Food Sci. Biotechnol. Vol. 14, No. 6, pp. 778 ~ 782 (2005)

Food Science and Biotechnology

Variation of Anthocyanins and Isoflavones between Yellow-Cotyledon and Green-Cotyledon Seeds of Black Soybean

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Abstract Analysis of black soybeans [*Glycine max* (L.) Merr.; 59 Korean varieties] revealed that 100-seed weights of green cotyledon seeds (33.5 g, n=31) were higher than those of yellow ones (28.9 g, n=28). Contents of delphinidin-3-glucoside (D3G), cyanidin-3-glucoside (C3G), petunidin-3-glucoside (P3G), and total anthocyanins in seed coats of black soybeans were 0.03-4.15, 0.74-18.36, 0.02-1.60, and 0.87-23.52 mg/g, respectively, among which most prominent anthocyanin was C3G (80.9% of total content), followed by D3G (13.6%) and P3G (5.5%). No significant differences were observed in color parameters a* and b* between black soybeans with yellow cotyledon (BYC) and green cotyledon (BGC). Total isoflavone content of BGC was higher than that of BYC, and negative correlation was found between total anthocyanin and isoflavone contents.

Keywords: black soybean, yellow cotyledon, green cotyledon, delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, color value, isoflavone

Introduction

Soybean [*Glycine max* (L.) Merr.] has traditionally been utilized in many ways in Korea. Recently, emphasis has been placed on the chemical composition of soybeans for improving various functional ingredients as well as for developing new soybean products and traditional soyfoods. Improvement of soybean components is expected not only to improve food-processing properties but also to increase the quality of soybean products. Seed coat of the soybean determines its important quality traits, such as luster, permeability, and nutritional value. In addition, seed coat color affects the visual appearance of soyfoods (1). Soybean seed shows diversity in color including yellow seed coat with yellow cotyledon, green seed coat with green cotyledon, brown seed coat with yellow cotyledon, and black seed coat with yellow or green cotyledon (2, 3). Black soybeans are sold at a premium price in the market for cooking with rice, and for producing black soymilk and traditional medicines among others, because they contain considerable amount of functional ingredients including anthocyanins, which have been used widely as natural coloring agents in the food and pharmaceutical industries, in the seed coat (4-8). Anthocyanins play important roles as dietary antioxidants in the prevention of oxidative damage, have several biological activities such as anticonvulsant, anticarcinogenic, antiatherosclerotic, and anti-inflammatory actions, and reduce the risk of coronary heart disease (9-14).

Soybean isoflavones are phytoestrogens, which have either weak estrogen-like or antiestrogenic activity, and have been reported to reduce the risk of breast cancer and heart disease as well as total cholesterol level through several mechanisms, and show some antioxidant activities (15-17). Due to these properties, black soybeans are currently receiving much attention as potential therapeutic agents against some pathological diseases.

With increasing soyfood consumption, black soybeans are in much demand for production of soy products such as black soymilk, black colored tofu, and various traditional soy sources. Although many studies have been performed on the isolation and identification of anthocyanins, researches on the contents of anthocyanin and isoflavone in various cotyledon phenotypes of black soybeans have not been reported. Therefore, this study was carried out to investigate the variation of anthocyanins among black soybeans with different cotyledon colors, and to determine their relationship with isoflavone contents.

Materials and Methods

Soybean seeds Black soybeans (59 varieties) were collected from nine provinces, where slight differences were observed depending on the variations in ecological environment in the Korean peninsula. Two black soybean varieties, '*Chongjakong*' and '*Geomjeongkong 1*', were selected as the control for the determination of anthocyanins, and 30 varieties with yellow seed coats were selected for isoflavones contents, respectively. Soybean seeds were milled to flour and defatted with hexane using automatic fat extraction system (Gerhardt Soxtherm 2000, Munchen, Germany) for analysis of isoflavones.

