Comparison of nutritional components (isoflavone, protein, oil, and fatty acid) and antioxidant properties at the growth stage of different parts of soybean [*Glycine max* (L.) Merrill]

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Abstract This is the first study to investigate antioxidant capacities of isoflavones prepared using microwave-assisted hydrolysis method from different parts (seeds, leaves, leafstalks, pods, stems and roots) of soybean at growth stages. In addition, the fluctuations in the isoflavone, protein, fatty acid, and oil contents in R6-R8 (R6: beginning; R7: beginning maturity; R8: full maturity) seeds were confirmed. The R7 seeds exhibited the most predominant contents of isoflavones ($1218.1 \pm 7.3 \mu\text{g/g}$) in the following order: daidzein (48%)>genistein (35%)>glycitein (17%). The second highest isoflavone content was found in the leaves ($1052.1 \pm 10.4 \mu\text{g/g}$), followed by R8 seeds>roots>R6 seeds>leafstalks> pods; the stems exhibited the lowest isoflavone content ($57.2 \pm 1.7 \mu\text{g/g}$). Interestingly, daidzein showed the highest individual isoflavone content with remarkable variations ($57.2\text{--}766.8 \mu\text{g/g}$), representing 46–100% of the total isoflavone content. R8 exhibited higher protein, fatty acid, and oil contents than R6 or R7. Moreover, the antioxidant capacities against two radicals in different parts of soybean plant showed considerable differences depending upon the isoflavone content. Our results suggested that soybean leaves and seeds might be useful materials for functional foods.

Keywords: soybean seeds, soybean leaves, isoflavone, antioxidant activity, growth stage

Introduction

Soybean [*Glycine max* (L.) Merrill, Leguminosae] is a staple human health crop that is highly popular in Korea because of its high-nutritional-value components such as phytochemicals (isoflavone, anthocyanin, saponin, terpenoid, and phenolic acid), protein, oil, and fatty acid (1–5). It is well-documented that soybean growth is categorized as indeterminate, semi-determinate, and determinate with vegetative stages (VE, VC, V1–V3, Vn, and V6) and reproductive stages (R1–R8) (6). The reproductive stages are categorized into four parts: 1) R1 and R2 represent flowering, 2) R3 and R4 represent pod development, 3) R5 and R6 represent seed development, and 4) R7 and R8 represent plant maturation (7). In particular, the R6–R8 stages are known as the growth periods of soybean seeds, i.e., beginning full seed (R6), beginning maturity (R7), and full maturity (R8) (6–8). Regarding the nutritional composition of soybean, interest in isoflavones has grown tremendously on account of their various biological properties (9–11). Soybean isoflavones play leading roles in preventing chronic diseases such as cardiovascular diseases, osteoporosis, menopausal symptoms, and cancer (8–10). They also possess potential beneficial effects

owing to their antioxidative, antibacterial, antimutagenic, and anticarcinogenic activities (2,9). In general, soybean isoflavones can be divided into four groups: aglycone (daidzein, glycitein, and genistein), glucoside, glucoside malonate, and glucoside acetylate (2,11). Among them, isoflavone aglycones are known to have higher bioavailability against coronary heart disease and cancer than glucosides because of their faster absorption in the human body (12,13). Therefore, many researchers have been searching for effective hydrolysis methods for isoflavone extraction from soybean and soy products (14,15). Food researchers and manufactures have been also focused on soybean protein, oil, and fatty acid because of their potential health benefits as a dietary supplement, nutraceutical source, and functional food as well as their nutrient quality and pharmaceutical properties (16–18).

To date, several studies have aimed at the promotion of health and prevention of diseases through various biological methods that use natural sources such as soybean. In particular, studies on functional materials have been conducted to identify natural antioxidants because of the safety issues associated with synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated

hydroxytoluene (BHT) (3,19). Numerous methods with distinctive mechanisms can be used to evaluate antioxidant capacity. In particular, radical-scavenging methods have been extensively used to screen antioxidants from crops, fruits, vegetables, and natural plants owing to their good reproducibility and easy quality control (8,11,19–21). Based on the above background, previous studies have evaluated the variations in phytochemical contents and antioxidant capacities at different growth stages of natural materials (4,22). We recently reported that soybean seeds and their seed-coat colors comprised significantly different isoflavones, primary metabolites (protein, fatty acid, and oil), and antioxidant activities (2,4). In addition, numerous studies have demonstrated that various factors of environmental and genetic conditions could significantly affect the contents of isoflavones (4,7,8,22). However, there is little information available on the variations in the isoflavone contents and antioxidant capacities of soybean seeds at different growth stages or in various parts of soybean plant. Therefore, our study was designed to investigate radical scavenging abilities of isoflavone aglycones in soybean seeds against DPPH and ABTS according to different growth stages. Furthermore, five different parts of soybean plant were examined to determine their potential beneficial effects as functional foods and pharmaceuticals.

