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ARTICLE

Bioconversion of γ-aminobutyric acid and isoflavone contents during the fermentation of high-protein soy powder yogurt with *Lactobacillus brevis*

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Abstract This study evaluated the changes in γ-aminobutyric acid (GABA) and isoflavone aglycone contents from soy powder yogurt (SPY) due to sprouting of soybean (1 cm) and fermentation with *Lactobacillus brevis*. The levels of GABA and the aglycone form increased, and the glutamate decarboxylase and β-glucosidase activities increased; however, the isoflavone glycoside and malonylglycoside contents decreased after fermentation for 72 h. In particular, after 60 h, the SPY presented the highest GABA content (120.38 mg/100 mL). The highest daidzein (179.93 μg/g), glycitein (44.10 μg/g), and genistein (126.24 μg/g) contents were present after 72 h of fermentation. In addition, the 2,2-diphenyl-1-picrylhydrazyl, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and hydroxyl radical scavenging activities increased from 69.65, 97.94, and 70.90% during this fermentation, respectively. This result suggests that SPY may be used for the preparation of high-protein soybean with high GABA and isoflavone aglycone contents, which can then be used as a natural ingredient of functional foods.

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Introduction

As a nutritious plant food material, soybean (*Glycine max* L.) is widely used in the Asia area. Soybean contains approximately 40% protein, 20% oil, and 30% carbohydrate. The amino acids of soybean not only have a high nutritional value, but also provide several human health benefits [1, 2]. Isoflavones are present in four chemicals, namely glycosides (25%), malonylglycosides (70–80%), acetylglycosides (5%), and aglycones (2%), in raw soybeans [3]. The chemical structures of the isoflavones and metabolites influence the extent of absorption of aglycone derivatives, which are more readily absorbed and bioavailable than highly polar conjugated glycoside derivatives [4]. In particular, soybean has been germinated for human consumption because germination can decrease the content of anti-nutritional factors while increasing the amount of nutrients and phytochemicals such as vitamin E and isoflavone aglycone derivatives [5, 6]. Additionally, *Saedanbaek* of the soybean cultivar contains approximately 48% protein, including 25–30% glutamic acid (GA) [3].

The γ-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is produced primarily by L-glutamate decarboxylase and pyridoxal 5′-phosphate to succinate semialdehyde using enzymes with GABA transaminase activity [7, 8]. GABA has beneficiary functions in animal physiology, such as neurotransmission, hypertension, and decreasing blood pressure secretion, making GABA attractive as an active material in functional

foods [9]. Although GABA is often widely found in many plants and microorganisms, its contents are very low [7]. However, it was previously reported that GABA can be increased by soaking, germination of the soybean [6, 10]. In particular, several microorganisms generally recognized as safe including lactic acid bacteria (LAB) such as *Lactobacillus brevis* [11–15], *Lactobacillus paracasei* [16], and *Enterococcus raffinosus* [17] have been widely studied and applied in GABA production over the last few years. Interestingly, GA is used to produce GABA, which is contained in most soybeans. Therefore, GABA production via fermentation and germination is believed to be convenient and efficient and has been applied in food technology. The health effects of soybean-based foods are due to the numerous functional ingredients in soybean, especially isoflavones [4].

The main purpose of the present research was to investigate the changes in the functional factors, including GABA and isoflavone aglycone derivatives, during sprouting of high-protein soy powder yogurt (SPY) upon fermentation with *L. brevis*. In addition, changes in the β-glycosidase and glutamate decarboxylase activities and the antioxidant activity of soy powder milk (SPM) during fermentation were evaluated.

Materials and methods

Soybean, medium, and chemicals

The high-protein soybean (HPS) cultivar, "*Saedanbaek*," was provided by the National Institute of Crop Science (Miryang, Korea) in 2014. *Lactobacillus brevis* KCTC 3320 was collected from a Korean culture-type collection. Three glycosides and aglycones, including daidzin, glycitin, genistin, daidzein, glycitein, and genistein, were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Three malonyl- and acetylglycosides (malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin) were obtained from LC Laboratories (Woburn, MA, USA). GA, GABA, bromocresol green, pyridoxal-5-phosphate (PLP), glacial acetic acid, 2 N Folin–Ciocalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, *p*-nitrophenol-β-D-glucopyranoside (*p*-NPG), *p*-nitrophenol-β-butyric acid (*p*-NPB), and *p*-nitrophenol were obtained from the Sigma-Aldrich Chemical Co. HPLC-grade H_2O, methanol, and acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ, USA). All other reagents were of analytical grade.

Preparation of HPS sprouts

The sprouted HPS was following partially modified methods of Huang et al. [1]. Briefly, whole HPSs were washed and soaked in water at room temperature for 12 h. After soaking, the soybeans were put into a semi-automatic sprouting machine (model HANCELL, Gwangmyeong, Korea). The HPSs were automatically watered every hour for 8 h and were sprouted for 5 days (0, 1, 2, and 4 cm) in an incubator at 20 °C. The sprouted HPS samples were steamed for 30 min at 121 °C. The steamed HPSs were harvested and stored in a deep freezer at − 70 °C until further analysis.

Preparation and the fermentation of soy powder yogurt

The different processing conditions of HPSs, namely fresh, steamed and sprouting of steamed HPSs, were dried at 55 ± 2 °C for 3 days after and were crushed for the production of soy powder. The 10 g of soy powder was mixed with 100 mL of 2% sucrose solution in different containers. This mixture, namely soy powder milk (SPM, unfermented high-protein soybean sprouts), was then sterilized in an autoclave at 121 °C for 15 min. After the seed culture contained approximately 8.0 log cfu/mL with *L. brevis*, the SPM was fermented at 30 ± 1 °C for 72 h (soy powder yogurt, SPY, fermented high-protein soybean sprouts), and sampling was carried out at 0, 12, 24, 36, 48, 60, and 72 h. The SPM and SPY samples were stored at − 70 °C until analysis.

pH, titratable acidity (TA), and viable cell numbers

The pH values of the SPM and SPY samples were measured using a pH meter (MP 200, London, UK), whereas the titratable acidity (TA) was determined by titration with 0.01 M NaOH and expressed as lactic acid (%) according to the methods previously described by Hwang et al. [18]. To measure the viable cell numbers, 1 mL of each sample was dissolved in 9 mL of sterilized distilled water at room temperature and the diluted suspension was spread on MRS agar plates. The plate was incubated at 30 °C for 48 h, after which colony counts were conducted.