Anthocyanin analysis in the seed coat of black soybeans To determine the anthocyanin contents, seed coats of black soybeans were peeled manually. The separated seed coats (0.1 g) were then extracted with 10 mL of 1% HCl in methanol for 24 h in darkness.

Anthocyanins in black soybeans were analyzed using a Waters CapLC XE pump (Waters, Milford, MA, USA) equipped with a photodiode array detection system Waters

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Received June 7, 2005; accepted October 5, 2005

2996. The column used was a Symmetry C_{18}, 5 μm (0.32 × 150 mm) column from Waters operated at 25°C. Mobile phase consisted of 0.1% TFA in water (eluent A) and 0.1% TFA in 95% acetonitrile (eluent B). The gradient program was from 3 to 43% B for 80 min. The injection volume for all samples was 0.2 μl. Spectra were recorded from 200 to 600 nm at a flow- rate of 0.5 μl min^{-1}. ESI-MS was carried out with a Micromass electrospray interface ZMD 4000 (Micromass, Manchester, UK). Nitrogen was used as the nebulizing gas. Source block and desolvation temperature were 110 and 20°C, respectively. Mass condition: electrospray ionization (positive mode); cone voltage ramp 10-50 V; acceleration lens potential 0.5 kV; scan rate 0.4 scan s^{-1}; multiplier voltage 650 V. Prior to analysis, all samples were filtered through a 0.45-μm membrane filter. The standard anthocyanins were purchased from Extrasythese (Genay, France).

Isoflavone analysis in black soybean seeds One gram of defatted soybean flour was put into a test tube, suspended with 30 ml of 1 N HCl, and heated for 2 hr at 100°C with a reflux condenser in the water bath. After digestion, the extract was volume up to 100 ml with methanol, and the supernatant was filtered through a PTFE 0.45 μm syringe filter (Waters). The filtrate was injected for HPLC analysis. Isoflavone was analyzed by reverse-phase HPLC (Waters 2690 Alliance System) equipped with YMC-Pack ODS-AM303 (250 × 4.6 mm) connecting a guard column packed with Bonda C18 Waters guard-Pak pre-column (Waters). Acetic acid (0.1%) in 35% acetonitrile was employed for analysis of isoflavone mobile phase. The solvent flow rate was 1.0 ml min^{-1}. Following the injection of 20 μl sample, the eluted isoflavones were detected at 254 nm using a Waters 2487 dual absorbance detector. All HPLC analyses were performed at ambient temperature. The standard isoflavones were purchased from Sigma (St. Louis, MO, USA).

Color value Hunter's color values such as L* (lightness), a* (redness), and b* (yellowness) were measured using a color & color difference meter (Minolta Chromameter CR-200, Tokyo, Japan), which had been adjusted with a standard white plate (L*=97.38, a*=-0.02, b*=1.66).

Statistical analysis All measurements were triplicated. The data obtained were statistically analyzed using SAS release ver. 8.0 for Windows (Statistical Analysis Systems Institute Inc., Raleigh, NC, USA).

Results and Discussion

Seed characteristics The collection of 59 black soybean varieties from nine provinces in Korea and their 100-seed weights are presented in Table 1. The collected black soybeans were classified into two phenotypes base on their cotyledon colors, 28 varieties into 'black seed coat with yellow cotyledon' (BYC) and 31 varieties into 'black seed coat with green cotyledon' (BGC) (Fig. 1).

BGC (33.5 g) showed higher100-seed weights than BYC (28.9 g), except those from Jeju-do, which was probably mainly due to the differences in ecological environment of Jeju island.

