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High Expression of β-Glucosidase in *Bifidobacterium bifidum* BGN4 and Application in Conversion of Isoflavone Glucosides During Fermentation of Soy Milk

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In spite of the reported probiotic effects, *Bifidobacterium bifidum* BGN4 (BGN4) showed no β-glucosidase activity and failed to biotransform isoflavone glucosides into the more bioactive aglycones during soy milk fermentation. To develop an isoflavone-biotransforming BGN4, we constructed the recombinant *B. bifidum* BGN4 strain (B919G) by cloning the structural β-glucosidase gene from *B. lactis* AD011 (AD011) using the expression vector with the constitutively active promoter 919 from BGN4. As a result, B919G highly expressed β-glucosidase and showed higher β-glucosidase activity and heat stability than the source strain of the β-glucosidase gene, AD011. The biotransformation of daidzin and genistin compounds using the crude enzyme extract from B919G was completed within 4 h, and the bioconversion of daidzin and genistin in soy milk during fermentation with B919G also occurred within 6 h, which was much faster and higher than with AD011. The incorporation of this β-glucosidase-producing *Bifidobacterium* strain in soy milk could lead to the production of fermented soy milk with an elevated amount of bioavailable forms of isoflavones as well as to the indigenous probiotic effects of the *Bifidobacterium* strain.

Keywords: β-Glucosidase, soy milk fermentation, soy isoflavones, biotransformation, recombinant *Bifidobacterium bifidum* BGN4

Introduction

Enzymatic transformation techniques using food microbial glycosidases have a profound potential for the production of biologically active aglycones *via* the hydrolysis of various sugar moieties of the various glycosides in foods [39]. Many attempts have been made to improve the nutritive value and beneficial effects of plant-derived foods containing diverse types of glycones, by fermentation with various food microorganisms that produce high levels of glycosyl-hydrolases [2, 17]. Specifically, *Bifidobacterium* has been reported to possess many glycosyl-hydrolases such as amylase, α- and β-glucosidase, α- and β-galactosidase, α-L-fucosidase, α-L-arabinofuranosidase, and β-fructofuranosidase, which play a major role in the intestinal hydrolysis of the various oligosaccharides, polysaccharides, and glyco-conjugated phytochemicals [34].

The potential health benefits of soy milk and soy-derived isoflavones have been emphasized by many researchers. In soybeans, the most abundant isoflavones exist predominantly as glycoside forms, whereas the aglycone forms such as daidzein, genistein, and glycitein comprise a minor content of isoflavone compounds. Various reports showed that the isoflavones in plants need to be converted to aglycones to be absorbed in the intestine, and the biological effects of isoflavones are not due to the glucoside forms but mainly to the aglycone forms, such as daidzein and genistein [8, 28, 43]. The ability to hydrolyze isoflavone glycosides in the intestine decreases with age, causing health problems in older populations [6, 33]. Approximately 5 mg of aglycones is contained in 100 g of dried soybeans, and this is much less than the amount (30–40 mg/day) required for achieving

health benefit [25].

As both a probiotic microorganism and a β-glucosidase (β-Glu) producer, *Bifidobacterium* has been applied to the production of fermented soy milk with enhanced levels of bioavailable forms of isoflavones as well as enriched viable cells of probiotic bacteria. The microbiological transformation methods using probiotic *Bifidobacterium* could be considered to have the advantage of enhancing the functionality of soy milk through the combined effects of probiotics and isoflavone aglycones. However, β-Glu activity and the ability to transform the isoflavones significantly vary among the species and the strains of *Bifidobacterium* [21, 26, 31]. The reported probiotic effects of *Bifidobacterium*, including preventing infectious diseases, allergic disorders, diarrhea, and inflammatory diseases, also vary and can be strain-specific [1, 4, 27]. Therefore, the capacity of specific strains to exert probiotic effects needs to be demonstrated and validated by experimental evidence in order to attain the enhanced functionality of *Bifidobacterium*-fermented soy milk.

Bifidobacterium bifidum BGN4 (BGN4) was isolated from the fecal sample of a healthy, breast-fed infant and exhibited a prominent adhesive capacity to intestinal epithelial cells, which is one of the desirable properties for a probiotic effect [10]. This strain has been reported to show immune-modulatory functions such as the suppression of inflammatory cell infiltration and the reduction of inflammatory cytokine release in an inflammatory bowel disease model [13, 16] and to exert the preventive effect of IgE-mediated ovalbumin-induced allergy in a C3H/HeJ mice model [9]. In two separate double-blind, randomized placebo-controlled human trials, the products containing BGN4 significantly lowered the prevalence of atopic dermatitis and pain scores in infants and increased the bowel movement comfortableness in irritable bowel syndrome patients [7, 12].

