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RESEARCH NOTE

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Biotransformation of Free Isoflavones by Bacillus Species Isolated from Traditional *Cheonggukjang*

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Abstract Our previous study showed that isoflavone profile of soybean undergoes a significant change during *cheonggukjang* preparation. In particular, the content of metabolite(s) with similar retention time to glycitein under the high performance liquid chromatography (HPLC) condition was significantly increased while the levels of genistein and its derivatives were notably lowered. Therefore, we hypothesized that genistein and its derivatives might be converted to genistein glucosides with similar elution time to glycitein. Our current data suggest that genistein and its derivatives are extensively metabolized into various compounds including genistein glycosides, but not glycitein or its derivatives, by Bacillus species isolated from traditional *cheonggukjang*. Some of daidzein was also converted into a derivative with shorter retention time by *Bacillus amyloliquefaciens* 51 and 86-1 but not by *Bacillus subtilis* 3-5 and 3-17. As metabolism of soy isoflavones, major health-promoting components in soy products, is widely variable depending upon Bacillus species, it is essential to select microorganism that minimizes the breakdown or modification of soy isoflavones in the process of fermented soy product manufacture.

Keywords: *cheonggukjang*, isoflavone, genistein, fermentation, bioconversion, soybean

Introduction

There is ample evidence that isoflavones present in soybeans and soy foods are associated with lowered risks for some chronic diseases including sex hormone-dependent cancers (1-3), cardiovascular diseases (4-7), and postmenopausal complications (8). The three isoflavones in soybeans and soy products, genistein, daidzein, and glycitein, occur in four possible forms: the free phenolic form, the glucoside, the malonyl glucoside, and the acetyl glucoside (9,10). The distribution of the forms can be altered by fermentation process of soy such as preparation of *cheonggukjang, doenjang*, and *kochujang* (11,12). Among three isoflavones, genistein has been reported to have stronger physiological activity such as inhibition of tyrosine kinase than the others, while the others contain relatively low biological activity.

In the previous study we found that the levels of glycitein and its glycosides were increased during *cheonggukjang* preparation while the concentrations of the other isoflavones are reduced (11). Since genistein, daidzein, and their glycosides are believed to have better health benefits, their conversion to other metabolites in the process of soybean products will impair the health functional value of soybean. Accordingly, the screening of Bacillus species which minimizes metabolism of genistein is highly demaned.

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In order to characterize and screen Bacillus species that bring about minimum metabolism of genistein we analyzed the isoflavone profile of *cheonggukjang* prepared by fermenting cooked soy with Six strains of Bacillus species isolated from traditional *cheonggukjang* (12). Also pure genistein and daidzein were cultured with several pure strains of Bacillus species, followed by isoflavone analysis of culture supernatant to see if any biotransformation occurred.

Materials and Methods

***Cheonggukjang* samples** *Cheonggukjang* was prepared in the following way; soybeans ('Taekwang' variety) were soaked in water at 20°C for 18-20 hr, and steamed for 60 min at 121°C. The steamed soybeans were inoculated with various Bacillus species (12) isolated from traditional *cheonggukjang* obtained from Sunchang Fermented Soybean Products Valley (Sunchang, Jeonbuk, Korea) and incubated in a jar maintained with 100% humidity at 42°C for 48 hr. Samples were taken at 48 hr fermentation time, freeze-dried, and subjected to solvent extraction for isoflavone analysis (11).

Isoflavone fermentation Various Bacillus species were cultured in the presence of pure genistein, daidzein for 48 hr in Lubria-Bertani (LB) broth, followed by isoflavone analysis.

Isoflavone standards Isoflavones standards were obtained as follows: genistein, daidzein, and glycitein from Sigma-

Aldrich (St. Louis, MO, USA), genistin, daidzin, and glycitin from Indofine (Hillsborough, NJ, USA), and malonyl genistein, malonyl daidzin, malonyl glycitin, acetyl genistein, acetyl daidzin, and acetyl glycitin from LC Labs (Woburn, MA, USA).

