Isolation of the Antioxidant Pyranonigrin-A from Rice Mold Starters Used in the Manufacturing Process of Fermented Foods

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Rice mold starters prepared from Aspergillus species are commonly used for the manufacture of koji in the production of oriental fermented foods. Methanol extracts of rice mold starters fermented by the Aspergillus species, A. awamori, A. kawachii, A. oryzae, A. saitoi, and A. sojae, were examined for their antioxidative activity by using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging system. The extracts of A. awamori, A. kawachii, and A. saitoi exhibited higher activity than those of A. oryzae and A. sojae. An antioxidant was isolated from the extract of A. saitoi and identified as pyranonigrin-A by ¹H-NMR, ¹³C-NMR, and FAB-MS analyses. The antioxidative activity of pyranonigrin-A was approximately equivalent to that of ferulic acid, an antioxidant in cereal grain. It was present in rice mold starters prepared by A. awamori, A. kawachii, and A. saitoi, although there was no pyranonigrin-A in the A. oryzae and A. sojae starters. The results suggest that the content of pyranonigrin-A in rice mold starters has a correlation with the antioxidative activity, and that it is induced in rice mold starters at the sporulation stage.

Key words: Aspergillus; fermentation; mold starter; pyranonigrin-A

Reactive oxygen species such as oxygen-free radicals occur both in the human body and in the food system. In addition to inducing lipid peroxidation, which causes the deterioration of foods, these radicals cause oxidative damage by oxidizing biomolecules, leading to cellular death and tissue damage. Indeed, many diseases such as atherosclerosis, cancer, rheumatism, and complications of diabetes are correlated with oxidative damage, and oxidative damage is thought to play a significant pathological role in human disease.⁵ It has been suggested that the intake of antioxidants in food may reduce oxidative damage and have a corresponding beneficial effect on human health. Antioxidants in food as phenolic compounds are plant-derived antioxidants that possess radical-scavenging properties, and have been reported to help prevent diseases such as atherosclerosis and cancer.⁶⁻⁷ Traditional Oriental fermented foods such as rice wine (sake, yakju), fermented soybeans (miso, dou-chi), soy sauce (shoyu), and distilled spirits (shochu) have been consumed widely in the East from ancient times. They are known to contain valuable nutrients (amino acids, carbohydrates, and vitamins) that are produced by microorganisms. Fermented foods and their products have recently received attention for their biofunction in promoting health and preventing diseases.⁴⁻⁷ Koji and its mold starter are used in the early stages of the fermentation process for manufacturing various Japanese fermented foods such as sake, miso, shoyu, and shochu. Koji and its mold starter are prepared by the fermentation of rice, barley, and soybean with GRAS (generally recognized as safe) filamentous fungi such as the Aspergillus species. Koji is made by fermenting steamed cereals at about 30°C for 2–3 days after inoculation with mold starter, and it is important for the digestion of starch in cereals during the manufacture of fermented foods. The mold starter is generally prepared by fermenting steamed rice with Aspergillus spp. at about 30°C for 5–7 days, and it produces epiphytic spores on rice of more than 10⁶ per weight (g) of rice. Fermented foods and their koji have been reported to show antioxidative activity, and have been evaluated for their role in the promotion of health or the prevention of life-style diseases such as cardiovascular disease or diabetes.⁸⁻¹⁰

The phenolic compounds as ferulic acid and its derivatives in cereals have been shown to have antioxidative activity and to have a suppressive effect on carcinogenesis in experimental animals.¹¹,¹² Soybeans contain isoflavone glycosides as daidzin and genistin,
and fermentation is known to enhance their antioxidative activity. It has been reported that miso, a fermented soy paste prepared in Japan, and dou-chi, a fermented soybean food prepared in China and Taiwan, contain potent antioxidants as o-hydroxyisoflavonones which are produced from isoflavone glycosides by fermentation with *Aspergillus* species.\(^{13-15}\) Soybean koji, prepared by *Aspergillus awamori* as a starter for fermented foods made of soybeans, has been reported to have high antioxidative activity and antimutagenic activity.\(^{10,16}\)

