Anthocyanins isolated from purple corn (*Zea mays* L.)

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Summary

Six anthocyanins were isolated from Peruvian purple corn seed (*Zea mays* L.) as raw material for a food colorant, and their complete structures were determined by means of spectroscopic analyses. The major anthocyanin was cyanidin 3-*O*-*β*-D-glucoside. The other five anthocyanins were pelargonidin 3-*O*-*β*-D-glucoside, peonidin 3-*O*-*β*-D-glucoside, cyanidin 3-*O*-*β*-D-(6-malonyl-glucoside), pelargonidin 3-*O*-*β*-D-(6-malonyl-glucoside) and peonidin 3-*O*-*β*-D-(6-malonyl-glucoside). Pelargonidin 3-*O*-*β*-D-(6-malonyl-glucoside) was found in purple corn for the first time. The cyanidin derivatives constitute around 70% in purple corn seed.

1. Introduction

There are various kinds of corn in the world, and they have various colors such as white, yellow, red, purple, brown, green and blue. Purple corn (*Zea mays* L., Photograph 1) has been cultivated in Latin America, mainly in Peru, and Peruvian people have been utilizing purple corn as a food material for centuries. “Chicha Morada” is a typical drink in Peru, which is made with water in which purple corn has been simmered.

The color of purple corn is due to anthocyanin. The anthocyanin colorant obtained from purple corn is approved in Japan and listed in the “Existing Food Additive List” as purple corn color. Purple corn color has been using for coloring beverages, jellies, candies and so on in Japan.

Recently, anthocyanins have been reported to have various biological activities, such as antioxidant [1~3], anti-mutagenic [4], and anticancer activities [5, 6]. Especially, purple corn colorant has been reported to decrease the carcinogenesis in rat colon induced by PhIP (2-amino-1-methyl-6-phenyl-imidazo [4,5-*b*] pyridine) [7]. In this way, anthocyanins have been noted not only as a food colorant but also as a health food material.

In the "Existing Food Additive List", purple corn color is described as obtained by extraction from purplish seeds of *Zea mays* L. in hot water or weak acidic aqueous solution, and that the main anthocyanin is cyanidin-3-glucoside. There are several reports concerning anthocyanin in purple corn plants. Cyanidin 3-glucoside, cyanidin galactoside and pelargonidin glucoside were found in purple corn seeds [8~12]. It was reported that purple corn leaves contain cyanidin 3-rahmnoside [13] and cyanidin 3-glucoside acylated with malonic acid [8]. Recently, Fossen *et al*. identified cyanidin 3-glucoside, peonidin 3-glucoside and their derivatives acylated with malonic acid in purple corn leaves and flowers [14].

In this study, we elucidated the chemical structures of anthocyanins that are contained in purple corn seed as raw material for a food colorant using MS and ^{1}H-, ^{13}C-NMR.

2. Materials and Methods

2-1. Materials

Dried purple corn seeds (*Zea mays* L.) collected in Peru in 2000 were used. Trifluoroacetic acid (TFA)-*d* and dimethylsulfoxide (DMSO)-d_6 were obtained from Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemicals Industry (Osaka, Japan). HPLC grade acetonitrile (MeCN) was used. Water was purified with the Easy Pure RO System (Barnstead, IA, USA) and the Ultra Pure Water System CPW-101 (ADVANTEC, Tokyo,

Japan).

2-2. Extraction anthocyanins from purple corn

One kg of purple corn seeds were pulverized and extracted with 10 l of 0.1 % TFA aqueous solution for 12 hours at 40°C. The extracts were filtered through filter paper (ADVANTEC, Filter Paper No.5C).

2-3. HPLC analysis

The equipment consisted of an injector with a 100 μl loop (JASCO, AS-1555), two pumps (JASCO, PU-1580), a degasser (JASCO, DG-980-50), and a detector (JASCO, UV-1570). Chromatograms were recorded at the absorbance of 515 nm. HPLC was carried out on a Symmetry C30 column (250×4.6mm i.d.; 5 μm, Waters, MA, USA).

