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Characteristics and Antioxidant Effect of Garlic in the Fermentation of *Cheonggukjang* by *Bacillus amyloliquefaciens* MJ1-4

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The changes in the β-glucosidase activity, total phenolic contents, isoflavone contents, and antioxidant activities during the fermentation of *cheonggukjang* by *Bacillus amyloliquefaciens* MJ1-4 with and without garlic were investigated. The levels of total phenolic and isoflavone-malonylglycoside, -acetylglycoside, and -aglycone contents increased, whereas the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing/antioxidant power (FRAP) assay results increased, but isoflavone-glycoside levels decreased during *cheonggukjang* fermentation. The levels of total phenolic and total isoflavone contents and the antioxidant activities were higher in *cheonggukjang* fermented without garlic (CFWOG) than in *cheonggukjang* fermented with garlic (CFWG) after 24 h of fermentation, but they were lower in CFWOG than in CFWG after 72 h of fermentation. In particular, the highest levels of total phenolic, daidzein, glycitein, and genistein were present at concentrations of 15.18 mg/g, 264.4 μg/g, 16.4 μg/g, and 31.1 μg/g after 72 h of fermentation in CFWG, showing 82.89% in DPPH radical scavenging activity, 106.32% in ABTS radical scavenging activity, and 1.47 ($OD_{593\ nm}$) in FRAP assay, respectively. From these results, we suggest that the high antioxidant activity of CFWG might be related to the markedly higher levels of total phenolic contents, isoflavone-malonylglycosides, -acetylglycosides, and -aglycones achieved during fermentation.

Keywords: *Cheonggukjang*, *Bacillus amyloliquefaciens* MJ1-4, garlic, total phenolic contents, isoflavones, antioxidant activity

Introduction

Soybeans [*Glycine max* (L.) Merrill] and soy products have long been consumed as an important protein source to complement grain protein in Asian countries. Besides protein, they are notably rich in isoflavones, anthocyanins, saponins, lipids, and oligosaccharides [13]. Various products, such as *cheonggukjang*, *doenjang*, *douche*, *tempeh*, and *tofu*, have been produced from soybeans in Asian countries, and fermentation is one of the major processes. The chemical structure and concentration of isoflavones in soy foods are dependent on many factors, including genotype, crop year, crop location, storage period, thermal processing, processing type, and the presence of microorganisms [1, 2, 13, 18–20,

32]. Isoflavone-aglycones are reported to be absorbed faster and in higher amounts than their corresponding glycoside forms [2, 13, 19, 20, 32].

The market size scale of soybean fermented foods was approximately 980 billion Korean won in 2012. Of these, *gochujang*, *doenjang*, *kanjang*, and others (including *cheonggukjang*) occupied 30.9%, 15.7%, 15.7%, and 24.5%, respectively (http://www.aTFIS.or.kr). *Cheonggukjang* is made from cooked whole soybeans fermented with microorganisms, including *Bacillus* sp. that is usually contained in the air or in the rice straw, at about 40°C without adding salts over two to three days [2]. In particular, *cheonggukjang* is traditionally produced in the house using different processes depending on the region; thus, its physicochemical and functional properties vary according to differences in the soybeans, microorganisms, and fermentation time [21]. The incorporation of β-glucosidase has been employed in an attempt to increase the content of isoflavone-aglycones in *cheonggukjang* [1, 2, 32]. In addition, several researchers have reported that the total phenolic and isoflavone-aglycone contents increased depending on whether the antioxidant activities increased after *cheonggukjang* fermentation [1, 2, 5].

Garlic (*Allium sativum*. L) is widely used as a seasoning for food, as well as having long-standing application as a medicinal agent for the treatment of a variety of human diseases and disorders. Garlic is one of the most highly antioxidant and hypoglycemic foods, and it is used for both culinary and medicinal purposes because it contains polyphenol-based antioxidant materials [12].

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense, is associated with a number of pathological conditions, such as inflammation, carcinogenesis, aging, atherosclerosis, and reperfusion injury [11]. These "free radicals" are usually removed or inactivated *in vivo* by a team of antioxidants. Individual members of the antioxidant defence team are deployed to prevent generation of ROS, to destroy potential oxidants, and to scavenge ROS. Thus, oxidative stress-induced tissue damage is minimized. Antioxidants have been used for the prevention of cardiovascular disease, cancer, and diabetes, whose prevalence has been increasing sharply in recent years [3].

The purposes of the present research were to investigate changes in the total phenolic and isoflavone contents and antioxidant effects *via* 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing/ antioxidant power (FRAP) assay during *cheonggukjang* fermentation with and without garlic. Moreover, we investigated the possibility that the enhancing effect on the antioxidant activities observed during *cheonggukjang* fermentation with and without garlic may be related to the total phenolic content and isoflavone composition of this product.