Isoflavone analysis Freeze-dried *cheonggukjang* was powdered in a food mixer (FM-909T; Hanil, Seoul, Korea), and 2 g samples were extracted in 10 mL of acetonitrile, 2 mL of 0.1 N HCl, and 7 mL of water in a 125-mL screw-top Erlenmeyer flask with stirring for 2 hr at room temperature according to Murphy's procedure (10). The residues were dissolved in 80% high performance liquis chromatography (HPLC) grade methanol. An aliquot was filtered through a 0.45-μm nylon filter (Nunc, Rochester, NY, USA) and analyzed by HPLC. A Jasco chromatograph with a Model AS 2055 autosampler (Jasco, Tokyo, Japan) a Model PU 1580 dual pump, and a Model UV-2077 UV-visible detector was used to analyze each sample. A Phenomenex Gemini C18 column (5 μm, 150×2.00 mm) was employed for chromatographic separations. A linear gradient composed of A (0.1% phosphoric acid in water) and B (acetonitrile) was used. After injection of a 10 μL sample, the system was increased from 10 to 35% B over 40 min, returned to 10% in 5 min, and maintained at 10% B for another 10 min. The system was recycled to 10% B at the end of 55 min. The flow rate was 0.8 mL/min. The ultraviolet (UV) absorbance was monitored at 280 nm. UV spectra were recorded and peak areas were integrated using Young-Lin Autochro 2000 software (Anyang, Korea). Analyses were repeated 3 times and data were expressed as the mean±standard deviation (SD, n=3). To determine the recovery of isoflavones, stock solutions of authentic isoflavone standards and fluorescein were added to *cheonggukjang* powder before isoflavone extraction (13).

The full-scan mass spectra were obtained within a range of *m/z* 50-1,500 using 3 microscans (14). Utilizing the capacity of the LCQ mass spectrometer to provide continuous polarity switching, data acquisition was conducted in positive and negative modes. The data dependent tandem mass spectrometry (MS/MS) experiments were controlled using the menu-driven software provided with the Xcalibur system. All the experiments were performed under automatic gain control conditions. The high resolution mass spectrometer (HR-MS) was measured in methanol with a PEG400 on a JMS-700 Mstation (Jeol Ltd., Tokyo, Japan) consisting of a high-resolution 2-sector mass spectrometer using the FAB ionization method from the Korea Basic Science Institute (KBSI, Daejeon, Korea). The liquid chromatography (LC)-MS analytical data were optimized using a background subtraction technique of chromatography with the metabolite ID 2.0 software system. In the subsequent optimization for the chromatography and spectrum analyses, data containing more real secondary metabolites were observed, along with more ions present in the processed mass spectra. After exporting the process, the LC-MS data were manually sorted to list such information as the retention time, *m/z* values for [M+H]+, MS/MS fragmentation pattern, and UV spectra from base peak chromatograms.

Results and Discussion

In this study we investigated changes of isoflavone content during the preparation of c*heonggukjang* using different Bacillus species. As shown in Table 1, isoflavone profile of *cheonggukjang* was widely different depending upon Bacillus species used. For instance, *cheonggukjang* prepared using *Bacillus amyloliquefaciens* 86-1 did not significantly accumulate isoflavone metabolite coeluting with glycitein or its derivatives while the product made by fermenting with *Bacillus subtilis* 3-5 caused significant increase in the

Table 1. Isoflavone contents (μg/g dry weight) of *cheonggukjang* prepared with various Bacillus strains

	Steamed soy	*B. subtilis* 3-5	*B. licheniformis* 3-17	*B. subtilis* 3-25	*B. amyloliquefaciens* 51	*B. amylolique-faciens* 86-1	*B. subtilis* 97
Daidzin	481[1)]	316	178	268	430	262	611
Glycitin	177	143	114	108	114	99	150
Genistin	948	445	362	468	473	469	681
M-Daidzin	272	171	111	219	223	330	92
M-Glycitin+IM[2)]	74	9	358	105	15	20	144
M-Genistin	299	345	268	351	296	353	211
A-Daidzin	130	271	51	32	174	35	3
A-Glycitin	77	53	105	53	110	71	82
A-Genistin	148	25	34	11	7	9	20
Total glycosides	**2,607**	**1,779**	**1,581**	**1,615**	**1,841**	**1,649**	**1,995**
Daidzein	36	172	156	192	141	242	158
Glycitein+GM[3)]	11	254	317	110	218	50	135
Genistein	37	325	277	342	275	402	264
Total aglycones	**83**	**751**	**751**	**644**	**634**	**695**	**557**
Total isoflavones	**2,690**	**2,530**	**2,331**	**2,259**	**2,475**	**2,344**	**2,551**

