Potent Antioxidant and Radical-Scavenging Activities of Traditional Japanese Cereal Grains

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Abstract: To estimate the preventive potential of Japanese traditional cereals against oxygen radical-related chronic diseases such as cardiovascular diseases and diabetes, antioxidant and radical-scavenging activities in the extracts of five Japanese traditional cereal grains were analyzed by using an assay system of lipid peroxidation and a radical compound, 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH radical-scavenging activities in the extracts of Japanese cereal grains were divided into two groups. One group including Japanese sorghum, black rice and red rice showed strong radical-scavenging activities, but the other group including Japanese barnyard millet and foxtail millet did not exhibit significant radical-scavenging activities. The DPPH radical-scavenging activities of these extracts were closely correlated to the contents of phenolic compound in the extracts, but not to the sugar or protein content in the extracts. In contrast, all the methanol and water extracts of the cereal grains caused significant antioxidant activities against hydroperoxide generation in the peroxidation of linoleic acid, in which the water extracts of these cereal grains caused much higher antioxidant activities than the methanol extracts of the same cereals. These results suggest that Japanese traditional cereals contain qualitatively different principles associated with antioxidant and radical-scavenging activities, and possible principles responsible for the antioxidant and radical-scavenging activities in the cereal grains are discussed.

Key words: antioxidant, radical-scavenging activity, Japanese traditional cereal grains.

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Introduction

There is an important current issue in the role of reactive oxygen radicals (ROS) in the damage of body cells, which is involved in the causation and progression of chronic diseases such as diabetes and cardiovascular diseases [1,2].

In contrast, various edible plants, including vegetables, fruits and cereals, have been considered as preventive fighters against these chronic diseases, and antioxidant constituents in these foods are important, because they possess the scavenging activity against ROS and protect body cells against oxidative damage to cellular biomolecules such as proteins, nucleic acids and lipids [3].

Among them, in Japan, traditional cereals have been believed to have beneficial effects on human health. However, the details of the antioxidant or ROS-scavenging activities of these cereals have not been analyzed except for a few studies. For example, one variety of Japanese barnyard millet showed a strong antioxidant activity against lipid peroxidation and several active components were identified in the extract of this cereal grain [4]. Furthermore, finger millet is a potent source of antioxidants and has a radical-scavenging activity that is higher than that of wheat, rice and other millets, which corresponded to their phenolic contents [5].

In our previous study, Japanese brown rice bran showed strong antioxidant and radical-scavenging activities, and the active principles in the extract responsible for the activities were identified to be endogenous peroxidase [6] and some phenolic acids [7].

In our present study, we analyzed the antioxidant and radical-scavenging activities of Japanese traditional cereals such as black rice, red rice, sorghum, Japanese barnyard millet and foxtail millet by using an assay system of lipid peroxidation and DPPH radical.

Materials and Methods

Chemicals and reagents
1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, Bradford protein assay kit and bovine serum albumin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Linoleic acid and 1,10-phenanthroline were purchased from Kanto Chemical Co.(Tokyo, Japan). Ferulic acid was gifted from Tsuno Food Industory (Wakayama, Japan). Two g of aluminum chlorohydrate (Kishida Chemical Co., Tokyo, Japan) and 20 μg of 1,10-phenanthroline were dissolved with 100 ml of ethanol. One g of soluble starch (Wako Pure Chemical, Osaka, Japan) and 20 g of NaCl were dissolved with 100 ml of distilled water.

Preparation of cereal grain extracts
Cereal grains such as sorghum (Sorghum bicolor Benikibi) and black rice (Oryza sativa japonica Asamurasaki) were purchased from Rice Island Co. (Gifu, Japan). Red rice (Oryza sativa japonica, Benizome-mochi), foxtail millet (Setaria italica Ohtsui-10) and Japanese barnyard millet (Echinonolou utilis Daruma) were purchased from Katsuya Co. (Hirosima, Japan).

The cereal grains were milled by an electric milling apparatus (IEM-100, Iwatani Sangyo, Tokyo, Japan) and the cereal grain powder was mixed with 20 times weight of distilled water.
or methanol and kept for 2 days at 4°C. Each extract of the cereal grain was separated from the precipitable residue by a centrifugation at 1,000 × g for 15 min and stored at −20°C.

