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Short Communication

Evaluation of Isoflavones from the Leaves of Soybean (*Glycine max* L.) Cultivars

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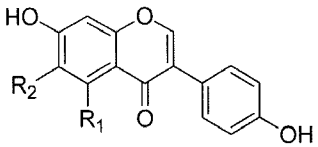
Recently, soybean [*Glycine max* (L.) Merr.] and soy foods have been receiving a great deal of attention due to their potential health benefits. Among various phytochemicals of soybean, it is well established that isoflavones are responsible for preventing cardiovascular diseases [Anthony *et al.*, 1996; Anthony *et al.*, 1998] and cancers [Wu *et al.*, 1998; Lamartiniere, 2000], as well as lowering of blood pressure [Jenkins *et al.*, 2002]. In numerous studies on isoflavone contents and compositions of soybean, focus have been placed on the seeds of the soybean cultivars [Wu *et al.*, 2004; Kim *et al.*, 2007]. Among the different parts of soybean, leaves have been used in Korean folk medicine as a detoxifying agent for snake poisoning and as food and dietary supplement sources in the southern province of Korea. However, the evaluation of the isoflavone contents in the leaves has not yet been attempted. Thus, characterization of the isoflavones in soybean leaves is of great importance to enhance the values of leaves as functional materials and soybean as pharmaceutical.

The leaves of soybean (cv. Heugcheongkong) (1.0 kg) were extracted at room temperature with MeOH (2 L×2) and filtered to remove the precipitate. After evaporation, the combined methanol extracts were suspended in water, and then successively partitioned with EtOAc and BuOH to afford EtOAc-soluble (62.1 g) and BuOH-soluble (38.9 g) fractions. The EtOAc phase was chromatographed on silica gel (6.0×50 cm, 230-400 mesh) using hexane/acetone gradient to yield five fractions (A-E). Fraction C (6.4 g) was subjected to the silica gel column chromatography, eluted with a hexane/acetone gradient (20:1→4:1) to give 25 subfractions. Subfractions 20-24 were rechromatographed to give isoflavone **3** (16.2 mg) ($CHCl_3$/acetone 5:1). Fraction E (12.5 g) was chromatographed using a gradient of $CHCl_3$/acetone (15:1→1:2), resulting in 45 subfractions. Subfractions 33-41 were combined and purified using the second flash silica gel column (1.5×50 cm, 230-400 mesh) at the gradient of $CHCl_3$/acetone (8:1→2:1) to yield isoflavone **1** (25.0 mg) ($CHCl_3$/acetone 3:1). Subfractions 43-45 were combined on the basis of their comparative TLC profiles and subjected to preparative TLC ($CHCl_3$/acetone 3:1) to give isoflavone **2** (19.4 mg). The structures of three isoflavones **1-3** were confirmed by ^{1}H NMR in comparison with the values previously reported (Fig. 1) [Lee *et al.*, 2006a; Lee *et al.*, 2006b; Lee *et al.*, 2007; Lee *et al.*, 2008].

On the basis of the isolated isoflavones **1-3**, HPLC



1 R_1 = H, R_2 = H

2 R_1 = H, R_2 = OCH_3

3 R_1 = OH, R_2 = H

Fig. 1. Chemical structures of the isolated isoflavones 1-3 from the leaves of soybean.

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Abbreviations: ESI/MS, electro spray ionization/mass spectrometry; HPLC, high-performance liquid chromatography; DAD, diode-array detector

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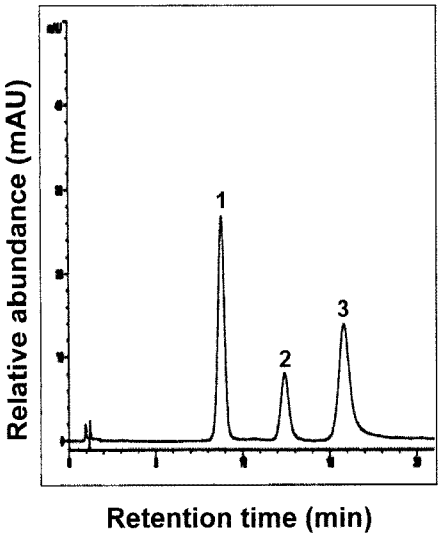

