Characterization of Acylated Anthocyanins in Callus Induced From Storage Root of Purple-Fleshed Sweet Potato, *Ipomoea batatas* L

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Four anthocyanins were isolated from a highly pigmented callus induced from the storage root of purple-fleshed sweet potato (*Ipomoea batatas* L) cultivar Ayamurasaki. The anthocyanins were respectively identified as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-caffeoyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside, and peonidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranoside)-5-*O*-*β*-D-glucopyranoside by chemical and spectroscopic analyses. These anthocyanins were examined with respect to the stability in neutral aqueous solution as well as the radical scavenging activity against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. These acylated anthocyanins exhibited both higher stability and higher DPPH radical scavenging activity than corresponding nonacylated cyanidin and peonidin 3-*O*-sophoroside-5-*O*-glucosides.

INTRODUCTION

A purple-fleshed sweet potato, *Ipomoea batatas* L cultivar Ayamurasaki, accumulates high levels of anthocyanin pigments in the storage root [1]. The major anthocyanins are cyanidin and peonidin 3-*O*-sophoroside-5-*O*-glucosides acylated with caffeic, ferulic, or *p*-hydroxybenzoic acids [2, 3, 4]. The sweet potato anthocyanins possess not only moderate stability in neutral aqueous solution [5] and high thermostability [6] but also various health benefits like antioxidative activity [7, 8, 9], antimutagenicity [10, 11], and antidiabetic action [12, 13, 14]. Therefore, the Ayamurasaki pigment is utilized as a high-quality natural food colorant with potentially preventive function against life-style-related diseases.

Recently, from the storage root of the cultivar Ayamurasaki we have established a high-anthocyanin accumulating cell line. In the crude pigment extract, some cell-line-specific anthocyanins have been detected which were different from those in the original storage root [15]. Previously, we have isolated and determined the molecular structures of the two anthocyanins, in which one was a known anthocyanin, cyanidin 3-sophoroside-5-glucoside, and the other was a new one, cyanidin 3-(*p*-coumaroyl) sophoroside-5-glucoside [5]. In sequence, we have isolated four more callus anthocyanins. The purpose of this study is to characterize the structures and the stability in a neutral aqueous solution and the antioxidative activity to utilize the pigment for food or other material.

MATERIALS AND METHODS

Chemicals

All the reagents and solvents employed were of analytical grade and used without further purification. 1,1-diphenyl-2-picrylhydrazyl (DPPH, Wako Pure Chemicals, Japan), *α*-tocopherol (Kishida Chemicals, Japan), and 2,6-di-*tert*-butyl-4-methylphenol (BHT, Wako Pure Chemicals) were used for DPPH radical scavenging assay. Open column chromatographies were carried out on Amberlite XAD-2000 (Rohm and Haas, USA) and ODS (Silica Gel Chromatorex, Fuji Silysia Chemical Ltd, Japan) resins.

HPLC methods

Solvent A (1.5% H_3PO_4 in H_2O) and solvent B (1.5% H_3PO_4, 20% CH_3COOH, 25% MeCN in H_2O) were used for analytical high-performance liquid chromatography (HPLC), and solvent A (15% CH_3COOH in H_2O) and solvent B (15% CH_3COOH, 30% MeCN in H_2O) were used for preparative HPLC. Analytical HPLC was run on LC-10AD intelligent pump (Shimadzu, Japan) with a linear gradient elution of solvent A : B = 75 : 25 to 15 : 85 for 40 minutes on Luna column (4.6 id × 100 mm, Phenomenex, USA) at 35°C with a flow rate of 1.0 mL/min monitoring at 520 nm equipped with SPD-10A diode array detector (Shimadzu). For purity check of anthocyanins, other analytical HPLC was performed on L-6200 intelligent pump system (Hitachi, Japan) with a linear gradient elution of solvent A : B = 75 : 25 to 45 : 55 for 60 minutes on Inertsil ODS-3 column (4.6 id × 250 mm, GL Sciences, Japan) at 30°C with a flow rate of 1.0 mL/min monitoring at 520, 310, or 280 nm equipped with MD-1510 multiwavelength detector (Jasco, Japan). Preparative HPLC was run on L-6200 intelligent pump system with isocratic elutions of solvent A : B = 80 : 20 for **1**, 75 : 25 for **2**, 60 : 40 for **3**, and 50 : 50 for **4** on Inertsil ODS column (20 id × 250 mm, GL Sciences) at room temperature with a flow rate of 7.0 mL/min monitoring at 310 nm with L-4200 UV-Vis detector and D-2000 integrator (Hitachi).

