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Caffeoylsophorose in a Red Vinegar Produced through Fermentation with Purple Sweetpotato

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Recently, a new red vinegar has been developed via fermentation with the storage root of purple-fleshed sweetpotato, *Ipomoea batatas* L. cv. Ayamurasaki. The red vinegar had a higher antioxidative activity than white or black vinegars. The red vinegar contained some new components possibly derived from the original purple sweetpotato. A major component was isolated using preparative HPLC, and the chemical structure was determined to be 6-*O*-(*E*)-caffeoyl-(2-*O*-β-D-glucopyranosyl)-α-D-glucopyranose (caffeoylsophorose) by MS and NMR. Because the caffeoylsophorose showed a high antioxidative activity, it plays an important functional role in red vinegar as do anthocyanins and other components. Examination of the mechanism of formation is now in progress.

KEYWORDS: Red vinegar; purple-fleshed sweetpotato; polyphenolics; acylated anthocyanin; 6-*O*-(*E*)-caffeoyl-(2-*O*-β-D-glucopyranosyl)-α-D-glucopyranose; radical scavenging activity

INTRODUCTION

Vinegars are widely used for seasonings or drinks all over the world and are made from apple, rice, sweetpotato, and so on. They are also considered to be components of a healthy diet with beneficial effects for the maintenance of health and prevention of diseases. For example, a black vinegar made from rice by a long brewing time is traditionally manufactured in Kagoshima prefecture, Japan. It is known to have action in improving blood fluidity (*1*), etc. Generally, white vinegars are well-known to have various physiological functions.

Recently, a new red vinegar has been developed (*2*), produced through acetic fermentation with the steamed and crushed storage roots of the purple-fleshed sweetpotato cultivar Ayamuraski (*3*). As a raw material, the red vinegar has been applied as a healthy drink and a red dressing. It was considered that in addition to the components of common white vinegar, the red vinegar also contained those of the sweetpotato and unknown fermented products. Indeed, along with anthocyanins and polyphenolics such as caffeoylquinic acids (*4*), characteristic components of sweetpotato, new UV-absorbing constituents have been detected in the vinegar (*2*), which are probably derived from original sweetpotato substances during fermentation. The purple-fleshed sweetpotato anthocyanins are acylated with one or two acyl groups such as *p*-hydroxybenzoic, caffeic, and ferulic acids (*5*−*8*). Therefore, the sweetpotato anthocyanins have not only high stability (*8*) but also multifaceted actions including antioxidative activity (*2*, *8*−*10*), antimutagenicity (*11*, *12*), hepatoprotective properties (*13*), and antidiabetes activity (*14*, *15*). Caffeoylquinic acid derivatives are also known to exhibit antioxidative activity (*16*, *17*), antihepatotoxic effects (*18*), invasion of hepatoma cells (*19*), and inhibition of HIV (*20*). Moreover, newly generated UV-absorbing compounds are likely to have similar functional roles. Thus, the red vinegar is expected to have additive or synergistic physiological effects of these functional components. This study investigated the structure of the major UV-absorbing compound in the red vinegar and its 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

MATERIALS AND METHODS

General Procedures. All reagents and solvents employed were of analytical grade and used without further purification. Thin-layer chromatography (TLC) was performed on microcrystalline cellulose plates (Funakoshi Co., Ltd.). High-performance liquid chromatography (HPLC) was run on an L-6200 intelligent pump system (Hitachi Co., Ltd.). Analytical HPLC was with linear gradient elution for 50 min from 20 to 45% solvent B (0.4% formic acid, 50% acetonitrile in H_2O) in solvent A (0.4% formic acid in H_2O) on a 250 mm × 4.6 mm i.d. Inertsil ODS-3 column (GL Sciences Inc.) at 30 °C with a flow rate of 1.0 mL/min and monitoring at 310 or 280 nm with an MD-1510 multiwavelength detector (Jasco Co., Ltd.). Preparative HPLC was run with isocratic elution of solvent A (15% acetic acid in H_2O)/solvent B (15% acetic acid, 30% acetonitrile in H_2O) (90:10) on a 250 mm × 20 mm i.d. Inertsil ODS column (GL Sciences Inc.) at room temperature

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with a flow rate of 7.0 mL/min and monitoring at 310 nm with an L-4200 UV−vis detector and D-2000 integrator (Hitachi). UV−vis spectra were recorded on a V-550 spectrophotometer (Jasco Co.) for structural determination and for colorimetric measurements of antioxidative activity assay. Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) analysis was performed on a Mariner Workstation system (Applied Biosystems Co., Ltd.) in 1% acetic acid in 50% aqueous acetonitrile. The high-resolution mass spectrum was measured using angiotensin I and neurotensin as internal standards. ^{13}C (100 MHz)- and ^{1}H (400 MHz) nuclear magnetic resonance (NMR) spectra were run on an Alpha-400 instrument (JEOL Co., Ltd.) in DMSO-d_6/CF_3COOD (9:1) with tetramethylsilane as an internal standard.