The study aimed to evaluate and compare the properties of the seeds as well as different parts of soybean plant at the growth stages to determine their potential as functional food sources. We isolated three representative isoflavone aglycones from hydrolyzed extracts of soybean seeds and determined their structures using high-performance liquid chromatography (HPLC) with diode array detector (DAD) and electrospray ionization/mass spectroscopy (ESI/MS) and nuclear magnetic resonance (NMR) spectroscopy. In addition, isoflavone contents in the hydrolyzed extracts were determined for different parts of soybean plant using a microwave-assisted method and the antioxidant capabilities of DPPH and ABTS radicals were evaluated. We also investigated the changes in the isoflavone, protein, fatty acid, and oil contents as well as the antioxidant activities of soybean seeds at different growth stages for the first time.

Materials and Methods

Plant material and chemicals The most popular Korean soybean (cv. Taekwangkong) was obtained from the National Institute of Crop Science, Rural Development Administration. This species was sown during a field research in 2014. The samples of six parts of soybean plant, i.e., seeds, leaves, leafstalks, stems, pods, and roots, were collected from each replicate of three different soybean plants. Soybean seeds (R6–R8 stages) were air-dried at 25°C for five days and stored at –40°C prior to analysis. Different parts (leaves, leafstalks, stems, pods, and roots) of soybean plant were collected in the beginning maturity stage (R7) of maturation (8). The collected parts were immediately washed with sterile water and air-dried for

three days at 25°C. These samples were freeze-dried and then stored at –40°C. For quantitative analysis, three isoflavone aglycones (daidzein, genistein, and glycitein) were separated using seeds, as described previously (4). DPPH, BHT, ABTS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, acetic acid, and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). The standard fatty acid compositions [C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), and C18:3 (linolenic acid) methyl esters] were purchased from Merck (Darmstadt, Germany). HPLC-grade water and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Other solvents and chemicals of analytical grade were bought from Sigma-Aldrich.

HPLC-DAD-ESI/MS and HPLC analyses HPLC-DAD-ESI/MS analysis was performed using an HPLC system (Agilent 1100 series; Agilent Technologies, Santa Clara, CA, USA) coupled online with Esquire 400 (Bruker Daltonik GmbH, Bremen, Germany) and an ion-trap mass analyzer, which were controlled by the Esquire 4000 control software. Isoflavone aglycones were evaluated under the following parameters of mass spectrometry: a capillary voltage of 300 V, fragmentation voltage of 80 V, drying-gas temperature of 300°C, gas flow (N₂) of 2 L/min, nebulizer pressure of 50 psi, and collision gas pressure of He 6×10⁶ mbar. The equipment was operated in the negative-ion mode, scanning from *m/z* 100 to *m/z* 500 at 1.5 s/cycle. Other conditions were maintained as per the general protocol for HPLC analysis. Isoflavone contents were evaluated using the Agilent 1100 series (Agilent Technologies) HPLC comprising a DAD, binary pump, 1100-well plate autosampler, a ChemStation software, and a LichroCART 125-4 C₁₈ column (Lichrophore 100 RP-18e, 125 mm×4 mm, 5 µm, Merck). The column temperature was 25°C, and the absorbance was monitored at 254 nm. The mobile rate was 1.0 mL/min under the isocratic condition of 40% methanol (v/v) in water, and the sample injection volume was 20 µL.