Spectral analyses

UV-Vis spectra were recorded on V-550 spectrophotometer (Jasco) in 0.01% HCl-MeOH for structural determination and for colorimetric measurements of antioxidative activity assay. The bathochromic shift test was carried out by the addition of 5% $AlCl_3$-MeOH. Electrospray ionization time-of-flight mass spectrometry (ESI-TOFMS) spectra were recorded on Mariner Workstation system (Applied Biosystems, USA) in 1% CH_3COOH-50% MeCN with a positive mode by an infusion-injection. High-resolutional electrospray ionization Fourier-transform ion-cyclotron resonance mass spectrometry (ESI/FT-ICRMS) spectra were recorded on APEX II 70e (Bruker Daltonics, Germany) in aqueous MeOH containing CH_3COOH with a positive mode. ^{1}H (800 MHz) and ^{13}C (200 MHz) NMR spectra were measured on Avance 800 spectrometer (Bruker BioSpin, Germany) in DMSO-d_6 : TFA-d_1 = 9 : 1 with tetramethylsilane (TMS) as an internal standard at 30°C.

Plant materials

A previously established high-anthocyanin-accumulating callus culture generated from the storage root of sweet potato cultivar Ayamurasaki has been used for this study [15]. Suspended cell cultures were initiated by transferring about 1.0 g (fresh weight) of callus to 50 mL of liquid medium in 250 mL Erlenmeyer flasks. Basal Murashige and Skoog (MS) medium [16] supplemented with 1.0 mg/L 2,4-D, and 3% sucrose has been used as a maintenance medium (MM). Medium pH has been adjusted to 5.8 before autoclaving. Subcultures have been done in 7-day intervals. The cultures were incubated on a rotary shaker (130 rpm) at 25°C in the dark. For the purpose of present analysis, suspension cultures have been produced under two different medium conditions: MM and a high-anthocyanin producing medium (APM).

The APM was a modified MS medium with 9.4 mM KNO_3, without NH_4NO_3, with 5% sucrose and nil growth regulators [17]. Medium pH has been adjusted to 5.8 before autoclaving and suspension cultures were incubated in 250 mL flasks. Five hundred mg of cell aggregates were placed in flasks containing 50 mL medium. The cultures were harvested after 7 days' growth on MM (200 g) and after 14 days on APM (199 g).

Extraction and isolation of anthocyanins

The aggregates were removed from the culture media, rinsed with distilled water, separated from the liquid by vacuum filtration, and weighed. A total amount of 399 g of fresh tissue was steeped in 15% CH_3COOH (1 L) for one day and filtered. This operation was repeated three more times (1, 1, and 0.5 L). The combined crude extract (3.5 L) contained nineteen or more anthocyanins [18]. The extract solution was applied on two XAD-2000 resin columns (30 id × 385 mm), the columns were washed with water (3 L), eluted stepwise with 10%, 20%, 30%, 40%, 60%, or 70% EtOH all with 1% CH_3COOH (1 L). Pigments containing 30%, 40%, 60%, and 70% EtOH fractions were combined and evaporated to dryness under reduced pressure. Subsequently, the residue was separated using ODS column (60 id × 320 mm) with 10%, 20%, 30%, 40%, 60%, or 70% EtOH all with 1% CH_3COOH (1 L). HPLC analysis confirmed that **1** was contained in 20% EtOH fraction, **2** and **3** in 60% EtOH fraction, and **4** in 70% EtOH fraction, and each fraction was evaporated to dryness under reduced pressure. Finally, **1**, **2**, **3**, and **4** were isolated on purification of the individual fractions by preparative HPLC monitoring at 310 nm. The elutions were evaporated to dryness, dissolved in minimum amount of TFA, precipitated with excess ether, and dried in a silica gel desiccator under reduced pressure.

Chemical analysis

Alkaline hydrolysis of isolated pigment was performed as follows. The pigment powder (3 mg) was dissolved in 2N NaOH, left for 15 minutes with a sealed cap, and then acidified with CH_3COOH. The components in the reaction mixture were identified by analytical HPLC.

Cyanidin
3-O-(2-O-(6-O-(E)-caffeoyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-O-β-D-glucopyranoside(1)

UV-Vis λ_{max} (0.01% HCl-MeOH) nm: 526 (bathochromic shift with $AlCl_3$ into 39 nm), 331, 281, $E_{440}/E_{vis.max} = E_{440}/E_{526} = 0.14$, $E_{acyl.max}/E_{vis.max} =$