Production of Red Vinegar. Red vinegar was produced according to the following process. Ethanol (200 L) was mixed with water (2600 L) and seed vinegar (800 L) and the steamed and mashed storage root of purple-fleshed sweetpotato cv. Ayamurasaki (400 kg). The mixture was fermented for 3 months with acetic bacteria in the seed vinegar, filtered, and then sterilized by heating and stored. The red vinegar was a vivid red color and had no unpleasant flavor.

Isolation of Compound 1. Red vinegar (3 L) was evaporated and freeze-dried under reduced pressure, and the powder (4.9 g) was dissolved in methanol and centrifuged. The precipitate was dried to obtain a reddish brown powder (fraction 1: 1.5 g, mixture), and the supernatant was precipitated with excess diethyl ether; the precipitate was centrifuged and dried. The red powder was separated into three fractions, 2−4, by preparative HPLC. Fraction 2 contained several UV-absorbing substances and a part of anthocyanins, fraction 3 contained compound **1** in a pure form, and fraction 4 mainly contained anthocyanins from the red vinegar. Each fraction was dried and redissolved in the smallest amount of methanol and then precipitated with excess diethyl ether. A reddish powder (0.4 g, mixture) was obtained from fraction 2 and a red powder (1.34 g, mixture) from fraction 4. From fraction 3, compound **1** was obtained in almost pure form as a slightly reddish powder (0.11 g, 2.2% yield from the red vinegar crude powder).

Chemical Analysis of 1. Acid and alkaline hydrolyses of isolated **1** were performed according to a previous paper (*21*). The acid hydrolysis was done in 2 N HCl in boiling water for 45 min. The alkaline hydrolysis was done in 2 N NaOH for 15 min with a sealed cap and acidified with acetic acid. The components in the reaction mixture were identified by TLC for sugars and by analytical HPLC for acids.

Radical Scavenging Activity Assay. The radical scavenging activity of each sample was tested according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) HPLC method developed by Yamaguchi et al. (*22*) with some modifications. (−)-Epigallocatechin 3-*O*-gallate (Wako Pure Chemicals), 5-caffeoylquinic acid (chlorogenic acid) (ICN Biochemicals Inc.), 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Wako Pure Chemicals), and α-tocopherol (Kishida Chemicals) were used as authentic antioxidants. Tris-HCl buffer (0.1 M, pH 7.4) was prepared by dissolving 2-amino-2-hydroxymethyl-1,3-propanediol (12.11 g) in ∼500 mL of distilled water, adjusting to pH 7.4 with 2 N HCl, and diluting to 1 L with distilled water. DPPH (Wako Pure Chemicals) (10 mg) was dissolved in 50 mL of ethanol by stirring to prepare a 500 μM DPPH−ethanol solution. HPLC was run with isocratic elution in 50% acetonitrile in H_2O on a 75 mm × 4.6 mm i.d. Wakosil ODS column (Wako) at room temperature with a flow rate of 1.0 mL/min, monitoring at 520 nm with an L-4200 UV−vis detector (Hitachi). Vinegar samples (pH ∼3) were adjusted to pH 7.4 with 1 N NaOH solution and diluted 2-fold with water. The powders from fractions 1−4 were dissolved in Tris-HCl buffer to 1 mg/mL concentration, and pure substrates such as compound **1** and other authentic antioxidants were prepared to 1 mM concentration. Fifty microliters of each vinegar solution was added to 150 μL of ethanol and 300 μL of Tris-HCl buffer, 25 μL of each solution of fractions 1−4 was added to 150 μL of ethanol and 325 μL of Tris-HCl buffer, or 25 μL of each pure substance solution was added to 125 μL of ethanol and 350 μL of Tris-HCl buffer, respectively. To each mixture was added 500 μL of DPPH−ethanol solution, and it was immediately shaken and then kept standing for 20 min in the dark at room temperature. The control for this experiment employed only distilled water (25 μL) instead of sample solution. An aliquot (15 μL) of the reaction mixture was analyzed four times by HPLC. Under the HPLC conditions, the DPPH peak appeared at a retention time of ∼10 min. Radical scavenging ability (RS%) was calculated as RS% = 100-($A_c - A_s$)/A_c, where A_s and A_c are the peak areas of residual DPPH of sample and control results, respectively.

