Antihypertensive Effects of Peptide in Sake and Its By-products on Spontaneously Hypertensive Rats

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Systolic blood pressure (SBP) decreased significantly when the hydrolysate of sake lee (HSL) and peptide fraction of sake (PFS) were orally administrated to spontaneously hypertensive rats (SHR). SBP of “young” SHR decreased significantly after orally administering Val-Tyr, His-Tyr, Arg-Phe, Val-Trp, and Tyr-Trp that were isolated from PFS and HSL. The hypotensive effect of Val-Tyr and His-Tyr that was observed in “young” SHR disappeared as they got older, but PFS and HSL maintained their antihypertensive effect on “aged” SHR.

SHR fed on a diet with HSL replacing half of the protein source for 3 weeks showed a significant decrease in SBP after 10 days of feeding.

We have been studying the functions of sake and its by-products for the development of their new markets.\(^{1,2}\) These studies identified several angiotensin I-converting enzyme (ACE)-inhibitory substances in the products, enabling the isolation of several ACE-inhibitory peptides, Tyr-Gly-Gly-Tyr, Val-Tyr, and His-Tyr from sake, and Ile-Tyr-Pro-Arg-Tyr, Phe-Trp-Asn, Val-Trp-Tyr, Arg-Phe, Val-Trp, and Tyr-Trp from sake lee.\(^{3,4}\) We have also confirmed that the strong ACE-inhibitory substance existing in rice bran is phytin.\(^{5}\)

Among these ACE-inhibitory peptides, some peptide fragments existed in rice protein; for example, Val-Tyr existed in glutelin and 16 KDa prolamin, and His-Tyr existed in glutelin and 13 KDa prolamin. The fact that these peptides are found in sake suggest that they may be released from rice protein during sake fermentation. It may be for the same reason that hot-water extracts of sake lee also have ACE inhibitory effects. Such phenomena were also observed in ferulic acid, the antioxidative substance in sake, which is thought to exist in the rice cell wall and be isolated from rice during sake fermentation.\(^{6}\)

In a recent study, many kinds of functional substances were found in various foods, but these substances commonly existed as elements in the food composition. Therefore, an extraction or purification process needed to be applied to these foods in order to utilize those functional substances. However, as already stated, in the case of fermented foods like sake, functional substances are separated to some extent during the fermentation period. For this reason, fermented foods can be expected to be physiologically functional foods by themselves, and by-products such as sake lee could be useful as a raw material for physiologically functional foods.

Recent studies of ACE-inhibitory substances as one of the functional components in foods have advanced, and various ACE-inhibitory peptides have been found in corn,\(^{7}\) soybean,\(^{8}\) fish,\(^{9}\) milk,\(^{10}\) garlic,\(^{11}\) and lactobacilli.\(^{12}\) However, some peptides only serve as a substrate for ACE and are not true inhibitory substances. In this case, we must confirm whether the peptide is a true inhibitor or not.\(^{13,14}\) Even when the true inhibitor is orally administrated, it is sometimes ineffective because of the low efficiency of digestion and absorption.

We report here whether our ACE inhibitory substances are efficacious in the living body or not by using SHR, and confirm that sake and its by-products have an antihypertensive effect on SHR.

**Methods**

**Samples and preparation.** We used PFS and HSL as samples.\(^{15}\) Sake brewed in our factory was concentrated in vacuo.\(^{16}\) Washed with water, it was eluted with 0.5 M NH₄OH and then lyophilized (Fig. 1). It was rich in amino acids and peptides, so we named this fraction “PFS”. Lyophilized lee from the process,\(^{17}\) using a liquefied product of rice slurry as the raw material (water content, 5%; crude protein, 78%; sugars, 15%, others,

![Diagram](https://via.placeholder.com/150)

**Fig. 1.** Purification Procedure for ACE Inhibitors from Sake. Details are shown in Method section.

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**Abbreviations:** ACE, angiotensin I-converting enzyme; SHR, spontaneously hypertensive rat; SBP, systolic blood pressure; HSL, hydrolysate of sake lye; PFS, peptide fraction of sake.
Antihypertensive Effects of Sake and Its By-products

Sake lees

Lyophilized hydrolysate

Thermoase (2%)

Toyopearl HW-40

Hot water x 20

Puresil C18

60 °C, 1.5 hr

Capcell pak C18

centrifuged

lyophilized

ACE - inhibitory peptides

Lyophilized hydrolysate

Fig. 2. Purification Procedure for ACE Inhibitors from Sake lees. Details are shown in Method section.

