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Note

Isoflavone aglycone contents of commercial fermented soybean products in Korea and the isoflavone aglycone-producing ability of *Bacillus subtilis* isolated from the products

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The isoflavone aglycone (IA) contents of commercial fermented soybean products in Korea, such as *cheonggukjang* and *doenjang*, and the IA-producing ability of the *Bacillus subtilis* strains isolated from the products were examined. Among three commercial products, product C (*doenjang*), which is produced using a long fermentation method, showed the highest conversion rate of isoflavone glucosides to aglycones. Of all eight *B. subtilis* strains isolated from the products, four strains had the same or higher level of IA production than *B. subtilis* (natto), with the C-3 strain showing the highest level. Hence, the difference in the conversion rate among the products might be attributed not only to fermentation time during the manufacturing process, but also the IA-producing ability of the *B. subtilis* strains. Moreover, the C-3 strain produced IA during the early stage of culture with soybean liquid medium, and the IA-producing ability of the C-3 strain in the fermentation with soybean solid medium was higher than that of *B. subtilis* (natto). Thus, the rapid IA production by the *B. subtilis* C-3 strain might contribute to the high IA content of product C.

Keywords: fermented soybean food, *Bacillus subtilis*, isoflavone, aglycone, β-glucosidase activity

Introduction

Cheonggukjang and *doenjang* are known as traditional fermented soybean foods with some health benefits in Korea (Soh *et al.*, 2008; Shim *et al.*, 2016). Since the production of these foods is traditionally performed using rice straw, *Bacillus* species of bacteria (especially *B. subtilis*) attached to the rice straw are involved in the fermentation and are also contained in the end product. Those products are characterized by the presence of multiple *B. subtilis* strains. On the contrary, in *natto*, a traditional Japanese fermented soybean product, only one strain of *B. subtilis* (natto) is found because a highly industrialized manufacturing process purely using the *natto*-producing strain is utilized.

Soy isoflavones, known as functional ingredients, have various health-promoting effects, such as prevention of bone loss and anticancer effects (Messina *et al*., 1994; Ishimi *et al*., 2002). It has been reported that the conversion of isoflavone glucosides to aglycones is important for exerting their physiological functions (Izumi *et al.*, 2000; Kobayashi *et al*., 2013). Although there are some findings on the changes in isoflavone content in the manufacturing process of *cheonggukjang* (Piao *et al.*, 2020; Lee *et al.*, 2022), no report has addressed the ability of the *B. subtilis* strains contained in these products to produce isoflavone aglycone (IA).

In a previous report, we revealed that the IA-producing ability of *B. subtilis* (natto) is significantly lower than that of *B. subtilis* strain 168, which is an experimental strain, and discussed its mechanism (Inagaki *et al.*, 2022). In this study, to elucidate further details of the mechanism of IA production by *B. subtilis,* we measured the IA content in commercial *cheonggukjang* and *doenjang* products and evaluated the IA-producing ability of the *B. subtilis* strains contained in these products.

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Materials and Methods

Materials Soybeans (small grain; Suzumaru variety) were obtained from Shimaya Co. Ltd. (Toyama, Japan). Commercial *cheonggukjang* products A and B (powder type) were purchased from Paldo Trading Company (Saitama, Japan) and Geumjung Tapgol Farm (Daejeon, Korea), respectively. Commercial *doenjang* product C (paste type) was from Aram Co. Ltd. (Saitama, Japan). *B. subtilis* (natto) (Miyagino strain) was from Miyagino *natto* factory (Miyagi, Japan).

Isolation of B. subtilis strains from commercial products One gram of each commercial product and 20 mL sterile saline (0.9 % NaCl) were mixed in a 50-mL centrifuge tube, followed by shaking at room temperature for 1 h. Then, the mixtures were left standing at room temperature for 30 min to separate the solid and liquid layers, the liquid layers were diluted incrementally to 10^{-6} in 10-fold increments, and the diluted solutions (0.1 mL) were spread onto LB medium agar plates. From plates that formed 50-100 colonies, *B. subtilis*-like colonies with different morphologies were selected for subsequent experiments.

