Antioxidant Activity of Hydrophilic Compounds of Defatted Soybean Fermented with Neurospora intermedia (D-Ontjom)

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Defatted soybean-ontjom (D-ontjom, defatted soybean fermented with Neurospora intermedia) was prepared to utilize defatted soybean (DSB) as a food source. The antioxidant activity of hydrophilic components of D-ontjom and the mechanism were investigated. The 1,1-diphenyl-2-picryl-hydrazyl-radical scavenging activities of three extracts from D-ontjom, hexane, methanol and water extracts, were all stronger than those from DSB. Among them, the water extract from D-ontjom showed the strongest activity. I prepared an 80% methanol soluble fraction (hydrophilic fraction) from the water extract, which showed much stronger DPPH-radical scavenging activity. This hydrophilic fraction strongly suppressed the generation of superoxide anion and glutathione-autoxidation. To confirm the antioxidant activity of the hydrophilic fraction in vivo, rats were fed the fraction prepared from D-ontjom or DSB. The serum and liver thiobarbituric acid reactive substance (TBARS) values, hepatic superoxide dismutase (SOD, EC 1.15.1.1) activity and serum glutathione peroxidase (GSH-PX, EC 1.11.1.9) activity in rats fed the D-ontjom diet were significantly lower compared with those in rats fed the DSB diet, while their serum levels of α-tocopherol and glutathione were remarkably higher than those of rats fed the DSB diet. These results suggest that some hydrophilic components of D-ontjom have a high antioxidant activity in vivo, perhaps due to their radical scavenging activity.

Keywords: in vivo antioxidant activity, defatted soybean ontjom, ontjom, SOD, GSH-Px., TBARS

Defatted soybean (DSB) contains about 50% crude protein and is an excellent vegetable protein source, but only about 10% of DSB is utilized as food material. In Indonesia, okara (the insoluble residue of homogenized soybean) has been utilized as okara-ontjom (Bogor red oncom), which is a traditional indigenous food fermented with Neurospora intermedia, and is usually consumed as well as tempe (Sastramadja & Saono, 1984). Generally, fermented soybean foods such as miso (Yamaguchi et al., 1979), tempe (Esaki et al., 1996) and natto (Esaki et al., 1990) have high antioxidant activities, and many kinds of hydrophobic antioxidants have been identified from soybean products.

We previously prepared DSB-ontjom (D-ontjom, DSB fermented with N. intermedia) in order to utilize DSB as a food source (Matsuo & Yamoto, 1999). Recently, I reported the antioxidant activity of hydrophobic components of D-ontjom in rats and assumed its mechanism to be a collaborative contribution of isoflavone-aglycones and carotene (Matsuo, 2001). In this paper, I investigated the antioxidant activity of hydrophilic components of D-ontjom and its antioxidative mechanism.

Materials and Methods

Preparation of D-ontjom (Matsuo and Yamoto, 1999) DSB was mixed with 0.4% acetic acid to 60% moisture content (pH 5.4) to sterilize it for a short time, and autoclaved for 5 min. After cooling, DSB was shaped into a 2 cm-thick cake and the ontjom starter (Matsuo, 1997a) was seeded on the cake-surface. The inoculated cake was incubated at 30°C for 18 h while a tightly covered with plastic sheeting. After spores had germinated, the cake was fermented at 27°C for another 24 h at 65% humidity of without a covering.

Preparation of antioxidant extracts The lyophilized powder of DSB or D-ontjom 25 g was refluxed with 200 ml of hexane and 200 ml of methanol, successively, in a Soxhlets extractor for 6 h. The hexane or methanol extract was concentrated to 5 ml with a rotary evaporator. After removal of methanol, 125 ml of water was added to the residue and the mixture was vigorously stirred for 3 h at room temperature. After centrifugation at 1000 ×g for 20 min, the supernatant of the water extract was heated at 121°C for 5 min and the resulting precipitate was removed by centrifugation at 43,000 ×g for 25 min. The secondary supernatant was lyophilized and vigorously stirred with 80% methanol (5 ml) for 5 min. After removal of methanol, the 80% methanol soluble fraction was lyophilized as the hydrophilic fraction. The yields of these fractions from DSB and D-ontjom were 6.0% and 13.5%, respectively. No isoflavones or α-tocopherol was detected in these fractions.

