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Structural Analysis of Black Common Bean (*Phaseolus vulgaris* L.) Anthocyanins

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Abstract Two anthocyanins were isolated from 1% HCl-20% methanol extracts of KG 97287 black common bean (*Phaseolus vulgaris* L.) using semipreparative, high-performance liquid chromatography (HPLC). The anthocyanins were identified using a combination of LC/ES-mass spectrometry (MS) and spectroscopic methods of UV-Vis, ^{1}H- and ^{13}C- nuclear magnetic resonance (NMR). The chemical structures of these two anthocyanins were elucidated as delphinidin 3-glucoside and petunidin 3-glucoside and their contents in KG 97287 black common bean seed coats were determined to be 2.614±0.11 and 0.167±0.01 mg/g, respectively. These contents were lower than reported internationally and we recommend the introduction into Korea of high anthocyanin varieties of black common bean.

Keywords: black common bean, *Phaseolus vulgaris* L., anthocyanin, HPLC, NMR

Introduction

In recent years research interest has increased in the health benefits of anthocyanins found in fruits and vegetables (1). Anthocyanins, the largest group of water-soluble pigments in the plant kingdom, are responsible for most of the red, purple, and blue colors exhibited by flowers, fruits, and other plant tissues and have found application in the food industry as natural colorants (2). Current medical interest in the anthocyanins remains high, with, for example, the crude extracts of several types of fruits having replaced rutin and its derivatives in the treatment of illnesses involving tissue inflammation or capillary fragility (3). There is currently strong evidence indicating that free radicals and other oxidants cause oxidative damage to lipids, proteins, and nucleic acids. These oxidants may be an important factor in the development of a number of diseases, including cancer and atherosclerosis (4). Antioxidants, which can neutralize free radicals, may therefore be of central importance in the prevention of these disease states (5). Recent studies on the total antioxidant capacity of fruits and vegetables at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University revealed that a large group of colorful compounds, some of which are flavonoids such as anthocyanins, flavones, isoflavones, catechin, and epicatechin, may be responsible for much of the antioxidant protection against peroxyl radicals (6, 7). Wang *et al.* (8) recently confirmed the potent antioxidant properties of anthocyanins and their aglycons against peroxyl radicals.

The common bean (*Phaseolus vulgaris* L.) is the world's second most important bean after soybean (9) and is cultivated for both its pod and seed (10). Among the many varieties of common bean, the seed size, shape, and color show large variety (10). The color variation is especially great in the common bean, with red, black, brown and white colors being common (11). Among these colors, the black color pigments in the seed coats of the common bean are an attractive potential source for natural food colorants (9). A more understanding of the composition of anthocyanins in the black common bean may aid in their further utilization as a resource material for anthocyanin. We therefore investigated the structures of anthocyanins in Korean, cultivated, black common beans.

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Materials and Methods

Plant materials The KG 97287 black common beans (*P. vulgaris* L.) were grown at the experimental fields of Gyeonggi-do Agricultural Research & Extension Services, Hwasong, Korea, in 2004. Cultivation and field management followed the standard cultivation procedure undertaken at the service center. After harvesting, the seeds were cleaned with distilled water to remove extraneous matters and dried at 105°C for 2 hr. The dried seeds were stored at 4°C until use.

Chemicals Methanol, acetonitrile, formic acid, and water were purchased from Merck Chemical Co. (Darmstadt, Germany). TMS (Tetramethylsilane), CD_3OD, and DCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All laboratory chemicals used in this study were of reagent grade.

Instrumentation and conditions ^{1}H nuclear magnetic resonance (NMR) (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured on a Varian Unity Plus 500 NMR (Varian Inc., Palo Alto, CA, USA) instrument in 0.5% DCl-CD_3OD containing TMS as the internal standard. LC/ES-mass spectrometry (MS) analyses were carried out on a Thermo Finnigan AQA single-quadrupole MS system (Thermo Electron Inc., Somerset, NJ, USA), equipped with a Spectra P-4000 high-performance liquid chromatography (HPLC) system (Thermo Electron Inc., Somerset, NJ, USA). The semipreparative HPLC system comprised a

Spectra system P-4000 pump, Spectra system UV-1000 UV-Vis variable wavelength detector, and Hitachi D-2500 integrator (Hitachi Ltd., Tokyo, Japan). Injections were carried out with a Rheodyne 7725i injector (IDEX Co., Rohnert Park, CA, USA) equipped with a 2 mL loop. The analytical HPLC system consisted of a G1311A Agilent 1100 quaternary pump, G1313A Agilent auto sampler equipped with a 20 μL sample loop, and G1315B Agilent diode array detector (all Agilent Technologies, Wilmington, DE, USA). The instrument control and data were processed by a G2180AA Agilent ChemStation for LC 3D Spectral SW Module (version A.08.01).