Preparation of sample and calibration curves To determine isoflavone contents, the samples (six parts of soybean plant) were grounded for 3 min using a grinder (HR 2860; Philips, Amsterdam, Netherlands). The pulverized samples (1.0 g, 60 mesh) were extracted with 10 mL of 1 N HCl at 100°C for 3 h using the method of Lee and Choung (15) via microwave-assisted hydrolysis. In summary, the sample (1.0 g) was poured into a 25 mL cap-reaction vial with 10 mL of 1 N HCl solution and heated at 100°C for 2 h of hydrolysis time in a microwave extractor. After being cooled to room temperature 15 mL of EtOH was added to reaction mixture. The mixture solution was filtered using filter paper (Whatman No. 42, Sigma-Aldrich, St. Louis, MO, USA) and a 0.45 µm membrane filter (Millex-HN, Millipore, Bedford, MA, USA) prior to HPLC analysis. The isolated isoflavones were identified from ¹H and ¹³C NMR data using a Bruker AM 500 spectrometer (Bruker Daltonik GmbH). Next, 2 mg of isoflavone was accurately weighted and diluted in dimethyl sulfoxide (DMSO) to obtain a 1,000 µg/mL concentration (stock solution). Calibration

curves were determined by diluting the stock solution with DMSO to seven concentrations of 1, 2, 5, 10, 20, 60, and 100 µg/mL. The correlation coefficients (r^2) of three curves were observed to be higher than 0.999.

Analyses of protein, fatty acid, and oil The content of protein and oil were evaluated using a B-339 Auto Kjeldahl analyzer and a BUCHI B-811 Extraction System (Buchi, Schweiz) according to a previously described method (4). To determine the protein content, powdered soybean seeds (0.2 g, 60 mesh) were mixed using a Buchi B-412 scrubber and a Buchi B-435 system with 20 mL H_2SO_4 and 3.0 g catalyst ($CuSO_4 \cdot K_2SO_4=1:9$, w/w) according to the Kjeldahl nitrogen procedure in a distillation unit. The content was calculated as a percentage of nitrogen multiplied by 6.25. Oil content was examined using a Buchi B-811 extraction system. The grounded seeds (2.0 g, 60 mesh) were added to *n*-hexane (200 mL) in an extraction thimble and boiled for 2 h at 105°C. The mixture was cooled for 30 min at 25°C and weighted. The total content was presented as the seed dry mass. Fatty acid content was examined using a gas chromatograph (Agilent 7890A; Agilent Technologies). The individual composition of fatty acid was determined using the extracted oil as described below. Oil (100 µL) was placed in a screw-capped vial, and 5 mL of the methylation solution with a $H_2O:MeOH:toluene$ mixture (1:20:10, v/v/v) was added. The mixture was heated on a water bath (100°C) and cooled at room temperature. The organic layer was injected into the gas chromatograph as per the method described in our previous study (4). Each fatty acid was expressed as a percentage of total fatty acid.

Evaluation of antioxidant properties using radical scavenging methods To determine the radical scavenging effects, ultraviolet-visible absorption spectra were recorded at 517 (for DPPH) or 734 (for ABTS) nm using a Beckman DU650 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The DPPH radical scavenging capacity was analyzed according to the procedures reported by Lee *et al.* (4,19); 0.1 mL of the sample or BHT (positive control) in various concentrations (100, 90, 70, 50, 40, 20, 10, and 5 µg/mL) was mixed with methanol (0.49 mL) and 1 mM DPPH (0.39 mL). After 30 min of incubation at 25°C, the absorbance was measured using a spectrophotometer. The scavenging effect was calculated as a percentage using the following equation: DPPH radical scavenging ability (%) = $(1 - \text{absorbance of the sample} / \text{absorbance of the control}) \times 100$. The ABTS radical scavenging capacity was evaluated using a method described previously (4,19). In summary, this evaluation was based on the capacity of different substances to scavenge the ABTS^{•+} in comparison to Trolox (positive control). A radical cation was produced by reacting a 7 mM ABTS^{•+} solution with 2.45 mM potassium persulfate; the cation was then incubated for 12 h at 25°C. The ABTS^{•+} solution was dissolved in ethanol to an absorbance of 0.7 for measurement. For each analysis, 0.1 mL of the sample solution (100, 90, 70, 50, 40, 20, 10, and 5 µg/mL) was added to 0.9 mL of the

ABTS^{•+} solution. Then, the mixture was allowed to react for 1 min. The result was determined as a percentage using the following formula: ABTS radical scavenging ability (%) = $([\text{absorbance of the control} - \text{absorbance of the sample}] / \text{absorbance of the control}) \times 100$.