Analysis for 16S rDNA and insertion sequence (IS) Bacterial cells were cultured in LB liquid medium at 37 °C overnight, and the cultures were incubated in boiling water for 3 min. Using the supernatant after centrifugation (3 000 × *g*, 5 min) as the template, DNA fragments of approximately 1.5 kb were amplified by polymerase chain reaction (PCR) with universal primers F4 (5'-ctgaagagttgatcctggc-3') and R1504 (5'-tacggctaccttgttacgac-3'). The fragments were purified using a HiYeld Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience; New Taipei City, Taipei) according to the manufacturer's instructions. Sequences were determined by a sequencing service (Eurofins Genomics; Tokyo, Japan) using the purified DNA fragments and universal primer (F4). Partial 16S rDNA sequences (approximately 800 bp) were analyzed by alignment with the GeneBank database using BLAST.

Insertion of IS in the *B. subtilis* genomic DNA (Nagai *et al.*, 2000) was examined by performing PCR. The region of IS*4Bsu*1 was amplified with the primers IS4F (5'-agtgccgactgaagtgttcc-3') and IS4R (5'-atggtgccgctttgagtaag-3'). The region of IS*256Bsu*1 was amplified with the primers IS256F (5'-taacaagattatcgaacagtatcagcc-3') and IS256R (5'-agctagagtggatactttcaacggc-3'). The PCR was carried out for 35 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, and elongation at 72 °C for 60 sec. The expected lengths of the PCR products for IS*4Bsu*1 and IS*256Bsu*1 were 800 bp and 1 012 bp, respectively. The PCR products were size-fractionated by electrophoresis on 2 % agarose gels and visualized by ethidium bromide staining. The presence of IS in the genomic DNA was determined by the appearance of each DNA product.

Evaluation of biotin auxotrophy The *B. subtilis* isolate was cultured in LB liquid medium at 37 °C overnight, and the cell suspension (5 µL) was spotted on E9 minimal medium (MP Biochemicals; Solon, OH, USA) agar plates supplemented with or without biotin (0.5 µg/mL). After three days of incubation at 37 °C, growth on the agar plates was visually confirmed.

Fermentation test To prepare pre-cultured solution, the *B. subtilis* isolate was cultured in LB liquid medium at 37 °C overnight. The cells were precipitated by centrifugation (3 000 × *g*, 5 min), and the obtained pellet was washed by suspending it with sterile saline and then dissolved in an appropriate amount of sterile saline. The solution was diluted incrementally and spread onto the LB medium agar plates at 37 °C overnight. Colony forming units (CFUs) were estimated by measuring the number of colonies formed on the plate.

For the cultivation with soybean liquid medium, soybeans (40 g) and tap water (100 mL) were mixed in a 500-mL glass beaker and autoclaved for 1 min to obtain soaked soybeans. The soaked soybeans and fresh tap water (100 mL) were mixed in the same glass beaker and autoclaved at 121 °C for 20 min. The mixture was filtrated through gauze to separate the soybeans and the boiled extract. Addition of water to the soybeans, heat treatment by autoclaving, and filtration were performed again. The accumulated extract (approximately 200 mL) was sterilized by autoclaving at 121 °C for 20 min and used as the soybean liquid medium. One milliliter portions of the medium were measured into 15-mL centrifuge tubes with aseptic manipulation, and the pre-cultured solution was inoculated to the medium to be at the concentration of 1×10^5 CFU/mL. Cultivation was carried out with agitation at 38 ± 0.5 °C in a thermostatic incubator. The incubated samples were removed from the incubator every 6 h (up to 72 h) for each experiment.

