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Research Article

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Comparison of Isoflavone Contents and Antioxidant Effect in *Cheonggukjang* with Black Soybean Cultivars by *Bacillus subtilis* CSY191

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Abstract

BACKGROUND: Soybeans are the rich sources of isoflavones. To date, the changes of isoflavone contents in various black soybeans *cheonggukjang* during fermentation by *Bacillus subtilis* CSY191 has not been investigated.

METHODS AND RESULTS: This study investigated the changes of total phenolic and isoflavone contents and antioxidant effects during *cheonggukjang* fermentation made with four black soybean (BS) cultivars including *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* with a potential probiotic *Bacillus subtilis* CSY191. The total phenolic contents, isoflavone-malonylglycoside and -aglycone contents, and antioxidant activity were increased in *cheonggukjang* at 48 h fermentation, while the content of isoflavone-glycosides was decreased during *cheonggukjang* fermentation. In particular, the *Cheongja*#3 soybean fermented at 37℃ for 48 h displayed the highest antioxidant activities, compared to those of the other BS cultivars tested. Also, the highest levels of total phenolic, daidzein, glycitein, and genistein were present at concentrations of 17.28 mg/g, 283.7 g/g, 39.9 g/g, and 13.2 g/g at the end of *Cheongja*#3 soybean fermentation.

CONCLUSION: The results from this study suggested that the enhanced antioxidant activity of *cheonggukjang* of BS might be related to increased levels of total phenolic, isoflavon-aglycone, and malonyl-glycoside contents achieved during fermentation. Furthermore, fermented *Cheongja*#3 soybean showed the highest levels of enhanced antioxidant activities than the other BS cultivars.

Key words: Antioxidant, *Bacillus subtilis* CSY191, Black soybean, *Cheonggukjang*, Isoflavone

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Introduction

In general, soybeans [*Glycine max* (L.) Merril] have been consumed as an important protein source to complement grain protein in Asian countries. However, they are also enriched with isoflavones, anthocyanins, saponins, lipids, and oligosaccharides (Kim *et al.*, 2011). The several soy-foods, such as *cheonggukjang*, *doenjang*, *douche*, *temphe*, and *tofu*, are being prepared from soybeans through fermentation process.

Among several soybean varieties, black soybean (BS) is increasingly sought after in food and medicinal industries because of their various beneficial effects (Lee *et al.*, 2012). The beneficial properties of BS are due to the many phytochemicals present in the crop, including isoflavone, flavanol, flavan-3-ol, anthocyanin, and saponin (Lee *et al.*, 2012; Lee *et al.*, 2014). BS is very much popular in Korea and are used to prepare several different traditional fermented foods, including *meju* (soybean cake), *cheonggukjang* (soybean cook), *kanjang* (soybean sauce), and *doenjang* (soybean paste). In particular, *cheonggukjang* is manufactured in a traditional way in homes using different types of processes, depending on the region: thus its physicochemical and functional properties vary due to differences in soybeans, microorganisms, and fermentation time (Nam *et al.*, 2012).

In raw soybeans, isoflavones are present in four chemical forms: malonylglycosides (70-80%), acetylglycosides (5%), glycosides (25%), and aglycones (2%) (Lee *et al.*, 2011). Isoflavones conjugated glycoside are converted to aglycones under acidic or alkaline conditions or by the action of -glycosidase. Importantly, the aglycone forms show greater potential for absorption in the intestine than the glycoside forms (da Silva *et al.*, 2011). Thus, incorporation of β-glycosidase has attempted to increase the content of isoflavone- aglycones in *cheonggukjang* in several studies (Yang *et al.*, 2006; Cho *et al.*, 2011). Moreover, some studies reports that total phenolic and isoflavone-aglycone contents increased, depending on whether antioxidant activities increased after *cheonggukjang* fermentation (Cho *et al.*, 2009; Hu *et al.*, 2010). Recently, we reported that the total phenolic and isoflavone- aglycone contents were enhanced during *cheonggukjang* fermentation made with two wild varieties of black soybeans (Hwang *et al.*, 2013) and brown soybean (Shin *et al.*, 2014) using a potential probiotic *Bacillus subtilis* CSY191 (Cho *et al.*, 2009; Cho *et al.*, 2011).

In this study, the antioxidant activities in *cheonggukjang* made with four BS cultivars including *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and Ilpumgeomjeong by the potential probiotic *B. subtilis* CSY191 were investigated. Moreover, the possibility of antioxidant enhancing effect during *cheonggukjang* fermentation made with BS cultivars that may be related to the total phenolic contents and isoflavone compositions of this product were also investigated.