Furthermore, the mold of *Monascus anka*, which is one of the molds used to manufacture the fermented soybean product “tofuyo” eaten in the Okinawa region of Japan, has been reported to produce dimerumic acid, an antioxidant.\(^{17}\) Rice mold starters of the *Aspergillus* species are used widely in the manufacture of koji for fermented foods such as sake, miso, shoyu, and shochu. However, their antioxidative activity or efficacy as antioxidants has never been reported. We attempted in this study to examine the antioxidative activity and antioxidative properties of rice mold starters prepared by fermentation with the *Aspergillus* species for the purpose of evaluating their function in the production of fermented foods and materials.

### Materials and Methods

**Materials.** Ten *Aspergillus* species used for the manufacture of mold starters were obtained from Bioc Industries, Osaka, Japan. *A. oryzae* KBN1010 (mold starter for sake), *A. oryzae* KBN1022 (mold starter for sake), *A. oryzae* KBN919 (mold starter for miso), *A. oryzae* KBN943 (mold starter for miso), *A. oryzae* KBN616 (mold starter for shoyu), *A. oryzae* KBN630 (mold starter for shoyu), *A. sojae* KBN622 (mold starter for shoyu), *A. awamori* KBN2012 (mold starter for shochu), *A. kawachi* KBN2001 (mold starter for shochu), and *A. saitoi* KBN2024 (mold starter for shochu) were obtained. The reagents used in this study were of analytical or HPLC grade (Wako Pure Chemical Industries, Osaka, Japan).

**Preparation of the rice mold starters.** Polished rice (2–3% degree of milling) was soaked overnight in water, fully treated for 1 h in a steamer basket, and then cooled to 35 °C. Test cultures were cultured in a PDA medium for 7 days at 30 °C and their spores were collected. The spores of the *Aspergillus* species (1 ml, \(10^6\) spores/ml) were inoculated into 10 g of steamed rice and cultured for 7 days at 30 °C. The number of spores of the rice mold starters after 7 days was more than \(5 \times 10^8\) spores per weight (g) of rice.

**Isolation of antioxidant from the rice mold starters.** A rice mold starter (50 g) was prepared by fermenting rice with *A. saitoi* and extracted with 1.0 liter of methanol. The extract was concentrated under reduced pressure, and the concentrate was put into an absorbent resin column (Φ37 × 500 mm, Amberlite XAD-2 resin, Rohm and Haas Co., Philadelphia, USA). The column was washed with 2.0 liter of water and eluted with 2.0 liter of 40% methanol, the eluate being concentrated under reduced pressure. The concentrated extract was applied to preparative HPLC (LC-8A, Shimadzu Co., Kyoto, Japan), which was carried out with YMC-ODS column (YMC-Pack ODS-A, Φ20 × 250 mm, S-5 μm, YMC Co., Kyoto, Japan). UV detection at 280 nm, a mobile solvent of 35% methanol, and a flow rate of 10 ml/min at room temperature. The fractions from each of the peaks separated by preparative HPLC were checked for antioxidative activity by a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, and the antioxidative fraction (peak-A) was concentrated under reduced pressure. An antioxidant (14.6 mg) was isolated and its purity was more than 99% by an HPLC analysis.