For the fermentation with soybean solid medium, soybeans (30 g) and tap water (100 mL) were mixed in a 500-mL glass beaker, and the mixtures were allowed to stand at 4 °C for 24 h before the residual water was removed. The soaked soybeans were autoclaved at 121 °C for 20 min to prepare the boiled soybeans, 5 g each of which were measured into 50-mL centrifuge tubes with aseptic manipulation. The pre-cultured solution was inoculated to the boiled soybeans to be at the concentration of 1×10^5 CFU/g. Fermentation was performed in a thermostatic incubator at 38 ± 0.5 °C, and the fermented samples were removed from the incubator once a day (up to 4 days) for each experiment.

Proteolytic activity and poly-γ-glutamic acid (γ-PGA) content Proteolytic activity and γ-PGA content were measured in the samples fermented with soybean solid medium for 1 day. Sterile saline (20 mL) was added to a 50 mL centrifuge tube containing the sample, and the mixture was

shaken at 4 °C for 1 h. The tubes were allowed to stand for 5 min to settle the solids, and the supernatant was used for the assays. Proteolytic activity was measured via casein hydrolysis (Yasunobu *et al.*, 1970). The amount of enzyme that releases the tyrosine equivalent (1 μg) of trichloroacetic acid solubilized matter per minute was defined as one unit of proteolytic activity. Values of the proteolytic activity were expressed as the units per each sample (gram). Gamma-PGA content was measured via the cetyltrimethylammonium bromide method and calculated as the glutamic acid equivalent (Kanno *et al*., 1995; Kiuchi *et al*., 2010). The contents were expressed as milligrams per each sample (gram).

Pre-treatment for isoflavone determination One gram of each powdered commercial product (*doenjang* paste was dried with a freeze dryer) was measured in a 50-mL centrifuge tube. Methanol (20 mL) was added to the tube, and the mixture was shaken at room temperature for 24 h. Extracted solid matter was removed after centrifugation (3 000 × *g*, 5 min), and a portion of the supernatant (0.5 mL) was concentrated by centrifugal evaporation in a micro-centrifuge tube. For preparation of the test samples, the extract was dissolved in dimethyl sulfoxide (DMSO) (0.1 mL) and filtrated using a membrane filter (Advantech, Tokyo, Japan).

In the culture test with soybean liquid medium, bacterial cells were removed from the culture product after centrifugation (3 000 × *g*, 5 min), and the supernatant was transferred to a new 15-mL centrifuge tube. An equal volume of ethyl acetate to the supernatant was added to the tube, and the mixture was shaken with a shaker at room temperature for 20 min. The ethyl acetate layer resulting after centrifugation (3 000 × *g*, 5 min) was transferred to a micro-centrifuge tube. The ethyl acetate extract was concentrated by centrifugal evaporation of the solvent. For preparation of the test sample, the extract was dissolved in DMSO (0.1 mL) and filtrated using a membrane filter.

For the fermentation test with soybean solid medium, fermented samples in 50-mL centrifuge tubes were lyophilized and powdered using a mill. The powder and methanol (20 mL) were mixed in 50-mL centrifuge tubes, and the mixture was shaken at room temperature overnight. Extracted solid matter was removed after centrifugation (3 000 × *g*, 5 min), and a portion of the supernatant (0.5 mL) was concentrated by centrifugal evaporation of the solvent in a micro-centrifuge tube. For preparation of the test sample, the extract was dissolved in DMSO (0.1 mL) and filtrated using a membrane filter.

