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Anthocyanins in callus induced from purple storage root of *Ipomoea batatas* L.

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Abstract

Two anthocyanins were isolated from the highly pigmented callus derived from the storage root of purple sweet potato (*Ipomoea batatas* L.) cultivar 'Ayamurasaki'. One was identified as cyanidin 3-*O*-sophoroside-5-*O*-glucoside, and the other as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, by chemical and spectroscopic analysis. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Ipomoea batatas* L; Convolvulaceae; Purple callus; Acylated anthocyanins; Cyanidin 3-*p*-coumaroylsophoroside-5-glucoside; Natural food colorant; Antioxidative activity

1. Introduction

A new sweet potato (*Ipomoea batatas* L.) cultivar 'Ayamurasaki', which highly accumulates anthocyanins in the storage root, has been developed from purple cultivar 'Yamagawamurasaki' in Kyushu National Agricultural Experiment Station, Japan. Though the anthocyanin content in the new cultivar is ca. fourfold higher than in the Yamagawamurasaki cultivar, their anthocyanin compositions are similar. The major anthocyanins in these storage roots are acylated with aromatic acids (Odake et al., 1992; Goda et al., 1997; Terahara et al., 1999). These anthocyanins not only have moderate stability but also contribute towards antioxidative activity (Suda et al., 1997) and antimutagenicity (Yoshimoto et al., 1999). Therefore, the sweet potato pigment is expected to be a high quality natural food colorant which may aid in the prevention of lifestyle-related diseases.

Recently, we succeeded in culturing the deep purple callus from Ayamurasaki storage root by successive selection and subculture under optimum conditions. The pigmented callus contained four anthocyanins which were different from those in the original storage root (Konczak-Islam et al., 2000). To clarify the anthocyanin biosynthetic route during callus development and to characterize the pigment for possible food use, the structures of the Ayamurasaki callus anthocyanins were determined by chemical and spectroscopic analysis.

2. Results and discussion

The deep purple callus derived from purple sweet potato cultivar 'Ayamurasaki' was extracted with 15%

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HOAc. The crude extract contained mainly early-eluting anthocyanins, as determined by ODS-HPLC (Konczak-Islam et al., 2000), and was successively purified by an absorbing resin CC, PVP CC, and prep. ODS-HPLC to afford two pure pigments **1** and **2** (named as YGM-0a and -0f′, respectively as red powders of TFA salts. Anthocyanin **2** was found only in the callus, while **1** was common in both the callus and the original storage root.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data for sweet potato purple callus anthocyanins **2** (δ ppm from TMS in DMSO-d_6:TFA-d_1 = 9:1)[a,b]

Position		**2** (YGM-0f′)		1 (YGM-0a)[c]	
		δ_c	δ_H	δ_C	δ_H
Aglycon	2	162.63			
	3	144.55			
	4	133.95	8.94 *s*		
	5	155.31			
	6	104.34	7.04 *d* (1.9)		
	7	167.96			
	8	96.25	6.98 *d* (1.9)		
	9	155.31			
	10	111.71			
	1′	119.65			
	2′	117.67	8.06 *d* (2.2)		
	3′	146.53			
	4′	155.51			
	5′	117.21	7.13 *d* (8.8)		
	6′	127.98	8.32 *dd* (2.2, 8.8)		
Glucose-a	1	100.01	5.57 *d* (7.3)	99.76	5.62 *d* (7)
	2	81.48	4.01 *t* (8.2)	81.18	4.08 *t* (8)
	3	76.10	3.93 *t* (8.8)	76.05	3.69 *t* (9)
	4	69.49	3.39 *t* (8.8)	69.66	3.42 *t* (9)
	5	77.68	3.57 *m*	76.03	3.59 *m*
	6a	60.71	3.75 *m*	60.72	3.77 *m*
	6b	60.71	3.75 *brd* (12.0)	60.72	3.79 *m*
Glucose-b	1	104.44	4.85 *d* (7.7)	103.89	4.70 *d* (7)
	2	74.78	3.13 *t* (8.4)	74.64	3.00 *t* (8)
	3	76.33	3.28 *t* (8.1)	76.32	3.11 *t* (9)
	4	69.65	3.26 *t* (8.8)	69.26	3.05 *t* (9)
	5	74.16	3.18 *m*	77.48	2.70 *m*
	6a	62.68	3.98 *m*	60.60	3.19 *m*
	6b	62.68	3.98 *m*	60.60	3.19 *m*
Glucose-c	1	101.57	5.10 *d* (7.7)	101.54	5.16 *d* (7)
	2	73.22	3.52 *t* (8.4)	73.21	3.52 *brt* (8)
	3	76.60	3.42 *t* (9.2)	76.61	3.41 *brt* (7)
	4	69.72	3.32 *t* (9.2)	69.76	3.32 *t* (9)
	5	77.67	3.49 *m*	77.63	3.52 *brt* (8)
	6a	60.77	3.60 *dd* (5.9, 10.3)	60.72	3.62 *m*
	6b	60.77	3.79 *brd* (10.3)	60.72	3.62 *m*
G_b-6-pC	1	125.17			
	2	130.37	7.35 *d* (8.8)		
	3	116.05	6.80 *d* (8.8)		
	4	160.10			
	5	116.05	6.80 *d* (8.8)		
	6	130.37	7.35 *d* (8.8)		
	α	113.99	6.06 *d* (15.8)		
	β	144.84	7.28 *d* (15.8)		
	C=O	166.52			

