Radical Scavenging Activity of a Purple Pigment, Hordeumin, from Uncooked Barley Bran-Fermented Broth

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A novel purple pigment called hordeumin, a type of anthocyanin-tannin pigment, was produced from barley bran-fermented broth. The radical scavenging activity of hordeumin was analyzed by using an electron-spin resonance (ESR) spectrometer. The hordeumin scavenged superoxide radical in a concentration-dependent manner. Superoxide dismutase-like activity values were 118 and 195 units/mg for crude and partially purified hordeumin, respectively. The two types of hordeumins also scavenged the DPPH radical. Furthermore, barley bran-fermented filtrate before pigment formation and extract of barley bran also scavenged the DPPH radical. However, the DPPH radical scavenging activity of a filtrate, fermented over a long period, was stronger than that fermented over a short period. Thus, it was considered that radical scavenging activity of hordeumin resulted from barley bran polyphenol such as proanthocyanidins.

Keywords: *Hordeumin; barley bran; DPPH radical; superoxide radical; radical scavenging activity*

INTRODUCTION

Recently, many studies of the physiological functions of the polyphenol compound contained in dietary plants and food have been performed. It has been reported that tannin derived from plants (Hatano et al., 1989; Uchida et al., 1987), procyanidin from azuki beans (Ariga et al., 1988; Ariga and Hamano, 1990), and catechin in tea (Yamamoto-Maeda, 1996; Yokozawa et al., 1998) scavenged radicals to protect living systems from active oxygens. Furthermore, the antioxidative effect of anthocyanin, a natural pigment, has also been reported (Igarashi et al., 1989, 1993; Tsuda et al., 1994).

We have been trying to effectively use barley to produce a useful product (pigment). In our previous studies (Ueda et al., 1987; Ohba et al., 1992, 1993), a large amount of precipitate of a novel purple pigment, hordeumin, was formed as a secondary product in addition to ethanol in an uncooked alcohol fermentation using barley bran as the fermented material. It was found that hordeumin was produced when cereals such as barley or wheat, in particular bran, were used as the raw materials (Ohba et al., 1992). Hordeumin contained cyanidin and delphinidin (Ohba et al., 1993). Hordeumin also contained a polyphenol such as tannin. Barley bran, a hordeumin material, contained various polyphenols such as procyanidin and prodelphinidin (Tamagawa et al., 1998, 1999). In previous studies, physiological functions of barley bran extract was reported (Tamagawa et al., 1997, 1998, 1999). Furthermore, hordeumin also had an antimutagenicity (Deguchi et al., 2000). However, the radical scavenging activity of hordeumin, which is produced from barley bran-fermented filtrate, was not studied.

We are trying to effectively apply hordeumin to foods, cosmetics, dyeing agents, etc. To clarify the physiological functions of hordeumin, the present paper describes the radical scavenging activity of hordeumin.

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MATERIALS AND METHODS

Materials and Chemicals. Barley bran (from Australian barley, husk) was provided by Torigoe Seifun Ltd. (Fukuoka, Japan). The baker's yeast used was saf-instanto (instanto-dry yeast) from S. I. Lesaffre (France). Hypoxanthine was purchased from Sigma Chemical Co. (St. Louis, MO). Xanthine oxidase and superoxide dismutase (SOD) were purchased from Boehringer Mannheim Co. (Tokyo, Japan). 5, 5-Dimethyl-1-pyroline *N*-oxide (DMPO) was obtained from Labotec Company Ltd. (Kyoto, Japan). Diethylenetriamin-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DETAPAC) was purchased from Dojindo Laboratories (Kumamoto, Japan). Cyanidin chloride was purchased from Funakoshi Co. (Tokyo, Japan). All other chemicals were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Preparation of Crude and Partially Purified Hordeumins. Uncooked barley bran (30 g), baker's yeast (3 g), and deionized water (200 mL) were placed in an Erlenmeyer flask (300 mL) and blended well. The pH of the mixture was adjusted to 3.5 using a 1 N HCl solution. A rubber stopper with a fermentation tube was inserted in the mouth of the Erlenmeyer flask, and the fermentation was performed at 30 °C for 6 d. Then the fermented broth was filtered through Toyo filter paper (No. 5C) and then refiltered through a membrane filter (pore size, 0.65 μm, Advantec Toyo Ltd., Tokyo, Japan). The pH of the fermented filtrate was adjusted to 3.5 in an Erlenmeyer flask (300 mL) equipped with a cotton plug and kept for 2 weeks in cold storage (5 °C, dark room). The yellow fermented filtrate became purple during the storage period, and a hordeumin precipitate was formed. The stored filtrate was centrifuged (8000 rpm, 30 min) to recover the precipitate. The precipitate was dried by using a vacuum oven (VOS-300SD, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). This purple precipitate was named crude hordeumin. The crude hordeumin was dissolved in 10 mL of 1% HCl in methanol, and the resulting solution was centrifuged (3000 rpm, 5 min) to remove insoluble matter. Diisopropyl ether (30 mL) was added to the supernatant to precipitate the pigment. The mixture was centrifuged (3000 rpm, 5 min), and then the supernatant was removed. This precipitate was again dissolved in 1% HCl in

