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Antioxidant Activity of Anthocyanin Extract from Purple Black Rice

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ABSTRACT

Antioxidant activity was studied for anthocyanins extracted from purple black rice (PBR) by a 3% aqueous trifluoroacetic acid solution (TFA), as well as for anthocyanins extracted from blueberry (Bluetta, high bush type). Capillary zone electrophoresis revealed that the PBR extract contained almost exclusively a single anthocyanin, which was identified as cyanidin 3-*O*-β-D-glucoside (Cy 3-Glc) after purification by polyvinylpyrrolidone column chromatography. In contrast, 11 anthocyanins were identified in the blueberry extract. PBR extract showed slightly weaker superoxide scavenging and crocin bleaching activities than blueberry extract did. Both PBR and blueberry extracts, however, showed 10 to 25 times stronger activity than the same concentration of Trolox used as a reference antioxidant. It was further noted that the purified Cy 3-Glc from PBR extract retained approximately 74% of the antioxidant activity (both crocin bleaching and superoxide scavenging) observed in the original TFA extract. The hydroxyl radical scavenging activity of both extracts was several times weaker than that of the same concentration of Trolox, although the PBR extract showed approximately two times stronger activity than blueberry extract did. The hydroxyl radical scavenging activity of the purified Cy 3-Glc from PBR, however, decreased to approximately 20% of that of the original PBR extract. These results indicate that the anthocyanin Cy 3-Glc contributes to the antioxidant activity of PBR through its strong superoxide radical but not hydroxyl radical scavenging activity.

INTRODUCTION

IT IS WELL KNOWN that reactive oxygen species are implicated in so-called lifestyle- and age-related diseases such as atherosclerosis, diabetes mellitus, and cancer.[1,2] Therefore, dietary intake of antioxidants is considered to be important for the prevention of these pathophysiological conditions. Much attention has been paid to the antioxidant ingredients in food sources such as lignans in common *Sinopodophyllum* fruit,[3] proanthocyanidins in red wine,[4] and catechins in tea.[5] Reddish purple pigment, anthocyanin, is an antioxidant that is distributed widely in colored vegetables and fruits such as blueberry and strawberry.[6] It has been studied for its possible physiological functions such as vision improvement,[7] anticholesterolemia,[8,9] and anticancer activities.[10]

Rice (*Oryza sativa L.*) is one of the world's ma-

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jor food sources. Therefore, it is valuable to consider certain functional components in rice that might deliver health benefits beyond rice's basic nutritional value. Colored rice might be expected to have enhanced antioxidant activity, because colored wild rice has been reported to contain anthocyanin pigments such as crisanthermin and keracyanin in the seed skin.[11]

We studied the antioxidant properties of anthocyanins extracted from an anthocyanin-rich mutant of purple black rice (PBR) that was developed by Hokuriku National Agricultural Experimental Station in Japan as a second-generation food source. The results were compared with those of anthocyanins extracted from a typical anthocyanin-rich fruit, blueberry.

MATERIALS AND METHODS

Reagents and anthocyanin sources

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec Co., Ltd., Japan. Ferrous sulfate ($FeSO_4$) was purchased from Kanto Chemical Co, Ltd., Japan. Xanthine oxidase (XOD; 20 U/ml, from cow's milk) was purchased from Boehringer Mannheim Co., Germany.

Dried saffron was purchased from a local supermarket. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co., U.S.A. CLEAN99K200 was purchased from Clean Chemical Co. Ltd., Japan. All other reagents, including sodium borate ($NaBO_4$), *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid monohydrate (CyDTA), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), and diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) were purchased from Wako Pure Chemical Industries Co, Ltd., Japan.

PBR and blueberry (Bluetta strain) were provided respectively by Hokuriku National Agricultural Experiment Station in Joetsu-city, Niigata, Japan, and Suzuki Farm in Atsumi-city, Yamagata, Japan.

Anthocyanin extraction from purple black rice and blueberry

Blueberry fruit and PBR grain, 100 g respectively, were gently shaken in 50 ml of 3% aqueous trifluoroacetic acid (TFA) for 24 hours at 5°C. The supernatant was collected, dried under reduced pressure, and then dissolved in an aliquot of 1% TFA. The solutions were filtered through a 0.25-mm diameter membrane filter and diluted with 1% TFA to adjust the absorbency at 560 nm to 2.0.