Materials and methods

Black soybeans, microorganism, and chemicals

Four BS cultivars, including *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong*, were harvested in 2012 and were procured from the National Institute of Crop Science (NICS) of the Rural Development Administration (RDA) in Miryang, Korea. The collected BS samples were packaged in labeled vacuum pouches to prevent their degradation. The potential probiotic *Bacillus subtilis* CSY191 previously isolated from Korean traditional soybean paste (*doenjang*), were used as the starter organism (Cho *et al.*, 2011). The twelve standard isoflavones were purchased as described previously (Hwang *et al.*, 2014). Glacial acetic acid, Folin-Cicalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, ferric chloride, sodium acetate, 2,4,6-tripyridyl-s-triazine (TPTZ), were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-grade H_2O, methanol, and acetonitrile were purchased from Fisher Scientific Korea ltd. (Gangnam-gu, Seoul, Korea). All other reagents were of analytical grade.

Cheonggukjang preparation

Approximately 1,000 g of BS from each cultivar were separately washed and soaked with 2.5 volume tap water at 25±2℃ for 12 h, and steamed for 15 min at 121±1℃. The steamed HBS were left to stand for 1 h at 40±2℃ to cool down. After that, cooked BS were inoculated with 5.0% (w/w) strain CSY191 (7.65 log cfu/mL) and fermented for 48 h at 37±2℃ in an incubator and sampled at 0 and 48 h. The method was adapted as previously described (Hwang *et al.*, 2013).

Viable cell number, pH, and β-glucosidase activity

One gram of *Cheonggukjang* was mixed with 9 mL of 0.85% NaCl solution and the diluted suspension (0.1 portions) was spread on a TSA plate. The plate was incubated at 37±1℃ for 48 h and after which colony counts were carried out. A 10 g portion of the *cheonggukjang* of BS samples was dissolved in 90 mL of distilled water at room temperature for 12 h and was filtered through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). The residue pH was measured by pH meter (MP 200, UK). Moreover, the β-glucosidase activity of the *Cheonggukjang* crude extract was measured as previously described (Hwang *et al.*, 2013).

Isoflavone extraction and analysis

The four BS *Cheonggukjang* samples isoflavone were extracted and analyzed by HPLC according to previously described method (Hwang *et al.*, 2013).

Total phenolic contents (TPCs)

A method based on gallic acid equivalents (GAE) was used to quantify the TPCs of 50% methanol extract of four BS *Cheonggukjang* samples according to Cho *et al.* (2009).

Diphenyl picrylhydrazyl (DPPH) radical scavenging activity

The 50% methanol extracts of BS *Cheonggukjang* (0.2 mL) were prepared and mixed with 0.8 mL of 1.5×10^{-4} mM DPPH methanolic solution. The mixture was vortexed vigorously and allowed to stand for 30 min at room temperature in the dark. The absorbance of the mixture at 517 nm was determined using a spectrophotometer. The scavenging activity was expressed as a percentage using the following formula: DPPH radical scavenging activity (%) = (1-absorbance of sample/absorbance of control)×100. The DPPH radical scavenging activity of *Cheonggukjang* extracts was carried out according to Kim *et al.* (2013) that was adapted from Cho *et al.* (2011).

ABTS radical scavenging activity

$ABTS^+$ was dissolved in methanol to a finial concentration of 7 mM. This radical cation was produced by reacting the $ABTS^+$ stock solution with 2.45 mM potassium persulfate (final concentration) and by leaving the mixture for 12-16 h until the reaction was complete and the absorbance was stable. The ABTS+ stock solution was diluted in ethanol to an absorbance of 0.7±0.02 at 734 nm. After adding 0.9 ml of the diluted $ABTS^+$ solution to 0.1 mL of the sample and mixing them, the absorbance was taken 3 min later. The ABTS radical scavenging activity (%) of four cultivars of *Cheonggukjang* extracts was expressed as a percentage using the following formula: ABTS radical scavenging activity (%) = (1-absorbance of sample / absorbance of control)×100 (Kim *et al.*, 2014).

Ferric reducing/antioxidant power assays

1.5 mL of working ferric reducing/antioxidant power (FRAP) reagent pre-warmed to 37℃ was mixed with 50 μL of the test samples and standards. After vortexing, the mixture absorbance was read at 593 nm against a reagent blank. The assay was conducted at 37℃ for 15 min. The FRAP assay was conducted according to Choi *et al.* (2012).