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methanol and further precipitated using the diisopropyl ether. This operation was repeated two times to remove ether-soluble matter, and the pigment precipitate was dried by using a vacuum oven. This purple precipitate was named partially purified hordeumin.

Superoxide Radical Scavenging Activity of Hordeumin. Analysis of the superoxide radical was carried out with the hypoxanthine−xanthine oxidase superoxide generating system by an electron-spin resonance (ESR) spectrometer (Jeol RE Series, Jeol Ltd., Tokyo, Japan), using manganese as the internal standard (Mitsuta et al., 1990; Sato et al., 1996). Crude hordeumin, partially purified hordeumin, and cyanidin chloride were dissolved in dimethyl sulfoxide (DMSO) at various concentrations (0−500 μg/mL). Fifty microliters of hypoxanthine (2 mM) was placed in a test tube, and 35 μL of DETAPAC (5.5 mM), 15 μL of DMPO, 20 μL of hordeumin solution, 30 μL of phosphate buffer (0.1 mM, pH 7.8), and 50 μL of xanthine oxidase (0.4 U/mL) were added successively. The reaction mixture was stirred for 10 s. Exactly 60 s later, the superoxide radical was measured using an ESR spectrometer. ESR analysis conditions were as follows: temperature, room temperature (25 °C); frequency, 9.4500 GHz; power, 8.00 mW; sweep width, 5.0 mT; sweep time, 2.0 min; field, 335.8 mT; modulation width, 0.1 mT; gain, 400; time constant, 0.1 s. The blank used was DMSO instead of the hordeumin solution. The SOD-like activity was evaluated by a standard curve of SOD produced under the same ESR conditions. One unit of SOD inhibits the antioxidation of pyrogallol (uninhibited rate: $\Delta A_{420nm} = 0.02$) by 50% under assay conditions (25 °C; Tris−succinate buffer, pH 8.2, containing 1 mM EDTA) (Minami and Yoshikawa, 1979).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of Hordeumin (Hatano et al., 1989; Uchida et al., 1987). Crude hordeumin and partially purified hordeumin were also dissolved in methanol at various concentrations (0−200 μg/mL). One hundred microliters of 100 μL of 100 μM DPPH solution in methanol and 100 mL of hordeumin were mixed, and then the reaction mixture was stirred for 10 s. Exactly 60 s later, the DPPH radical was measured by ESR spectrometer. ESR analysis was performed under similar conditions as described above. The blank used was methanol instead of the hordeumin solution.

DPPH Radical Scavenging Activity of Barley Bran-Fermented Filtrate and Extract. The fermented filtrates (1 or 6 d) were prepared as described above. Barley bran (30 g) was soaked in phosphate buffer (0.04 M, pH 7.0, 200 mL) for 1 h, filtered through Toyo filter paper (No. 5C), and then refiltered through a membrane filter (pore size, 0.65 μm, Advantec Toyo Ltd., Tokyo, Japan). Ethanol (4 mL) was added to the sample (4 mL) and then centrifuged (2800 rpm, 5 min). The resultant supernatant was diluted 10-fold using 50% ethanol. The resultant solution was used for the DPPH radical scavenging assay. DPPH radical scavenging assay was carried out by spectrometry (Yamaguchi et al., 1998). The DPPH solution (100 μM, 0.6 mL) in 50% ethanol and the sample solution (0.6 mL) were mixed, and then the reaction mixture was stirred for 10 s. Exactly 2 min later, DPPH radical scavenging activity was measured for absorbance at 520 nm by using a spectrophotometer (DU-70, Beckman). The blank used was 50% ethanol instead of the sample solution.