Extraction and isolation of anthocyanins from the black common bean The seed coats of KG 97287 black common beans were peeled manually and extracted in 100 g masses with 1% HCl-20% CH_3OH (5 L) at 4°C for 48 hr. The extracts were filtered through Advantec Toyo No. 2 filter paper and concentrated at 30°C *in vacuo*. The crude anthocyanin extracts were 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, USA), with column temperature 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. The solvents were delivered at a flow rate of 3 mL/min in the following cycle: 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. Two anthocyanins were purified from the black common beans by semipreparative HPLC. To determine the purity, the purified anthocyanins were analyzed by RP-HPLC using a 150 × 4.6 mm i.d. TSK gel ODS-120T column (Supelco Inc, Bellefonte, PA, USA). The flow rate was set at 0.7 mL/min by gradient elution, using the same solvent system as that for semipreparative HPLC. The photodiode array detection wavelength was set over the range 200-700 nm, and the injection volume of extract was 20 μL. Prior to analysis, samples were filtered through a 0.45 μm membrane filter. To protect the analytical column, a Nova-Pak C_{18} guard insert column (Waters, Milford, MA, USA) was used. The anthocyanin contents of the black common beans 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 μg to 1 μg of purified anthocyanins in 20 μL of 1% HCl-20% CH_3OH.

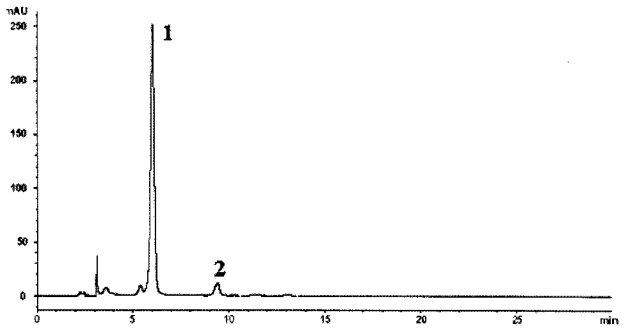

Fig. 1. HPLC chromatogram of anthocyanin obtained from the seed coats of the black common bean (peak 1: delphinidin 3-glucoside, peak 2: petunidin 3-glucoside).

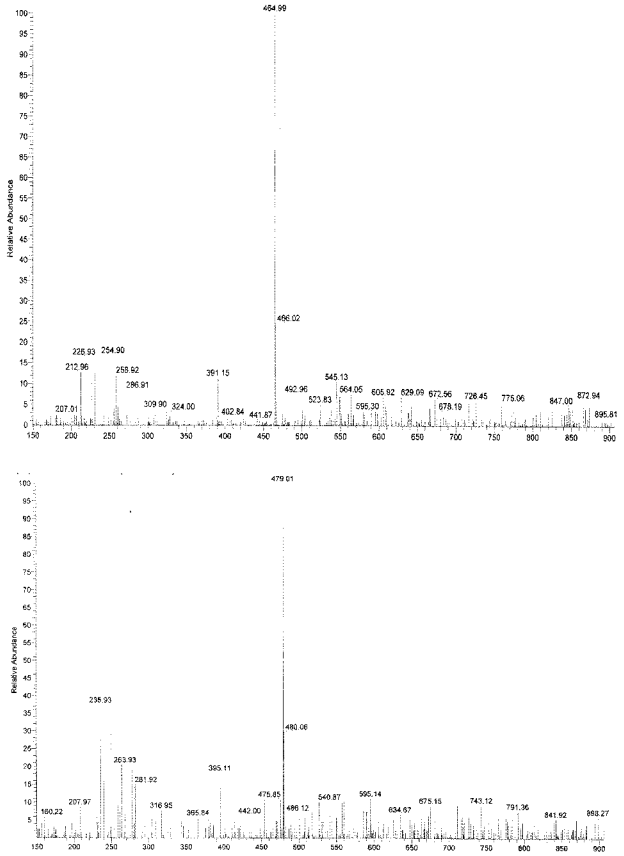

Fig. 2. LC/ES-MS spectra of anthocyanin 1 (upper) and 2 (lower) obtained from the seed coats of the black common bean.

