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Identification of Anthocyanin from The Extract of Soybean Seedcoat

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Anthocyanins are naturally occuring phytochemicals and the main components of the coloring of plants, flowers and fruits. They are known to elicit antioxidative, anti-inflammatory and cancer preventive activity. In this study, we investigated anthocyanins in black / yellow soybean seedcoats using different methods of detection - thin layer chromatography (TLC), capillary zone electrophoresis (CZE) and HPLC analysis. The anthocyanins in soybean seedcoats were extracted by five independent methods of extraction and the aglycons (anthocyanidins) of the corresponding anthocyanins were prepared by acid mediated hydrolysis. The anthocyanin / anthocyanidin in black soybean seedcoat showed characteristic TLC mobility, CZE electrophoretic retention and HPLC migration time while little of anthocyanins were detected from yellow soybean seedcoat. The extracted anthocyanins showed pH dependent retention time in CZE and spectral change in UV-Vis spectrum. HPLC analysis of the hydrolyzed extract of black soybean seedcoat identified the presence of four anthocyanidins. The major anthocyanin in black soybean seedcoat was cyanin (cyanidin-3-O-glucoside), with the relative order of anthocyanidin in cyanidin > delphinidin > petunidin > pelargonidin.

Key words: anthocyanin / anthocyanidin, soybean seedcoat, TLC, HPLC, CZE

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Introduction

Anthocyanins are naturally occuring phytochemicals and the main components of the coloring of plants, flowers, fruits and beans. They are widely consumed in our daily dine and known to elicit antioxidative, anti-inflammatory activity and cancer preventive activity (Hui *et. al.*, 2010; Koide *et. al.*, 1997; Lin,Chou, 2009; Shin *et. al.*, 2009; Slavin *et. al.*, 2009). Particularly, soybean has been one of the main nutritional rich sources for the intake of anthocyanins in our daily diet.

Anthocyanidins, aglycons of anthocyanins with the sugar moieties removed, also show anti-cancer effects against multiple cancer cell types, inhibiting the growth of certain cancer cells or the transformation of cells (Bin Hafeez *et. al.*, 2008; Cvorovic *et. al.*, 2010; Hyun,Chung, 2004; Meiers *et. al.*, 2001). Although the exact mechanisms in cancer preventive activity are not fully understood, the suppression of proliferation and angiogenesis and the induction of cancer cell apoptosis have been reported in many articles (Fernandes *et. al.*, 2010; Matsunaga *et. al.*, 2010; Stoner *et. al.*, 2010; Yun *et. al.*, 2009).

Anthocyanins have a group of phenolic compounds (called flavonoids) and their structures were characterized by the basic flavylium cation and various substituents. A variety of protonated, deprotonated, hydrated and isomeric forms exist and the relative proportion of these molecules is strongly dependent on pH environment. At low pH (< pH 2), anthocyanin exists in solution as an orange to purple flavilyum (red color species). As the pH is raised to 4.5, hydration and proton-transfer reactions occur and colorless carbinol pseudobase (or chalcone) becomes the main chemical species. At pH 7 or higher condition, unstable blue quinoidal forms become the main species in solution.

Cancer in the oral cavity is an aggressive tumor with high mortality (Lee *et. al.*, 2010; Lee *et. al.*, 2008). Despite of many studies on the anti-tumor effects of anthocyanin / anthocyanidins in various human cancer cell types, little is known about the fate and the anti-tumor effect of anthocyanin / anthcyanidin in oral cavity (Thomasset *et. al.*, 2009). Thus, our interest in molecular pathway of tumor suppression in oral cavity motivated us to initiate the analysis of anthocyanin / anthocyanidin in soybean seedcoat. In this study, anthocyanin rich extracts of black / yellow soybean seedcoat were prepared by different methods of extraction and the extract was exposed to aqueous acidic condition for the analysis of aglycons of anthocyanins. Anthocyanins / anthocyanidins were detected by TLC, CZE, HPLC analysis and UV-Vis spectrum.

