Antioxidant Activity of a Medicine Based on Aspergillus oryzae NK Koji Measured by a Modified t-Butyl Peroxy Radical Scavenging Assay

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A koji-based medicine composed of powder of Aspergillus oryzae NK koji, dried yeast, and lactobacilli koji had high antioxidant activity measured by a modified t-butyl peroxyl radical scavenging assay. This activity was mainly derived from A. oryzae NK koji. Digestion of koji-making grain germ medium with several commercial enzymes also increased antioxidant activity. By two weeks of oral administration of A. oryzae NK koji, the serum lipid peroxide levels elevated in STZ-induced diabetic rats could be decreased significantly.

Key words: antioxidant; t-butyl peroxyl radical scavengers; medicine; Aspergillus oryzae; koji

There is currently great interest in free radical mediated damage associated with many diseases, and much effort has gone into the study1-6 of antioxidant substances especially of plant origin (vegetables, fruits, spices, herbs, etc.) detected in foods, and of their therapeutic effect against various diseases which are caused by in vivo radical reactions by ingestion in raw or after processing (heating, mixing, fermentation, etc.). In addition to daily foods, medical products (gastrointestinal preparations, nutritional supplements, etc.) containing natural compounds involving microbial cells, digestive enzymes, extracts from plants or Chinese herbs, etc. could be expected to play a role in general health care or have mild therapeutic effects. In this report we describe the antioxidative activity of a test sample of a commercial preparation for gastrointestinal regulation and a nutritional supplement derived from a kind of cereal germ koji fermented with Aspergillus oryzae, and also the effect on serum lipid peroxide levels in Streptozotocin (STZ)-induced diabetic rats after its oral administration.

As a test sample, we used Wakamoto Strong (Wakamoto Pharmaceutical Co., Ltd.) which is comprised of A. oryzae NK koji, dried yeast, and lactobacilli koji and contains no antioxidative additives such as ascorbic acid. Grain germ medium is used for the koji production. The test sample was used after being powdered. A lot of A. oryzae NK koji was the same component of the test sample used. Total reducing activity (TRA) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity were measured by the methods of Tsushida et al.6 and Kato et al.,7 respectively. t-Butyl peroxyl radical (t-BuOO·) scavenging activity was measured by the bioassay method of Akaie et al.5,9 with some modifications (Kawasumi et al., preceding paper in this issue). Cold water extracts and gastrointestinal pH extract (designated as Gp extract) from the test sample, A. oryzae NK koji, dried yeast, lactobacilli koji powder, and sterilized grain germ medium were prepared as follows: Ground sample (2.5 g) was suspended in 50 ml of cold distilled water, and stirred on ice for 4 h to obtain the cold extract. In the latter case, ground sample (2.5 g) was suspended in 10 ml of distilled water and adjusted to pH 5.0 with 1.0 N HCl. Then, the pH was gradually decreased to 2.0 with 1 N HCl under shaking for 4 h at 37°C. The extracted solution was centrifuged (2800 rpm, 15 min, 4°C), and supernatant (sup1) was recovered. The residue was re-extracted with 25 ml of cold water for 30 min, centrifuged at the same condition as the first extraction, and supernatant (sup2) was collected. This extraction procedure was repeated again, and supernatant (sup3) was collected. Sup1-3 were pooled, adjusted to pH 7.0, filled up to 100 ml with cold water, and used for analysis. For ethanol extract preparation, one gram of ground sample was suspended in 30 ml of ethanol and statically extracted at room temperature overnight. Supernatant was collected after centrifugation (2800 rpm, 15 min, 4°C). The residue was re-extracted twice by suspending in 20 ml of ethanol at room temperature for one hour and centrifuged. Pooled supernatants were dried in vacuo. Dried extracted material was solubilized twice by 2.5 ml of ethanol, filled up to 5 ml with ethanol, and used for analysis. Activities of α-amylase, pH 6-protease, pH 3-protease, and lipase were measured by the method described in the Pharmacopoeia of Japan. Leucine aminopeptidase activity was measured by using a “LAP C Test Wako” (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The effects of A. oryzae NK koji administration on serum lipid peroxide level in STZ-induced diabetic rats was done as follows: Sixty-two male Crj:CS (SD) rats, 6 weeks old (Charles River Japan Inc.) were maintained on laboratory chow pellets (MF, Oriental Yeast) and water ad libitum. Seven-week-old rats weighing 220-

