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Article

Identification of Anthocyanins from Pigmented Rice Seeds

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Anthocyanins pigments were isolated from the pigmented rice seeds (cultivar Killimhuk-mi) by a combination of Sephadex LH-20 and preparative high performance liquid chromatographies. Four anthocyanins were identified by their chromatographic, spectral and chemical properties. The major pigment was cyanidin 3-glucoside and cyanidin 3-oxalyl-glucoside was found in the rice for the first time.

Key words : *anthocyanin, rice pigment, cyanidin 3-glucoside, cyanidin 3-oxalylglucoside.*

Anthocyanins are probably the most widespread water-soluble pigments in plant kingdom. With the current trend of avoiding synthetic colorants as food additive due to their suspected toxicity, the use of natural pigments in foods, pharmaceuticals, and cosmetics is expected to increase.[1)] However, unlike synthetic dyes, anthocyanins have not been widely used as food colorant because of their instability during storage and processing. In recent years, it has been found that anthocyanins containing two or more aromatic acyl groups are stable in weakly acidic and neutral aqueous solutions.[2)]

Pigmented rice, also known as black rice is actually red or purple colored and an important crop in Asia. Color components of pigmented rice are mainly anthocyanins.[3)] Cyanidin 3-glucoside is the major anthocyanin isolated from Japanese black rice.[4] There were previous reports on the structure and thermal stability of the anthocyanins from pigmented rice seeds[5-7] grown in Korea. The authors have also reported optimal extraction conditions for the rice pigments.[8]

In this study, we attemped to identify the anthocyanins from one of the less-examined cultivar, killimhuk-mi, and found cyanidin glucoside as major pigment. Cyanidin 3-oxalylglucoside, which was not previously found in the pigmented rice, was found to be occuring as a minor component.

Materials and Methods

Rice cultivars. *Oryza sativa* cutivar Suwon 415 (S-415), S-425, Sanhaehyanghyolla (上海香血糯), Jajin (紫珍), LKB-2-1-1, Jakwang-do (紫光稻), Killimhuk-mi (吉林黑米) and Heukjin-mi (黑珍米) were from the stock grown in 1996 at Crop Experiment Station, Rural Development Admisnistration, Suwon, Korea.

Extraction and separation. Dried rice seeds, *ca* 10 g, were suspened in 100 ml of 0.035% HCl in EtOH, and stirred on a magnetic stirrer for 1 hour and centrifuged for 30 minutes at 8,300 g. The volume of the supernatant was reduced to *ca* 15 ml on a rotatory evaporator and was chromatographed on a Sephadex LH-20 column (3×60 cm; 25~100 μ, 1.0 ml/min) with 50% EtOH as eluent. Fraction volume was 20 ml, and tube number 15 through 25 were pooled and named fraction II. The fraction was dried on a rotatory evaporator and finally in a desiccator. To purify the anthocyanins, the dried crude pigment was redissolved in EtOH and chromatographed on HPLC equipped with μBondapak C-18 column [19× 300 mm; eluent A (MeOH : HCOOH : H_2O=5 : 4 : 1) and B (H_2O : HCOOH=9 : 1); linear gradient A from 15% to 90% in B for 25 minutes, detection at 280 nm; flow rate, 10 ml/min].[9)] Fraction containing pure pigment was collected and freeze-dried after removing most of the organic solvent.

Acid hydrolysis and derivatization. The procedure of acid hydrolysis and derivatization for gas chromatographic analysis was based on Gao and Mazza's method.[10)]

Gas chromatographic conditions. Sugar was analyzed as follows: HP-101 capillary column, 0.22 mm×25 m; temperature programed from 120°C to 180°C at 20°C/min, followed by linear increase at 5°C/min upto 200°C; split ratio of 1:20; temperature of detector and injector at 250°C; flow rate, 1 ml/min. Methyl oxalate was analyzed

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as follows: temperature programming from 35°C for 2 min, followed by linear increase to 200°C at 20°C/min.[10] The other conditions were same as those for sugar analysis.

Spectral analysis. UV-VIS spectra were obtained on an HP8452A diode-array spectrometer (Hewlett Packard, Waldbronn, Germany). Mass spectra were obtained on a VG70-VSEQ (VG analytical, UK) spectrometer operating in FAB-MS mode with 35 KeV Cs^+ ion beam. NMR spectra were determined on a JNM LA 400 spectrometer (JEOL, Japan) operating at 400 MHz for ^1H and 100 MHz for ^{13}C on samples dissolved in CD_3OD with a trace of DCl.