Materials and Methods

Materials, Chemicals, and Instruments

Yellow soybeans and garlics were harvested in 2011 and 2012 and were provided by the Processing Establishments of Hamyang Agricultural Cooperative in Hamyang-gun and Saenamhae Agricultural Cooperative in Namhae-gun, Korea, respectively. The tryptic soy (TS) medium was purchased from Difco (Becton Dickinson Co., Sparks, MD, USA). Three isoflavone aglycones, including daidzein, genistein, and glycitein, were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), and three isoflavone glycosides, including genistin, daidzin, and glycitin, were purchased from Indofine (Hillsborough, NJ, USA). Three malonyl- and three acetyl-isoflavone glycosides (malonylgensitin, malonylglycitin, malonyldaidzin, acetylgenistin, acetylglycitin, and acetyldaidzin) were purchased from LC Laboratories (Woburn, MA, USA). HPLC-grade H_2O, methanol, and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, USA). Glacial acetic acid, Folin-Ciocalteu phenol reagent, 2,2-diphenyl-2-picrylhydrazyl hydrate (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), and rutin were obtained from Sigma-Aldrich Chemical Co. All other reagents were of analytical grade.

The UV spectra were measured with a Spectronic 2D spectrophotometer (Thermo Electron Co., CL, USA). HPLC was performed using an Agilent 1200 series system (Agilent Co., Forest Hill, Victoria, Australia) equipped with a quaternary HPLC pump, a degasser, and an Agilent 1200 series diode array detector (DAD). The isoflavones were analyzed on a LiChrospher 100 RP C_{18} column (4.6 × 150 mm, 5 μm; Merck, Germany), whereas the phenolic acids were analyzed on an XTerra RP C8 column (4.6 × 250 mm, 5 μm; Waters Corp., Milford, MA, USA).

Growth Test of *Bacillus* sp. on *Cheonggukjang* with Garlic

Bacillus subtilis groups, such as *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus*, were previously isolated and collected from Korean *cheonggukjang*, *doenjang*, and *kanjang*, respectively [1, 2, 17, 27]. *B. subtilis* groups were kindly provide by the Laboratory of Food Microbiology, Gyeongnam National University of Science and Technology and Gyeongsang National University, Jinju, Korea, respectively. The bacterial cultures were grown in TS broth at 37 ± 2°C overnight, and garlic at 0%, 5%, 10%, and 20% (w/w) was added to the soaking soybeans, steamed, and cooled down. Then, the cooked soybeans were inoculated with 5% (w/w) of various *Bacillus* sp. and fermented for 72 h at 37 ± 2°C in an incubator. The cell concentrations were checked by a viable count determination on TS agar plates after 48 h of incubation.

***Cheonggukjang* Preparation**

The *cheonggukjang* fermentation procedure described by Cho *et al.* [2] was followed to prepare the soybeans. Garlic was washed with running tap water before being chopped into pieces. CFWOG (*cheonggukjang* fermentation without garlic): Whole soybeans (1,000 g) were washed and soaked with three volumes of tap water at 20 ± 2°C for 12 h and steamed for 30 min at 121 ± 1°C. CFWG (*cheonggukjang* fermenting with garlic): Whole soybeans (1,000 g) were washed and soaked with three volumes of tap water at 20 ± 2°C for 12 h, and then 10% garlic was added and the soybeans were steamed for 30 min at 121 ± 1°C. The steamed soybeans with and without garlic were allowed to stand for 1 h at 37 ± 2°C to cool down. Then, the cooked soybeans with and without garlic were inoculated with 5% (w/w) of the MJ1-4 strain (5.9×10^9 CFU/ml) and fermented for 72 h at 37 ± 2°C and sampled at 0, 12, 24, 48, and 72 h.

Viable Cell Numbers and β-Glucosidase Assay

A 1 g sample was mixed with 9 ml of 0.85% NaCl solution, and the diluted suspension (0.1 ml aliquots) was spread on tryptic soy agar (TSA) plates. The plates were incubated at 37°C for 24 h, and then the colony counts were performed.

The method described by Cho *et al.* [2] was used to determine the β-glucosidase activity. Ground *cheonggukjang* (1.0 g) was mixed with 20 ml of 50 mM sodium phosphate buffer (pH 7.0), vortexed for 1 min, and centrifuged at 6,000 ×*g* at 4°C for 30 min. The supernatant was collected and filtered through a 0.45 μm filter before analysis. The β-glucosidase activity in the crude extract was assayed by determining the rate of hydrolysis of *p*-NPG. The crude extract (250 μl) was added to 250 μl of substrate (5 mM *p*-NPG) in 50 mM sodium phosphate buffer (pH 7.0). After 30 min of incubation at 37°C, the enzymatic reaction was stopped by adding 500 μl of 0.2 M glycine-NaOH (pH 10.5) and the contents were immediately measured in a spectrophotometer at 405 nm. The blank solution was composed of 2.5 ml of 50 mM glycine-NaOH, 2.0 ml of substrate solution, and 0.5 ml of 50 mM citric buffer (pH 4.5) containing 0.1 M NaCl. The *p*-NP released by the action of the enzyme was determined by referring to a calibration curve prepared from the *p*-NP in concentrations that varied from 5 to 300 mmol. One unit of β-glucosidase activity was defined as the amount of enzyme that liberated 1 μM of *p*-NPG.

Extraction of Isoflavones and Analysis

The isoflavone extract and analysis were performed as previously described [2]. Briefly, each of the ground powders (10 g) was extracted with 100 ml of 50% methanol by shaking (320 rpm) at 30°C for 12 h; the extracts were filtered through Whatman No. 2 filter paper (GE Healthcare Life Sci., Piscataway, NJ, USA) and then filtered through a 0.45 μm Millipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany). Samples of the filtrates were used for HPLC analysis. The rest of the filtrates were dried under a vacuum. The dried samples were stored at −70°C in the dark until further use in antioxidant activity assays, and then the dried materials were redissolved in 50% methanol at 1 mg/ml.