**DPPH radical scavenging activity.** The antioxidative activity of each sample was measured by a DPPH radical scavenging assay. A 0.5-g sample of fermented rice was extracted with 10 ml of methanol for 12 h at room temperature after an ultrasonic treatment (US-5R, Asone Co., Osaka, Japan) for 55 min. The DPPH radical scavenging activity of each extract was then measured. 100 μl of 0.13 mg/ml of DPPH dissolved in ethanol, 80 μl of 0.1 M Tris-HCl buffer (pH 7.4), and 20 μl of the sample were mixed on a microplate.\(^{18}\) After a 1-h incubation at room temperature, the absorbance was recorded at 517 nm with a microplate reader (Sunrise Rainbow, Wako Pure Chemical Industries, Osaka, Japan). An antioxidant (pyranonigrin-A), isolated from the rice mold starter, was dissolved in DMSO to prepare 10 mM solution. Ferulic acid, trolox, and \(\alpha\)-tocopherol were also each dissolved in DMSO to prepare 10 mM samples as reference antioxidants and were used for positive control. The samples were assayed at a final concentration of 40 μM or 80 μM. The negative control was run with a sample solvent in place of the test sample. Each result is expressed as the percentage decrease with respect to the negative control value. Each data value is presented as the mean ± SD (N = 3). A statistical analysis was conducted by two-way ANOVA and subsequent Fisher PLSD test to identify significant differences.

**Superoxide radical scavenging activity.** Samples of 10 mM used in the DPPH radical scavenging assay were diluted to 0.2 mM with methanol. The superoxide radical scavenging effect of phenolic compounds was measured with a commercial kit (SOD assay kit-WST, Dojindo Molecular Technologies, Gaithersburg, USA), using a 96-well microplate.\(^{19}\) The microplate, with the added reagents, was incubated at 37 °C for 30 min, and the absorbance was measured at 450 nm with a microplate reader. The negative control was run with a sample solvent, and the antioxidants, trolox and \(\alpha\)-tocopherol, served as the positive controls. Each sample and
standard antioxidant was assayed at a final concentration of 8.3 μM and 16.7 μM. Results are expressed as the percentage decrease with respect to the negative control value. Each data value is conducted as the mean ± SD (N = 3). A statistical analysis was conducted by two-way ANOVA and subsequent Fisher PLSD test to identify significant differences.

**Determination of pyranonigrin-A by HPLC.** The content of pyranonigrin-A in rice or fermented rice was determined by HPLC (900 series, Jasco Co., Tokyo, Japan), using a YMC-ODS column (YMC-pack Φ4.6 × 150 mm, S-5 μm, YMC Co., Kyoto, Japan). UV detection at 311 nm, mobile solvents of methanol and water containing 5% acetic acid, a flow rate of 1 ml/min, and a column temperature of 40°C. The concentration of methanol in the mobile phase condition was changed from 10% to 90% in 15 min, and 100% methanol was eluted for 5 min. The retention time of pyranonigrin-A was 7.8 min. The content of pyranonigrin-A in the fermented rice (mg/g of fermented rice) is shown as the mean ± SD (N = 3). Levels of less than 1 μg/g of fermented rice are shown as “ND” (no detection).

**Instrumental analysis of the antioxidant isolated from the rice mold starter.** The UV–vis absorption spectrum for the sample dissolved in methanol was recorded by a spectrophotometer (Hitachi U-2000, Hitachi High-Technologies Co., Tokyo, Japan). 1H-NMR and 13C-NMR spectra for the sample were recorded on a Jeol JNM-A-600 NMR instrument (600 MHz for 1H and 150 MHz for 13C, Jeol, Tokyo, Japan). Chemical shifts for 1H- and 13C-NMR are given in parts per million (δ) relative to the solvent signal (DMSO-d6: δH 2.49 and δC 39.5). The FAB-MS data were obtained with a Jeol JMS HX-110 spectrometer (Jeol, Tokyo, Japan).

Pyranonigrin-A: UV (MeOH): λ max: 311, 250, 209. 1H-NMR (DMSO-d6, 600 MHz) δ: 1.92 (3H, br d, J = 7.0, H-3'), 5.72 (1H, br d, J = 8.8, H-7), 6.45 (1H, dq, J = 15.8, 8.1, H-2'), 6.57 (1H, br d, J = 15.8, H-1'), 6.78 (1H, d, J = 8.8, OH-7), 8.62 (1H, br s, NH), 9.69 (1H, s, OH-3). 13C-NMR (DMSO-d6, 150 MHz) δ: 18.6 (C-3'), 75.0 (C-7), 111.6 (C-4a), 118.9 (C-1'), 131.5 (C-2'), 142.1 (C-3), 145.8 (C-2), 164.9 (C-5), 168.9 (C-4), 175.1 (C-7a). FAB-MS: m/z 224 [M + H]+.