Quantative analysis of anthocyanins in other cultivars. One hundred milligrams of dried rice seeds were crushed and extracted with 0.1% trifluroacetic acid in methanol with monitoring at 500 nm until most pigment was extracted, and the extract was filtered through a syringe filter (0.22 μm). The filtrate was dried on a rotary evaporator and analyzed by HPLC: Bakerbond standard octadesyl (C_{18}) column, 5 μm, 4.6×250 nm; monitoring at 280 nm; flow rate 0.9 ml/min; HCOOH : H_2O= 1 : 9 (A), MeOH : HCOOH : H_2O=5 : 1 : 4 (B); gradient of B from 15% to 90% in solvent A for 25 min then held isocratic for 10 min.

Results and Discussion

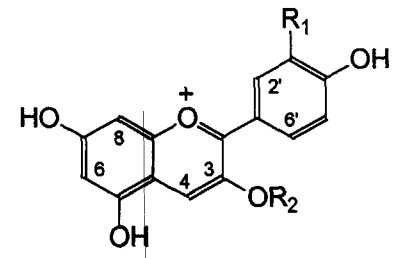
The Sephadex LH-20 chromatography of ethanol extract of the pigmented rice gave the complex mixture of anthocyanins. The total content of anthocyanins in Killimhuk-mi was measured quantitatively by HPLC analysis (2.53 mg/g, dried seed). The structure of each compound was elucidated by NMR, FAB-MS, UV-VIS spectrometries and chemical degradation followed by chromatographic analysis.

Cyanidin 3-*O*-β-D-glucoside[11] (**1**), peonidin 3-*O*-β-D-glucoside[12] (**2**), cyanidin *O*-β-D-(6"-*O*-β-D-rhamnosyl)glucoside[13] (**4**) and quercetin 7-*O*-β-D-glucoside[14] (**5**) were identified from the comparision of the spectral data with the previously reported ones (Table 1). It was reported

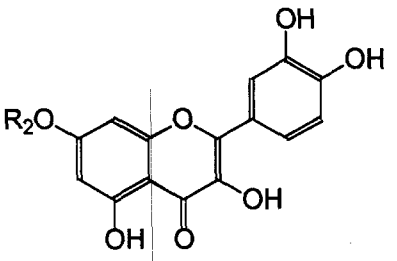
Table 1. Spectral data of the isolated compounds.[a]

	1		2		3	4	5
	^1H	^{13}C	^1H	^{13}C	^1H	^1H	^1H
2		163.2		163.2			
3		145.6		145.5			
4	8.97(s)	136.7	9.01(s)	137.2	8.99(s)	8.94(m)	
5		159.1		159.2			
6	6.62(s)	103.2	6.64(s)	103.3	6.65(s)	6.67(s)	6.21(d,*J*=2.0)
7		170.3		170.1			
8	6.86(d,*J*=1.1)	95.0	6.92(d,*J*=1.0)	95.3	6.909(d,*J*=1.2)	6.90(s)	6.40(d,*J*=2.0)
9		157.6		157.7			
10		113.2		113.5			
1'		121.1		121.0			
2'	7.99(s)	118.2	8.08(s)	117.6	8.03(s)	8.03(d,*J*=2.2)	7.71(d,*J*=2.4)
3'		147.3		149.4			
4'		155.7		156.6			
5'	6.97(d,*J*=8.8)	117.3	7.03(d,*J*=8.8)	115.2	7.02(d,*J*=8.8)	7.03(d,*J*=8.8)	6.87(d,*J*=8.4)
6'	8.23(d,*J*=8.8)	128.3	8.23(d,*J*=8.4)	128.8	8.26(d,*J*=8.8)	8.28(d,*J*=8.8)	7.59(d,*J*=8.4)
-OMe			4.00(s)	56.9			
Glucose							
1"	5.29(d,*J*=7.2)	103.6	5.32(d,*J*=7.6)			5.29(d,*J*=7.6)	5.29(d,*J*=7.2)
2"		74.7		103.8	5.27(d,*J*=7.8)		
3"		78.0		74.9			
4"	3.92-3.29(m)	71.0	3.93-3.30(m)	78.1		3.78-3.32(m)	3.92-3.29(m)
5"		78.7		71.1	3.88-3.16(m)		
6a"	3.90(m)	62.3		78.8		4.01(m)	
6b"	3.75(m)			62.3	4.25(m)	3.90(m)	
Rhammnose					4.15(m)		
1"'						4.63(s)	
2"'							
3"'						3.78-3.32(m)	
4"'							
5"'							
-CH_3						1.15(d,*J*=6)	
FAB-MS, m/z	449 $[M]^+$		463 $[M]^+$		613[M+glycerol]$^+$	597 $[M+2]^+$	
λ_{max}, nm (0.01%MeOH-MeOH)	530		528		529	530	
$AlCl_3$ shift	+				+	+	

[a]Chemical shifts are measured at 400 MHz and expressed in δ (ppm). Coupling constants are expressed in Hz.