Table 1. Change of viable cell, pH, and β-glucosidase activity during four black soybean cultivars with *cheonggukjang* fermentation by *B. subtilis* CSY191

Soybean cultivars	Samples	Contents[1)]		
		Viable cell numbers (log cfu/g)	pH	β-Glucosidase activity (Unit/g)
Cheongja	UFBS[2)]	4.83±0.17[b]	6.57±0.12[b]	3.91±0.1[b]
	FBS[3)]	11.74±0.63[a]	8.39±0.10[a]	18.8±0.18[a]
Cheongja#3	UFBS	4.81±0.22[b]	6.63±0.12[b]	3.94±0.12[b]
	FBS	11.47±0.53[a]	8.48±0.15[a]	20.5±0.2[a]
Geomjeong#5	UFBS	4.92±0.19[b]	6.54±0.15[b]	4.01±0.13[b]
	FBS	11.58±0.51[a]	8.51±0.16[a]	21.1±0.25[a]
Ilpumgeomjeong	UFBS	4.95±0.17[b]	6.61±0.13[b]	3.98±0.12[b]
	FBS	11.62±0.58[a]	8.42±0.16[a]	19.2±0.22[a]

1) Values indicate the mean's of three replications (n=3). Means with different lowercase letters (a and b) indicate significant differences of fermentation times by Tukey's multiple range test (p<0.05).
2) Unfermented black soybeans
3) Fermented black soybeans at 37℃ for 48 h.

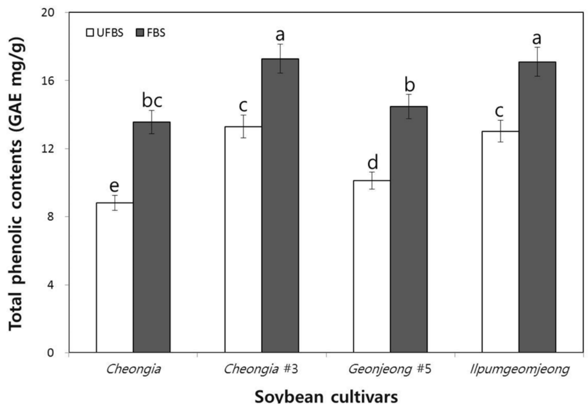

Fig. 1. Change of total phenolic contents during *cheonggukjang* fermentation with four black soybean cultivars by *B. subtilis* CSY191. UFBS, unfermented black soybeans; and FBS, fermented black soybeans at 37℃ for 48 h. All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, d, and e) indicate significant differences of fermentation times by Tukey's multiple range test (p< 0.05).

Statistical analysis

Data were expressed as the means SD (standard deviation) of three replicates. The results were subjected to analysis of variance followed by the Tukey's multiple range tests at p<0.05 using the SAS program (Version 9, USA).

Results and discussion

Viable cell numbers, pH, and β-glucosidase activity during black soybean fermentation

The viable cell numbers, pH, and β-glycosidase activities in each *cheonggukjang* made with four BS cultivars were increased during fermentation (Table 1). As the results, the bacterial cell numbers in fermented *cheonggukjang* made with BS of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivar were increased to 2.43-, 2.38-, 2.35-, and 2.35-fold, respectively. In addition, the β-glucosidase activities in *cheonggukjang* made with BS of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivars were increased to 4.8-, 5.2-, 5.26-, and 4.82-fold by the end of fermentation (48 h), respectively. In fact, the pH of fermented *cheonggukjang* made with BS from all four cultivars was raised approximately to 8.39 to 8.51. The viable cell numbers and β-glycosidase activities were markedly increased during *cheonggukjang* fermentation in previous studies (Yang *et al.*, 2006; Cho *et al.*, 2011; Hwang *et al.*, 2013; Shin *et al.*, 2014), which is a very good agreement with the current study.

Changes of total phenolic and isoflavone contents during BS fermentation

The change in the TPCs in *cheonggukjang* made with BS of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivars is shown in Fig. 1. The TPCs in each fermented *cheonggukjang* made with BS from all cultivars were increased than that of the unfermented BS. In particular, the TPCs in fermented *cheonggukjang* made with BS were increased to 1.53-, 1.3-, 1.43-, and 1.31-folds by the end of fermentation (48 h) for the *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivars, respectively (Fig. 1). Phenolics are usually found in conjugated forms through hydroxyl groups with sugars and glycosides in plant materials (Juan & Chou, 2010). Catalyzing the release of the total phenolic contents from the BS during fermentation may thus lead to an increase in the content of those compounds, as shown in Fig. 1. Our previous study reported that the TPCs in fermented *cheonggukjang* made with brown soybean (*Galmi*) and wild BS cultivars (*Seoritae* and *Seomoktae*) were increased in amount with potential probiotic *Bacillus subtilis* CSY191 by the end of fermentation (Hwang *et al.*, 2013; Shin *et al.*, 2014). These results suggested that *Bacillus subtilis* CSY191 is a strong potential candidate strain for the biotransformation of soybean biopolymers into beneficial phenolics during *cheonggukjang* fermentation made with wild or breeding soybean seeds. Meanwhile, some studies reported that the total phenolic content increased during soybean fermentation in foods, such as *cheonggukjang* and *natto* (Cho *et al.*, 2009; Cho *et al.*, 2011; Shon *et al.*, 2007).

