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RESEARCH NOTE

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Succinyl Daidzin and Succinyl Genistin are New Isoflavone Derivatives Found in *Cheonggukjang*

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Abstract Two new fermentation products were separated from *cheonggukjang* by high performance liquid chromatography (HPLC) and tentatively identified as succinyl daidzin and succinyl genistin by HPLC-mass spectrometry (MS). Both succinyl daidzin and succinyl genistin were detected in all 4 commercially available and one laboratory prepared *cheonggukjang* and 1 commercially available *natto*. However, these compounds were not detected in commercially available 4 home-made and 4 factory-produced *doenjang*. Peak areas of succinyl genistin were about 1.95-2.45 times higher than those of succinyl daidzin in *cheonggukjang*, which may be due to the higher concentration of genistin derivatives than daidzin derivatives in soybeans. This is the first report on the succinyl derivatives as isoflavone metabolites from *cheonggukjang* and these 2 isoflavone derivatives could be characteristic indicators for *cheonggukjang*.

Keywords: *cheonggukjang*, isoflavone fermented product, succinyl daidzin, succinyl genistin

Introduction

Cheonggukjang is made of cooked whole soybeans which are fermented with mainly *Bacillus subtilis* and/or *Bacillus natto* for short period (1). *Cheonggukjang* has gained popularity among Koreans due to its health beneficial functions such as increasing immune responses and fibrinolytic activity (2,3). Isoflavones are phytoestrogenic compounds in soybeans and consumption of isoflavone has been associated with the reduction of the risk of various cancers, several chronic inflammatory diseases, and osteoporosis (4-6). The distributions of isoflavones during *cheonggukjang* preparation are also reported in the literature. Depending on the fermentation conditions, β-glucosides or aglycones are major chemical forms of isoflavones in *cheonggukjang* (7-9). Generally, cooked soybean contains high percentage of β-glucosides which are converted from malonyl derivatives during cooking. Microorganisms possessing high β-glucosidase activity can convert β-glucosides into corresponding aglycones during *cheonggukjang* fermentation.

Our previous study showed that as fermentation time proceeded two new unidentified peaks were detected and increased in laboratory prepared *cheonggukjang* (9). In this study, those two new peaks were tentatively identified and other fermented soy products were screened for these new peaks.

The objectives of this study were to identify the two new peaks detected in *cheonggukjang* using liquid chromatography/mass spectrometry (LC/MS) and to monitor these two peaks in fermented soy foods such as 4 *cheonggukjang*, 4 home-made *doenjang*, 4 factory-produced *doenjang*, and 1 sample of *natto*.

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Materials and Methods

Materials Soybean was purchased from a local market (Seoul, Korea) and *B. subtilis* MYCO10001 was donated from Myco Corp. (Gyeongju, Korea). Home-made *doenjang*, which were fermented for 4, 16, 28, 40, or 52 months after decanting soy sauce, were donated from Yeosudak Co. (Jeonnam, Korea). Commercially available 4 *cheonggukjang*, 1 *natto*, and 4 factory-produced *doenjang* were purchased from a local grocery market (Seoul, Korea). Six isoflavone standards including glycitein, β-glycitin, acetyl-β-daidzin, acetyl-β-genistin, acetyl-β-glycitin, and malonyl-β-genistin were purchased from LC Laboratories Co. (Woburn, MA, USA) and 3 standard compounds including daidzein, genistein, and β-genistin were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile, hydrochloric acid (HCl), and acetic acid were purchased from Fisher Scientific Ltd. (Fairlawn, NJ, USA).

Sample preparation *Cheonggukjang* was prepared with slight modification of the previous report (9). Briefly, whole soybean of 300 g was soaked for 10 hr at room temperature and cooked for 2 hr. Cooked whole soybean was cooled down and inoculated with 10%(w/w) *B. subtilis* MYCO10001 (2.7×10^7 CFU/mL) and fermented for 36 hr at 40°C in a incubator. Four commercially available *cheonggukjang*, 1 laboratory-prepared *cheonggukjang*, 1 *natto*, and 8 *doenjang* samples were freeze-dried (Ilshin Lab Co., Yangju, Korea).

