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Anthocyanin Profile of Korean Cultivated Kidney Bean (*Phaseolus vulgaris* L.)

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This investigation was conducted to determine the structures and amounts of anthocyanins obtained from seed coats of kidney bean (*Phaseolus vulgaris* L.) cultivated in Korea. Anthocyanins in the seed coat of kidney bean were extracted with 1% HCl/20% CH_3OH, and the crude anthocyanin extracts were purified by semipreparative HPLC. Five major anthocyanins were isolated, and their chemical structures were identified by spectroscopic methods (UV−vis, LC/ES-MS, and ^1H and ^{13}C NMR). The structures of these five anthocyanins were elucidated as cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, and pelargonidin 3-glucoside. Using RP-HPLC with photodiode array detection, each of the five anthocyanins was separated within 12 min by using a gradient elution. It was proved that the application of RP-HPLC could be an excellent method for determining the composition and contents of anthocyanins in kidney bean. The preponderance of pelargonidin 3-glucoside and delphinidin 3-glucoside are observed in red and black kidney beans, respectively. However, in this study, it is reported for the first time that the contents and composition of anthocyanins in speckled seed depend on the classes of speckle color. The contents of cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, pelargonidin 3-glucoside, and total anthocyanins in seed coats of 16 kidney beans cultivated in Korea were in the ranges of 0−0.04, 0−2.61, 0−0.12, 0−0.17, 0−0.59 and 0−2.78 mg/g of dried seed coats, respectively.

KEYWORDS: Kidney bean (*Phaseolus vulgaris* L.); anthocyanins; HPLC

INTRODUCTION

The kidney bean (*Phaseolus vulgaris* L.) is the world's second most important bean after soybean (*1*) and is cultivated for its pod and seed (*2*). There are many varieties of kidney bean, and it is known that the seed size, shape, and color in each variety generally differ (*2*). The color variation is especially great in kidney bean, and red, black, brown, and white colors are common (*3*). Among these colors, red and black color pigments in the seed coats of kidney bean are an attractive potential source for natural food colorants (*1*).

Anthocyanins have been used for coloring foodstuffs and snack foods, beverages, and pharmaceutical and cosmetic products as well as textiles, paper, and leather (*4*). Also, medical interest has been focused on anthocyanins; for example, crude extracts of several types of fruits appear to have replaced rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility (*5*). Anthocyanins have received further attention for promising activities as antioxidants and free radical scavengers (*6*). These activities are interesting as they may prevent degenerative diseases due to oxidative stress such as heart disease, cancer, and Alzheimer's disease.

Anthocyanins in the seed coat of kidney bean (*P. vulgaris* L.) were first reported by Feenstra (*7*) in 1960, and subsequently several investigators have identified different kinds of anthocyanins from diverse kidney bean varieties. Feenstra (*7*) found four anthocyanins, malvidin 3-glucoside, petunidin 3-glucoside, delphinidin 3-glucoside, and delphinidin 3,5-diglucoside, in black violet beans (*P. vulgaris* L.). Another anthocyanin, malvidin 3,5-diglucoside, together with delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside, was reported in the black bean cultivar Kurodanekinugasa by Okita et al. (*8*). In another study, Stanton and Francis (*9*) reported that delphinidin 3-glucoside was the major anthocyanin in the Canadian Wonder cultivar, although small amounts of cyanidin

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Table 1. Comparison of Anthocyanin Contents in Seed Coats of 16 Korean Cultivated Kidney Beans

seed color	speckle color	cultivar	seed wt (mg/seed)	anthocyanin content[a] (mg/g)					
				1	**2**	**3**	**4**	**5**	total
red	none	KG98001	480.5	0.042	0.030	0.081		0.588	0.741
		IT100952	437.6	0.032	0.017	0.065		0.347	0.461
		Kangnang 1	428.8	0.037	0.097	0.125		0.174	0.443
	black	IT100875	408.0	0.038	0.106	0.080		0.050	0.274
		KG96127	363.5	0.041	0.182	0.104		0.053	0.380
		IT110356	490.0	0.043	0.127	0.113		0.110	0.393
black	none	KG98010	359.8		2.022		0.122		2.144
		KG97287	477.6		2.614		0.167		2.781
brown	black	IT168080	476.4	0.016	0.038	0.029		0.015	0.098
	red	Sundoo	510.3			0.024		0.080	0.104
		IT103412	489.0	0.015		0.019		0.033	0.067
	none	KG97135	312.1						
		KG97123	307.7						
white	none	KG96013	113.8						
		KG97128	373.9						
		KG97621	309.3						