Materials and Methods

Materials

Cyanidin, delphinidin, and other anthocyanidins were purchased from Extrasynthese (Genay, France) and their purities were greater than 99%. Stock solution of delphinidin was prepared at concentration of 5 mg/ml in ethanol. The soybeans (*Glycine max (L.) Merr.*), which had been harvested in 2005, were provided by National Agricultural Cooperative Federation (NACF, Yeocheon, Chonnam, Korea). Cellulose TLC and silica gel TLC and other chemicals were purchased from Sigma (St Louis, MO).

Preparation of anthocyanin-rich extract from soybean seedcoat

Soybean seeds were washed with distilled water and heated for 2 h at 100°C prior to taking off seedcoat. Extraction of anthocyanin from soybean seedcoat was carried out by five different methods: 1) 70% ethanol (80°C, 3 h), 2) Hot water (100°C, 3 h), 3) 70% acetone (80°C, 3 h), 4) 1% HCl in 20% methanol in distilled water (4°C, 48 h), 5) 1% HCl, 40% methanol in distilled water (4°C, 48 h). 100 mg of black / yellow soybean seedcoat was placed in the media of 1 ml extraction solution for 48 h. The extracted solution was centrifuged for 3 min at 13,000 rpm and the supernatant was transferred into a new tube prior to the sep-pak purification. Finally the extract was vacuum-evaporated at 30°C.

Preparation of anthocyanidins

The aglycons of anthocyanins were prepared by acid hydrolysis of anthocyanins isolated from seedcoats of black (or yellow) soybean. The anthocyanin mixtures (200 μl) in 100 μl of 2 N HCl were hydrolyzed under an atmosphere of nitrogen for 3 h at 98°C.

TLC analysis

Samples taken before / after hydrolysis were spotted on TLC plate of 20 × 60 mm glass coated with cellulose or silica gel with a layer thickness of 0.1 mm. TLC plates were developed in mobile phase of hydrogen chloride : formic acid : water (volume ratio, 7.1 : 51.4 : 41.4) for cellulose TLC and ethylacetate: formic acid: water (volume ratio, 50 : 3 : 2) for silica gel TLC, respectively.

CZE analysis of anthocyanin extracts

CZE was carried out with a constant voltage (25 kV) at 20°C using a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Brea, CA) equipped with a standard cassette containing an uncoated fused-silica capillary (50 μm I.D. and 375 μm O.D.; × 72.5 cm long with effective length of 60.0 cm) and a photodiode array detector. The capillary was conditioned before injection by washing with 0.1 M sodium hydroxide, and then with ultra pure water. The running buffer used for analysis was (1) 30 mM borate, 100 mM SDS, pH 9.0; (2) 200 mM phosphate : acetonitrile, 2:1 volume ratio, pH 1.5; (3) 200 mM phosphate : acetonitrile, 2:1 volume ratio, pH 1.8. The sample solutions (1 mg/ml in running buffer) were loaded onto the capillary with a pressure mode (0.5 psi for 5.0 sec) (Saenz-Lopez *et. al.*, 2003).

Analytical HPLC separation

The analysis of anthocyanidins in soybean seedcoats was carried out using a HPLC system (Waters 2487, USA) equipped with a C_{18} reverse phase column (4.6 mm × 250 mm). The extract (200 μl) was mixed with 100 μl of 2 N HCl in 40% methanol solution, and then incubated at 100°C. Samples were taken at various time periods prior to injection. HPLC was run by isocratic elution mode using 18% solvent B (0.4% TFA in acetonitrile) in solvent A (0.4% TFA in distilled water) at a flow rate of 1.0 ml/min. The elution profile was monitored by UV-detection at 530 nm.