Results and Discussion

HPLC separation of black common bean anthocyanins The HPLC chromatogram of the black common bean extract detected in the visible spectral region (520 nm) revealed two major anthocyanins with retention times of 6.5 and 9.4 min, which were designated as anthocyanin 1 and 2, respectively (Fig. 1). Trace amounts of an unidentified anthocyanin appeared as a small peak eluting before peak 1. The UV-Vis on-line spectra using a diode array HPLC detector showed λmax of the two anthocyanins in the 525-526 nm region. None of the pigments were acylated with aromatic acids, as evidenced by the absence of peaks in the 300-350 nm region (12, 13).

Identification of black common bean anthocyanins Anthocyanin 1 was identified as delphinidin 3-glucoside (Fig. 3) by analysis of its NMR (Tables 1 and 2), LC/ES-MS (Fig. 2), and UV-Vis spectral data. The aromatic region in the one-dimension proton NMR spectrum (Table 1) of anthocyanin 1 showed a 1H singlet at δ 8.95 (H-4), a 2H singlet at δ 7.76 (H-2', 6'), and a 2H AX system at δ 6.65 (H-6) and 6.87 (H-8), in accordance with a delphinidin nucleus (14, 15). The spectral region δ 60-80 in the ^{13}C spin echo Fourier transform NMR spectrum (Table 2) contained five resonances which

Table 1. ^{1}H NMR spectral data of isolated anthocyanins from the seed coats of the black common bean (0.5% DCl-CD_3OD at 25°C, 500 MHz)

Proton No.	Anthocyanin 1	Anthocyanin 2
Aglycone	--------- ppm --------	
4	8.95 s	8.92 s
6	6.65 d (1.5 Hz)	6.64 d (1.5 Hz)
8	6.87 d (1.5 Hz)	6.84 d (1.5 Hz)
2'	7.76 s	7.86 d (2.2 Hz)
6'	7.76 s	7.71 d (2.2 Hz)
OMe	-	3.95 s
3-Glucoside(H")		
1	5.33 d (8.0 Hz)	5.31 d (7.8 Hz)
2	3.71 dd (9.2, 7.8 Hz)	3.67 t (9.5 Hz)
3	3.58 m	3.58 t (9.5 Hz)
4	3.46 t (9.0 Hz)	3.43 t (9.5 Hz)
5	3.58 m	3.61 m
6_a	3.92 dd (12.0, 2.5 Hz)	3.91 dd (12.1, 2.2 Hz)
6_b	3.74 dd (12.0, 5.8 Hz)	3.75 dd (12.2, 5.9 Hz)

Table 2. ^{13}C NMR spectral data of isolated anthocyanins from the seed coats of the black common bean (0.5% DCl-CD_3OD at 25°C, 125 MHz)

Carbon No.	Anthocyanin 1	Anthocyanin 2
Aglycone	--------- ppm --------	
2	164.1	162.6
3	144.8	145.1
4	136.2	135.3
5	159.2	158.8
6	103.3	103.3
7	170.3	170.3
8	95.1	95.4
9	157.6	157.2
10	112.6	113.1
1′	120.0	119.5
2′	113.2	109.2
3′	147.5	149.5
OMe	-	57.2
4′	145.8	145.6
5′	147.5	147.2
6′	112.6	113.4
3-Glucoside		
1	103.6	103.5
2	74.8	74.8
3	78.8	78.6
4	71.1	71.2
5	78.1	78.2
6	62.3	62.5

together with the anomeric carbon were in agreement with one hexose (9, 15). The ^{1}H and ^{13}C shift values, assigned by the one-bond heteronuclear shift correlation NMR experiment and the ^{1}H-^{1}H coupling constant of the sugar ring protons (7-10 Hz) revealed by iterative spin simulation (PANIC) were in agreement with those of β-D-glucopyranoside (15, 16). The binding site of the sugar was confirmed, through a long-range correlation peak

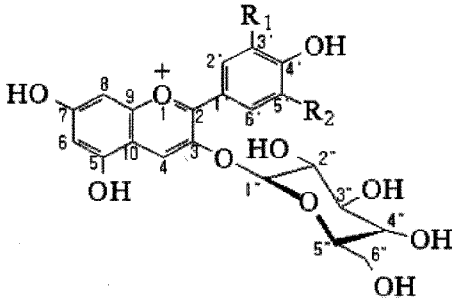

Fig. 3. Chemical structures of anthocyanin obtained from the seed coats of the black common bean (delphinidin 3-glucoside, R_1=R_2=H; petunidin 3-glucoside, R_1=CH_3, R_2=H).