Glutamate decarboxylase assay

The enzyme assays for the glutamate decarboxylase activity were spectrophotometrically analyzed according to the method of Yu et al. [19]. The standard reaction medium in each well consisted of 200 μL of acetate buffer (20 mM, pH 5.5) containing 50 μmol of bromocresol green, 10 mM of PLP, 10 μL of 1% glutamic acid, and crude enzyme extract (2.5 unit). After reaction at 48 °C for 30 min, the

absorbance of the mixture was then determined at 620 nm (Spectronic 2D, Thermo Co., Petaluma, CL, USA).

β-Glucosidase assay

The enzyme assay for the β-glycosidase activity was spectrophotometrically analyzed according to the method of Cho et al. [20]. The extract (250 μL) was added to 250 μL of the substrate (5 mM p-NPG or 5 mM p-NPB) 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 mL of 0.2 M glycine–NaOH (pH 10.5) and the contents were immediately measured in a spectrophotometer (Spectronic 2D) at 405 nm.

Free amino acid (FAA) contents

Free amino acids (FAAs) were analyzed according to the method described by Kim and Ji [11]. One milliliter of sample was added to 4 mL of distilled water, and then a heating block (HB-48P, DAIHAN Scientific, Seoul, Korea) was used to drive hydrolysis at 60 °C for 1 h. After 1 mL of 5-sulfosalicylic acid (10%) was added, the mixture was vortexed for 1 min and maintained at 4 °C for 2 h. After centrifuging at 3000 rpm for 3 min, the supernatant was collected and syringe-filtered using a rotary vacuum evaporator at 60 °C. The lithium buffer (pH 2.2) was dissolved by applying membrane filtration. The free amino acids content was determined using an amino acid analyzer (Hitachi L-8900, Tokyo, Japan).

Production of extracts from SPM and SPY

The SPM and SPY samples were freeze-dried into a powder (1.0 g) and extracted with 10 mL of 50% methanol (MeOH) by shaking (280 rpm) at 25 °C for 12 h and filtered through Whatman No. 42 filter paper. The extract solution was dissolved in 10 mL of 50% MeOH and filtered through a 0.45-μm Minipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany).

Analysis of total phenolic contents

The 0.5 mL of 50% MeOH extract was mixed with 0.5 mL of a 25% sodium carbonate (Na_2CO_3) solution and 0.25 mL of Folin–Ciocalteu reagent in a test tube and was kept at 30 °C for 1 h. The absorbance of the mixtures was determined at 750 nm (Spectronic 2D). A gallic acid equivalent standard curve was prepared according to the method of Lee et al. [3].

Analysis of isoflavone contents

The quantification of the isoflavone in the 50% MeOH extracts was carried out by a high-performance liquid chromatography (HPLC, Agilent Co., Santa Clara, CL, USA) equipped with a diode array detector. The extracts were separated on a 100 RP C_{18} column (4.6 × 250 mm, 5.0 μm, Merck, Germany) at 30 °C. The injection volume was 20 μL for all extracts, and the flow rate was 1.0 mL/min. The following binary mobile phase consisting of (A) 0.2% acetic acid in water and (B) 0.2% acetic acid in acetonitrile was used for the separation of isoflavone: 0 min, 100% A; 15 min, 90% A; 25 min, 80% A; 35 min, 75% A; 45 min, 65% A; 50 min, 65%. All isoflavone peaks were detected and monitored at 254 nm [2].

DPPH radical scavenging activity

The DPPH radical scavenging activity of the fermented 50% MeOH extracts was performed according to the method described by Hwang et al. [18]. Specifically, DPPH solution (1.5×10^{-4} mM, 0.8 mL) was mixed with the SPM and SPY extracts. After standing at room temperature for 30 min, the absorbance of the mixture was determined at 525 nm using a spectrophotometer (Spectronic 2D).

ABTS radical scavenging activity

The ABTS radical scavenging activity was following the modified methods of Hwang et al. [18]. The ABTS stock solution diluted 50 times with SPY extract (0.1 mL) was added to 0.9 mL of $ABTS^{\bullet+}$ solution. After being kept in the dark at room temperature for 3 min, the absorbance was determined at 730 nm using spectrophotometry (Spectronic 2D).

Hydroxyl radical scavenging activity

The hydroxyl (·OH) radical scavenging capacity was performed using 50% methanol extracts, as recently described by Herraiz and Galisteo [16]. All of the reagents, such as 10 mM $FeSO_4 \cdot 7H_2O$-EDTA, 10 mM 2-deoxyribose, 2.8% trichloroacetic acid, and 1% thiobarbituric acid, were dissolved in KH_2PO_4-KOH buffer (10 mM, pH 7.4), and the reaction was initiated upon the addition of H_2O_2 (10 mM). After incubation at 37 °C for 1 h, the reaction was stopped by adding 0.7 mL of 2.8% trichloroacetic acid and 0.7 mL of 1% barbituric acid. The mixture was heated in a water bath at 100 °C for 10 min and then cooled in water at room temperature. The absorbance of the resulting solution was measured at 532 nm (Spectronic 2D).

Table 1 Comparison of GA and GABA, total phenolic, and isoflavone glycoside and aglycone contents on the SPM and SPY for different sprouting conditions and fermentation of high-protein soybean with *L. brevis*