UV-Vis spectral study

UV-Vis spectra of anthocyanin extract from black soybean seedcoat were obtained in buffer solutions with different pH values (Choung *et. al.*, 2001). Small aliquots of the extract were diluted in either 200 mM phosphate : acetonitrile, 2:1 volume ratio, pH 1.5 or 30 mM borate, 100 mM SDS, pH 9.0. The spectrum was recorded in the range of 300 nm to 700 nm using a UV-Vis spectrophotometer (Shimadzu, Japan).

Results

Thin layer chromatography analysis

Extracts of black / yellow soybean seedcoats were prepared as mentioned in Materials and methods. In TLC analysis, the anthocyanin rich extract of black soybean seedcoat was hydrolyzed to the anthocyanidins which appeared as a second spot with different R_f values from the anthocyanin. The acid hydrolysis to convert anthocyanin into anthocyanidin was completed within 60 minutes with higher R_f value of anthocyanidin in silicagel chromatography. On the contrary, anthocyanidins in cellulose TLC were appeared with lower

value of R_f than anthocyanins (Fig. 1). Similar type of migration patterns were observed from the extracts prepared by other methods (data not shown).

Capillary zone electrophoresis analysis of the extract

In order to study electrophoretic property of anthocyanin / anthocyanidin in CZE, the stock solution of the extract was applied to the P/ACE MDQ Capillary Electrophoresis System, using running buffers of 30 mM borate, 100 mM SDS, pH 9. The electropherogram of the extracts of black soybean seedcoat, prepared by five different conditions, showed similar peak retention time of anthocyanins while the extracts from yellow soybean seedcoat did not show peaks (Fig. 2). Under the acidic buffer solution (200 mM phosphate : acetonitrile, 2:1 volume ratio with pH 1.5 or pH 1.8), electropherograms of extracts of black soybean seedcoat showed a broaden peak with almost identical retention time except the extract prepared by the hot water. Again no detectable peak was observed from the extracts of yellow soybean seedcoat. Also the migration time of anthocyanin under the buffer condition of pH 1.8 was longer than the one of pH 1.5 probably due to the equilibrium shift to the form of neutral quinoidal base (Fig. 3).

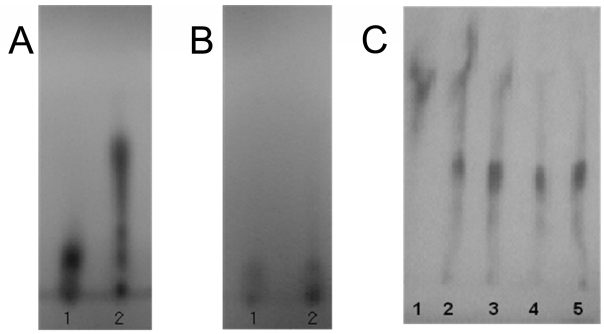

Fig. 1. TLC analysis of extract. Acid hydrolysis of black (A) / yellow (B) soybean seedcoat extracts in silica gel plate: 1, before hydrolysis; 2, after hydrolysis. Mobile phase for silica gel TLC was ethylacetate : formic acid : water (volume ratio of 50 : 3 : 2). (C) Cellulose chromatography of acid hydrolysis of black soybean seed coat extracts; 1, non-hydrolyzed black soybean seedcoat extraction; 2 - 5, hydrolyzed from 10 to 40 min. Mobile phase was hydrogen chloride : formic acid : water (volume ratio of 7.1 : 51.4 : 41.4).

HPLC analysis of anthocyanidin in soybean seedcoat extract

The anthocyanidins of soybean seedcoats were analyzed by a reversed phase HPLC system monitored at 530 nm. The extract of soybean seedcoat was acid hydrolyzed with 2 N HCl in 40% methanol. Samples were taken at various time intervals and run by isocratic elution mode as described in Materials and methods. HPLC analysis of hydrolyzed extract of black soybean seedcoat (Fig. 4) showed a characteristic peak of individual anthocyanidin which was identified by validating with the retention time of the corresponding standard. The initial peak of anthocyanin was disappeared gradually as the hydrolysis is processed. The migration time was fast in order of delphinidin > cyanidin > petunidin. Contrary to the black soybean, the anthocyanin extract hydrolyzed from yellow soybean seedcoat showed barely detectable peaks which were identified to be cyanidin and delphinidin. In addition, further analysis with the HPLC chromatogram of black soybean seedcoat allowed us to identify the presence of another anthocyanidin, a pelargonidin, as shown in Fig. 5.

