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High Performance Liquid Chromatographic Determination of Anthocyanin Pigments in Some Varieties of Black Rice

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ABSTRACT

Anthocyanin pigments in ten pigmented rice cultivars were isolated with cellulose thin-layer chromatography and preparative high performance liquid chromatography (HPLC), and further identified with conventional chromatographic techniques. Individual anthocyanins were then quantified by HPLC using a reversed ODS-5 column and detected at 280 nm. Ten rice varieties contained two major anthocyanins, cyanidin 3-glucoside and peonidin 3-glucoside. Total anthocyanin contents varied greatly in the range of 0-493 mg/100 g grain among varieties. Cyanidin 3-glucoside, an agent having important oxygen radical absorbing capacity (ORAC), was most abundant in Suwon #415 variety (470 mg/100 g grain), while Jawangdo (10 mg/100 g grain) and Ilpumbyeo (not detected) had the smallest amount of anthocyanin among the samples examined. As for the distribution ratio of individual anthocyanin, Suwon #415 was characterized by a relatively high percentage of cyanidin 3-glucoside (95.3%); whereas, Suwon #425 and Heugjinmi were characterized by a relatively larger amount of peonidin 3-glucoside.

Key words: **anthocyanins, pigmented rice, black rice, cyanidin 3-glucoside, peonidin 3-glucoside, HPLC analysis.**

INTRODUCTION

Rice (*Oryza Sativa* L.) is the principal cereal food in Asia and the staple food of nearly half of the world's population. The quality of rice grains selected for breeding and cultivation is an important concern of the agricultural industry, so is the improvement of the nutritional value of the harvested grain.

Black rice (*Oryza Sativa* L. Indica), having dark purple-colored grains, is a major rice crop in the South Asia and Mainland China. It is broadly known as an enriched rice with medicinal effects. Besides, its purple pigment is widely used as food colorants when processing bread, ice creams and liquor[1-3]. Pigments of rice varieties vary greatly

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in content and distribution, thus provide a fascinating array of topics for taxonomic and genetic studies. Anthocyanin and nonanthocyanin pigments, found in bran or hull part of rice, characterize various pigmented rices based on their red or black grains. Cultivating pigmented rice grains through genetic engineering in the early 1970's began a surge in world production of various kinds of rice grains[4].

Anthocyanins have only recently begun to be regarded as biologically active substances, as well as colorants. For example, their anti-inflammatory activity[5], redox potentials[6] and antioxidative activity[7-10] have all been studied.

Recently, antioxidative activity of human low-density lipoprotein caused by phenolic substances in red wine (at pH 7.4) was also reported by Frankel *et al*[11]. Anthocyanins are now being isolated from the residue of pressed grapes used in red wine manufacturing to make colorants for food and pharmaceuticals[12].

There is considerable interest in developing food colorants from natural sources to replace synthetic ones. In particular, the role of anthocyanins as food coloring agents becomes very important since they are universally associated with attractive, colorful, and flavorful fruits[13]. Cyanidin 3-glucoside (C3G), which is abundant in pigmented rice, has been known to effect diverse physiological function in addition to its strong antioxidant activity. Antioxidative properties have been extensively investigated in rice[14].

The purpose of our work was to isolate, and characterize anthocyanin pigments in newly bred black rice using a rapid and efficient method.

MATERIALS AND METHODS

I. *Material and Reagents*

For this study, ten rice varieties -- Jawangdo, Ilpumbyeo, Sanghaehyeolla, Hongmi, Heugjinmi, Suwon #405, Suwon #415, Suwon #420, Suwon #425 and Kilimheugmi (var, originally from Mainland China) -- were cultivated under controlled conditioned in the experimental field of the National Crop Experiment Station, Republic of Korea. Rice grains were cleaned and stored at 4 ℃ until use, also were polished through a sieve to obtain uniform grain fractions. Trifluoroacetic acid (TFA), 99%, was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All laboratory chemicals used were of analytical grade and obtained from Merck-Clevenot Corp. (Darmstadt, Germany).