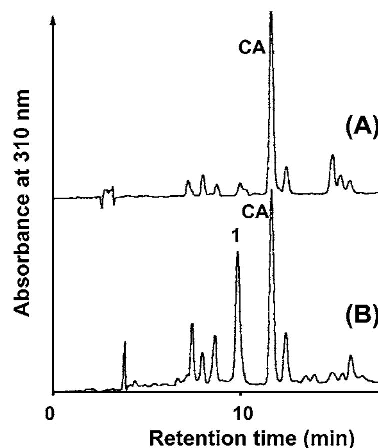

Figure 1. Typical HPLC profiles of the sweetpotato crude pigment (A) and the red vinegar (B). Analytical HPLC was carried out with linear gradient elution for 50 min from 20 to 45% solvent B (0.4% formic acid, 50% acetonitrile in H_2O) in solvent A (0.4% formic acid in H_2O) on a 250 mm × 4.6 mm i.d. Inertsil ODS-3 column at 30 °C with a flow rate of 1.0 mL/min and monitoring at 310 nm. CA = chlorogenic acid.

RESULTS AND DISCUSSION

To confirm the change of components from purple-fleshed sweetpotato during the red vinegar production process and/or preserving, we conducted an HPLC analysis of the red vinegar at 310 nm and compared the chromatogram with that of the sweetpotato crude pigment. The red vinegar contained some newly generated UV-absorbing compounds, which were probably derived from the original purple-fleshed sweetpotato components (**Figure 1**). Other than anthocyanins, two major UV-absorbing components were detected as shown in **Figure 1B**, one with a retention time at 9.9 min (peak area ∼11%) and the other at 11.7 min (peak area ∼13%). The former was an unknown compound **1**, which was contained only in the red vinegar. The latter was contained both in the red vinegar and in the sweetpotato crude pigment and was identified as chlorogenic acid by its UV spectroscopic pattern (λ_{max} = 330 nm) on a multiwavelength detection and HPLC coelution test with an authentic chlorogenic acid. Anthocyanins and chlorogenic acid contents in the red vinegar were relatively lower, but the composition ratios were almost the same, indicating that they underwent some degradation during acetic fermentation and sterilization by heating or during storage. We tried to isolate the major UV-absorbing compound **1** for structural analysis. Freeze-dried red vinegar powder was fractionated with methanol by centrifugation. The supernatant was precipitated with excess diethyl ether, and the red powder was separated into three fractions (2−4) by preparative HPLC. From fraction 3 was obtained compound **1** as an almost pure powder in 2.2% yield.

Compound **1** gave glucose on acid hydrolysis and gave caffeic acid on alkaline hydrolysis (data not shown). The presence of the caffeoyl residue in **1** was also supported by characteristic UV absorption (in 0.01% HCl−MeOH at λ_{max} = 330 nm, ϵ = 18900), which agreed closely with caffeic acid (λ_{max} = 328 nm, ϵ = 18800) and chlorogenic acid (λ_{max} = 330 nm, ϵ = 18900). Therefore, **1** was suspected to be a caffeic acid−glucose derivative. On electrospray ionization time-of-flight mass

Table 1. ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR Chemical Shifts of Compound **1** in DMSO-d_6 and CF_3COOD (9:1, v/v) with Tetramethylsilane as Internal Standard

	δ_C[a]	δ_H[a]
caffeoyl moiety		
1	125.9	
2	114.3	7.10 (1H, br s)
3	145.8	
4	148.6	
5	116.1	6.81 (1H, d, J = 8.1 Hz)
6	121.7	7.04 (1H, br d, J = 8.1 Hz)
α	115.2	6.30 (1H, d, J = 15.6 Hz)
β	145.5	7.57 (1H, d, J = 15.6 Hz)
C=O	166.9	
glucose a		
1	91.9	5.23 (1H, d, J = 3.3 Hz)
2	82.3	3.30 (1H, dd, J = 3.3, 9.3 Hz)
3	71.9	3.72 (1H, t, J = 9.3 Hz)
4	69.5	3.24 (1H, t, J = 9.3 Hz)
5	65.2	3.90 (1H, m)
6a	64.1	4.21 (1H, dd, J = 5.9, 11.2 Hz)
6b	64.1	4.41 (1H, br d, J = 11.2 Hz)
glucose b		
1	105.5	4.35 (1H, d, J = 7.3 Hz)
2	74.2	3.08 (1H, br t, J = 8.8 Hz)
3	76.6	3.21 (1H, t, J = 8.8 Hz)
4	70.4	3.07 (1H, t, J = 8.8 Hz)
5	77.2	3.16 (1H, m)
6a	61.5	3.47 (1H, dd, J = 5.9, 11.0 Hz)
6b	61.5	3.71 (1H, br d, J = 11.0 Hz)