**Results and Discussion**

**Antioxidative activity of the extracts of rice mold starters**

Ten rice mold starters were prepared by fermenting rice with Aspergillus species, A. oryzae, A. sojae, A. awamori, A. kawachii, and A. saitoi. The methanol extracts of these ten rice mold starters were examined for their antioxidative activity by a DPPH radical scavenging system (Fig. 1). The extracts of A. awamori, A. kawachii, and A. saitoi, which are used as the rice mold starter of Japanese distilled liquor, shochu, had significantly high antioxidative activity in comparison with that of 40 μM trolox (P < 0.001). Their extracts had higher antioxidative activity than other Aspergillus species which are used as the rice mold starters for sake, miso, and shoyu. The activity of A. saitoi had the highest activity of all the rice mold starters. A. saitoi and A. awamori are used to manufacture “awamori,” which is a distilled liquor, shochu, produced in the Okinawa region of Japan. Soybeans fermented with A. saitoi have been reported to exhibit the highest antioxidative activity of 30 kinds of Aspergillus strains.20 Potent antioxidants such as 6-hydroxydaidzein, 8-hydroxydaidzein and 8-hydroxygenistein of the o-dihydroxysflavones have been produced by fermenting soybeans with A. saitoi.21,22 Moreover, o-dihydroxysflavones, which have been isolated from fermented soybean miso, are reported to have DPPH radical scavenging activity, antiproliferative activity, and antimutagenic activity.13,14 Furthermore, the production of the potent antioxidant, 8-hydroxyhesperetin, by the fermentation of hesperidin (hesperetin 7-O-β-rutinoside) with A. saitoi has been reported.23 The filamentous fungus of A. saitoi of used in fermented foods has been shown to have the capability for hydrolysis to the aglycone from a flavonoid glycoside and for hydroxylation to o-dihydroxylflavonoid.21,22 The koji made by the fermentation of soybeans with A. awamori reportedly has high antioxidative activity, although the antioxidants linked to this activity are not clear.19 Our study found high antioxidative activity in the rice mold starters prepared by fermenting rice with A. awamori, A. kawachii, and A. saitoi. This suggests that the antioxidants were produced by the fermentation of rice with their filamentous fungi.

**Isolation and identification of the antioxidant**

The extract of A. saitoi, which exhibited the highest antioxidative activity in comparison with other A. awamori trolox (P < 0.001). Their extracts had higher antioxidative activity than other Aspergillus species which are used as the rice mold starters for sake, miso, and shoyu. The activity of A. saitoi had the highest activity of all the rice mold starters. A. saitoi and A. awamori are used to manufacture “awamori,” which is a distilled liquor, shochu, produced in the Okinawa region of Japan. Soybeans fermented with A. saitoi have been reported to exhibit the highest antioxidative activity of 30 kinds of Aspergillus strains.20 Potent antioxidants such as 6-hydroxydaidzein, 8-hydroxydaidzein and 8-hydroxygenistein of the o-dihydroxysflavones have been produced by fermenting soybeans with A. saitoi.21,22 Moreover, o-dihydroxysflavones, which have been isolated from fermented soybean miso, are reported to have DPPH radical scavenging activity, antiproliferative activity, and antimutagenic activity.13,14 Furthermore, the production of the potent antioxidant, 8-hydroxyhesperetin, by the fermentation of hesperidin (hesperetin 7-O-β-rutinoside) with A. saitoi has been reported.23 The filamentous fungus of A. saitoi of used in fermented foods has been shown to have the capability for hydrolysis to the aglycone from a flavonoid glycoside and for hydroxylation to o-dihydroxylflavonoid.21,22 The koji made by the fermentation of soybeans with A. awamori reportedly has high antioxidative activity, although the antioxidants linked to this activity are not clear.19 Our study found high antioxidative activity in the rice mold starters prepared by fermenting rice with A. awamori, A. kawachii, and A. saitoi. This suggests that the antioxidants were produced by the fermentation of rice with their filamentous fungi.