between the anomeric proton and aglycone C-3 in the HMBC experiment, to be the 3 position of the aglycone (15, 17). In addition, the UV-Vis spectrum of anthocyanin 1 taken during on-line HPLC showed a visible maximum at 525 nm with an E_{440}/E_{525} ratio of 29%, indicating it to be a 3-glycoside with delphinidin nucleus (9, 15). The ratio of E_{uv}/E_{vis} (60%) and E_{acyl}/E_{vis} (9%) indicating that anthocyanin 1 was simple an anthocyanin without any complex acylation (13, 17). The LC/ES-MS spectrum (Fig. 2) of anthocyanin 1 gave a molecular ion at *m/z* 465, supporting the molecular formula $C_{21}O_{12}H_{21}^+$ and corresponding to delphinidin hexose (10, 13, 18). The NMR data (Tables 1 and 2) of anthocyanin 1 were in agreement with the literature values for delphinidin 3-glucoside (9, 10, 13, 15, 19).

The UV-Vis spectrum of anthocyanin 2 taken during on-line HPLC showed a visible maximum at 526 nm with an E_{440}/E_{526} ratio of 27%, indicating it to be a 3-glycoside of an anthocyanidin (9, 13, 15). In addition, the ratios of E_{uv}/E_{vis} (59%) and E_{acyl}/E_{vis} (7%) indicated that anthocyanin 2 was a simple anthocyanin without any complex acylation (13, 17).

The NMR spectra (Tables 1 and 2) of anthocyanin 1 and 2 showed many similarities. After assigning the proton resonance, anthocyanin 2 revealed an asymmetric anthocyanidin B-ring with one methoxy group (δ 3.95) and two hydroxyl groups in accordance with petunidin (9, 13, 18). The cross peak at 5.31/145.1 (H-1"/C-3) in the HMBC spectrum confirmed that the glucose unit was attached to the aglycone at the 3-OH (15). Thus, anthocyanin 2 was identified as petunidin 3-glucoside (Fig. 3, (13, 15, 19)). A molecular ion m/z 479 (Fig. 2), supporting the molecular formula $C_{22}O_{12}H_{23}^+$ and corresponding to petunidin glucoside in the LC/ES-MS spectrum of anthocyanin 2 confirmed this identity (9, 13, 15, 18).

Takeoka *et al.* (9) identified delphinidin 3-glucoside, petunidin 3-glucoside, and malvidin 3-glucoside in the black common bean (*P. vulgaris* L. cv. UI 911). In another study, Tsuda *et al.* (10) reported that the black common bean (*P. vulgaris* L. cv. Yamashirokurosando) contained anthocyanin delphinidin 3-glucoside only. However, in the present study, the black common bean (KG 97287) clearly contained both delphinidin 3-glucoside (anthocyanin 1) and petunidin 3-glucoside (anthocyanin 2), but we were not able to detect the presence of malvidin 3-glucoside. It is possible to conclude that the compositional differences

in anthocyanins depend on the variety of the black common bean germplasm, even though they share the same seed coat color.

Anthocyanin content of the black common bean Anthocyanins were extracted from KG 97287 black common beans using 1% HCl-20% CH_3OH. The total anthocyanin content of the black common beans (average of three determinations) was 2.781±0.12 mg/g of dried seed coats, which was comprised of 2.614 and 0.167 mg/g for delphinidin 3-glucoside and petunidin 3-glucoside, respectively. Delphinidin 3-glucoside comprised 94% of the total anthocyanin content in Korean black common beans.

Using HPLC, Yoshida *et al.* (19) analyzed the anthocyanin contents in 26 kinds of colored legumes of different species or from different production districts. In their study, the anthocyanin contents of 2 kinds of black common beans (*P. vulgaris* L.), produced in Japan or North America, ranged from 3.3 to 4.0 mg/g. The legumes of the same species produced in different areas or countries contained the same anthocyanin types and only varied in their contents depending on the cultivated region. In another study, Takeoka *et al.* (9) reported that the UI 911 black common beans (*P. vulgaris* L.) produced in the USA contained 3 kinds of anthocyanins, and that the anthocyanin content in the seed coats of UI 911 was about 23.7 mg/g of seed coats. In the present study, the black common bean cultivated in Korea contained far less anthocyanins than that of Japan and the USA (9, 19).

The introduction of high anthocyanin varieties in black common bean breeding programs is needed in Korea in order to increase the anthocyanin contents. We are presently undertaking quantification analysis of anthocyanins contained in the seed coats from a wide range of black common bean germplasms in order to investigate black common bean germplasms with high anthocyanin content.

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