Indexes[a]	Sprouting length (cm)							
	0		1		2		4	
	SPM[b]	SPY[c]	SPM	SPY	SPM	SPY	SPM	SPY
GA and GABA contents (mg/100 mL)								
Glutamic acid	23.35 ± 1.17^{e}	13.19 ± 0.66^{f}	100.31 ± 5.02^{a}	45.09 ± 2.25^{d}	92.66 ± 4.63^{b}	17.33 ± 0.87^{f}	68.45 ± 3.42^{c}	21.88 ± 1.09^{e}
γ-Aminobutyric acid	17.85 ± 0.89^{g}	32.46 ± 1.62^{e}	29.31 ± 1.47^{f}	101.60 ± 5.08^{a}	50.46 ± 2.52^{d}	77.32 ± 3.87^{c}	36.92 ± 1.85^{e}	85.94 ± 4.30^{b}
Total phenolic acid contents (mg/g d.w.)	1.42 ± 0.07^{a}	1.63 ± 0.08^{a}	1.76 ± 0.10^{a}	2.92 ± 0.16^{a}	1.44 ± 0.07^{a}	2.30 ± 0.12^{a}	1.58 ± 0.08^{a}	2.54 ± 0.13^{a}
Isoflavone glycoside contents (μg/g d.w.)								
Daidzin	364.70 ± 15.59^{c}	107.24 ± 5.36^{e}	341.82 ± 17.09^{d}	22.51 ± 1.13^{f}	410.77 ± 20.54^{b}	20.57 ± 1.03^{f}	468.46 ± 23.42^{a}	24.19 ± 1.21^{f}
Glycitin	151.88 ± 6.08^{b}	138.81 ± 6.94^{c}	179.76 ± 8.99^{a}	28.50 ± 1.43^{e}	179.05 ± 8.95^{a}	17.03 ± 0.85^{f}	122.67 ± 6.13^{b}	25.27 ± 1.26^{e}
Genistin	397.89 ± 15.92^{c}	33.76 ± 1.69^{d}	388.07 ± 19.40^{c}	2.35 ± 0.12^{f}	409.08 ± 20.45^{b}	1.56 ± 0.03^{f}	471.52 ± 23.58^{a}	4.49 ± 0.22^{e}
Isoflavone aglycone contents (μg/g d.w.)								
Daidzein	8.99 ± 0.45^{g}	138.55 ± 6.93^{d}	26.05 ± 1.30^{f}	186.08 ± 9.30^{c}	59.71 ± 2.99^{e}	243.78 ± 12.19^{b}	53.07 ± 2.65^{e}	277.71 ± 13.89^{a}
Glycitein	38.25 ± 1.91^{d}	40.05 ± 1.62^{c}	12.24 ± 0.61^{e}	46.01 ± 2.30^{b}	19.37 ± 0.97^{e}	62.00 ± 3.10^{a}	16.24 ± 0.81^{e}	48.34 ± 2.42^{b}
Genistein	6.83 ± 0.34^{e}	120.55 ± 6.03^{c}	10.00 ± 0.50^{d}	129.52 ± 6.48^{b}	16.04 ± 0.80^{d}	139.27 ± 6.96^{b}	11.49 ± 0.57^{d}	165.20 ± 8.26^{a}

[a]All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

[b]*SPM* soy powder milk (unfermented high-protein soybean sprout)

[c]*SPY* soy powder yogurt (fermented high-protein soybean sprout)

Statistical analysis

All experimental values are presented as the mean ± SD of triplicate determination. Differences in the means of each value were confirmed by one-way ANOVA followed by the Tukey's multiple range tests at $p < 0.05$ using the Statistical Analysis System.

Results and discussion

Confirmation of optimum sprouting conditions

A comparison of the GA, GABA, total phenolic, and isoflavone aglycone contents in the SPM and SPY samples is shown in Table 1. As shown, the GA contents decreased from 100.31 to 45.09 mg/100 mL and the corresponding GABA contents increased to a maximum of 101.60 mg/100 mL at 72 h upon the fermentation of one sprouting soybean (SPY-1 cm) (Table 1). In particular, the highest concentration of GA in the SPY-1 was responsible for the highest concentration of GABA in the SPY-1. Also, the levels of isoflavone glycoside (genistin, glycitin, and daidzin) decreased, while the total phenolic and isoflavone aglycone (genistein, glycitein, and daidzein) contents increased throughout fermentation for 72 h; however, the glycitein content slightly increased (Table 1).

Liao et al. [21] reported that the increasing commercial demand for GABA has led to diverse foods containing both biologically and chemically produced GABA. For example, GA- and GABA-enriched green tea and the germination of unpolished rice are produced by an anaerobic treatment, and in rice germ, it is produced by soaking in water via a high-pressure treatment. Upon germination in brown rice, in tempeh-like fermented soybean and in black raspberry juice, GABA enrichment is achieved by fermentation by *L. brevis* [11, 21, 22]. The highest concentration of GA in the SPM-1 was responsible for the highest concentration of GABA in the SPY-1. Additionally, Koo et al. [12] recently reported that the total phenolic contents (TPCs) were higher in soybean sprouts than in soybean seeds, corresponding to the higher antioxidant activity that appeared. Several researchers previously reported that the total phenolic and isoflavone aglycone contents were enhanced during the lactic acid fermentation of soymilk by *Lactobacillus plantarum* P1201 [18].

Change in pH, acidity, and enzyme activities during the fermentation of SPY-1

Changes in the pH, acidity, and glutamate decarboxylase and β-glucosidase activities in during the fermentation SPY-1 with *L. brevis* are shown in Table 2. As shown, the pH decreased during fermentation from 6.82 to 5.03 after 72 h, whereas the acidity increased 0.07–0.54% after 72 h of fermentation. The viable cell numbers gradually increased from 12 h (9.47 cfu/mL) to 36 h (13.39 cfu/mL), whereas they negligibly decreased from 48 h (12.48 cfu/mL) to 72 h (11.47 cfu/mL) of fermentation. The glutamate decarboxylase activity was the highest (4.87 unit/mL and 36 h), whereas it slightly decreased during fermentation times of 48 h (4.26 unit/mL), 60 h (3.95 unit/mL), and 70 h (3.85 unit/mL). The β-glucosidase activity rapidly increased during the first 12 h of SPY-1 fermentation, reaching 1.78 unit/mL of SPY-1. Then, it increased gradually with a longer fermentation time until 48 h (2.76 unit/mL) and thereafter slightly decreased (Table 2).

Table 2 Changes in the pH, acidity, viable cell numbers, and *enzyme activity during* the fermentation of SPY-1 with *L. brevis*

Fermentation time (h)	Contents[a]				
	pH	Acidity (%, as lactic acid)	Viable cell numbers (log cfu/mL)	Glutamate decarboxylase activity (unit/mL)	β-glucosidase activity (unit/mL)
0	6.82 ± 0.41^{a}	0.07 ± 0.00^{b}	7.06 ± 0.60^{b}	0.14 ± 0.00^{d}	0.12 ± 0.00^{c}
12	5.59 ± 0.34^{b}	0.40 ± 0.02^{a}	9.47 ± 0.63^{b}	0.88 ± 0.03^{d}	1.78 ± 0.02^{b}
24	5.39 ± 0.32^{b}	0.43 ± 0.03^{a}	10.68 ± 0.64^{a}	2.94 ± 0.04^{c}	2.04 ± 0.03^{a}
36	5.37 ± 0.32^{b}	0.44 ± 0.03^{a}	13.39 ± 0.80^{a}	4.87 ± 0.06^{a}	2.37 ± 0.03^{a}
48	5.26 ± 0.32^{b}	0.47 ± 0.03^{a}	12.48 ± 0.75^{a}	4.26 ± 0.05^{a}	2.76 ± 0.05^{a}
60	5.18 ± 0.31^{b}	0.50 ± 0.03^{a}	11.52 ± 0.69^{a}	3.95 ± 0.04^{b}	2.72 ± 0.04^{a}
72	5.03 ± 0.30^{b}	0.54 ± 0.03^{a}	11.47 ± 0.69^{a}	3.85 ± 0.04^{b}	2.67 ± 0.05^{a}