Statistics Isoflavone aglycones and other nutritional compositions were reported as the mean values of three replicates with standard derivations. All data were analyzed statistically via Duncan's multiple-range test using the statistical analysis software (SAS) 9.2 PC package (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Elucidation of isoflavone aglycones using NMR and HPLC-DAD-ESI/MS

Three representative isoflavone aglycones **1–3** were isolated via chromatographic techniques reported by Lee *et al.* (4). Next, they were confirmed using NMR and HPLC-DAD-ESI/MS analyses. Isoflavone structures (Fig. 1A) and their HPLC chromatographic profile (Fig. 1B) are shown in Fig. 1. Their retention times (t_R) were as follows: peak 1, $t_R=8.8$ min; peak 2, $t_R=10.4$ min; and peak 3, $t_R=15.7$ min. HPLC-MS offers significant information on phytochemicals in natural products and foods because of the fragmentation of a precursor ion (23). Thus, isoflavone aglycones in the hydrolyzed extracts from different parts of soybean plant were elucidated in the negative-ion mode using HPLC-DAD-ESI/MS. A full mass scan of peak 1 exhibited a pseudomolecular ion at m/z 253 ($[M-H]^-$) (Fig. 2A); this ion was elucidated as daidzein (**1**), as reported in a previous study (4). The MS analysis of peak 2 detected a deprotonated ion $[M-H]^-$ at m/z 283 and a fragment ion at m/z 268, which was formed by the loss of the m/z 15 fragment (Fig. 2B). The fragmentation ion at m/z 268 was characterized by the loss of a methyl moiety ($[M-H]^- - 15$; m/z 268). Based on this evidence, peak 2 was assumed to be glycitein (**2**). Subsequently, peak 3, which comprised a molecular ion $[M-H]^-$ at m/z 269 (Fig. 2C), was tentatively assigned as genistein (**3**). These peaks were in agreement with previously reported data (4).

Changes in isoflavone aglycone contents at different growth stages of soybean seeds

It is well-known that extraction is the first step in the isolation of secondary metabolites from natural plants, crops, and edible sources (2,14,15). Although several researchers have investigated the optimal extraction conditions (time, organic solvent, and temperature) for soybean isoflavones, the evaluation method for determining isoflavone aglycone content has not been studied extensively. In this study, we investigated isoflavone content under the acid hydrolysis condition using a microwave-assisted hydrolysis method based on previous studies (15). In summary, we evaluated isoflavone aglycone contents under the extraction condition of microwave-assisted acid hydrolysis with 1 N HCl at 100°C for 50 min. Isoflavone aglycones were determined based on each peak area in an HPLC. As illustrated in Fig. 3, isoflavone peaks in three seed stages

Fig. 1. (A) Chemical structures of the three representative isoflavone aglycones isolated from the hydrolyzed extract of soybean seeds. (B) Typical HPLC chromatogram of the isolated aglycones mixtures: (1) daidzein, (2) glycitein, and (3) genistein.

Fig. 2. Mass-fragmentation patterns of the three isoflavone aglycones: (A) daidzein, (B) glycitein, and (C) genistein.

(R6: full seed, R7: beginning maturity, and R8: full maturity) were detected in the HPLC chromatograms (Fig. 3F-3H). Moreover, the obtained values of isoflavone contents in the hydrolyzed extracts are summarized in Table 1. The highest isoflavone content was $1218.1 \pm 7.3 \mu\text{g/g}$ in R7 seeds (Fig. 3G), whereas R6 stage (Fig. 3F) exhibited the lowest content at $497.1 \pm 4.5 \mu\text{g/g}$. The total isoflavone content ($1038.0 \pm 9.8 \mu\text{g/g}$) of R8 seeds (Fig. 3H) was lower than that of R7 seeds. Our results were consistent with those of earlier research regarding total isoflavone contents at different stages (22). The predominant individual isoflavone was daidzein, which showed

significantly high isoflavone content, followed by genistein and glycitein (Table 1). The isoflavone contents in daidzein showed remarkable variations ($270.5 \rightarrow 562.3 \rightarrow 495.7 \mu\text{g/g}$ in R6, R7, and R8, respectively) compared to those in glycitein ($159.0 \rightarrow 236.1 \rightarrow 175.0 \mu\text{g/g}$ in R6, R7, and R8, respectively) and genistein ($67.6 \rightarrow 419.7 \rightarrow 367.3 \mu\text{g/g}$ in R6, R7, and R8, respectively) in the three growth stages of the seeds. In particular, daidzein increased by ~ 2.0 times ($270.5 \rightarrow 495.7 \mu\text{g/g}$) during the transition period from the R6 to R8 stage. Moreover, genistein rapidly increased by 5.4 times ($67.6 \rightarrow 367.3 \mu\text{g/g}$) during the same period. In general, it has been