Identification of anthocyanins The anthocyanin compositions of black soybeans were determined by ESI-MS. Three anthocyanins, delphinidin-3-glucoside (D3G), cyanidin-3-glucoside (C3G), and petunidin-3-glucoside (P3G) were

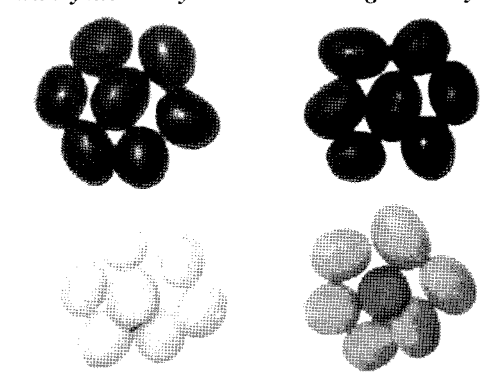
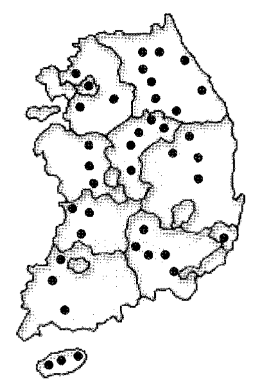


Fig. 1. Black soybean seeds with different cotyledon colors.

Table 1. Local collection and 100-seed weights of black soybeans from the nine provinces in Korea

Provinces	Collected samples		100-seed weights (g)	
	BYC[1]	BGC[2]	BYC	BGC
Gyeonggi-do	4	1	25.6±1.18	32.9±1.02
Gangwon-do	6	6	27.2±0.95	32.1±1.10
Chungcheongnam-do	-	4	-	33.0±0.87
Chungcheongbuk-do	5	5	22.7±1.31	34.9±1.54
Gyeongsangnam-do	2	4	30.7±0.76	32.3±1.42
Gyeongsangbuk-do	3	5	31.5±0.91	36.7±1.71
Jeollanam-do	3	2	31.9±1.12	33.6±1.32
Jeollabuk-do	4	2	30.9±0.89	34.3±1.26
Jeju-do	1	2	31.4±0.88	31.3±0.92
Mean±SD	(n=28)	(n=31)	28.9±1.02	33.5±1.23

[1]BYC, black seed coat with yellow cotyledon.
[2]BGC, black seed coat with green cotyledon.

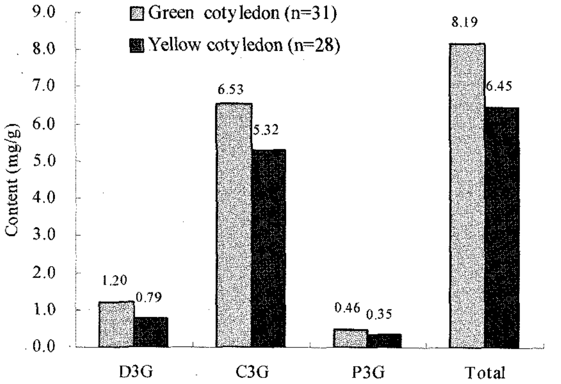


Each spot represents the regions of black soybean collection in the nine provinces of Korea.

Table 2. Anthocyanin contents in the seed coat of control varieties '*Chongjakong*' and '*Geomjeongkong 1*'

Varieties	Seed coat color	Cotyledon color	Anthocyanin contents (mg/g)				
			D3G[1]	C3G[2]	P3G[3]	total	CV(%)[4]
Chongjakong	black	green	1.41	8.14	0.51	10.06	2.85
Geomjeongkong 1	black	yellow	0.82	6.89	0.45	8.16	1.94

[1]D3G, delphinidine-3-glucoside.
[2]C3G, cyanidine-3-glucoside.
[3]P3G, petunidine-3-glucoside.
[4]CV, coefficient of variation.



Cotyledon color	Ranges of anthocyanin contents (mg/g)			
	D3G[1]	C3G[2]	P3G[3]	total
Green	0.09-4.15	0.74-18.36	0.03-1.60	0.87-23.52
Yellow	0.03-1.59	2.20-11.28	0.02-0.72	2.28-12.39

Fig. 2. Comparison of anthocyanin contents between yellow- and green-cotyledon seeds of black soybeans collected from nine provinces in Korea. [1]D3G, delphinidine-3-glucoside; [2]C3G, cyanidine-3-glucoside; [3]P3G, petunidine-3-glucoside, respectively.