[a] δ_C and δ_H: ^{13}C and ^{1}H NMR chemical shifts, respectively.

spectrometry (ESI-TOFMS) measurement, **1** gave a high-resolution mass m/z of 527.14777 [M + Na = $C_{21}H_{28}O_{14}$ + Na]$^+$ corresponding to the Na^+ adduct peak of the molecule. All of the above data suggest that compound **1** is constructed of caffeic acid and glucose in a 1:2 ratio.

^{13}C and ^{1}H NMR analyses, including homonuclear double quantum filtered correlation spectroscropy (DQF-COSY), homonuclear nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single quantum coherence spectroscopy (HSQC), and heteronuclear multiple-bond correlation spectroscopy (HMBC) pulse techniques, determined the complete structure, and the assignment data are summarized in **Table 1**. ^{1}H NMR spectra showed the presence of an (E)-caffeoyl group based on the 1,2,4-trisubsututited benzene and the (E)-olefinic α- and β-proton signals with sugars to have a D-glucopyranosyl configuration due to the large J values (J = 8.8−9.3 Hz) of the ring protons. Anomeric protons of glucose a (G_a) and glucose b (G_b) were oriented in α- and β-configurations, respectively, because anomeric protons of G_a and G_b had small (J = 3.3 Hz) and large (J = 7.3 Hz) coupling constant values, respectively. Because G_b was linked on the 2-hydroxyl of G_a (G_a-2OH) as determined from the glycosylation shifts of δ_{Ga-2H} 3.30 and δ_{Ga-2C} 82.3 (**Table 1**), the interglycosidic linkage was β-G_b-(1→2)-G_a, confirming this sugar moiety to be sophorose. The connectivity was also supported by observation of the NOESY cross-peak between G_b-1H and G_a-2H as shown in **Figure 2**. The deshielding shifts of G_a-6Hs (δ_H 4.21 and 4.41) and G_a-6C (δ_C 64.1) suggested that the caffeoyl group was attached to G_a-6OH. Therefore, compound **1** was elucidated as having a 6-caffeoylsophorose structure. ^{13}C NMR data of **1** also supported this elucidation (**Table 1**). The connecting position of an acyl residue in **1** was confirmed by HMBC experiments. In the HMBC spectrum of **1**, the correlation was observed between G_a-6Hs and a carbonyl carbon signal (δ_C 166.9) as shown in **Figure 2**. On the basis of all the above analytical data,

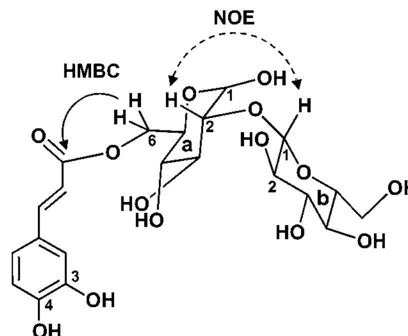

Figure 2. Structure of compound **1** in red vinegar.