High-performance liquid chromatography (HPLC) analysis Isoflavone measurement was performed using HPLC. Each sample was separated using a Shimazu (Kyoto, Japan) HPLC system with a COSMOSIL 5C18-MS-II column (4.6 × 150 mm, 5-μm particle size; Nacalai Tesque). The solutions used for the mobile phase had the following composition: (A) H_2O (0.025 % TFA) and (B) CH_3CN. Elution was performed under the following conditions: 0 to 5 min, isocratic elution with 100 % A; 5 to 10 min, a linear gradient of 0 to 20 % B; 10 to 40 min, a linear gradient of 20 to 40 % B; 40 to 45 min, a linear gradient of 40 to 100 % B; and 45 to 50 min, isocratic elution with 100 % B. The flow rate was 1.0 mL/min. The column temperature was maintained at 40 °C. The absorbances for detecting each isoflavone were monitored using a photodiode array detector (SPD-M20A). Each isoflavone in the samples was identified by comparing its relative retention time and UV spectrum with authentic compounds (FUJIFILM-Wako Pure Chemical Corporation; Osaka, Japan); except for succinyl isoflavones, which were identified with reference to already known information (Toda *et al.*, 1999) because their standard compounds are unavailable. The content of the isoflavones was calculated from the standard curves of the peak areas according to isoflavone standards and expressed as micrograms per each sample (mL or gram). The conversion rate of isoflavone glucosides to aglycones (hereafter referred to as the aglycone conversion rate) was calculated using the following equation: [Total amounts of all detectable isoflavone aglycones (mol)/Total amounts of all detectable isoflavones (mol)] × 100.

Measurement of β-glucosidase activity In the culture test with soybean liquid medium, bacterial cells were precipitated by centrifugation (3 000 × *g*, 5 min) and washed by suspending them with sterile saline (2 mL). The cells were suspended in phosphate buffer (50 mM, pH 7.0; 0.1 mL) to prepare the test sample.

In the fermentation test with soybean solid medium, sterile saline (20 mL) was added to a 50-mL centrifuge tube containing fermented sample, and the mixture was shaken at 4 °C for 1 h. After the mixtures were left standing for 5 min to allow the solid matters other than bacterial cells to settle, a part of the supernatant (5 mL) was transferred to a 15-mL centrifuge tube. Bacterial cells were precipitated by centrifugation (3 000 × *g*, 5 min) and washed by suspending them with sterile saline (3 mL). The cells were suspended in phosphate buffer (50 mM, pH 7.0; 0.5 mL) to prepare the test sample.

The test sample (20 μL) and 1 mM *p*-nitrophenyl-β-glucopyranoside (Nacalai Tesque; Kyoto, Japan) solution (0.1 mL) were mixed and incubated at 37 °C for 2 h. After the reaction, the cells were removed after centrifugation (10 000 × *g*, 5 min), and the supernatant (0.1 mL) was transferred to wells in a microplate. Absorbance at 405 nm was measured using a microplate reader (Bio-rad, California, USA). The *p*-nitrophenol content in the reaction solution was calculated from the standard curve of the standard *p*-nitrophenol. The amount of enzyme that released 1 μmol of *p*-nitrophenol per

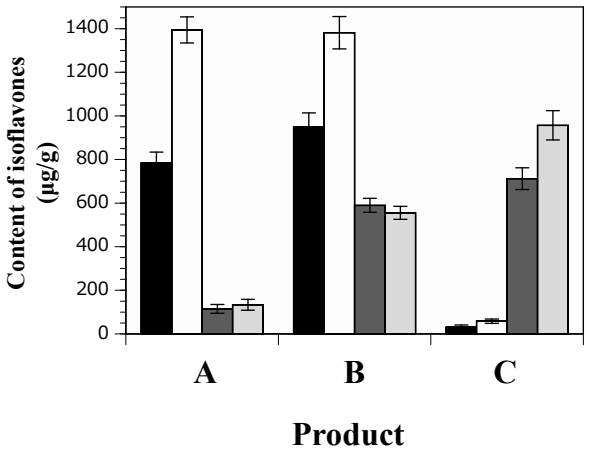

Fig. 1. Content of each isoflavone in commercial fermented soybean products in Korea.
Black bar, Daidzin; White bar, Genistin; Dark gray bar, Daidzein; Light gray bar,Genistein. Values are means (± SEM) of three independent experiments.

Table 1. Aglycone conversion rates of commercial fermented soybean products in Korea and *natto* products in Japan.

Product name	Aglycone conversion rate (%)
Product A	8.9
Product B	33.7
Product C	81.5
Natto product A (Whole grain type)	5.8
Natto product B (Whole grain type)	3.6
Natto product C (Groats type)	16.0
Natto product D (Groats type)	14.5

Values are means of three independent experiments.

minute was defined as one unit of enzyme. Values of this activity were expressed as the units per each sample (mL or gram).

