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Polymeric Procyanidins as Radical-Scavenging Components in Red-Hulled Rice

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The extracts from white-, black-, and red-hulled rice were prepared by sequential extraction with six different polar solvents, and their radical-scavenging activities were measured by methods using 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•) and *tert*-butyl hydroperoxyl radical (*t*-BuOO•). The extracts prepared with highly polar solvents, methanol and deionized water, exhibited higher DPPH• and *t*-BuOO• scavenging activities in all three cultivars. In addition, the acetone extract from red-hulled rice exhibited a high DPPH• and *t*-BuOO• scavenging activity, while no such activity was detected for the acetone extracts from white- and black-hulled rice. The major components responsible for the radical scavenging in the acetone extract from red-hulled rice were identified as procyanidins by acidic hydrolysis, vanillin assay, and Sephadex LH-20 chromatography. GPC analysis of the acetylated procyanidins revealed that the average molecular weight is about 5000, in a range of about 500−18 000.

KEYWORDS: Red-hulled rice; radical-scavenging activity; procyanidin

INTRODUCTION

Rice (*Oryza sativa*) is widely consumed around the world, and the most common type (>85%) is white-hulled. Other types have colored hulls, the most common of which are green, black, and red. The black and red varieties are planted mainly in South Asia and in countries such as Italy, Greece, and the United States (*1*). Rice with colored hulls has long been consumed in Japan and China and is considered to be a healthy food. Our focus is now on the physiological functions of rice with colored hulls when consumed by humans and the components responsible for those functions in the rice. We were especially interested in the antioxidative and radical-scavenging properties of rice because of the potential of such properties to provide protection against reactive oxygen species and free radicals, which have been implicated in more than 100 diseases (*2*).

There have already been some reports concerning the antioxidative compounds found in rice. γ-Oryzanol, which is a mixture of ferulate esters of triterpene alcohol, is well known as an antioxidant in rice bran (*3*). In black-hulled rice, cyanidin-3-glucoside has been reported to be one of the major antioxidant compounds (*4*). However, little information exists on the antioxidative effects of red-hulled rice, although the presence of catechin and tannin has been reported (*5*).

In the present paper, extracts from rice with hulls of three different colors (white, black, and red) were prepared by using solvents with various polarities and subjected to a radical-scavenging assay. The rice with black and red hulls came from new varieties released by the National Agricultural Research Center for Kyushu Okinawa Region (KONARC). Furthermore, the components in the acetone extract obtained from the red-hulled rice, which exhibited the highest DPPH• scavenging activity, were investigated.

MATERIALS AND METHODS

Reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and luminol were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI). *tert*-Butyl hydroperoxide (*t*-BuOOH) is a product of Nacalai Tesque (Kyoto, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) was purchased from Dojin (Kumamoto, Japan) and methemoglobin from Sigma Chemical Co. (St. Louis, MO). Other reagents were of analytical grades and used without further purification.

Rice. Three rice cultivars, Hinohikari, Saikai-225, and Beniroman, which have white, black, and red hulls, respectively, were harvested at KONARC (Chikugo Branch, Fukuoka, Japan) in 1998. Each cultivar was milled with an ultracentrifugal mill (type ZM1000, Retsch GmbH & Co., KG, Haan, Germany). The milled rice was stored at 4 °C and used within 2 days of milling.

Preparation of the Rice Extract. Milled rice powder (2.0 g) was sequentially extracted with six different polar solvents in a manner similar to that reported by Przybylski et al. (*6*). Extraction was done using 5.0 mL of solvent and the following sequence of solvents with increasing polarity: *n*-hexane, diethyl ether, ethyl acetate, acetone, methanol, and deionized water. After each extraction, the residue was dried under reduced pressure at room temperature for 1 h to remove

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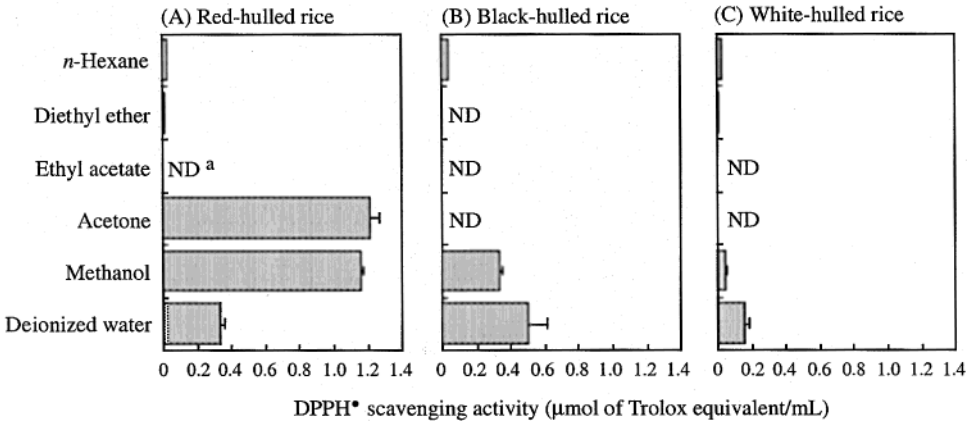