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from fermentation extract of marine fungus *Aspergillus niger* (culture LL-LV3020). This study found pyranonigrin-A in the rice mold starter, which is safe and practical for use in food processing, for the first time.

**Antioxidative activity of pyranonigrin-A**

The antioxidative activity of pyranonigrin-A was measured by the radical scavenging activity toward DPPH and superoxide (Tables 1 and 2). It was compared with ferulic acid, which is known as an antioxidant in such cereals as rice, corn, wheat, and oats.25) The radical scavenging activity of pyranonigrin-A toward DPPH and superoxide was approximately equivalent to that of ferulic acid, although pyranonigrin-A tends to have weaker activity toward DPPH and stronger activity toward superoxide in comparison with ferulic acid. The antioxidative activity of pyranonigrin-A has not previously been reported and was found in this study for the first time.

**Distribution of pyranonigrin-A in rice mold starters fermented by Aspergillus species**

The content of pyranonigrin-A in rice mold starters fermented by *Aspergillus* species was determined by HPLC (Table 3). Although there was no pyranonigrin-A in non-fermented rice, it was found in rice mold starters of *A. awamori* (0.70 ± 0.04 mg/g of rice mold starter), *A. kawachii* (1.59 ± 0.02 mg/g of rice mold starter), and *A. saitoi* (4.05 ± 0.02 mg/g of rice mold starter). Pyranonigrin-A was found to be produced by the fermentation of rice with filamentous fungi which are used for the manufacturing the distilled liquor, shochu, in Japan. However, it was not detected in rice mold starters prepared with *A. oryzae* and *A. sojae*, which are used for the manufacture of sake, miso, and shoyu. The distribution in rice mold starters was found to be specific to the *Aspergillus* species. The amount of pyranonigrin-A in the rice mold starter of *A. saitoi*, which exhibited high antioxidative activity in Fig. 1, was the highest of all. It was found that, the higher the pyranonigrin-A content, the higher the antioxidative activity of the extract for the rice mold starter. The content of pyranonigrin-A in the rice mold starters was found to have a correlation with the antioxidative activity. Pyranonigrin-A is suggested to be the main antioxidant in rice mold starters.

**Content of pyranonigrin-A and antioxidative activity toward fermented rice during culturing**

The content of pyranonigrin-A in rice fermented by *A. saitoi* during the culture period was determined by HPLC (Fig. 4). There was no pyranonigrin-A in fermented rice for the first two days, and then a small
quantity was detected in fermented rice during day 3. The amount increased until day 5 and remained constant after that. The DPPH radical scavenging activity of the extract of fermented rice during the culture period increased until day 5 and remained at an approximately constant level after that. These results suggest that pyranonigrin-A was the main antioxidant in rice mold starters of A. saitoi and that the pyranonigrin-A content has a correlation with the antioxidative activity of the rice mold starter. Furthermore, A. saitoi spores could be observed by the naked eye on fermented rice on day 2, being $2.5 \times 10^6$ in number per weight (g) of fermented rice. The spores on the rice mold starters were found to increase in number until day 5 ($2.5 \times 10^8$ spores/g of fermented rice) and then remain constant after that. The content of pyranonigrin-A in fermented rice was found to be correlated with the spore level on the rice. It has been reported that $\alpha$-dihydroxyisoflavone, a potent antioxidant in fermented soybeans, was produced at the sporulation stage for A. saitoi.28

This suggests that pyranonigrin-A may also be produced at the spore formation stage. Pyranonigrin-A, which has a pyrano pyrrole skeleton, has not previously been reported to be present in rice. It is possible that pyranonigrin-A may not be converted from a compound in rice, but be a secondary metabolite of such Asper-