Statistical analysis Values are shown as means of three independent experiments with or without ± standard error (SEM). The difference between groups was evaluated by a two-sided Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results and Discussion

IA contents of commercial products The contents of daidzein and genistein (as the aglycones mainly contained in fermented soybean products) and the aglycone conversion rate of product C (*doenjang*) tended to be higher than those of products A and B (*cheonggukjang*) among the three commercial products (Fig. 1 and Table 1).

As a reference, even the aglycone conversion rate of product A, which had the lowest value among the three products, was higher than that of commercial *natto* products (whole grain type) in Japan (Table 1). *Meju*, a fermented soybean product that is fermented while wrapped in rice straw for a long period of time (supposedly one to three months), is the main raw material of *doenjang* (Yang *et al.*, 2012). In contrast, short-time fermentation (approximately one to three days) is applied for the production of *cheonggukjang* (Piao *et al.*, 2020). Fermentation time for *natto* products in Japan is approximately 20 hours. Thus, differences in the fermentation time of the soybeans used as a raw material seem to significantly affect the amount of IAs contained in the end products. However, there was also a difference between the *cheonggukjang* products A and B, and the result suggested that the IA-producing ability of the *B. subtilis* strains may vary from product to product.

Characteristics of B. subtilis isolates Of all 10 isolates, eight strains (two from product A, three from product B, and three from product C) exhibited the highest homology (98 to 100 %) to *B. subtilis* via 16S rDNA analysis, while the other two isolates were identified as *B. amyloliquefaciens* and *B. licheniformis*. We also confirmed that there were minor differences in the 16S rDNA sequences among these *B. subtilis* strains. Some characteristics of these strains are shown in Table 2. Of the eight strains, two had both IS*4Bsu*1 and IS*256Bsu*1, five had only IS*256Bsu*1, and one strain had neither type of IS. These results support the findings from a previous study showing that *B. subtilis* strains involved in soybean fermentation often have an IS (Kimura *et al.*, 2002). None of these eight strains required biotin for growth, although the *natto*-producing *B. subtilis* strains are known to require biotin frequently for growth (Kubo *et al.*, 2011). In the evaluation of protease activity and γ-PGA-producing ability, which are involved in their growth potential on soybeans and physical properties of the fermented products, some strains showed values similar to or higher than those of *B. subtilis* (natto), suggesting that the strains may have *natto*-producing characteristics.

IA-producing ability of B. subtilis isolates The β-glucosidase activity and IA-producing ability of each *B. subtilis* isolate cultured with the soybean solid medium were

Table 2. Some characteristics of the *B. subtilis* strains isolated from commercial fermented soybean products in Korea.

Strain name	Presence of IS [a]		Biotin auxotrophy [b]	Protease activity (U/g) [c]	γ-PGA (mg/g) [c]
	IS*4Bsu*1	IS*256Bsu*1			
A-1	+	+	N	358.2	2.70
A-2	+	+	N	515.3	3.08
B-1	−	+	N	475.1	0.29
B-2	−	−	N	460.5	0.86
B-3	−	+	N	215.6	N.D.
C-1	−	+	N	581.1	0.43
C-2	−	+	N	504.4	1.10
C-3	−	+	N	310.7	0.19
Miyagino	+	+	P	394.7	1.36
168	−	−	N	76.8	N.D.

