JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Aging of Whiskey Increases 1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Activity

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1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of Japanese whiskey after various aging periods in oak barrels was measured to evaluate the antioxidative effects of whiskey. The activity of the whiskey increased with the aging period with high correlation. The activity of various types of whiskey was measured and shown to be correlated to the potentiation of the $GABA_A$ receptor response measured in a previous paper. However, the fragrant compounds in the whiskey which potentiated the $GABA_A$ receptor response had low DPPH radical scavenging activity, while phenol derivatives had high radical scavenging activity. The whiskey was extracted by pentane. The aqueous part showed the scavenging activity, whereas the pentane part did not. Thus, both the DPPH radical scavenging activity and the potentiation of the $GABA_A$ receptor response increased during whiskey aging in oak barrels, but were due to different components. The whiskey protected the H_2O_2-induced death of *E. coli* more than ethanol at the same concentration as that of the whiskey. The changes that occurred in the whiskey during aging may be the reason aged whiskies are so highly valued.

KEYWORDS: Aging of whiskey; antioxidant; DPPH radical; polyphenol; radical scavenging

INTRODUCTION

Fresh distillates of whiskey are stored in oak barrels for long periods of time. During aging, the sharp or raw odor typical of fresh whiskey distillates is modified to a rounded, soft, and mellow one, and the colorless distillates turn to amber ones, suggesting changes and the production of flavor and pigment components in whiskey (*1*−*3*).

In a previous paper (*4*), we found that most fragrant components in whiskey potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes. Whiskey itself also potentiated the $GABA_A$ receptor response more than ethanol at the same concentration as that of the whiskey (*5*). Inhalation of whiskey in mice increased the sleeping time induced by pentobarbital more than ethanol at the same concentration as that of the whiskey. Further, the potentiation of the $GABA_A$ receptor response increased with the aging period of the whiskey. These results suggest that not only ethanol but also minor fragrant components in whiskey play an important role in the potentiation of the $GABA_A$ receptor response and possibly the sedative effect of whiskey.

Antioxidants are very important for human health, since the production of reactive oxygen species is thought to be a significant cause of aging and carcinogenesis (*6*, *7*). Many laboratories reported that phenolic constituents present in red wine, such as querecetin, resveratol, and catechin derivatives, possess antioxidant, anticancer, and anti-inflammatory properties, and suggested that red wine may impact mortality more favorably than other alcohol beverages (*8*). Various antioxidants such as phenolic constituents were isolated in whiskey, and their antioxidant status was measured by Goldberg et al. (*9*). They suggested that the antioxidants might be due to the aging process in oak barrels.

In this paper, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (*10*, *11*) of Japanese whiskey after various aging periods was measured to find the relationship between the radical scavenging activity and the aging period of whiskey. The radical scavenging activity of various types of whiskey was also measured to examine the relationship between scavenging activity and the potentiation of the $GABA_A$ receptor response. To clarify which components in whiskey were contributing to the antioxidative activity, the DPPH radical scavenging activities of fragrant components and phenol derivatives were measured. The effect of whiskey on the H_2O_2-induced death of *E. coli* was also measured to determine the antioxidative activity of the whiskey.

MATERIALS AND METHODS

Materials. Japanese whiskey of various aging periods in barrels of North American white oak was supplied by Suntory Ltd., Osaka, Japan. The single-malt (S) whiskey was produced from only barley malt, while

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10.1021/jf049817s CCC: $27.50
Published on Web 07/17/2004

the blended (B) one was produced by blending barley malt whiskey with grain whiskey produced by a mixture of barley malt and grain such as corn or wheat. Various types of whiskey, grain, bourbon, Tennessee, Canadian, Irish, S Scotch, and B Scotch, were purchased from a local market in Osaka, Japan. These were the same as those used previously in an experiment of the potentiation of the $GABA_A$ receptor response (*5*). The whiskey contained 40−43% (v/v) ethanol. Beer, oolong tea, and red wine were products of Suntory and were purchased from a local market in Yamaguchi, Japan. Sake (Japanese liquor fermented from rice; Gokyo, Sakai Brewery, Yamaguchi, Japan), shochyu (Japanese distilled liquor fermented from potato; Oharu, Fukuda Brewery, Hirado, Japan), yomeishyu (Japanese medicinal liquor with various herbs; Yomeishyu Co., Ltd., Tokyo, Japan), and Hua Tuo (Chinese medicinal liquor with various herbs; Shanghai Guanshengyuan Huaguang Breing and Medicine Co. Ltd., China) were purchased from local markets in Japan and China.