Table 1. Composition of Diets

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>41.5</td>
<td>37.7</td>
<td>34.0</td>
<td>30.2</td>
<td>20.0</td>
</tr>
<tr>
<td>Casein</td>
<td>25.0</td>
<td>18.8</td>
<td>12.5</td>
<td>6.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Hydrolysate of sake lees</td>
<td>10.0</td>
<td>20.0</td>
<td>30.0</td>
<td>40.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Starch</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Vegetable oil</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Mineral mixture (AIN-76)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Saccharose</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(AIN-76 + choline bitartrate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Constituents are presented as%. Crude protein was adjusted to 21.6%.

2% was digested by Thermase (Daiai Kase) at 60°C for 1.5 hr, and the hydrolysate was then lyophilized (Fig. 2). Hydrolysis was conducted so that the substrate concentration was 5% in water and the enzyme concentration was 2% of the substrate. The ACE inhibitory peptides isolated from PFS and HSL were also synthesized by a solid-phase method with a peptide synthesizer (VEGA PEPTICULAR 2200) or were purchased from Sigma.

Diet. A modified AIN-76 mixture containing 25% casein (crude protein, 21.6%) was used as the control diet (Table I). During a long-term feeding test, HSL was substituted for casein in a fixed proportion. Since HSL already contained K and Na (0.55% and 0.7%, respectively), these insufficient dietary quantities were made up by adding potassium dihydrogenphosphate and sodium chloride.

Breeding conditions and blood pressure measurements. SHR and Wistar rats were purchased from Hoshino Laboratory Animals and Japan SLC, respectively. The rats were bred in a room with a 12-h light-dark cycle (lights on 7-19h). Temperature and humidity were controlled to 23±2°C and 50±5% RH, respectively, and the diet and tap water were available ad libitum. SBP was measured by the tail-pulse pick up method with a programmable electrophysymomanometer (UR-5000, Ueda) after warming the rat in a chamber maintained at 40°C for 5-15 min. This manometer appeared to give 10 to 20 mmHg higher readings for SBP when compared with the reported values.

Single oral administration. Samples were dissolved in 2 ml of distilled water and orally administrated to SHR by intubation. SBP was measured before (9 a.m.), as well as 4 and 6 hours after administering the sample. SBP of the control group that was administered distilled water alone was measured by the same method.

Experiment a.) PFS, HSL, His-Tyr, and Val-Tyr were administrated to ten SHR at the age of 11 weeks and then at intervals of 6-7 weeks, and the reduction in blood pressure was studied. The doses were 1 g/kg, 1 g/kg, 0.1 g/kg, and 0.1 g/kg, respectively, and body weights were 292, 357, and 36 g at 11, 18, and 24 weeks, respectively.

Experiment b.) Val-Tyr, Arg-Phe, Val-Trim, and Tyr-Trp were administrated to 10 SHR of 16-18 weeks of age (body weight, 316-390 g), and the hypotensive activity was investigated. Captopril was administered as a positive control, the doses being 0.1 g/kg of peptide and 0.003 g/kg of Captopril.

Long-term feeding of HSL

a. Growth test. A growth test was conducted to confirm whether HSL was appropriate as a protein source or not, the composition of each tested diet being shown in Table I. A modified AIN-76 mixture containing 25% casein (crude protein, 21.6%) was used as the control diet. For the test group, HSL was substituted for casein in proportions of 25, 50, 75, and 100% as crude protein in tests 1, 2, 3, and 4, respectively. 4-Week-old male Wistar rats were acclimatized to the control diet for 5 days, and then divided into 5 groups of 4 rats, each group having approximately the same mean weight (average body weights of each group were 115, 113, 118, 114, and 115 g), respectively, and fed for 19 days. Body weight was measured 2 times every week.

b. Hypotensive effect of HSL. The diets with HSL supplying 50% of crude protein, which produced no significant difference in growth, were fed to SHR and the hypotensive effect was studied. 4-Week-old male SHR were acclimatized to the control diet for 5 days, divided into 2 groups of 4 animals each (average body weights were 83 and 84 g, respectively), and each group fed with a different diet for 3 weeks. The body weight and blood pressure were measured 2 times every week between 1 and 3 p.m.

Statistical analysis. Data are presented as mean ± SD. A statistical analysis was performed by using Students paired or unpaired t-test after checking whether the variance was equal among each of the two groups by an F-test. A statistical inspection was performed to evaluate individual changes in SBP before and after dosing with a single oral administration (paired t-test), and for differences between the two groups after the long-term feeding test (unpaired t-test).