In the case of *chenoggukjang* made with BS of *Cheongja* cultivar, the isoflavone-malonylglycoside and-aglycone contents increased throughout fermentation to approximately 1.24- and 1.85-fold relative to their starting amounts (23.1% and 6.8%, respectively), but the isoflavone-glycoside contents decreased from 70.1 to 58.7% at the end of fermentation. Similarly, the levels of isoflavone-malonylglycoside and -aglycone in the *chenoggukjang* made with BS of *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivars increased throughout fermentation to approximately 1.47- and 2.52-fold, 1.02- and 2.48-fold, and 1.27- and 1.72-fold relative to their starting amounts (20.4 and 7.5%, 32.9

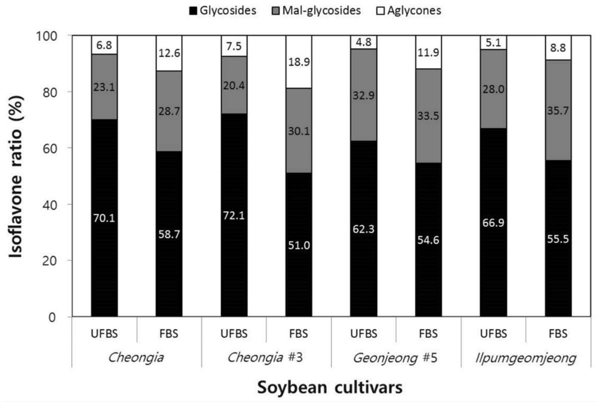

Fig. 2. Change of isoflavone β-glucoside, -malonyl- β-glucoside, and -aglycones and total isoflavones contents during *cheonggukjang* fermentation with four black soybean cultivars by *B. subtilis* CSY191. UFBS, unfermented black soybeans; and FBS, fermented black soybeans at 37℃ for 48 h.

and 8.8%, and 28.0 and 5.1%), but the isoflavone-glycoside contents decreased from 72.1 to 51.0%, 62.3 to 54.6%, and 66.9 to 55.5%, respectively, at the end of the fermentation (Fig. 2). In particular, daidzin of the glycoside type decreased from 432.9 μg/g to 258.7 μg/g and the corresponding daidzein of the aglycone type increased from 96.5 μg/g to 283.7 μg/g in *chenoggukjang* made with BS of *Cheongja*#3 cultivar at the end of fermentation (Table 2). In fact, the total amount of isoflavone was higher in unfermented and fermented (*chenoggukjang*) BS of *Cheongja*#3 cultivar than those of the other BS cultivars examined. However, it is apparently shown that the isoflavone-glycosides decreased, while the isoflavone-aglycones increased during *cheonggukjang* fermentation made with all the four BS cultivars (Fig. 3A-H). These results suggested that the glycoside type isoflavone content daidzin, glycitin, and genistin concentrations were decreased in *chenoggukjang* made with all BS cultivars, while the aglycones type isoflavone content daidzein, glycitein, and genistein concentrations were increased at the end of fermentation. In addition, the malonylglycosides concentrations were also increased in the fermentation of BS *chenoggukjang* than that of the unfermented BS. The content and composition of these isoflavones vary in soybean foods depending on the soybean varieties and processing techniques used, such as fermentation. It has been reported that the isoflavone levels in soybean-containing foods, such as *tofu*, *douchi*, and *cheonggukjang*, decrease depending on the processing conditions (Yang *et al.*, 2006; Cho *et al.*, 20011; Coward *et al.*, 1998; Prabhakaran *et al.*, 2006). Jang *et al.* (2006) reported that the total isoflavone content in raw soybeans was 2.87 μg/g, which decreased by approximately 50% during cooking prior to *cheonggukjang* fermentation. In a related study, Yang *et al.* (2006) reported that the total isoflavone content decreased from 1,055 μg/g (0 h) to 870 μg/g (36 h) during *cheonggukjang* fermentation by *B. subtilis*. Meanwhile, Cho *et al.* (2011) reported that the total isoflavone contents in *cheonggukjang* fermentation decreased approximately 64% from an initial 2923.21 μg/g to 1051.59 μg/g after 60 h of fermentation. Hwang *et al.* (2013) reported that the total isoflavone content in *cheonggukjang* made with wild soybeans was decreased by 13.15% and 8.3% for