[1)]Values are mean (n=3).
[2)]IM: isoflavone metabolites.
[3)]GM: genistein metabolites.

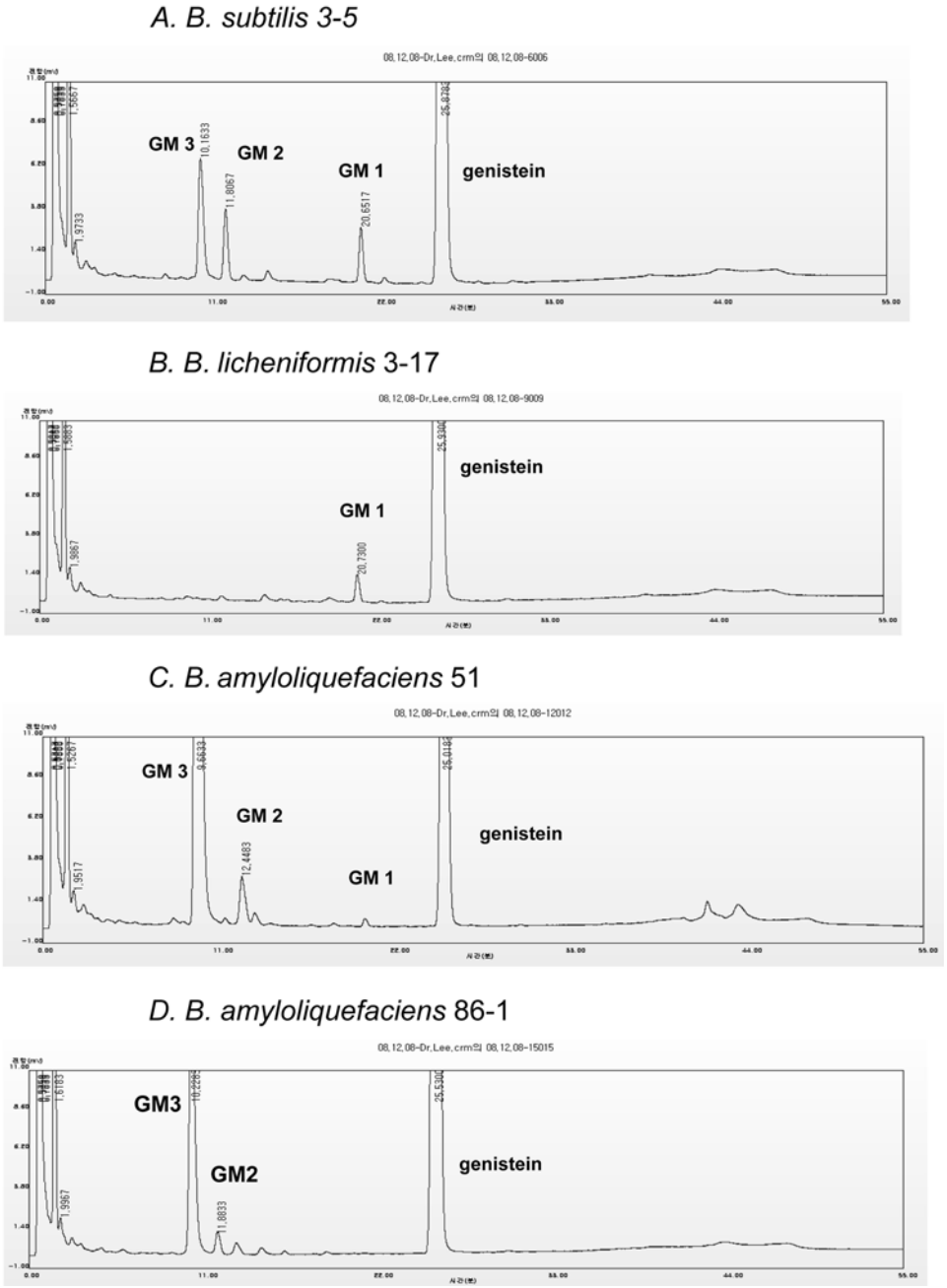

Fig. 1. Biotransformation of genistein by Bacillus species. GM1-3 represents genistein metabolite(s).

level of isoflavone metabolite coeluting with glycitein. Since genistein is the possible source for production of glycitein during *cheonggukjang* preparation, we tested that genistein is converted into glycitein by Bacillus species. As shown in Fig. 1, *B. subtilis* 3-5 metabolized pure genistein into a metabolite coeluting with glycitein (GM1) and others while *B. amyloliqufaciens* 86-1 did not generate a metabolite coeluting with glycitein from genistein. In addition to *B. subtilis* 3-5, *Bacillus licheniformis* 3-17, and *B. amyloliqufaciens* 51 produced a metabolite coeluting with glycitein from genistein during culturing bacteria with pure genistein.

Mass spectrometrical analysis demonstrated that a peak coeluting glycitein (GM1) is, in fact, not glycitein but appears to be more polar genistein metabolite with molecular weight 532 which is higher than one of the parent compound genistein (Mw 270).

Culture of genistein with *B. subtilis* 3-5, *B. amyloliqufaciens* 51 and 86-1 also led to accumulation of metabolites eluted around 10.2 (GM3) and 11.8 min (GM2) after injection of the sample under the HPLC operation conditions used. While the metabolite being eluted at 11.8 min had Mw 350 and could not be identified, the compound eluted at 10.2 min was estimated to be genistein glucoside (4',5,7-trihydroxyisoflavone-7-glucoside) as mass analysis showed molecular weight of 432 and mass fragment profile which matched with standard genistin. In fact, it has been reported that UDP glycosyltransferase isolated from *B.*