Purification of anthocyanins from black rice extract

The 3% TFA extract of PBR was dried under reduced pressure, dissolved in a small volume of 70% ethanol containing 1% TFA, and then applied onto a polyvinylpyrrolidone (PVP) column, as reported elsewhere.[12] Purified anthocyanin collected with the use of methanol as an elution solvent was dried under reduced pressure and dissolved in 0.1% TFA/methanol. The purified anthocyanin was subjected to capillary zone electrophoresis (CZE), nuclear magnetic resonance (NMR) and high-performance liquid chromatography–mass spectometry (HPLC-MS) measurements to identify the anthocyanin type.

Anthocyanins separation by capillary zone electrophoresis

CZE analysis of anthocyanins was carried out according to the method reported previously,[13] using a CAPI-3100 capillary electrophoresis system (Otsuka Electronics Co., Ltd., Japan) equipped with a fused-silica capillary, 75-μm diameter, 62.5 cm long (effective length, 50.0 cm).

Hydroxyl radical scavenging assay

Hydroxyl radicals were generated by the Fenton reaction.[14] The reaction mixture (total volume 300 μl) containing distilled water, 20 mM DMPO, 10 mM H_2O_2, 0.1 mM $FeSO_4$, and anthocyanin test samples was quickly transferred into a hematocrit capillary tube. Exactly 1 minute after the addition of $FeSO_4$, the DMPO-OH signal was determined at room temperature by JEOL model JES-TE 200 electron spin resonance (ESR) spectrometer (X-band Microwave Unit). Stock solutions of anthocyanin extracts were prepared as 1.0 mM delphinidin-equivalent aqueous solutions in

1% TFA and Trolox as 1.0 mM methanol solution. The spectrometer settings were as follows: microwave power, 8 mW; microwave frequency, 9.18 GHz; modulation amplitude, 0.1 mT; time constant, 0.03 sec; sweep time, 30 sec; center fields, 331.6/321.6 mT.

Superoxide radical scavenging activity measurement

Superoxide radical scavenging activity was measured as previously reported,[15] using the hypoxanthine-XOD system as a superoxide radical generator. The reaction mixture (total 300 μl) containing 0.2 M phosphate (pH 7.8), 1 mM DTPA, 0.5 mM hypoxanthine, various concentrations of anthocyanin samples or Trolox as a reference antioxidant, 33 mM DMPO, and 0.1 U/ml XOD was quickly transferred into a hematcrit capillary tube. Exactly 1 minute after the addition of XOD, DMPO-OOH signals were determined by ESR. The ESR settings were the same as for DMPO-OH measurement.

Crocin bleaching activity assay

Crocin extraction and the bleaching assay were carried out according to the method of Wolf et al.[16] with minor modifications. The reaction mixture (3.0 ml) contained 0.1 M phosphate (pH 7.0), 10 μM crocin extracted from saffron, and various amounts of anthocyanins or