### Table 1. DPPH Radical Scavenging Activity of Pyranonigrin-A

<table>
<thead>
<tr>
<th>Concentration</th>
<th>40$\mu$m</th>
<th>80$\mu$m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyranonigrin-A</td>
<td>$17.2 \pm 1.9^a$</td>
<td>$35.6 \pm 2.2^a$</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>$20.2 \pm 0.7^a$</td>
<td>$45.3 \pm 3.6^a$</td>
</tr>
<tr>
<td>$\alpha$-Tocopherol</td>
<td>$15.5 \pm 1.8^a$</td>
<td>$56.7 \pm 5.1^a$</td>
</tr>
<tr>
<td>Trolox</td>
<td>$28.4 \pm 5.2^a$</td>
<td>$61.2 \pm 3.3^a$</td>
</tr>
</tbody>
</table>

Samples were examined for their radical scavenging activity toward DPPH at final concentrations of 40$\mu$m and 80$\mu$m. Each data value is presented as the mean $\pm$ SD (N = 3). Values within the same row that do not share a common superscript letter are significantly different at $P < 0.05$.

### Table 2. Superoxide Radical Scavenging Activity of Pyranonigrin-A

<table>
<thead>
<tr>
<th>Concentration</th>
<th>8.3$\mu$m</th>
<th>16.7$\mu$m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyranonigrin-A</td>
<td>$5.6 \pm 0.9^a$</td>
<td>$17.2 \pm 0.1^b$</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>$4.1 \pm 0.2^a$</td>
<td>$9.6 \pm 0.6^b$</td>
</tr>
<tr>
<td>$\alpha$-Tocopherol</td>
<td>$10.2 \pm 1.0^a$</td>
<td>$42.7 \pm 0.9^b$</td>
</tr>
<tr>
<td>Trolox</td>
<td>$4.4 \pm 0.2^a$</td>
<td>$7.4 \pm 1.4^a$</td>
</tr>
</tbody>
</table>

Samples were examined for their radical scavenging activity toward superoxide at final concentrations of 8.3$\mu$m and 16.7$\mu$m. Each data value is presented as the mean $\pm$ SD (N = 3). Values within the same row that do not share a common superscript letter are significantly different at $P < 0.05$.

### Table 3. Content of Pyranonigrin-A in the Rice Mold-Starter Prepared by Aspergillus species

<table>
<thead>
<tr>
<th>Pyranonigrin-A content in rice mold-starter (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No fermented rice</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN1010</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN1022</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN943</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN630</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN616</td>
</tr>
<tr>
<td>Aspergillus oryzae KBN622</td>
</tr>
<tr>
<td>Aspergillus awamori KBN2012</td>
</tr>
<tr>
<td>Aspergillus kawachii KBN2001</td>
</tr>
<tr>
<td>Aspergillus saitoi KBN2024</td>
</tr>
</tbody>
</table>

Samples were examined for their radical scavenging activity toward superoxide at final concentrations of 8.3$\mu$m and 16.7$\mu$m. Each data value is presented as the mean $\pm$ SD (N = 3). Values within the same row that do not share a common superscript letter are significantly different at $P < 0.05$. The concentration of methanol in the mobile phase was changed from 10% to 90% for 15 min, and 100% methanol was eluted for 5 min.
Elucidation of the biosynthesis of pyranonigrin-A in rice mold starters from A. saitoi, A. awamori, and A. kawachii, is a subject for further research.

This study investigated the antioxidant in rice mold starters produced by several Aspergillus species. Pyranonigrin-A was isolated for the first time from rice mold starters used in the manufacturing process for the Japanese distilled liquor, shochu. Pyranonigrin-A was found to have antioxidative activity as a biofunction, although this function will need further research. We expect that functional research on pyranonigrin-A will be connected to elucidating the biofunction in oriental fermented foods.

Acknowledgments

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References

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