UV-Vis spectral property of anthocyanin rich extract

The UV-Vis spectrophotograms were obtained using buffer

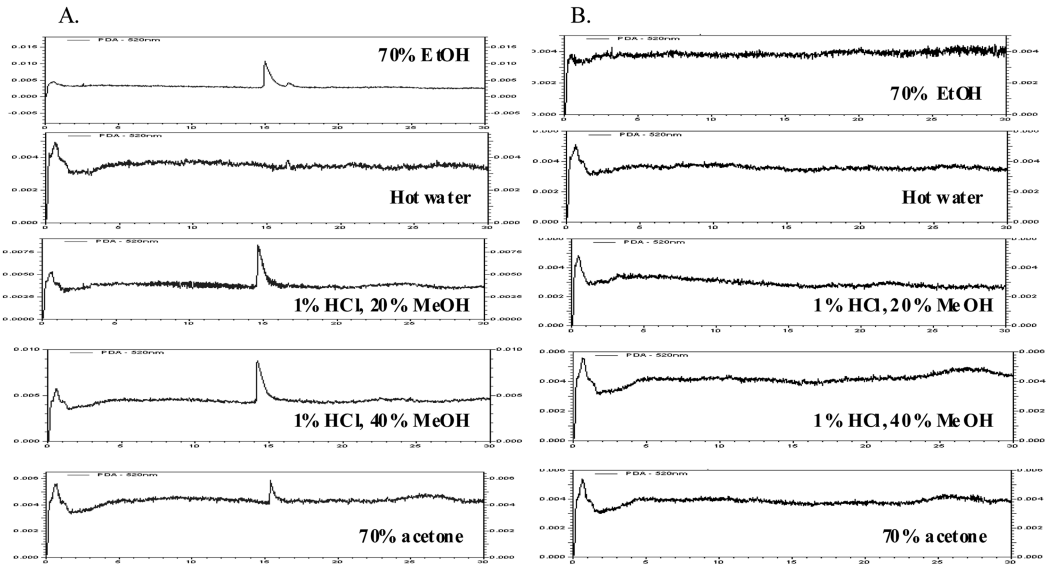

Fig. 2. CZE electropherogram of extracts of soybean seedcoats at 520 nm, using running buffers of 30 mM borate, 100 mM SDS, pH 9. A, extracts of black soybean seedcoats; B, extracts of yellow soybean seedcoats.

