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Differences in the metabolic profiles and antioxidant activities of wild and cultivated black soybeans evaluated by correlation analysis

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ABSTRACT

Wild soybeans are considered a potential resource for soybean domestication and an important source of genetic diversity for soybean crop improvement. Understanding metabolite-caused bioactivity differences between cultivated and wild soybeans is essential for designing a soybean with enhanced nutritional traits. In this study, the non-targeted metabolic profiling of 26 soybean varieties, 15 wild black soybeans (WBS) and 11 cultivated black soybeans (CBS), using liquid chromatography-mass spectrometry (LC-MS) in combination with multivariate analysis revealed significant differences in 25 differential metabolites. Among these, the soyasaponins Ab and Bb were found to be characteristic metabolites expressed more substantially in CBS than in WBS. Three different antioxidant assays and correlation analysis identified major and minor antioxidants that contributed to WBS having an antioxidant activity 4- to 8-fold stronger than that of CBS. Epicatechin, procyanidin B2, and cyanidin-3-*O*-glucoside were identified by both association analysis and the online LC-ABTS radical scavenging assay as being major antioxidants.

1. Introduction

According to the Food and Agriculture Organization (FAO), approximately 350 million tonnes of soybeans were produced globally in 2015/2016 (www.fao.org/faostat/en/#data/QC). Soybeans are a main crop in terms of economic and nutritional aspects, containing rich amounts of protein, starch, and essential minerals, as well as secondary metabolites, such as lipids and phenolic compounds (Kim et al., 2006; Xiao, 2008). Specifically, phenolic compounds, including isoflavones and anthocyanins, are considered important bioactive compounds that contribute to the health benefits associated with soybean seed consumption (Astadi, Astuti, Santoso, & Nugraheni, 2009). Numerous *in vivo* and *in vitro* studies have been performed on soybean seeds, highlighting their antiobesity (Kwon et al., 2007), antidiabetic (Kurimoto et al., 2013; Yao et al., 2013), antifungal (Kim et al., 2010), and chemopreventive (Galvez, Chen, Macasieb, & de Lumen, 2001; Lim, Jeong, & Song, 2016) activities. Additionally, several epidemiological studies have shown that soybean consumption is associated with a positive effect on cardiovascular disease (Choquette et al., 2011; Rebholz et al., 2013) breast cancer (Chen et al., 2014; Ko et al., 2013), chronic obstructive pulmonary disease and respiratory symptoms (Engelen et al., 2007; Hirayama et al., 2009) in diverse populations.

Generally, black soybean seeds demonstrate antioxidant activity stronger than those of other colours due to their higher anthocyanin content. Zhang et al. (2011) analysed the seed coats of 60 Chinese black soybeans to investigate their total phenolic compounds, focusing on six anthocyanin profiles that were found to vary with seed variety. Although individual phenolic compounds were not analysed, the authors reported a strong correlation between total phenolic content and antioxidant activity.

Cultivated soybeans (*Glycine max*) are thought to have domesticated from wild soybeans (*Glycine soja*) in China approximately 5000 years ago, and they became widespread in Korea and Japan before being introduced to North, Central, and South America (Chen, Hu, & Zhang, 2009; Guo et al., 2012). Wild soybeans are considered a potential resource for soybean domestication and an important source of genetic

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diversity for crop improvement (Wen, Ding, Zhao, & Gai, 2009; Zhou et al., 2015). Understanding the metabolic profiles of cultivated and wild soybeans is essential for designing a soybean with enhanced nutritional traits. Various genomic and biochemical analyses of both cultivated and wild soybeans have been performed to better decipher the molecular mechanisms associated with agronomic traits, such as drought stress, viral infection, oil content, and plant height (Phan, Seo, Choi, Lee, & Kim, 2014; Wang et al., 2016; Zhou et al., 2015). However, limited studies have investigated the differences in the comprehensive metabolic profiles and antioxidant compounds between cultivated black soybeans (CBS) and wild black soybeans (WBS).

Non-targeted metabolomics analysis based on liquid chromatography-high resolution mass spectrometry (LC-HRMS) allows the simultaneous detection of a wide spectrum of metabolites and rapidly provides valuable chemical information regarding molecular candidates for differentiating between species, such as CBS and WBS. Furthermore, when combined with bioactivity profiling and multivariate analysis, LC-HRMS can directly identify bioactive compounds in extracts prepared from different crop varieties.

In the present study, we analysed the secondary metabolites of 26 black soybean seeds and investigated the differences in the metabolic profiles and antioxidant activities of CBS and WBS using correlation analysis. Finally, an online high-performance liquid chromatography (HPLC)-2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay was performed to confirm the major soybean antioxidants identified by correlation analysis.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol, acetonitrile, and water were obtained from Fisher Scientific (Pittsburgh, PA, USA). 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrochloric acid, and formic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The chemical standards for delphinidin-3-*O*-glucoside (purity: > 95%), cyanidi-3-*O*-galactoside (> 90%), cyanidin-3-*O*-glucoside (> 95%), procyanidin B2 (> 90%), epicatechin (> 98%), daidzin (> 95%), daidzein (> 98%), catechin (> 99%), rutin (> 95%), isoquercetin (> 98%), quercetin (> 95%), hesperidin (> 98%), glycitein (> 97%), glycitin (> 95%), cyanidin (> 95%), peonidin (> 96.5%), genistin (> 95%), genistein (> 97%), and phlorizin (99%) were obtained from Sigma Aldrich.