Figure 1. DPPH• scavenging activities of the extracts prepared from rice with red hull (A), black hull (B), and white hull (C) by using solvents with various polarities. Values are means ± SD of four experiments. [a]ND, not detected.

the residual solvent. Each extract was filled up to 5.0 mL with the extraction solvent. An aliquot (1.0 mL) was dried under reduced pressure at 35 °C, and the residue was redissolved in 1.0 mL of dimethyl sulfoxide (DMSO).

Assay for DPPH• Scavenging Activity. The DPPH• scavenging activity was examined by the method described previously (*7*). Briefly, the sample solution (50 μL), ethanol (50 μL), and a 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0, 50 μL) were pipetted into a 96-hole microplate. When necessary, the sample solution was diluted with DMSO. The reaction was initiated by adding 50 μL of 800 μM DPPH in ethanol. After the reaction mixture was left to stand for 20 min at ambient temperature, its absorbance at 520 nm was measured (CS9300PC, dual-wavelength flying spot scanning densitometer, Shimazu Co., Ltd., Kyoto, Japan). The DPPH• radical-scavenging activity was estimated from the decrease of absorbance at 520 nm and expressed as Trolox equivalents per milliliter of sample solution by using a standard Trolox curve. In this assay, (+)-catechin, which is one of the constituents of procyanidins, exhibited 1.53 times higher DPPH• scavenging activity than Trolox on a mole basis.

Assay for *t*-BuOO• Scavenging Activity. The *t*-BuOO• scavenging activity was measured using a modification of the procedure described by Maeda et al. (*8*). The sample solution (120 μL) and the mix solution (1060 μL) were pipetted into a 24-hole microplate. The sample solution was diluted 32 times with DMSO. The mix solution consisted of 120 μL of 6 mM DTPA, 120 μL of 12 mM *t*-BuOOH, 120 μL of 60 μM luminol, and 600 μL of phosphate-buffered saline (pH 7.3). The reaction was initiated by adding 120 μL of 0.6 mg/mL methemoglobin. The chemiluminescence was measured with an analyzer equipped with a charge-coupled device (CCD) camera (type CLA-IMG2, Tohoku Electronic Industrial Co., Sendai, Japan). The accumulation and interval times were set at 180 and 2 s, respectively. The image of chemiluminescence intensity with pseudo-color (1024 × 1024 pixels) was converted into a luminance with 254 gradients (1−255) by using Scion-Image software (Scion Corp., Frederick, MD). The total luminance in a hole was calculated by multiplying the area of a hole (2255 square pixels) by the mean luminance computed by using Scion-Image software. The *t*-BuOO• scavenging activity was estimated from the decrease in luminance and expressed as Trolox equivalents per assay by using a standard Trolox curve.

Qualitative Analysis and Determination of Proanthocyanidin. The following methods were used:

Acid Hydrolysis. This was performed with hydrochloric acid according to the method described by Todd and Vodkin (*9*). Briefly, 1.0 M HCl was added to the sample after removal of the solvent, and the mixture was hydrolyzed in a boiling water bath for 1 h. The hydrolysate was concentrated until dry in a vacuum and redissolved in 1.0% trifluoroacetic acid solution. The identification of the hydrolysate was performed using a JASCO 900 series HPLC equipped with a photodiode array detector. An aliquot (20 μL) of the hydrolysate was applied to a Wakosil-II AR column (4.6 mm × 250 mm, Wako Pure Chemicals Industries, Ltd.) and eluted with a linear acetonitrile gradient (5% to 25%, 40 min) containing 1.5% phosphoric acid at a flow rate of 0.75 mL/min. The column temperature was maintained at 35 °C.