An aqueous solution containing 0.1% TFA and MeCN were used as the eluents. The column was eluted at 40°C using consecutive linear gradients of 0-15% MeCN for 10 minutes, 10-30% MeCN for 40 minutes, and 80% MeCN for 10 minutes at a flow rate of 1 ml/min.

2-4. Isolation of anthocyanin from purple corn

The filtrate of purple corn extract was applied to 1 l of a Sepabeads SP-207 resin column (Mitsubishi Chemical, Japan). The resin was washed with 3 l of water, and then eluted with 50 % of ethanol aqueous solution containing 0.1 % of TFA. The eluate was dried under *vacuum* at 40°C.

The high-speed counter-current chromatography (HSCCC) system consisted of a horizontal flow-through planar centrifuge with an Ito Multilayer coil (Pharma-Tech Research Co., Model CCC-1000, MD, USA), a pump (JASCO, 880-PU), a Microflow pH sensor (Broadley-James, Model 14, CA, USA), a manual injection valve with a 20 ml loop, and a fraction collector (JASCO,

SF-212N). The upper phase, consisting of a mixture of *tert*-butylmethylether: 1-butanol: MeCN: water (2:2:1:5 v/v) containing 0.2% of TFA, was used as the stationary phase, while the lower phase was used as the mobile phase. A total of 300 mg of crude anthocyanin extract was dissolved in 20 ml of a mixture of stationary phase: mobile phase (3:1 v/v), and introduced through the injection port. The mobile phase was pumped at 2.5 ml/min, while centrifugation was carried out at 1000 rpm. Four ml of each fraction was collected.

Each fraction was analyzed by HPLC as mentioned before, and further purification was carried out using preparative HPLC. The equipment consisted of a sample loader (Waters, 170), a pump (Waters, 400F), a system controller (Waters, 4000), and a multiwavelength detector (Waters, 490E). Preparative HPLC was carried out on an L-column ODS column (250×10 mm i.d.; 5 μm, Chemicals Evaluation And Research Institute, Tokyo, Japan). The mobile phase consisted of 0.1 % TFA aqueous solution and MeCN and liner gradient elution was used at flow rate of 10 ml/min and monitored at 515 nm.

2-5. Identification of anthocyanin

^{1}H and ^{13}C NMR, ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple bond coherence (HMBC), and heteronuclear multiple quantum coherence (HMQC) of isolated anthocyanins in TFA-*d*/DMSO-d_6 (1:9) with tetramethylsilane (TMS) as an internal standard were measured using a JEOL Datum JMN-LA400 spectrometer (JEOL, Tokyo, Japan). LC/MS analysis was performed with a VG Biotech Platform (Biotech, UK) mass spectrometer with electrospray ionization in positive ion mode. The isolated anthocyanins were introduced directly into the MS apparatus. The cone voltage (90 eV) was applied, and the flow rate of ESI nebulising gas (N_2) was 25 l/hour.

3. Results and Discussion

3-1. Identification of anthocyanin from purple corn

Typical HPLC chromatogram at 515 nm of crude extract of purple corn is shown in Fig. 1 and eight anthocyanins were found (named from PCA-1 to 8 in elution order). The results of MS analysis for purple corn anthocyanins are summarized in Table 1. The fragment ion peaks corresponding to three anthocyanidins, cyanidin at *m/z* 287, pelargonidin at *m/z* 271 and peonidin at *m/z* 301 were detected. The analysis of $[M]^+$ peaks indicated the presence of malonic acid moiety in PCA-4 ~ 8.

