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Isolation and Identification of DPPH Radical Scavenging Compounds in Kurosu (Japanese Unpolished Rice Vinegar)

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Dihydroferulic acid (DFA) and dihydrosinapic acid (DSA) were isolated from Kurosu (unpolished rice vinegar) as the major constituents responsible for Kurosu's radical scavenging activity. The levels of antioxidative activity of DFA and DSA in DPPH radical scavenging were higher than those of their respective structurally related compounds, ferulic acid and sinapic acid. The concentrations of DFA and DSA were low in common rice vinegar (polished rice vinegar), suggesting that Kurosu is more advantageous than rice vinegars as an antioxidative food item. As the concentrations of DFA and DSA were low in unpolished rice, too, these acids are thought to be produced in Kurosu through the process of the fermentation from ferulic acid and sinapic acid, respectively.

KEYWORDS: Antioxidative activity; Kurosu; vinegar; dihydroferulic acid; dihydrosinapic acid

INTRODUCTION

Vinegar, which can be made from rice, apples, wine, and various other materials, is a widely used acidic seasoning (1). Vinegar also has medicinal uses by virtue of its physiological effects, such as aiding digestion, stimulating the appetite, and promoting recovery from exhaustion (2). Kurosu, which is produced from unpolished rice containing rice bran through static surface acetic acid fermentation, is one of the most common traditional vinegars in Japan and is characterized by higher concentrations of amino acids and organic acids than other vinegars. This type of vinegar has been shown to improve blood fluidity and to prevent hypertension (3).

Recently, Kurosu was found to exhibit higher levels of antioxidative activity than rice vinegar, grain vinegar, and wine vinegar in both a DPPH radical scavenging system and a linoleic acid autoxidation system (4). EtOAc extract of Kurosu was also reported to be effective in preventing skin carcinogenesis in mice (4). We are interested in Kurosu's antioxidative activity, and in this study we isolate and identify the DPPH radical scavenging compounds contained in Kurosu. On the basis of those scavenging compounds, the physiological characteristics of Kurosu are also described.

MATERIALS AND METHODS

General. The following analytical instruments were used: HPLC, Hitachi D-7000; UV, Hitachi U-2001; ¹H NMR and ¹³C NMR, Bruker

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ARX-400; MS, Hitachi M-80B. The following chromatographic materials were used: DEAE-Toyoparl 650M from Tosoh Co., Ltd. (Tokyo, Japan); Amberlite XAD-4 from Organo Co., Ltd. (Tokyo, Japan); Wako gel C-300 from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan); Divergan F from BASF Japan Co., Ltd. (Tokyo, Japan); Develosil C30-UG-5 (4.6 mm i.d. × 250 mm) from Nomura Chemical Co., Ltd. (Aichi, Japan); and Cosmosil 5C18-AR-II (4.6 mm i.d. × 150 mm) from Nacalai Tesque Co., Ltd. (Kyoto, Japan).

Vinegar Samples and Materials. Kurosu and rice vinegar were obtained from Tamanoi Vinegar Co., Ltd. (Nara, Japan). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Nacalai Tesque Co., Ltd. (Kyoto, Japan).

Measurement of DPPH Radical Scavenging Activity. The DPPH radical scavenging activity was evaluated as reported previously (5).

Isolation and Identification of Active Constituents. Kurosu (50 L) was loaded onto a XAD-4 column equilibrated with distilled water. The adsorbed fraction was eluted with MeOH and concentrated under reduced pressure at room temperature. The concentrate was partitioned between EtOAc and water.

The EtOAc-soluble part (20.5 g) was loaded onto a DEAE-Toyoparl equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The nonadsorbed fraction showed radical scavenging activity. The active fraction was extracted with EtOAc and concentrated under reduced pressure at room temperature. The EtOAc extract (9.76 g) was chromatographed on silica gel (Wako gel C-300) with a hexanes–EtOAc gradient. In this step, the EtOAc extract was divided into two active fractions (A and B). Fraction A (899.6 mg) was chromatographed on silica gel (Wako gel C-300) with CHCl₃–MeOH (9:1). The obtained active fraction (110.6 mg) was applied to preparative TLC (CHCl₃–MeOH, 9:1; *R_f*, 0.76) to give compound I (33.0 mg). ¹H NMR (400 MHz, CDCl₃): 2.65 (2H, m), 2.89 (2H, m), 3.82 (3H, s), 6.69 (1H, d, *J* = 1.89 Hz), 6.71 (1H, s), 6.84 (1H, dd, *J* = 6.75, 1.35 Hz). ¹³C NMR (400 MHz, CDCl₃): 30.4, 35.9, 55.9, 111.0, 114.6, 120.9, 132.1,