**Measurement of protein, sugar and phenolic content**

The protein content was determined by the Bradford method [8], using bovine serum albumin as a standard protein. The sugar content, including sugar monomer, oligosaccharide or carbohydrate, was analyzed by the phenol-sulfuric acid method [9] using glucose as a standard sugar. The phenolic content, including flavonoid or phenolic acid, was measured by a modified Folin-Ciocalteu method of Zielinski and Kozlowska [10], using ferulic acid as a standard phenolic substance.

**Assay for radical-scavenging activity**

The radical-scavenging activity was measured using DPPH radical according to the modified method of Brand-Williams [11]. An aliquot of water or methanol extract of the cereal grain (200 μl) was mixed with 3.8 ml of 0.1 mM DPPH in methanol prepared daily. The extract was diluted with methanol or distilled water, and a control experiment was performed with methanol or distilled water alone. The decrease of absorbance at 530 nm was measured by a Shimadzu UV-265 spectrophotometer. The background absorbance value derived from the test sample itself before the assay reaction was subtracted from the apparent absorbance value.

**Assay for the hydroperoxide generation in lipid peroxidation**

The amount of hydroperoxide generated from oxidized linoleic acid was measured by the modified method of Asakawa and Matsusita [12]. One ml of test solution was vigorously mixed with 1 ml of linoleic acid in ethanol solution and 2 ml of 50 mM phosphate buffer (pH 7.5) in a glass tube and stood for two weeks at 33°C. As a negative control experiment, 1 ml of distilled water or methanol was used in place of the test solution. After two weeks reaction, the test solution mixture mentioned above (200 μl), 250 μl of 2% KI solution, 2% aluminum chloride solution and 1 ml of hexane were mixed in a glass test tube and incubated for 5 min at 37°C under dark condition, then 250 μl of 1% starch solution and 7.5 ml of 10 mM HCl were mixed and shaken vigorously. The solution was centrifuged at 1,500 × g for 5 min, and the absorbance of the lower layer was measured at 560 nm using a Shimadzu UV-265 spectrophotometer.

**Expression of experimental results and statistical analysis of data**

The experimental result of antioxidant or radical-scavenging activity was expressed as the mean and standard deviation (SD) of triplicate assays. The antioxidant or radical-scavenging activity(%) was expressed by the following equation: ((the mean value of control experiment − the mean value of test experiment) / the mean value of the control experiment) × 100%. The amount of protein, sugar or phenolic content in the test solution was determined by the average value of duplicate assays. The statistical comparison of the data between the negative control and test experiment was carried out using Student’s t test. A P value less than 0.05 was considered to be statistically significant.
Results

First, when DPPH radical-scavenging activities in the extracts of Japanese cereal grains were analyzed, their activities were roughly divided into two groups. One group, which included Japanese sorghum, red rice and black rice, showed relatively strong radical-scavenging activities, but the other group, which included Japanese barnyard millet and foxtail millet, exhibited very weak radical-scavenging activities (Fig. 1). Among these active extracts, methanol extracts caused stronger radical-scavenging activities than water extracts of the same cereal grains except for the extracts of black rice. The water and methanol extracts of black rice showed nearly equal radical-scavenging activities.

Fig. 1. DPPH radical-scavenging activity of cereal grain extracts.
The white and gray columns and bars represent the mean values and SD of three radical scavenging activities of methanol and water extracts of the cereal grains. Two hundreds μl of each extract or control solvent for the extraction was added to the assay mixture of DPPH radical at a final volume of 4 ml and incubated for 60 min at room temperature. The radical scavenging activity was calculated by the following equation: ((Absorbance of the control experiment – Absorbance of the test experiment) / Absorbance of the control experiment) × 100%.

☐ : Methanol, ■ : Water.
Then, to analyze the active principles responsible for the radical-scavenging activity in the cereal extract, we assumed phenolic substances including flavonoids or phenolic acids as the active principles according to the previous reports describing the radical-scavenging activity of millet and sorghum varieties [5, 13, 14]. We measured the phenolic content in the extracts of the cereal grains by the Folin-Ciocalteau method. As indicated in Fig. 2, the active group, including sorghum, black rice and red rice, contained relatively high amounts of phenolic substance, but the inactive group, including foxtail millet and barnyard millet, showed less content of phenolic substance. The methanol extracts of the active cereal grains showed much higher concentrations of phenolic substance than those of the water extracts. The extractability of methanol for phenolic substance seems to be more efficient than that of water.