[a]All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

Table 3 Changes in the free amino acid contents during the fermentation of SPY-1 with *L. brevis*

Contents[a] (mg/100 mL)	Fermentation time (h)						
	0	12	24	36	48	60	72
Non-essential amino acids							
Phosphoethanolamine	28.67 ± 1.43[a]	16.35 ± 0.82[b]	16.23 ± 0.81[b]	15.98 ± 0.80[b]	14.25 ± 0.71[b]	18.59 ± 0.93[b]	10.39 ± 0.52[c]
Urea	115.37 ± 5.77[a]	20.73 ± 1.04[b]	18.81 ± 0.94[b]	21.14 ± 1.06[b]	17.64 ± 0.88[b]	23.84 ± 1.19[b]	11.61 ± 0.58[c]
Proline	17.70 ± 0.89[c]	30.29 ± 1.51[b]	35.56 ± 1.78[b]	35.78 ± 1.79[b]	32.03 ± 1.60[b]	43.83 ± 2.19[a]	21.89 ± 1.09[c]
Aspartic acid	47.82 ± 2.39[a]	36.92 ± 1.85[c]	41.21 ± 2.06[b]	40.96 ± 2.05[b]	36.84 ± 1.84[c]	45.55 ± 2.2[b]	21.03 ± 1.05[d]
Serine	17.65 ± 0.88[a]	4.11 ± 0.21[b]	3.79 ± 0.19[b]	3.55 ± 0.18[b]	3.03 ± 0.15[b]	4.48 ± 0.22[b]	2.39 ± 0.12[b]
Glutamic acid	113.59 ± 5.68[a]	43.75 ± 2.19[b]	36.86 ± 1.84[c]	34.12 ± 1.71[c]	31.24 ± 1.56[c]	30.37 ± 2.02[c]	21.08 ± 1.05[d]
Aminoadipic acid	17.18 ± 0.86[c]	34.16 ± 1.71[b]	39.01 ± 1.95[b]	40.71 ± 2.04[b]	37.17 ± 1.86[b]	51.50 ± 2.58[a]	33.54 ± 1.68[b]
Glycine	7.58 ± 0.38[b]	14.16 ± 0.71[a]	15.71 ± 0.79[a]	16.72 ± 0.84[a]	14.99 ± 0.75[a]	20.73 ± 1.04[a]	11.97 ± 0.60[b]
Alanine	59.90 ± 3.00[a]	43.78 ± 2.19[b]	41.32 ± 2.07[b]	40.95 ± 2.05[b]	39.70 ± 1.99[b]	48.87 ± 2.44[b]	24.07 ± 1.20[c]
Citrulline	47.46 ± 2.37[a]	10.00 ± 0.50[b]	8.60 ± 0.43[b]	8.79 ± 0.44[b]	7.45 ± 0.37[b]	9.72 ± 0.49[b]	5.70 ± 0.29[b]
α-Aminobutyric acid	1.35 ± 0.07[b]	1.25 ± 0.06[b]	1.13 ± 0.06[b]	1.22 ± 0.06[b]	37.17 ± 1.86[a]	1.34 ± 0.07[b]	0.83 ± 0.04[b]
Cystine	38.96 ± 1.95[a]	34.95 ± 1.75[a]	5.34 ± 0.27[c]	37.61 ± 1.88[a]	4.65 ± 0.23[c]	9.09 ± 0.45[b]	4.44 ± 0.22[c]
Cystathionine	nd[b]	nd	nd	8.05 ± 0.40[a]	nd	9.53 ± 0.48[a]	nd
Tyrosine	18.94 ± 0.95[a]	11.63 ± 0.58[b]	5.78 ± 0.29[c]	10.22 ± 0.51[b]	2.46 ± 0.12[d]	11.64 ± 0.58[b]	1.43 ± 0.07[d]
β-Alanine	22.89 ± 1.14[b]	36.08 ± 1.80[a]	22.16 ± 1.11[b]	27.08 ± 1.35[b]	21.47 ± 1.07[b]	30.59 ± 1.53[a]	16.52 ± 0.83[c]
β-Aminoisobutyric acid	12.05 ± 0.60[b]	17.08 ± 0.85[b]	19.67 ± 0.98[b]	31.20 ± 1.56[a]	19.42 ± 0.97[b]	35.87 ± 1.79[a]	15.58 ± 0.78[b]
γ-Aminobutyric acid	31.86 ± 1.59[f]	79.44 ± 3.97[d]	89.66 ± 4.48[c]	99.93 ± 5.00[b]	113.16 ± 4.16[a]	120.38 ± 6.02[a]	57.51 ± 2.88[e]
Hydroxyproline	2.36 ± 0.12[a]	nd	nd	nd	nd	nd	nd
Ornithine	38.72 ± 1.94[d]	205.29 ± 10.26[a]	211.91 ± 10.60[a]	211.90 ± 10.60[a]	192.34 ± 9.62[b]	242.50 ± 12.13[a]	117.12 ± 5.86[c]
1-Methylhistidine	nd	28.76 ± 1.44[a]	nd	nd	nd	nd	nd
Arginine	213.56 ± 10.68[a]	nd	nd	nd	nd	nd	nd
Total	853.56 ± 42.68[a]	668.71 ± 33.44[d]	612.71 ± 30.64[e]	685.87 ± 34.29[c]	594.97 ± 29.75[f]	768.38 ± 38.42[b]	377.07 ± 18.85 [g]
Essential amino acids							
Threonine	10.57 ± 0.53[a]	1.57 ± 0.08[b]	1.86 ± 0.09[b]	1.64 ± 0.08[b]	1.51 ± 0.08[b]	2.02 ± 0.10[b]	0.64 ± 0.03[c]
Valine	23.04 ± 1.15[b]	23.29 ± 1.16[b]	23.53 ± 1.18[b]	24.06 ± 1.20[b]	21.89 ± 1.09[b]	29.48 ± 1.47[a]	16.34 ± 0.82[c]
Methionine	8.38 ± 0.42[b]	6.91 ± 0.35[b]	6.19 ± 0.31[b]	11.80 ± 0.59[a]	5.48 ± 0.27[b]	13.31 ± 0.67[a]	3.85 ± 0.19[b]
Isoleucine	16.48 ± 0.82[b]	16.50 ± 0.83[b]	17.41 ± 0.87[b]	27.27 ± 1.36[a]	16.75 ± 0.84[b]	32.85 ± 1.64[a]	12.42 ± 0.62[b]
Leucine	24.44 ± 1.22[d]	36.42 ± 1.82[c]	44.04 ± 2.20[c]	53.62 ± 2.68[b]	43.72 ± 2.19[c]	67.22 ± 3.36[a]	31.43 ± 1.57[d]
Phenylalanine	47.99 ± 2.40[d]	66.61 ± 3.33[c]	68.62 ± 3.43[c]	82.81 ± 4.14[b]	64.63 ± 3.23[c]	99.63 ± 4.98[a]	43.94 ± 2.20[d]
Lysine	37.52 ± 1.88[a]	32.29 ± 1.61[a]	34.03 ± 1.70[a]	33.02 ± 1.65[a]	30.18 ± 1.51[a]	39.69 ± 1.98[a]	19.78 ± 0.99[b]
Histidine	27.80 ± 1.39[a]	28.76 ± 1.44[a]	29.91 ± 1.50[a]	30.29 ± 1.51[a]	27.66 ± 1.38[a]	35.68 ± 1.78[a]	18.68 ± 0.93[b]