Isoflavone extraction Isoflavones were extracted from 1 g of freeze dried samples according to Lee *et al.* (10). Mixtures of 2 mL of 100 mM HCl, 7 mL acetonitrile, and 3 mL deionized water were added 1 g of *cheonggukjang* and shaken for 2 hr using a shaker (cute mixer CM-1000; Eyela, Tokyo, Japan). Samples were centrifuged at 2,208×

g for 10 min (Hanil Co., Incheon, Korea) and the supernatant was dried under nitrogen gas flow at room temperature. Dried samples were stored at −40°C in the dark until use. Extraction yields were confirmed using an internal standard, formononetin.

Separation of 2 compounds by HPLC Two new isoflavones in sample extracts were separated using a high performance liquid chromatograph (HPLC) equipped with an ultraviolet detector (Hitachi Co., Tokyo, Japan). Conditions of HPLC analysis were the same as those reported previously (9).

Identification using HPLC-mass spectrometry (MS) Two new isoflavones in the sample extracts were identified using LC/MS. Samples dried under nitrogen gas were dissolved in 1 mL methanol and analyzed by LC/MS. Separation of the sample was performed using Agilent 1100 series HPLC (Waldbronn, Germany) equipped with a 4-μm Waters Novapak C18 reverse phase column (150× 3.9 mm i.d.). Conditions of mobile phase were the same as those of HPLC analysis. The sample injection volume was 10 μL and the flow rate was 0.6 mL/min. Electrospray (ESI) MS data were acquired using a Agilent 1100 series

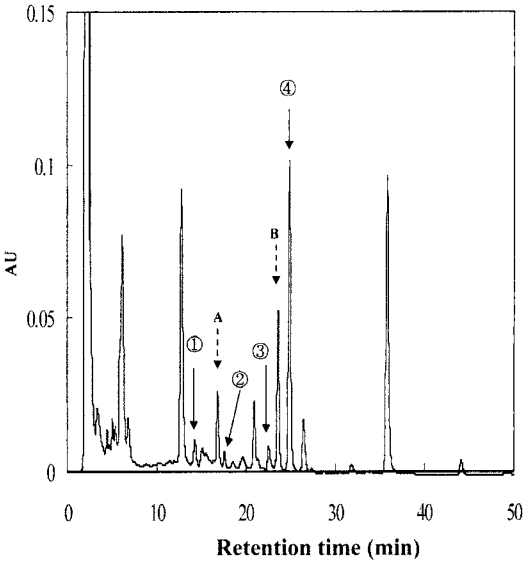

Fig. 1. HPLC chromatograms of peak A and peak B in *cheonggukjang*, which were tentatively identified as succinyl daidzin and succinyl genistin, respectively. ① Malonyl-β-glycitin, ② 6"-*O*-Acetyl-β-daidzin, ③ 6"-*O*-Malonyl-β-genistin, ④ Daidzein.