The isoflavones were analyzed by HPLC. A 20 μl sample of the crude 50% methanol extracts was injected onto an analytical C_{18} column with the column temperature set to 30°C. The isoflavones were detected by monitoring the elution at 254 nm using a diode array detector. The isoflavones in the samples were identified by comparing their retention times with those of standards. The mobile phase was composed of 0.1% glacial acetic acid in water (solution A) and 100% acetonitrile (solution B). The gradient conditions were as follows: 0–20 min, 10% B; 30 min, 20% B; 40 min, 25% B; and 50 min, 35% B. The solvent flow rate was maintained at 1 ml/min.

Total Phenolic Contents (TPCs)

A method based on that of Cho *et al.* [2], which uses gallic acid equivalents (GAE), was used to quantify the TPCs in the 50% methanol extracts. A 500 μl aliquot of each isoflavone extract was mixed with 250 μl of 2 N Folin-Ciocalteu's reagent. After standing at room temperature for 3 min, a 500 μl aliquot of a 25% sodium carbonate (Na_2CO_3) solution was added, and that mixture was allowed to stand at room temperature for 1 h. Quantification was performed using a linear regression equation on the gallic acid standard curve. Five gallic acid standard solutions of 100, 250, 500, 750, and 1,000 mg/l were prepared in deionized water, and 500 μl of each standard solution was collected and prepared using the same procedure described above. The absorbance of the solution was measured at 750 nm, and a standard curve was obtained by plotting the concentration against absorbance.

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the samples was measured by the method of Cho *et al.* [2] with a slight modification. Briefly, the various concentrations of extract samples (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.

ABTS Radical Scavenging Activity

$ABTS^{\bullet+}$ was dissolved in methanol to a final concentration of 7 mM. This radical cation was produced by reacting the $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ solution to 0.1 ml of the sample and mixing them, the absorbance was taken 3 min later [3]. This scavenging activity (%) was expressed as a percentage using the following formula: ABTS radical scavenging activity (%) = (1 – absorbance of sample/absorbance of control) × 100.

FRAP Assays

The FRAP assay developed by Choi *et al.* [3] was used. Briefly, 1.5 ml of working FRAP reagent pre-warmed to 37°C (300 mM acetate buffer (pH 3.6):10 mM TPTZ in 40 mM HCl:20 mM FeCl3 = 10:1:1 (v/v/v)) was mixed with 50 µl of the test samples and standards. This mixture was vortexed, and the absorbance at 593 nm was read against a reagent blank at a predetermined time after the sample-reagent mixing. The test was performed at 37°C, and a 0−4 min reaction time window was used.

Statistical Analysis

All values are means of determinations in three independent experiments. Differences in the means of each value were determined by one-way ANOVA followed by the Tukey's multiple range tests at $p < 0.05$ using the Statistical Analysis System software ver. 9.0 (SAS Institute, Cary, NC, USA).

Results and Discussion

Change in the Viable Cell Numbers and β-Glucosidase Activity During *Cheonggukjang* Fermentation

The bacterial cell concentrations of *B. amyloliquefaciens* MJ1-4 were 10.23, 10.45, 10.77, and 9.97 log CFU/ml after *cheonggukjang* fermentation with 0%, 5%, 10%, and 20% garlic at 37°C for 72 h, respectively (data not shown). The bacterial population and β-glucosidase activity during CFWOG and CFWG are shown in Table 1. The viable cell numbers and β-glucosidase activity increased with fermentation. As the results show, the viable cell numbers of bacteria in CFWOG ranged from 7.19 to 10.59 CFU/ml, while the β-glucosidase activity increased greatly to a maximum of 24.8 unit/g after 48 h and then decreased gradually during fermentation. On the other hand, the viable cell numbers of bacteria in the CFWG ranged from 7.11 to 12.52 CFU/ml, while the β-glucosidase activity increased greatly to a maximum of 24.2 unit/g after 72 h during fermentation (Table 1). *Bacillus* species are known to be responsible for producing *cheonggukjang* [1, 2]. Several studies reported that the viable cell numbers of *Bacillus* spp. increased depending on the increased β-glucosidase activities during *cheonggukjang* fermentation [1, 2, 32].

Change of Total Phenolic and Isoflavone Contents During *Cheonggukjang* Fermentation

The change in the total phenolic contents and isoflavone-glycoside, -malonylglycoside, and -aglycone rates during CFWOG and CFWG are shown in Figs. 1 and 2, respectively. The CFWOG and CFWG at 37°C for 0, 12, 24, 48, and 72 h showed 5.05 (CFWOG) and 5.49 (CFWG) mg GAE/g, 6.39 and 5.98 mg GAE/g, 10.12 and 9.64 mg GAE/g, 12.67 and 13.52 mg GAE/g, and 13.58 and 15.18 mg GAE/g dry weight, respectively (Fig. 1). Phenolic compounds are known to possess antioxidant properties [3]. The phenolics are secondary plant metabolites that are present in all plants. Phenolics are usually found in conjugated forms through hydroxyl groups with sugars and glycosides in plant materials [25]. Catalyzing the release of the total

Table 1. Change of viable cell numbers and β-glucosidase activity during *cheonggukjang* fermentation without and with garlic by *Bacillus amyloliquefaciens* MJ1-4 starter.