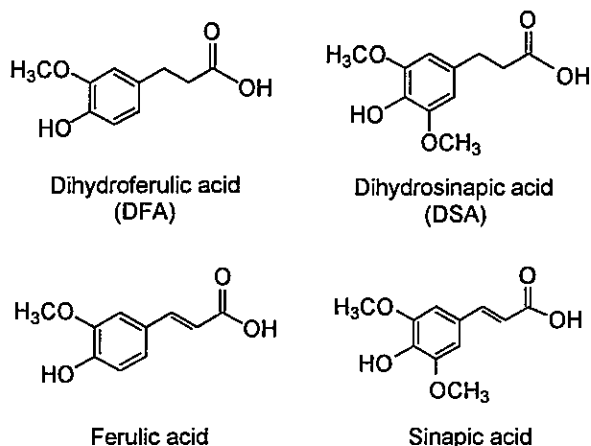


Figure 1. Structures of DFA, DSA, and their related compounds in Kurosu.

144.2, 146.5, 178.4. LC-MS m/z : 196.3 [M]⁺. The final structure of compound I was confirmed by HMBC and HMQC (data not shown).

Fraction B (348.3 mg) was chromatographed on silica gel (Wako gel C-300) with hexanes-EtOAc (5:5). The obtained active fraction (150 mg) was applied to preparative TLC (hexanes-EtOAc, 4:6; R_f , 0.27) to give the isolated compound II (14.3 mg). ¹H NMR (400 MHz; CDCl₃): 2.67 (2H, t, J = 8.6 Hz) 2.89 (2H, t, J = 8.0 Hz), 3.87 (6H, s), 6.43 (2H, s). ¹³C NMR (400 MHz, CDCl₃): 30.9, 35.9, 56.3, 105.4, 131.3, 133.4, 147.9, 178.0. LC-MS m/z : 226.4 [M]⁺. The final structure of compound II was also confirmed by HMBC and HMQC (data not shown).

Quantitative Analysis of Antioxidative Compounds in Kurosu and Rice Vinegar. The quantities of dihydroferulic acid (DFA) and dihydrosinapic acid (DSA) were measured as follows. Kurosu was extracted with CHCl₃. The CHCl₃ layer was analyzed by an HPLC equipped with the diode array detector L-7450H on Develosil C30-UG-5 (flow rate, 1 mL/min; mobile phase, CH₃CN/H₂O/CH₃COOH, 20:80:0.1; retention time, 10.86 min, 10.30 min).

The quantities of ferulic acid, sinapic acid, vanillic acid, and *p*-hydroxycinnamic acid were measured by the following method. Kurosu was adsorbed on a Divergan F column, and the adsorbed fraction was eluted with 4 N NaOH. The eluted fraction was adjusted to pH 2.5 with HCl and extracted with EtOAc. The EtOAc layer was analyzed by an HPLC equipped with the diode array detector L-7450H on a Cosmosil 5C18-AR-II (flow rate, 1 mL/min; mobile phase, CH₃CN/H₂O/CH₃COOH, 5:95:0.1 ~ 75:25:0.1; gradient for 30 min; retention times, 11.14, 10.91, 8.43, and 10.46 min).

Comparison of Antioxidative Activity between Kurosu and Rice Vinegar. Concentrates of Kurosu and rice vinegar were obtained by evaporation under reduced pressure at 50 °C. Their antioxidative activities in DPPH radical scavenging were measured using the method described above.

Quantitative Analysis of DFA and Ferulic Acid in Unpolished Rice. The quantities of DFA and ferulic acid were measured as follows. NaOH (500 mL, 0.5 N) was added to 10 g of unpolished rice, which were then stirred well and kept 90 min at 60 °C. The liquid was adjusted to pH 2.5 with HCl and extracted with CHCl₃. The CHCl₃ layer was analyzed by HPLC as described above.