Fig. 2. HPLC chromatogram of isoflavones 1-3 isolated from the leaves of soybean.

separation was performed on an Agilent 1100 series instrument equipped with a diode array detector (DAD) operating at 254 nm and a C_{18} column [LichroCART 125-4, 5 μm, 125 mm×4 mm (Lichrophore 100 RP-18e)]. The column temperature was set at 30°C, and methanol: water mixture [40:60 (v/v)] was isocratically eluted at 1.0 mL/min. The sample injection volume was 20 μL. A typical HPLC chromatogram of isoflavones **1-3** is presented in Fig. 2. Retention times were as follows: isoflavone **1** (daidzein, 8.8 min), isoflavone **2** (glycitein, 12.4 min), and isoflavone **3** (genistein, 15.8 min).

To quantitatively analyze isoflavones **1-3**, calibration curves were constructed in the range 0.38-100 μg/mL. The concentrations of the isoflavones were determined on the basis of the peak areas in the chromatogram as follows:daidzein, $y = 146.50x - 19.78$, $R^2 = 0.999$; glycitein, $y = 98.91x - 3.75$, $R^2 = 0.999$; and genistein, $y = 200.20x - 151.20$, $R^2 = 0.999$.

The isoflavones present in the leaves of soybean were characterized by HPLC-DAD-ESI/MS analysis. Mass spectrometer was performed on an ion trap mass analyzer (Bruker Daltonick GmbH, Bremen, Germany) equipped with an ESI source. Conditions of HPLC-DAD-ESI/MS detection were as follows: negative ion mode; drying gas, N_2 from a generator; flow rate, 0.2 mL/min; column, LichroCART 125-4, 5 μm, 125 mm×4 mm; gas temperature, 350°C; nebulizer pressure, 30 psi; He (collision gas) pressure, 6×10^{-6} mbar. Other conditions were the same as those used for HPLC/DAD analysis. Data was acquired in the ESI mode at 1.10 kV with a scan range of *m/z* 100-350. Retention times of the sample peaks were compared to those of the pure standards. Peak identity was confirmed when peak retention time and HPLC-DAD-ESI/MS value were identical to those of the pure standard in the mobile phase. The mass spectra of isoflavones **1-3** in soybean leaf extracts (cv. Cheongja 1) are presented in Fig. 3. Analysis revealed compounds **1-3** were daidzein, glycitein, and genistein with $[M-H]^-$ at *m/z* 253.1 (Fig. 3A), 283.0 (Fig. 3B), and 269.1 (Fig. 3C), respectively.