![Fig. 2. Phenolic content of cereal grain extracts.](image)

Phenolic content was measured by Folin-Ciocalteau method. The white and gray columns represent the phenolic contents in methanol and water extracts of cereal grains as the average value of duplicate assays.

□ : Methanol, □ : Water.
In contrast, the protein or sugar contents in these extracts did not show a proportional relationship to radical-scavenging activities in the same extracts. As shown in Fig. 3, all extracts contained a considerable amount of proteins, and the water extracts of the cereal grains showed higher concentrations of protein than those of the methanol extracts. Furthermore, as illustrated in Fig. 4, the water extracts of the cereal grains exhibited much higher concentrations of sugar substance, including sugar monomer, oligosaccharide or another carbohydrate, than the methanol extracts, and especially the methanol extracts of foxtail millet and Japanese barnyard millet did not contain significant sugar substance.

![Graph showing protein content in cereal grains](image)

**Fig. 3.** Protein content in the extracts of cereal grains.
Protein content of the cereal grain extract was measured by Bradford method. The white and gray columns show the protein contents in methanol and water extracts of the cereal grains, respectively, as the average of duplicate assays.

- **: Methanol,  : Water.
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Fig. 4. Sugar content in the cereal grain extracts. Sugar content of the cereal grain extract was measured by phenol-sulfuric acid method. The white and gray columns show the sugar contents in methanol and water extracts of the cereal grains, respectively, as the average of duplicate assays.

□: Methanol, ■: Water.

To analyze in more detail the correlation between phenolic content and radical scavenging activity of the extracts of the active cereal group, we performed a kinetic analysis study of radical-scavenging activity by using various dilutions of extracts. A typical result is shown in Fig. 5. Surprisingly, the 5-fold diluted methanol extract of sorghum caused a very strong activity (95%) in 60 min reaction, but red rice and black rice showed 52% and 39%, respectively, under the same experimental condition. Furthermore, the sorghum extract exhibited remarkable initial radical-scavenging activity, which showed about 88% in 5 min reaction, but red rice and black rice showed 23% and 15%, respectively, under the same condition (Fig. 5). Similarly, the 5-fold diluted water extract of sorghum exhibited 63% in 5 min reaction, but black rice and red rice caused 18% and 7%, respectively, under the same condition (Fig. 6). The considerable difference of the radical scavenging activities of diluted extracts of sorghum and other active cereal grains seems to correspond to the qualitative and quantitative difference of phenolic substances of these extracts.
Fig. 5. Kinetic analysis study of radical-scavenging activity of the methanol extracts of the cereal grains.
Two hundreds μl of 5-fold diluted methanol extract of the cereal grain was added to the assay mixture including DPPH radical at a final volume of 4 ml and the absorbance at 530 nm of the assay mixture was successively measured at the indicated times in figure.

Fig. 6. Kinetic analysis study of radical-scavenging activity of the water extracts of the cereal grains.
Two hundreds μl of 5-fold diluted water extract of the cereal grain was added to the assay mixture including DPPH radical at a final volume of 4 ml and the absorbance at 530 nm of the assay mixture was successively measured at the indicated times in figure.
Next, we analyzed the effects of the extracts of cereal grains on hydroperoxide generation in linoleic acid peroxidation by the aluminum chloride method. Unexpectedly, the effects of the cereal grains were considerably different from the effects on the DPPH radical-scavenging activities described above. As shown in Fig. 7, all extracts of the cereal grains showed significant antioxidant activities against hydroperoxide generation in the linoleic acid peroxidation, and the water extracts of the cereal grains exhibited much higher antioxidant activities than the methanol extracts of the same cereals. Especially, although the water extracts of Japanese barnyard millet and foxtail millet did not show significant DPPH radical-scavenging activities (Fig. 1), they caused strong antioxidant activities against lipid peroxidation (Fig. 7).

Fig. 7. Antioxidant activity of the cereal grain extracts against lipid peroxidation. Effect of the extracts of cereal grains on hydroperoxide generation in linoleic acid peroxidation was analyzed by aluminum chloride method as described in Materials and Methods. The white and gray columns and bar represent the mean values and SD of three antioxidant activities of methanol and water extracts of the cereal grains. The antioxidant activity was calculated by the following equation: ((Absorbance of the control experiment - Absorbance of the test experiment) / Absorbance of the control experiment) × 100%.