Samples	Fermentation time (h)	Contents[c]	
		Viable cell number (log CFU/g)	β-Glucosidase activity[d] (Unit/g)
CFWOG[a]	0	7.19 ± 0.32[c]	5.4 ± 0.30[d]
	12	8.39 ± 0.40[bc]	12.4 ± 0.52[c]
	24	9.07 ± 0.51[b]	16.6 ± 0.84[b]
	48	9.25 ± 0.52[b]	24.8 ± 1.22[a]
	72	10.59 ± 0.43[ab]	22.0 ± 1.13[a]
CFWG[b]	0	7.11 ± 0.30[c]	5.2 ± 0.22[d]
	12	7.35 ± 0.52[c]	11.6 ± 0.64[c]
	24	8.31 ± 0.56[bc]	15.7 ± 0.85[b]
	48	9.08 ± 0.62[b]	23.8 ± 1.10[a]
	72	12.52 ± 0.51[a]	24.2 ± 1.23[a]

[a]CFWOG : *Cheonggukjang* fermented without garlic.

[b]CFWG : *Cheonggukjang* fermented with 10% garlic.

[c]Data are presented as the mean ± SD ($n = 3$) of triplicate determinations. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test ($p < 0.05$).

[d]One unit of β-glucosidase activity was defined as the amount of enzyme that liberated 1 µM of *p*-NPG.

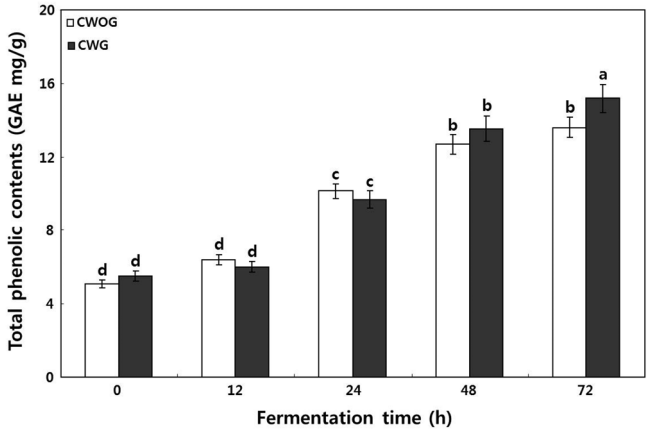

Fig. 1. Change of the total phenolic contents during *cheonggukjang* fermentation with *Bacillus amyloliquefaciens* MJ1-4 starter.
Data are presented as the mean ± SD (n = 3) of triplicate determinations. Means with different lowercase letters (a, b, and c) indicate significant differences of fermentation times by Tukey's multiple range test ($p < 0.05$).

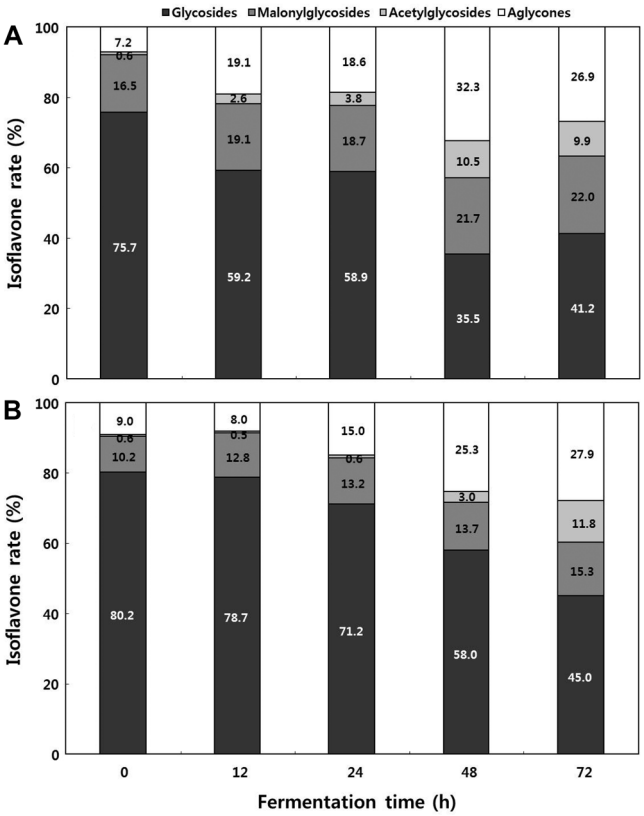

Fig. 2. Change of β-glycoside, malonyl-β-glycoside, acetyl-β-glycoside, and aglycone rates during *cheonggukjang* fermentation without and with garlic by *Bacillus amyloliquefaciens* MJ1-4 starter.
(**A**) *Cheonggukjang* without garlic. (**B**) *Cheonggukjang* with garlic.

phenolic contents from the soybean substrate during fermentation may thus lead to an increase in the content of those compounds, as shown in Fig. 1. Similar previous studies reported that the total phenolic content increased during soybean fermentation in foods, such as *cheonggukjang* and *natto* [1, 2, 8, 28]. Few studies have examined the phenolic acid groups in soybean and soybean-based products, and those studies were carried out from a physiological point of view [1, 2, 10]. Gallic, protocatechuic, *p*-coumaric, caffeic, chlorogenic, gentisic, *p*-hydroxylbenzoic, vanillic, and ferulic acids are the main components of the soybean phenolic acids, and they possess strong antioxidant activity [1, 2, 10, 24, 26]. In addition, Kim *et al.* [12] reported that gallic, *p*-coumaric, *o*-coumaric, *m*-coumaric, caffeic, and ferulic acids are the components of the garlic phenolic acids.