E_{331}/E_{526} = 0.49; ESI-TOFMS: m/z 935 (M^+ = $C_{42}H_{47}O_{24}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 935.24620 (calculated 935.24518 for M^+ = $C_{42}H_{47}O_{24}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Cyanidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-caffeoyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (2)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 528 (bathochromic shift with $AlCl_3$ into 44 nm), 320, 297, 283, $E_{440}/E_{vis.max}$ = E_{440}/E_{528} = 0.14, $E_{acyl.max}/E_{vis.max}$ = E_{330}/E_{528} = 1.02; ESI-TOFMS: m/z 1081 (M^+ = $C_{51}H_{53}O_{26}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1081.28241 (calculated 1081.28196 for M^+ = $C_{51}H_{53}O_{26}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Cyanidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-p-coumaroyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (3)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 529 (bathochromic shift with $AlCl_3$ into 48 nm), 315, 299, $E_{440}/E_{vis.max}$ = E_{440}/E_{529} = 0.15, $E_{acyl.max}/E_{vis.max}$ = E_{315}/E_{529} = 1.21; ESI-TOFMS: m/z 1065 (M^+ = $C_{51}H_{53}O_{25}^+$), 287 (Cy^+ = $C_{15}H_{11}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1065.28613 (calculated 1065.28704 for M^+ = $C_{51}H_{53}O_{25}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Peonidin 3-O-(2-O-(6-O-(E)-p-coumaroyl-β-D-glucopyranosyl)-6-O-(E)-p-coumaroyl-β-D-glucopyranoside)-5-O-β-D-glucopyranoside (4)

UV-Vis λ_{max} (0.01% HCl–MeOH) nm: 528 (no bathochromic shift with $AlCl_3$), 315, 297, $E_{440}/E_{vis.max}$ = E_{440}/E_{528} = 0.13, $E_{acyl.max}/E_{vis.max}$ = E_{315}/E_{528} = 1.25; ESI-TOFMS: m/z 1079 (M^+ = $C_{52}H_{55}O_{25}^+$), 301 (Pn^+ = $C_{16}H_{16}O_6^+$); high-resolutional ESI/FT-ICRMS: m/z 1079.30256 (calculated 1079.30269 for M^+ = $C_{52}H_{55}O_{25}^+$); ^{13}C and ^1H NMR data were listed in Table 1.

Stability test

Stability of anthocyanins **1**, **2**, **3**, and **4** in neutral aqueous solution was compared with that of nonacylated anthocyanins cyanidin 3-O-sophoroside-5-O-glucoside (Cy3S5G) and peonidin 3-O-sophoroside-5-O-glucoside (Pn3S5G) isolated from original storage root according to a previously reported method [19]. Each anthocyanin TFA salt was dissolved in McIlvaine (pH 7.0, 0.1 M citrate–0.2 M phosphate) buffer solution to make 50 μM test solution, and the Vis spectra (400–700 nm) were measured automatically at appropriate time intervals. Based on the absorbance at $\lambda_{vis.max}$ of each spectrum, the residual color (%) was calculated as percent of the initial absorbance (= 100%). The stability of the anthocyanin
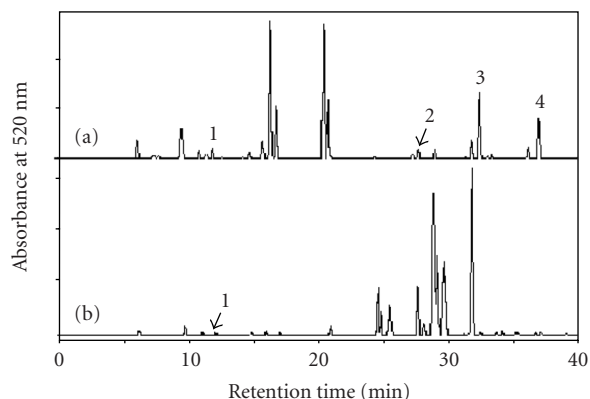

FIGURE 1. (a) HPLC chromatograms of the crude pigments of the purple sweet potato callus, and (b) storage root of sweet potato (*Ipomoea batatas* L), cv Ayamurasaki. Analytical HPLC was run with a linear gradient elution of solvent A : B = 85 : 15 to 65 : 35 for 100 minutes on Luna column at 35°C with a flow rate of 1.0 mLmin^{-1} monitoring at 520 nm.

could be evaluated on the basis of the half-life ($t_{1/2}$), defined as the time required to reach 50% residual color.

DPPH radical scavenging activity assay

Radical scavenging activity of **1**, **2**, **3**, and **4** was tested according to the DPPH-colorimetric method developed by Yamaguchi et al [20] and compared with Cy3S5G, Pn3S5G, and α-tocopherol as natural antioxidants and BHT as a synthetic antioxidant. Each sample was dissolved in EtOH to 500 μM concentration. Sample solution (25 μL) was added 375 μL of EtOH, 350 μL of Tris-HCl buffer solution (pH 7.4, 0.1 M), and 250 μL of 500 μM DPPH-EtOH solution (to obtain a final sample concentration was 12.5 μM), and immediately shaken and then kept standing for 20 minutes in the dark at room temperature. The absorbance of residual DPPH in sample solution was measured at 520 nm. Initial and blank were measured without substrate and without DPPH, respectively. The DPPH radical scavenging activity (RS%) was calculated as RS% = $100(A_i - A_s + A_b)/A_i$, in which A_i, A_s, and A_b were the absorbances at 520 nm of initial, sample, and blank solutions, respectively. The experiment was conducted with four replicates.