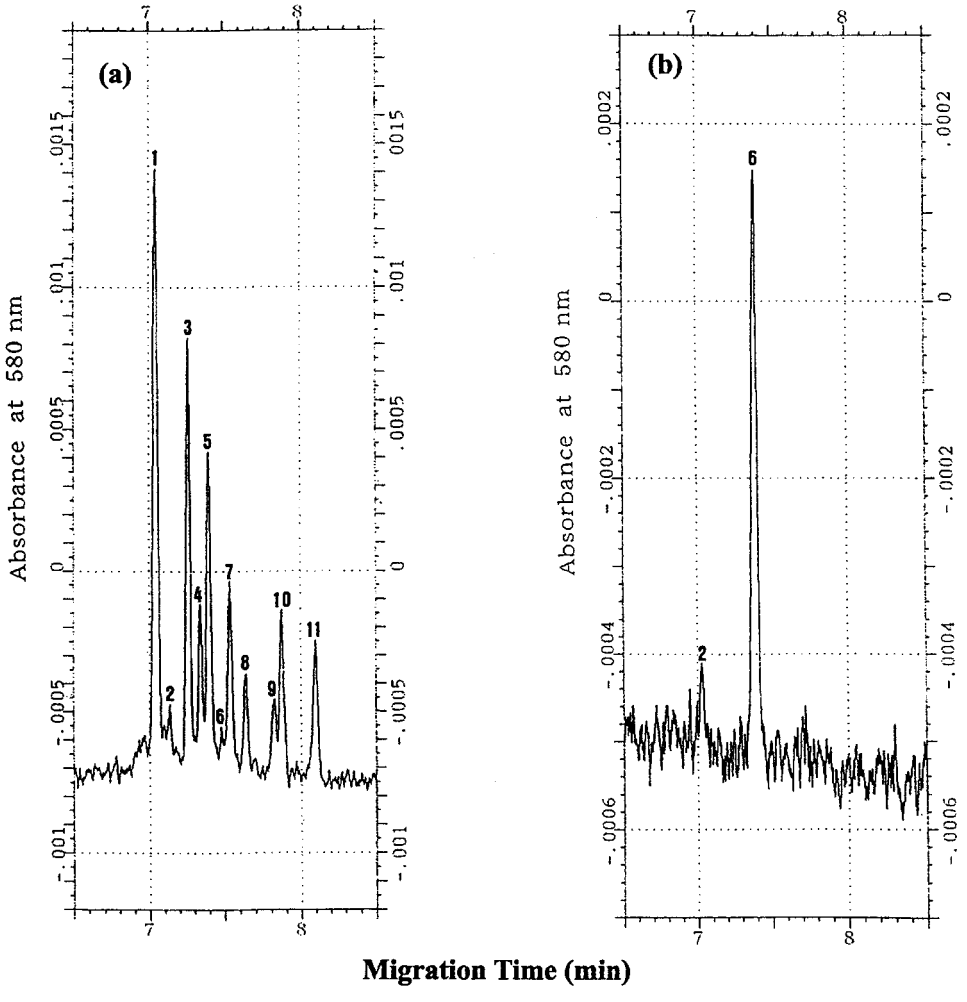

FIG. 1. Capillary zone electrophoresis of anthocyanins from blueberry and PBR. Anthocyanins were extracted from blueberry fruit and grained PBR with 3% TFA aqueous solution and subjected to CZE. (**a**) Blueberry (Bluetta). (**b**) PBR extract. 1, Mv 3-Glc; 2, Pn 3-Glc; 3, Mv 3-Gal; 4, Pt 3-Glc; 5, Pt 3-Gal; 6, Cy 3-Glc; 7, Dp 3-Glc; 8, Pt 3-Ara; 9, Cy 3-Gal; 10, Dp 3-Gla; 11, Dp 3-Ara.

Trolox as a reference antioxidant. The reaction was started by the addition of 75 μl of AAPH (0.5 mM stock solution) to the reaction mixture kept at 40°C, and the decrease in the absorbency at 442 nm was kinetically recorded.

RESULTS

The anthocyanins extracted with 3% TFA aqueous solution from blueberry (Bluetta) and PBR grain were analyzed by CZE (Fig. 1). It was found that 11 anthocyanins were present in the Bluetta extract. From comparison of the mobilities recorded with those of standard anthocyanins as reported previously,[14] these anthocyanins were identified as malvidin-3-*O*-β-D-glucoside (Mv 3-Glc), malvidin-3-*O*-β-D-galactoside (Mv 3-Gal), peonidin 3-*O*-glucoside (Pn 3-Glc), petunidin-3-*O*-β-D-glucoside (Pt 3-Glc), petunidin-3-*O*-β-D-galactoside (Pt 3-Gal), petunidin-3-*O*-β-D-arabinoside (Pt 3-Ara), delphinidin-3-*O*-β-D-glucoside (Dp 3-Glc), delphinidin-3-*O*-β-D-galactoside (Dp 3-Gla), delphinidin-3-*O*-β-D-arabinoside (Dp 3-Ara), cyanidine-3-*O*-glucoside (Cy 3-Glc), and cyanidin-3-*O*-galactoside (Cy 3-Gal) (Fig. 1A). In contrast, in the extract from PBR, essentially one peak was determined. From the CZE mobility, the major peak was identified as Cy 3-Glc (Fig. 1B). A minor small peak was also identified as Pn 3-Glc. From the peak intensity, the Cy 3-Glc was found to comprise approximately 94% of total PBR anthocyanins. The major peak component (Cy 3-Glc) was further purified by PVP column chromatography and analyzed by HPLC-MS. The mass-to-charge ratio (456) thus determined by negative ion mode was consistent with the molecular mass of Cy 3-Glc (Fig. 2). The structure of the purified component was