RESULT

Superoxide Radical Scavenging Activity of Hordeumin. When crude hordeumin or partially purified hordeumin was added to the superoxide generating system, hordeumin scavenged the superoxide radical in a concentration-dependent manner (Figure 1). Superoxide radical scavenging activity of partially purified hordeumin was stronger than that of crude hordeumin in every concentration. On the basis of the SOD standard curve, the SOD-like activity value of crude hordeumin, partially purified hordeumin, and cyanidin chloride were 118, 195, and 199 units/mg, respectively.

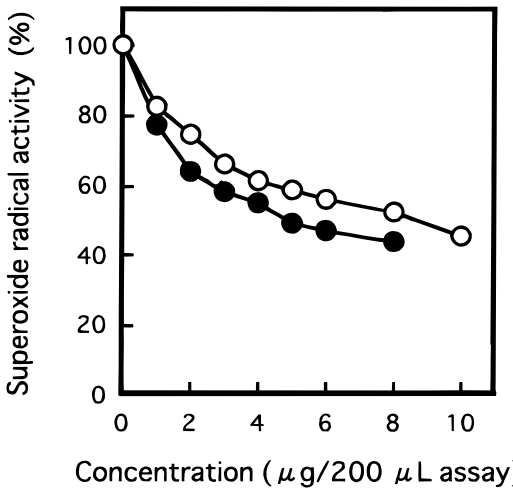

Figure 1. Effect of hordeumin on the superoxide radical. Symbols: ○, crude hordeumin; ●, partially purified hordeumin.

The SOD-like activity value of partially purified hordeumin was the same as that of cyanidin chloride.

DPPH Radical Scavenging Activity of Hordeumin. The DPPH radical was a stable radical in solution. DPPH radical scavenging activity of hordeumin was determined by using an ESR spectrometer. ESR spectra of DPPH radical are shown in Figure 2. Crude hordeumin and partially purified hordeumin scavenged the DPPH radical. Inhibition of the DPPH radical was dependent on concentration of hordeumin (Figure 3). Furthermore, DPPH radical scavenging activity of partially purified hordeumin was stronger than that of crude hordeumin at every concentration.

DPPH Radical Scavenging Activity of Barley Bran-Fermented Filtrate and Extract. It was found that hordeumin had radical scavenging activity. Next, we studied the radical scavenging activity of hordeumin derived from barley bran as a raw material or that which occurred during hordeumin formation. Barley bran-fermented filtrate (1 or 6 d) and barley bran extract were analyzed for DPPH radical scavenging activity. Barley bran uncooked alcohol fermented filtrate, which fermented during 1 or 6 d, scavenged the DPPH radical in a concentration-dependent manner (Figure 4). However, DPPH radical scavenging activity of 6-day-fermented filtrate was stronger than that of 1-day-fermented filtrate. The barley bran extract, which was produced by phosphate buffer different from fermentation pH of hordeumin formation, also had DPPH radical scavenging activity, but it was lower than those of the two fermented filtrates.

DISCUSSION

Uncooked alcohol fermentation was performed by mixing raw barley bran, baker's yeast, and water. When the barley bran-fermented filtrate was stored at low temperature in an oxidative environment, hordeumin, a purple pigment, was formed on oxidative polymerization of precursors such as anthocyanidins and tannins (Deguchi et al., 1999). Furthermore, hordeumin had an antimutagenicity (Deguchi et al., 2000). Therefore, the radical scavenging activity of hordeumin was investigated in the present study. Crude hordeumin and partially purified hordeumin had radical scavenging activity of the superoxide radical and DPPH radical. Radical scavenging activity of partially purified hor-