RESULTS AND DISCUSSION

The deep-purple callus induced from the storage root of purple-fleshed sweet potato cultivar Ayamurasaki was extracted with 15% CH_3COOH. The crude extract contained 19 or more anthocyanins as detected by analytical HPLC (Figure 1(a)) [18]. The crude extract was successively purified by an absorbing resin and ODS column chromatographies and then preparative HPLC to afford four anthocyanins **1**, **2**, **3**, and **4** (named as YGM-0d, -3′, -7a, and -7e, resp) as red powders of TFA salts which were respectively obtained in amounts of 46, 28, 114, and 22 mg (yield of 0.0115%, 0.007%, 0.0286%, and 0.006%, resp).

Table 1. NMR chemical shifts purple-fleshed sweet potato callus anthocyanins **1**, **2**, **3**, and **4** from tetramethylsilane in DMSO-d_6/TFA-d_1 (9/1), 800 MHz.

Position	1 δ_C	1 δ_H	2 δ_C	2 δ_H	3 δ_C	3 δ_H	4 δ_C	4 δ_H
Aglycon								
2	162.57	—	162.58	—	162.55	—	162.69	—
3	144.59	—	144.36	—	144.28	—	144.39	—
4	133.65	8.92 s	133.35	8.27 s	133.35	8.84 s	135.62	8.95 s
5	155.31	—	155.41	—	155.37	—	155.63	—
6	104.32	6.99 d (1.8)	105.02	6.95 d (2.0)	105.12	6.95 d (1.6)	104.89	6.98 d (1.9)
7	167.80	—	167.92	—	167.91	—	168.24	—
8	96.22	7.06 d (1.8)	96.36	6.97 d (2.0)	96.38	6.93 d (1.6)	96.70	7.05 d (1.9)
9	111.74	—	111.88	—	111.87	—	112.00	—
10	155.20	—	155.24	—	155.29	—	155.54	—
1′	119.66	—	119.64	—	119.63	—	119.42	—
2′	117.73	8.08 d (2.3)	117.76	8.03 d (2.3)	117.74	8.03 d (2.3)	114.11	7.98 d (2.1)
3′	146.41	—	146.46	—	146.48	—	148.60	—
4′	155.48	—	155.41	—	155.42	—	155.99	—
5′	117.18	7.13 d (8.7)	117.16	7.11 d (8.7)	117.15	7.11 d (8.7)	116.84	7.10 d (8.7)
6′	127.87	8.31 dd (2.3, 8.7)	127.79	8.27 dd (2.3, 8.7)	127.80	8.27 dd (2.3, 8.7)	129.40	8.36 dd (2.1, 8.7)
OMe	—	—	—	—	—	—	56.19	3.90 s
Glucose-a								
1	99.85	5.59 d (7.6)	99.67	5.67 d (7.4)	99.50	5.68 d (7.4)	100.65	5.64 d (7.3)
2	81.54	4.02 t (8.3)	81.86	4.05 t (8.1)	81.84	4.06 t (8.0)	81.74	3.95 t (7.6)
3	76.56	3.69 t (8.9)	76.06	3.76 t (8.8)	76.05	3.76 t (8.8)	75.95	3.75 t (8.7)
4	69.43	3.39 t (8.9)	69.94	3.49 t (9.3)	69.98	3.48 t (9.3)	69.83	3.47 t (9.2)
5	77.65	3.56 m	74.27	3.93 m	74.26	3.94 m	74.44	3.87 m
6a	60.65	3.57 m	63.20	4.32 dd (6.8, 12.2)	63.24	4.31 dd (7.1, 12.1)	63.16	4.27 dd (6.8, 12.6)
6b	60.65	3.74 brd (10.1)	63.20	4.42 brd (10.0)	63.24	4.42 brd (12.1)	63.16	4.40 brd (11.3)
Glucose-b								
1	104.44	4.84 d (7.8)	104.71	4.81 d (7.8)	104.69	4.82 d (7.8)	104.47	4.78 d (7.8)
2	74.75	3.14 t (8.4)	74.85	3.15 t (8.7)	74.86	3.15 t (8.4)	74.65	3.19 t (8.4)
3	76.26	3.27 t (9.1)	76.23	3.26 t (8.7)	76.24	3.27 t (8.7)	76.28	3.29 t (8.9)
4	69.55	3.24 t (8.8)	69.61	3.25 t (8.7)	69.66	3.26 t (8.8)	69.77	3.26 t (9.4)
5	74.18	3.14 m	74.20	3.18 m	74.21	3.19 m	74.30	3.30 m
6a	62.58	3.99 dd (6.2, 12.5)	62.66	3.99 dd (4.3, 11.8)	62.67	4.00 dd (4.3, 11.7)	63.23	4.03 dd (5.4, 11.6)
6b	62.58	3.92 brd (10.1)	62.66	3.95 brd (10.0)	62.67	3.96 brd (10.4)	63.23	4.13 brd (10.1)
Glucose-c								
1	101.59	5.12 d (7.7)	102.24	5.07 d (7.7)	102.35	5.05 d (7.7)	102.01	5.10 d (7.7)
2	73.19	3.51 t (8.5)	73.39	3.56 t (8.5)	73.41	3.56 t (8.9)	73.35	3.56 t (8.5)
3	76.02	3.41 t (8.9)	76.29	3.41 t (9.0)	76.30	3.41 t (9.0)	76.37	3.42 t (9.0)
4	69.70	3.31 t (9.2)	69.88	3.29 t (9.2)	69.94	3.30 t (9.3)	69.83	3.31 t (9.4)
5	77.65	3.49 m	77.86	3.50 m	77.85	3.48 m	77.82	3.50 m
6a	60.74	3.58 dd (5.7, 12.3)	61.08	3.59 dd (6.2, 12.3)	61.04	3.58 dd (5.8, 12.0)	60.93	3.59 dd (5.7, 12.2)
6b	60.74	3.78 brd (11.8)	61.08	3.83 brd (10.1)	61.04	3.81 dd (1.9, 11.9)	60.93	3.80 dd (2.0, 10.0)

