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Isolation and Identification of Anthocyanins from Purple Sweet Potatoes

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Abstract

Anthocyanin pigments of purple sweet potato roots(*Ipomoea batatas* L.) were extracted with 0.5% TFA (trifluoroacetic acid) in 95% EtOH, and further isolated and purified by Amberlite XAD-7 and ODS column chromatography, and final preparative HPLC. Among nine anthocyanins isolated, the structure of three major anthocyanins were identified as 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-caffeylglucopyranosyl)-*β*-D-glucopyranosyl-5-*O*-(*β*-D-glucopyranosyl)-peonidin, 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-feruloyl-glucopyranosyl)-*β*-D-glucopyranosyl-5-*O*-(*β*-D-glucopyranosyl)-peonidin, and 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*p*-hydroxylbenzoylglucopyranosyl)-*β*-D-glucopyranosyl-5-*O*-(*β*-*D*-glucopyranosyl)-peonidin, by using UV-visible absorption spectra, ^{1}H-NMR and FAB-MS analysis.

Key words: sweet potatoes, anthocyanins, chromatographic and spectral analyses

INTRODUCTION

There is much attention in the development of food colorants from natural sources to replace synthetic food colorants. In particular, anthocyanins have considerable potential in the food industry as safe and effective food additives due to apparent harmlessness to human(1).

Compared to the synthetic colorants, anthocyanins have been less extensively used because of their instability towards a variety of chemical and physical factors(2,3). However, since the novel polyacylated anthocyanins displaying marked stability were recently found in several plants(4-6), further studies on the production of these stable anthocyanins by cell cultures, and their application in foods and beverages have been performed(7-9).

Recently, anthocyanins have been recognized as biologically active substances(10-12) and natural colorants. Tsuda et al.(13,14) suggested that anthocyanins may play an important role as dietary antioxidants for prevention of oxidative damage caused by active oxygen radicals in living systems. Anthocyanins together with other flavonoids are also known to have an important impact on inhibiting the lipid peroxidation of human low-density lipoprotein(15).

Many researches on the isolation and structural elucidation of anthocyanins from sweet potatoes are available. The presence of several cyanidin and peonidin glycosides, as well as several hydroxybenzoyl and hydroxycinnamoyl acylated anthocyanins was reported in several cultivars and tissues of sweet potatoes(16-20). These anthocyanin pigments were found to be more stable than other anthocyanins in a neutral or weakly acidic aqueous solution(21,22). Especially, much interest has recently received on the acylated anthocyanins in sweet potato as natural food colorants due to high stability and antioxidative activity(23).

The purpose of this study was to isolate and characterize anthocyanin pigments of newly bred purple sweet potatoes by using several chromatographic, and ^{1}H-NMR and FABMS techniques.

MATERIALS AND METHODS

Materials and reagents

The roots of purple sweet potatoes, Yamagawa Murasaki, (*Ipomoea batatas* L.) came from Kushui in Japan. These were grown on the farm of Mokpo Experiment Station in Honam Agricultural Experiment Station. Fresh roots of sweet potatoes were harvested, washed and

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stored at incubator(HB-105S, Hanbaek, RH 85%, 13°C) until used. Trifluoroacetic acid(TFA), CF_3COOD, DMSO-d_6, were obtained from Sigma Chem. Co.(St. Louis, MO, USA). Acetonitrile and other solvents for HPLC analysis were supplied by Merck(Darmstadt, Germany). All laboratory chemicals used in this study were of reagent grade.

Isolation and purification of anthocyanin pigments

The schematic procedure for isolation and purification of anthocyanins from purple sweet potato roots is shown in Fig. 1. The sliced purple sweet potato roots(100g) were twice extracted with 1L of 0.5% TFA in 95% EtOH overnight. filtered and then concentrated to a small volume below 40°C *in vacuo*. The aqueous solution was extracted with n-hexane to remove the lipid, and then applied on the Amberlite XAD-7(Organo Co. Ltd., Tokyo, Japan) column(25mm×500mm) with 100ml of 0.5% TFA solution, and final anthocyanin was eluted with 1.5L of 0.1% TFA in MeOH-H_2O(70 : 30) solution. The pigment fraction was evaporated under vacuum to dryness, and 100ml of 1% HCl in MeOH and 300ml of ethylether were