II. *Extraction, Isolation and Identification of Anthocyanin*

The flow chart for preparation of anthocyanin pigments from black rice is shown in Figure 1. Ground rice grains (50 g) were defatted with 400 ml of *n*-hexane and dried. Then anthocyanin pigments were extracted twice with 500 ml of 0.5 % TFA in 95% ethanol overnight, filtered through a Sephadex LH-20 column (length 300 mm, i.d. 20 mm) (Pharmicia Biotech, Uppsala, Sweden). They were further purified on a Lobar glass prepacked column (Licreprep RP-8 column, length 310 mm, i.d. 25 mm, Merck-Clevenot Corp.) The crude pigment solution was loaded onto a Amberlite XAD-7 (Organo Co. Ltd., Tokyo, Japan, 20-50 mesh) column (25 mm i.d. × 500 mm). The column was washed stepwise with glass-distilled water, MeOH-H_2O (40 : 60) to remove sugars, amino acids, organic acids, low molecular phenols and polymerized dark brown pigments. Finally, the bright purple-colored anthocyanin pigments were eluted with 0.1% TFA in MeOH-H_2O (70:30). The anthocyanin fraction of 0.1% TFA in 70% methanol were collected, and concentrated as before. These partially purified aqueous anthocyanin pigments were further isolated, separated by preparative HPLC. Anthocyanins were identified by their retention times or standard addition. Anthocyanins were purchased from Indofine Chemical Co., Inc. (Somerville, NJ, USA) and were >99% pure as indicated by HPLC analysis.

Thin-Layer Chromatogram (TLC) on the crude extracts and on the partially purified pigments was carried out on a reversed-phase RP_{18}, F_{254}, (10×20 cm, 0.25 mm layer thickness, Merck-Clevenot, Darmstadt, Germany) with three differ-

ent solvent systems: i-BAW (isobutanol: acetic acid: water = 8:2:3, v/v); AHW (acetic acid: hydrochloric acid:water =15:3:82, v/v) and 1% HCl, respectively (Table 2). TLC standard mixture was applied as a reference on one side of each plate. Partially purified pigment extract was further purified by paper chromatography as described by Du and Francis[15-16]. Identification of pigments in general followed the chromatographic methods described by Strack and Wray [17].

III. *Separation of Anthocyanin Using Preparative HPLC*

Separation of anthocyanins was achieved by preparative HPLC. Partially purified, aqueous anthocyanin pigments were adsorbed onto a Waters Sep-Pak C_{18} Cartridge (Milford, MA, USA), washed with water, eluted with acidified methanol, and concentrated by flushing with nitrogen gas. Furthermore, purification of pigments was conducted with HPLC on a JASCO Twincle HPLC (Japan Spectroscopic Co. Ltd., Tokyo, Japan) using a reversed-phase Develosil ODS-10 column (20 mm i.d. × 250 mm, Nomura Chemical Co. Ltd., Seto, Japan) and an UV spectrophotometric detector (JASCO UVIDEC-100; Japan Spectroscopic, Tokyo, Japan) at 530 nm. The solvent was 3% H_3PO_4 in AcOH-CH_3CN-H_2O (6.0 : 7.5 : 86.5) at a flow rate of 5.0 ml/min over 80 min.