TABLE 1. Continued.

Ga-Acid I								
1	—	—	125.74	—	125.20	—	125.19	—
2	—	—	115.40	6.94 d (2.0)	130.35	7.33 d (8.6)	130.30	7.28 d (8.7)
3	—	—	145.70	—	115.98	6.78 d (8.7)	115.95	6.76 d (8.7)
4	—	—	148.53	—	160.08	—	160.05	—
5	—	—	115.88	6.77 d (8.7)	115.98	6.78 d (8.7)	115.95	6.76 d (8.7)
6	—	—	121.61	6.86 dd (2.0, 8.7)	130.35	7.33 d (8.6)	130.30	7.28 d (8.7)
α	—	—	113.98	6.02 d (15.8)	113.96	6.026 d (15.9)	113.79	5.96 d (15.9)
β	—	—	145.53	7.24 d (15.8)	144.82	7.24 d (15.8)	144.76	7.23 d (15.8)
Carbonyl	—	—	166.77	—	166.78	—	166.68	—
Gb-Acid II								
1	125.72	—	125.19	—	125.17	—	125.12	—
2	115.15	6.99 d (2.0)	130.36	7.33 d (8.6)	130.58	7.38 d (8.6)	130.52	7.38 d (8.7)
3	145.25	—	116.03	6.78 d (8.7)	116.03	6.79 d (8.7)	115.97	6.80 d (8.7)
4	148.48	—	160.08	—	160.08	—	160.08	—
5	116.01	6.77 d (8.3)	116.03	6.78 d (8.7)	116.03	6.79 d (8.7)	115.97	6.80 d (8.7)
6	121.39	6.83 dd (2.0, 8.3)	130.36	7.33 d (8.6)	130.58	7.38 d (8.6)	130.52	7.38 d (8.7)
α	113.94	6.00 d (15.8)	113.86	6.17 d (15.8)	113.86	6.23 d (15.9)	113.79	6.18 d (15.9)
β	145.65	7.22 d (15.8)	144.83	7.32 d (15.8)	145.30	7.37 d (15.8)	145.24	7.36 d (15.7)
Carbonyl	166.46	—	166.48	—	166.49	—	166.47	—

*Values in parentheses indicate coupling constants (J in Hz).

**Abbreviations: s = singlet, d = doublet, t = triplet, m = multiplet, dd = double doublet, and brd = broad doublet.

On alkaline hydrolysis, **1**, **2**, and **3** gave cyanidin 3-*O*-sophoroside-5-*O*-glucoside (Cy3S5G) [19], while only **4** gave peonidin 3-*O*-sophoroside-5-*O*-glucoside (Pn3S5G). Moreover, in the hydrolysates of **1**, **2**, **3**, and **4**, caffeic acid from **1**, caffeic acid and *p*-coumaric acid from **2**, *p*-coumaric acid from **3**, and *p*-coumaric acid from **4** were detected as acylating acid(s) by HPLC co-chromatographic analysis with authentic cinnamic acids. In the UV-Vis spectra, the absorptions around 331 and 320 nm of **1** and **2**, 315 nm of **3** and **4** also respectively supported the presence of caffeoyl and *p*-coumaroyl residues in their molecules. Numbers of the aromatic acids were respectively estimated one for **1**, and two for **2**, **3**, and **4**, on the basis of $E_{acyl.max}/E_{vis.max}$ (absorbance at $\lambda_{acyl.max}$/absorbance at $\lambda_{vis.max}$) values of 0.49 for **1**, and 1.02–1.25 for **2**, **3**, and **4**, respectively.