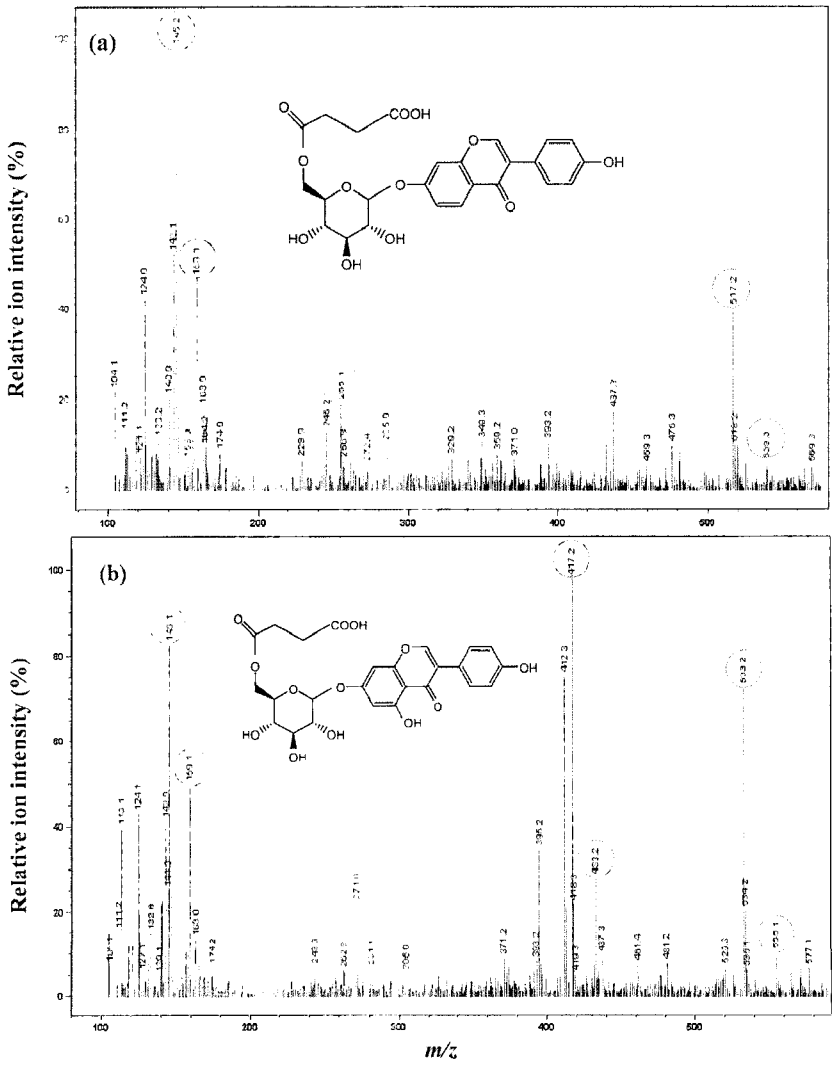

Fig. 2. Mass spectra of peak A (a) and peak B (b) and suggested structures.

LC/MSD (Palo Alto, CA, USA) quadrupole instrument in positive ion mode. Nitrogen was used as nebulization gas at flow rate of 11 L/min. The capillary voltage was 4 kV.

Results and Discussion

Identification of 2 new peaks in *cheonggukjang* HPLC chromatogram of factory-produced *cheonggukjang* is shown in Fig. 1. From the previous report of our group, one peak was eluted between malonyl glycitin and acetyl daidzin and the other peak appeared between malonyl genistin and daidzein, which were designated as peak A and B, respectively. Mass spectra of peak A and peak B from LC/MS with quadrupole as mass analyzer are shown in Fig. 2. The mass spectrum of peak A displays *m/z* 517, 255, 163, and 145 as shown in Fig. 2a and can be assigned to succinyl daidzin. The *m/z* 517, 255, and 163 are the $[\text{succinyl daidzin}+H]^+$, $[\text{daidzein}+H]^+$, and $[\text{Glucose - OH}]^+$, respectively. The *m/z* 163 and 145 come from the glucose moiety. The $[\text{succinyl daidzin}+Na]^+$ (*m/z* 539) can be observed in the mass spectrum.

The mass spectrum of peak B displays *m/z* 533, 433, 417, 271, 163, and 145 as shown in Fig. 2b and can be assigned to succinyl genistin. The *m/z* 533, 433, and 271 are the $[\text{succinyl genistin}+H]^+$, $[\text{genistin}+H]^+$, and $[\text{genistein}+H]^+$, respectively. The *m/z* 417 can be assigned to the $[\text{genistin - OH}]^+$. The *m/z* 163 and 145 also come from the glucose moiety. The $[\text{succinyl genistin}+Na]^+$ (*m/z* 555) can be observed in the mass spectrum.