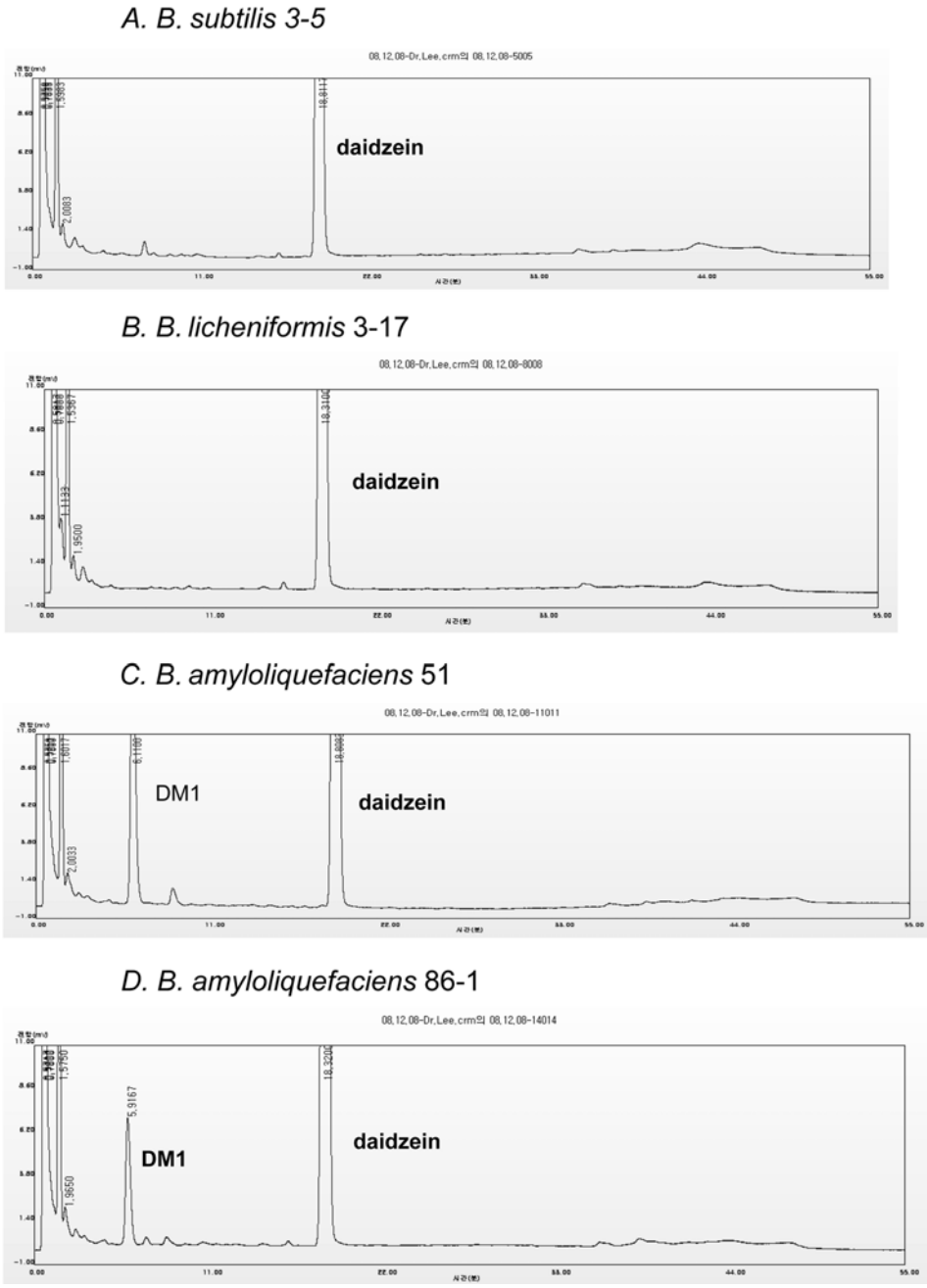

Fig. 2. Biotransformation of daidzein by Bacillus species. DM represents daidzein metabolite(s).

cereus could glycosylate genistein at 3- or 7-hydroxyl group of the isoflavone (15). Furthermore, genistin could be further glycosylated and become more water-soluble by alkalophilic Bacillus species and *Thermus scotoductus* (16).

Thus most of Bacillus strains present in traditional *cheonggukjang* appear to contain relatively strong glycosylation enzyme activity as well as β-glucosidase activity.

Since genistein is believed to have the most beneficial health effect among soy isoflavones, its metabolism during soy processing such as *cheonggukjang* preparation is recommended to be minimized. Our data suggest that bioavailability and health benefit of soy products might be differential depending upon microorganism used for manufacturing *cheonggukjang*. For instance, certain bacteria can bring about extensive metabolism of genistein, one of the most bioactive components in soy, leading to depreciation of health beneficial value of the soy products. Isoflavones have been known to undergo extensive metabolism during processing of soy. In particular, conversion of glycoside forms of isoflavones into aglcones by β-glucosidase activity present in bacteria, yeast, or fungi is commonly observed when traditional fermented soy foods were made (11,13).

Not only genistein but also daidzein was metabolized into a compound coeluting with daidzein glucoside (DM1) by Bacillus species, in particular, *B. amyloliqufaciens* 51 and 86-1 (Fig. 2). However, when pure daidzein was cultured with *B. subtiltis* 3-5 and *licheniformis* 3-17, it remained

unchanged. Some Bacillus species, therefore, appear to express glycosylation enzyme activity as well as β-glucosidase activity. The balance between these 2 enzymes may determine the ratio of glycosides to aglycones of isoflavones.

While there are many reports of hydrolysis of isoflavone glycosides by Bacillus sp. in the process of *natto* preparation, glycosylation reaction did not have proper attention while the reaction is, in fact, universally occurring during *cheonggukjang* manufacture.

In conclusion, the capability of microorganism to metabolize genistein is widely variable and is recommended to be taken into cosideration in selecting culture seed for *cheonggukjang* manufacture.

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