Although the β-Glu from several *Bifidobacterium* strains has been screened and utilized for the hydrolysis of various substrates, only one from *B. lactis* has been cloned, characterized, and expressed in *E. coli* and *Bifidobacterium* [41]. The main obstacles to the wide application of recombinant gene expression in *Bifidobacterium* are the need to construct efficient expression vectors and to overcome the low transformation efficiency of the bifidobacterial strains. In the last few years, we attempted to develop optimized expression vectors and transformation techniques for a high-level expression system to express foreign genes in *Bifidobacterium* as a host [14, 23, 35, 41]. Kim *et al*. [14] cloned the structural gene of β-Glu from AD011 with the pBES-16PR bifidobacterial expression vector; however, the expression level of β-Glu in bifidobacteria was unexpectedly low compared with corresponding *E. coli* strains [14]. Recently, a novel strong promoter of BGN4 that induced constitutively high-level expression was selected through microarray analysis based on the genome sequences of BGN4 [35]. Based on a series of efforts, in this study, we cloned the structural β-Glu gene from *B. lactis* AD011 into the expression vector placed under the control of the constitutively active promoter 919 (pBES2-919G), and expressed in it a β-Glu-negative strain, *B. bifidum* BGN4. The enzymatic properties of the recombinant *B. bifidum* BGN4 strain (B919G) with regard to the pH, temperature, and incubation time were assessed and the practical application of this recombinant strain to soy milk fermentation was confirmed. The biotransformation of isoflavone glucoside standards by crude enzyme extracts from B919G and AD011 was compared, and the bioconversion of soy isoflavones from soy milk during fermentation by B919G was also analyzed.

Materials and Methods

Materials

Standards of isoflavones (genistin, daidzin, glycitin, genistein, daidzein, glycitein) were obtained from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). All restriction enzymes were purchased from Promega (Madison, WI, USA). Ampicillin, chloramphenicol, and all other reagents were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA) unless specifically described. Soybeans (*Glycine max*) were purchased from a local market (Seoul, Korea).

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this work are listed in Table 1. The cloning host *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) was grown in Luria-Bertani (LB) medium (Becton-Dickinson Company, Detroit, MI, USA) at 37°C with vigorous shaking. The β-Glu-deficient *B. bifidum* BGN4 [16, 40] was used to obtain the newly screened promoter (P919) and used as an expression host. *B. animalis* subsp. *lactis* AD011, which was used as the source of the β-Glu gene (BLA_0141), was isolated from the fecal sample of a healthy breast-fed infant and its complete genome sequence was previously reported [11]. *Bifidobacterium* strains were grown in Lactobacilli MRS medium (Becton-Dickinson Company) supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C anaerobically. When needed, 50 μg/ml ampicillin and 3.6 μg/ml chloramphenicol were used for the selection of the transformants in LB and MRS medium, respectively.

Molecular Cloning and Vector Construction

The chromosomal DNA of *B. animalis* subsp. *lactis* AD011 (AD011) and *B. bifidum* BGN4 (BGN4) was isolated with the PureLink

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Characteristics	Source or reference
Strains		
DH5α	*E. coli* DH5α, cloning host	Invitrogen
AD011	*B. animalis* subsp. *latis* AD011 containing BLA_0141	[11]
BGN4	*B. bifidum* BGN4, expression host β-Glucosidase-negative strain Source of promoter 919	[16]
B919G	Recombinant *B. bifidum* BGN4 containing pBES2-919G	This work
Plasmids		
pSC-B-amp/kan	Subcloning vector in *E. coli*	Stratagene
pBES2	Cm^r, Amp^r: *E. coli-Bifidobacterium* shuttle vector	[23]
pBES2G	Recombinant plasmid containing β-glucosidase gene	[35]
pGEM-T easy	Amp^r, M13ori pBR322ori, linear T-overhangs vector	Promega
pBES2-919G	pBES2 derivative containing the P919 promoter upstream of β-glucosidase	This work

Genomic DNA Kit (Invitrogen) according to the manufacturer's instructions and used as template for PCR amplification. The target β-Glu gene (BLA_0141; GenBank Accession No. CP001213.1) was amplified using PfuUltra II HS DNA polymerase (Stratagene, La Jolla, CA, USA) with primers BLA_0141-F (5′-CGCGGATCCATG ACGATGACGTTCCCGAAGGGC-3′) and BLA_0141-R (5′-CCG GAATTCCTACTTGGCGGAGTGCTCGGC GAT-3′) [15]. *Bam*HI and *Eco*RI recognition sequences are underlined. The amplified DNA was recovered using a Qiagen Mini Elute Gel extraction kit (Qiagen Korea Ltd., Seoul, Korea) and subcloned into the pSC-B-amp/kan vector with a StrataClone Blunt PCR cloning kit (Stratagene, La Jolla, CA, USA), and transformed into *E. coli* DH5α by the $CaCl_2$ method. Correct clones were selected by spreading on LB agar supplemented with 50 μg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; BioBasic, Ontario, Canada). Plasmids from white colonies were purified using the Plasmid Purification Mini Kit (NucleoGen Inc., Siheung, Korea) and confirmed by restriction enzyme digestion using *Bam*HI and *Eco*RI. Each cloned gene was excised from pSC-B-amp/kan and ligated into *Bam*HI–*Eco*RI-digested pBES2 to establish pBES2G [35].

The P919 promoter region of *B. bifidum* BGN4 was amplified using primers P919-F (5′-TCTAGATGAAGTGTGTCGTGTGGCGT-3′) and P919-R (5′-GGATCCTGGTGTACCTTTTCTTGCTT-3′) containing *Xba*I and *Bam*HI restriction sites (underlined), respectively [35]. The PCR products were ligated into pGEM-T Easy vector (Promega) and transformed into *E. coli* DH5α by the $CaCl_2$ method. Plasmid DNA was isolated and purified from *E. coli* DH5α, and P919 was inserted upstream of the β-glucosidase gene in pBES2G to construct pBES-919G.