Table 3 continued

Contents[a] (mg/100 mL)	Fermentation time (h)						
	0	12	24	36	48	60	72
Total	196.21 ± 9.81[d]	212.33 ± 10.62[c]	225.57 ± 11.28[c]	264.50 ± 13.23[b]	211.80 ± 10.59[c]	319.86 ± 15.99[a]	147.06 ± 7.35[e]
Total amino acids	1049.77 ± 52.49[a]	881.04 ± 44.05[c]	838.28 ± 41.91[c]	950.37 ± 47.52[b]	806.77 ± 40.34[c]	1088.24 ± 54.41[a]	524.12 ± 26.21[d]
Ammonia	40.82 ± 2.04[c]	50.97 ± 2.55[b]	54.72 ± 2.74[b]	54.54 ± 2.73[b]	49.94 ± 2.50[c]	63.81 ± 3.19[a]	34.08 ± 1.70[c]

[a]All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

[b]*nd* not detected

General yogurts were reported to have a pH range of 4.2–4.4 [23]; however, the pH value of produced SPY-1 in this study was 5.03 at 72 h after fermentation. Many papers have reported that there is a significant positive correlation between the GABA yield and GAD activity due to *L. rhamnosus* [21] and *L. brevis* [22] during germination and fermentation, which is in agreement with the results in this study. Pyo et al. [13] found that β-glucosidase activity (55.5 unit/g dry weight) of soybean with *L. thermophilum* KFRI 00748 occurred upon 48 h of fermentation. Chung et al. [14] previously reported that GAD activity may be stimulated by an increase in GA, and the increased GA may be an important cause for the increase in GABA. However, GABA is mainly due to the bioconversion of GA, whereas GA is mainly derived from the breakdown of proteins during germination [7], although it may be provided by GABA-T activity [8]. Komatsuzaki et al. [24] reported that during germination, GAD enzymes are activated, and as a result, GA is effectively converted to GABA.

Change in free amino acids (FAAs), GA, and GABA contents during the fermentation of SPY-1

The total FAAs decreased during fermentation from 1049.77 to 524.12 mg/100 mL after 72 h of fermentation (Table 3). Similarly, after fermentation for 72 h, the non-essential amino acid (phosphoethanolamine, urea, aspartic acid, serine, alanine, citrulline, α-aminobutyric acid, cysteine, tyrosine, and β-alanine) contents in the SPYs decreased, whereas the essential amino acid (threonine, valine, methionine, isoleucine, phenylalanine, lysine, and histidine) contents decreased due to the fermentation process. In addition, the essential amino acids, including leucine, slightly increased during fermentation from an initial 24.44 to 31.43 mg/100 mL after 72 h of fermentation. However, the proline, aminoadipic acid, glycine, and ornithine contents slowly increased upon 72 h of fermentation (Table 3). In particular, with an increase in fermentation period, the GA contents slightly decreased: 113.59 mg/100 mL (0 h), 43.75 mg/100 mL (12 h), 36.86 mg/100 mL (24 h), 34.12 mg/100 mL (36 h), 31.24 mg/100 mL (48 h), 30.37 mg/100 mL (60 h), and 21.08 mg/100 mL (72 h). However, the GABA contents gradually increased from 12 h (79.44 mg/100 mL), 24 h (89.66 mg/100 mL), 36 h (99.93 mg/100 mL), 48 h (113.16 mg/100 mL), and 60 h (120.38 mg/100 mL), and they significantly decreased upon 72 h (57.51 mg/100 mL) of fermentation (Table 3 and Fig. 1A). Figure 1B shows the amino acid chromatograms of the GA and GABA peaks obtained, which exhibited significant differences during the different fermentation periods (0, 12, 36, and 60 h).