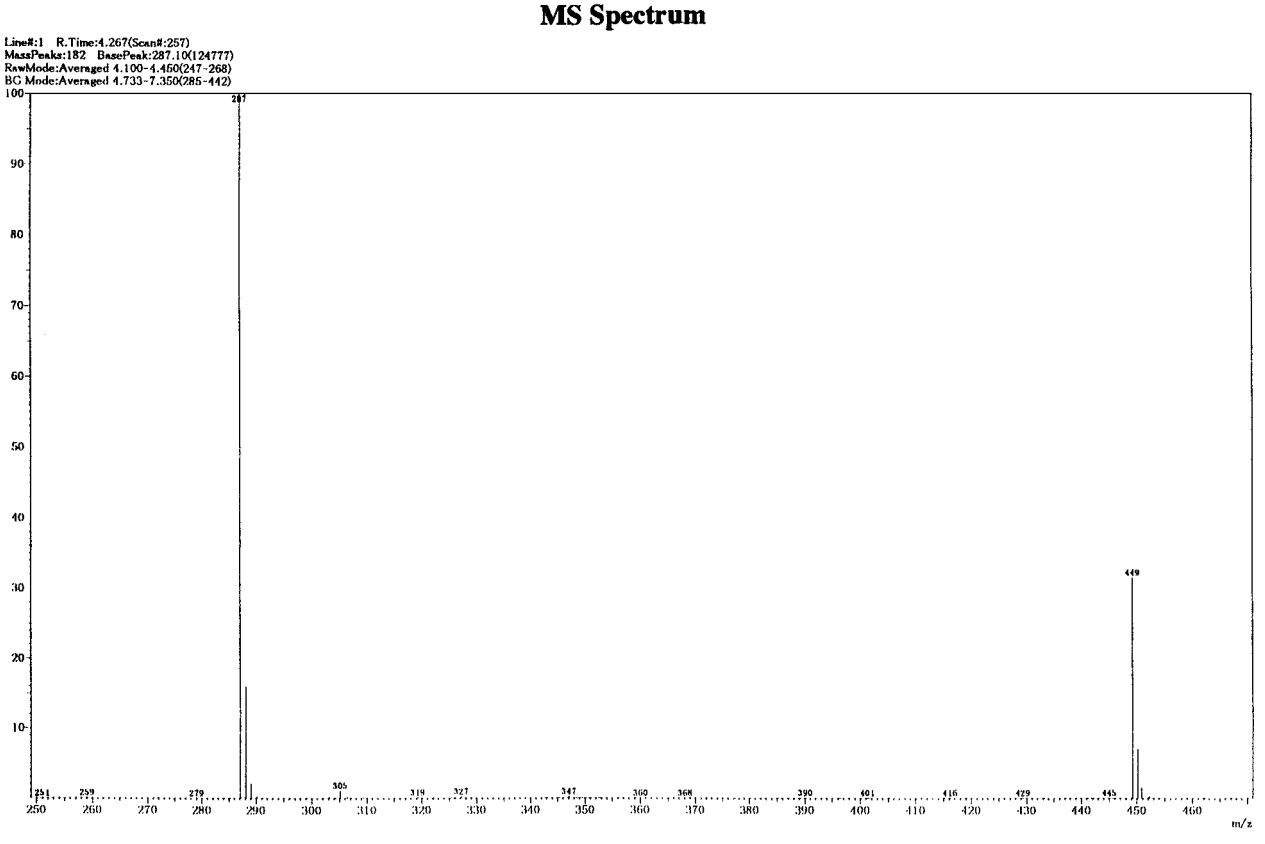

FIG. 2. Mass spectrum of major anthocyanin purified from PBR. Anthocyanin extract from PBR was further purified by PVP column chromatography, and then the structure was identified by HPLC-MS and NMR (data not shown). The purified PBR anthocyanin was subjected to HPLC-MS, and the mass spectrum was recorded by negative ion mode.

further identified by NMR spectroscopy (data not shown).