Transformation of *B. bifidum* BGN4 with pBES-919G

The recombinant expression vectors were prepared from *E. coli* DH5α using an Axyprep Midi kit (Axygen Biosciences, Union City, CA, USA) and methylated using GpC methyltransferase (M.CviPI) (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's instruction. After methylation, the expression vectors were transformed into *B. bifidum* BGN4 by electroporation [14]. The fructose-6-phosphate phosphoketolase (F6PPK) test and plasmid preparation were carried out to confirm the correct transformants among the developed colonies. The β-glucosidae-positive *B. bifidum* BGN4 (B919G) strain was confirmed

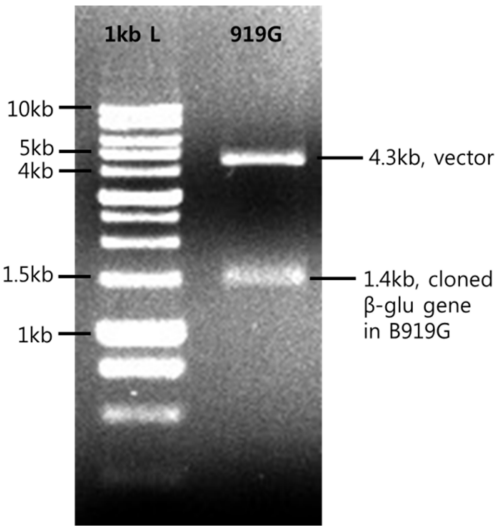

Fig. 1. Confirmation of cloned β-glucosidase gene in recombinant B919G strain by gel electrophoresis after *Eco*RI and *Bam*HI double digestion.

Lane 1, 1 kb DNA ladder. Lane 2, B919G plasmid DNA digested to 1.4 kb β-glu insert and 4.3 kb vector.

by PCR and gel electrophoresis after restriction enzyme digestion. The correct ORF, β-Glu gene (1.4 kb), was observed after double digestion with *Eco*RI and *Bam*HI, as shown in Fig. 1. The nucleotide sequence was analyzed using the Applied Biosystems 3730 DNA Analyzer in the Genome Research Facility in Seoul National University.

β-Glucosidase Activity Assay

The β-Glu activities of B919G and AD011 were compared using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. Various biotransformation conditions with respect to incubation time, pH, temperature, duration of heat treatment, and sonication time were assessed. *B. bifidum* BGN4 and *B. lactis* AD011 were anaerobically cultured in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl at 37°C. *B. bifidum* BGN4 transformants harboring pBES2-919G were cultivated in MRS medium supplemented with 0.05% (w/v) L-cysteine·HCl and 3.6 μg/ml of chloramphenicol at 37°C, if necessary. During cultivation, 1.0 ml of culture broth was harvested at indicated times and centrifuged at 10,000 ×*g* for 2 min. The cell pellet was washed twice with 200 μl of ice-cold 0.1 M phosphate buffer (pH 6.0), disrupted by sonication in 500 μl of the same buffer, and centrifuged at 10,000 ×*g* for 2 min at 4°C to obtain the supernatant as crude enzyme. Fifteen microliters of enzyme solution was mixed with 10 μl of 5 mM *p*NPG and incubated at 37°C for 20 min. The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. The released *p*-nitrophenols (*p*NPs) was measured at 405 nm, using *p*NPs standard solution. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*NP per minute at 37°C.

Biotransformation of Isoflavone Glucosides to Aglycones by Crude Enzyme Extracts

Biotransformation of isoflavone standards (daidzin, genistin, and glycitin) to respective aglycones (daidzein, genistein, and glycitein) by crude enzyme extracts from BGN4, AD011, and B919G was analyzed. Prepared enzyme extracts (10% (v/v)), 1 mM isoflavone glucosides solution (50% (v/v)), and phosphate buffer (40% (v/v)) were mixed and incubated at 37°C for 4 h. The reaction was terminated by boiling for 10 min and freeze-dried before HPLC analysis.

Preparation of Soy Milk Medium

The preparation of soy milk medium was conducted as previously described [37]. Briefly, whole soybeans were washed and soaked in distilled water at 25°C for 8 h. The water was then decanted and the soaked soybeans were ground for 3 min in distilled water (10 times the dry weight of soybeans). The resulting soybean slurry was filtered through double-layered cheese cloth to produce the soy milk. For the fermentation, soy milk was supplemented with 0.05% (w/v) cysteine·HCl and autoclaved at 121°C for 15 min and then inoculated with the experimental bifidobacteria. The pH of the soy milk medium was adjusted to pH 6.4 ± 0.2 prior to the fermentation.

Soy Milk Fermentation and Viable Cell Counts

At every 3 h until 12 h during fermentation, each of the fermented soy milk was serially diluted with phosphate-buffered saline containing 0.05% (w/v) cysteine·HCl, and viable cell counts of bifidobacteria were determined in triplicate by using the surface plating method with MRS agar. Confirmed colonies after anaerobic culture at 37°C for 3 days were counted. Results are expressed as $\log_{10}$ CFU/ml of viable cell count. For the measurement of the biotransformed isoflavones during fermentation, fermented soy milk was boiled for 10 min and freeze-dried until the HPLC analysis.