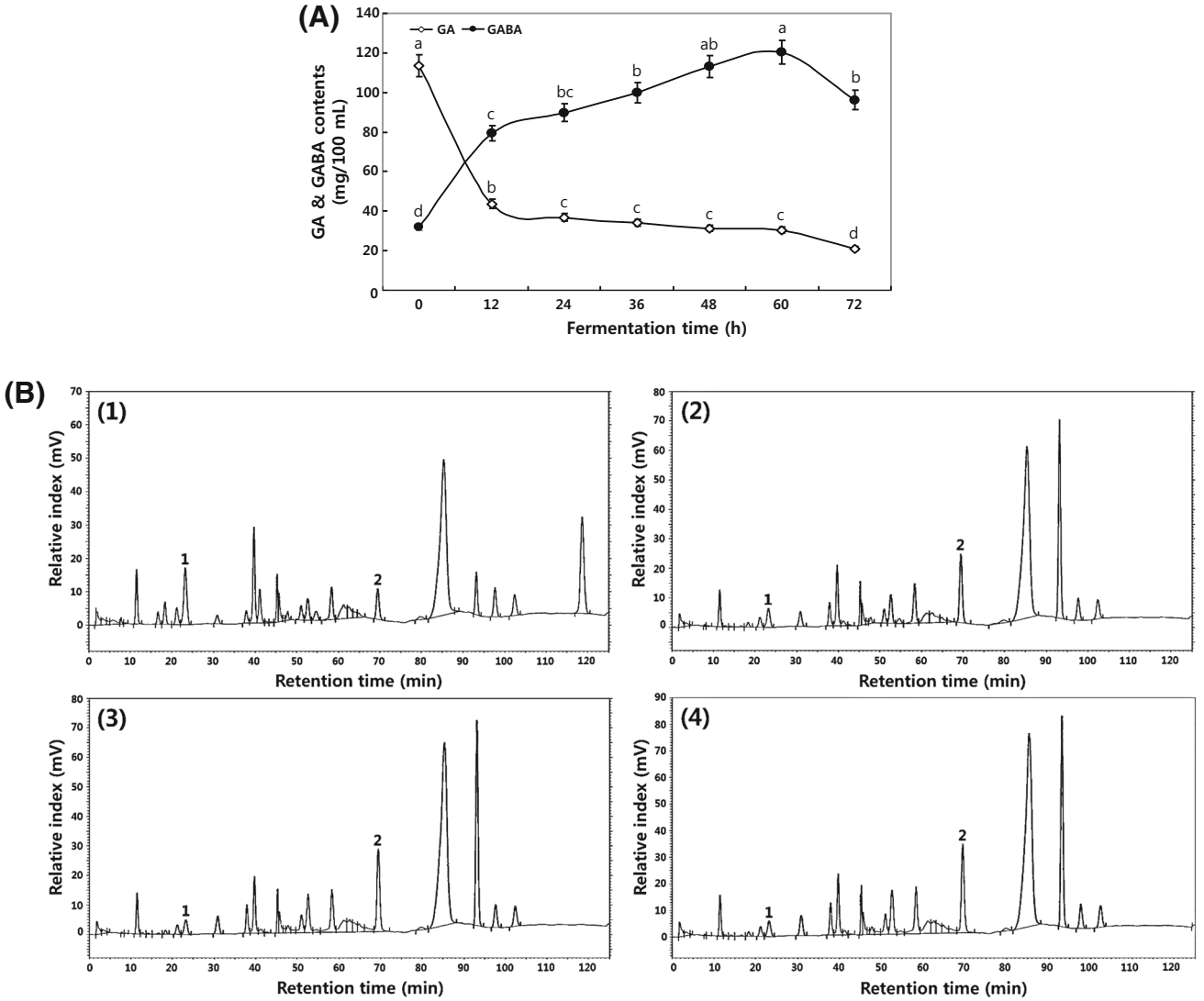

Fig. 1 Change in the glutamic acid (GA) and γ-aminobutyric acid (GABA) contents during the fermentation of SPY-1 with *L. brevis*. (**A**) Graph of GA and GABA profiles and (**B**) typical chromatogram of GA and GABA. B1; fermentation period of 0 h, B2; fermentation period of 12 h, B3; fermentation period of 36 h, and B4; fermentation period of 60 h. 1; GA and 2; GABA. All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

The GA metabolic rate results in GABA creation, and serine, alanine, valine, and leucine have activities as vasodilators, which lower blood pressure and fatigue recovery [25]. It has been reported that microbial fermentation can produce GABA [26]. GAD is required for organisms to produce GABA because it can catalyze the decarboxylase of glutamate to GABA and CO_2. Microbial GAD is the most extensively studied and has been purified and produced from *L. brevis* [25] and *L. paracasei* [27]. Liao et al. [21] reported that the GABA contents were 201.2 and 68.2 mg/100 g upon cold shock and fermentation, respectively, by lactic acid bacteria with adzuki bean. Lee et al. [28] reported that the GA yield was 2789 mg/L before fermentation and significantly decreased during fermentation, and the GABA yield significantly increased in sea tangle during fermentation with *L. brevis* BJ20 after

5 days of fermentation. After fermentation, most of the GA was converted to GABA, and some contents of free amino acids, such as aspartic acid, serine, and threonine, decreased during fermentation, which is in agreement with our results [28].

Changes in the total phenolic and isoflavone contents during the fermentation of SPY-1

The TPCs increased from 3.61 to 5.89 mg/g during the fermentation of SPY-1 with *L. brevis* (Fig. 2). As shown in Fig. 3, the content of isoflavone glycoside and malonyl-glycoside decreased, whereas the isoflavone aglycones (daidzein, glycitein, and genistein) increased. In the case of SPY, the isoflavone aglycone contents increased throughout fermentation to approximately 24.5-fold relative to the starting amounts after 72 h of fermentation (3.33–81.68%). However, the glycoside contents decreased from 87.51 to 8.09% at 72 h (Fig. 3A). Figure 3B shows the HPLC chromatograms of the isoflavone peaks obtained, showing significant differences during the different fermentation periods (0, 12, 36 and 60 h). Importantly, with an increase in fermentation period, the total isoflavone contents slightly decreased upon fermentation from 0 h (898.12 μg/g) to 72 h (428.85 μg/g). In particular, daidzin of the glycoside contents decreased from 305.24 to 22.06 μg/g, and the corresponding daidzein of the aglycone contents increased to 179.93 μg/g at 72 h of fermentation (Table 2).

Germination and fermentation can increase the total phenolic and isoflavone aglycone contents and change the isoflavone compositions of soybean. Fermented processing disintegrates the cell wells and cell membranes and releases soluble phenolic contents from the insoluble ester bonds by acid and estrolytic enzymes [18]. Paucar-Menacho et al. [29] reported that the composition of isoflavone profiles significantly changed after fermentation and germination effects on the soybean cultivars and the process of germination. Ewe et al. [4] reported that biotin-supplemented fermented soymilk had isoflavone aglycone contents that increased from 22.9 to 131.5% due to fermentation. This result enhanced the bioconversion of glycosides to aglycones due to the β-glycosidase activity during fermentation by *L. brevis* in soymilk [4]. In addition, Hwang et al. [18] reported an increase in TPCs and isoflavone aglycones, such as daidzein and genistein, in SPM as the fermentation time increased. Additionally, Lin and Lai [30] reported that the ratio of isoflavone aglycones and total isoflavones improved by germination in black soybeans. Meanwhile, Devi et al. [31] reported that germinated soybean had the highest isoflavone content among soybean products, such as soy milk, soy sauce, soy meals and soy flour and soy seeds. Thus, the bioconversion rates of glycosides to aglycones in SPY are different according to the soybean cultivar and germination conditions (Table 4).

Change in the radical scavenging activities during the fermentation of SPY-1

In this study, the ascorbic acid (a positive control) showed DPPH, ABTS, and hydroxyl radical scavenging activities of 80.22, 98.46, and 78.22%, respectively, at 0.5 or 0.25 mg/mL. Similarly, the SPY-1 exhibited stronger radical scavenging activities upon fermentation periods of 72 h (Fig. 4). The activity of the DPPH radical increased steadily from 48.97% at 0 h of fermentation to 69.65% upon 72 h of fermentation (Fig. 4A). The ABTS radical activities upon fermentation for 0 and 72 h significantly increased by 69.66 and 97.64%, respectively (Fig. 4B). Similarly, the hydroxyl radical scavenging activity increased from 36.22 to 70.90% upon 72 h (Fig. 4C).