Results

Hypotensive effect of a single oral administration

Experiment a. Up to 19 weeks, SBP was significantly decreased by 10 to 30 mmHg after a single administration of His-Tyr and Val-Tyr (each dose was 100 mg/kg), just like PFS and HSL did (each dose was 1 g/kg respectively) (Table II). However, by 24 weeks, His-Tyr and Val-Tyr had lost their hypotensive effect. On the other hand, the decrease in blood pressure 6h after administering HSL and PFS was 23 and 22 mmHg, respectively, and they maintained their antihypertensive effect at the same age. Hypotensive effect of HSL was still significantly.

Experiment b. SBP values for 16–18-week-old male SHR were significantly decreased after a single administration of Val-Tyr, Arg-Phe, Val-Trim, and Tyr-Trp (each dose was 100 mg/kg) (Table III). The reduction in blood pressure (15 to 30 mmHg) was similar to that from Captopril at a dose of 3 mg/kg.

Long term feeding of HSL

Growth test. Tests 1 and 2 showed growth equal to that of the control group, while test 3 showed inferior growth in the early stage, and test 4 showed similar growth to that in test 3 in the early stage and lower growth in the latter stage. At the 19th day, the end of the experiment, the body weights of the control and in tests 1 to 4 were 262 ± 8, 253 ± 5, 252 ± 23, 238 ± 7, and 211 ± 13 g, respectively.
Table II. Influence of SHR Age on the Susceptibility to Various Hypotensive Substances

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (g/kg)</th>
<th>Age (weeks)</th>
<th>Before administration</th>
<th>Time after administration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>11-12</td>
<td>218±15</td>
<td>215±16</td>
</tr>
<tr>
<td>Hydrolysat of sake lee</td>
<td>1</td>
<td>11-12</td>
<td>224±11</td>
<td>209±17*</td>
</tr>
<tr>
<td>Peptide fraction of sake</td>
<td>1</td>
<td>11-12</td>
<td>221±16</td>
<td>209±20</td>
</tr>
<tr>
<td>His-Tyr</td>
<td>0.1</td>
<td>11-12</td>
<td>224±13</td>
<td>221±19</td>
</tr>
<tr>
<td>Val-Tyr</td>
<td>0.1</td>
<td>11-12</td>
<td>219±10</td>
<td>207±17</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>18-19</td>
<td>254±13</td>
<td>245±11</td>
</tr>
<tr>
<td>Hydrolysat of sake lee</td>
<td>1</td>
<td>18-19</td>
<td>251±17</td>
<td>242±20</td>
</tr>
<tr>
<td>Peptide fraction of sake</td>
<td>1</td>
<td>18-19</td>
<td>240±15</td>
<td>233±17</td>
</tr>
<tr>
<td>His-Tyr</td>
<td>0.1</td>
<td>18-19</td>
<td>not tested</td>
<td>not tested</td>
</tr>
<tr>
<td>Val-Tyr</td>
<td>0.1</td>
<td>18-19</td>
<td>251±12</td>
<td>234±22</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>24-25</td>
<td>246±16</td>
<td>243±12</td>
</tr>
<tr>
<td>Hydrolysat of sake lee</td>
<td>1</td>
<td>24-25</td>
<td>257±13</td>
<td>246±17</td>
</tr>
<tr>
<td>Peptide fraction of sake</td>
<td>1</td>
<td>24-25</td>
<td>246±17</td>
<td>233±17</td>
</tr>
<tr>
<td>His-Tyr</td>
<td>0.1</td>
<td>24-25</td>
<td>247±19</td>
<td>238±22</td>
</tr>
<tr>
<td>Val-Tyr</td>
<td>0.1</td>
<td>24-25</td>
<td>247±22</td>
<td>244±25</td>
</tr>
</tbody>
</table>

Systolic blood pressure is shown as mean±SD, n=10. Significant differences from "before administration": *p<0.05; **p<0.01.

Table III. Changes in Blood Pressure after Administering Various Peptides

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (mg/kg)</th>
<th>Systolic blood pressure (mmHg)</th>
<th>Before administration</th>
<th>Time after administration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td></td>
<td>245±13</td>
<td>245±11</td>
</tr>
<tr>
<td>Arg-Phe</td>
<td>100</td>
<td>246±12</td>
<td>222±15**</td>
<td>229±9*</td>
</tr>
<tr>
<td>Val-Trp</td>
<td>100</td>
<td>241±13</td>
<td>215±20**</td>
<td>231±24</td>
</tr>
<tr>
<td>Tyr-Trp</td>
<td>100</td>
<td>243±16</td>
<td>228±15</td>
<td>215±8**</td>
</tr>
<tr>
<td>Val-Tyr</td>
<td>100</td>
<td>251±12</td>
<td>234±22</td>
<td>220±17**</td>
</tr>
<tr>
<td>Captopril</td>
<td>3</td>
<td>244±13</td>
<td>223±16</td>
<td>218±16**</td>
</tr>
</tbody>
</table>

Systolic blood pressure is shown as mean±SD, n=10. Significant differences from "before administration": *p<0.05; **p<0.01.