First, we studied the antioxidant activity of these anthocyanin extracts and the purified major PBR anthocyanin (Cy 3-Glc) by the crocin bleaching method, which is used to evaluate overall antioxidant potentials of biological fluid samples such as blood plasma.[16] The crocin bleaching rate increased linearly with the anthocyanin concentration up to 1.0 μM expressed as delphinidin equivalents calculated from the absorbency at 560 nm (ϵ = 34,000), and then gradually became saturated (data not shown). Therefore, the crocin bleaching activities were compared at the same anthocyanin concentration (0.5 μM as delphinidin equivalent) for both blueberry and PBR extracts and for the purified PBR Cy 3-Glc (Fig. 3). Both PBR and blueberry anthocyanin samples showed strong crocin bleaching activity, at least 20 times stronger than that shown by the same molar concentration of Trolox used as a reference antioxidant. The highest activity was attained by blueberry extract, but PBR extract showed almost the same level of activity as blueberry. The purified Cy 3-Glc of PBR also showed strong activity, as high as approximately 75% of the activity in the original PBR extract.

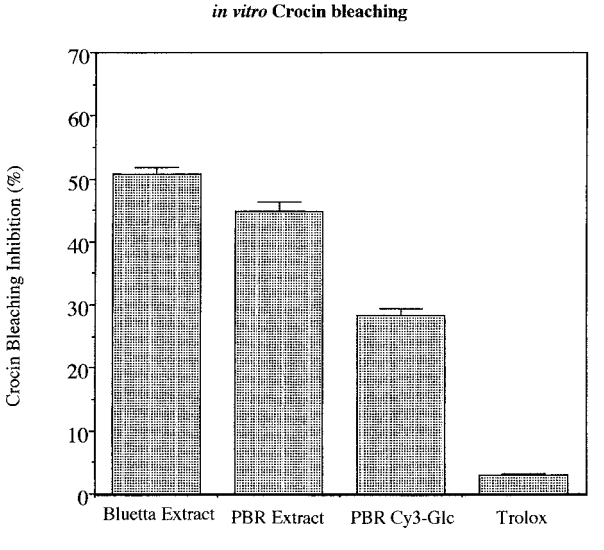

FIG. 3. Inhibition of AAPH-induced crocin bleaching by anthocyanin extracts from PBR and blueberry. Crocin was extracted from commercially available saffron. Anthocyanin concentration of the reactants was normalized to delphinidin equivalent using the molecular absorption coefficient (ϵ = 34,000) of delphinidin at 560 nm. Trolox was dissolved in MeOH and used as a reference antioxidant. Relative activity was compared by determining the concentrations that provided 20% inhibition of control (IC_{20}), obtained from the experiments in this figure.

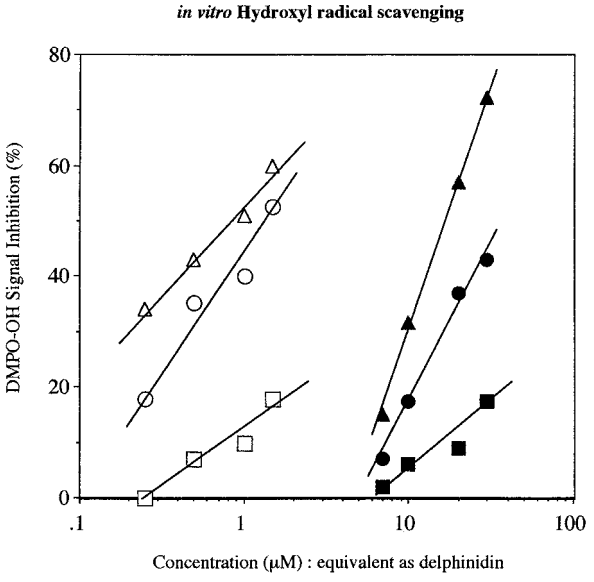

FIG. 4. Dose dependence of hydroxyl radical scavenging activity of anthocyanin extracts from PBR and blueberry determined at various DMPO concentrations. Hydroxyl radical was generated by the Fenton reaction and determined by ESR using DMPO as a spin trap. Open symbols represent the data obtained at 2 mM DMPO; closed symbols, at 20 mM DMPO concentrations. *Circles*, blueberry; *triangles*, PBR extract; *squares*, PBR Cy 3-Glc.