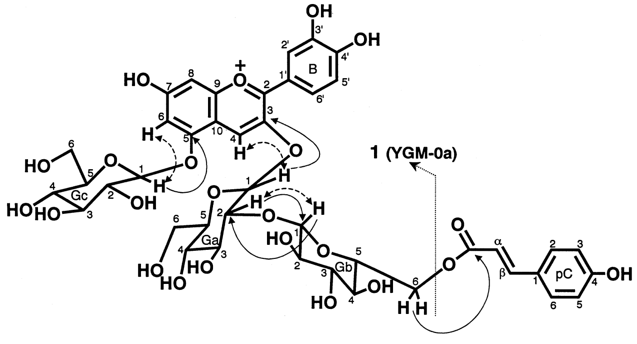
[a] Abbreviations: *s*, *d*, *t*, *m*, *dd*, *brd*, *brt* = singlet, doublet, triplet, multiplet, double doublet, broad doublet and broad triplet.

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

[c] Only sugar data are cited from the reference (Terahara et al., 1996).

On alkaline hydrolysis, **2** gave the previously characterized cyanidin 3-*O*-sophoroside-5-*O*-glucoside (Cy-3-S-5-G) **1** (Terahara et al., 1996). The hydrolysate of **2** also contained *p*-coumaric acid (pC), detected as an acylating acid by HPLC co-chromatographic analysis. In the UV–Vis spectra, the absorption around 313 nm also supported the presence of a pC group in **2**. FABMS of **1** and **2** showed molecular ion peaks at *m*/*z* 773 and 919 corresponding to $C_{33}H_{41}O_{21}^+$ and $C_{42}H_{47}O_{23}^+$, respectively, and also showed fragment ion peaks of Cy^+ at *m*/*z* 287. These findings indicated that **1** and **2** were Cy 3-S-5-G and mono-*p*-coumaroylated Cy 3-S-5-G, respectively.

The complete structure of **2** was established by ^{1}H and ^{13}C NMR analysis, using DQF-COSY, HMQC, NOE difference spectroscopy and HMBC techniques. Assignment of ^{1}H and ^{13}C signals was performed with the aid of DQF-COSY, HMQC, NOEDS and HMBC, and is summarized in Table 1. In the low field region (δ_H 6–9 ppm), the ^{1}H NMR spectrum of **2** shows the presence of a Cy moiety based on proton signals of the benzopyrylium nucleus and 1, 2, 4-trisubstituted aromatic B-ring. The presence of a *trans* (*E*)-pC group was confirmed by the presence of signals corresponding to a 1,4-disubstituted benzene and (*E*)-olefinic proton signals with large coupling constant ($J_{\alpha,\beta}$ = 15.8 Hz). In the high field region (δ_H 3–6 ppm), the spectra also showed all sugars of **2** to have β-D-glucopyranosyl configuration, as shown by the relatively low field chemical shifts (δ_H 4.85–5.57 ppm)



2 (YGM-0f′)

Fig. 1. Structures of anthocyanins **2** (YGM-0f′) and **1** (YGM-0a) in purple callus induced from the storage root of purple sweet potato.

of all anomeric protons, and the large J values ($J =$ 7.3–9.2 Hz) of the anomeric and ring protons. Since glucose b (G_b) is linked to G_a-2OH as shown by the glycosylation shifts of G_a-2H (at δ_H 4.01 ppm) and G_a-2C (at δ_C 81.5 ppm) as shown in Table 1, the interglycosidic linkage is β-D-G_b (1 → 2) G_a, a sophoroside.