[a] Average of three determinations.

3-glucoside, cyanidin 3,5-diglucoside, pelargonidin 3-glucoside, and pelargonidin 3,5-diglucoside were also found.

Although some reports are available, research on the exact composition and contents of anthocyanins in the seed coat of kidney bean has been limited. Also, their genetic study and other related features such as anthocyanin composition and content are more limited in the speckled kidney bean, although this type is very common. An understanding of the composition and contents of anthocyanins in the kidney bean may aid in their further utilization as anthocyanin resource materials. We have therefore investigated the structures of anthocyanins in Korean cultivated kidney beans and especially the anthocyanin contents of speckled kidney beans.

MATERIALS AND METHODS

Plant Materials. Sixteen kidney beans cultivated in Korea with different seed coat colors were selected for this study (**Table 1**). Three red kidney beans speckled with black and three brown kidney beans speckled with black or red were studied together with 10 kidney beans colored with red (3 genotypes), black (2 genotypes), brown (2 genotypes), and white (3 genotypes). The kidney beans were grown at the experimental field of Gyeonggi-do Agricultural Research and Extension Services, Hwasong, Korea, in 2001. Cultivation and field management followed a standard cultivation procedure of the service center.

After harvest, the seeds were cleaned with distilled water to remove extraneous matter and dried at 105 °C for 2 h. The dried seeds were stored at 4 °C until they were used. For structural identification of anthocyanins in kidney bean seed coats, KG98001 (red) and KG97287 (black) seeds, which have been identified as containing different kinds of anthocyanins in our laboratory, were used. All 16 kidney bean cultivars were also used for the determination of total anthocyanin contents.

Chemicals. Methanol, acetonitrile, formic acid, and water were purchased from Merck Chemical Co. (Darmstadt, Germany). Tetramethylsilane (TMS), CD_3OD, and DCl were obtained from Sigma Chemical Co. (St. Louis, MO). A 0.45 μm membrane filter was purchased from Waters Co. (Milford, MA). All laboratory chemicals used in this study were of reagent grade.

Instrumentation and Conditions. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured on a Varian Unity Plus 500 NMR instrument in 0.5% DCl/CD_3OD containing TMS as the internal standard. LC/ES-MS analyses were carried out on a ThermoFinnigan AQA single-quadrupole mass spectrometer system, equipped with a Spectra system P-4000 HPLC system. The semipreparative HPLC system was composed of a Spectra system P-4000 pump, a Spectra system UV-1000 UV−vis variable wavelength detector, and a Hitachi D-2500 integrator. Injections were carried out with a Rheodyne 7725i injector equipped with a 2 mL loop. The analytical HPLC system consisted of a G1311A Agilent 1100 quaternary pump, a G1313A Agilent autosampler equipped with a 20 μL sample loop, and a G1315B Agilent diode array detector. Instrument control and data processing were performed by a G2180AA Agilent ChemStation for LC 3D Spectral SW module (version A.08.01).