In the case of CFWOG, the isoflavone-malonylglycoside, -acetylglycoside, and -aglycone rates increased throughout fermentation to approximately 1.3-, 16.5-, and 3.7-fold relative to their starting amounts at 72 h (22.0%, 9.9%, and 26.9%, respectively), but the isoflavone-glycoside contents decreased from 75.7% to 41.2% at the end of fermentation (72 h) (Fig. 2A). During CFWG, the levels of isoflavone-malonylglycoside, -acetylglycoside, and -aglycone increased throughout fermentation to approximately 1.5-, 19.6-, and 3.1-fold relative to their starting amounts at 72 h (15.3%, 11.8%, and 27.9%, respectively), but the isoflavone-glycoside contents decreased from 80.2% to 45.0% at the end of the fermentation time (72 h) (Fig. 2B). In particular, daidzin of the glycoside type decreased from 450.0 to 152.1 μg/g, and the corresponding daidzein of the aglycone type increased to a maximum of 264.4 μg/g at 72 h during the fermentation process (Table 2). The isoflavone-glycosides decreased, while the isoflavone-aglycones increased during *cheonggukjang* fermentation (Figs. 3A–3D).

Generally, thermal conversion of isoflavone-malonylglycoside into the corresponding isoflavone-glycoside is the major change in the isoflavone profile that occurs during the cooking process. Yang *et al.* [32] reported that thermal cooking of raw-soaked soybeans significantly increased the isoflavone-glycoside level by 57.1% and decreased the isoflavone-malonylglycosides by 57.6%. Additionally, the isoflavone-glycoside and -malonylglycoside contents in raw soybeans were 328 and 2,277 mg/kg, which changed by 1,103 and 139 mg/kg, respectively, during the soaking and steaming process [7]. *B. amyloliquefaciens* MJ1-4 is capable of producing β-glycosidase in soybeans with and

Table 2. Change of 12 isoflavone contents during *cheonggukjang* fermentation by *Bacillus amyloliquefaciens* MJ1-4 starter.

Samples	Fermentation time (h)	Isoflavone contents[c] (μg/g)												Total
		Aglycosides			Malonylglycosides			Acetylglycosides			Aglycones			
		Daidzin	Glycitin	Genistin	Daidzin	Glycitin	Genistin	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	
CFWOG[a]	0	469.4 ± 23.53[a]	244.9 ± 14.74[ab]	490.6 ± 24.52[a]	96.6 ± 4.93[a]	34.6 ± 1.40[ab]	130.8 ± 6.52[a]	tr[d]	9.1 ± 0.46[c]	nd[e]	34.6 ± 1.73[d]	59.6 ± 2.98[a]	20.7 ± 0.83[c]	1,590.9
	12	274.0 ± 13.72[b]	147.9 ± 7.42[c]	323.8 ± 19.71[b]	86.2 ± 5.22[a]	37.4 ± 1.85[ab]	116.8 ± 6.93[a]	23.7 ± 0.96[c]	9.5 ± 0.48[c]	nd	159.9 ± 6.40[b]	57.5 ± 2.87[a]	23.1 ± 1.15[ab]	1,259.8
	24	291.0 ± 14.64[b]	184.4 ± 9.23[b]	340.9 ± 13.65[b]	93.4 ± 4.76[a]	46.7 ± 1.84[b]	119.1 ± 4.85[a]	43.2 ± 2.15[b]	10.1 ± 0.30[c]	nd	184.0 ± 9.20[ab]	43.6 ± 2.18[b]	28.5 ± 1.43[a]	1,384.9
	48	114.2 ± 5.61[c]	91.9 ± 4.61[d]	103.3 ± 5.24[d]	59.3 ± 3.02[b]	56.0 ± 3.36[b]	74.4 ± 3.72[b]	68.1 ± 2.56[ab]	23.7 ± 0.95[b]	nd	243.9 ± 12.19[a]	22.3 ± 1.12b[c]	15.1 ± 0.08[d]	872.2
	72	104.9 ± 5.23[c]	135.5 ± 8.21[c]	98.7 ± 4.97[d]	52.5 ± 2.12[b]	51.2 ± 2.04[b]	77.1 ± 3.85[b]	51.8 ± 2.55[b]	29.4 ± 1.47[b]	nd	190.5 ± 5.72[ab]	13.8 ± 0.65[c]	16.7 ± 1.00[d]	822.1
CFWG[b]	0	450.0 ± 22.56[a]	237.0 ± 9.53[ab]	493.9 ± 24.72[a]	51.0 ± 2.02[b]	27.5 ± 1.35[c]	71.6 ± 3.45[b]	nd	9.2 ± 0.46[c]	nd	38.8 ± 2.31[d]	68.8 ± 4.13[a]	24.8 ± 0.99[ab]	1,472.6
	12	405.4 ± 24.32[a]	227.7 ± 13.42[ab]	428.0 ± 17.23[ab]	60.0 ± 3.08[b]	28.5 ± 0.84[c]	83.7 ± 4.11[b]	tr	6.7 ± 0.33[d]	nd	31.8 ± 1.59[d]	57.7 ± 2.59[a]	17.7 ± 0.89[cd]	1,347.2
	24	305.4 ± 12.24[b]	187.7 ± 9.45[b]	313.6 ± 18.89[b]	48.0 ± 2.43[b]	28.0 ± 1.12[c]	73.2 ± 2.92[b]	0.2 ± 0.01[d]	6.5 ± 0.19[d]	nd	111.3 ± 5.65[c]	39.0 ± 1.95[b]	19.1 ± 0.96[c]	1,132.0
	48	213.9 ± 12.76[bc]	301.3 ± 18.11[a]	228.2 ± 11.42[c]	58.7 ± 2.34[b]	53.2 ± 1.59[b]	64.9 ± 3.90[b]	18.0 ± 0.90[c]	20.3 ± 1.01[ab]	nd	250.6 ± 12.53[a]	49.3 ± 2.46[ab]	24.3 ± 1.21[ab]	1,282.7
	72	152.1 ± 9.11[c]	197.8 ± 9.93[b]	154.1 ± 7.76[cd]	59.5 ± 2.93[b]	72.8 ± 2.22[a]	40.1 ± 2.00[c]	87.8 ± 4.39[a]	43.9 ± 2.19[a]	nd	264.4 ± 10.58[a]	16.4 ± 0.82[c]	31.1 ± 1.56[a]	1,120.0