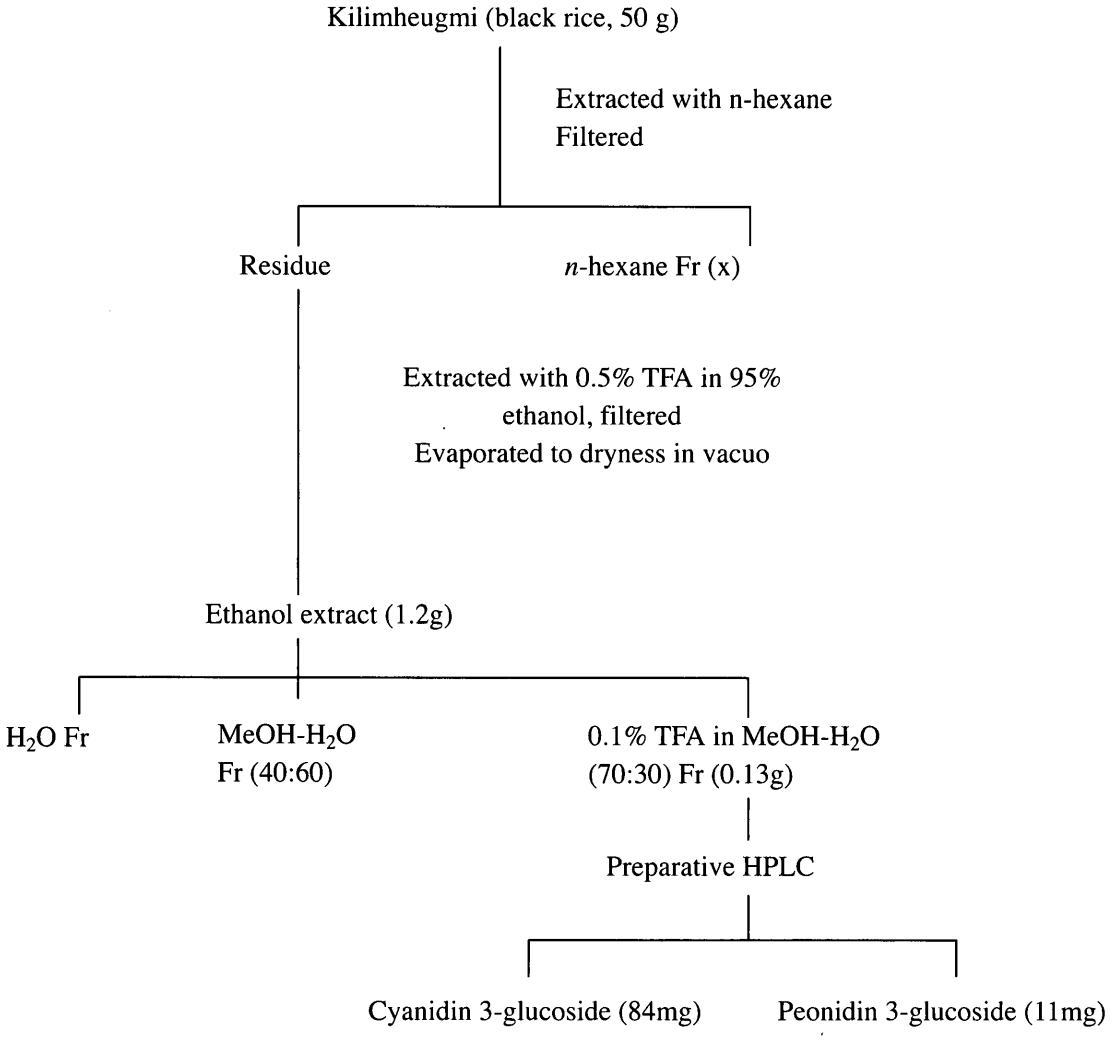


Figure 1. Flow chart for preparation of anthocyanins from pigmented rice.

IV. *Quantification of Anthocyanin Pigment Levels*

HPLC analysis was performed with a P-2000 pump (TSP Co., Tokyo, Japan), Datajet integrator, and a model of Spectro Focus Spectrophotometric detector set at 280 nm. About 1 g of sample was accurately weighed and shaken with 20 ml of 0.5% TFA- 95% EtOH for 9 hrs at room temperature. The crude sample was separated by decantation and filtrated with Sep-Pak C_{18} SPE cartridges attached to 10 ml syringes. Pigment content of this filtrate was determined by HPLC. HPLC was performed in a Develosil ODS-5 column (4.6 × 250 mm) with a linear gradient from 0.1% TFA-H_2O to 0.1% TFA-MeOH for 30 min as an elution solvent at a flow rate of 1.0 ml/min. Standard solutions of 4, 15, 40 and 60 ppm of cyanidin 3-glucoside and peonidin 3-glucoside were injected on HPLC respectively. Standard calibration curves were made using the average value of peak areas for triplicate determinations of cyanidin 3-glucoside and peonidin 3-glucoside standards.

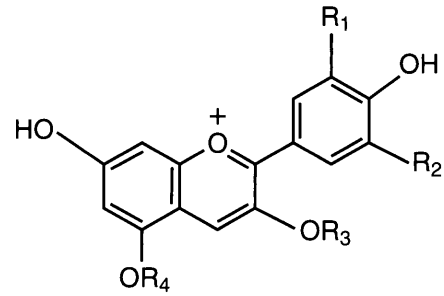
RESULTS AND DISCUSSION

I. *Extraction, Isolation and Identification of Anthocyanin from Pigmented Rice*

As anthocyanins are very reactive compounds, easily degraded or condensed to polymeric pigments, a rapid and efficient method is necessary to isolate and purify anthocyanin pigments[18]. Recently, a column chromatography technique using several synthetic resins has been developed to isolate and purify anthocyanins in food and plants[19-20]. In particular, it was suggested that, the reason Amberlite XAD resins are of more general use probably because they offered a better resolution and a higher recovery rate of pigments by means of gradient elution of anthocyanins with alcohol[19-20]. However, we found that black rice pigments do not adsorbe onto polystyrene resin, such as Amberlite XAD-2 or XAD-4 (Organo Co. Ltd., Tokyo, Japan), but do readily adsorb onto acrylester resin, like Amberlite XAD-7 (Organo Co. Ltd., Tokyo, Japan). Figure 1 shows the flow chart for extraction and partial purification of anthocyanin from rice grains. The yield of anthocyanins was 1.5-2% by weight. Structures of two major anthocyanins have been identified based on FAB-MS and NMR spectral and chemical evidences as shown in Table 1 [21].