Vanillin Assay. This assay was performed with vanillin and sulfuric acid according to the method described by Sun et al. (10). Specifically, 100 μL of 1.0% (w/v) vanillin in methanol and 100 μL of 9.0 M H_2SO_4 in methanol were added to 40 μL of the sample solution dissolved in methanol. The mixture was allowed to stand for 15 min at 30 °C, and the absorbance was then measured at 500 nm. The influence of the interference, such as anthocyanin, was eliminated by using a blank that was the same as the reaction medium but without vanillin. For the determination of the proanthocyanidin content, (+)-catechin was used as a standard, and the proanthocyanidin content was expressed as (+)-catechin equivalents.

Fractionation of the Extract from Red-Hulled Rice. The acetone extract obtained from red-hulled rice (2.0 mL) was dried under reduced pressure and redissolved in 2 mL of 80% ethanol solution. The sample solution was put on a Sephadex LH-20 column (12 mm × 40 mm) and successively eluted with 20 mL of ethanol, methanol, and a 70% acetone solution. The DPPH• scavenging activity and the proanthocyanidin content in each fraction (1.0 mL per tube) were measured by the method described above.

GPC Analysis of Proanthocyanidins in Red-Hulled Rice. The molecular weight of proanthocyanidins after acetylation was estimated by GPC analysis according to the report by Williams et al. (*11*). Specifically, the fractions containing proanthocyanidins eluted from a Sephadex LH-20 column (fractions 44−49) were combined and concentrated by evaporation under reduced pressure. After 2.0 mL of pyridine−anhydrous acetic acid (1:1) was added to the concentrate, the reaction mixture was left to stand overnight. The acetylated procyanidin was precipitated by adding excess deionized water. The precipitate was recovered by centrifugation and then dried under reduced pressure. An aliquot of the product dissolved in tetrahydrofuran (THF) was put directly on a TSKgel GMH_{HR}-N column (7.8 mm × 300 mm, Tosoh Co., Tokyo, Japan) at 40 °C. The mobile phase was THF, and the flow rate was 0.8 mL/min. The monitoring absorbance was set at 280 nm. For the calibration curve, TSK standard polystylene (Tosoh Co.) was used.

RESULTS AND DISCUSSION

Rice powder was sequentially extracted each with *n*-hexane, diethyl ether, ethyl acetate, acetone, methanol, and deionized water, and the DPPH• scavenging activity of each extract is shown in **Figure 1**. The total DPPH• scavenging activity, which was calculated from the sum of the activity in each extract, varied according to the hull color. The highest activity was observed in rice with a red hull (2.77 μmol of Trolox equivalents/mL), followed by black (0.92) and white (0.26). In all varieties, the extracts prepared with highly polar solvents,

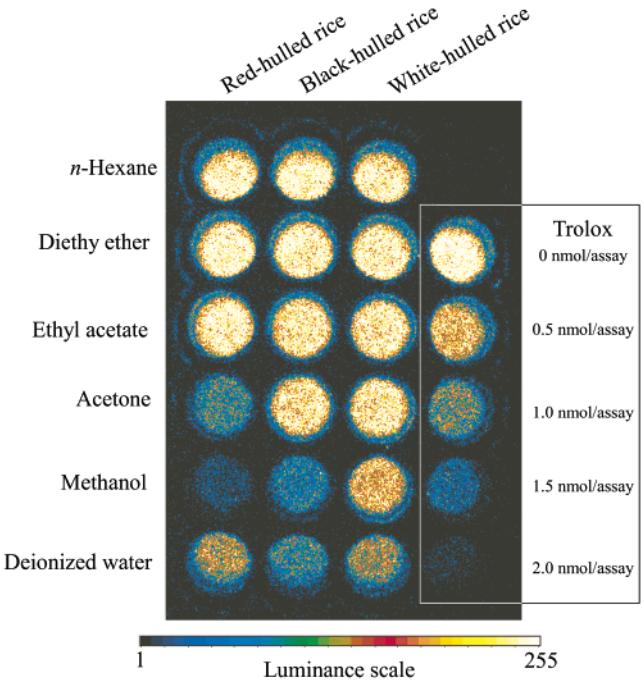

Figure 2. Chemiluminesence generated by the reaction between *t*-BuOO• and luminol in the presence of Trolox or extracts from rice with hulls of various colors.