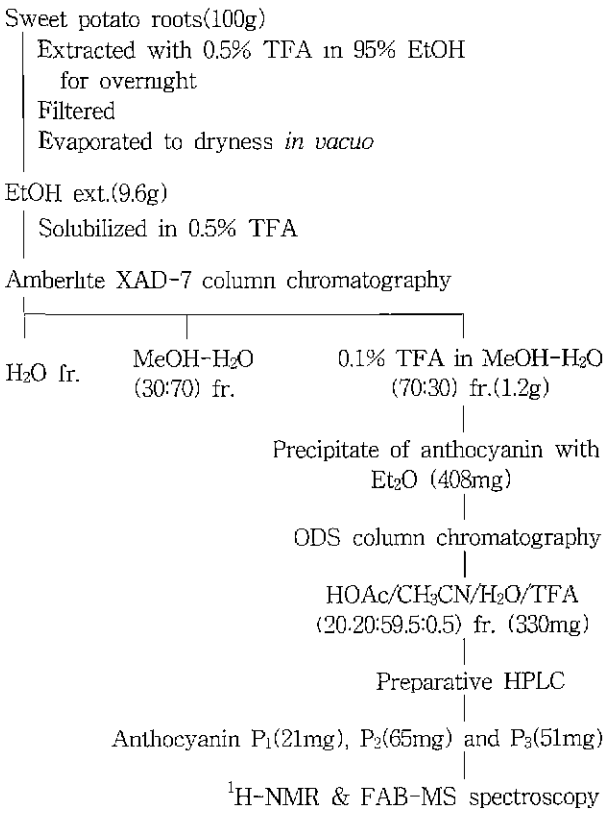

Fig. 1. Schematic procedure for extraction, separation and purification of three major anthocyanins from purple sweet potato roots.

further added stepwise to the residue to precipitate the anthocyanin pigments. The precipitates were collected by centrifugation(2,500rpm, 20min) and then dried in a vacuum desiccator. These partially purified anthocyanins were adsorbed on the ODS(Sigma Chem. Co. 40~63μ) open column(20mm×300mm) and then fractionated into 4 fractions using following solvent systems; solvent A (HOAc-CH_3CN-H_2O=20 : 30 : 50) : solvent B(0.1% TFA) (1 :1, 2 :1, 3 :1 and 4 :1, v/v). The last fraction was further purified by preparative HPLC(Delta Prep-4000, Waters) using ODS-5 column(20mm×250mm, Nomura Chem. Co. Ltd., Seto, Japan) at 40°C with a flow rate of 5ml/min monitoring at 525nm. Solvent systems used were as follows; a linear gradient elution for 40min from 40 to 85% solvent B(1.5% H_3PO_4, 20% HOAc, 25% CH_3CN in H_2O) in solvent A(1.5% H_3PO_4 in H_2O). Three major anthocyanin pigments, P_1(21mg), P_2(65mg), P_3(51mg) were obtained.

Thin-layer chromatography(TLC) analyses

TLC of three purified anthocyanins from sweet potato roots was carried out on cellulose plate(20×20cm, 0.2mm, Merck, Darmstadt, Germany) by using following solvent systems; BAW(n-BuOH-HOAc -H_2O, 4 : 1 : 5), BuHCl (n-BuOH-2M HCl, 1 : 1), AHW(HOAc-HCl-H_2O, 15 : 3 : 82), and 1% HCl(HCl-H_2O, 3 : 97).

Instrumental analyses

(a) UV-visible spectrometry: UV-visible absorption spectra of purified anthocyanins were recorded on a spectrophotometer(Spectronic Genesys, Milton Roy, U.S.A.) in 0.1% HCl in MeOH.

(b) ^{1}H-NMR and FAB-MS spectrometry: ^{1}H-NMR (500MHz) was measured on a Varian Unity Plus spectrometer(California, U.S.A.) in CF_3CO_2D-DMSO-d_6(1 : 9) containing tetramethylsilane(TMS) as an internal standard, and the chemical shifts were given as δ values. The fast atom bombardment mass spectra(FAB-MS) was recorded on a JEOL JMS-AX-505 WA(Japan Electron and Optics Laboratory Co. Ltd., Tokyo, Japan) with glycerol as the mounting matrix.