RESULTS AND DISCUSSION

Antioxidative Compounds Derived from Kurosu. Two active fractions (A and B) were obtained from the Kurosu by the procedure described above. DPPH radical scavenging compounds I and II were isolated from fractions A and B, respectively, and they were identified respectively as dihydroferulic acid (3-(4-hydroxy-3-methoxyphenyl) propanoic acid) (DFA) and dihydrosinapic acid (3-(4-hydroxy-3, 5-dimethoxyphenyl) propanoic acid) (DSA) by their spectral data, as shown in Figure 1.

Table 1. Content of Antioxidative Compounds in Kurosu and Rice Vinegar and Their IC₅₀ in DPPH Radical Scavenging Activity

	content (mg/L) in Kurosu	content (mg/L) in rice vinegar	IC ₅₀ (μM)	[IC ₅₀ (μg/mL)]
concentrate of Kurosu	29400			[1710]
concentrate of rice vinegar		23800		[3340]
dihydroferulic acid	24.8	0.09	77.0	[15.1]
dihydrosinapic acid	4.68	n.d. ^a	44.3	[10.0]
ferulic acid	0.95	0.03	113.9	[22.1]
sinapic acid	1.15	n.d.	77.2	[17.3]
vanillic acid	1.44	n.d.	250.0	[42.0]
<i>p</i> -hydroxycinnamic acid	0.17	n.d.	2130	[350]

^a n.d. is not detected.

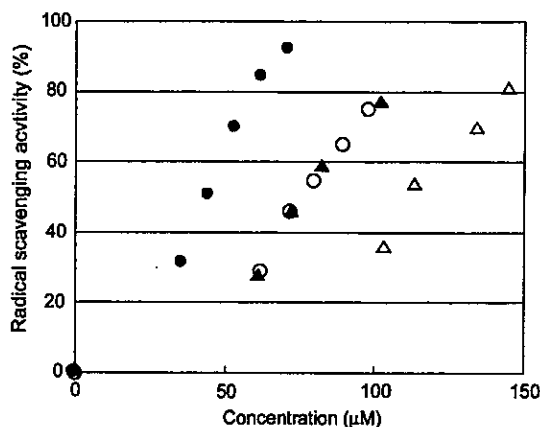


Figure 2. DPPH radical scavenging activities of DSA, DFA, ferulic acid, and sinapic acid: ●, DSA; ▲, DFA; ○, sinapic acid; △, ferulic acid.

Quantitative analysis showed that 1 L of Kurosu contained 24.8 mg of DFA and 4.68 mg of DSA (Table 1). Although DFA and DSA have been found in the nonvolatile acidic part of dent corn silage (6), this is the first finding that they occur in Kurosu. DFA and DSA are the homologues of ferulic acid and sinapic acid, which are known as natural antioxidants occurring in rice, wheat, unpolished rice, and other grains (7).

The antioxidative activities of DFA and DSA were evaluated by IC₅₀ in DPPH radical scavenging activity. As shown in Figure 2, the DPPH radical scavenging activities of DFA (IC₅₀: 77.0 μM) and DSA (IC₅₀: 44.3 μM) were stronger than those of ferulic acid (IC₅₀: 113.9 μM) and sinapic acid (IC₅₀: 77.2 μM), respectively. Previous studies on the antioxidative mechanism for BHT (2,6-di-*tert*-butyl-4-methylphenol) have indicated that BHT can be converted to the corresponding quinone methide by two-electron oxidation because it is possible for an aryl proton at the 4-methyl group to be subtracted by free radicals (8). We, therefore, speculated that "side-chain-saturated" DFA and DSA would show more potent radical scavenging activity than ferulic acid and sinapic acid, respectively, according to their capacity to donate protons.