The total phenolic, isoflavone, anthocyanin, and tocopherol contents represent important biological activities, such as antioxidant activity, a capillary protective effect, and human health benefits, in various stages of tumors [32]. The increased free radical scavenging activity of fermented soymilk observed in the present study is consistent with previous reports [18, 33]. Chun et al. [23] reported a significant correlation between the antioxidant activity of isoflavone aglycone and phenolic contents in soymilk. Therefore, it is expected that the high antioxidant activity of SPY from high-protein soybean cultivars may be related

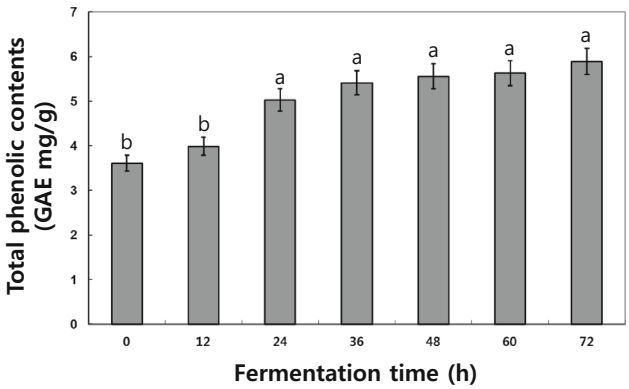

Fig. 2 Change in the total phenolic contents during the fermentation of SPY-1 with *L. brevis*. All values are means from three independent experiments. All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

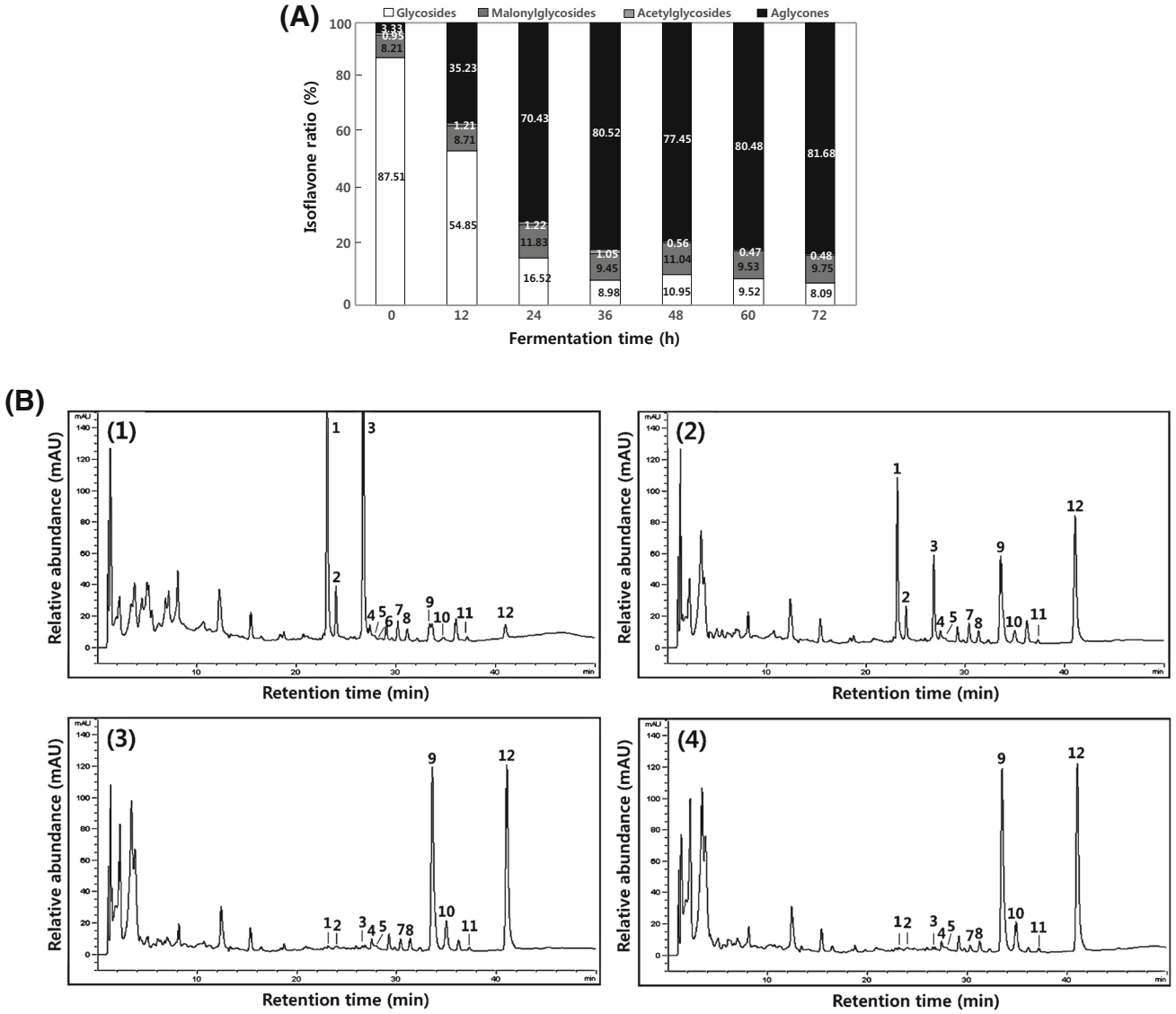

Fig. 3 Change in the isoflavone contents during the fermentation of SPY-1 with *L. brevis.* (**A**) Graph of isoflavone ratio and (**B**) typical chromatogram of isoflavone. B1; fermentation period of 0 h, B2; fermentation period of 12 h, B3; fermentation period of 36 h, and B4; fermentation period of 60 h. 1; daidzin, 2; glycitin, 3; genistin, 4; malonyldaidzin, 5; malonylglycitin, 6; acetyldaidzin, 7; acetylglycitin, 8; malonylgenistin, 9; daidzein, 10; glycitein, 11; acetyl genistein, and 12; genistein

to the significant isoflavone aglycone contents achieved during fermentation.

In conclusion, the contents of GABA, total phenolic, and isoflavone aglycones and radical (DPPH, ABTS, and hydroxyl) scavenging activities were increased, while the isoflavone glycoside and malonylglycoside contents decreased during the SPY due to sprouting of soybean (1 cm) fermentation with *L. brevis*. The level of GABA (120.38 mg/100 mL) was the highest at 60 h. On the other hand, the contents of daidzein (179.93 μg/g), glycitein (44.10 μg/g), and genistein (126.24 μg/g) were the highest after the end fermentation time (72 h), respectively. These results suggest that SPY can be used for the production of HPS with the high GABA, total phenolic, and isoflavone aglycone contents, which can be used as a natural component of functional foods.