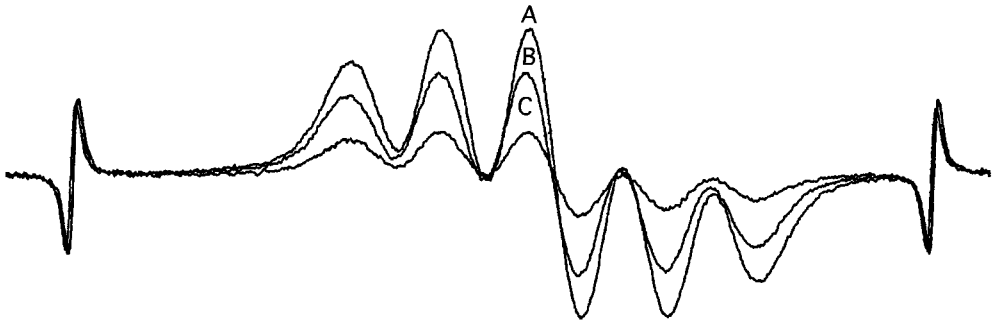

Figure 2. ESR spectra of DPPH radical. Crude hordeumin or partially purified hordeumin was added to reaction mixture at concentration of 12 μg/200 μL assay: (A) blank; (B) crude hordeumin; (C) partially purified hordeumin.

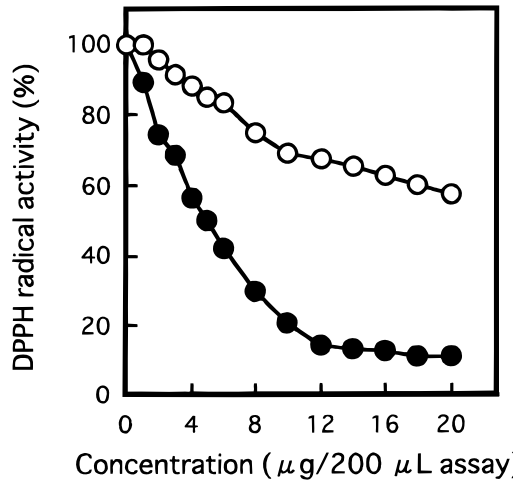

Figure 3. Effect of hordeumin on the DPPH radical. Symbols: ○, crude hordeumin; ●, partially purified hordeumin.

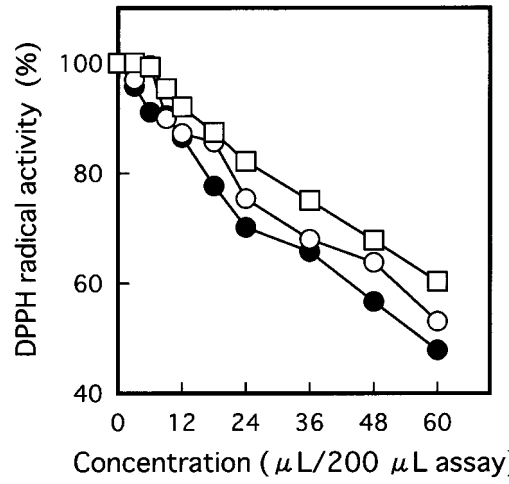

Figure 4. Effects of extracts and fermented filtrate from barley bran on DPPH radical. Symbols: ○, fermented filtrate (1 d); ●, fermented filtrate (6 d); □, barley bran extract.

deumin was stronger than that of crude hordeumin in every concentration, since the polyphenol content of partially purified hordeumin (235.0 mg/g) was higher than that of crude hordeumin (80.7 mg/g).

Furthermore, we studied the radical scavenging activity of hordeumin that was derived from barley bran or that occurred during hordeumin formation. As a result, all of the tested samples were shown to have DPPH radical scavenging activity. Tamagawa et al. (1997) studied antioxidative activity of barley bran extracts and reported that proanthocyanidins separated from barley bran extracts were shown to have antioxidative activity (Tamagawa et al., 1998, 1999). These findings suggest that the radical scavenging activity of hordeumin is attributable to polyphenol compounds such as procyanidins and anthocyanidins from barley bran as raw materials rather than generation during hordeumin formation.

Since polyphenol compounds show various physiological functions, hordeumin might also be expected to have physiologically functional properties because it contains polyphenol. Further study is necessary in order to clarify the other physiological functions.

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