Comparison of Antioxidative Activity between Kurosu and Rice Vinegar. It has been reported that the EtOAc extract from Kurosu exhibits stronger antioxidative activity against lipid autoxidation than do the EtOAc extracts of other types of vinegar (4). The total phenolic content in the EtOAc extract of Kurosu is greater than it is in the EtOAc extracts of other types of vinegar, including rice vinegar (4). In DPPH radical scavenging activity, concentrate of Kurosu is 1.95 times as strong as that of rice vinegar (Table 1). Therefore, we compared the contents of six major phenolic compounds (DFA, DSA, ferulic acid, sinapic acid, vanillic acid, and *p*-hydroxycinnamic acid) between Kurosu and rice vinegar, and found that all six of the compounds

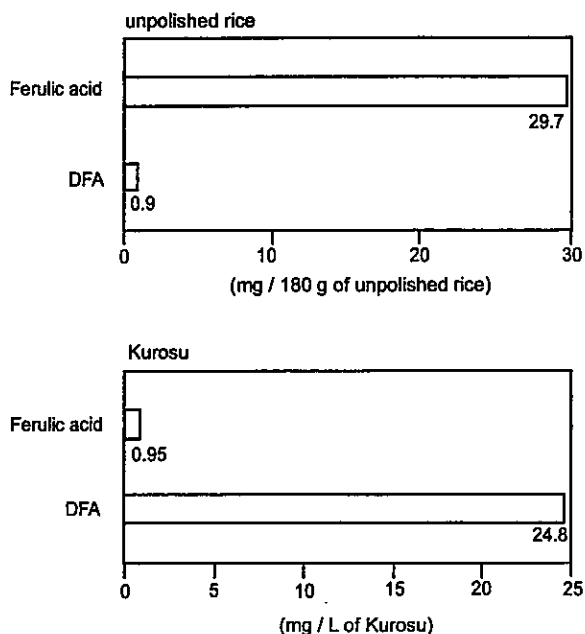


Figure 3. Contents of ferulic acid and DFA in Kurosu and unpolished rice. 180 g of unpolished rice was used to get 1 L of Kurosu. The quantities of these compounds were measured by HPLC.

were more abundant in the former than in the latter (Table 1). This may reflect that Kurosu is produced from unpolished rice containing rice bran. Also, this result strongly suggested that phenolic compounds such as DFA and DSA, occurring specifically in Kurosu, play a major role in Kurosu's strong antioxidative activity.

The Process of DFA Generation. As ferulic acid exists mainly in the cell walls of plants, rice bran, in which a large percentage of the overall mass comes from the cell walls (approximately 25% (9)), contains a significant amount of ferulic acid (7). Though Kurosu is made from unpolished rice containing rice bran, the quantitative analysis proved that it contains much less ferulic acid than DFA (Table 1). Thus we hypothesized that ferulic acid may be changed into DFA through the process of the fermentation. It is known that baker's yeast reduces conjugated C=C double bonds (10, 11). Additionally, it has been suggested that DFA is formed in the human via the reduction of ferulic acid (12). To verify our hypothesis, we quantified the DFA contained in unpolished rice, the starting material of Kurosu, by HPLC. We detected a small amount of a compound whose retention time in HPLC was the same as that of DFA. Because the content of this compound, calculated in terms of the DFA equivalent, was 0.05 mg/10 g of unpolished rice, its structure could not be identified. As shown in Figure 3, 29.7 mg of ferulic acid and 0.9 mg of DFA were contained in 180 g of unpolished rice (equivalent to 1 L of Kurosu), whereas Kurosu contains much more DFA than ferulic acid. From these results, it is possible to judge that ferulic acid should be reduced to DFA through the process of the fermentation. Fermentation may play an essential role in increasing DPPH radical scavenging activity. Further mechanistic study on the reduction of ferulic acid during fermentation is currently under way.

Conclusions. The reason Kurosu exhibits stronger DPPH radical scavenging activity than other vinegars is that it contains

higher concentrations of phenolic compounds than the other vinegars. DFA and DSA were isolated from Kurosu for the first time and identified as the major DPPH radical scavenging compounds of Kurosu. We previously reported that the EtOAc extract of Kurosu inhibited TPA-induced inflammatory responses and oxidative stress in mouse skin (4). The EtOAc extract of Kurosu also contains phenolic compounds such as DFA and DSA. This inhibitory effect may be partly explained by the free radical scavenging activity, and hence DFA and DSA may contribute to antitumor activity. Further investigation is necessary to clarify how the antioxidative effects of phenolic compounds such as DFA and DSA act on *in vivo* antitumor activity.

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