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Abbreviations: t-BuOO·, t-Butyl peroxyl radical; TRA, total reducing activity; DPPH, 1,1-diphenyl-2-picrylhydrazyl; STZ, Streptozotocin
260 g were grouped by the randomized classification by body weight into six groups containing 10 rats in each group. After being starved overnight, one group was injected i.v. 1 ml/kg body weight of 0.05 M citrate buffer (pH 4.5), and the others were injected with 60 mg/kg of STZ prepared in 0.05 M citrate buffer (pH 4.5) immediately before injection. Injections were done via a tail vein under mild ether anesthesia. Then, after rats were kept on MF for one week, the urinary sugar of the injected 52 rats was measured with Tes·Tape A (Shionogi Pharmaceutical Co., Ltd). Urinary sugar-positive rats were again grouped into five groups having 8 rats in each group by randomized classification by body weight. Then, after they were kept for 2 weeks on mixed chow (Control and Diabetic control groups: 100% MF chow; 1%, 5%, and 15% test sample groups: 1, 5, and 15% test sample and MF chow 99, 95, and 85%, respectively; 7.5% A. oryzae NK koji group: 92.5% MF chow and 7.5% A. oryzae NK koji), starved overnight before killing, and blood was collected via the abdominal aorta under ether anesthesia. Sera were separated from blood samples, and lipid peroxides were measured by the method of thiobarbituric acid. Measurement was ordered at Bozo Research Center Inc. (Gotenba, Shizuoka). Results of statistical analysis are shown in mean±S.D. (standard deviation). The significance of the result was examined on Wilcoxon rank sum test between the control group and the diabetic control (DC) group. After testing in homoscedasticity of each group by the Bartlett method, in case of homoscedasticity, analysis of variance on one-way layout by the parametric Dunnett method was done between the DC group and the test sample or A. oryzae NK koji, or in case of non-homoscedasticity, the difference of mean values between the DC group and the test sample or A. oryzae NK koji was analyzed by non-parametric Dunnett method after doing Kruskal-Wallis test. The significant level of these analysis was fixed at 5% or 1% on one side.

Three kinds of components of the test sample: A. oryzae NK koji (50%), dried yeast (38%), and lactobacilli koji powder (10%); and sterilized grain germ medium were extracted by three different conditions (cold water extraction, Gp extraction, and ethanol extraction), and their antioxidant activity was analyzed. Table 1 shows that among three components A. oryzae NK koji had the highest antioxidant activity, and that lactobacilli koji powder had almost no activity, at the same level as the sterilized grain germ medium, based on the \( t \)-BuOO· scavenging assay (these tendencies were also observed with TRA or DPPH measurements). A. oryzae NK koji powder was considered to be a main source of antioxidant activity in the test sample. Antioxidant activities in koji or similar products fermented with A. oryzae were reported elsewhere. In 1974, Yamaguchi et al. found that an antioxidative peptide with molecular weight of 2500–3000 was derived from digestion of soy proteins by a protease from A. oryzae. Niwa and Minamiyama speculated that antioxidant activity in koji could be derived from low molecular weight compounds liberated by digestion of high molecular weight polymers existing in the native products during koji fermentation. Rashid et al. reported antioxidative substances produced extracellularly by the liquid-state cultivation of A. oryzae. The purification and characterization of the substances indicated that they were branched chain peptides with molecular weight of about 700 and able to suppress the oxidation of fish oil.

To clarify that the high antioxidative activity of A. oryzae NK koji was caused by digestion of grain germ medium with secreted enzymes of A. oryzae during koji making, we tried to produce substances to show antioxidative activity by digestion of grain germ medium with commercial enzyme preparations, instead of fermentation with A. oryzae. In Table 2, the amounts of enzymes added to the medium and antioxidative activity changes of the medium are shown. After the media were mixed with each enzyme preparation and were maintained under the same conditions as for koji making, they were extracted with cold water for 4 hours. Biodiastase 2000 (Amano Pharmaceutical Co., Ltd., Tokyo, Japan) and Sumizyme SAL or LP (Sin Nihon Chemical Co. Tokyo, Japan) were found to be effective for generating antioxidative activities in koji-making medium, while Newlase and Lipase AP6 (Amano Pharmaceutical Co., Ltd., Tokyo, Japan) had little effect on the increase of the activity. There was a relatively high positive correlation between activities of pH 6-protease or leucine aminopeptidase and generated \( t \)-BuOO· scavenging activity, but \( \alpha \)-amylase and lipase activity had a weak but negative correlation. These results suggested that enzymes (especially proteases) secreted by A. oryzae degraded medium proteins (protein content of the test sample was calculated as 37.3% based on the Kjeldahl method) into low molecular weight substances that could give high antioxidative activity. Other experiments also showed that it is acid- and alkaline-stable, heat-stable, and low molecular weight molecule(s), and that at least polyphenolic.
Table 2. Antioxidant Activity of Grain Germ Medium Digested with Commercial Enzyme Preparations