Table 2. Distributions of isoflavone contents in *cheonggukjang* made of four black soybean cultivars by the *B. subtilis* CSY191

Soybean Cultivars	Samples	Isoflavone contents[1)] (μg/g)												
		Glycosides			Malonylglycosides			Acetylglycosides			Aglycones			Total
		Daidzin	Glycitin	Genistin	Daidzin	Glycitin	Genistin	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	
Cheongja	UFBS[2)]	243.2±8.6^{b}	187.0±7.1^{b}	421.2±13.1^{c}	65.5±3.1^{d}	45.6±1.6^{c}	169.1±4.2	nd[4)]	nd	nd	39.9±1.5^{d}	42.7±1.9^{ab}	tr[5)]	1214.1^{d}
	FBS[3)]	189.9±5.1^{c}	173.8±6.8^{b}	302.0±10.2^{d}	80.0±4.8^{bc}	71.1±2.5^{b}	175.0±5.6^{ab}	nd	nd	nd	114.7±3.2^{bc}	27.9±1.6^{bc}	tr	1134.4^{d}
Cheongja#3	UFBS	432.9±12.7^{a}	254.6±9.3^{a}	737.5±15.8^{a}	108.4±6.1^{b}	58.3±2.1^{ab}	236.1±5.3	nd	nd	nd	96.5±2.7^{c}	51.4±2.7^{a}	nd	1975.5^{a}
	FBS	258.7±7.3^{b}	233.1±6.1^{ab}	416.9±11.4^{c}	133.1±6.6^{ab}	116.9±3.4^{a}	285.7±8.7	nd	nd	nd	283.7±4.1^{a}	39.9±2.4^{b}	13.2±1.3^{a}	1781.2^{b}
Geomjeong#5	UFBS	267.5±7.8^{b}	155.4±5.2^{bc}	475.6±13.2^{b}	124.0±5.3^{ab}	53.6±1.8^{ab}	297.3±9.2	nd	nd	nd	29.1±1.2^{d}	39.6±2.5^{b}	tr	1442.1^{d}
	FBS	235.5±7.5^{b}	146.9±4.5^{c}	400.1±10.1^{c}	131.8±4.0^{ab}	64.0±1.8^{b}	284.4±8.8	nd	nd	nd	126.3±3.5^{b}	33.1±1.8^{b}	10.2±1.1^{b}	1432.3^{c}
Ilpumgeomjeong	UFBS	384.7±10.4^{a}	277.3±7.1^{a}	493.0±12.8^{b}	154.6±4.5^{a}	67.3±2.3^{b}	261.7±6.8	nd	nd	nd	42.4±1.1^{d}	45.7±2.1^{ab}	tr	1726.7^{b}
	FBS	237.3±9.0^{b}	267.4±6.4^{a}	286.2±8.8^{d}	166.4±4.7^{a}	105.2±2.6^{a}	252.5±5.2	nd	nd	nd	127.9±2.5^{b}	21.4±1.8^{c}	3.3±.05^{c}	1467.5^{c}

[1)] Values indicate the mean's of three replications (n=3). Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test (p<0.05).
[2)] Unfermented black soybeans.
[3)] Fermented black soybeans at 37℃ for 48 h.
[4)] nd, not detected.
[5)] tr, trace <0.002 μg/g.