Fragrant compounds, ethyl phenylpropanoate, ethoxy derivatives, lactone derivatives, and phenol derivatives, were supplied by Suntory and were the same as those used in a previous paper (*4*). DPPH was purchased from Wako Pure Chemical Industry, Ltd., Osaka, Japan. Syringic acid, gallic acid, ellagic acid, and furan were purchased from Nacalai Tesque, Kyoto, Japan. All chemicals used were of guaranteed reagent quality.

A 2 mL sample of Japanese blended whiskey aged for 30 years was mixed with 2 mL of pentane, and the mixture was shaken vigorously. After separation with a separating funnel, the pentane in the pentane part was evaporated using a suction pump, and the residue was dissolved in 2 mL of 43% (v/v) ethanol aqueous solution for measurement. The aqueous part was used for measurement without further treatment.

Measurement of DPPH Radical Scavenging Activity. The reaction mixture (total volume 3 mL), consisiting of 0.5 mL of 0.5 M acetic acid buffer solution at pH 5.5, 1 mL of 0.2 mM DPPH in ethanol, and 1.5 mL of 50% (v/v) ethanol aqueous solution, was shaken vigorously with various samples (*10*, *11*). After incubation at room temperature for 30 min, the remaining DPPH was determined by absorbance at 517 nm, and the radical scavenging activity of each sample was expressed using the ratio of the absorption decrease of DPPH (%) to that of the control DPPH solution (100%) in the absence of the sample. When the diluted sample itself has a 517 nm absorption of more than 1% of the control absorption, it is subtracted from the 517 nm absorption of the sample reaction mixture. That is, the radical scavenging activity (%) = $100(A - B)/A$, where A and B are the 517 nm absorption of the control and the corrected absorption of the sample reaction mixture.

Mean values were obtained from triplicate experiments. A Student's *t* test was used to evaluate the significance of the mean values, in comparison with that of the control.

Measurement of Antioxidative Activity. *E. coli* W3110Y cells were kindly given by Prof. M. Yamada of Yamaguchi University. The cells were cultured in the LB medium (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCl; pH 7.5) with shaking at 37 °C.

For measurements of the antioxidative effect of whiskey, the following mixtures were prepared: 400 μL of stationary-phase *E. coli* cells in the LB medium, ±10 μL samples (whiskey or 43% ethanol), ±10 μL of 3.5% H_2O_2, adjusted to 1 mL by addition of the LB medium. The mixtures were incubated for 90 min at 37 °C. Then the mixtures were diluted 20 times by addition of the LB medium. The cell growth with shaking at 37 °C was monitored by the optical density of the culture at 600 nm after 0, 1, 2, and 3 h. Mean values were obtained from four experiments. A Student's *t* test was used to evaluate the significance of the mean values.

RESULTS

Figure 1 shows the dose dependency of the DPPH radical scavenging activities of Japanese blended whiskey after aging for 8.5, 17, and 30 years. Scavenging activity increased almost proportionally with whiskey concentration at low concentrations; however, the activity showed a tendency to become saturated at high concentrations. The scavenging activity of the whiskey depended on the aging period.