<table>
<thead>
<tr>
<th>Enzymes(a)</th>
<th>Amount of enzyme ((mg/g \text{ medium}))</th>
<th>(t)-BuOO(^.-) scavenging(^b) ((\text{mg AsA eq./g \text{ medium}}))</th>
<th>Enzyme activities in commercial enzymes(^c) ((\text{unit/g \text{ medium}}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>42.4 (100)</td>
<td>Amylase pH 6 (^d) pH 3 (^e) LAAk (^f) Lipase</td>
</tr>
<tr>
<td>Biodiastase 2000</td>
<td>31.7 (10.5) (^g)</td>
<td>99.6 (188) (^h)</td>
<td>1000 1070 434 1240 0</td>
</tr>
<tr>
<td>Newlase</td>
<td>6.34 (2.1)</td>
<td>73.8 (166)</td>
<td>200 214 87 247 0</td>
</tr>
<tr>
<td>Lipase AP6</td>
<td>8.30 (1.45)</td>
<td>54.6 (125)</td>
<td>87 81 430 39 29</td>
</tr>
<tr>
<td>Lipase SAL</td>
<td>1.68 (0.294)</td>
<td>51.1 (120)</td>
<td>17 16 86 8 5.8</td>
</tr>
<tr>
<td>Sumizyme LP</td>
<td>4.34 (0.358)</td>
<td>49.8 (116)</td>
<td>1 4 4 20 29</td>
</tr>
<tr>
<td>Sumizyme HP</td>
<td>0.86 (0.071)</td>
<td>37.2 (88)</td>
<td>0.2 0.8 0.8 4 5.8</td>
</tr>
<tr>
<td></td>
<td>8.70 (1.7)</td>
<td>60.4 (137)</td>
<td>198 52 15 8.4 0</td>
</tr>
<tr>
<td></td>
<td>31.0 (8.37)</td>
<td>109.5 (216)</td>
<td>29 1287 1070 2960 0</td>
</tr>
<tr>
<td></td>
<td>6.20 (1.67)</td>
<td>96.2 (218)</td>
<td>6 257 214 592 0</td>
</tr>
</tbody>
</table>

Correlation coefficient\(^i\) 0.43 0.84 0.67 0.76 –0.55

\(a\) Each enzyme preparation was added to the medium for \(koji\) making (water content: 55%), and incubated for 48 h at 30°C.
\(b\) Activities of samples extracted with cold water for 4 hours were determined.
\(c\) Figures in parentheses in a column mean \(t\)-BuOO\(^.-\) scavenging activity of added enzyme expressed as mg AsA eq./g medium.
\(d\) Figures in parentheses in a column mean percentage of \(t\)-BuOO\(^.-\) scavenging activity compared with sum of that of the grain medium and added enzyme.
\(e\) pH 6-Protease.
\(f\) pH 3-Protease.
\(g\) Leucine aminopeptidase.
\(h\) Correlation coefficient between \(t\)-BuOO\(^.-\) scavenging activity and each enzyme activity contained in commercial enzymes.

**Fig.** Effects of Test Sample and \(A. oryzae\) NK \(koji\) on Serum Lipid Peroxide in STZ-Induced Diabetic Rats.

compounds displaying coloration by FeCl\(_3\) reagent\(^{16}\) were absent in the medium (data not shown). These data suggested that the degraded product during \(A. oryzae\) NK \(koji\) making might be one of the antioxidative sources in the test sample, though some substances secreted by \(A. oryzae\) which were described by such as Rashid et al.\(^{14}\) could also be candidates.

The effects of the test sample and its component, \(A. oryzae\) NK \(koji\), on serum lipid peroxide elevated in STZ-induced diabetic rats is shown in the Figure. The serum lipid peroxide of the normal control group was 2.4±0.2 nmol/ml, while that of the diabetic control (DC) group increased to 5.7±2.0 nmol/ml and found to be statistically significant (p<0.01). Sera lipd peroxide of the test sample group and \(A. oryzae\) NK \(koji\) group in diabetic rats could be decreased as follows: 15% test sample group was 3.2±0.7 nmol/ml and 7.5% \(A. oryzae\) NK \(koji\) group was 3.6±1.4 nmol/ml, and the differences between these groups and DC group were found to be statistically significant (p<0.01, p<0.05, respectively). Identification of a compound responsible for high antioxidant activity or elevated serum lipid peroxide decrease in \(A. oryzae\) NK \(koji\) is under investigation. In addition, the antioxidative activities of other medical product samples (gastrointestinal preparations, nutritional supplements, etc.) containing natural compounds also become a subject of considerable interest.

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**References**