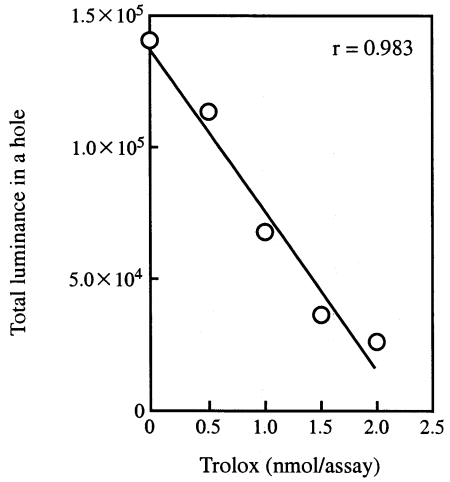

Figure 3. Standard Trolox curve for calculating *t*-BuOO• scavenging activity.

methanol and deionized water, showed the highest radical-scavenging activity. In rice with black and white hulls, the sum of the activities in two extracts corresponded to more than 90% of the total DPPH• scavenging activity. In red-hulled rice, high radical-scavenging activity (1.22 μmol of Trolox equivalents/mL) was observed in the acetone extract in addition to the methanol and deionized water extracts.

The chemiluminescence intensities of the Trolox standard and the extracts from the rice with hulls of various colors are shown in **Figure 2**. The total luminance decreased with an increase of the Trolox concentration. The correlation between the total luminance and the Trolox concentration was 0.983 (**Figure 3**), indicating that this assay was acceptable for measuring *t*-BuOO• scavenging activity. As the activities of the extracts from the rice with hulls of various colors were calculated (**Figure 4**), it could be observed that the extracts prepared with highly polar solvents exhibited higher *t*-BuOO• scavenging activity, which was similar to the results of the study of the DPPH• scavenging activity (**Figure 1**). The total *t*-BuOO• scavenging activity, which was calculated from the sum of the activity in each extract, decreased in the order of rice with red hull (4.15 μmol of Trolox equivalents/assay), black (2.83), and white (2.09). In addition, the acetone extract from red-hulled rice exhibited a high *t*-BuOO• scavenging activity, while no such activity was detected for the acetone extracts of the other cultivars (white- and black-hulled).

To obtain useful information about the radical-scavenging components contained in the acetone extract from red-hulled rice, the extract was subjected to acid hydrolysis and a vanillin assay. In both reactions, the tested solution turned red, indicating the presence of proanthocyanidin in the extract. Following this study, HPLC analysis of the acid hydrolysate was performed to elucidate the constituents of proanthocyanidin. As shown in **Figure 5**, only one peak (retention time 24.8 min) was observed in the chromatograms of the acid hydrolysate. This peak was identified as cyanidin by comparison of the retention time and the photodiode array spectrum with those of authentic anthocyanidins. In addition, it was found that no anthocyanins and anthocyanidins were contained in the acetone extract from red-hulled rice by HPLC analysis of the acetone extract without acid hydrolysis (data not shown). These results revealed that the proanthocyanidin in the extract from red-hulled rice was the procyanidin type, that is, (+)-catechin and/or (−)-epicatechin derivatives.

The acetone extract from red-hulled rice was fractionated using a Sephadex LH-20 column. **Figure 6** shows the elution patterns for the DPPH• scavenging activity and the proanthocyanidin content. It was apparent that the two elution patterns were similar and that potent DPPH• scavenging activity was observed in the fraction eluted with the 70% acetone solution, accompanied by a high proanthocyanidin content. In further analysis, the apparent molecular weight distribution of procyanidin-containing fractions 44−49 was estimated by GPC measurement (**Figure 7**). The acetylated procyanidins eluted in the molecular weight range of about 500−18 000, and the average molecular weight was 5000. Assuming that the molecular weight of the acetylated monomer unit was 500 (*11*), the degree of polymerization ranged from 1 to 38. Many studies have reported that procyanidins exist in various plants, such as the azuki bean (*12*), grape seed (*13*), black and brown soybean (*14*), and cocoa (*15*). Furthermore, the studies have reported that extracts containing procyanidin exhibit potent antioxidative activities.

As a result of the studies noted above, our interest turned to how procyanidins are absorbed by the human body. Furthermore, we wanted to know how the antioxidative effect takes place in the human body after the administration of a fraction containing procyanidin prepared from red-hulled rice. However, not all of these questions have been thoroughly answered in this study. There is currently no satisfactory explanation for how the procyanidin polymer is absorbed into the bloodstream. Richelle et al. (*16*) have reported that epicatechin from chocolate was found to reach a concentration of 0.7 μM in plasma after the intake of 80 g of black chocolate, which is rich in procyanidin oligomers. Recent work by Spencer et al. (*17*) has shown that, in an acid environment, such as that found in the gastric milieu, procyanidin oligomers were hydrolyzed to a mixture of an epicatechin monomer and a dimer. On the other hand, Ling et al. (*18*) have reported that, when a hypercholesterolemic rabbit had red-hulled rice as a part of its diet, the total antioxidative properties of the serum and liver increased.