[a] -, absent; +, present. [b] N, negative; P, positive.
[c] Values are means of three independent experiments. N.D., not detected.

measured, and compared to *B. subtilis* (natto) (Table 3). As a result, four strains (A-1, A-2, B-2, and C-3) showed IA-producing ability equal to or higher than that of *B. subtilis* (natto). Among them, the C-3 strain isolated from product C, which had the highest aglycone conversion rate, showed the most superior IA-producing ability among the isolates. There were no differences between the growth potential of the *B. subtilis* strains used in the experiment (data not shown). In addition, microorganisms other than *Bacillus* spp. such as mold were not detected in the culture using LB or PDA (potato dextrose agar) medium from the commercial products, and two isolates of *Bacillus* spp. other than *B. subtilis* did not have the ability to produce IAs (data not shown). From these results, the differences in aglycone conversion rates observed among the commercial products might be attributed not only to the fermentation time of the products, but also to the IA-producing ability of the *B. subtilis* strains involved in the fermentation. The *B. subtilis* strains contained in product B, which showed a higher aglycone conversion rate than product A, did not have much IA-production ability, suggesting that product B may have a longer fermentation time than product A. Hereafter, it would be of interest to clarify how the IA-producing ability of *B. subtilis* is involved in the health properties found in the various fermented soybean foods in Korea.

IA-producing ability of C-3 strain As seen in Table 3, the β-glucosidase activity levels of the *B. subtilis* strains did

Table 3. β-glucosidase activity and IA production by the *B. subtilis* strains isolated from commercial fermented soybean products in Korea.

Strain name	β-glucosidase activity (mU/g) [a]	IA production (μg/g) [b]
A-1	451.5 ±32.9	261.5 ±17.6
A-2	503.2 ±38.7	336.0 ±24.6
B-1	N.D.	N.D.
B-2	666.8 ±45.5	272.5 ±18.8
B-3	N.D.	N.D.
C-1	94.4 ±8.3	38.6 ±4.2
C-2	547.2 ±42.5	78.1 ±7.4
C-3	345.4 ±31.2	602.6 ±46.4
Miyagino	363.5 ±27.6	222.3 ±14.2
168	2862.9 ±120.2	998.2 ±53.5

Values are means (± SEM) of three independent experiments. N.D., not detected. [a] Data from the day of highest activity in a 4-day fermentation (sampled every day) with soybean solid medium are shown. [b] Data from the day with the highest IA content in the soybean solid medium (total IAs) during a 4-day fermentation (sampled every day) are shown.