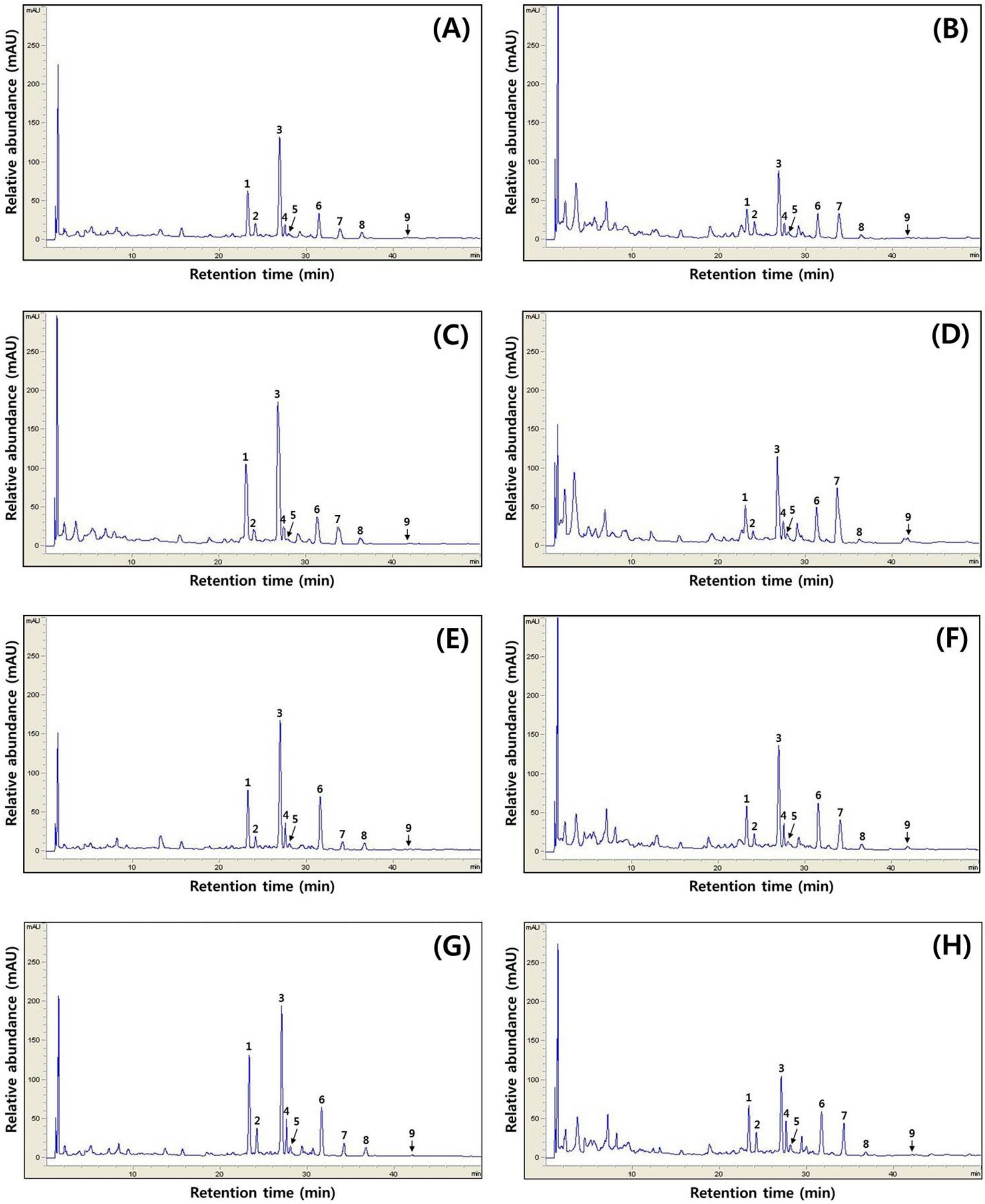

Fig. 3. Typical HPLC chromatograms of isoflavones. HPLC chromatogram of isoflavone extract in *cheonggukjang* made with black soybean of (A) *Cheongja* cultivar according to fermentation period (0 h), (B) *Cheongja* cultivar according to fermentation period (48 h), (C) *Cheongja*#3 cultivar according to fermentation period (0 h), (D) *Cheongja*#3 cultivar according to fermentation period (48 h), (E) *Geomjeong*#5 cultivar according to fermentation period (0 h), (F) *Geomjeong*#5 cultivar according to fermentation period (48 h), (G) *Ilpumgeomjeong* cultivar according to fermentation period (0 h), and (H) *Ilpumgeomjeong* cultivar according to fermentation period (48 h). 1, diadzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; 5, malonyl glycitin; 6, malonyl genistin; 7, daidzein; 8, glycitein; and 9, genistein.

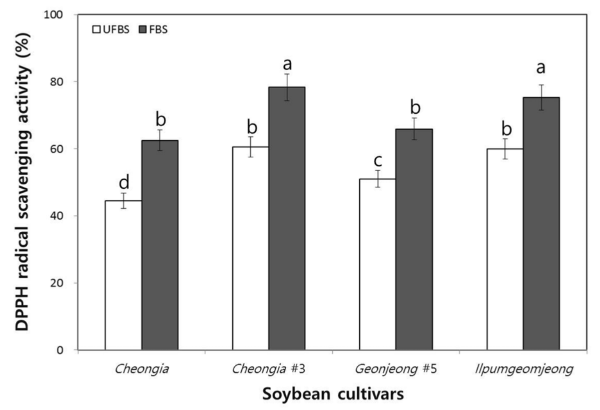

Fig. 4. Change of diphenyl picrylhydrazyl (DPPH) radical scavenging activity during *cheonggukjang* fermentation with four black soybean cultivars by *B. subtilis* CSY191. UFBS, unfermented black soybeans; and FBS, fermented black soybeans at 37℃ for 48 h. All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test (p<0.05).