HPLC Analysis of Isoflavones During Biotransformation of Standards and Soy Milk Fermentation

The stock solutions of each of the standard compounds of daidzein, genistein, glycitein, daidzin, genistin, and glycitin were prepared by dissolving 1 mg of each in 10 ml of 80% aqueous methanol and were stored in the refrigerator. Each isoflavone standard solution was injected into the HPLC, and the peak areas were determined. The lyophilized samples were dissolved in methanol, filtered through a 0.45 μm syringe filter (Pall, Ann Arbor, MI, USA), and used for HPLC analysis. HPLC was conducted with a Dionex P680 instrument (Dionex Corporation, Sunnyvale, CA, USA) equipped with an ASI-100 auto sampler (Dionex) and a UVD 170 UV-vis detector (Dionex). A Sunfire C18 column (150 mm × 4.6 mm, 3.5 μm particle size) from Waters (Milford, MA, USA) and TCC-100 thermostatted column compartment (Dionex) were used, and the column was maintained at 30°C during the separation. The mobile phase consisted of solvent A (0.1% (v/v) trifluoroacetic acid in water, pH 2.5) and solvent B (acetonitrile) with the following gradient: 0–5 min, 15% B; 5–45 min, linear gradient from 15% to 40% B. The injection volume of standards and samples was 20 μl, and the flow rate was 1 ml/min. The retention times of daidzein, genistein, glycitein, daidzin, genistin, and glycitin were 25.7, 35.5, 28.1, 8.6, 15.6, and 10.3 min, respectively.

Statistical Analysis

The mean values and the standard deviation were calculated by the triplicate independent trials. One-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test was used to test statistically significant differences (p value <0.05) using the SPSS Statistical package (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of β-Glucosidase Expressed in Recombinant *B. bifidum* BGN4 (B919G) Strain

The β-glucosidase activities of bifidobacterial strains were measured by the degradation of *p*NPG substrate to produce free *p*NPs. As shown in Fig. 2A, the β-glucosidase activities of B919G and AD011 reached a maximum level at

18 h, and decreased (B919G) or maintained (AD011) thereafter up to 30 h of fermentation in MRS media. The β-Glu activity of B919G enzyme extract at 18 h was 1.9 U/ml, whereas AD011 showed 1.1 U/ml. In order to evaluate the effect of pH on the β-glucosidase activities from B919G, AD011, and BGN4, the pH was varied from 3 to 8 at 37°C. Maximum activity was observed at pH 6.0 in both B919G and AD011 strains. At pH values of 5.0 and 7.0, the activity of B919G enzyme extract was approximately 81% and 57% of the maximum activity, respectively (Fig. 2B). The effect of temperature on the β-glucosidase activity was investigated at pH 6 at the range of 20°C–70°C. Maximal activity was shown at 40°C in both strains (Fig. 2C).

The thermal stability of bifidobacterial β-glucosidase was examined by measuring the enzyme activity over time at pH 6.0 and at the temperatures from 20°C to 70°C (Fig. 3). Both strains showed sharp decreases in enzyme activity at over 50°C. However, the stability of β-glucosidase at 40°C was higher in strain B919G than in strain AD011.

The localization of the enzyme activities was also examined by separating the culture supernatants, whole-cell extract without disruption, and the cytoplasmic enzyme fraction. The β-glucosidase activities of all three bifidobacterial strains were shown only in the cytoplasmic enzyme fraction (data not shown).