Preparation of anionic fraction The hydrophilic fraction of D-ontjom was adsorbed on an Amberlite IRA400 (24×150 mm) column and then eluted with 0.05N acetic acid.

DPPH-radical scavenging activity Sample solution (0.3 ml) was added to a mixture of 0.2 mM 1,1-diphenyl-2-picryl-hydrazyl (DPPH in ethanol; 0.3 ml) and 0.1 M acetate buffer (pH 5.5, 0.3 ml). The decrease in absorbance at 517 nm for 1 min was measured at room temperature.

Glutathione (GSH) oxidation (Matsuo et al., 1997) An
aqueous solution of 0.9 mM GSH in 50 mM phosphate buffer (pH 8.0, 0.9 ml) was incubated with sample at 30°C for 30 min. The remaining GSH was reacted with 2.5 mM of hydrogen peroxide (0.2 ml) and liver supernatant. The absorbance at 412 nm was measured.

Glutathione level of serum (Harrap, 1967) Serum was deproteinized with salicylic acid and reacted with DTNB reagent. The absorbance at 412 nm was measured.

Thiobarbituric acid reactive substance (TBARS) value The TBARS values of liver and serum were measured at 532 nm by the method of Ohkawa et al. (1979) and Hitomi et al. (1999), respectively, using 1,1,3,3-tetraethoxypropane as the standard substance.

α-Tocopherol α-Tocopherol of serum was extracted with ethanol/water/hexane (2/1/0, v/v; Igarashi, 1989) and then analyzed by HPLC (Matsu, 1997b) and a mineral mixture according to Harper (1959). These diets were included in 1000 g of each diet. Mixtures of a vitamin E-free vitamin mixture and a mineral mixture were added to the experimental diets.

Enzyme preparations Liver was homogenized with 4 fold 0.02M tris (hydroxymethyl) aminomethane buffer (pH 8.0) and then centrifuged at 43,000×g for 20 min at 4°C. The supernatants of liver homogenate and serum were used as crude enzymes.

Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity (Gunzler et al., 1984) A mixture of 20 mM GSH (0.1 ml), 4U of GSH-reductase (0.1 ml), 1 mM nicotinamide adenine dinucleotide phosphate reduced form (NADPH, 0.1 ml), 0.05 M Tris-HCl buffer (pH 8.0, 0.3 ml) and 10 mM NaN₃ (0.1 ml) was incubated with 2.5 mM of hydrogen peroxide (0.2 ml) and liver enzyme (5 μl) or serum (10 μl) at 37°C. The consumption of NADPH was monitored by the absorbance at 340 nm.

Superoxide dismutase (SOD, EC 1.15.1.1) activity (Oyanagi, 1984) A mixture of 65 mM phosphate-borate buffer (pH 8.2, 0.1 ml), 0.5 mM xanthine (0.1 ml), 10 mM hydroxylamine (0.05 ml), serum or liver supernatant, and xanthinoxidase (EC 1.2.3.2, Wako Pure Chemical Ind., diluted 100 fold with water, 50 μl), was incubated at 37°C for 30 min. After the naphytylethylenediamine reagent (1 ml) was added to the reaction mixture, the absorbance at 550 nm was measured.

Transaminase activity The activities of glutamic oxaloacetic transaminase (GOT, EC 2.6.1.1) and glutamic pyruvic transaminase (GPT, EC 2.6.1.2) were measured by commercial kits (S-T’-a-test Wako, Wako Pure Chemical Industries, Osaka).

Superoxide anion (O₂⁻) scavenging activity The same method described for the SOD activity was used, except that hydrophilic fractions were used instead of serum or liver supernatant.