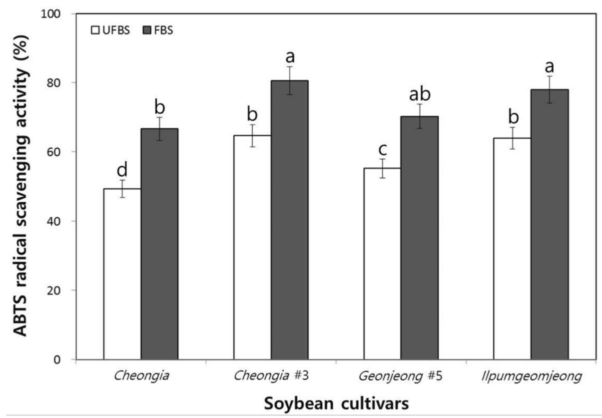

Fig. 5. Change of ABTS radical scavenging activity during *cheonggukjang* fermentation with black soybean cultivars by *B. subtilis* CSY191. UFBS, unfermented black soybeans; and FBS, fermented black soybeans at 37 C for 48 h. All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test (p< 0.05).

the *Seoritae* and *Seomoktae* cultivars using *Bacillus subtilis* CSY191. In this study, the total isoflavone content decreased by approximately 6.6, 9.84, 0.7, and 15.1% after fermentation processing in BS of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* cultivars at the end of fermentation (48 h), respectively (Table 2).

In general, most isoflavones in soybean are present in glycoside form, and they are converted into aglycones during fermentation by microbial β -glycosidase activity (Velioglu *et al.*, 1998; Yang *et al.*, 2006; Cho *et al.*, 2011). It is important to note that the levels of isoflavone-aglycones and the β-glycosidase activity increased and isoflavone-glycosides decreased during *cheonggukjang* fermentation by the potential probiotic *B. subtilis* CS90 (Cho *et al.*, 2011; Hwang *et al.*, 2013). In this study, we found that the starter potential probiotics *B. subtilis* CSY191 had the effect of increasing the β-glycosidase activity, and the algycone contents increased at the end of fermentation. In contrast, Yang *et al.* (2006) reported that the addition of *B. subtilis* had no effect on β-glycosidase activity, and the aglycone contents did not increase during *cheonggukjang* fermentation.

Change of antioxidant activities during Black Soybean fermentation

To examine the hydrogen donating activity, the DPPH radical scavenging activity of the unfermented black soybean (UFBS) and fermented black soybean (FBS) were tested. As the result, the DPPH radical scavenging activities of *chenoggukjang* made with different BS cultivars were increased about to 1.4-, 1.3-, 1.3-, and 1.25-folds at the end of fermentation, respectively (Fig. 4). This result suggested that the hydrogen donating activities of BS were increased after *chenoggukjang* fermentation. Importantly, the *chenoggukjang* made with BS of *Cheongja*#3 cultivars has shown the greater DPPH radical scavenging activity (78.3%) than those of the *chenoggukjang* made with other BS cultivars in this study.

Moreover, to determine the hydrogen-donating antioxidants and chain-breaking antioxidants, the ABTS radical scavenging ability of *chenoggukjang* made with UFBS and FBS were tested. The levels of ABTS radical scavenging activity in *chenoggukjang* of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong* was increased about to 1.35-, 1.25-, 1.27-, and 1.22-folds at 48 h of fermentation (Fig. 5). However, like DPPH radical scavenging activity, the *chenoggukjang* of *Cheongja*#3 has shown the greater ABTS radical scavenging activity (80.64%) than those of the other *chenoggukjang* examined in this study.