Therefore, peak A and peak B are tentatively identified as daidzein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside or succinyl daidzin and genistein 7-*O*-β-(6"-*O*-succinyl)-D-glucoside or succinyl genistin, respectively based on LC/MS results.

This study is the first report on the identification of succinyl daidzin and succinyl genistin in *cheonggukjang*. However, the presence of succinyl derivatives of isoflavones has been reported in *natto*, a similar fermented soy food consumed in Japan (11). Succinyl glycitin, which was detected in *natto*, was not found in this study. 6-*O*-Succinylated isoflavone glycosides from *natto* have been reported to show preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet (11). These isoflavone metabolites could play important roles in the health beneficial effects of *cheonggukjang*.

Detection of succinyl daidzin and succinyl genistin in *cheonggukjang*, *natto*, and *doenjang* Peak areas of succinyl daidzin and succinyl genistin from *cheonggukjang*, *natto*, and *doenjang* are shown in Table 1. All the 5 *cheonggukjang* samples and 1 *natto* sample possessed both succinyl daidzin and succinyl genistin. However, peaks of succinyl daidzin and succinyl genistin were not detected in *doenjang* samples irrespective of home-made or factory-produced.

Peak areas of succinyl genistin were about 1.95-2.45 times higher than those of succinyl daidzin (Table 1), which may be due to the higher concentration of genistin derivatives than daidzin derivatives in soybeans. Commercial *natto* possessed the highest peak areas of succinyl derivatives compared to 5 *cheonggukjang* samples and peak ratios of succinyl genistin to succinyl daidzin in *natto* was 1.44, which was lower than *cheonggukjang*. Yang *et al.* (9) reported that both succinyl derivatives increased as fermentation period increased in *cheonggukjang*. Higher relative peak areas of succinyl derivatives may indicate longer or more active fermentation during *cheonggukjang*

Table 1. Peak areas of succinyl daidzin and succinyl genistin in tested *cheonggukjang*, *natto*, and *doenjang*

Fermented soy foods		Succinyl daidzin ($\times 10^5$)	Succinyl genistin ($\times 10^5$)	Ratio of succinyl genistin to succinyl daidzin
Cheonggukjang	Factory-produced A	1.992±0.170[1] (2.08%)[2]	3.902±0.012 (4.07%)	1.95
	Factory-produced B	1.848±0.064 (7.02%)	4.300±0.181 (16.32%)	2.32
	Factory-produced C	2.359±0.032 (4.29%)	5.793±0.097 (10.53%)	2.45
	Factory-produced D	2.156±0.0073 (4.66%)	5.001±0.068 (10.82%)	2.32
	Laboratory made	3.175±0.034 (12.01%)	6.343±0.069 (24.01%)	1.99
Natto		10.443±0.136 (10.39%)	15.051±0.197 (14.97%)	1.44
Doenjang	Home-made A	ND[3]	ND	-[4]
	Home-made B	ND	ND	-
	Home-made C	ND	ND	-
	Home-made D	ND	ND	-
	Factory-produced A	ND	ND	-
	Factory-produced B	ND	ND	-
	Factory-produced C	ND	ND	-
	Factory-produced D	ND	ND	-

[1]Mean±SD in peak areas.
[2]Relative peak area of succinyl daidzin or succinyl genistin/total peak areas of isoflavones except succinyl derivatives.
[3]Not detected.
[4]Not available.

preparation. Therefore, these 2 peaks could be used to predict the degree of fermentation in *cheonggukjang* and/or characteristic indicators to differentiate *cheonggukjang* from other soy fermented foods like *doenjang*.

This is the first report on the succinyl derivatives as isoflavone metabolites from *cheonggukjang*. Any unidentified peak from isoflavone extracts during *cheonggukjang* preparation could be either succinyl genistin or succinyl daidzin depending on the conditions of isoflavone analysis and of fermentation. Further studies on the functionality and/or stability of succinyl derivatives are needed.

Acknowledgments

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