Isolation of Anthocyanins from Kidney Bean Seed Coats. The seed coats of KG98001 (red) and KG97287 (black) were peeled manually. Each of the separated seed coats (200 g) was extracted with 1% HCl/20% CH_3OH (10 L) at 4 °C for 48 h. The extracts were filtered through Advantec Toyo no. 2 filter paper and concentrated at 30 °C in vacuo. Each of the crude anthocyanin extracts was fractionated by semipreparative HPLC with monitoring at 520 nm using a 250 × 9.4 mm i.d. Zorbax SB-C_{18} column (Agilent Technologies, Wilmington, DE), and the column temperature was set at 30 °C. Gradient elution was performed with solvent A, consisting of 5% aqueous formic acid, and solvent B, comprising 5% formic acid/acetonitrile, and delivered at a flow rate of 3 mL/min as follows: 0−10 min, 10−18% B; 10−18 min, 18−28% B; 18−19 min, 28−40% B; 19−21 min, 40% B; 21−23 min, 40−10% B; 23−25 min, 10% B. A sample volume of 2 mL was used for injection. Five anthocyanins were purified from kidney bean by semipreparative HPLC.

Determination of Anthocyanin Contents in Seed Coats of Kidney Bean. To determine the anthocyanin contents, seed coats of each kidney bean variety were peeled manually. The separated seed coats (0.1 g) were extracted with 30 mL of 1% HCl/20% CH_3OH at 4 °C with standing for 48 h. The anthocyanin contents of 16 kidney beans were analyzed by RP-HPLC using a 150 × 4.6 mm i.d. TSK gel ODS-120T column (Supelco Inc., Bellefonte, PA). The flow rate was set at 0.7 mL/min by gradient elution, using the same solvent system as for semipreparative HPLC. The photodiode array detection wavelength was set at 200−700 nm, and the injection volume of extract was 20 μL. Prior to analysis, all samples were filtered through a 0.45 μm membrane filter. For protection of the analytical column, a Nova-Pak C_{18} guard insert column (Waters, Milford, MA) was used. The anthocyanin contents were calculated by HPLC peak areas compared with external standard calibration curves. The linear standard calibration curves (r = 0.999**) were generated by injecting 0.05−1 μg of purified anthocyanins in 20 μL of 1% HCl/20% CH_3OH.

RESULTS AND DISCUSSION

HPLC Analysis. The HPLC chromatogram of the seed coat extract obtained from cv. KG98001 (red bean) showed four distinct major anthocyanin peaks (**Figure 1A**), and cv. KG97287 (black bean) showed only two major anthocyanin peaks (**Figure 1B**). These chromatograms showed clearly that the class of anthocyanins contained in seed coats of red kidney bean differs from that in black kidney bean. No anthocyanin peak was observed in either brown or white kidney beans. Under our analytical HPLC conditions, the anthocyanin peaks eluted at 4.9, 6.5, 8.1, 9.4, and 10.4 min, respectively (**Table 2**). The results obtained in this study showed that the HPLC conditions could be an excellent method to determine the composition and contents of anthocyanins in kidney bean seed coats within 12 min.

Identification of Anthocyanins. The further characterization of anthocyanins contained in seed coats of kidney bean based on the on-line HPLC spectroscopic analysis are presented in **Table 2**. The UV−vis of peak 1 showed a visible maximum at 518 nm with E_{440}/E_{vis} of 19%, indicating a 3,5-diglycoside of an anthocyanidin (*10*). Peaks 2−5 showed a visible maximum at 503−526 nm with E_{440}/E_{vis} of 27−44%, indicating a 3-glycoside of an anthocyanidin (*10*, *11*). The ratios of E_{UV}/E_{vis} (59−66%) and E_{acyl}/E_{vis} (7−14%) of peaks 1−5 indicate