RESULTS AND DISCUSSION

Isolation and purification of anthocyanins

The typical HPLC chromatogram of crude anthocyanin

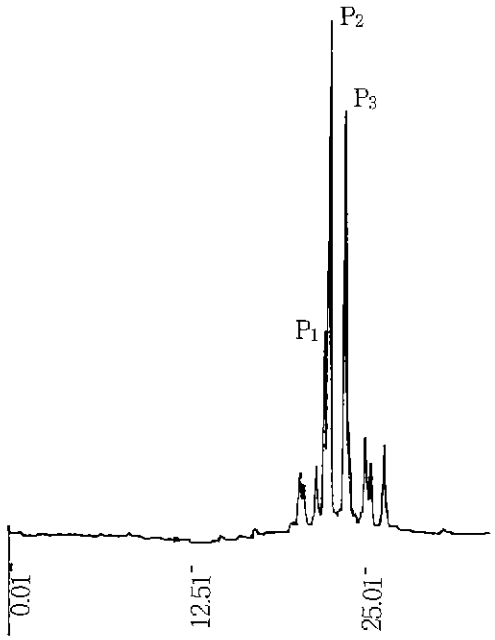

Fig. 2. HPLC chromatogram of crude anthocyanins extracted from purple sweet potato roots.
HPLC(Waters Delta Prep 4000) conditions: column, ODS-5(20×250mm); flow rate, 10ml/min; detection at 525nm; column Temp., 40°C. Solvent systems used were as follows; a linear gradient elution for 40min from 40 to 90% solvent B(1.5% H_3PO_4, 20% HOAc, 25% CH_3CN in H_2O) in solvent A(1.5% H_3PO_4 in H_2O).

pigments from sweet potato roots extracted with 0.5% TFA in 95% ethanol is shown in Fig. 2. Three major anthocyanin pigments. P_1~P_3, which represented 10.7~12.2%, 33.8~35.5%, and 24.5~26.8% of the total peak area, respectively, and six minor pigments were observed. Among these anthocyanin peaks, three major anthocyanins were isolated, purified and identified, as described in Materials and Methods.

Chromatographic and spectral data of three anthocyanins are shown in Table 1. The strong intensities of UV absorption at 330nm suggest that three anthocyanin pigments are acylated by cinnamate derivatives such as caffeic and ferulic acids. The ratio of $E_{acyl}/E_{vis\,max}$ of three anthocyanins was higher than that of simple anthocyanin glycosides, indicating that the presence of one or two acylated anthocyanins(19). In addition, the UV-visible absorption maxima of three anthocyanins were shifted towards lower wavelengths compared to that of cyanidin anthocyanidin, and they did not display bathochromic shifts at 529nm on the addition of $AlCl_3$, indicating that the presence of peonidin nucleus in three anthocyanins. Meanwhile, from ^{1}H-NMR spectra, benzene A ring of three anthocyanins has substituted group at H-5 and H-7 position, which was deduced from the 3 part of proton nucleus at 6.95~6.97(H-6), 7.06~7.08(H-8) and 8.91~8.94(H-4)ppm. Benzene B ring was identified as phenol with one methoxyl group at H-3′ position and one hydroxyl group at H-4′ position, which was deduced from the 3 part of proton nucleus at 7.04~7.09(H-5′), 7.96~7.99(H-2′) and 8.30~8.36(H-6′)ppm, and the 3H part at 3.90~3.93ppm representing $-OCH_3$ group. Other spectral data and R_f values of three major pigments were given in Table 1.

Meanwhile, the precise composition of three major anthocyanins was identified by FABMS and ^{1}H-NMR spectroscopy, as well as by analogy with previous studies.

FABMS of P_1 gave its molecular ion peak[M^+] at 1111m/z. Its ^{1}H-NMR spectrum, as measured in trifluoroacetic-d_1-DMSO-d_6(1 : 9), showed the presence of a peonidin nucleus, two caffeic acids, and three hexoses. Three hexoses must be β-glucopyranoside forms based on the vicinal coupling constants with J values($J_{1,2}$ $J_{2,3}$ $J_{3,4}$ and $J_{4,5}$) of 7.0~10.0 Hz. By application of the negative NOE difference spectroscopy(24,25), anomeric protons at δ5.66, 5.13 and 4.76 were assigned as glucose A, B and C, respectively. Two caffeic acids were determined to be bonded to C-6 hydroxyl groups of glucose A and C by the observation of low-field shifts of both methylene protons(H-6a & H-6b) of glucose A and C at δ4.29, 4.35 and 4.02, 4.09. Moreover, the

Table 1. R_f values, spectral properties, and FAB-MS spectra of three major anthocyanins isolated from purple sweet potato roots

Anthocyanin	R_f values[1)](×100)				Spectral data[2)]			FAB-MS[3)]
	BAW	BuHCl	1%HCl	AHW	λ_{max}(nm)	E_{acyl}/E_{max}(%)	$AlCl_3$	$[M]^+$
P_1	30	15	15	68	529, 296, 330	98	0	1111
P_2	40	18	16	74	529, 295, 330	95	0	1125
P_3	43	25	23	75	526, 285, 331	51	0	1069