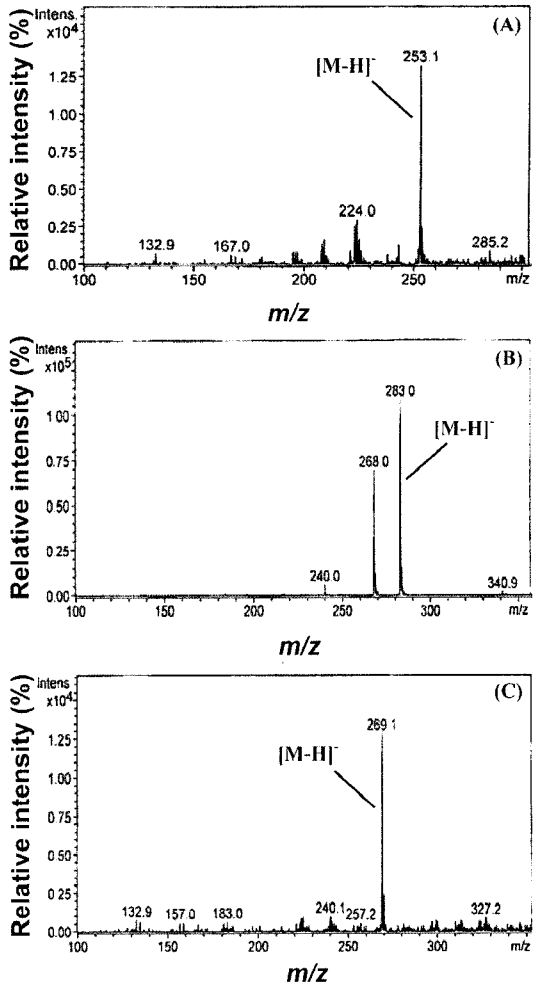

Fig. 3. HPLC-DAD-ESI/MS fragmentations of isolated isoflavones 1-3 from soybean leaf extracts (cv. Cheongja 1). HPLC operating conditions: column, LichroCART 125-4, 5 μm, 125 mm×4 mm; solvent, methanol:water [40:60 (v/v)]; flow rate: 0.2 mL/min; detection at 254 nm; HPLC system, Agilent 1100; mass system, Bruker Daltonick DE/ESQUIRE 4000 (negative ion mode).

On the basis of the above results, the leaves of 14 Korean soybean cultivars were evaluated. The soybean leaves were collected on September 10, 2006 at the experimental field of Yeongnam Agricultural Research Institute, Miryang, Korea and air-dried in the shade for 5 days. The dried leaves were ground using a coffee grinder (Brabender, Germany). One gram each of the leaves from different cultivars was hydrolyzed in the presence of 10 mL of 1 N HCl at 105°C for 3 h. After the hydrolysis was completed, the mixture was cooled to room temperature,

Table 1. Contents of isofalvones 1-3 from the leaves of soybean cultivars

Cultivar	Compound (μg/g)[a]		
	1	2	3
Geomjeongkong 2	124.23±3.55	134.46±3.40	79.36±0.90
Seonheukkong	44.20±1.40	126.11±3.99	66.57±1.28
Milyang 168	97.25±1.64	174.29±3.97	79.65±2.29
Milyang 181	65.59±3.69	129.47±2.13	82.39±1.89
Suwon 235	131.10±2.57	122.11±0.95	111.56±1.20
Hobankong	122.63±15.50	137.85±5.78	122.74±3.40
Jinpum 2	101.27±3.58	150.66±9.82	157.68±6.41
Galmikong	106.87±8.89	116.88±2.35	102.74±1.15
Heugcheongkong	163.61±4.15	143.27±2.51	82.46±0.41
Jinyulkong	145.66±1.03	147.63±3.76	96.76±1.91
Cheongja 1	162.14±6.69	292.94±2.43	173.14±7.35
Cheongja 2	36.17±0.74	95.11±2.06	73.09±0.11
Cheongja 3	82.66±0.22	188.90±2.38	67.72±0.11
Geomjeongkong 1	29.94±0.13	133.11±1.40	81.66±0.96

[a]Values indicate the means of three replications.

followed by the addition of 15 mL methanol. The hydrolysate obtained from the acid hydrolysis was filtered using a Millipore (Millipore, MSI, Westboro, MA) with 0.45-μm membrane filter. The isoflavone contents in leaves of 14 soybean cultivars harvested from Miryang are shown in Table 1.

Differences between isoflavones and cultivars were found. The isoflavone concentrations of soybean leaves ranged from 29.94±0.13 to 163.61±7.35 μg/g (daidzein), 95.11±2.06 to 292.94±2.43 μg/g (glycitein), and 67.72± 0.11 to 173.14±7.35 μg/g (genistein) (Table 1). Among the 14 Korean cultivars, the highest total isoflavone content was 628.22 μg/g in Cheongja 1 leaves. Glycitein (**2**) (292.94±2.43 μg/g) was the predominant isoflavone, followed by daidzein (**1**) (162.14±6.69 μg/g) and genistein (**3**) (173.14±7.35 μg/g). The lowest was found