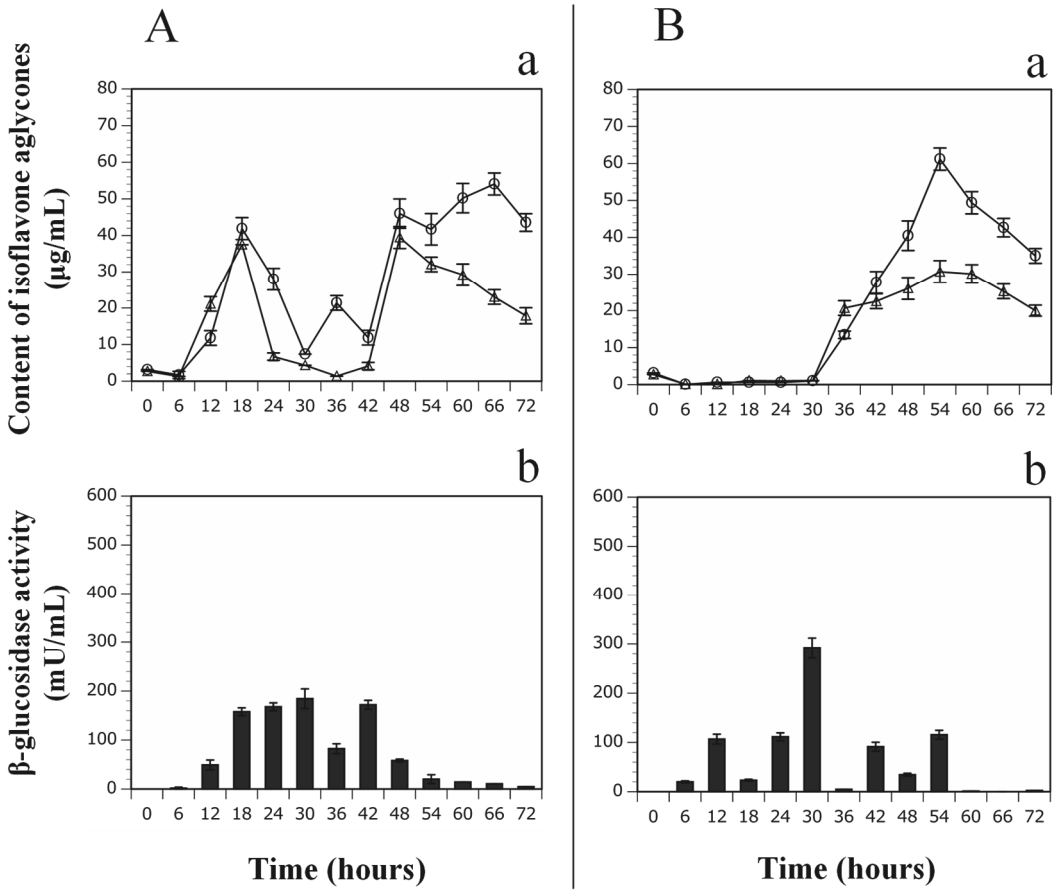

Fig. 2. Comparison of IA production (a) and β-glucosidase activity (b) between *B. subtilis* C-3 (A) and Miyagino strain (B) cultured with soybean liquid medium.
Values are means (± SEM) of three independent experiments. (a) Circle, Daidzein; Triangle, Genistein.

not necessarily correlate with the IA content of the medium: for example, the B-2 and C-2 strains had higher β-glucosidase activity than the C-3 strain, but the IA-producing ability of these strains was lower than that of the C-3 strain. Hence, we examined the rapidity of IA production in a culture test using soybean liquid medium as a possible factor for the IA-producing ability other than β-glucosidase activity. Interestingly, of all the isolates, the C-3 strain produced IAs during the early incubation stage (12 to 24 h); thereafter, the IA content decreased, and subsequently increased again during the late incubation stage (Fig. 2A-a). Other strains, including *B. subtilis* (natto) and *B. subtilis* strain 168, did not produce IAs until 24 h (data not shown). Additionally, there was no increase in isoflavone glucosides due to the decrease in IAs (data not shown). From these results, we presume that the uptake and release of IAs by *B. subtilis* cells are purposefully regulated regardless of the induction of β-glucosidase activity, and there are differences in their mechanisms among *B. subtilis* strains. Moreover, the C-3 strain had higher ability for IA production than *B. subtilis* (natto) in the fermentation test using soybean solid medium, despite having the same level of β-glucosidase activity (Fig. 3). In the soybean solid medium, the immediate decrease after an increase of IAs in the medium fermented with the *B. subtilis* C-3 strain as seen during the fermentation at the early stage with soybean liquid medium (Fig. 2A-a) was not observed (Fig. 3A-a). We consider that these results were caused by the difference in the mechanism of IA production by *B. subtilis* depending on the types of medium used. These results suggest that the amount of IAs in the soybean solid medium is attributed not only to the β-glucosidase activity but also the rapidity of IA production by the *B. subtilis* strains involved in the fermentation. However, the detailed mechanism of IA production by *B. subtilis* remains unclear.

Application to natto products Previously, we reported that a delay in IA production was observed during the fermentation of soybeans with *B. subtilis* (natto) (Inagaki *et al*., 2022). In that paper, we discussed that the utilization of a *B. subtilis* strain that produces IAs rapidly, and the use of a short-time (within one day) fermentation method are required for