[1)]Developed in cellulose plate by descending method at 25°C using following solvent systems; BAW(n-BuOH-HOAc-H_2O. 4:1:5); BuHCl(n-BuOH-2M HCl, 1:1); 1%HCl(HCl-H_2O, 3:97); AHW(HOAc-HCl-H_2O, 15:3:82)
[2,3)]Determined in 0.1% HCl-MeOH, and glycerol as a matrix, repectively

Table 2. ^{1}H-NMR spectra data of three major anthocyanins isolated from purple sweet potato roots (in CF_3CO_2D-DMSO-d_6, 1 : 9 at 25°C)

Proton	Anthocyanin		
	P_1	P_2	P_3
Peonidin			
4	8.93 s	8.94 s	8.91 s
6	6.96 br s	6.97 br s	6.95 d (1.5)
8	7.06 d (1.7)	7.08 d (1.7)	7.08 br s
2	7.99 d (1.8)	7.97 d (1.8)	7.96 d (2.7)
5′	7.06 d (8.9)	7.04 d (8.8)	7.09 d (9.3)
6′	8.36 dd (1.8, 8.9)	8.34 dd (1.8, 8.8)	8.30 dd (2.7, 9.3)
3′-OMe	3.91	3.90	3.93
Caffeic acid (I)			
2	6.96 br s	6.97 d (1.8)	6.96 br s
5	6.76 br s	6.78 d (9.0)	6.77 d (8.7)
6	6.85 dd (1.8, 9.0)	6.84 dd (1.8, 9.0)	6.85 dd (1.8, 8.7)
H_α	6.12 d (15.3)	6.12 d (15.6)	6.11 d (16.2)
H_β	7.32 d (15.3)	7.31 d (15.6)	7.31 d (16.2)
Caffeic acid (II)		Ferulic acid (II)	*p*-Hydroxybenzoic acid (II)
2	6.87 br s	6.85 br s	
5	6.75 d (9.0)	6.75 d (9.0)	3′& 5′- 6.64 d (8.4)
6	6.78* dd (2.0, 9.0)	6.77* dd (2.0, 9.0)	2′& 6′- 7.49 d (9.3)
H_α	5.90 d (15.5)	6.05 d (15.3)	
H_β	7.16 d (15.5)	7.21 d (15.3)	
3-OMe		3.80	
Glucose			
Sophorose-glucose A			
1	5.66 d (7.0)	5.62 d (7.0)	5.61 d (7.5)
2	3.83 t (8.1)	3.95 t (8.1)	3.96 t (8.6)
3	3.75 t (8.8)	3.76 t (8.9)	3.79 t (8.9)
4	3.40 t (8.8)	3.49 t (8.9)	3.54 t (8.9)
5	3.90[1)] m	3.89* m	3.83* m
6a	4.29 dd (8.8, 11.0)	4.28 dd (8.9, 11.0)	4.29 dd (8.8, 11.0)
6b	4.35 d (11.0)	4.38 d (11.0)	4.39 d (11.0)
Sophorose-glucose C			
1	4.76 d (7.7)	4.77 d (7.0)	4.76 d (7.8)
2	3.18 t (8.4)	3.19 t (8.5)	3.18 t (8.6)
3	3.25* m	3.29* m	3.27* m
4	3.42 t (9.5)	3.42 t (9.5)	3.42 t (9.6)
5	3.28* m	3.31* m	3.30* m
6a	4.02 dd (8.0, 11.4)	4.03 dd (8.0, 11.4)	4.09 dd (8.0, 11.5)
6b	4.09 dd (11.4)	4.09 dd (11.4)	4.13 d (11.5)
5-Glucose B			
1	5.13 d (7.7)	5.12 d (7.5)	5.15 d (7.8)
2	3.57 t (8.4)	3.58 t (8.5)	3.57 t (8.5)
3	3.42 t (11.0)	3.42 t (11.0)	3.45 t (11.1)
4	3.30 t (10.0)	3.30 t (10.0)	3.31 t (10.2)
5	3.50* m	3.50* m	3.50* m
6a	3.59* dd (7.8, 11.7)	3.59* dd (7.8, 11.7)	3.57* dd (7.8, 11.7)
6b	3.82 d (11.7)	3.82 d (11.7)	3.82 d (11.7)