The connectivities of the aglycone, three sugars and one acyl group in **2** were confirmed by NOE and HMBC measurements (Fig. 1). In the NOE difference spectra of **2**, three informative NOE signals between Cy-4H and G_a-1H (Cy-4H/G_a-1H), G_a-2H/G_b-1H and Cy-6H/G_c-1H indicated that G_a and G_b, and G_c connected at Cy 3-OH, at G_a-2OH and at Cy 5-OH through a glycosyl bond, respectively. In the 2D-HMBC spectra of **2**, the clear ^1H–^{13}C cross peaks between G_a-1H and Cy-3-carbon signals (G_a-1H/Cy-3C), G_b-1H/G_a-2C, G_a-2H/G_b-1C, and G_c-1H/Cy-5C verified the connections G_a/Cy 3-OH, G_b/G_a-2OH, and G_c/Cy 5-OH, respectively. Moreover, the distinct cross peak between G_b-6H and pC carbonyl carbon signal provided decisive proof that pC was linked at G_b-6OH (Goda et al., 1997). The 6-acylation was also supported by the comparison of the NMR data of **2** and **1** already reported by Terahara et al. (1996). As shown in Table 1, deshielding shifts of G_b-6Hs (chemical shift difference $\Delta\delta_H$ +0.9 ppm) and G_b-6C ($\Delta\delta_C$ +2 ppm) in **2** clearly indicated the presence of a *p*-coumaroyl moiety on G_b-6OH. In conclusion, anthocyanin **2** was unambiguously determined as cyanidin 3-*O*-(2-*O*-(6-*O*-(*E*)-*p*-coumaroyl-β-D-glucopyranosyl)-β-D-glucopyranoside)-5-*O*-β-D-glucopyranoside, a new anthocyanin (Fig. 1). This is the first report of a *p*-coumaroylated and G_b-6-monoacylated anthocyanin in sweet potato related pigments.

The major anthocyanins in sweet potato storage root are cyanidin- and peonidin-based and diacylated, whereas the callus ones are cyanidin-based and non- or monoacylated. Therefore, anthocyanin **1** (Cy 3-S-5-G) is considered as the common precursor in the biosynthesis of both the callus and the storage root anthocyanins. While methylation of cyanidin B-ring-3′-OH and/or acylation on both G_a-6OH and G_b-6OH of **1**, and probable 3(5)-hydroxylation of th *p*-coumaroyl residue of **2** might occur in the storage root while being repressed in the callus.

The pigment concentration of the callus is high and close to that of Ayamurasaki's storage root (Konczak-Islam et al., 2000). In addition, the major callus anthocyanins were cyanidin-based, which showed stronger antioxidative activity than pelargonidin- and delphinidin-based anthocyanins in the linoleic acid system at neutral condition (Tsuda et al., 1994). Thus, the callus pigment could be utilized as food additives which provide protective action from oxidative damage.

3. Experimental

3.1. Spectroscopic procedures

UV–Vis spectra were recorded on an MPS-2000 (Shimadzu, Japan) spectrophotometer in 0.01% HCl–MeOH. The bathochromic shift test was carried out by the addition of 5% $AlCl_3$–MeOH. The FABMS spectra were recorded on JMS SX-102A (JEOL. Japan) with *m*-nitrobenzyl alcohol as a matrix on a positive mode. ^1H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on A-600 spectrometer (JEOL, Japan) in DMSO-d_6:TFA-d_1 = 9:1 with tetramethylsilane (TMS) as an internal standard at 30°C.