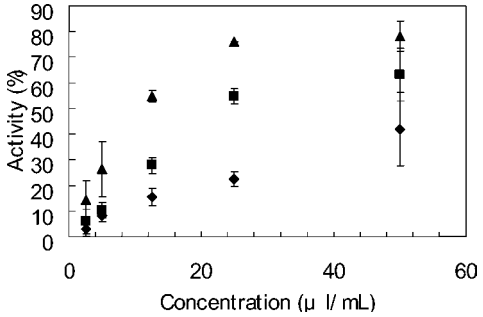

Figure 1. Dose dependency of the DPPH radical scavenging activities of Japanese blended whiskies after aging for 8.5 (◆), 17 (■), and 30 (▲) years. Data are mean ± SD (bars) values from three or four experiments. $p < 0.05$ by Student's *t* test for values between the whiskey and the control except the values at 2.5 μL/mL whiskey after aging for 8.5 and 17 years.

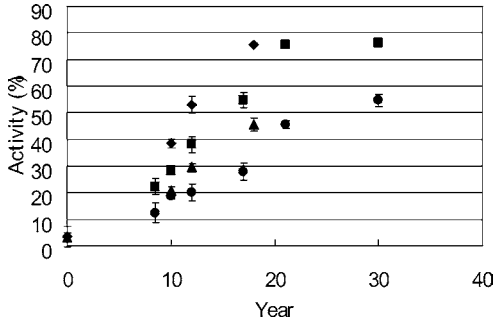

Figure 2. DPPH radical scavenging activities of Japanese single-malt and blended whiskies after various aging periods. The correlation factors between the DPPH radical scavenging activities and aging for single-malt whiskies and blended whiskies were estimated to be 0.995 and 0.960 when the whiskey concentration was 25 μL/mL and 0.989 and 0.977 when the whiskey concentration was 12.5 μL/mL, respectively. Data are mean ± SD (bars) values from three experiments. $p < 0.02$ by Student's *t* test for values between the whiskey and the control except the value of the whiskey without aging (0 years). Key: ◆, 25 μL/mL single-malt whiskey; ■, 25 μL/mL blended whiskey; ▲, 12.5 μL/mL single-malt whiskey, ●, 12.5 μL/mL blended whiskey.

Figure 2 shows the DPPH radical scavenging activities of Japanese single-malt and blended whiskey after various aging periods, which were the same as those used in the previous experiment (*5*). Activity increased with whiskey age. The correlation factors between the DPPH radical scavenging activity and age for single-malt whiskies and blended whiskies were estimated to be 0.995 and 0.960 when the whiskey concentration was 25 μL/mL and 0.989 and 0.977 when the whiskey concentration was 12.5 μL/mL, respectively. The activity of single-malt whiskey increased with age more than the blended one.

To find out if DPPH radicals were scavenged only by Japanese whiskey, we measured the scavenging activities of various types of whiskey at 25 μL/mL (**Figure 3**), which were the same as those used in the previous experiment (*5*). As expected, all types of whiskey examined scavenged DPPH radicals. However, the degree of activity showed some variation, from 36% (Irish whiskey) to 74.8% (single-malt Scotch whiskey).

To find out which compounds in whiskey scavenged the DPPH radicals, we measured the radical scavenging activities of 0.25% (v/v) fragrant compounds whose effect on the $GABA_A$ receptor response we measured in a previous study (*4*). Most compounds whose effects on the receptor response were

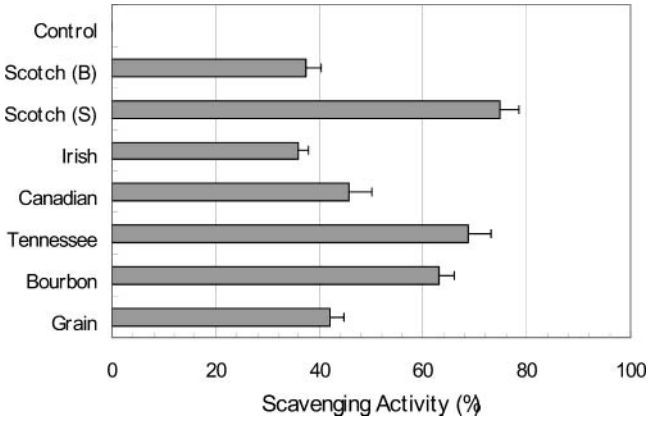

Figure 3. DPPH radical scavenging activities of various types of 25 μL/mL whiskey. The potentiation of the $GABA_A$ receptor response by these whiskies was measured in a previous paper (*5*). Data are mean ± SD (bars) values from three experiments. $p < 0.01$ by Student's *t* test for values between the whiskey and the control.