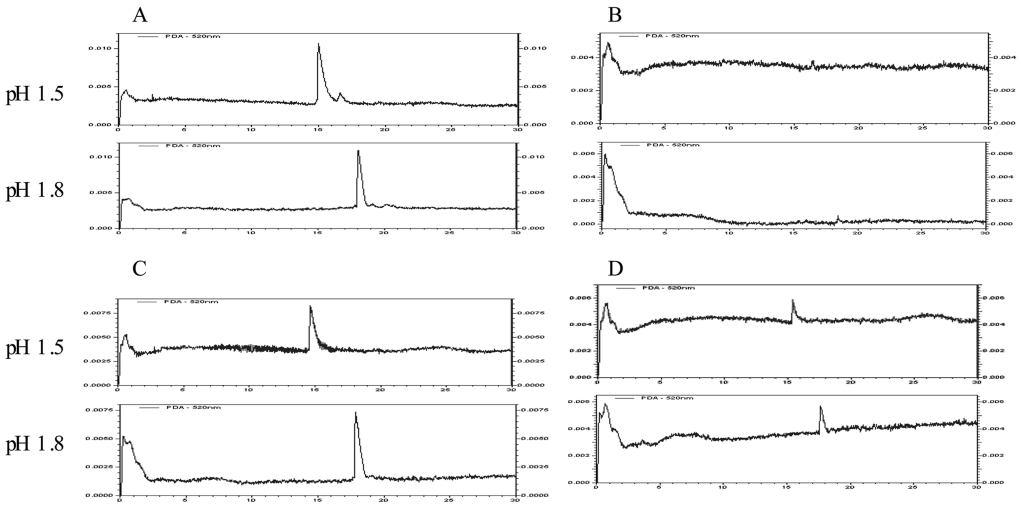

Fig. 3. pH dependence on CZE migration of anthocyanin at low pH buffer solution. The running buffer was 200 mM phosphate : acetonitrile, 2 : 1 volume ratio with pH 1.5 or pH 1.8. Samples for CZE analysis were taken from the extract as described in Materials and methods: A, 70% ethanol; B, hot water; C, 20% methanol in distilled water; D, 40% methanol in distilled water.