For further study on the scavenging activities of these anthocyanin samples toward hydroxyl radical, ESR spin-trapping experiments were carried out using 20 mM DMPO as a spin-trapping reagent and the Fenton reaction as the hydroxyl radical source. All samples showed clear dose-dependent inhibition of DMPO-OH formation (Fig. 4). When the measurement was carried out with a low concentration of DMPO (2 mM), the dose-response of the extracts shifted to the left without changing the profile. At 2 mM DMPO, IC_{20} (50% inhibitory concentration) values were approximately one tenth of those obtained at 20 mM DMPO.

When the hydroxyl radical scavenging activities were compared among the three anthocyanin samples at 30 μM expressed as delphinidin equivalents, PBR extract showed the strongest activity (approximately 31% inhibition), followed by the blueberry extract (16% inhibition) (Fig. 5). The inhibitory activity of purified PBR Cy 3-Glc was significantly low

(7% inhibition) compared with other two anthocyanin extracts. Moreover, the activities of all of the anthocyanin samples were considerably weak compared with that of Trolox.

Next, the superoxide anion scavenging activity was studied for the three anthocyanin samples (Bluetta extract, PBR extract, and purified PBR Cy 3-Glc) by ESR using DMPO as a spin trap. All of the samples showed marked inhibition of DMPO-OOH signal production; the inhibitory activities increased with the anthocyanin concentration up to 1.0 μM expressed as delphinidin equivalents and then became saturated (data not shown). Therefore, the relative scavenging activity was compared at 0.4 μM concentration together with Trolox as a reference antioxidant (Fig. 6). PBR extract showed almost the same activity as blueberry extract (approximately 27% and 30% inhibition, respectively). Further, the purified PBR Cy 3-Glc was found to retain approximately 75% of the activity observed in the original PBR extract. Contrary to the case of hydroxyl radical scavenging activity, all of the anthocyanin samples showed several times stronger superoxide radical scavenging activity than Trolox, which showed only 3% inhibition.

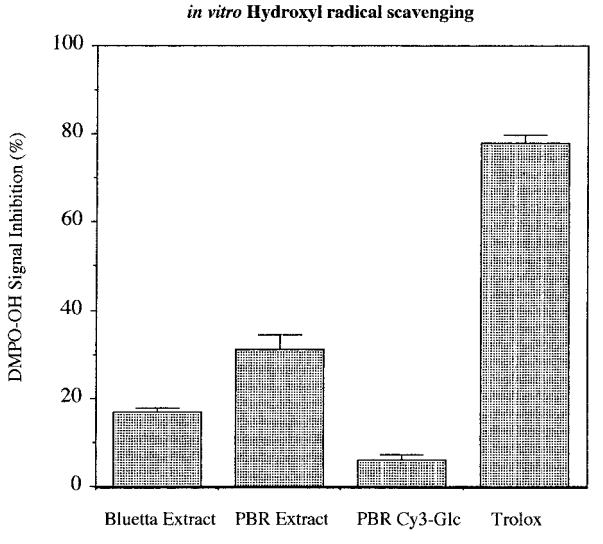

FIG. 5. Relative hydroxyl radical scavenging activities of anthocyanin extracts from PBR and blueberry. Relative activity was compared at 30 μM concentrations of Trolox and anthocyanins as delphinidin equivalent.

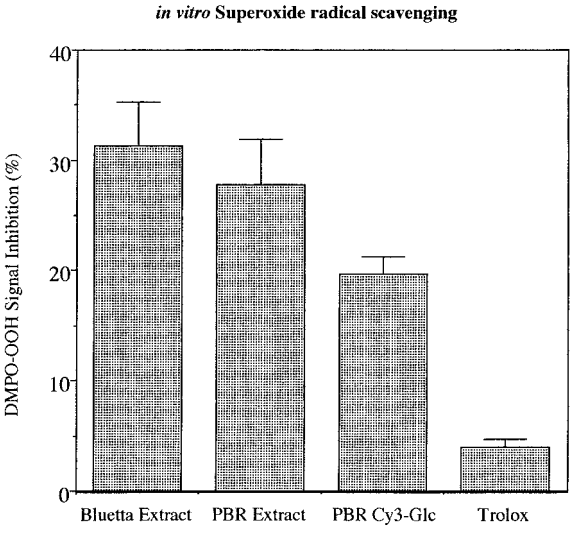

FIG. 6. Superoxide radical scavenging activity of anthocyanin extracts from PBR and blueberry. Relative activity was compared at 0.4 μM concentrations of Trolox and anthocyanins as delphinidin equivalent.