Table 1. DPPH Radical Scavenging Activity of Various Compounds[a]

compound	activity (%)	signif
cysteine (control)		
10 μmol/L	6.3 ± 0.8	
20 μmol/L	13.4 ± 0.5	*b*
50 μmol/L	42.6 ± 0.6	*b*
fragrant compounds (2.5 μL/mL)		
1,1-diethoxypropane	1.9 ± 1.9	
1-diethoxy-2-methylpropane	2.0 ± 2.0	
1-diethoxy-3-methylbutane	3.3 ± 0.6	
1,1-diethoxyheptane	6.2 ± 1.0	
ethylphenylpropane	6.6 ± 2.0	
damascenone	−3.7 ± 4.9	
lactone derivatives (2.5 μL/mL)		
Quercus lactone b	10.7 ± 0.5	*b*
A-ionone	16.8 ± 2.4	*b*
B-ionone	22.0 ± 1.7	*b*
nonalactone	−2.2 ± 0.6	
decanolactone	0.0 ± 0.0	
dodecalactone	−2.2 ± 0.6	
phenol derivatives and furan		
4-ethylguaiacol (2.5 μL/mL)	82.4 ± 0.6	*b*
2-methyl-4-methylphenol (2.5 μL/mL)	82.8 ± 0.1	*b*
syringic acid (0.25 mM)	80.0 ± 1.3	*b*
gallic acid (0.25 mM)	78.3 ± 1.4	*b*
furan (25 μL/mL)	39.1 ± 0.0	*b*

[a] Data are mean ± SD values from three experiments. [b] $p < 0.05$ by Student's *t* test.

examined before showed no scavenging activities (**Table 1**). Only Quercus lactone showed a slight radical scavenging activity; therefore, we measured the radical scavenging activity of various types of lactone derivatives. A- and B-ionone showed some activity, while nonalactone, decanolactone, and dodecalactone showed no activity (**Table 1**). Since antioxidative activity of phenolic constituents in whiskey has been reported (*9*), the DPPH radical scavenging activities of some phenol derivatives were examined. Syringic acid and gallic acid had very strong DPPH radical scavenging activity (**Table 1**). Ellagic acid also showed high activity, but we did not show the exact value because of its insolubility, even in ethanol solution.

The extract of whiskey by pentane was shown to potentiate the $GABA_A$ receptor response in a previous paper (*4*). To examine whether antioxidative activity comes from the same components in whiskey as those that potentiated the $GABA_A$ receptor response, we measured the DPPH radical scavenging activity of both the aqueous part and the pentane part of the whiskey extract. Colored components remained in the aqueous part. As expected, the aqueous part of the blended whiskey aged for 30 years showed high DPPH radical scavenging activity, whereas the pentane part showed only minimal activity (25 μL/mL whiskey, 82.5 ± 0.1%; aqueous part, 80.6 ± 1.4%; pentane part, 1.3 ± 1.0%).

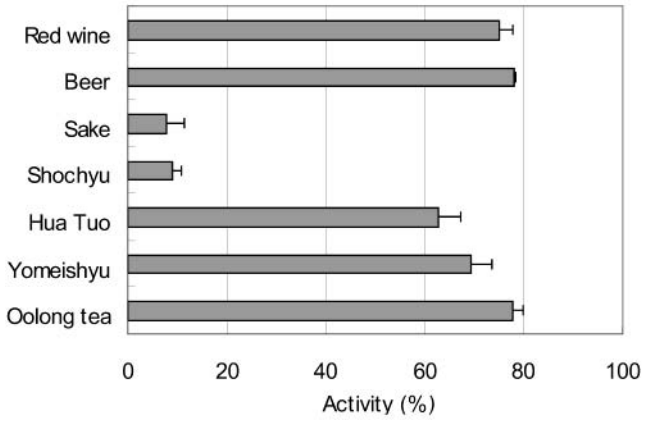

Figure 4. DPPH radical scavenging activities in various types of 25 μL/mL beverages. Sake is a colorless Japanese liquor fermented from rice. Shochyu is a colorless Japanese distilled liquor fermented from potato. Yomeishyu is a dark brown Japanese medicinal liquor with various herbs. Hua Tuo is a yellow-brown Chinese medicinal liquor with various herbs. Data are mean ± SD (bars) values from three experiments. $p < 0.05$ by Student's *t* test for values between the liquor and the control except the value of Sake ($p = 0.07$).