Animals and diets Ten 6-week-old male rats of Wistar strain (Japan SLC Inc, Hamamatsu), weighing 207±10 g, were divided into two groups. They were individually kept in stainless steal cages with screen bottoms and maintained on an automatic lighting schedule from 08:00 to 20:00, at 25±1°C, with humidity at 60±10%. The rats were fed two kinds of experimental diets (Table 1) for 2 weeks. Purified fish oil (from tuna, containing 23.7% docosapentaenoic acid and 6.0% eicosapentaenoic acid, and free of tocopherol-isomer) was used as the fat source to accelerate lipid peroxidation in vivo. The hydrophilic fractions obtained from 500 g of D-ontjom and DSB were included in 1000 g of each diet. Mixtures of a vitamin E-free vitamin mixture and a mineral mixture were included in 1000 g of each diet. These diets were im-

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**Table 1.** Composition of experimental diets for rats.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DSB D-ontjom</td>
</tr>
<tr>
<td>SPI</td>
<td>200.0 200.0</td>
</tr>
<tr>
<td>Fish oil</td>
<td>200.0 200.0</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>6.0 6.0</td>
</tr>
<tr>
<td>Salt mixture⁴</td>
<td>40.0 40.0</td>
</tr>
<tr>
<td>Vitamin mixture⁵</td>
<td>10.0 10.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2.5 2.5</td>
</tr>
<tr>
<td>DSB-hydrophilic fraction</td>
<td>30.0⁶</td>
</tr>
<tr>
<td>D-ontjom-hydrophilic fraction</td>
<td>67.5⁶</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0 100.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>361.5 324.0</td>
</tr>
<tr>
<td></td>
<td>50.0 50.0</td>
</tr>
</tbody>
</table>

⁴Harper mixture (Oriental Yeast Industries Co., Tokyo).
⁵prepared from 300 g of DSB or D-ontjom.

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Fig. 1. DPPH-radical scavenging activities of three extracts from DSB and D-ontjom. The DPPH-radical scavenging activities of hexane (A), methanol (B) and water (C) extracts from D-ontjom (a) and DSB (c) were measured by the bleaching of DPPH’s purple color at 517 nm. Values are means±SE of three determinations. DSB, defatted soybean.

Fig. 2. Scavenging of superoxide anion and inhibition of glutathione autoxidation by the hydrophilic fractions of DSB and D-ontjom. GSH was autoxidized with the hydrophilic fractions of D-ontjom (a) and DSB (c) at 30°C for 30 min. The remaining GSH was reacted with DTNB and the absorbance at 412 nm was measured. Values are means±SE of three determinations. DSB, defatted soybean.
Hydrophilic Antioxidant of Defatted Soy Ontjom

Table 2. Food ingestion, body weight gain, liver weight and serum transaminase activities of rats.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Food ingestion (g/14 d)</th>
<th>Body weight gain (g/14 d)</th>
<th>Liver weight (g/rat)</th>
<th>Serum transaminase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSB</td>
<td>228±2</td>
<td>91±2</td>
<td>12.3±0.6</td>
<td>41±4</td>
</tr>
<tr>
<td>D-ontjom</td>
<td>224±13</td>
<td>89±11</td>
<td>12.2±1.2</td>
<td>38±4</td>
</tr>
</tbody>
</table>

No significant difference was detected between these values of the DSB group and D-ontjom group by Duncan’s multiple range test. Mean±SE (n=5). DSB, defatted soybean.

Table 3. TBARS, serum GSH and α-tocopherol levels, GSH-Px activity and SOD activity of rats.

<table>
<thead>
<tr>
<th>Diet</th>
<th>TBARS (nmol MDA/g)</th>
<th>Glutathione (µmol/ml)</th>
<th>α-Tocopherol (µg/ml)</th>
<th>SOD activity (U/ml)</th>
<th>GSH-Px activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSB</td>
<td>57.0±3.8</td>
<td>1.8±0.2</td>
<td>2.7±0.2</td>
<td>86.6±1.7</td>
<td>83.2±6.9</td>
</tr>
<tr>
<td>D-ontjom</td>
<td>37.0±9.5</td>
<td>1.4±0.2</td>
<td>3.4±0.3</td>
<td>79.0±4.3</td>
<td>67.4±3.7</td>
</tr>
</tbody>
</table>

Values in the column not sharing a common superscript letter are significantly different at p<0.05 by Duncan’s multiple range test.