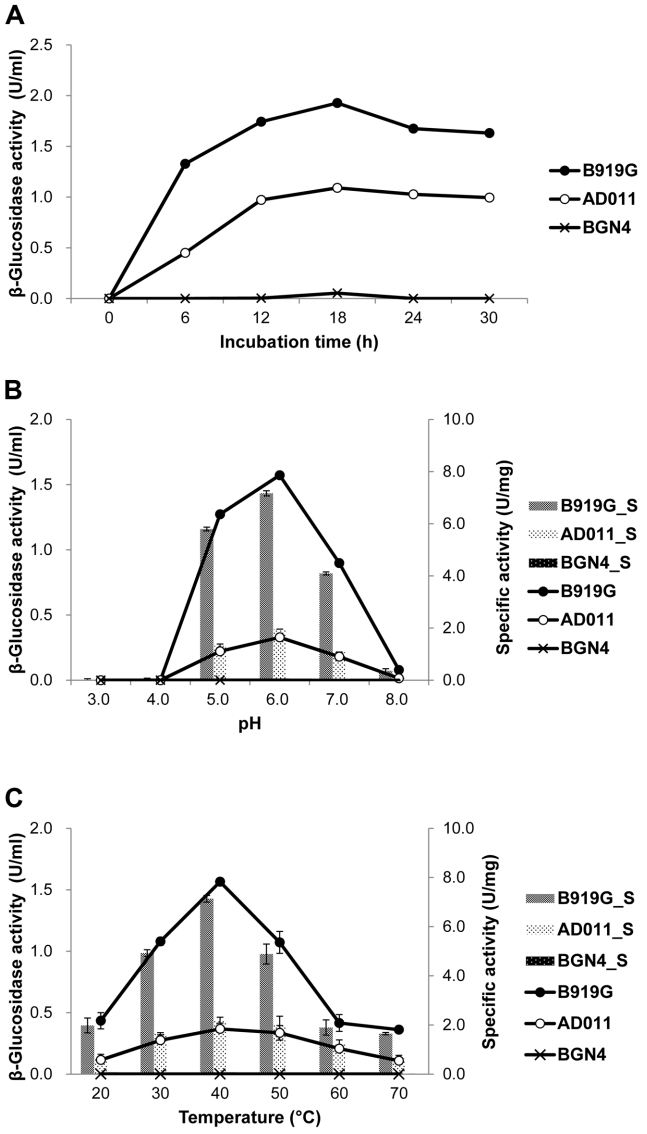

Fig. 2. Effects of incubation time (**A**), pH (**B**), temperature (**C**) on β-glucosidase activity.

Biotransformation of Isoflavone Glucosides by Crude Enzyme Extracts from Bifidobacterial Strains

The ability of all wild-type and recombinant *Bifidobacterium* strains to hydrolyze the three basic isoflavone glucosides was investigated by measuring the contents of daidzin, genistin, and glycitin compounds after the biotransformation with crude enzyme extracts of B919G, AD011, and BGN4 (Fig. 4). The initial concentration of each compound was

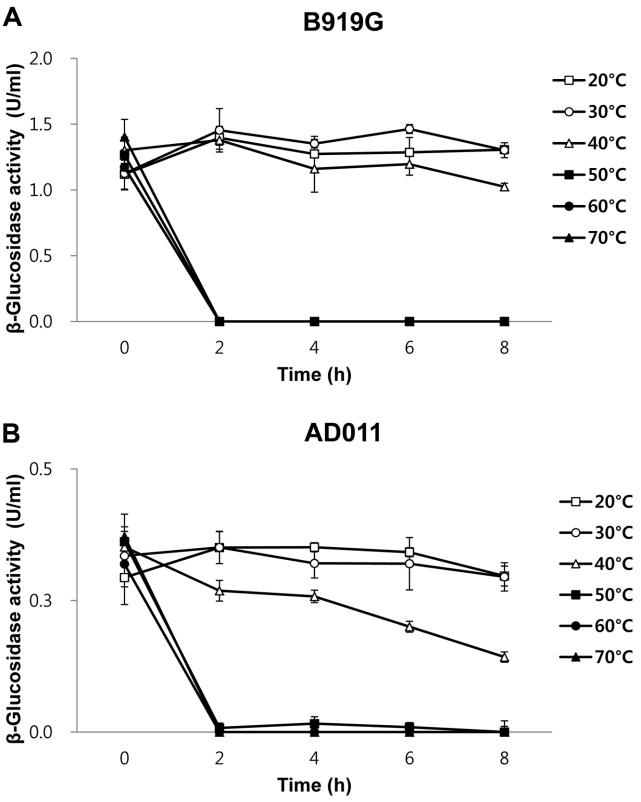

Fig. 3. Stability of β-glucosidase from B919G (**A**) and AD011 (**B**) against heat treatment.

0.5 mM, and no by-products or degraded metabolites of these except aglycone forms were found in the HPLC chromatograms. There was no production of daidzein, genistein, and glycitein after biotransformation with enzyme extracts from the β-glucosidase-negative BGN4 strain (Fig. 4, empty and filled triangles). The crude enzyme extract from B919G showed the fastest and strongest hydrolyzing activities against all of the isoflavone glucosides. In accordance with daidzin reduction, 58.4%, 84.0%, and 96.0% of daidzein yields were observed in the B919G-transformed group after 1, 2, and 4 h, respectively (Fig. 4, empty and filled circles). In the case of AD011, the daidzein yields at different time points were 23.7%, 44.0%, and 66.5% after 1, 2, and 4 h of fermentation. Genistin was more easily hydrolyzed by both AD011and B919G enzyme extracts than daidzin or glycitin. Glycitein yields at 1, 2, and 4 h of biotransformation were 75.7%, 91.2%, and 97.3% for the B919G group and 34.6%, 56.3%, and 71.5% for the AD011 group, respectively. The B919G enzyme extract showed 2.46-fold and 2.19-fold higher biotransformation activity than AD011 enzyme extract in the conversion of daidzin and genistin, respectively.

