Further elaborated studies are necessary to investigate the *in vivo* function of the renin inhibitory activity in miso and soybean.

### Table 1. Effects of the Miso Extracts on Renin Activity\textsuperscript{a}

<table>
<thead>
<tr>
<th>Sample\textsuperscript{b}</th>
<th>n</th>
<th>Mean (% renin activity)</th>
<th>Standard deviation</th>
<th>Control</th>
<th>Miso 1</th>
<th>Miso 2</th>
<th>Miso 3</th>
<th>Miso 4</th>
<th>Miso 5</th>
<th>Miso 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control \textsuperscript{c}</td>
<td>9</td>
<td>100.23</td>
<td>4.68</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 1</td>
<td>4</td>
<td>94.55</td>
<td>10.12</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 2</td>
<td>5</td>
<td>75.36</td>
<td>5.44</td>
<td>**</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 3</td>
<td>5</td>
<td>92.26</td>
<td>9.43</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 4</td>
<td>5</td>
<td>83.04</td>
<td>6.91</td>
<td>**</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 5</td>
<td>5</td>
<td>89.98</td>
<td>6.93</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miso 6</td>
<td>6</td>
<td>91.86</td>
<td>1.43</td>
<td>n.s.</td>
<td>n.s.</td>
<td>*</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

\textsuperscript{a 商业可用的味噌样本被使用。}

\textsuperscript{b 最终味噌提取液浓度为2 mg/ml。}

\textsuperscript{c 蛋白水解酶抑制率。}

\textsuperscript{d renin activity without the miso extract was used as a control.}

n.s., not significant.
Conclusion

Miso has been a traditional seasoning for several centuries in Japan. In the present study, we discovered renin inhibitory activity in miso that was derived from soybean. Soybeans contained relatively high renin inhibitory activity. Our studies provide the possibility to develop antihypertensive functional foods with soybean.

Acknowledgment

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References


Table 2. Effects of the Miso, Koji, and Soybean Extracts on Renin Activity

<table>
<thead>
<tr>
<th>Samplea</th>
<th>n</th>
<th>Renin activity (%)</th>
<th>Significant difference by the Scheffé testb (**p = 0.01, *p = 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Controlb</td>
<td>9</td>
<td>100.23</td>
<td>4.68</td>
</tr>
<tr>
<td>7-day misoa</td>
<td>5</td>
<td>67.10</td>
<td>13.28</td>
</tr>
<tr>
<td>30-day misoa</td>
<td>5</td>
<td>83.52</td>
<td>4.87</td>
</tr>
<tr>
<td>Koji</td>
<td>5</td>
<td>90.01</td>
<td>3.70</td>
</tr>
<tr>
<td>Soybean</td>
<td>5</td>
<td>49.42</td>
<td>3.16</td>
</tr>
<tr>
<td>Steamed soybean</td>
<td>5</td>
<td>37.38</td>
<td>3.78</td>
</tr>
</tbody>
</table>

n.s., not significant

---

aFinal concentration of the miso extract was 2 mg/ml.

bRenin activity without the miso extract was used as a control.

cExtract from the 7-day cultivated miso was used.

dExtract from the 30-day cultivated miso was used.