Since absorption maxima ($\lambda_{vis.max}$) of **1**, **2**, and **3** showed bathochromic shift by addition of $AlCl_3$, but not in **4**, **1**, **2**, and **3** could be elucidated as cyanidin-(Cy)-based anthocyanins and **4** as a peonidin-(Pn)-based one. ESI–TOFMS measurement of **1**, **2**, **3**, and **4** showed molecular ion peaks at m/z 935, 1081, 1065, and 1079 corresponding to $C_{42}H_{47}O_{24}^+$, $C_{51}H_{53}O_{26}^+$, $C_{51}H_{53}O_{25}^+$, and $C_{52}H_{55}O_{25}^+$, respectively, and also showed fragment ion peaks of Cy^+ at m/z 287 in **1**, **2**, and **3** and of Pn^+ at m/z 301 in **4**. The molecular formulas of **1**, **2**, **3**, and **4** were confirmed by high-resolutional ESI/FT–ICRMS at m/z 935.24620, 1081.28241, 1065.28613, and 1079.30256, respectively. All these findings indicated **1** as mono-caffeoyl Cy3S5G, **2** as caffeoyl-*p*-coumaroyl Cy3S5G, **3** as di-*p*-coumaroyl Cy3S5G, and **4** as di-*p*-coumaroyl Pn3S5G.

The complete structures of **1**, **2**, **3**, and **4** were established by ^{13}C and ^1H NMR analyses containing 2D pulse experiments such as a homonuclear double quantum filtered correlation spectroscopy (DQF-COSY), a total correlation spectroscopy (TOCSY), a heteronuclear single-quantum correlation (HSQC), a nuclear Overhauser and exchange spectroscopy (NOESY), and a heteronuclear multiple bond correlation spectroscopy (HMBC) technique. Assignment of ^{13}C- and ^1H-signals was summarized in Table 1. ^1H-signals in low magnetic field (δ_H 6–9 ppm) shows characteristic aglycons and cinnamic acids. Anthocyanins **1**, **2**, and **3** and **4** have Cy and Pn moieties, respectively, due to the corresponding signals of benzopyrylium nucleus and 1, 3, 4-trisubstituted aromatic B-ring. Only **4** has an additional methoxyl signal at high magnetic field (δ_H 3.90 ppm, δ_C 56.19 ppm). The presence of *trans* (*E*)-caffeoyl residue in **1** and **2** spectra and (*E*)-*p*-coumaroyl residue in **2**, **3**, and **4** spectra is confirmed with the 1, 3, 4-trisubstituted and 1, 4-disubstituted benzenes, respectively having the (*E*)-olefinic proton signals with large coupling constant (about $J_{\alpha,\beta}$ = 16 Hz). In high magnetic field (δ_H 3–6 ppm), the spectra also show all sugars of **1**, **2**, **3**, and **4** to be β-D-glucopyranosyl configuration because of the resonances at lower magnetic field (δ_H 4.78–5.68 ppm) of all anomeric protons and the large J values (J = 7.2–9.4 Hz) of the anomeric protons and the ring protons. As shown in Table 1,
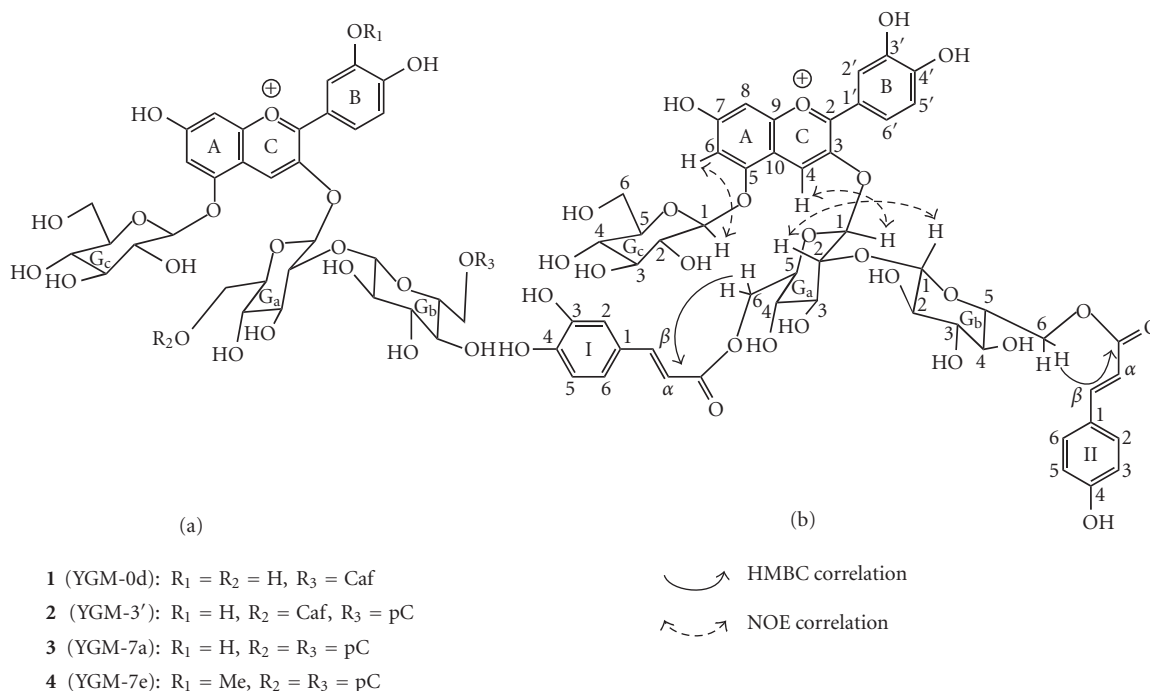
FIGURE 2. Purple sweet potato callus anthocyanins. (a) Structures of **1**, **2**, **3**, and **4**; Caf = caffeic acid and pC = *p*-coumaric acid, (b) typical NOE and HMBC correlations of caffeoyl-*p*-coumaroylated anthocyanin **2**.