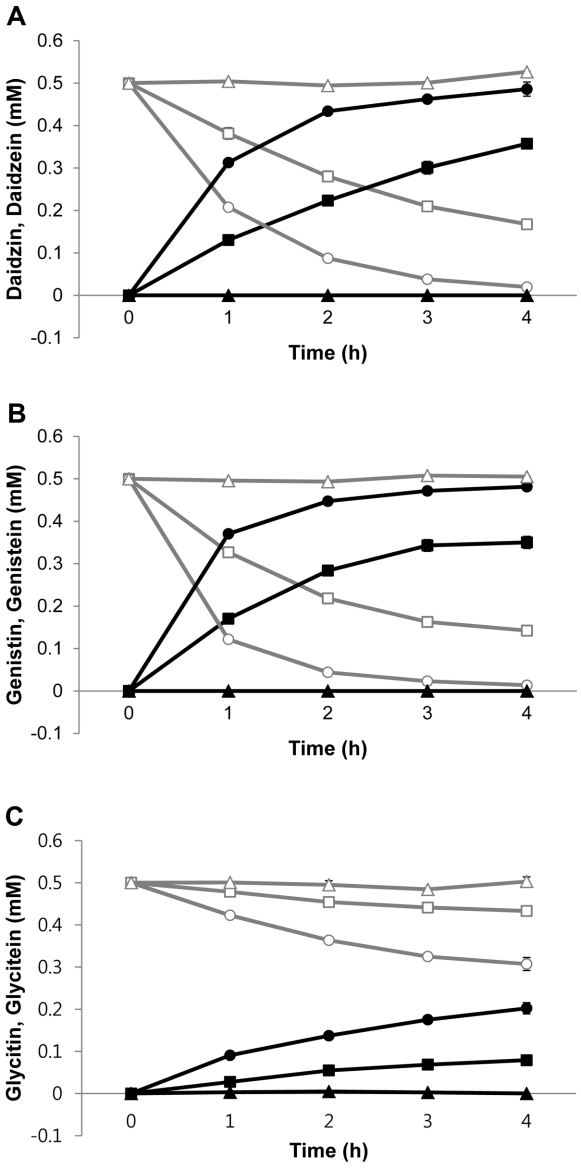

Fig. 4. Time courses for the bioconversion of daidzin (**A**), genistin (**B**), and glycitin (**C**) by crude enzyme extracts from *Bifidobacterium* strains.

Isoflavone glucoside (0.5 mM) was hydrolyzed at 37°C for 4 h by the enzyme extracts from the recombinant *B. bifidum* BGN4 (*circle*), *B. lactis* AD011 (*square*), and *B. bifidum* BGN4 (*triangle*). Residual isoflavone glucosides (*empty, grey lines*) and converted aglycones (*filled, black lines*) are shown.

Deglycosylation levels between the different glucosides (daidzin, genistin, and glycitin) were not identically affected by enzymatic bioconversion. Glycitin showed the lowest level of deglycosylation after fermentation with each strain, whereas genistin was most susceptible to the hydrolysis by β-glucosidase in all the groups. After 4 h of biotransformation with B919G enzyme extract, 38.6% of glycitin was transformed to glycitein (in the case of AD011, 13.4%). The biotransformation activity of B919G in conversion of glycitin was 3.6-fold higher than that of AD011.

Changes in Isoflavone Levels and Viable Cell Counts During 12 h Fermentation of Soy Milk

The concentration of six isoflavones (daidzin, genistin, glycitin, daidzein, genistein, and glycitein) in soy milk was measured by HPLC analysis during the 12 h fermentation at 3 h intervals. Changes of glucoside and aglycone isoflavone contents are expressed as a percentage of the total isoflavones, which was obtained by dividing the content of individual isoflavone by the sum of glycone and its aglycone content. As shown in Fig. 5, there was a marked decrease of daidzin and genistin contents until 6 h of fermentation in the B919G-fermented soy milk group. At 6 h, 94.2% and 94.8% of daidzin and genistein, respectively, were converted to its aglycones by B919G, whereas there were slight decreases of daidzin and genistin contents in the AD011-fermented group (9.6% and 16.2%, respectively). The contents of isoflavone glycones decreased in correspondence to the increase of aglycones content in the fermented soy milk. The amounts of glycitin and glycitein in soy milk media were at a minimal level and no significant change was observed during the fermentation with all three *Bifidobacterium* strains (data not shown).