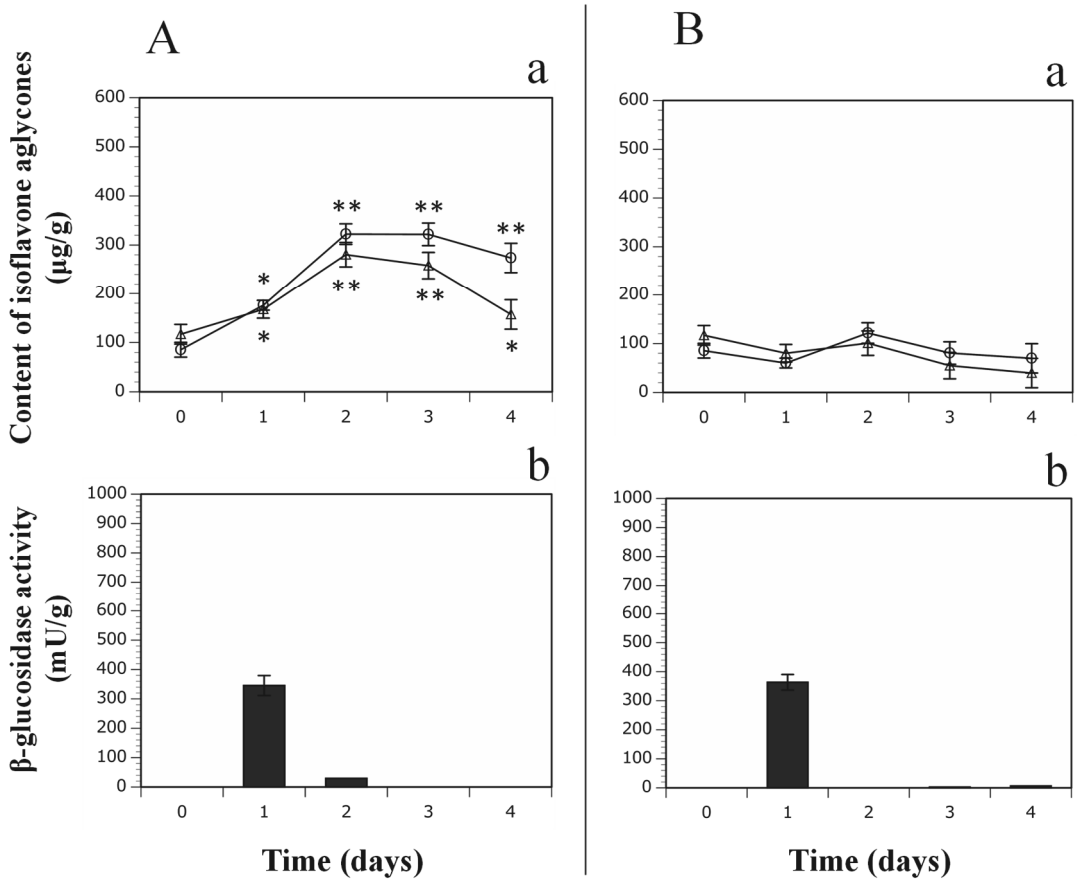

Fig. 3. Comparison of IA production (a) and β-glucosidase activity (b) between *B. subtilis* C-3 (A) and Miyagino strain (B) fermented with soybean solid medium.
Values are means (± SEM) of three independent experiments. $^{**}p < 0.01$; $^{*}p < 0.05$ (C-3 *versus* Miyagino strain at the same fermentation time and isoflavone species). (a) Circle, Daidzein; Triangle, Genistein.

producing an IA-rich fermented soybean product (*natto*), because prolonged fermentation produces undesirable components such as ammonia. In the present study, the characteristic of the *B. subtilis* C-3 strain that can rapidly produce IAs suggested that the delay in IA production is not an event necessarily caused by *B. subtilis* strains and it is possible to produce an IA-rich fermented soybean product through short-time fermentation. However, even the C-3 strain did not produce abundant IAs in the early period during the fermentation with soybean solid medium (Fig. 3A-a), and seemed to be unsuitable for *natto* production because of a lack of viscous material (γ-PGA) production (Table 2). Therefore, the exploitation of *B. subtilis* strains, which possess the abilities of both rapid IA production and higher β-glucosidase activity in solid-state fermentation and can give physical *natto* characteristics to boiled soybeans, would be essential for the development of an IA-rich soybean product (*natto*).

Conflict of interest There are no conflicts of interest to declare.

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