Fig. 3. HPLC chromatograms of the hydrolyzed extracts from different parts of soybean plant. (A) leaves, (B) leafstalks, (C) stems, (D) pods, (E) roots, (F) R6 stage seeds, (G) R7 stage seeds, and (H) R8 stage seeds.

reported that isoflavone contents in soybean seeds were in the order of malonyl glucoside>glucoside>aglycone>acetyl glucoside (2,7,24). It has also been reported that the main isoflavone aglycones of this species were genistein, followed by daidzein and glycitein (4,25). However, in this study, we observed isoflavone contents in the order of daidzein, genistein, and glycitein. This phenomenon could be attributed to the fact that isoflavone concentration and distribution in soybean might be altered by several factors, including cultivars, growth conditions (year, temperature, and soil), and environmental

stress (2,4,7,11,25). Overall, isoflavones in soybean seeds during the R7 and R8 growth stages were elevated by ~2.5 and 2.0 times, respectively, compared with those at the R6 stage. Here, for the first time, we report that the content of isoflavone aglycones can change during different growth stages of soybean seeds.

Changes in protein, fatty acid, and oil contents at different growth stages of soybean seeds Several studies have revealed that soybean contains various nutritional components for that are beneficial for

Table 1. Comparison of total and individual isoflavone aglycones in the hydrolyzed extracts from different parts of soybean plant

Parts of soybean plant	Total isoflavone aglycone (mg/g) ¹⁾			
	Daidzein	Glycitein	Genistein	Total
Leaves ³⁾	529.4±7.3b	68.7±3.5d	454.0±5.6b	1052.1±10.4a
Leafstalks ³⁾	125.9±4.3d	30.2±1.9e	ND ²⁾	156.1±2.5d
Stems ³⁾	57.2±1.7e	ND	ND	57.2±1.7e
Pods ³⁾	141.4±3.6d	ND	ND	141.4±3.6d
Roots ³⁾	766.8±9.4a	ND	ND	766.8±9.4b
R6 stage seeds	270.5±3.8c	159.0±3.1c	67.6±2.4d	497.1±4.5c
R7 stage seeds	562.3±6.5b	236.1±4.7a	419.7±3.2a	1218.1±7.3a
R8 stage seeds	495.7±6.7b	175.0±2.3b	367.3±3.9c	1038.0±9.8a

¹⁾All values are presented as the mean±SD of triplicate determinations. The same letters are not significant at the 0.05 level of Duncan's multiple range test (DMRT).

²⁾ND, not detected.

³⁾Soybean parts at the R7 growth stage.

human health (1,2,4,5). This crop is of great interest to food researchers and manufactures owing to its primary metabolites such as protein, fatty acid, and oil (4,16,26). Recently, we reported that soybean protein, fatty acid, and oil contents exhibited a slight decrease during storage periods at room temperature (4). These results suggested that protein possibly degraded into an amino acid and small peptides (27) and that oil and fatty acid possibly oxidize and degraded because of environmental stress (air, light, heat, and time) during storage (28). Although soybean has been known as a good source of protein (average 30–42%) and oil (average 18–20%) with high unsaturated fatty acid content (26), little information was available on the changes in their contents during different growth stages. Therefore, our research was designed to analyze the changes in protein, oil, and fatty acid contents at different growth stages of soybean seeds. In protein analysis, the highest content was observed in seeds at the R8 growth stage (44.5±2.1%), followed by those at the R7 growth stage (41.3±2.7%) and R6 growth stage (30.7±1.9%) (Table 2). In other words, protein content was significantly different in the R6 and R7 stages. Our results were similar to the previously reported data, which indicated that the compositional components of soybean increased with growth time because of metabolite accumulation through maturity (29). Oil contents at the R6 and R7 stages were found to be significantly different (13.6±1.3→18.9±1.5%), and the variation in oil content at the R8 stages increased slightly, i.e., 18.9±1.5→20.7±0.7% (Table 2). Consequently, soybean protein and oil contents increased when the seed-growth stage progressed from R6 to R8. These results were similar to those reported in earlier