[a]CFWOG: *Cheonggukjang* fermented without garlic.

[b]CFWG: *Cheonggukjang* fermented with 10% garlic.

[c]Data are presented as the mean ± SD (n = 3) of triplicate determinations. Means with different lowercase letters (a, b, c, and d) indicate significant differences of fermentation times by Tukey's multiple range test ($p < 0.05$).

[d]tr, trace < 0.002 μg/g.

[e]nd, not detected.

without garlic. The fermentation seemed to cause a significant increase in the amount of isoflavone-aglycones accompanied by a decrease in isoflavone glycosides. The phenomenon of a sharp increase in β-glucosidase activity corresponds well with the marked increase in isoflavone aglycone contents in the fermented *cheonggukjang*.

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 [1, 2, 4, 7, 23, 32]. Jang *et al.* [7] reported that the total isoflavone content in raw soybeans was 2.87 μg/g, and this content decreased by approximately 50% during cooking prior to *cheonggukjang* fermentation. The total isoflavone content decreased from 1,055 μg/g (0 h) to 870 μg/g (36 h) during *cheonggukjang* fermentation by *B. subtilis* [32]. Recently, Cho *et al.* [2] showed that the total isoflavone content in *cheonggukjang* fermentation decreased approximately 64% from an initial 2,923.21 to 1,051.59 μg/g after 60 h of fermentation. In this study, the total isoflavone content decreased by approximately 15% after steam and fermentation processing in raw soybeans from 1,182.7 to 1,001.1 μg/g at the end of fermentation (48 h). Yang *et al.* [32] suggested that cooking soybeans in the presence of water can greatly decrease the total isoflavone content.

In general, most isoflavones in soybean are present in glycoside form, and they are converted into aglycones during fermentation by microbial β-glucosidase activity [1, 2, 7, 22, 23, 31, 32]. Cho *et al.* [2] recently reported that the levels of isoflavone-aglycones increased, while the β-glucosidase activity and isoflavone-glycosides decreased, during *cheonggukjang* fermentation by the potential probiotic *B. subtilis* CS90. In this study, we found that the starter *B. amyloliquefaciens* MJ1-4 had the effect of increasing the β-glucosidase activity, and the alglycone contents increased over 48 h. In contrast, Yang *et al.* [32] reported that the