DISCUSSION

Several anthocyanin pigments have been reported in red rice grain, including Cy 3-Glc, cyanidin 3,5-diglucoside (Cy 3,5-Glc), cyanidin 3-rutinoside (Cy 3-Rut), Mal 3-Gal, and Pn 3-Glc.[17] It was also reported that two anthocyanins are the major pigments in aromatic red rice grains of *O. sativa* var *Indica Tapol* (approximately 56% Cy 3-Glc and 16% Pn 3-Glc).[18]

The present studies revealed that the PBR, newly developed by Hokuriku National Experiment Station in Niigata, Japan, as a second-generation rice source, contains Cy 3-Glc as the major anthocyanin (>90% of total anthocyanins) when calculated from the CZE peak intensity monitored at 580 nm (Fig. 1). On the other hand, 11 anthocyanins were determined in the blueberry (Bluetta) extract examined here. The highest content among them was found for Mv 3-Glc, followed by Mv 3-Gal and Pt 3-Gal; Cy 3-Glc was a minor anthocyanin in the Bluetta extract.

When the antioxidant activities of PBR and blueberry extracts were compared, the blueberry extract showed slightly stronger activity in both superoxide radical scavenging and crocin bleaching activities, but PBR extract showed stronger hydroxyl radical scavenging activity.

The major anthocyanin in PBR (Cy 3-Glc) was purified with the use of a PVP column, and its radical scavenging activity was compared with that of the original extract. Both superoxide radical scavenging and crocin bleaching activities were not significantly reduced even after the purification; that is, the purified Cy 3-Glc retained approximately 75% of the activity observed in the original PBR anthocyanin extract in both cases. In contrast, the hydroxyl radical scavenging activity was remarkably reduced after purification. These results suggest that Cy 3-Glc is the active component contributing to the superoxide radical scavenging and crocin bleaching activities of PBR extract, but not to the hydroxyl radical scavenging activity.

Although the hydroxyl radical scavenging activity of anthocyanin extracts was not as strong as that of Trolox as a reference, a considerable contribution of anthocyanin to hydroxyl radical scavenging potential of the 3% TFA extract, especially from blueberry, was suggested.

Dose-response profiles observed at various DMPO concentrations further indicated that the observed inhibition of DMPO-OH formation was mainly caused by direct scavenging of hydroxyl radicals. However, inhibition of hydroxyl radical generation in the Fenton reaction[19] was also expected for the extracts both from PBR and blueberry, because the profiles of the dose-response curves were not strictly identical at high and low concentrations of DMPO (Fig. 3). On the other hand, the parallel shift of the dose-response curve indicated that Cy 3-Glc does not interact with Fe^{2+} in the Fenton reaction to inhibit hydroxyl radical generation, although other anthocyanins such as nasnin, a delphinidin aglycon, have been reported to interact with Fe^{2+}.[20] The original PBR anthocyanin extract was suggested to contain one or more minor ingredients other than anthocyanin that affected both hydroxyl radical scavenging and generation processes based on the change in dose-response profiles observed with low and high concentrations of DMPO. Blueberry extract–containing delphinidins also showed significant changes in the dose-response curves determined at high and low DMPO concentrations (Fig. 4).

All of the anthocyanin samples showed remarkably high crocin bleaching activity, higher than that of Trolox. The crocin bleaching activity of PBR extract was almost the same as that of blueberry extract, although the types of anthocyanin presented in each extract were completely different. Cy 3-Glc was almost the only anthocyanin in the PBR extract, but its content was little in the Bluetta extract. Although the antioxidant activity of Cy 3-Glc has been studied previously,[21,22] few studies have been carried out for other anthocyanins because of the lack of purified anthocyanin samples. Our data indicate that anthocyanins other than Cy 3-Glc, such as malvidin and delphinidin, also contribute to the antioxidant potential of the fruit extract. The results also suggest that the crocin bleaching test could be a reasonable indicator to evaluate the overall antioxidant potential of multicomponent samples such as food material.