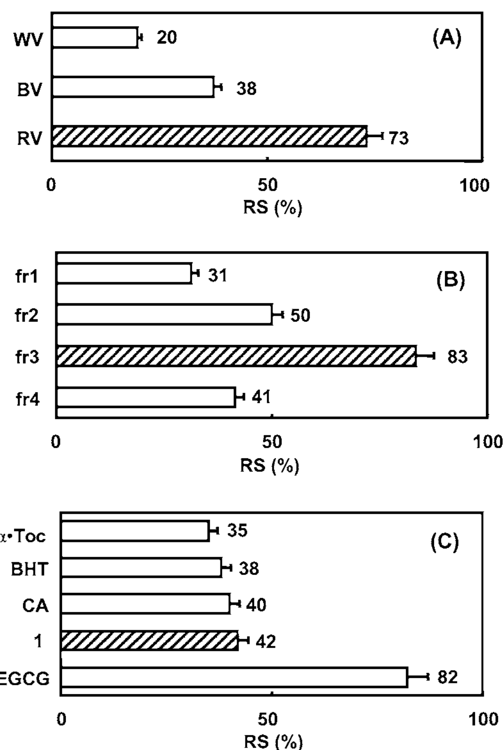

Figure 3. DPPH radical scavenging activity (RS%): (A) RV, BV, and WV = red, black, and white vinegars, respectively; (B) fr1–4 = powders from fractions 1–4 of red vinegar, respectively; (C) compound **1** and other authentic antioxidants [α-Toc, BHT, CA, EGCG = α-tocopherol, 2,6-di-*tert*-butyl-4-methylphenol, chlorogenic acid, and (–)-epigallocatechin 3-*O*-gallate, respectively]. Each value is the mean (RS%) ± standard deviation (n = 4).

compound **1** was unambiguously determined as 6-*O*-(*E*)-caffeoyl-(2-*O*-β-D-glucopyranosyl)-α-D-glucopyranose (**Figure 2**).

In general, caffeic acid esters with sugars or quinic acid occurred widely in many plants (*23*, *24*). Indeed, the storage root of the purple-fleshed sweetpotato cv. Ayamurasaki contains caffeoyl anthocyanins and caffeoylquinic acids such as chlorogenic acid. Caffeic acid derivatives are well-known to have potent antioxidant properties because the catechol structure donates the phenolic hydrogens or electrons to acceptors such as reactive oxygen species or lipid peroxyl radicals (*25*, *26*). Hence, we evaluated the antioxidative activity of the red vinegar by the DPPH−HPLC method to check the radical scavenging activity (22). The red vinegar showed a significantly higher activity than did a white vinegar and a black vinegar (**Figure 3A**). Similary, on the inhibition test of lipid peroxidation by β-carotene bleaching method (*21*, *27*, *28*), the red vinegar also demonstrated the highest activity (data not shown). DPPH

radical scavenging activities of powders from fractions 1−4 were also measured, and the results are shown in **Figure 3B**. The powder (compound **1**) from fraction 3 shows a higher activity than those from fractions 1, 2, and 4 of the red vinegar. Moreover, the DPPH radical scavenging activity of compound **1** was examined by comparison with authentic antioxidants at the same molar concentration. As shown in **Figure 3C**, the activity of compound **1** was not higher than that of (−)-epigallocatechin 3-*O*-gallate but was almost the same as that of antioxidants such as chlorogenic acid, BHT, and α-tocopherol. Thus, we conclude that the high antioxidative activity of the red vinegar is attributable to the major components, compound **1**, chlorogenic acid, and possibly anthocyanin polymers, in addition to purple-fleshed sweetpotato anthocyanin monomers.

The structure of compound **1** (6-caffeoylsophorose) suggests that it may be formed via hydrolysis from a purple-fleshed sweetpotato anthocyanin, YGM-2, and a major anthocyanin, YGM-5b, because they have the same 6-*O*-(*E*)-caffeoyl-(2-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranosyl structure in their sugar moieties (*6*). As reported by Tsuda (*29*, *30*), a nonacylated anthocyanin (cyanidin 3-glucoside) was a potent antioxidative food factor, because not only itself but also protocatechuic acid, the oxidative degradation product or in vivo metabolized product of the aglycon moiety, had stronger antioxidative activity. In our case, compound **1**, a hydrolytic degradation product of acylated anthocyanins, had a high antioxidative activity as did the original acylated anthocyanins.

Thus, the red vinegar has been shown to have characteristic components such as antioxidative caffeoylsophorose (**1**) in addition to many functional anthocyanins and chlorogenic acids other than original vinegar components. Probably, the lower molecular size of caffeoylsophorose is easier to absorb than anthocyanins that were found to absorb partially as the intact (glycoside) forms in the human intestine (*30*−*33*). Therefore, intake of the red vinegar as a seasoning or healthy drink in our daily life is expected to be highly beneficial for the maintenance of health and prevention of diseases.

We are presently investigating other functions and the formation mechanism of compound **1** in the red vinegar.

ABBREVIATIONS USED

ESI-TOFMS, electrospray ionization time-of-flight mass spectrometry; DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DQF-COSY, homonuclear double quantum filtered correlation spectroscropy; NOESY, homonuclear nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple-bond correlation spectroscopy.

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