studies, showing that the nutritional components varied at different growth stages of soybean plant (5,30). In the R6 stage, the highest fatty acid component was C18:2 (33.0%), followed by C18:1 (16.8%), C16:0 (8.9%), and C18:3 (2.9%), whereas C18:0 content exhibited the lowest fatty acid content at 2.1% (Table 2). Although the individual composition had low contents, the rank order was consistent with that for soybean fatty acid (4). The results for the R7 seeds were also similar to those for the R6 seeds. Moreover, the individual fatty acid content increased significantly from the R6 to R7 stage. The order of the individual fatty acid composition at the R7 stage was as follows: C18:2 (42.1%)>C18:1 (21.6%)>C16:0 (11.4%)> C18:3 (4.5%)>C18:0 (3.9%) (Table 2). The individual and total fatty acid contents of R8 seeds exhibited slight increases compared to those of R7 seeds. The most predominant component was C18:2 (54.3%), followed by C18:1 (25.4%), C16:0 (12.7%), and C18:3 (6.0%), whereas C18:0 exhibited the lowest content at 4.5% (Table 2). Conclusively, soybean fatty acid significantly increased with the growth time of seeds. The individual and total fatty acid contents of the R8 seeds were higher than those of the R6 or R7 seeds. Furthermore, the total unsaturated fatty acid content [>53.4% (R6), 68.2% (R7), and 85.7% (R8)] was considerably higher than the total saturated fatty acid content [<11.0% (R6), 15.3% (R7), and 17.2% (R8)], as reported previously (4). Our results are in agreement with a previous study reporting the distribution of fatty acid content in soybean seeds (4). However, this study is the first to demonstrate the variations in fatty acid contents at different growth stages of soybean seeds.

Table 2. Comparisons of protein, oil, and fatty acid compositions at three different stages of soybean seeds

Soybean stage	Protein (%) ¹⁾	Oil (%) ¹⁾	Fatty acid composition (%) ¹⁾				
			C16:0	C18:0	C18:1	C18:2	C18:3
R6 growth	30.7±1.9b	13.6±1.3b	8.9±0.5b	2.1±0.1b	16.8±0.5b	33.0±1.6c	2.9±0.3c
R7 growth	41.3±2.7a	18.9±1.5a	11.4±0.6a	3.9±0.3a	21.6±0.9a	42.1±2.1b	4.5±0.9b
R8 growth	44.5±2.1a	20.7±0.7a	12.7±0.5a	4.5±0.3a	25.4±1.1a	54.3±2.7a	6.0±0.4a

¹⁾All values are expressed as mean±SD of triplicate experiments on a dry-weight basis. The same letters are not significant at the 0.05 level of Duncan's multiple range test.

Fig. 4. DPPH and ABTS radical scavenging activities in various soybean parts at a concentration of 1 mg/mL.

Comparison of the antioxidant capacities of DPPH and ABTS radicals at different growth stages of soybean seeds

To evaluate the antioxidant capacities of foods, crops, and natural plants, various analytical methods and substrates have been used (20,31-34). It is necessary to use at least two methods depending on the potential abilities of samples (2,4,20). Many researchers have focused on the DPPH and ABTS assays because of their reproducibility and simple quality control to screen antioxidants based on their preventative and therapeutic properties (2,4,11,20). Thus, we investigated the radical scavenging capacities of soybean seeds with stable references of DPPH and ABTS. These two radicals have been widely used as tools to estimate the scavenging capabilities with a decrease in color in the presence of antioxidant-containing samples. In this study, the antioxidant effects of DPPH and ABTS were evaluated as the inhibition percentage through radical formation by the hydrolyzed extracts of soybean seeds. The scavenging capacities of the sample extracts, BHT (DPPH positive control), and Trolox (ABTS positive control) increased with their concentrations. The order of the radical scavenging effects of DPPH was similar to that of ABTS (Fig. 4). In the hydrolyzed extracts (1.0 mg/mL), among three different growth stages (R6–R8 seeds), the highest DPPH and ABTS radical scavenging abilities were detected at the R7 stage with values of 84% and 89% (BHT; 89%, Trolox; 94%). Our results are similar to those reported for the antioxidant effects in soybean seeds (4). The seeds in the remaining growth stages also showed considerable differences in radical scavenging capacities in the following order: R8 seeds (DPPH: 75%; ABTS: 82%)>R6 seeds (DPPH: 46%; ABTS: 50%) (1.0 mg/mL) (Fig. 4). These results suggest that isoflavones in the hydrolyzed extracts of soybean seeds might be mainly responsible for their radical scavenging activities (2,4). Other phenolics in soybean might also play