TLC analysis, on the other hand, is useful for

Table 1. Structure of two anthocyanins in black rice



Peak No.	Compound	R_1	R_2	R_3	R_4
1	C3G[a]	OH	H	Glu	H
2	P3G[b]	OCH_3	H	Glu	H

[a] C3G: Cyanidin 3-glucoside.

[b] P3G: Peonidin 3-glucoside.

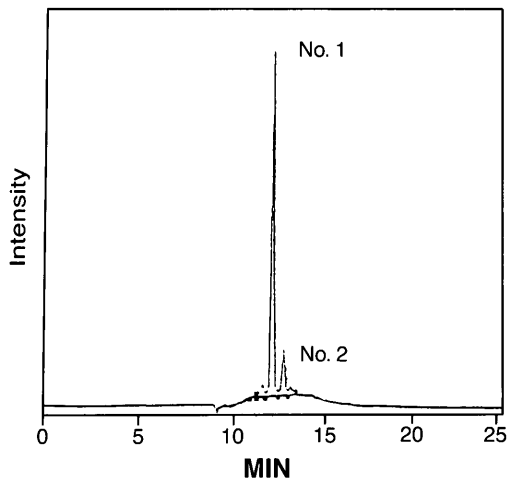


Figure 2. High performance liquid chromatogram of anthocyanins extracted from pigmented rices cv. "Kilimheugmi"; Peak No.1, cyanidin 3-glucoside; No. 2, peonidin 3-glucoside; HPLC condition: Column, Develosil ODS-5 (4.6×250 mm); Solvent, 0.1% TFA-H_2O to 0.1%TFA- MeOH (30 min); Flow rate, 1.0 ml/min; detection at 280 nm.

Table 2. Comparison of R_f values of anthocyanins in pigmented rice cv. "Kilimheugmi"

Anthocyanin	R_f values (x 100) in		
	i-BAW[a]	AHW[b]	1% HCl
No.1	30	22	5
No.2	41	30	11
No.3	1	21	15
C3G[c]	30	21	6
P3G[d]	41	30	10

[a] i-BAW, isobutanol : acetic acid : water= 8 : 2 : 3 (v/v).
[b] AHW, acetic acid : hydrochloric acid : water = 15:3:82 (v/v).
[c] C3G: Cyanidin 3-glucoside.
[d] P3G: Peonidin 3-glucoside.

Table 3. Retention times (tR) of anthocyanins studied, and relationship between peak area anthocyanin contents by HPLC

Anthocyanin (Chloride)	tR ± S.D[a] (min)	Relationship (y = ax + b)[b]		
		a	b	r[c]
C3G[d]	11.34 ± 0.11	2.84	- 2.65	0.996
P3G[e]	12.05 ± 0.13	2.95	- 9.41	0.997

[a] Mean values and standard deviations of retention times for two determination.
[b] Here, a and b are coefficients of the regression equation y = ax + b, where x is anthocyanin concentration (ppm) and y is peak area for concentrations ranging from 4 to 60 ppm.
[c] Correlation coefficients of the regression equation.
[d] C3G: Cyanidin 3-glucoside.
[e] P3G: Peonidin 3-glucoside.

isolation and identification of anthocyanins. As shown in Table 2, three pigment bands with R_f 0.01, 0.3, 0.41 using i-BAW as the solvent were isolated from crude pigments of black rice grains extracted with 0.5% TFA in 95 % ethanol. Among them, two major bands with R_f 0.3 and 0.41 could be separated on an Amberlite XAD-7 column with 0.1% TFA in 70% methanol. Band No.1 was the most abundant anthocyanin in rice grain hulls. When compared with rice pigments, Band No. 3 was present but at a low concentration. Co-chromatography with authentic anthocyanin confirmed that Band No.1 was cyanidin 3-glucoside and Band No.2 was peonidin 3-glucoside (Table 2).