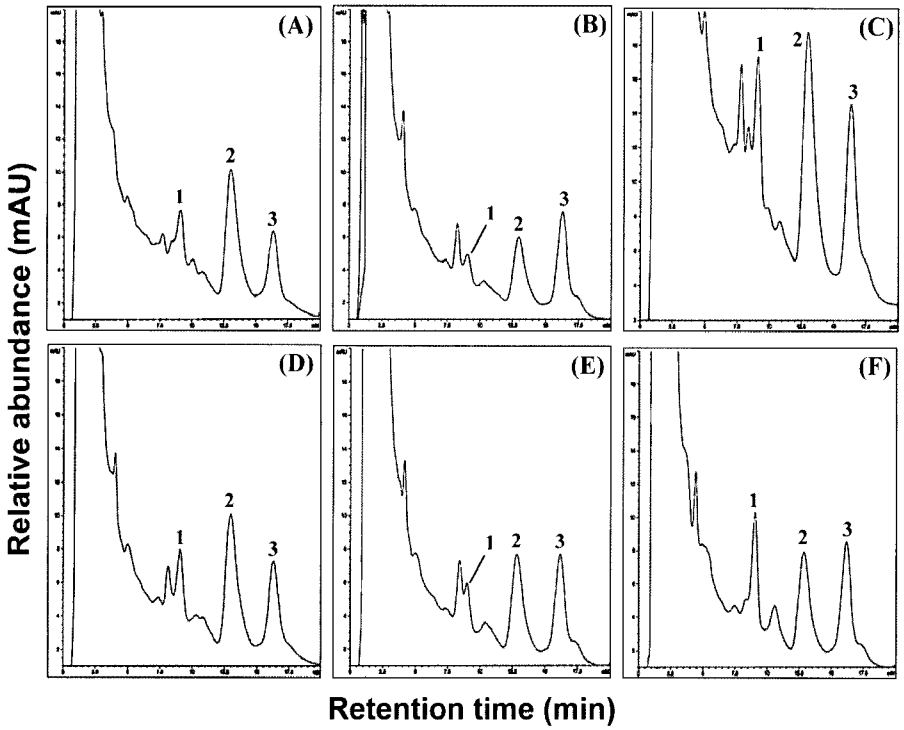

Fig. 4. HPLC chromatogram of 40% methanol extracts from the leaves of Korean soybean cultivars. (A) Cheongja 3, (B) Cheongja 2, (C) Cheongja 1, (D) Milyang 168, (E) Seonheukkong, and (F) Milyang 181 cultivars.

in Cheongja 2 leaves (204.37 μg/g) (**1**, 36.17±0.74; **2**, 95.11±2.06; and **3**, 73.09±0.11 μg/g). Between the two cultivars, Cheongja 1 (628.22 μg/g) showed three times higher isoflavone content than Cheongja 2 (204.37 μg/g) (Table 1, Fig. 4B, and 4C). A typical HPLC chromatogram of three isoflavones **1-3** is presented in Fig. 4. The isoflavone contents slightly differed among cultivars. Although the isoflavone contents of soybean leaves were lower than those of the soybean (Lee *et al.*, 2003; Lee *et al.*, 2004), the contents of the three representative isoflavones, daidzein, glycitein, and genistein, have not yet been analyzed.

Among the 14 cultivars, the leaves of Jinpum 2 (409.61 μg/g) and Cheongja 1 (628.22 μg/g) exhibited higher levels of the total isoflavone concentrations in comparison with other cultivars (Table 1). These results indicated that Jinpum 2 and Cheongja 1 cultivars are important sources for development of soybean leaves with high quality breeding and nutritional values.

In conclusion, we isolated and identified three representative isoflavones from soybean leaves. On the basis of the isolated isoflavones, the leaves of 14 Korean soybean cultivars were subjected to HPLC analysis. Jinpum 2 and Cheongja 1 cultivars showed high amounts of isoflavone, with the content of glycitein (**2**) being predominantly higher than those of daidzein (**1**) and genistein (**3**). Our results show these cultivars are important sources for development of better soybean leaves contains high quality breeding and nutritional value.

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