essential roles in the antioxidant effects concerning a radical scavenger (33,34). As a result, the R6-R8 soybean seeds were found to have mildly higher ABTS radical scavenging capacities than the results against DPPH. This phenomenon indicated that DPPH inhibition could be due to the scavenging effects of hydrogen-donating compositions in seeds and that the inhibition of ABTS radicals might be attributed to the presence of the scavenging activities of chain-breaking and hydrogen-donating compositions (4,20). Our work is the first to evaluate the antioxidant properties regarding the DPPH and ABTS radical scavenging effects of soybean seeds at different growth stages.

Comparison of isoflavone aglycones in different parts of soybean plant

Many studies have reported that the variations in the isoflavone content of soybean might be affected by various factors (4,11,29,31). However, there is a lack of systematic research to determine isoflavone contents in different parts of soybean plant. Because we found that the total isoflavone aglycone content in the R7 seeds was higher than that in R6 or R8 seeds, we focused on soybean seeds at the R7 growth stage. The isoflavones in the hydrolyzed extracts from five soybean parts (leaves, leafstalks, stems, pods, and roots) are summarized in Table 1, and their HPLC chromatograms are shown in Fig. 3. The individual and total isoflavones contents were found to be considerably different in the five soybean parts. Excluding the seeds (the R7 stage), the highest total isoflavone content was observed in leaves ($1052.1 \pm 10.4 \mu\text{g/g}$) (Fig. 3A), whereas the stems exhibited the lowest content ($57.2 \pm 1.7 \mu\text{g/g}$) (Fig. 3C). Isoflavone contents in the remaining parts were detected in the following order: roots ($766.8 \pm 9.4 \mu\text{g/g}$) (Fig. 3E)>leafstalks ($156.1 \pm 2.5 \mu\text{g/g}$) (Fig. 3B)>pods ($141.4 \pm 3.6 \mu\text{g/g}$) (Fig. 3D).

Interestingly, the predominant isoflavone in all parts was daidzein in the range of 57.2 ± 1.7 – 766.8 ± 9.4 $\mu\text{g/g}$, followed by genistein (ND – 454.0 ± 5.6 $\mu\text{g/g}$), and glycitein (ND – 236.1 ± 4.7 $\mu\text{g/g}$) (Table 1). The total isoflavone content in leaves (1052.1 ± 10.4 $\mu\text{g/g}$) was similar to that in the R7 seeds (1218.1 ± 7.3 $\mu\text{g/g}$) (Fig. 3G). Moreover, daidzein and genistein in leaves were found to be the major components (529.4 ± 7.3 and 454.0 ± 5.6 $\mu\text{g/g}$, respectively) compared with the R7 seeds (daidzein: 562.3 ± 6.5 and genistein: 419.7 ± 3.2 $\mu\text{g/g}$). In the remaining parts, the most abundant individual isoflavone was daidzein at 125.9 ± 4.3 $\mu\text{g/g}$ in leafstalks (81%), 57.2 ± 1.7 $\mu\text{g/g}$ in stems (100%), and 141.4 ± 3.6 $\mu\text{g/g}$ in pods (100%). In particular, soybean roots (glycitein: ND and genistein: ND) showed the highest daidzein content at 766.8 ± 9.4 $\mu\text{g/g}$ in all parts (Fig. 3E). This research provides important information on isoflavone distribution in different parts of soybean plant at the growth stages as published data (4,22). Moreover, our information concerning isoflavone in soybean may provide a better understanding of its various beneficial effects on human health.