	R_1	R_2
1,	OH	O-β-D-glucopyranosyl
2,	OMe	O-β-D-glucopyranosyl
3,	OH	O-β-D-(6"-O-oxalyl)-glucopyranosyl
4,	OH	O-β-D-(6"-O-β-D-rhamno-pyranosyl)gucopyranosyl



5, R_2 = O-β-glucopyranosyl

that cyanidin 3-glucoside, malvidin 3-galactoside, cyanidin 3-rhamnoside and cyanidin 3,5-diglucoside were present in Japanese black rice.[3,4] However, no anthocyanin with malvidin aglycone was found in the cultivars tested in this experiment. Position of sugar substitution at C-3 of **4** was confirmed from the value of $A_{440}/A_{vis \cdot max}$ at about 25%.[15] Evidence that rhamnose was attached to C-6 of glucose was attained by the observation that chemical shift of H-6 protons was down-shifted compared to compound **1** (Table 1).

The occurrence of cyanidin 3-*O*-β-D-(6"-*O*-oxalyl)glucoside (**3**) in the rice seeds had not been reported before. Because this compound was present in a small quantity, ^{13}C-NMR measurement was not satisfactory. However, physical measurements through ^{1}H-NMR and FAB-MS and chemical degradation followed by GC analysis were sufficient to identify the compound. In ^{1}H-NMR spectra, the down-field shift of the H-6" of glucose from δ 3.75 and δ 3.9 to δ 4.25 and δ 4.15 indicated the presence of an acyl moiety at *O*-6".[15] The ^{1}H-NMR spectra of this compound (**3**) showed almost the same pattern in proton chemical shift with the previously published data.[16] Small discrepancy in the chemical shift from the published data was probably due to concentration of added DCl. To confirm the presence of oxalic acid, acid hydrolysis of the pigment and derivatization with menthanol followed by GC analysis were attempted. The gas chromatographic data clearly indicated the presence of the oxalate moiety (Table 2). The sugar moiety was also identified through acidic hydrolysis and derivatization into trimethylsilyl compound followed by GC analysis (Table 2). The positive mode FAB-MS spectrum showed the peak at m/z, 613 [M+glycerol]$^+$, further confirming the structure as cyanidin 3-*O*-β-(6"-*O*-oxalyl)-glucoside. The compound *3* was known to be present as a major anthocyanin in the various members of Orchidaceae.[16]

Table 2. Retention time of sugars and oxalic acids in capillary gas chromatography.

	Retention time (min)	
	Methyl ester	TMS-sugar
Hydrolysate		
3	1.93	11.08, 13.66
4	·	6.57, 7.48, 11.12, 13.70
5	·	11.15 13.76
Standard		
Oxalic acid	1.94	·
Glucose	·	11.30, 13.77
Galactose	·	10.56, 11.50
Rhamnose	·	6.66, 7.53

Table. 3. Distribution of anthocyanins in rice cultivars.

Compound	Content (μg/g seed)			
Cultivar	**1**	**2**	**3**	**4**
Killimhuk-mi	1,810	600	67	44
S-415	460	140	0.13	139
S-425	1,630	380	24	12.4
Sanhaehyanghyolla	760	130	trace	trace
	retention time (min)[a]			
	19.23	25.80	15.9	22.16

[a]The chromatographic condition is described in the Materials and Methods section.

Distribution of anthocyanins in other colored rice cultivars such as S-415, S-425 and Sanghaehyanghyolla, Jajin, LKB-2-1-1, Jakwang-do and Hukjin-mi was determined. Cyanidin 3-glucoside was the major anthocyanin in the cultivar S-415 as reported previously[6] and other cultivars such as S-425, Killimhuk-mi and Sanghaehyanghyolla. Cyanidin 3-oxalylglucoside was found in Killimhuk-mi, S-415, S-425 and Sanghaehyanghyolla. This suggested that the oxalyglucoside is wide-spread among the colored rice cultivars. Anthocyanins were not dectected by HPLC from Jajin, LKB-2-1-1 and Jakwang-do, which were known to contain melanine-type pigments.[3] Red-purple pigment was extracted from the Heukjin-mi but the chromatogram of the pigment showed no peak matching with those of Killimhuk-mi (Table 3). Identity of pigments from this variety needs further attention in the future.

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