☐ : Methanol,  ■ : Water.
Thus, we analyzed another possible principle responsible for the antioxidant activity against hydroperoxide generation in lipid peroxidation. As a possible principle, we considered the proteinous molecule in the extracts, because the extraction pattern of proteins in the water and methanol extracts roughly resemble that of the antioxidant activity against lipid peroxidation in their extracts (Fig. 3 and 7). We examined the peroxidase activity in the extracts of cereal grains, because we previously identified a considerable peroxidase activity in the extract of Japanese rice bran, which is associated with the scavenging activity against hydroperoxide generation in lipid peroxidation[6]. Although significant peroxidase activity was observed in the water extracts of black rice and foxtail millet, a significant peroxidase activity could not be detected in other extracts of the cereal grains compared with the positive control activity of Japanese brown rice bran (data not shown). This result indicates that the hydroperoxide-scavenging activity in the extracts of the cereal grains is not associated with the peroxidase activity, and another unknown factor is responsible for the hydroperoxide-scavenging activity.

Discussion

As described in the experimental results, DPPH radical-scavenging activities in the extracts of Japanese cereal grains were divided into two groups. One group, which included Japanese sorghum, black rice and red rice, showed strong radical-scavenging activities, but the other group, which included Japanese barnyard millet and foxtail millet, did not exhibit significant radical-scavenging activities (Fig.1). However, the antioxidant activities against lipid peroxidation of the same extracts were considerably different from the DPPH radical-scavenging activities. As indicated in Fig.7, all the extracts of the cereal grains showed significant antioxidant activities against lipid peroxidation. Furthermore, these antioxidant and radical-scavenging activities showed different extractabilities by methanol and water (Fig. 1 and 7). These results indicate that the extracts of the cereal grains contain qualitatively different principles responsible for antioxidant and radical-scavenging activities.

As a possible principle responsible for DPPH radical-scavenging activity, we analyzed the content of phenolic compounds such as flavonoids or phenolic acids in the cereal grain extracts. As shown in Fig. 1, 2, 5 and 6, the concentrations of phenolic substance in the cereal grain extracts were associated with their DPPH radical-scavenging activities. However, the protein or sugar concentrations in the cereal grain extracts were not correlated with radical-scavenging activities (Fig. 3 and 4). This result is consistent with previous studies [5, 13, 14], which showed a positive relationship between phenolic content and antioxidant or radical-scavenging activities in the cereal grain extracts.

Then, we analyzed other principles responsible for the antioxidant activity against hydroperoxide generation in lipid peroxidation. As a possible principle responsible for the hydroperoxide-scavenging activity, we analyzed endogenous peroxidase activity in the cereal extracts according to our previous study describing peroxidase-dependent hydroperoxide-scavenging activity in the extract of brown rice bran [6], because the extraction pattern of proteins in the water and methanol extracts roughly resembles that of the antioxidant activity.
against lipid peroxidation in their extracts (Fig. 3 and 7). However, although significant peroxidase activity was observed in the water extracts of black rice and foxtail millet, a significant peroxidase activity could not be detected in other water and methanol extracts of the cereal grains compared with the positive control activity of Japanese brown rice bran (data not shown), which indicates that the antioxidant activity against lipid peroxidation is not derived from the endogenous peroxidase in their extracts, but that another unknown factor seems to be responsible for the hydroperoxide-scavenging activity in their extracts.

As another low possibility, superoxide dismutase (SOD) in the cereal grain extracts may be a candidate responsible for hydroperoxide-scavenging factor, because lipid peroxidation consists of the initiation reaction associated with the generation of an oxygen radical superoxide in some experimental systems [15], and SOD have a potent scavenging activity against superoxide [16]. However, the lipid peroxidation system in our present experiment mostly reflects the propagation reaction, but not an initiation reaction. Although we have no data of SOD activities in the cereal grain extracts at present, the analysis of SOD may be one subject in our next study.