Comparison of the antioxidant abilities of DPPH and ABTS radicals in different parts of soybean plant To better understand the functional properties concerning the development of the nutritional value of soybean plant, antioxidant capacities for various parts of it were comparatively evaluated. We investigated the scavenging activities of the different parts (leaves, leafstalks, stems, pods, and roots) using DPPH and ABTS radicals. These effects were also evaluated by comparing the inhibition percentages through the formation of DPPH and ABTS radicals from the hydrolyzed extract of each part, as described above for analyzing soybean seeds. Although the radical scavenging abilities of the positive controls and extracts increased with increasing concentration, the positive controls (BHT: 89%; ABTS: 94% at 1.0 mg/mL) exhibited higher activities than the various extracts (Fig. 4). The radical scavenging capacities of the five parts showed remarkable differences. The DPPH radical scavenging abilities were in the following order: leaves (80%)>roots (69%)>leafstalks (35%)>pods (31%)>stems (17%) at 1.0 mg/mL (Fig. 4). These results suggest that the scavenging activities of the DPPH radical might be because of their total isoflavone content, as reported in previous studies (2,4,31). Other metabolites may also play a role in the DPPH radical scavenging activities (4,33). The rank order of the ABTS radical scavenging activity was consistent with that of DPPH. The leaves had the highest ABTS radical scavenging ability of 85%, followed by roots (74%)>leafstalks (46%)>pods (41%), whereas the stems exhibited the lowest ability 25% (Fig. 4). Based on the above observations, the antioxidant capacities of soybean leaves may be positively correlated with various phytochemicals, as reported previously (35). The ABTS radical scavenging activities of all the extracts were higher than those of the DPPH assay. Moreover, these activities in various soybean parts were similar to those in soybean seeds. Our research suggests that isoflavone possibly contributes to

electron-donating and hydrogen-donating activities (4). In particular, the seeds, leaves, and roots are potent free-radical scavengers because of the isoflavone contents. Therefore, these parts may be widely used as important sources of natural antioxidants in pharmaceuticals and functional foods. This study determined for the first time the comparison of the antioxidant activities in different parts of soybean plant.

In conclusion, this study revealed for the first time that the isoflavone contents and antioxidant activities of different parts of soybean plant, including seeds, leaves, leafstalks, roots, stems, and pods had remarkable differences. We also demonstrated that soybean seeds exhibit considerable differences in isoflavone, protein, fatty acid, and oil contents as well as in the antioxidant activities depending on the growth stage. Three isoflavone aglycones were isolated from hydrolyzed extracts using a microwave-assisted method and identified via NMR spectroscopy and HPLC-DAD-ESI/MS. The highest isoflavone content (1218.1 ± 7.3 $\mu\text{g/g}$) was detected in soybean seeds at the R7 growth stage, including the contents of daidzein (562.3 ± 6.5 $\mu\text{g/g}$), genistein (419.7 ± 3.2 $\mu\text{g/g}$), and glycitein (236.1 ± 4.7 $\mu\text{g/g}$), whereas the seeds at the R6 growth stage showed the lowest content (497.1 ± 4.5 $\mu\text{g/g}$). Protein, fatty acid, and oil contents were the most abundant in the R8 seeds. Among different soybean parts, leaves had the predominant isoflavone content of 1052.1 $\mu\text{g/g}$, followed by roots (766.8 ± 9.4 $\mu\text{g/g}$), leafstalks (156.1 ± 2.5 $\mu\text{g/g}$), and pods (141.4 ± 3.6 $\mu\text{g/g}$), whereas the stems had the lowest isoflavone content (57.2 ± 1.7 $\mu\text{g/g}$). The order of the antioxidant effects of different parts of soybean plant determined using DPPH and ABTS radicals were similar to the order of the isoflavone contents in those parts. In particular, soybean seeds (R7 stage, DPPH: 84% and ABTS: 89%) and leaves (DPPH: 80% and ABTS: 85%) exhibited stronger DPPH and ABTS radical activities than the other soybean parts at a concentration of 1 mg/mL. Consequently, the isoflavone content and antioxidant capacities differed considerably according to the parts and growth stages. Soybean leaves and seeds can be considered as good sources of natural antioxidants for functional foods and commercial uses because of the high radical scavenging activities. Further research is needed to examine the various beneficial properties of different parts of soybean plant during the growth stages for the development of functional foods and pharmaceutical applications.

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