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REFERENCES

1. Laight DW, Carrier MJ, Anggard EE: Antioxidants, diabetes and endothelial dysfunction. *Cardiovasc Res* 2000;47:457–464.
2. Polidori MC, Mecocci P, Cherubiru A, Senin U: Physical activity and oxidative stress during aging. *Int J Sports Med* 2000;21:154–157.
3. Ng TB, Liu F, Wang TZ: Antioxidative activity of natural products from plants. *Life Sci* 2000;66:709–723.
4. Wakao M, Takio S, Ono K: Alkyl peroxyl radical-scavenging activity of catechins. *Phytochemistry* 1998;49: 2379–2382.
5. Kondo K, Kurihara M, Miyata N, Suzuki T, Toyoda M: Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch Biochem Biophys* 1999;362:79–86.
6. Joseph JA, Shukitt-Male B, Denisova N, Bielinski D, Martin A, McEwen JJ, Bickford PC: Reversals of age-related declines in neuronal signal transduction, cognitive, and motor behavioral deficits with blueberry, spinach, or strawberry dietary supplementation. *J Neurosci* 1999;19:8114–8121.
7. Mercier A, Perdriel G, Rozier J, Cheraleraud J: Note

concerning the action of anthocyanin glycoside on the human electroretinogram. *Bull Soc Ophtalmol Fr* 1965;65:1049–1053.
8. Igarashi K, Abe S, Satoh J: Effects of atsumi-kabu (red turnip, *Brassica campestris* L.) anthocyanin on serum cholesterol levels in cholesterol-fed rats. *Agric Biol Chem* 1990;54:171–175.
9. Satue-Gracia MT, Heinonen M, Frankel E: Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. *J Agric Food Chem* 1997;45:3362–3367.
10. Bomster J, Madhavi DL, Singletary K, Smith MAL: *In vitro* anticancer activity of fruit extracts from *Vaccium* species. *Planta Med* 1996;42:212–216.
11. Nawa Y, Otani T: The pigment qualities of colored rice. *Shyokuhin Kogyo* 1991;34:28–33.
12. Wrolstad RE, Barbara JS: Polyvinilpyrrolidone column chromatography of strawberry, rhubarb, and raspberry anthocyanins. *J Chromatogr* 1971;55:405–408.
13. Ichiyanagi T, Tateyama C, Oikawa K, Konishi T: Comparison of anthocyanin distribution in different blueberry sources by capillary zone electrophoresis. *Biol Pharm Bull* 2000;23:492–497.
14. Ali MA, Konishi T: Enhancement of hydroxyl radical generation in the Fenton reaction. *Biochem Mol Biol Int* 1998;46:137–145.
15. Eli F, Gerald MR, Elmer JR: Spin trapping of superoxide and hydroxyl radical: Practical aspects. *Arch Biochem Biophys* 1980;200:1–16.
16. Wolf B, Christa M, Manfred S: Inhibition of the bleaching of the carotenoid crocin: A rapid test for quantifying antioxidant activity. *Biochim Biophys Acta* 1984;796:312–319.
17. Takahashi K, Sugimoto T, Miura T, Washizu Y, Yoshizawa K: Isolation and identification of red rice pigments. *J Brew Soc Jpn* 1989;84:807–812.
18. Terahara N, Saigusa N, Ohba R, Ueda S: Composition of anthocyanin pigments in aromatic red rice and its wine. *Nippon Shokuhin Kogyo Gakkaishi* 1994;41: 519–522.
19. Sekine T, Masamizu T, Maitani Y, Takayama K, Kohno M, Nagai T: Effect of shikonin and alkannin on hydroxyl radical generation system concerned with iron ion. *Yakugaku Zasshi* 1998;118:609–615.
20. Kaneyuki T, Noda Y, Traber MG, Mori A, Packer L: Superoxide anion and hydroxy radical scavenging activities of vegetable extracts measured using electron spin resonance. *Biochem Mol Biol Int* 1999;47:979–989.
21. Tsuda T, Horio F, Kitoh J, Osawa T: Protective effects of dietary cyanidin 3-*O*-beta-D-glucoside on liver ischemia-reperfusion injury in rats. *Arch Biochem Biophys* 1999;368:361–366.
22. Tsuda T, Horio F, Osawa T: Dietary cyanidin 3-*O*-D-glucoside increases *ex vivo* oxidation resistance of serum in rats. *Lipids* 1998;33:583–588.

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