Although we have not identified the active principle responsible for the hydroperoxide-scavenging activity in the cereal grain extracts, as another possibility B group vitamins were postulated as water-soluble hydroperoxide-scavenging substances. We recently found that B group vitamins caused significant scavenging activity against hydroperoxide generation in lipid peroxidation [17, 18]. Furthermore, a previous reference [19] indicated that Japanese millets and sorghum contain a considerable amount of B group vitamins as water-soluble vitamins, but not ascorbic acid. Especially, a relatively abundant amount of nicotinic acid (1.7 – 6 mg/100 g cereal grains) was detected in these cereal grains compared with other B group vitamins (B1: 0.15 – 0.35 mg/100 g grains, B6: 0.17 – 0.31 mg/100 g grains). According to this reference, the minimal and maximal concentrations of endogenous B group vitamins in the extracts of cereal grains were calculated to be 2.2 and 24 μM, respectively. As in our separate study [17], nicotinic acid showed significant antioxidant activity against hydroperoxide generation at the concentrations of 2.5 – 25 μM. However, vitamin B6 did not exhibit significant scavenging activity at the same concentrations. Vitamin B1 showed significant scavenging activity at 2.5 μM, but not at 25 μM. This result suggests a possibility that several B group vitamins are responsible for the antioxidant activity against hydroperoxide generation. Additionally, vitamin B1, B6 and nicotinic acid did not show significant scavenging activities against DPPH radical at the same concentrations (data not shown). This result indicates a possibility that vitamin B1 and nicotinic acid may be responsible for the hydroperoxide-scavenging activity in traditional Japanese cereal grains, but not for DPPH-radical-scavenging activity. To confirm the above mentioned claim, we are studying the endogenous concentrations of B group vitamins in these extracts at present.

Although vitamin B1 and nicotinic acid seem to be possible principles responsible for the scavenging substance against hydroperoxide generation, their scavenging activity is insufficient to explain the strong scavenging activity of the water extracts of the cereal grains (Fig. 7). This result indicates that the hydroperoxide-scavenging activity in the extracts of the cereal grains is associated with other unknown substances besides B group vitamins. For example, protein fraction of proso millet caused a protective effect on D-galactosamine-induced rat liver
injury (20). Although the protective mechanism of protein fraction against liver injury was not elucidated in that study, the antioxidant or radical-scavenging activity of unknown factors in the protein fraction may have been associated with the protective activity against liver injury. Furthermore, other investigators reported that finger millet feeding caused an inhibitory effect on lipid peroxidation in the skin of wound healing of diabetic rats, which was associated with an increase of several antioxidants in the skin [21].

On the other hand, human epidemiological studies suggest that the consumption of whole cereal grains reduced the incidence rate of chronic diseases such as heart diseases and diabetes, which were associated with the decrease of serum LDL cholesterol and triglycerides [22, 23].

Possibly, the dietary intake of whole cereal grain including bran is important for its health-promoting effect, but not refined cereal flour (major endosperm). Several previous reports indicated the beneficial effects of brans separated from whole cereal grains. Rooney et al. reported that sorghum and millet brans have better cholesterol-lowering properties than wheat brans [24]. Nam et al. reported that pigmented rice brans inhibit tumor promotion in lymphoblastoid cells by phorbol ester [25]. These bran extracts seem to contain strong antioxidant substances similar to the extracts of the black and red rices in our present study. Turner et al. showed that sorghum brans reduced colon cancer in rats. Rats fed diets containing sorghum brans had fewer aberrant crypts than those fed diets containing cellulose, and the reduction in colon cancer could be due to the antioxidant or radical-scavenging activity of sorghum bran [24]. At present, we are studying the antioxidant and radical-scavenging activities of the brans separated from the traditional Japanese cereal grains mentioned above.

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日本産伝統雑穀類の抗酸化活性とラジカル消去活性

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要　旨： 日本産伝統雑穀類五種（黒米・赤米・高きび・あわ・ひえ）についてその冷水・メタノール抽出液の抗酸化・ラジカル消去活性の分析を行った。その結果ジフェニル・ビクリル（DPPH）ラジカル消去活性については、高きび・黒米・赤米が比較的強いラジカル消去活性を示し、あわ・ひえが弱い活性を示したが、特に高きびのメタノール抽出液がもっとも高い消去活性を示した。またそれらのラジカル消去活性は抽出液中のフラボノイドやフェノール酸の濃度に比例したが、抽出液中のタンパク質や糖類の濃度と消去活性の相関性は認められなかった。一方、リノール酸由来の過酸化脂肪の生成に対してすべてに雑穀の抽出液が比較的強い活性を示したが、冷水抽出液の方がメタノール抽出液より強い活性を示した。これらの活性と抽出液中のバーオキシダーゼ活性との相関関係は認められなかった。以上の実験結果から日本産伝統雑穀類には、質的に異なる抗酸化・ラジカル消去活性が存在することが示唆された。

キーワード： 日本産伝統雑穀類、抗酸化、ラジカル消去活性。

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