G_a-2H (at δ H 3.95–4.06 ppm) and G_a-2C (δ C 81.54–81.86 ppm) clearly shift to downfield more than G_b-2H (at δ H 3.14–3.19 ppm) and G_b-2C (δ C 74.65–74.86 ppm) or G_c − 2H (at δ H 3.51–3.56 ppm) and G_c-2C (δ C 73.19–73.39 ppm). On the basis of this data, we concluded that glucose b (G_b) links to glucose a (G_a)-20H. On the other hand, NOESY and HMBC spectra gave more direct and certain data on the presence of β-D-G_b $(1 \rightarrow 2)$ G_a bond of the sophorosyl residue.

The connecting relations among an aglycon, three sugars, and acyl groups in **1**, **2**, **3**, and **4** were confirmed by NOESY and HMBC measurements, for example, anthocyanin **2** shown in Figure 2(b). In the NOESY spectra of **1**, **2**, **3**, and **4**, three intensive NOE signals between aglycon-4H and G_a-1H (aglycon-4H/G_a-1H), G_a-2H/G_b-1H, and aglycon-6H/G_c-1H indicated that G_a, G_b, and G_c connected at aglycon-3-OH, at G_a-2OH, and at aglycon-5OH through glycosyl bond, respectively. In the HMBC spectra of **1**, **2**, **3**, and **4**, the clear ^{1}H-^{13}C cross peaks between G_a-1H and aglycon-3-carbon signals (G_a-1H/aglycon-3C), G_b-1H/G_a-2C, G_a-2H/G_b-1C, and G_c-1H/aglycon-5C verified the connections of G_a/aglycon-3OH, G_b/G_a-2OH, and G_c/aglycon-5OH, respectively. Moreover, the distinct correlation peaks between G_b-6H and acyl-carbonyl carbon signals (about δ_C 166.5 ppm) provided decisive proof that acylating acids were linked at G_b-6OH. In **2**, **3**, and **4**, the cross peak between G_c-6H and acyl-carbonyl carbon signals (about δ_C 166.8 ppm) also showed directly links aromatic

acids and G_c-6OH [3]. In conclusion, **1**, **2**, **3**, and **4** were unambiguously determined as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-caffeoyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-6-*O*-(*E*)-caffeoyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, and peonidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, respectively, by chemical and spectroscopic analyses (Figure 2(a)). Only anthocyanin **4** is a new compound, while **1**, **2**, and **3** are known ones. Anthocyanins **1** and **2** have already been identified in *Ipomoea cairica* flowers [21], but the exact binding sites of the acyl residues were not established. Similarly, anthocyanins **2** and **3** have been identified in the pigment of *Ipomoea asarifolia* flower [22], and in the pigment of *Ajuga reptans* flower and the corresponding cell cultures [23], respectively. Pigment **1** is also found in original purple-fleshed sweet potato storage root (Figure 1(b)) or sweet potato leaf pigment as a minor component, while *p*-coumaroylated **2**, **3**, and **4** are confirmed to be cell-line-specific pigments.