The DPPH radical scavenging activities of various beverages were examined and are shown in **Figure 4**. Colored beverages, red wine, beer, oolong tea, and medicinal liquors, showed high DPPH radical scavenging activity, whereas colorless ones, sake (Japanese liquor fermented from rice) and shochyu (Japanese distilled liquor fermented from potato), showed low scavenging activity.

In previous papers (*12*, *13*), we found that generation of free radicals caused cell death during oxidation by lipid hydroperoxide or H_2O_2. To examine the effect of whiskey on the H_2O_2-induced death of *E. coli*, H_2O_2-treated cell growth of the mixture with the Japanese blended whiskey aged for 30 years was compared with that with ethanol at the same concentration as that of the whiskey. The whiskey (1% v/v) and ethanol (0.43% v/v) caused no effect on *E. coli* cell growth. After oxidation of *E. coli* cells with 20 mM H_2O_2 in the presence and absence of the whiskey or ethanol for 90 min, the cell growth was monitored by the absorbance at 600 nm. As shown in **Table 2**, which was one example of three experiments, the whiskey showed significantly more antioxidative effect than the ethanol, though ethanol also had a small antioxidative effect. We repeated the experiments three times, since the effect was not so large in these experimental conditions. Every time, the whiskey showed significant antioxidative effect, though the cell growth after oxidation showed some variation because of the different growth stages of the cells used.

DISCUSSION

The aging of whiskey in oak barrels is an important process in the production of good-quality whiskey (*1*−*3*). During aging, the sharp or raw odor typical of fresh whiskey distillates is modified to a rounded, soft, and mellow one. In previous papers (*4*, *5*), we found that fragrant components in whiskey potentiated the response of $GABA_A$ receptors expressed in *Xenopus* oocytes

Table 2. Protective Effect of Japanese Blended Whiskey Aged for 30 Years against *E. coli* Cell Death by H_2O_2 Oxidation[a]

	absorbance at 600 nm			
	0 h growth time	1 h growth time	2 h growth time	3 h growth time
control (ethanol)	0.167 ± 0.001	0.458 ± 0.008	0.992 ± 0.016	1.265 ± 0.015
whiskey + H_2O_2	0.107 ± 0.000	0.121 ± 0.002	0.176 ± 0.005	0.265 ± 0.006
ethanol + H_2O_2	0.106 ± 0.000	0.113 ± 0.002	0.148 ± 0.004	0.201 ± 0.008
H_2O_2	0.105 ± 0.001	0.111 ± 0.001	0.141 ± 0.003	0.184 ± 0.003

[a] The *E. coli* cell growth was measured after H_2O_2 oxidation and the 20-fold dilution by LB solution as described under Materials and Methods. Data are mean ± SD values from four experiments. $p < 0.01$ by Student's *t* test for the values between whiskey + H_2O_2 and ethanol + H_2O_2. $p < 0.05$ by Student's *t* test for values between whiskey + H_2O_2 and only H_2O_2 except the values at 0 h.

and that the potentiation of the $GABA_A$ receptor response by whiskey itself increased with the aging period, possibly because of the increase in fragrant compounds in the whiskey.