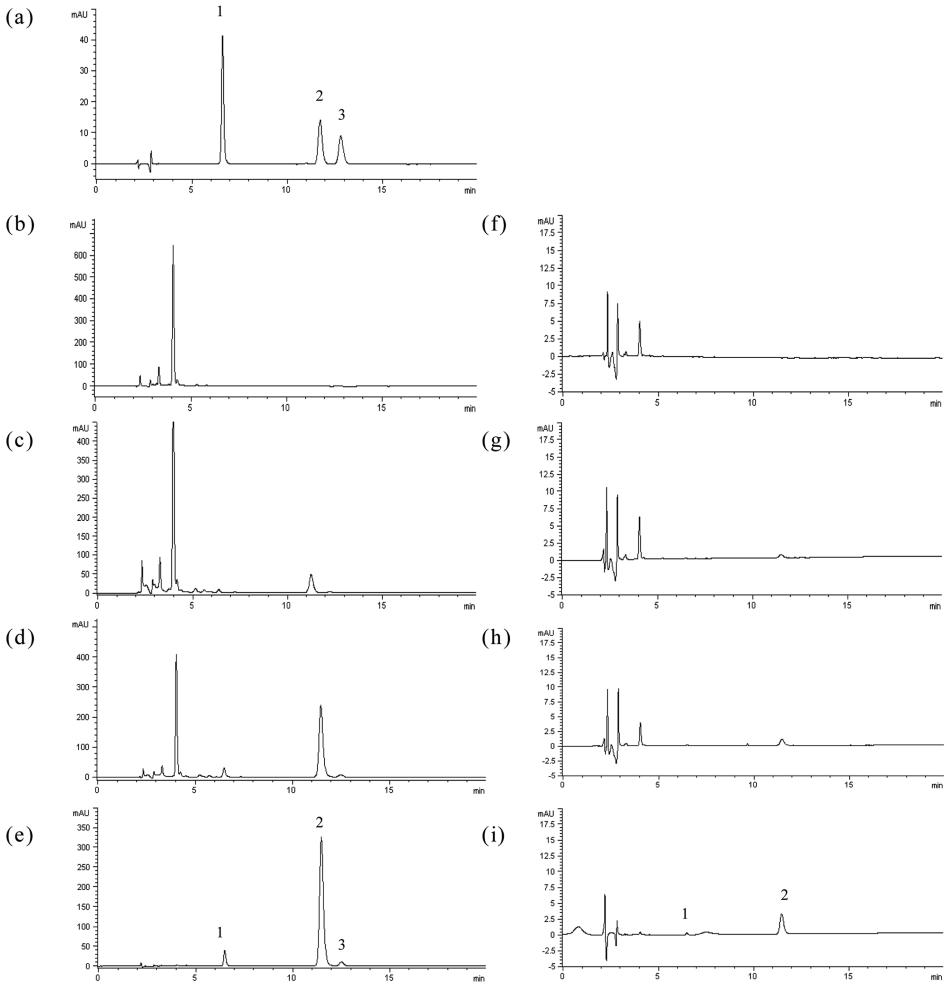

Fig. 4. HPLC analysis of soybean seedcoat extracts. The peaks were identified by the commercial standard anthocyanidin. Extracts (200 μl) of soybean seedcoats were mixed with 100 μl of 2 N HCl and incubated at 100°C. Samples were taken at several time intervals. Numbers were assigned based on the migration of standards: 1, delphinidin; 2, cyanidin; 3, petunidin. (a), standard mixture (10 mM); (b), crude extract of black soybean seedcoat; (c - f), hydrolysis of black soybean seedcoat extract at 10, 30, 90 min; (f), crude extract of yellow soybean seedcoat; (g - i), hydrolysis of yellow soybean seedcoat extract at 10, 30, 90 min.

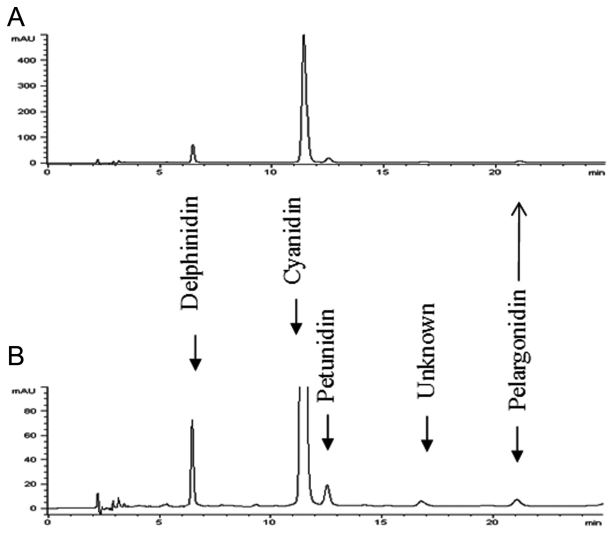

Fig. 5. Identification of pelargonidin by reversed phase HPLC analysis of black soybean seedcoat extracts. The individual peak was identified by the commercial standard anthocyanidin (10 mM). Extracts (200 μl) of soybean seedcoats were mixed with 100 μl of 2 N HCl and incubated for 2 h at 100°C. A, HPLC chromatogram of the extract of black soybean after hydrolysis; B, The same HPLC chromatogram scaled down for showing the pelargonidin peak.

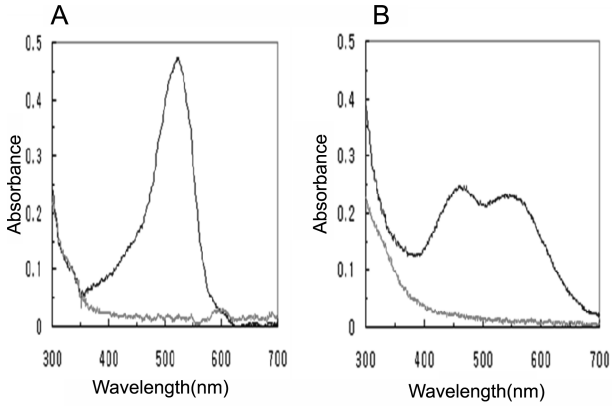

Fig. 6. pH dependent UV-Vis spectrum of the extract of black soybean seedcoat. (A), UV-spectrum in 200 mM phosphate acetonitrile, pH 1.5; (B), UV-spectrum in 30 mM borate, 100 mM SDS, pH 9.0. The UV-Vis spectrum was scanned in the presence / absence of the extract.

solutions with two different pH values (200 mM phosphate : acetonitrile, 2:1 volume ratio at pH 1.5; 30 mM borate, 100 mM SDS at pH 9.0). The absorbance spectra were obtained by adding small aliquots of the extract into buffer solutions. The spectrum of anthocyanidin was dependent on the pH value of the buffer solution with dramatic change in spectral shape (Fig. 6). The spectrum of the extract at pH 1.5 showed the maximum absorbance at 525 nm, which was similar to the maximum absorbances of cyanin (518 nm) and delphinidin (525 nm) (Choung *et. al.*, 2001). On the contrary, the spectrum in buffer solution of pH 9.0 was dramatically changed with two maximum absorbances at 455 nm and 552 nm.