In this study, six purple corn anthocyanins (PCA-1, 2, 3, 5, 6, and 7) were isolated from the crude purple corn extract using an absorbing resin, HSCCC and preparative HPLC. The complete structures of isolated anthocyanins were established by ^{1}H- and ^{13}C-NMR analysis using HMBC techniques. Full assignments of ^{1}H- and ^{13}C-NMR were performed with aid of COSY, HMQC and HMBC and the ^{1}H- and ^{13}C-NMR chemical shifts of purple corn anthocyanin are summarized in Tables 2 and 3. The spectra show all sugars to be *β*-D-glucopyranosyl configuration. The connecting positions of glucose to aglycon for each pigment were confirmed by HMBC. The higher chemical shifts obtained for the proton at the position 6 in glucose for PCA-5, 6 and 7 showed the presence of substitution on that position.

The structures of the PCAs estimated on the basis of spectral means are shown in Fig. 2. Six anthocyanins found in purple corn seed were identified as cyanidin 3-*O*-*β*-D-glucoside (PCA-1), pelargonidin 3-*O*-*β*-D-glucoside (PCA-2), peonidin 3-*O*-*β*-D-glucoside (PCA-3), cyanidin 3-*O*-*β*-D-(6-malonyl-glucoside) (PCA-5), pelargonidin 3-*O*-*β*-D-(6-malonyl-glucoside) (PCA-6) and peonidin 3-*O*-*β*-D-(6-malonyl-glucoside) (PCA-7), respectively. Of great interest is the pelargonidin 3-*O*-*β*-D-(6-malonyl-glucoside), this pigment is found in purple corn for the first time.

PCA-4 and PCA–8 were not analyzed by NMR. On the basis of MS spectrum, the authors considered that PCA-4 was malonylated cyanidin 3-glucoside. Also, the connecting position of the malonic acid moiety of PCA-4 would differ from that of PCA-5. The MS information obtained for PCA-8 indicated cyanidin-3-(dimalonylglucoside) as well. So we assumed that it might be cyanidin 3-(3,6-dimalonylglucoside) as previously reported [14]. Cyanidin galactoside was not detected in this study. Further study is necessary for PCA-4 and –8.

In this paper, the authors elucidated that cyanidin, pelargonidin, peonidin and their molonylated derivatives are present in purple corn seeds.

3-2. Relative quantitative anthocyanin content in purple corn

To estimate of the proportion of anthocyanin in purple corn seed, anthocyanins were extracted from several samples and analyzed by HPLC method. Cyanidin derivatives predominated in purple corn seed (around 70%) as shown in Table 4. Small amount of pelargonidin derivatives and peonidin derivatives were detected. Anthocyanins in flowers and leaves were reported to be more than 90% of cyanidin derivatives [14] and a little difference between the proportion of anthocyanins in seed and that in flowers/leaves was observed.

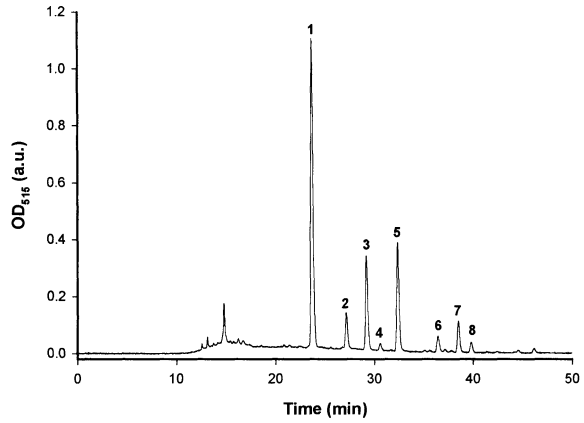

Fig. 1. HPLC chromatogram of purple corn extract

Table 1. MS spectral data for the purple corn anthocyanins

Compound	*m/z*	
PCA-1	449.0[Cy+Glc]$^+$	287.0[Cy]$^+$
PCA-2	433.0[Pg+Glc]$^+$	271.0[Pg]$^+$
PCA-3	463.1[Pn+Glc]$^+$	301.1[Pn]$^+$
PCA-4	535.0[Cy+Glc+Mal]$^+$	287.0[Cy]$^+$
PCA-5	535.1[Cy+Glc+Mal]$^+$	287.0[Cy]$^+$
PCA-6	519.1[Pg+Glc+Mal]$^+$	271.0[Pg]$^+$
PCA-7	549.1[Pn+Glc+Mal]$^+$	301.0[Pn]$^+$
PCA-8	621.1[Cy+Glc+2Mal]$^+$	287.1[Cy]$^+$