During whiskey aging, colorless distillates turn to amber ones, suggesting the movement of pigments from the oak barrels into the whiskey (*1*−*3*). Many antioxidants such as phenolic constituents or furans are reported to be present in whiskey (*9*); therefore, the antioxidative effects of whiskey and its components were evaluated by DPPH radical scavenging activity. We examined the DPPH radical scavenging activity of whiskey after aging for various time periods. As expected, the radical scavenging activity increased with the whiskey aging period with very high correlation factors (0.960−0.995). Since both the potentiation of the $GABA_A$ receptor response (*5*) and the DPPH radical scavenging activity of the whiskey showed very high correlation with the whiskey aging period, we examined the correlation between the potentiation of the $GABA_A$ receptor response reported in a previous paper and the DPPH radical scavenging activity of the whiskey. We also measured the DPPH radical scavenging activity in various types of whiskey. The DPPH radical scavenging activity of the whiskey showed very high correlation with the potentiation of the $GABA_A$ receptor response reported in the previous paper (*5*), suggesting that compounds which potentiated the $GABA_A$ receptor response or caused the DPPH radical scavenging activity of the whiskey were similarly produced during aging in oak barrels. However, since different types of whiskey must be stored under different storage conditions, such as cask-type, oak-type, or pre-cask-type, etc., their radical scavenging activities in **Figure 3** must not show their aging period proportionally. The scavenging activity of the blended whiskey was less than that of single-malt whiskey (**Figures 2** and **3**), possibly because blended whiskey was prepared by mixing single-malt whiskey with grain whiskey which had low radical scavenging activity.

To find out which components contributed to the antioxidative activity of the whiskey, we examined the DPPH radical scavenging activities of various components of whiskey. Though most fragrant components in whiskey potentiated the $GABA_A$ receptor response, these components did not display antioxidative activity, with the exception of Quercus lactone b. As previously reported (*9*), polyphenol derivatives showed high DPPH radical scavenging activity. Though we have not examined them in this study, lyoniresinol (*14*) or sulfur-containing compounds (*15*) may also show high DPPH radical scavenging activity. Both the potentiation of the $GABA_A$ receptor response and the DPPH radical scavenging activity of the whiskey increased with the aging period of whiskey, but were due to different components. This estimation was confirmed by the extraction of whiskey by pentane. The aqueous part of the whiskey extraction was colored and showed high radical scavenging activity. However, the pentane part of the extraction caused the potentiation of the $GABA_A$ receptor response (*4*), but showed no radical scavenging activity.

The effect of whiskey on the H_2O_2-induced death of *E. coli* cells was also measured to determine the antioxidative activity of the whiskey. As expected, the whiskey showed significantly more antioxidative effect than the ethanol. However, the protective effect of whiskey at this concentration (1% v/v) was not large enough to show dependence of the whiskey protective effect on its aging period. We tried to increase the concentration of the whiskey in the incubation mixture 10 times, but cell toxicity of the ethanol in the whiskey prevented it. So it is necessary to condense the whiskey by removing water and ethanol to examine the dose−activity relationship of radical scavenging by whiskey (*16*). However, condensed whiskey itself may induce some cell toxicity at high concentrations as Chinese medicinal liquor did (*16*).

Whiskey and wine often mature in wooden barrels for long periods of time, and aged ones are usually highly valued. This process may be performed because fragrant and antioxidative compounds in the wooden barrels are transferred to liquors and increase both the potentiation of the $GABA_A$ receptor response and the DPPH radical scavenging activity in liquors. The condensation of these components may also occur during aging because small molecules such as water and ethanol go through the oak barrel and the liquor volume decreases with storage period.

Many researchers have been studying antioxidants such as polyphenols in liquors and their effect on diseases such as coronary heart disease (*8*). The consumption of such liquors increased the total plasma phenol content and antioxidative capacity (*17*). The antioxidative compounds in liquors may offer protection against cancer, atherosclerosis, and inflammatory diseases. We do not recommend drinking whiskey or wine as a method of antioxidant intake, since other beverages such as green tea and oolong tea also include many antioxidants such as catechin derivatives. However, you can think of the antioxidative activity of whiskey or wine if you often drink liquors. More epidemiological studies are also necessary to clarify whether the antioxidants in liquors are related favorably to human mortality.

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Received for review February 3, 2004. Revised manuscript received May 20, 2004. Accepted May 23, 2004.

JF049817S