Partially purified anthocyanin pigments were finally separated by preparative reversed-phase HPLC on a Develosil ODS-10 column using 3% H_3PO_4 in AcOH-CH_3CN-H_2O (6.0:7.5:86.5, v/v) as a solvent. The HPLC conditions described in this experiment allowed good separation of all anthocyanins, as shown in Figure 2. Major peaks No. 1 and No. 2, which eluted near 11.34 min and 12.05 min, respectively, were separated and analyzed. Identity of the minor peak (peak No. 3) with an area percentage less than 5% was not considered. An HPLC condition using a Develosil ODS-5 column and a linear gradient from 0.1% TFA-H_2O to 0.1% TFA-MeOH (30 min) was

Table 4. Anthocyanin contents in pigmented rices investigated

Varieties	Anthocyanin (mg/100g grain weight)[a]			
	C3G[b]	P3G[c]	Total	C3G/Total (%)
Suwon #415	470	23	493	95.3
Kilimheugmi	240	26	266	90.2
Suwon #425	206	40	246	83.7
Heugjinmi	200	32	232	86.2
Sanghaehyanghyeolla	50	5	55	90.9
Hongmi	30	6	36	83.3
Suwon #405	16	4	20	80.0
Suwon #420	10	ND	10	—
Jagwangdo	10	tr[d]	10	—
Ilpumbyeo	ND[e]	ND	ND	ND

[a] Mean values in triplicate.
[b] C3G: Cyanidin 3-glucoside.
[c] P3G: Peonidin 3-glucoside.
[d] Trace.
[e] Not detected.

selected for separation and quantitative regression equation for cyanidin 3-glucoside and peonidin 3-glucoside (Table 3).

II. *Anthocyanin Composition of Pigmented Rice Varieties*

To get quantitative information on the extracts, cyanidin 3-glucoside and peonidin 3-glucoside were chosen as the reference compounds. The linearity of the response for cyanidin 3-glucoside and peonidin 3-glucoside is shown in Table 3. As an example, the results of the analysis of pigmented rice are reported in Table 4. Major anthocyanins of pigmented rice were identified as cyanidin 3-glucoside and peonidin 3-glucoside[22-23]. Further, it was found that cyanidin 3-glucoside, a predominant anthocyanin in pigmented rice grains, had a stronger antioxidative activity than peonidin 3-glucoside in both food and biological model systems[14,22,24]. Total anthocyanin contents varied greatly in the range of 0~493 mg/100 g grain among rice varieties. Dark purple rice grains such as Suwon #415, Kilimheugmi and Suwon #425 had the highest levels of the two anthocyanin pigments combined, from 246 to 493 mg/100 g rice grain, followed by Heugjinmi, Sanghaehyanghyeolla, Hongmi, Suwon #405, Jawangdo, and Suwon #420, in decreasing order. Ilpumbyeo, with a white color, had the lowest levels of anthocyanin pigments. In particular, Suwon #415 had the highest level of cyanidin 3-glucoside and Suwon #425 had the highest level of peonidin 3-glucoside. Cyanidin 3-glucoside is an important OARC and was most abundant in Suwon #415 (470 mg/100 g grain), while Jawangdo (10 mg/100 g grain) and Ilpumbyeo (not detected) had the smallest amount of anthocyanin among the samples examined. Judging by distribution ratio of individual anthocyanin, Suwon #415 was characterized by a relatively high percentage of cyanidin 3-glucoside (95.3%); whereas, Suwon #425 and Heugjinmi were characterized by a relatively greater amount of peonidin 3-glucoside. Rice varieties of Suwon #415 may also be related to high antioxidative activity. Further studies including isolation and identification of these compounds are needed.

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高效液相層析探討黑米之花青素

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摘 要

用纖維薄層層析及製備級的高效液相層析將不同品種的黑米中的花青素分離出來，並以色層分析來鑑定。Cyanidin 3-glucoside和penonidin 3-glucoside是黑米中最重要的兩種花青素。所有品種的花青素總量分析介於0～493 mg/100 g的米粒。Cyanidine 3-glucoside是一個重要的自由基收容物（ORAC）。在所有檢驗的黑米品種中以Suwon #415品種的Cyanidine 3-glucoside含量最高。

關鍵詞：花青素，黑米，高效液相層析，Cyanidin 3-glucoside，penonidin 3-glucoside。