Discussion

This study was initiated to identify the anthocyanin / anthocyanidin in the soybean seedcoat. Anthocyanins from the extract of black / yellow soybean seedcoat were detected by using three independent methods - TLC, CZE and HPLC. Anthocyanins exist in solution as various forms of chemical structure with characteristic color (Choung *et. al.*, 2001). The equilibrium in different chemical forms of anthocyanins depends on the pH. The most prevalent form of anthocyanins at low pH (< 2.0) is the red flavylium cation, a stable form of anthocyanin. The flavylium cation undergoes structural transformations as the pH is raised, which is due to the hydration and proton-transfer reactions. In TLC analysis before / after acid hydrolysis of extract, anthocyanins / anthocyanidins were detected from black soybean seed coat while the extract of yellow soybean seedcoat was only weakly detectable. Tailing spots on TLC made us difficult to identify an anthocyanin / anthocyanidin. The difference in the R groups of anthocyanidins, the same aglycone with different sugars and the fast shift in equilibrium between cationic quinoidal base and neutral quinoidal base caused a long tailing and different spots on TLC. CZE analysis of extracts, prepared by five different methods, showed a broaden peak with almost identical retention time at a given pH condition except the extract prepared by the hot water. Since flavilium cationic form (red color) of anthocyanins in acidic condition of running buffer would be the main structure in solution, we expected that the resolution on the migration of the anthocyanin would be improved in CZE analysis. However, a tailing broad peak was appeared in the electropherogram of extracts at low pH condition. This may be due to the difference of the mobility between anions of running buffer and solute anions. In contrast, HPLC system provided the excellent method to determine the composition of anthocyanidin in the extract. Reversed phase HPLC analysis of hydrolyzed extract of black soybean seedcoat allowed us to identify anthocyanidins by validating the peak with the retention time of the standard. The major anthocyanin in black soybean seedcoat was cyanin with the relative order of anthocyanidins in cyanidin > delphinidin > petunidin > pelargonidin. On contrast, weak peaks corresponding to cyanidin and delphinidin were detected from yellow soybean seedcoat indicating that little of anthocyanins are contained yellow soybean seedcoat. UV-vis spectral property of the extract showed pH dependence. The spectrum at a buffer solution of low pH value was dramatically changed into a different spectral shape at buffer solution of pH 9.0, indicating that the equilibrium between acidic quinoidal base and neutral quinoidal base of anthocyanins at pH 1.5 was shifted to the neutral quinoidal base form at buffer solution of pH 9.0. While our analysis work was in process,

another study characterizing anthocyanins in the black soybean was reported by Lee's group, using HPLC with diode array detection and electro spray ionization / mass spectrometry (HPLC-DAD-ESI/MS) (Lee *et. al.*, 2009). In their study, anthocyanins were extracted from the coat of black soybeans with 1% TFA in methanol and the anthocyanins in extracts were characterized without hydrolysis. Interestingly, the identified hydrolyzed anthocyanins (anthocyanidins) by our HPLC analysis were consistent with their finding which was based on the fragmentation patterns of HPLC-DAD-ESI/MS.

In conclusion, anthocyanins in soybean seedcoats, which were investigated by different methods of extraction and detection, revealed the presence of four anthocyanins in black soybean seedcoats while yellow soybean seedcoat had little of anthocyanins. The most major anthocyanin in black soybean seedcoat was cyanin (cyanidin-3-O-glucoside) with the relative order of anthocyanidin in cyanidin > delphinidin > petunidin > pelargonidin.

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