The stability of **1**, **2**, **3**, and **4** was compared with nonacylated Cy3S5G and Pn3S5G in neutral aqueous solution at room temperature. The stability was evaluated on the basis of the half-life ($t_{1/2}$) that was defined as the time required to reach 50% residual color. As shown in
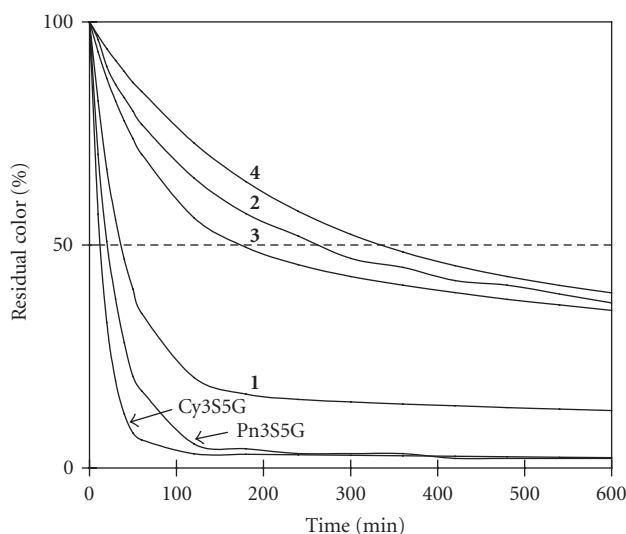
FIGURE 3. Stability of purple sweet potato callus anthocyanins **1**, **2**, **3**, and **4** in neutral aqueous solution at room temperature.
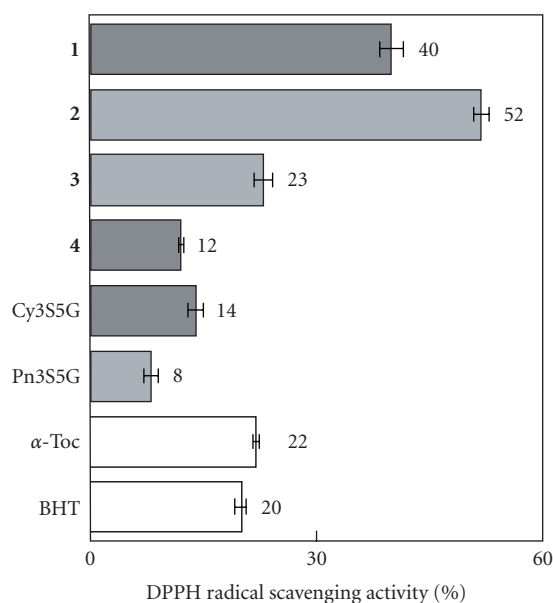

FIGURE 4. DPPH radical scavenging activity (%) of purple sweet potato callus anthocyanins **1**, **2**, **3**, and **4** (pH 7.4 at room temperature). α-Toc = α-tocopherol and BHT = 2,6-di-*tert*-butyl-4-methylphenol. Each value is the mean (RS%) ± standard deviation (n= 4).

Figure 3, diacylated anthocyanins ($t_{1/2}$ of **2**: 266 minutes, **3**: 180 minutes, and **4**: 337 minutes) were more stable than monoacylated **1** ($t_{1/2}$ = 37 minutes), and nonacylated Cy3S5G ($t_{1/2}$ = 15 minutes) and Pn3S5G ($t_{1/2}$ = 19 minutes). Effect of the structures on the stabilities of the anthocyanins suggested that the two aromatic acids protect the aglycon nucleus by a sandwich-type hydrophobic stacking mechanism and inhibit the attack of a water molecule that leads to a loss of color [24, 25].

These anthocyanins were evaluated for the antioxidant activity as scavenging ability against DPPH radical. As shown in Figure 4, activity of identically substituted but Cy-based **3** exhibited higher scavenging activity than Pn-based **4**. The caffeoyl-*p*-coumaroylated **2** and monocaffeoylated **1** showed higher activity than di-*p*-coumaroylated **3**. Therefore, anthocyanins with catechol group(s) in the aglycon and/or the acyl group(s) were thought to be very effective in radical scavenging. Moreover, since hydroxycinnamic acid acylation enhanced the activity (**1**, **2**, **3** > Cy3S5G, and **4** > Pn3S5G), the acylation might intramolecularly and synergistically achieve the radical scavenging activity of aglycon in addition to their own activity [26].

The cell line generated from Ayamurasaki storage root accumulates high levels of anthocyanin pigments. Depending on medium conditions under which the tissue is produced, the total amount of anthocyanins is equal to that of the original storage root on an MM [15] or 2.5-fold higher on a high-anthocyanin producing medium [27].

Thus the callus pigment has a potential to be utilized as a high-quality natural food colorant/natural food ingredient with protective action against oxidative damage [18].

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