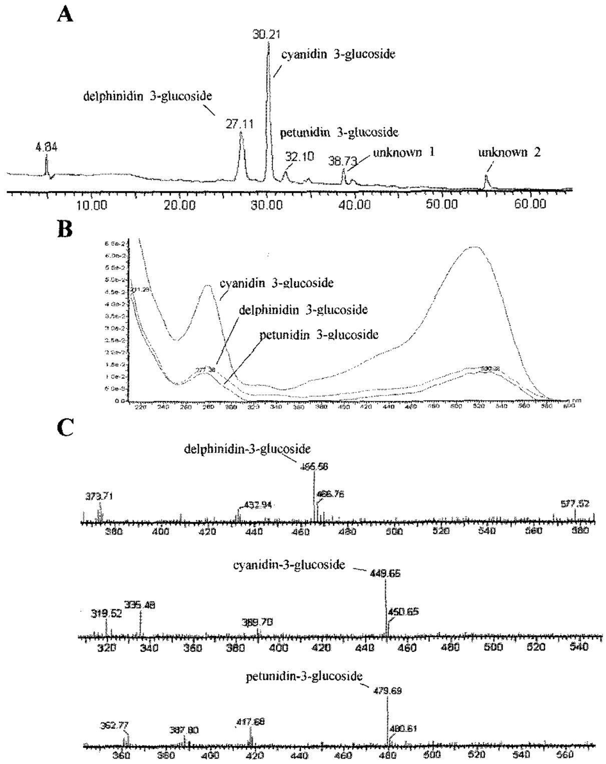


Fig. 3. Capillary HPLC chromatogram (A), UV-vis spectra of photodiode array detector (B), and ESI-MS spectral profiles (C) of anthocyanins in the seed coat of black soybean.

identified, with C3G being the major pigment in the black soybeans (Fig. 3). The λ_{max} range of the black soybean pigments on PDA spectrum showed a maximum absorbance at ranges of 520-530 and 275-280 nm.

There have been many reports on black soybean anthocyanins, with two anthocyanins C3G and D3G detected in the black soybeans (18-20). Recently, Choung et al. (4) reported that C3G and D3G were observed in all black soybeans of Korean varieties, whereas P3G was found only in some varieties, probably due to the different compositions of anthocyanins in black soybean seeds. In our study, however, three anthocyanins were observed not only in '*Chongjakong*' and '*Geomjeongkong 1*' but also in the collected black soybeans (Table 2 and Fig 2). However, notable variances in the anthocyanin contents were observed base on the cotyledon colors of black soybeans. This difference observed between our study results and those of previous reports (4, 18-20) was considered mainly due to the efficiency of separation conditions.

The dietary and health values of anthocyanins in black-colored crops such as black rice, black soybean, black waxy corn have received an increased attention in recent years. The antioxidant functions of anthocyanins have been ascribed to the aglycone moiety; however, the number of sugar residues at the 3-position, hydroxylation and methylation patterns, as well as acylation by phenolic acids are considered crucial factors for the expression of antioxidant effects (21).

Anthocyanin contents in the seed coats of 59 black soybeans are presented in Fig. 2. The total anthocyanin contents ranged from 0.87 to 23.52 mg/g, and D3G, C3G, and P3G contents were in the ranges of 0.03-4.15, 0.74-18.36, and 0.02-1.60 mg/g, respectively. The most prominent anthocyanin was C3G, which accounted for about 80.9% of the total anthocyanins, followed by D3G (13.6%) and P3G (5.5%). Nevertheless, inspite of the relatively lower contents of D3G and P3G than C3G in black soybeans, they merit special attention, because they also have antioxidant activities (22).