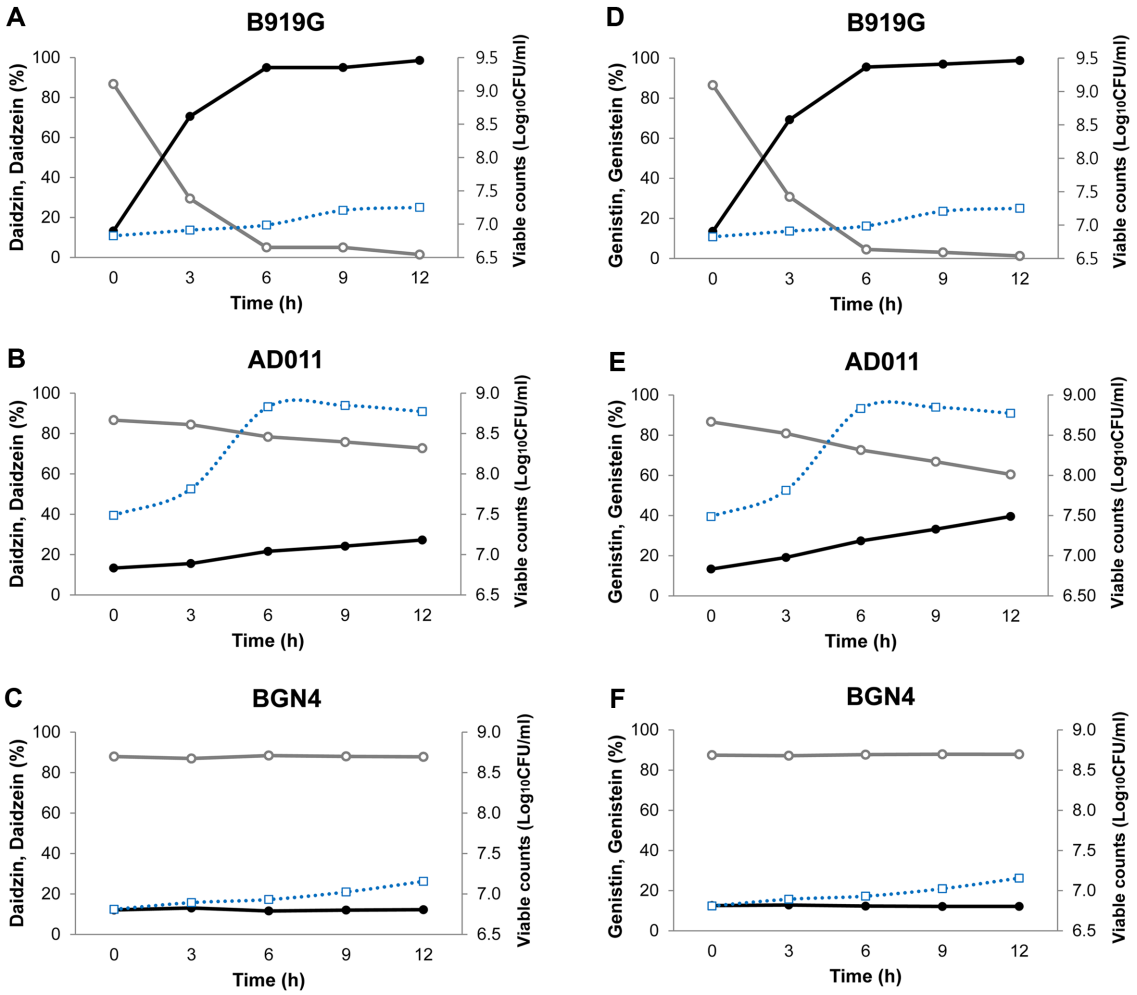

Fig. 5. Bioconversion of daidzin and genistin to the respective aglycones during soy milk fermentation with *Bifidobacterium* strains at 37°C for 12 h.

The data are expressed as the percentage of the total isoflavones (glucoside and its aglycone). Residual isoflavone glucosides (*empty circle*), and hydrolyzed aglycones (*filled circle*) are shown. Viable microbial cell counts ($\log_{10}$ CFU/ml) of *Bifidobacterium* strains during fermentation are also shown (*empty square*). (**A**) and (**D**), recombinant *B. bifidum* BGN4 (B919G); (**B**) and (**E**), *B. lactis* AD011 (AD011); (**C**) and (**F**), *B. bifidum* BGN4 (BGN4), the expression host. Data represent the means of three experiments and error bars represent standard deviation.

The growth patterns of B919G, AD011, and BGN4 are also shown in Fig. 5. During the fermentation of soy milk, the viable counts of AD011 were maintained significantly at a higher level than those of BGN4 and B919G. In spite of the same inoculation ratio, the bacterial cell number of AD011 at the beginning of the fermentation was 7.49 × $\log_{10}$ CFU/ml, compared with 6.82 × $\log_{10}$ CFU/ml and 6.81 × $\log_{10}$ CFU/ml of B919G and BGN4, respectively. The viable cell count for B919G did not differ significantly from that of BGN4. The highest difference between B919G and AD011 was found at 6 h when the viable counts of B919G and AD011 were 6.99 × $\log_{10}$ CFU/ml and 8.83 × $\log_{10}$ CFU/ml, respectively.

Discussion

The abilities of many bacterial and fungal β-glucosidases to convert isoflavone glucosides into the deglucosylated forms have been studied [18, 20, 21, 26, 30, 38]. Among the various bacteria, the biotransformation rate of isoflavone glucosides by probiotic bacteria in general was considerably low in fermented soy milk [3, 31, 36]. Several studies were performed to increase the level of biotransformed isoflavones by inducing the bacterial growth in soy milk using skim milk powder or lactulose supplementation [24, 25] and mixed cultures with different probiotic strains [2].

Bifidobacterium bifidum BGN4 (Beta-Glucosidase Negative 4)

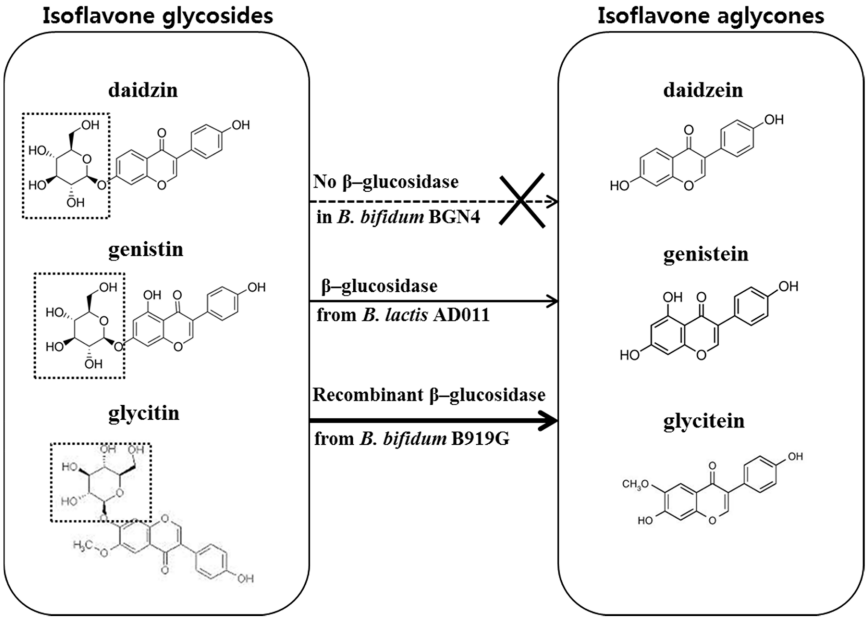

Fig. 6. Reaction scheme of bioconversion of isoflavone glycosides by bifidobacterial strains.