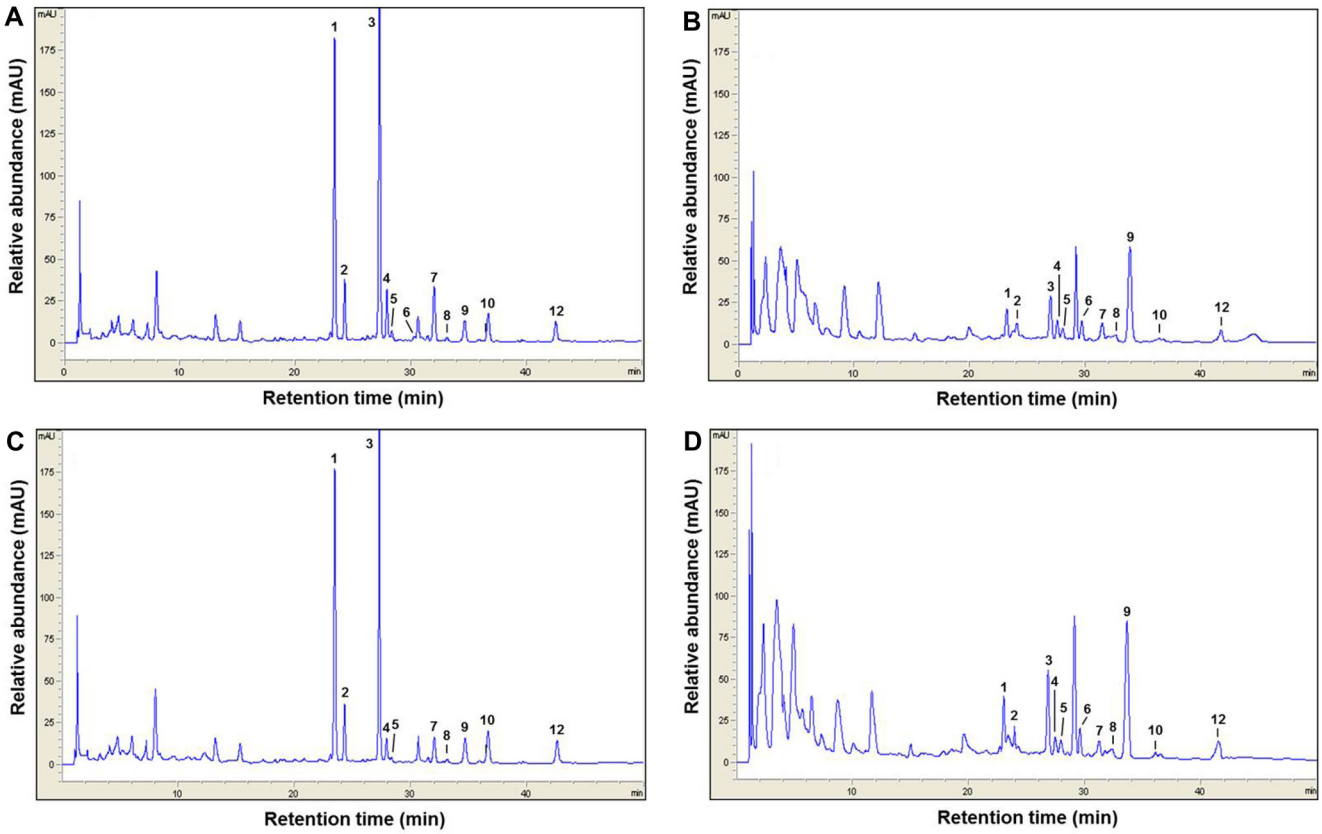

Fig. 3. Profile of HPLC chromatogram from isoflavone extracts.
(**A**) *Cheonggukjang* without garlic, by *Bacillus amyloliquefaciens* MJ1-4 (0 h), (**B**) *Cheonggukjang* without garlic, by *Bacillus amyloliquefaciens* MJ1-4 (72 h), (**C**) *Cheonggukjang* with garlic, by *Bacillus amyloliquefaciens* MJ1-4 (0 h), and (**D**) *Cheonggukjang* without garlic, by *Bacillus amyloliquefaciens* MJ1-4 (72 h). 1, Daidzin; 2, glycitin; 3, genistin; 4, malonyldaidzin; 5, malonylglycitin; 6, acetyldaidzin; 7, malonylgenistin; 8, acetylglycitin; 9, daidzein; 10, glycitein; and 12, genistein.

addition of *B. subtilis* had no effect on β-glucosidase activity, and the aglycone contents did not increase during *cheonggukjang* fermentation. Shon *et al.* [29] showed that the isoflavone content in the *cheonggukjang* made of yellow soybeans, large black soybeans, and small black soybeans was 499.15, 1,278.04, and 907.85 μg/g, respectively, and the large black soybeans had higher isoflavone content than yellow soybeans. Kim *et al.* [13] reported that the isoflavone contents were higher in *cheonggukjang* of *Daepung* cultivar than in *cheonggukjang* of other cultivars, such as *Nampung*, *Sodam*, and *Cheongja* Recently, Hwang *et al.* [6] reported that total isoflavone contents were higher in *cheonggukjang* of *Seoritae* than *cheonggukjang* of *Seomoktae* at 0 and 48 h of fermentation. These contrasting results suggest that the isoflavone concentrations and profiles in *cheonggukjang* vary depending on the thermal processing, soybean cultivar, fermentation period, and microorganism starter.

Change of Antioxidant Activities During *Cheonggukjang* Fermentation

The CFWG exhibited stronger antioxidant activities than the CFWOG during the fermentation periods of 0, 48, and 72 h, but the CFWG showed lower antioxidant activities than CFWOG during the fermentation periods of 12 and 24 h (Fig. 4). To examine the hydrogen-donating activity, we performed a DPPH radical scavenging assay. In the CFWOG, the DPPH radical activities of fermented soybean at 37°C for 0, 12, 24, 48, and 72 h were 32.02%, 37.87%, 49.95%, 63.26%, and 62.44%, respectively. In the case of the CFWG, the levels of DPPH radical activity of fermented soybeans at 37°C for 0, 12, 24, 48, and 72 h were 36.56%, 34.82%, 46.59%, 71.55%, and 82.29%, respectively (Fig. 4A).