Consequently we have used test 2 for evaluating the hypotensive effect of HSL.

Effect of HSL on blood pressure and body weight of SHR

The growth curve is shown in Fig. 3A. Test group had growth equal to that of the control group. At the end of the experiment, the body weight of the control group was 192±21 g and of the test group was 191±11 g. Changes in blood pressure are shown in Fig. 3B. The blood pressure of the test group was lower in comparison with that of the control group after 5 days of feeding, and had significantly decreased compared with the control group after 10 days. SBP for the test group was 184±6 mmHg and for the control group was 205±7 mmHg at the 14th day, so a 21 mmHg decrease in the blood pressure of the test group was observed. At the 21st day, the end of this feeding test, SBP values for the test group and control group were 196±11 and 225±6 mmHg.

Discussion

The SBP value for SHR in this study is about 10-20 mmHg higher than the reported value, so we investigated the reason for this difference. We obtained one result to indicate that our manometer generally gave higher readings for blood pressure. We tested SHR 7 times under the same conditions described here. Their SBP values had been increasing equally to those in this experiment, and at the age of age of 20 weeks, the SBP value had reached a reproducible 240-250 mmHg. We believe that a relative comparison of SBP based on ACE inhibition is possible; although we must clarify the reason why the SBP values in our SHR are so high.

We studied the reduction in blood pressure attributable to repeated administrations at intervals of 6-7 weeks of His-Tyr and Val-Tyr, which had been isolated from PFS, and of crude preparations such as PFS and HSL. It was confirmed that, up to 19 weeks of age, these purified dipeptides (His-Tyr and Val-Tyr) had the same antihypertensive effects as those of PFS and HSL but they lost the effects at 24 weeks of age. On the other hand, PFS and HSL retained their antihypertensive effects at the same age.

It is known that ACE inhibitors such as Captopril are safe and efficacious for hypertensive patients. However, Penner et al. have reported that young patients required a lower dose than elderly patients, and Saalbach et al. have reported that the reduction in SBP of young patients was greater than that of old patients at the same dose. It has been reported that SHR of more than about 20 weeks age showed hardening and fragility of the arteries and reduced immunity occurred. There are two possible hypotheses to explain this phenomenon. 1. Purified ACE-inhibitory peptides such as His-Tyr and Val-Tyr lost their hypotensive effect with the aging of SHR because of tissue...
In this study, we examined the hypotensive effect after administering the 4 dipeptides isolated, among which Val-Trp had the most potent inhibitory activity for ACE with an IC$_{50}$ value of 1.4 $\mu$M. Val-Tyr, Tyr-Trp, and Arg-Phe had IC$_{50}$ values of 7.1, 10.5, and 93.0 $\mu$M, respectively. Although these activities differ widely, they showed a similar hypotensive effect on SHR equal to that of Captopril at a dose of 3 mg/kg. A dipeptide is generally absorbed directly through the peptide transporter or absorbed as amino acids after being hydrolyzed by peptidase.\textsuperscript{25} The fact that no correlation was apparent between the results in vivo and the activities in vitro is considered to be due to the affinity of these peptides to the peptide transporter and the resistance of the peptides to dipeptidase not being similar.\textsuperscript{8,9}

It is known that a strain of rat developed fatty liver when fed on rice as the only protein source.\textsuperscript{26} However, in a recent study, it has been reported that specially purified rice protein with a protein content of more than 90% was not damaging in this way and in fact improved the metabolism of lipids and resulted in the reduction of cholesterol.\textsuperscript{27} Our sake lees, using the liquefied product of rice slurry as a raw material, has a high protein content (60–80% as dry matter), so it is thought that this sake lees would be a valuable protein source although it is a by-product of fermentation.

We conducted the long-term feeding test with HSL to display such physiological roles as hypotensive effect and maintaining efficiency as a protein source. When SHR were fed on the diets containing HSL as 50% of crude protein, SHR grew normally and the SBP value was significantly decreased.

We have thus confirmed that ACE-inhibitory peptides purified by using the ACE-inhibitory activity as an in vitro index were hypotensive for SHR. It was also confirmed that the SBP value for SHR was decreased by the direct administration of HSL and PFS, and that normal SHR growth and reduced SBP resulted by feeding on a diet containing HSL as 50% of the crude protein. These results indicate that sake lees could not only be useful as a protein source, but also for making foods intended to treat and prevent hypertension.

As already stated, it is thought that these hydrolysates contained many physiological components such as hypotensive substances other than ACE inhibitors, and we intend to extend our study further in the future.

\textbf{References}