[1)]Assigned by ^{1}H-^{1}H COSY
Coupling constants(J in Hz) in parentheses

glycosidic linkage of sophorose(glucose A and C) was deduced from a low-field shift(3.96ppm) of glucose A H-2, suggesting that C-1 of glucose C was bonded to C-2 of glucose A. Anomeric protons of glucose A and C were finally related to the two acylated methylene protons(δ4.29 and 4.35) and (δ4.02 and 4.09), respectively, by ^{1}H-^{1}H COSY spectrum(4). Other protons of P_1 were similar to those of acylated anthocyanin isolated from

Pharbitis nil(26). From the above data, P_1 was determined to be 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-caffeylglucopyranosyl)-β-D-glucopyranosyl-5-*O*-(β-D-glucopyranosyl)-peonidin. This anthocyanin pigment was first isolated and identified from purple sweet potato roots, although the pigment has already been reported to be present in the stems of sweet potato(16).

The FABMS of P_2 gave its molecular ion peak [M^+] at 1125m/z. Its ^{1}H-NMR spectrum exhibited six protons of a peonidin nucleus and three protons of a methoxyl group assignable to ferulic acid. Three anomeric protons of glucose and the glycosidic linkage of sophorose(glucose A and C) were in full agreement with those of P_1. Additionally, the bonding site of caffeic and ferulic acids to glucose A and C, as well as four olefinic protons of two acids were almost consistent with that of P_1, although the acid bonded to each glucose was not established firmly. Thus, P_2 was identified as 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*trans*-feruloylglucopyranosyl)-β-D-glucopyranosyl-5-*O*-(β-D-glucopyranosyl)-peonidin.

The FABMS of P_3 gave its molecular ion peak [M^+] at 1069m/z. Its ^{1}H-NMR spectrum exhibited the presence of a peonidin, one mole of caffeic and *p*-hydroxybenzoic acids. Three anomeric protons of glucose and the glycosidic linkage of sophorose(glucose A and C) were similar to those of P_1 and P_2. However, two olefinic protons at δ_H 6.64(d, J=8.4Hz) and δ_H 7.49(d, J=9.3Hz) assignable to *p*-hydroxybenzoic acid were specifically observed. Although we did not confirm correctly where the acid was bonded to the C-6 position of glucose A and C, the *p*-hydroxybenzoic acid could likely be bonded to the C-6 position of glucose C due to low-field shift of C-6a and C-6b position of glucose C in comparison with those of glucose C of P_1 and P_2. Thus, P_3 was identified as 3-*O*-(6-*O*-*trans*-caffeyl)-2-*O*-(6-*O*-*p*-hydroxylbenzoylglucopyranosyl)-β-D-glucopyranosyl-5-*O*-(β-D-glucopyranosyl)-peonidin. The assignments of ^{1}H-NMR and chemical structures of three anthocyanins are given in Table 2 and Fig. 3.

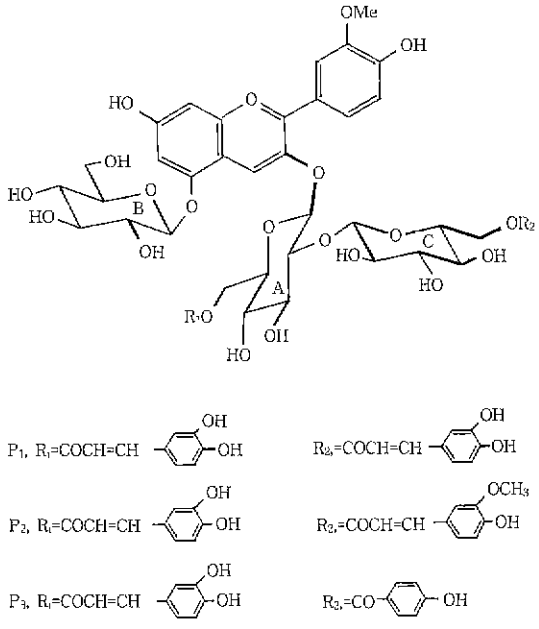

Fig. 3. Chemical structures of three major anthocyanins isolated from purple sweet potato roots.

Thus, the complete structural assignments of three major diacylated anthocyanins from purple sweet potato roots are reported here for the first time, although those anthocyanins were already found in several cultivars and tissues of sweet potato(16-20). It is very interesting to note that the newly bred purple sweet potato contained a fairly large amount of acylated peonidin glucosides(in fresh periderms around 70~80% against total anthocyanin contents). Therefore, future successful biotechnological processes using cell cultures for the production of these stable anthocyanins as potential source of natural food colorants is promising. Further isolation and identification of minor anthocyanin pigments from purple sweet potatoes are needed.

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