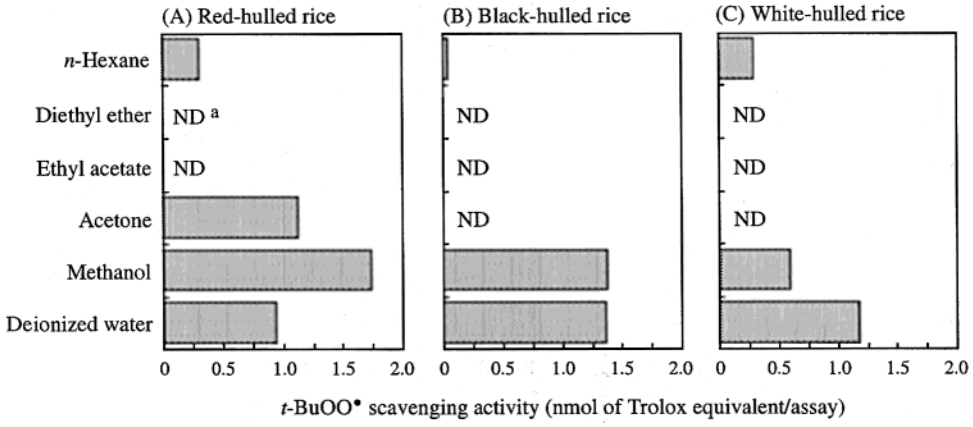

Figure 4. *t*-BuOO• scavenging activities of the extracts prepared from rice with red hull (A), black hull (B), and white hull (C) by using solvents with various polarities. [a]ND, not detected.

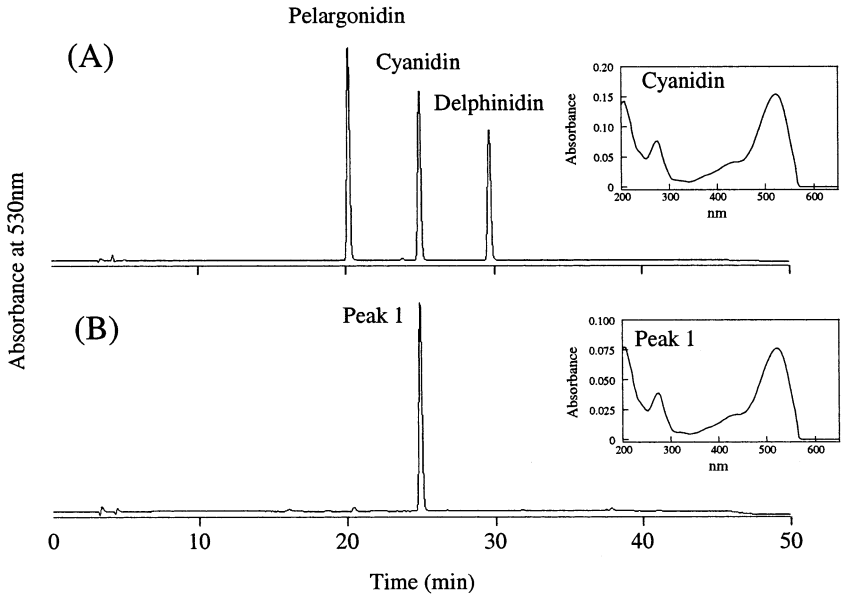

Figure 5. HPLC chromatograms of anthocyanidin standards (A) and the acetone extract from red-hulled rice after acid hydrolysis (B). Peak 1 was identified as cyanidin by a comparison of the retention time and the UV–vis spectrum.