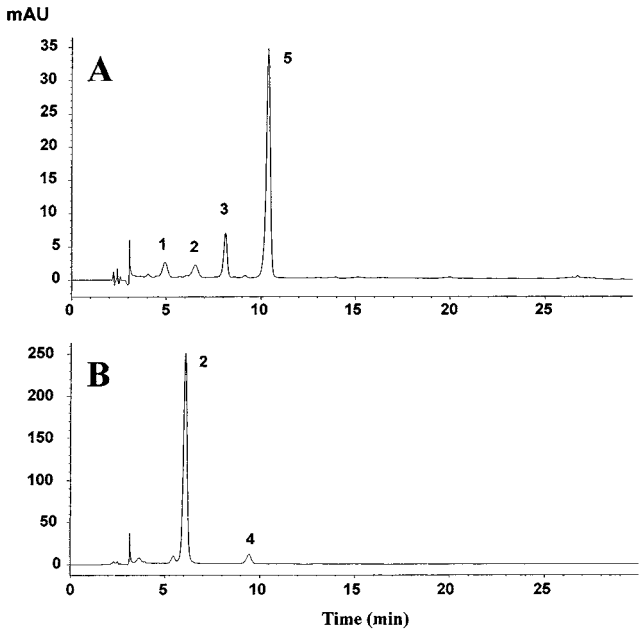


Figure 1. HPLC chromatograms of anthocyanins obtained from red kidney bean (**A**) and black kidney bean (**B**). For peak identity see **Figure 2**.

Table 2. Chromatographic and Spectroscopic Properties and LC/ES-MS Data of Purified Anthocyanins Obtained from the Seed Coats of Kidney Bean

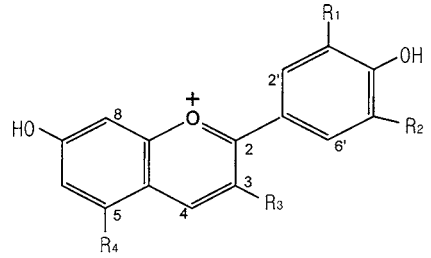
peak	HPLC retention (min)	LC/ES-MS (*m*/*z*)	UV–vis Λ_{max} (nm)	E_{UV}/E_{vis} (%)	E_{acyl}/E_{vis} (%)	E_{440}/E_{vis} (%)
1	4.9	611 $[M]^+$	278, 518	60	7	19
2	6.5	465 $[M]^+$	277, 525	60	9	29
3	8.1	449 $[M]^+$	280, 518	66	9	31
4	9.4	479 $[M]^+$	277, 526	59	7	27
5	10.4	433 $[M]^+$	278, 503	66	14	44

[a] E_{UV}, extinction coefficient of maximum absorption peak in UV region; E_{vis}, extinction coefficient of maximum absorption peak in visible region; E_{acyl}, extinction coefficient at 330 nm; E_{440}, extinction coefficient at 440 nm.

that these were simple anthocyanins without any complex acylation (*10*).

From LC/ES-MS (**Table 2**) and $^1H/^{13}C$ NMR (not shown) data, the purified anthocyanins obtained from red (KG98001) or black (KG97287) kidney bean were identified as cyanidin 3,5-diglucoside (**1**), delphinidin 3-glucoside (**2**), cyanidin 3-glucoside (**3**), petunidin 3-glucoside (**4**), and pelargonidin 3-glucoside (**5**), respectively, as shown in **Figure 2**. The UV−vis, MS, and NMR data were in good agreement with those reported in previous studies (*2*, *6*, *12*−*17*).

In this study, the red kidney bean cultivar (KG98001) was shown to have four anthocyanins, cyanidin 3,5-diglucoside (**1**), delphinidin 3-glucoside (**2**), cyanidin 3-glucoside (**3**), and pelargonidin 3-glucoside (**5**), whereas the black kidney bean cultivar (KG97287) contained only two anthocyanins, delphinidin 3-glucoside (**2**) and petunidin 3-glucoside (**4**). Takeoka et al. (*1*) identified delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside in black bean (*P. vulgaris* L. cv. UI 911). In another study, Tsuda et al. (*2*) reported that the black bean (*P. vulgaris* L. cv. Yamashirokurosando) contained the anthocyanin delphinidin 3-glucoside only. However, in this study, the black kidney bean cultivar (KG97287) clearly contains delphinidin 3-glucoside as well as petunidin 3-glucoside, and we were not able to detect malvidin 3-glucoside.