Table 4 Changes in the isoflavone contents during the fermentation of SPY-1 with *L. brevis*

Isoflavone contents[a] (μg/g d.w.)	Fermentation time (h)						
	0	12	24	36	48	60	72
Glycosides							
Daidzin	305.24 ± 18.31[a]	160.34 ± 9.62[b]	30.91 ± 1.85[c]	21.04 ± 1.26[c]	21.05 ± 1.26[c]	20.08 ± 1.20[c]	22.06 ± 1.32[c]
Glycitin	189.20 ± 11.35[a]	83.62 ± 5.02[b]	42.95 ± 2.58[c]	17.43 ± 1.05[e]	26.64 ± 1.60[d]	21.20 ± 1.27[d]	12.65 ± 0.76[e]
Genistin	291.59 ± 17.50[a]	66.27 ± 3.98[b]	3.04 ± 0.18[c]	tr	0.69 ± 0.04[d]	tr	tr
Malonylglycosides							
Daidzin	29.72 ± 1.78[a]	14.78 ± 0.89[b]	17.15 ± 1.03[b]	2.30 ± 0.14[c]	14.55 ± 0.87[b]	10.44 ± 0.63[b]	3.24 ± 0.19[c]
Glycitin	17.03 ± 1.02[a]	11.50 ± 0.69[a]	14.21 ± 0.85[a]	15.09 ± 0.91[a]	13.34 ± 0.80[a]	10.54 ± 0.63[a]	15.29 ± 0.92[a]
Genistin	26.95 ± 1.62[a]	22.96 ± 1.38[a]	23.71 ± 1.42[a]	23.07 ± 1.38[a]	20.87 ± 1.25[a]	20.33 ± 1.22[a]	23.27 ± 1.40[a]
Acetylglycosides							
Daidzin	tr[b]	nd[c]	nd	nd	nd	nd	nd
Glycitin	5.66 ± 0.34[a]	4.56 ± 0.27[a]	3.77 ± 0.23[a]	2.99 ± 0.18[a]	1.65 ± 0.10[a]	1.36 ± 0.08[a]	1.38 ± 0.08[a]
Genistin	2.83 ± 0.17[a]	2.28 ± 0.14[a]	1.89 ± 0.11[a]	1.50 ± 0.09[a]	0.83 ± 0.05[b]	0.68 ± 0.04[b]	0.69 ± 0.04[b]
Aglycones							
Daidzein	12.79 ± 0.77[c]	93.69 ± 5.62[b]	171.49 ± 10.29[a]	180.17 ± 10.81[a]	178.07 ± 10.68[a]	181.00 ± 10.86[a]	179.93 ± 10.80[a]
Glycitein	8.53 ± 0.51[c]	20.62 ± 1.24[b]	40.81 ± 2.45[a]	43.88 ± 2.63[a]	43.37 ± 2.60[a]	44.30 ± 2.66[a]	44.10 ± 2.65[a]
Genistrin	8.58 ± 0.51[c]	84.97 ± 5.10[b]	115.66 ± 6.94[a]	120.69 ± 7.24[a]	120.64 ± 7.24[a]	123.62 ± 7.42[a]	126.24 ± 7.57[a]
Total	898.12 ± 44.91[a]	565.59 ± 28.28[b]	465.59 ± 23.28[c]	428.16 ± 21.41[c]	441.70 ± 22.09[c]	433.55 ± 21.68[c]	428.85 ± 21.44[c]

[a]All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

[b]*tr* trace (< 0.002 μg/g)

[c]*nd* not detected

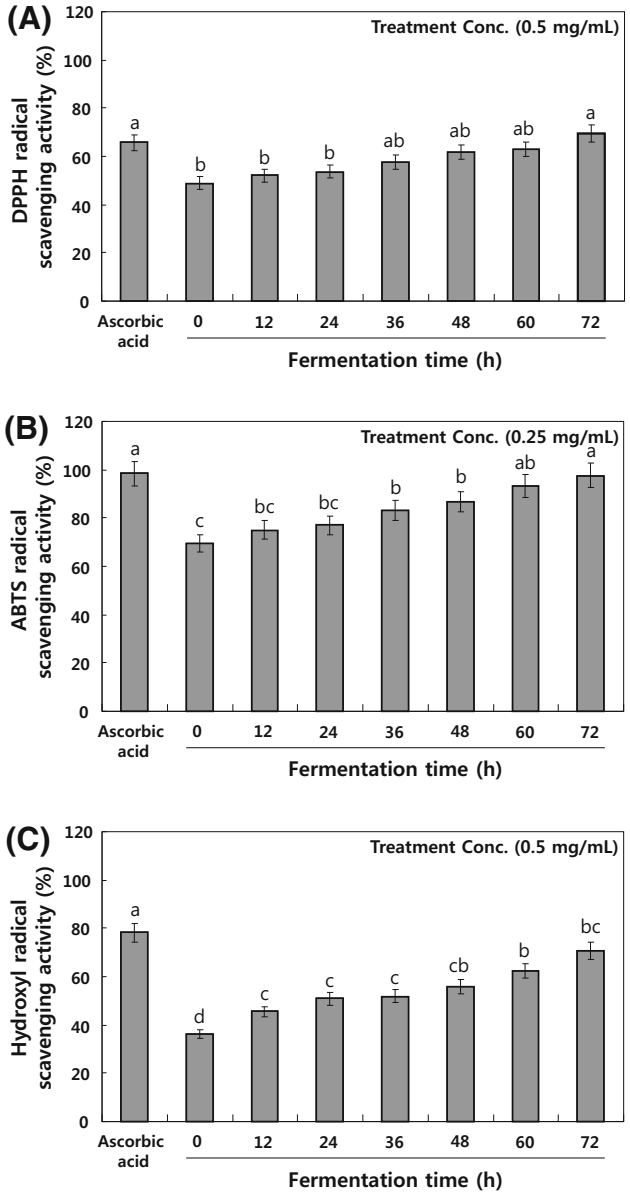

Fig. 4 Change in the antioxidant activity during the fermentation of soy powder yogurt (SPY) with *L. brevis*. (**A**) DPPH, (**B**) ABTS, and (**C**) hydroxyl radical scavenging activity. All values are means from three independent experiments. All values are presented as the mean ± SD of triplicate determination. All values within a column with different superscript letters are significantly different from each other at $p < 0.05$ by Duncan's multiple range tests

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