Cy, cyanidin; Pg, pelargonidin; Pn, peonidin; Glc, glucose; Mal, malonic acid.

Table 2. ^{1}H-NMR spectral data for the purple corn anthocyanins (δ_H ppm in TFA-*d*: DMSO-d_6=1:9)

Position	PCA-1	PCA-2	PCA-3	PGA-5	PGA-6	PCA-7
Aglycon						
4	8.85 *s*	9.02 *s*	8.99 *s*	8.79 *s*	8.91 *s*	8.91 *s*
6	6.66 *d* (2.0)	6.77 *d* (1.7)	6.76 *s*	6.69 *d* (2.0)	6.77 *d* (2.0)	6.78 *d* (1.2)
8	6.86 *d* (1.2)	7.02 *s*	7.05 *s*	6.87 *d* (1.2)	7.00 *d* (1.0)	7.05 *d* (1.0)
2'	7.97 *d* (2.4)	8.65 *d* (9.3)	8.22 *d* (2.2)	7.97 *d* (2.4)	8.61 *d* (9.3)	8.17 *d* (2.2)
3'		7.12 *d* (9.0)			7.09 *d* (9.0)	
5'	7.00 *d* (8.8)	7.12 *d* (9.0)	7.12 *d* (8.8)	7.01 *d* (8.8)	7.09 *d* (9.0)	7.12 *d* (8.8)
6'	8.19 *dd* (2.4, 8.8)	8.65 *d* (9.3)	8.30 *dd* (2.1, 8.7)	8.21 *dd* (2.4,8.8)	8.61 *d* (9.3)	8.31 *dd* (2.2, 8.8)
OMe			3.96 *s*			3.96 *s*
Glucose						
1	5.32 *d* (7.8)	5.40 *d* (7.6)	5.41 *d* (7.8)	5.37 *d* (7.8)	5.42 *d* (7.6)	5.45 *d* (7.6)
2	3.49 *m*	3.52 *m*	3.49 *m*	3.51 *t*	3.52 *t*	3.55 *t*
3	3.37 *t*	3.44 *t*	3.43 *t*	3.40 *t*	3.44 *t*	3.45 *t*
4	3.23 *t*	3.30 *t*	3.28 *t*	3.23 *t*	3.28 *t*	3.28 *t*
5	3.49 *m*	3.55 *m*	3.53 *m*	3.80 *m*	3.85 *m*	3.86 *m*
6a	3.49 *m*	3.56 *m*	3.54 *m*	4.11 *m*	4.16 *m*	4.17 *m*
6b	3.70 *m*	3.78 *m*	3.77 *m*	4.44 *m*	4.50 *m*	4.48 *m*
Malonyl						
2				3.35 *d* (2.2)	3.41 *d* (2.4)	3.40 *d* (1.7)

*Values in parentheses indicate coupling constant (*J* in HZ).