2.2. Plant materials

Fifteen wild and 11 cultivated black soybeans were selected from soybean germplasm collections maintained by the National Institute of Crop Science in Korea. Three biological sample sets were planted and grown in the normal soybean-growing season (June to October 2015) in the national agricultural research experiment field of Wanju-gun in JeonJu, Korea (latitude/longitude: 35° 50′ 42″ N/ 127° 8′ 51″ E). The field soil type was cray-loam, and the average monthly temperatures were recorded as follows: 22.7 °C in June, 25.1 °C in July, 25.9 °C in August, 21.6 °C in September, and 16.1 °C in October. A planting density of 15 cm was established between plants in a row, and the rows were 70 cm apart. Field management, including irrigation, fertilizer application, and pest control, followed essentially normal agricultural practices according to the standard protocol of the Rural Development Administration (http://www.nongsaro.go.kr/).

2.3. Sample preparation

Soybean extraction was performed according to previous reports with slight modifications (Lin et al., 2014). Dried black soybeans were ground into fine powder and sieved through a 55-mesh count screen, and 40 mg of the resulting powder was mixed with 70% aqueous methanol (4 mL, 1% HCl) containing 1.5 μg/mL phlorizin as an internal standard before being sonicated for 30 min. The mixture was retained at room temperature in the dark for approximately 16 h. An acidified solvent was then used to extract secondary metabolites, including anthocyanin compounds, from the black soybeans. Next, 2 mL of the supernatants was filtered through a 0.20-μm syringe filter with a GH Polypro membrane (Pall Corporation, Ann Arbor, MI, USA), vacuum freeze-dried (Ilshin lab, Korea), and reconstituted with 0.5 mL of 50% aqueous methanol for LC-MS analysis. Quality WBS and CBS controls were prepared by combining all the WBS and CBS extracts, respectively. For bioactivity analysis, soybeans were extracted with the same solvent used for LC-MS analysis without adding an internal standard. After being dried and weighed, the extracts were reconstituted with 50% aqueous methanol at 20 mg/mL, and the extract solutions were stored at − 80 °C until used for functional activity analysis.

2.4. Metabolite analysis

The non-targeted metabolites of the soybean extracts were analysed using a MicrOTOF-Q II (Bruker Daltonics, Bremen, Germany) mass spectrometer, which was coupled with a 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA). Resolving power of the MS instrument was 17,500 at *m/z* 922. The solvent system consisted of water/acetonitrile (95:5, v/v with 0.1% formic acid) as solvent A and acetonitrile/water (95:5, v/v with 0.1% formic acid) as solvent B. A 60-min linear gradient was used starting from 5% B to 20% for 20 min, 20% to 50% for 20 min, and 50% to 100% for 15 min. Chromatographic separation was achieved using a reversed-phase Prevail C_{18} Column (250 × 4.6 mm, 5-μm) at a 0.7-mL/min flow rate. The column was maintained at room temperature and was re-equilibrated for at least 10 min between analyses. A 10-μL aliquot of each reconstituted extract was injected, and mass spectra were recorded over the range of *m/z* 50 to *m/z* 1000 in positive and of *m/z* 500 to *m/z* 1500 every 1.0 s in negative ESI mode. The ion source parameters for high-resolution accurate mass measurements and automatic tandem mass spectra included a interpolating collision energy of *m/z* 100 at 7 eV, *m/z* 500 at 18 eV, *m/z* 1000 at 30 eV, *m/z* 1500 at 50 eV, a capillary voltage of + 4.5 kV for positive and − 3.0 kV for negative ESI mode, a nebulization gas flow of 10 L/min, a source gas temperature of 180 °C, and a cone voltage of 35 V. External calibration using a solution of sodium trifluoroacetate provided a mass accuracy within 5 ppm.

2.5. DPPH and ABTS radical-scavenging activities

DPPH radical-scavenging assay was carried out according to the modified method of Shang, Kim, and Um (2014). The DPPH solution was prepared by dissolving 15.77 mg of DPPH in 150 mL of ethanol. 100 μL of soybean extracts at various concentrations was added to 100 μL of the DPPH solution in a 96-well plate for 60 min at room temperature in the dark. The absorbance of each solution was measured using a Synergy HT Multi-microplate Reader (Bio-Tek Instruments) at 515 nm against an ethanol blank. The antioxidant activity was expressed according to the following equation: $S_{DPPH} = [1 - A_s / A_{bk}] \times 100$, where S_{DPPH} is the DPPH free radical-scavenging ability (%), A_s is the sample absorbance, and A_{bk} is the blank absorbance.

For the ABTS assay, ABTS radical reagent was prepared according to the previous procedures with minor modification (Re et al., 1999). The solution was prepared through the reaction of 7 mM of ABTS and 2.45 mmol/L of potassium persulfate after incubation at room temperature in the dark for 16 h. The ABTS solution was diluted with 100 mL of distilled water. To determine the scavenging activity, the soybean extract was added to a 96-well plate and reacted with the ABTS solution for 5 min in the dark. After incubation, the absorbance was measured at 734 nm using a Synergy HT multimode microplate reader.

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