The FRAP assay directly measure the total antioxidant power of plant extracts. Thus, we further determine the antioxidant activity of the unfermented and FBSs. In fact, the chenoggukjang of *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong*, the values resulting from the FRAP assay of the

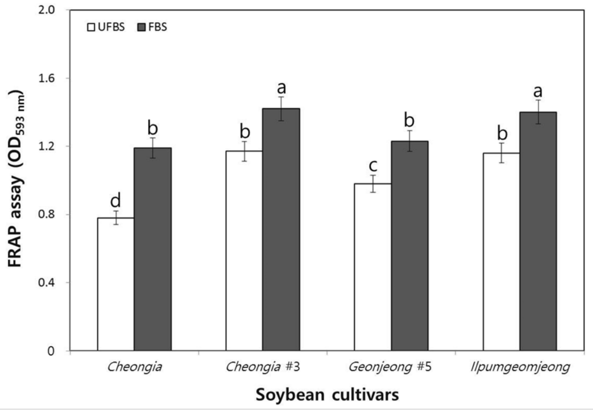

Fig. 6. Change of ferric reducing/antioxidant power value during *cheonggukjang* fermentation with black soybean cultivars by *B. subtilis* CSY191. UFBS, unfermented black soybeans; and FBS, fermented black soybeans at 37℃ for 48 h. All values are means of determinations in three independent experiments. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test (p< 0.05).

fermented soybeans increased about to 1.52-, 1.21-, 1.25-, and 1.2-fold at 48 h fermentation, respectively (Fig. 6). Importantly, like DPPH and ABTS radical scavenging activity, the FRAP assay value (1.42) of *chenoggukjang* made with BS of *Cheongja*#3 cultivar was greater than those of the other *chenoggukjang* examined.

Several studies revealed that phenolic compounds were responsible for the antioxidant activity in fruits, vegetables, and grains (Velioglu *et al*., 1998; Kwak *et al*., 2005). Pratt (1980) reported that the combined isoflavones and phenolic acids account for nearly all the *in vitro* antioxidant activity of soybean and soy product. In fact, the TPCs were measured as an overall indicator of the contents of these molecules with antioxidant properties (Slavin *et al*., 2009). It was reported that methanol extract of *cheonggukjang* exhibited radical-scavenging activity of 69-87% and total phenolic contents of 0.13-0.27 mg/g (Shon *et al*., 2007). Interestingly, fermentation enhances the TPC as well as antioxidant activity of the BS extract (Juan *et al*., 2010). However, isoflavones have direct free radical quenching ability, with daidzein and genistein being particularly effective (Shon *et al*., 2007; Cho *et al*., 2009; Cho *et al*., 2011). Moreover, Kim *et al*. (2008) reported that the *cheonggukjang* extract and its constituents, genistein and daidzein, exhibited significant antioxidant activity *in vitro*. In the present study, higher *in vitro* antioxidant activities were found shown in FBS in *cheonggukjang* than the UFBS of the four cultivars tested. This result indicates that enhanced antioxidant effects of *cheonggukjang* made with BS were caused by the increased amount of total polyphenol content and aglycone isoflavone than that of the unfermented BS. In addition, the increased malonyl glucoside concentration during fermentation may also contribute to enhance the antioxidant activity of FBS (*cheonggukjang*) compared to the UFBS. In our previous study, the radical scavenging activity was increased from 53.6% to 93.9% according to the total phenolic and isoflavone-aglycone (daidzein) contents during *cheonggukjang* fermentation with potential probiotic *B. subtilis* CS90 (Cho *et al*., 2011). Recently, we found that the stronger antioxidant activity of cheonggukjnag made with two wild cultivars of BS as well as brown soybeans might be related to the markedly higher TPCs and isoflavone-aglycones and -malonylgycosides achieved during fermentation (Hwang *et al*., 2013; Shin *et al*., 2014), which is consistent with the current study and with Kwak *et al*. (2007).

In conclusions, this study first documented the changes in the TPCs and in the contents of isoflavones during *cheonggukjang* fermentation made with BS cultivars with a potential probiotic *B. subtilis* CSY191. In the case of four BS cultivars, including, *Cheongja*, *Cheongja*#3, *Geomjeong*#5, and *Ilpumgeomjeong*, the TPCs and isoflavone-aglycone contents were markedly increased, while the isoflavone-glycosides were decreased according to the β-glycosidase activities. Importantly, the TPCs, total isoflavone contents, and antioxidant activities were higher in *cheonggukjang* made with BS of *Cheongja*#3 cultivar than those of the *cheonggukjang* made with *Cheongja*, *Geomjeong*#5, *Ilpumgeomjeong* cultivars at 48 h of fermentation. Therefore, it is supposed that high antioxidant activity of *cheonggukjang* made with BS might be related to the higher TPCs and isoflavone-aglycone contents achieved during fermentation with *B. subtilis* CSY191. Moreover, *cheonggukjang* extract made with hybrid BS seeds supposed to be used for the commercial production of functional foods in near future.

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