TBARS, thiobarbituric acid reactive substance; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

MDA, malondialdehyde.

immediately frozen after preparation and a small portion for each feeding was thawed every day. During the feeding, food ingestion and body weight gain of the rats were measured. On the final day, blood was collected from the abdominal aorta under light ether anesthesia and then liver was removed and weighed. The sera and liver were stored at −80°C until analyzed.

Statistical analysis Data obtained as the means±standard error were analyzed by Duncan’s multiple range test (Duncan, 1957) using the Statistical Analysis System.

Result and Discussion

In vitro antioxidant activity The DPPH-radical scavenging activities of hexane (A), methanol (B) and water (C) extracts from DSB and D-ontjom were compared (Fig. 1). The activities of the three extracts from D-ontjom were all stronger than those from DSB, respectively, with the activity of the water extract from D-ontjom being the strongest. This result suggests that D-ontjom has a strong antioxidative activity and that its main antioxidants are hydrophilic components. The fifty percent DPPH inhibitory concentrations (DPPH-IC50) of the hydrophilic fraction and the water extract from D-ontjom were 4.2 and 13.3 (dry D-ontjom mg/ml), respectively. The radical scavenging activity of the hydrophilic fraction was stronger than that of the water extract, and thereafter the hydrophilic fraction was used as the antioxidative fraction of D-ontjom.

Figure 2 showed the effect of the hydrophilic fraction on O2− scavenging (A) and GSH autoxidation (B). The hydrophilic fraction of D-ontjom demonstrated stronger O2− scavenging and GSH-autoxidation suppression activities than that of DSB. Some hydrophilic components of D-ontjom might be acting as a kind of antioxidant in vivo.

In vivo antioxidation To confirm the antioxidant activity of the hydrophilic components of D-ontjom in vivo, rats were fed diets containing the hydrophilic fractions of DSB and D-ontjom for 2 weeks (Table 1). Food ingestion, body weight gain, liver weight and serum transaminase activities are shown in Table 2. No significant differences in these values in these between the two rat-groups were found. The transaminase activities, GOT and GPT, were both within in the normal range (Tanimoto, 1989). Therefore, neither experimental diet, vitamin E deficient-fish oil diet, caused no disturbance of hepatic function of the rats.

To test the effect of the hydrophilic fractions of D-ontjom on oxidative stress, TBARS values, antioxidant levels (α-tocopherol and GSH) and enzyme activities (SOD and GSH-Px) were measured (Table 3). In the D-ontjom group of rats, the liver and serum TBARS values and the hepatic SOD and serum GSH-Px enzyme activities were lower than those of the DSB group, while the serum GSH and α-tocopherol levels were higher. The TBARS value would be promoted by products induced from oxidative stress such as lipid hydroperoxide, superoxide anion and hydroxyl radical. Activities of SOD and GSH-Px might be enhanced to respond to increased oxidative stress (Banerjee et al., 2002). Improvement of TBARS value, SOD activity and GSH-Px activity by the supplementation of antioxidants has been reported in cases of ischemia-reperfusion in pylorus-ligated rats (Tanaka & Yuda, 1993) and by the herb Epimedii in aged mice (Zeng et al., 1997). α-Tocopherol is known to be an effective radical scavenger (Niki, 1987). Since D-ontjom-hydrophilic fraction was able to scavenge DPPH-radical (Fig. 1) and O2− (Fig. 2), maintaining a high level of α-tocopherol (Table 3), this fraction might act as a lipoperoxyl radical scavenger in vivo resulting in a decrease in α-tocopherol consumption and suppressed rise in TBARS value. These results suggest that the hydrophilic fraction of D-ontjom has a high antioxidant activity in vivo. Based on D-ontjom having high antioxidative activities in its hydrophilic (Table 3) and hydrophobic fractions (Matsuo, 2001) and also a strong plasma cholesterol reduction activity (Matsuo, 2000), D-ontjom is expected to become popular as a useful and healthful food.

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References