Table 3. ^{13}C-NMR spectral data for the purple corn anthocyanins (δ_X ppm in TFA-*d*: DMSO-d_6=1:9)

Position	PCA-1	PCA-2	PCA-3	PGA-5	PGA-6	PCA-7
Aglycon						
2	161.9	162.2	161.8	162	162.3	161.9
3	144.4	144.3	144.3	144.4	144.2	144.3
4	135.1	136	135.9	134.7	135.5	135.4
5	157.9	157.9	157.8	157.7	157.6	157.6
6	102.5	102.5	102.5	102.6	102.5	102.5
7	168.7	168.9	168.8	168.6	168.7	168.7
8	94.3	94.5	94.6	94.4	94.5	94.7
9	156.1	156.3	156.2	156.1	156.2	156.2
10	111.1	112.3	112.3	112	112.2	112.2
1'	119.7	119.2	119.7	119.8	119.6	119.7
2'	117.7	134.8	114.5	117.6	134.8	114.5
3'	146.3	117.1	148.3	146.3	117.0	148.3
4'	154.6	164.9	155.1	154.6	164.9	155.1
5'	116.9	117.1	116.8	117.0	117.0	116.8
6'	127.1	134.8	127.9	127.2	134.8	128.1
OMe			56.2			56.2
Glucose						
1	102.3	102.5	102.6	101.9	102.1	102.2
2	73.3	73.4	73.6	73.1	73.1	73.3
3	76.7	76.7	76.7	76.3	76.3	76.3
4	69.9	69.9	69.8	70.1	70.0	70.0
5	77.9	77.9	77.9	74.5	74.5	74.5
6	61	61	60.9	64.6	64.6	64.6
Malonyl						
1				167.3	167.3	167.2
2				41.4	41.4	41.4
3				168.2	168.2	168.1

Table 4. Relative quantitative anthocyanin content in purple corn seed

	Compound	
PCA-1	Cy3-Glc	44.2±6.0
PCA-2	Pg3-Glc	4.9±0.9
PCA-3	Pn3-Glc	11.5±4.3
PCA-4	malonyl Cy3-Glc	2.5±0.9
PCA-5	Cy-3-(6-Mal-Glc)	23.2±4.5
PCA-6	Pg-3-(6Mal-Glc)	4.3±1.0
PCA-7	Pn-3-(6-Mal-Glc)	6.0±1.2
PCA-8	dimalonyl Cy-3-Glc	3.3±1.7
	Cyanidin derivatives	73.3 ± 4.7
	Pelargonidin derivatives	9.3±0.7
	Peonidin derivatives	17.5±5.1

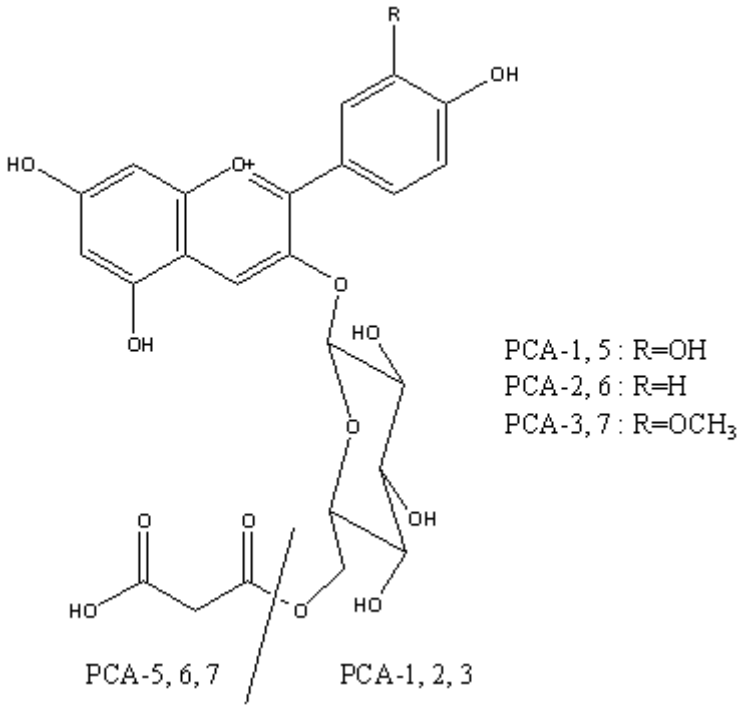

Fig. 2. Structures of purple corn anthocyanins

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