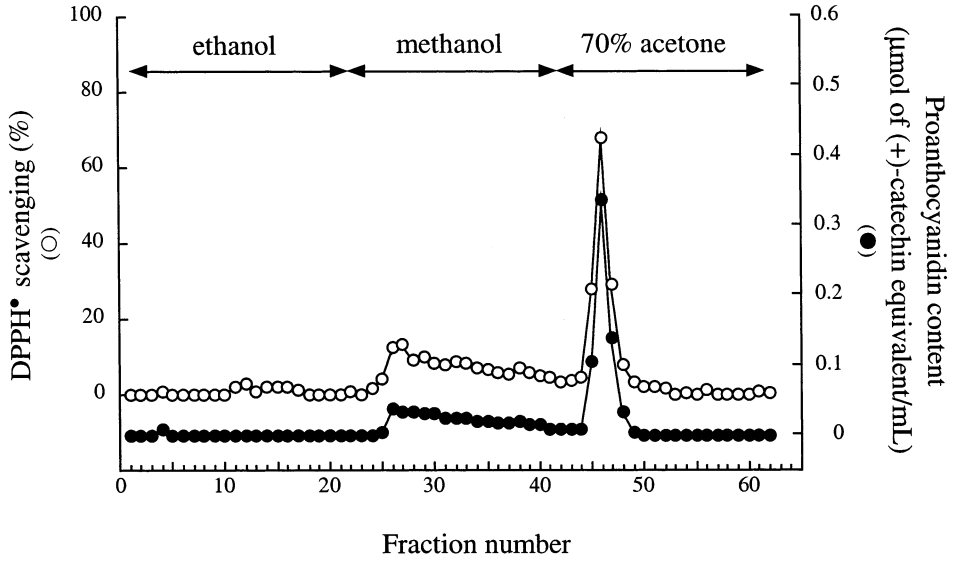

Figure 6. Chromatographic profiles in a Sephadex LH-20 column of the acetone extract from red-hulled rice.

They suggested that constituents of red-hulled rice with antioxidative activity could be responsible for the increased antioxidative properties in the body (*18*); however, they did not identify the antioxidant compounds in the red-hulled rice. These reports suggest that, after polymeric procyanidins in red-hulled rice are consumed, they could be absorbed by the body after

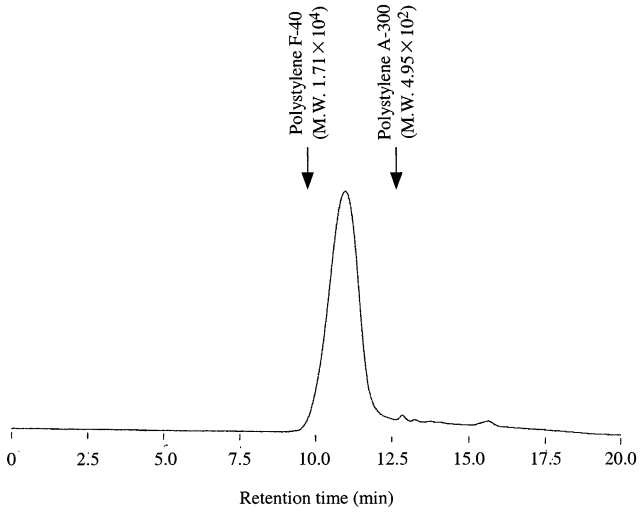

Figure 7. GPC analysis of the acetone extract from red-hulled rice after acetylation.

hydrolysis in the gastric milieu, which would elevate the antioxidative capacity.

Also of interest is the antioxidative activity that takes place in the intestine because alkyl peroxyl radicals, which have a tumor-promoter effect (*19*, *20*), are associated with the incidence of colon cancer when these radicals are generated in the intestine (*21*). The alkyl peroxyl radicals were generated by heme-iron-catalyzed decomposition of hydroperoxide. Fukushima et al. (*21*) have pointed out that alkyl peroxyl radicals could be generated from food materials such as fat and hemoferrum. In this study, the *t*-BuOO$^{\bullet}$ generation system was applied with *t*-BuOOH and methemoglobin as a model reaction, according to a report by Maeda et al. (*8*), and the *t*-BuOO$^{\bullet}$ scavenging activity of the extracts from rice with hulls of various colors was assayed. Only the extract from the red-hulled rice exhibited a high *t*-BuOO$^{\bullet}$ scavenging activity when acetone was used as an extraction solvent. This result indicated that polymeric procyanidins would exhibit *t*-BuOO$^{\bullet}$ scavenging activity as well as DPPH$^{\bullet}$ scavenging activity. Therefore, it appears that the consumption of red-hulled rice might have the potential to prevent colon cancer, even when the polymeric procyanidins present in the rice are not decomposed and absorbed into the bloodstream.

In this study, we demonstrated that polymeric procyanidins are the major radical-scavenging components in red-hulled rice. We are now researching the physiological functions of red-hulled rice in vivo. The useful information reported here will assist in the elucidation of the mechanisms at work in these functions.

ABBREVIATIONS USED

DPPH$^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl radical; *t*-BuOO$^{\bullet}$, *tert*-butyl hydroperoxide radical.

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