Using ESI mass, two unknown compounds, 336.8 and 307.26 *m/z*, were successfully identified. ESI mass spectra were recorded in the positive ion mode. Due to the ionization conditions applied, $[M+H]^+$ ions and minimum fragmentations could be obtained. The λ_{max} range of the unknown compounds 1 and 2 on PDA spectra showed maximum absorbances at 263 (Band II), 351 (Band I) and 255 (Band II), 355 nm (Band I), respectively (Fig. 4). The

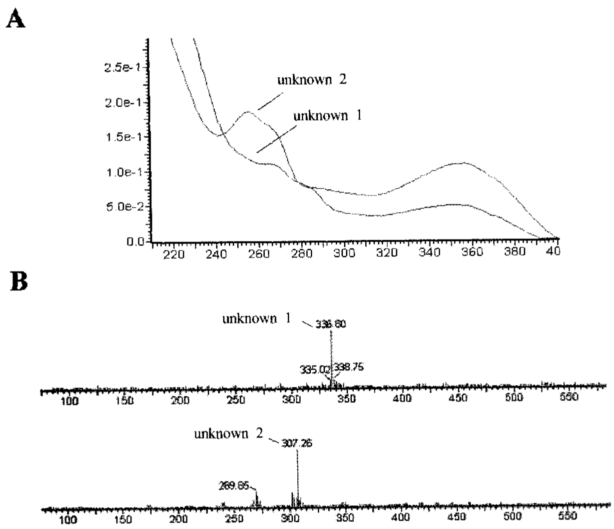


Fig. 4. UV-vis spectra of photodiode array detector (A) and ESI-MS spectral profiles (B) of unknown compound 1 and 2 observed in the seed coat of black soybean.

obtained results indicate these two unknown compounds belong to the flavonoid groups (23), although further study is required for confirmation.

Color characterization The color characteristics of the black soybean are shown in Table 3. Although in appearance the seed coats of black soybeans were deep black, their color values were significantly different. The L* value of BGC was significantly higher ($p < 0.05$) than that of BYC, whereas the a* and b* values showed no differences, indicating that the lightness parameter is one of the important characteristics between the BYC and BGC of black soybeans.

The statistical relationship between the color parameters and the anthocyanin contents was measured by means of simple correlations (Table 4). The anthocyanin contents showed low correlation with the color parameters (L*, a*, b*), suggesting that the color value is not an effective parameter for determining the anthocyanin intensity of the black soybeans. In our recent study, contrary to the results of this study, we have reported that the color values of black-colored rice were significantly correlated with the anthocyanin content (24). Therefore, further study is necessary to explain the different results obtained.

Recently, interest in anthocyanin-rich crops and foods

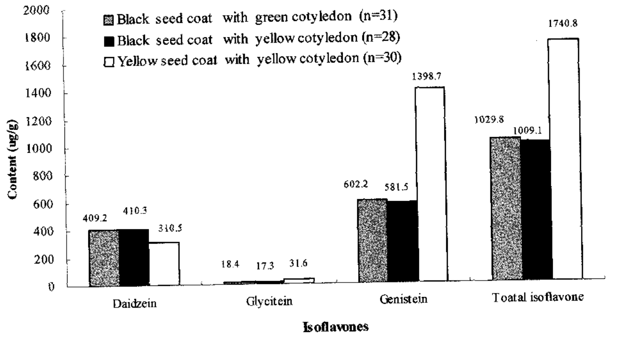


Fig. 5. Comparison on isoflavone contents between different cotyledon color seeds of black soybeans collected from the nine provinces in Korea.

has intensified due to their possible health benefits and use as safe food colorants. Development of a fast and accurate method for the determination of anthocyanin contents is now in progress.

Isoflavones The present study aimed at determining the effects of seed coat and cotyledon colors on the seed isoflavone levels. Isoflavone contents of 30 soybean varieties (developed in NICS), which have yellow seed coats, and 59 black seed coat soybean varieties collected from nine provinces in Korea were assayed (Fig. 5). Total isoflavone contents were 1740.8, 1029.8, and 1009.1 μg/g, respectively, in yellow seed coat soybeans, BGC, and BYC. These results indicated that yellow seed coat soybeans accumulate higher levels of isoflavones than black seed coat soybeans. Within black seed coat soybeans, isoflavone content of BGC was slightly higher, although not statistically significant, than that of BYC.