3.2. Chromatographic procedures

Open column chromatography was carried out on Amberlite XAD-2000 (Rohm and Haas, USA) and PVP (Polyclar SB-100, ISP) resins. HPLC analysis was performed using an L-6200 intelligent pump. (Hitachi, Japan) equipped with an L-4200 UV–Vis detector, D-2000 integrator and 655A-52 column oven (Hitachi, Japan). Analytical HPLC was performed on an ODS column (Luna eu C18 (2), 100 × 4.6 mm i.d., Phenomenex, USA) at 35°C using solvent A (H_3PO_4:H_2O = 1.5:98.5) and B (H_3PO_4:HOAc:MeCN:H_2O = 1.5:20:25:53.5) at a flow rate of 1.0 ml min^{-1}. Anthocyanins were eluted with a linear gradient from B 15 to 35% for 100 min in solvent A monitoring at 520 nm. The acylating aromatic acid was co-chromatographically analyzed with standard acids under a linear gradient from 25% to 55% solvent B in solvent A for 30 min at 310 nm. Preparative HPLC was performed on Inertsil ODS 5 column (250 × 20 mm i.d., GL Sciences, Japan) at 7 ml min^{-1} by an isocratic elution using a mixture of solvent A (TFA:H_2O = 0.5:99.5) and solvent B (TFA:MeCN:H_2O = 0.5:30:69.5), A:B = 70:30–50:50 at 520 nm.

3.3. Plant materials and callus culture

The storage roots of the purple sweet potato (*I. batatas* L.) cultivar 'Ayamurasaki' were used for the culture. The somatic pro-embryos were induced on the surface of the storage root explants that had been cultivated on Murashige and Skoog (MS) medium enriched with 2,4-D (1 mg l^{-1}). The pro-embryos produced a compact, slightly pigmented callus on MS medium with 2,4-D (0.5 mg l^{-1}). From this callus, a cell line that produced large amounts of pigments in the dark was selected (Konczak-Islam et al., 2000).

3.4. Extraction and isolation of anthocyanins

The fresh pigmented calli (160 g) were steeped in

15%, HOAc (1 l) for one day and filtered. This operation was repeated three more times (1, 1, and 0.5 l). The combined crude extract (ca., 3.5 l) contained 19 or more anthocyanins, among which the contents of **1** and **2** were 31.5% (R_t, 2.9 min) and 11.0% (R_t, 14.6 min), respectively, as determined by HPLC analysis. The extract solution was applied to an XAD-2000 resin column (320 × 60 mm i.d.) and the column was washed with water (3 l), and then eluted with 1% HOAc–70% EtOH (1 l). The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 0.1% TFA (ca., 50 ml), and the solution was chromatographed on PVP column (100 × 60 mm i.d.) with stepwise elution by an aqueous EtOH solution (0%, 30%, 40%, 60% and 70% EtOH containing 0.1% TFA). Seven pigment fractions were obtained, with fraction 2 containing mostly **1** and fraction 4 containing mostly **2**. On purification of fractions 2 and 4 by prep. HPLC with solvent A:B = 70:30–50:50, **1** and **2** were isolated, respectively. The elutions were evaporated to dryness, dissolved in minimum amount of TFA, and precipitated with excess ether. TFA salts of **1** and **2** were obtained in amounts of 80 and 60 mg (yield of 0.05% and 0.04%), respectively, as red powders.

3.5. Chemical analysis

Alkaline hydrolysis of isolated pigment was performed as follows: the pigment powder (ca., 1 mg) was dissolved in 2 N NaOH, left for 15 min with a sealed cap, and acidified with HOAc. The components in the reaction mixture were identified by analytical HPLC.

3.6. Anthocyanin 1

UV–Vis $\lambda_{\max}$ (0.01% HCl–MeOH) nm : 527 (bathochromic shift with $AlCl_3$ 41 nm), 278, $E_{440}/E_{\text{vis max}} = E_{440}/E_{527} = 13\%$; FABMS *m*/*z*: 773 [$M^+ = C_{33}H_{41}O_{21}^+$], 287 [$Cy^+ = C_{15}H_{11}O_6^+$].

3.7. Anthocyanin 2

UV–Vis $\lambda_{\max}$ (0.01% HCl–MeOH) nm : 527 (bathochromic shift with $AlCl_3$ 7 nm), 313, 295, 282, $E_{440}/E_{\text{vis max}} = E_{440}/E_{527} = 13\%$, $E_{313}/E_{\text{vis max}} = E_{313}/E_{527} = 69\%$; FABMS *m*/*z*: 919 [$M^+ = C_{42}H_{47}O_{23}^+$], 287 [$Cy^+ = C_{15}H^{11}O_6^+$]; ^{1}H and ^{13}CNMR spectral data: see Table 1.

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