contains no structural gene for β-Glu among the annotated 1,835 coding sequences in the whole genome [42] and does not hydrolyze β-linked glucosides. However, *B. animalis* subsp. *lactis* AD011 (AD011) contains 35 glycoside hydrolase family (GHF)-related genes out of a total of 1,603 genes [11]. Among the three ORFs (BLA_0039, BLA_0893, and BLA_0141), which were annotated as β-Glu from the genome of AD011, BLA_0141 showed high similarity to the β-Glu from *B. breve* clb [19] with 85% identity in amino acid sequence, and was classified as the GHF 1 group of glycoside hydrolase based on the protein domain and family database (http://www.ebi.ac.uk). Additionally, in our preliminary study, only BLA_0141 showed a broad range of hydrolyzing activities on diverse glycosides, including *p*NP-β-D-glucopyranoside and *p*NP-β-D-cellobioside, when expressed in *E. coli* DH5α.

Compared with the expression host (BGN4) and the source strain of the β-glucosidase gene (AD011), B919G showed the highest β-glucosidase activity in the hydrolysis of *p*NPG. The biotransformation of daidzin and genistin standards using the crude enzyme extract from B919G was completed within 4 h, and the bioconversion of daidzin and genistin of soy milk during fermentation of B919G also occurred within 6 h, which was much faster and higher than AD011. To the best of our knowledge, this is the fastest biotransformation of soy milk isoflavones. In most of the studies, bioconversion of the glucosides to the aglycones was incomplete even after 24 h of fermentation [21, 24–26, 31].

Champagne *et al*. [2] suggested that conditions that enhanced the bacterial growth also improved bioconversion levels. They showed that the growth of *Lactobacillus helveticus* up to the viable counts of 8×10^8 CFU/ml could allow the complete conversion to isoflavone aglycones, and the mixed culture with a *Streptococcus thermophilus* strain reduced the effectiveness of *L. helveticus* accompanied by the decrease in cell number. When the bacterial population a below 10^8 CFU/ml and the fermentation time was less than 8 h, isoflavone bioconversion did not go above 50%. In the present study, as shown in the viable cell counts during fermentation of soy milk up to 12 h, the viable cell count of B919G at 12 h was $7.25 \times \log_{10}$ CFU/ml, which was approximately 10-fold lower than that of AD011. Although the cell number of B919G was below 10^8 CFU/ml, 94.2% of daidzin and 94.8% of genistin were converted to the respective aglycones after 6 h of fermentation, compared with 9.6% of daidzin and 16.2% of genistin in the AD011 group. This suggested that the high expression of incorporated β-glucosidase in strain B919G was not dependent on the growth cycle, the viable cell counts, and/or the nutritional environment. Both the wild-type and recombinant BGN4 showed relatively low growth rate in soy milk; however, the biotransformation rate of isoflavones in the B919G-fermented soy milk group was much higher. This could be attributed to the insertion of the highly and constitutively expressed strong promoter 919.

Donkor and Shah [5] evaluated the β-glucosidase activity

of selected commercial probiotic bacteria, including *B. lactis* LAFTI B94. *B. lactis* B94 showed 77% increase in the level of aglycones compared with non-fermented soy milk. Chien *et al*. [3] showed that the β-glucosidase activity in *S. thermophilus*-fermented soymilk was 39.2 mU/ml at 12 h after fermentation and increased to 176.2 mU/ml at 24 h. However, corresponding with the results of Donkor and Shah, no significant increase in the content of isoflavone aglycones was observed during the initial 12 h fermentation. The percentages of daidzein, genistein, and glycitein to the total isoflavone were 37.00%, 28.80%, and 12.44%, respectively, after 24 h. Soy milk prepared using soy protein isolate supplemented with D-glucose and L-cysteine did not enhance the growth of bifidobacteria [32]. The β-glucosidase activity of the B919G enzyme extract was 1.9 U/ml, whereas that of AD011 was 1.1 U/ml. These results were approximately 10 times higher than that reported by Chien *et al*. [3], and 6 h faster than that reported by Marazza *et al*. [18] in which *L. rhamnosus* CRL981 achieved 100% bioconversion within 12 h.

In the present study, the recombinant *B. bifidum* BGN4 strain (B919G) expressing a highly increased level of β-glucosidase was constructed using the expression vector placed under control of the constitutively active promoter 919. The conversion of various glucosides using this recombinant *Bifidobacterium* would be of value in industrial processes. Specifically, the incorporation of this β-glucosidase-producing *Bifidobacterium* strain in soy milk could lead to a production of fermented soy milk with an elevated amount of bioavailable forms of isoflavones as well as to the indigenous probiotic effects of the *Bifidobacterium* strain.

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