The isoflavones of soybean seeds, daidzein, genistein,

Table 4. Correlation coefficients between anthocyains and color values in black soybeans (n=59)

Anthocyanins	Color values		
	L*	a*	b*
Delphinidin-3-glucoside	0.101^{ns}	0.165^{ns}	0.186^{ns}
Cyanidin-3-glucoside	-0.018^{ns}	0.092^{ns}	0.113^{ns}
Petunidine-3-glucoside	0.079^{ns}	0.177^{ns}	0.178^{ns}
Total anthocyanin	0.007^{ns}	0.113^{ns}	0.133^{ns}

ns: not significant

Table 3. Variation of color values in the yellow- and green-cotyledon seeds of black soybean

Seed phenotype	Color values		
	L*	a*	b*
Black seed coat with yellow cotyledon (n=28)			
mean±S.D.	25.70±3.36	0.84±1.64	4.42±5.82
range	16.51~33.83	-0.38~4.12	0.49~17.11
Black seed coat with green cotyledon (n=31)			
mean±S.D.	26.76±4.57	1.15±2.07	5.54±6.53
range	16.38~35.46	-0.39~6.54	0.43~19.52
LDS ($p \leq 0.05$) between yellow- & green-cotyledon	0.981	ns	ns

ns: not significant; L: lightness; a*: (+) redness, (0) gray and (-) greenness; b*: (+) yellowness, (0) gray and (-) blueness.

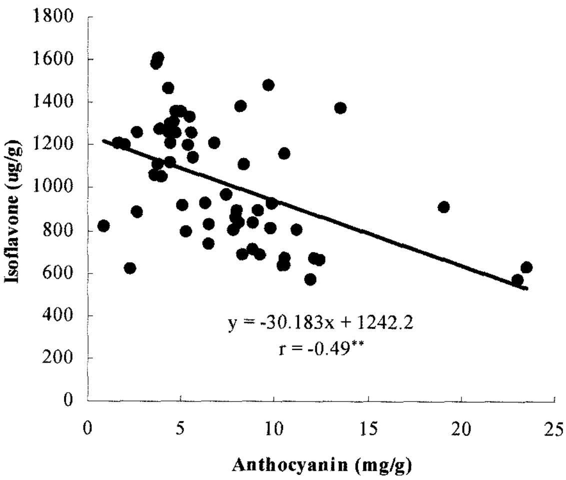


Fig. 6. Linear regression analysis of total anthocyanin contents versus isoflavone contents in black soybeans (n=59) collected from nine provinces in Korea.

and glycitein, are synthesized through the phenylpropanoid pathway and stored as glucoside conjugates (25); however, their contents vary significantly and are affected by both genotype and environmental conditions (26, 27).

Results of this study showed that the most dominant isoflavone is genistein, followed by daidzein and glycitein. However, the composition of isoflavones varied depending on the seed coat color. Genistein, daidzein, and glycitein accounted for 80.3, 17.8, and 1.9% of the total isoflavone in yellow seed coat soybeans, respectively. Although the compositions of isoflavones did not vary significantly within black seed coat soybean varieties, contents of genistein, daidzein, and glycitein were 58.5, 39.7, and 1.8% in BGC, and 57.6, 40.7, and 1.7% in BYC, respectively. These results suggested that isoflavone content varied among varieties with different seed coat colors, and the physiological responses of the isoflavones differed depending on the seed coat color of the soybeans. A significantly negative correlation (r=-0.49**) was found between anthocyanin and isoflavone contents (Fig. 6), suggesting that anthocyanins, which are also biosynthesized through the phenylpropanoid pathway (25), affect the biosynthesis of isoflavones in black soybeans, although no evidences are yet available to support this observation.

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