Anthocyanin		R_1	R_2	R_3	R_4
cyanidin 3, 5-diglucoside	(**1**)	OH	H	O-β -D-glucose	O-β -D-glucose
delphinidin 3-glucoside	(**2**)	OH	OH	O-β -D-glucose	OH
cyanidin 3-glucoside	(**3**)	OH	H	O-β -D-glucose	OH
petunidin 3-glucoside	(**4**)	OCH_3	OH	O-β -D-glucose	OH
pelargonidin 3-glucoside	(**5**)	H	H	O-β -D-glucose	OH

Figure 2. Chemical structures of anthocyanins in seed coats of kidney bean.

In the case of red kidney beans, Yoshida et al. (*18*) identified pelargonidin 3-glucoside and cyanidin 3-glucoside from *P. vulgaris* L. cv. Taishokintoki. Tsuda et al. (*2*) also reported the same anthocyanins in *P. vulgaris* L. cv. Honkintoki. However, in this study, we identified pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, and cyanidin 3,5-diglucoside in the red kidney bean (KG98001). It is possible to conclude that the differences in anthocyanins exist in the kidney bean germplasms, although they share the same seed color.

Determination of Anthocyanin Contents in Seed Coats of Kidney Bean Cultivated in Korea. The anthocyanin contents in seed coats of kidney beans, on the basis of HPLC peak area with monitoring at 520 nm, are presented in **Table 1**. Kidney beans showed variant colors of seed coats depending on the genotypes. Four colors are most common: black, red, brown, and white (*3*). However, various speckled, mottled, or eyed seeds with different colors also exist. In this study, the kidney beans cultivated in Korea consisted of six red beans (including three speckled beans with black color), two black beans, five brown beans (including one speckled bean with black color, and two speckled beans with red color), and three white beans.

A preponderance of pelargonidin 3-glucoside and delphinidin 3-glucoside was observed in red and black kidney beans, respectively. However, in this study, we report for the first time that the contents and composition of anthocyanin in speckled seed depend on the classes of speckle color. Thus, the pelargonidin 3-glucoside content was increased in the speckled beans with red-speckle color, whereas the delphinidin 3-glucoside content was increased in the speckled beans with black-speckle color. Among the 16 kidney beans cultivated in Korea, 2 brown (KG97135 and KG97123) and 3 white (KG96013, KG97128, and KG97621) kidney beans contained no anthocyanin.

The individual cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, and pelargonidin 3-glucoside contents in seed coats of 16 kidney beans cultivated in Korea were in the ranges of 0−0.04, 0−2.61, 0−0.12, 0−0.17, and 0−0.59 mg/g of dried seed coats, respectively. The total anthocyanin contents of seed coats in black kidney beans was high, >2.1 mg/g, whereas that of red kidney beans was low, <0.7 mg/g. The total anthocyanin contents in seed coats of 16 kidney beans ranged from 0 to 2.78 mg/g.

Using HPLC, Yoshida et al. (*18*) analyzed the anthocyanin contents in 26 kinds of colored legumes of different species or from different production districts. In their study, the antho-

cyanin contents of seven kinds of red or black kidney beans (*P. vulgaris* L.), produced in Japan or North America, ranged from 0.3 to 5.1 mg/g. The legumes of the same species produced in different areas or countries contained the same anthocyanin types and varied only in their contents, depending on the cultivated region. In another study, Takeoka et al. (*1*) reported that the UI 911 black beans (*P. vulgaris* L.) produced in the United States contained three kinds of anthocyanins, and the anthocyanin content in the seed coats of UI 911 was ~23.7 mg/g of seed coats.

In this study, the types of anthocyanins contained in the seed coats of kidney beans with variant colors such as red, black, brown, white, or speckled are different. In addition, kidney beans cultivated in Korea contained far less anthocyanin contents compared to the Japanese and U.S. kidney beans (*1*, *18*). Introduction of high anthocyanin and brilliantly colored foreign germplasms is needed in kidney bean breeding programs to increase the contents of anthocyanin and also to satisfy the demand for brilliantly colored kidney beans in Korea. Further studies for the determination of anthocyanin contents contained in seed coats of kidney beans from a wide range of germplasms are now in progress for the breeding of high-anthocyanin kidney bean in Korea.

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