To determine the hydrogen-donating antioxidants and chain-breaking antioxidants, we measured the ABTS radical scavenging ability of CFWOG and CFWG. The

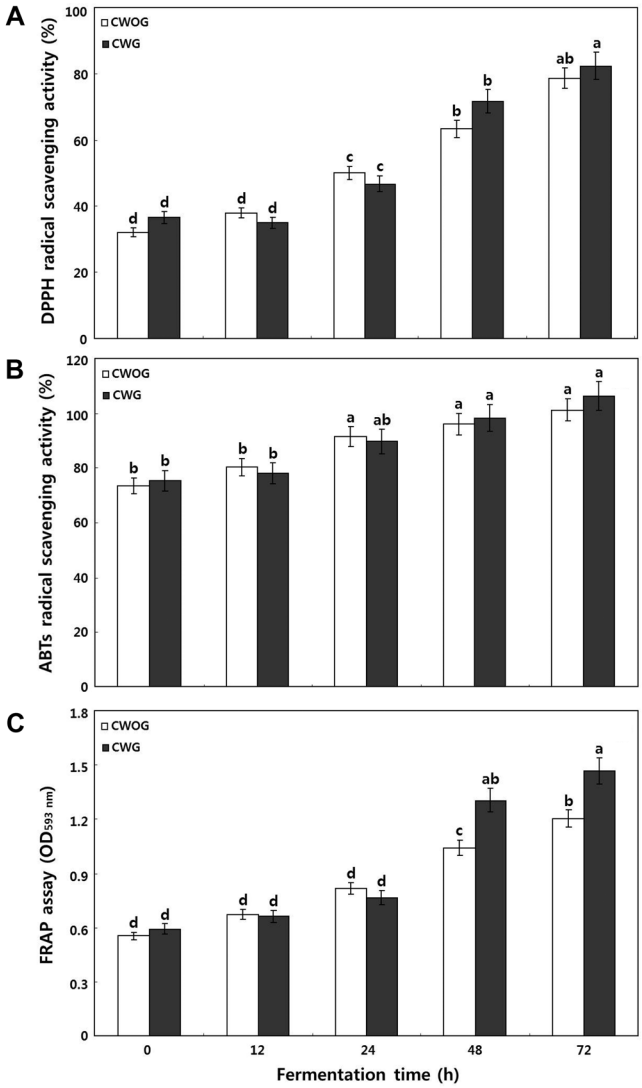

Fig. 4. Change of antioxidant activities during *cheonggukjang* fermentation by *Bacillus amyloliquefaciens* MJ1-4 starter.

(**A**) DPPH radical scavenging activity, (**B**) ABTs radical scavenging activity, and (**C**) FRAP assay. Data are presented as the mean ± SD ($n = 3$) of triplicate determinations. Means with different lowercase letters (a, b, and c) indicate significant differences of fermentation times by Tukey's multiple range test ($p < 0.05$).

levels of ABTS radical activity in CFWOG increased greatly from 73.41% at 0 h of fermentation to 101.11% at 72 h of fermentation. Additionally, the ABTS radical activities in CFWG increased greatly from 75.28% at 0 h of fermentation to 106.32% at 72 h of fermentation (Fig. 4B).

The FRAP assay is a direct test of the total antioxidant power. In the case of CFWOG, the values resulting from the FRAP assay of the fermented soybeans at 37°C for 0, 12, 24, 48, and 72 h increased by 0.55, 0.67, 0.82, 1.04, and 1.20, respectively. During CFWG, the values resulting from FRAP assay of fermented soybeans at 37°C for 0, 12, 24, 48, and 72 h increased by 0.59, 0.66, 0.76, 1.31, and 1.47 corresponding to increases of 15.59%, 23.33%, 29.77%, and 31.85%, respectively (Fig. 4C).

The total phenolic contents were measured as an overall indicator of the contents of these molecules with antioxidant properties [30]. Shon *et al.* [28] reported that a methanol extract of *cheonggukjang* exhibited a radical scavenging activity of 69–87% and total phenolic contents of 0.13–0.27 mg/g. In contrast, Kao and Chen [9] reported that the DPPH radical scavenging activity was not affected by the various isoflavone standards, and mixtures of two and four isoflavone standards were more effective in scavenging DPPH free radicals (yielding 5.6% and 10.5% scavenging activity, respectively). Similarly, many isoflavones were reported to have low scavenging potency for DPPH free radicals, with scavenging effects only half that of α-tocopherol and one-third that of epicatechin [16]. However, isoflavones have direct free radical quenching ability, with daidzein and genistein being particularly effective [1, 2, 28]. In addition, Kim *et al.* [14] reported that the *cheonggukjang* extract and its constituents, genistein and daidzein, exhibited significant antioxidant activity *in vitro*. We recently reported that the radical scavenging activity increased from 53.6% to 93.9% depending on the total phenolic and isoflavone-aglycone (daidzein) contents during *cheonggukjang* fermentation by the potential probiotic *B. subtilis* CS90 [2]. In particular, Kwak *et al.* [15] suggested that the stronger antioxidant activity of *cheonggukjnag* might be related to the markedly higher total phenolic contents and isoflavone-aglycones and -malonylglycosides achieved during fermentation. In addition, phenolic acids, such as gallic acid and its derivatives, are also reported to have antioxidant and antimutagenic activities, and daily intake is proposed to bring various health benefits, including reducing the risk of disease [9].

In conclusion, this study has documented for the first time that changes occurred in the total phenolic contents and in the contents of 12 isoflavones during *cheonggukjang* fermentation by *B. amyloliquefaciens* MJ1-4 with and without garlic. Among these changes, the total phenolic and isoflavone-aglycone contents were markedly increased, while the isoflavone-glycosides were decreased according to the β-glucosidase activities. The total phenolic and total isoflavone contents and antioxidant activities were higher in CFWOG than in CFWG at 24 h of fermentation, but they were lower in the CFWOG at 72 h of fermentation. These results suggest that the high antioxidant activity of *cheonggukjang*

fermented with garlic might be related to the markedly higher total phenolic and isoflavone-aglycone contents achieved during fermentation.

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