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Cloning, Expression, and Characterization of Two β-Glucosidases from Isoflavone Glycoside-Hydrolyzing *Bacillus subtilis* natto

LUN-CHENG KUO AND KUNG-TA LEE*

Institute of Microbiology and Biochemistry, National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei, Taiwan, Republic of China

On the basis of the genomic sequence of *Bacillus subtilis* 168, two β-glucosidase genes (*bglH* and *yckE*) from *B. subtilis* natto, which has been reported to have high isoflavone glucoside-hydrolyzing activity, were cloned and overexpressed in *E. coli* M15. The temperature for the optimal *p*-nitrophenyl-β-D-glucoside hydrolyzing activity of both enzymes was between 37 and 45 °C, but BglH had a higher thermal stability than YckE. Both showed high activity at pH 6.0, but YckE was stable over a wider pH range than BglH. Recombinant BglH was inhibited 73%, 63%, and 43% by 1.0 mM Cd^{2+}, Fe^{2+}, or Cu^{2+}, respectively, while other divalent metal ions resulted in 0–23% inhibition, whereas YckE was inhibited by less than 20% by any of the divalent metal ions we tested. Among the substrate we used, BglH showed the highest affinity for genistin and YckE showed the highest affinity for *p*-nitrophenyl-β-D-fructopyranoside. Both BglH and YckE hydrolyzed genistin and daidzin into their isoflavone aglycones, genistein and daidzein, but BglH was more efficient than YckE in isoflavone glucoside hydrolysis (20-fold higher k_{cat}). Our results suggest that recombinant BglH may be applicable in the process of isoflavones deglycosylation.

KEYWORDS: ***Bacillus subtilis*** **natto; isoflavone; β-glucosidases; BglH; YckE**

INTRODUCTION

Among the foods eaten by humans, soybeans contain the highest concentration of isoflavones. These soy isoflavones (e.g., daidzein and genistein) are implicated in some health-enhancing properties, such as prevention of certain cancers (*1–3*), lowering the risk of cardiovascular diseases (*4, 5*), and improvement in bone health (*6, 7*). In vitro studies of metabolites produced from the total microflora of volunteers have shown that the bioavailability of soybean isoflavonoids depends upon the ability of the gut microflora to metabolize these compounds (*8, 9*).

Numerous studies have shown that the biological effects of isoflavones are not due to the glycoside form, but mainly to their aglycones, such as daidzein and genistein (*10, 11*). Aglycone isoflavones are highly bioactive due to their unimpeded intestinal absorption, unlike their related glycosides, which are not absorbed across enterocytes because of their higher hydrophilicity and molecular weights (*12–14*). Isoflavone aglycones are present in high amounts in soy products, such as miso, natto, and tempeh, produced, respectively, by *Aspergillus oryzae* (*15*), *Bacillus subtilis* natto (*16, 17*), and *Rhizopus oligosporus* (*18*). *Bifidobacteria* β-glucosidase can convert isoflavone glucosides in soymilk to their aglycones (*19*), and β-glucosidase from *Saccharopolyspora erythraea* can hydrolyze genistin during fermentation of soy-based media (*20*). These bacteria with β-glucosidase activity are potentially important in the production of compounds with higher estrogenicity and better absorption, thus affecting their bioavailability and pharmacokinetics.

β-Glucosidase (β-glucoside glucohydrolase, EC 3.2.1.21) is the key enzyme for carbohydrate metabolism in bacteria that are able to cleave the β-glucosidic linkages of di- and/or oligosaccharides or other glucose conjugates (*21*). β-Glucosidases are widely distributed in living organisms and play pivotal roles in many biological processes, such as the degradation of cellulosic biomass (*22*), cyanogenesis (*23*), and the cleavage of glucosylated flavonoids (*24*).

According to previous reports, the isoflavone glycoside-hydrolyzing β-glucosidases of *B. subtilis* natto (*16, 25*) or lactic acid bacteria (*26, 27*) are cell-associated and difficult to purify; as a result, there have been no reports of the biochemical properties of these enzymes. In our previous study (*25*), a strain of *B. subtilis* natto NTU-18 with high isoflavone glycoside-hydrolyzing β-glucosidase activity was selected from commercial natto product (a traditional soy fermentation product in Japan and eastern Asia), and a fermentation process for deglucosylation of black soybean isoflavones was established. In the present study, two β-glucosidase genes (*bglH* and *yckE*) were cloned from *B. subtilis* natto NTU-18 and the characteristics of the recombinant enzymes expressed in *E. coli* were investigated.

* Corresponding author. E-mail: ktlee@ntu.edu.tw. Phone: +886-2-33664436. Fax: +886-2-23640961.

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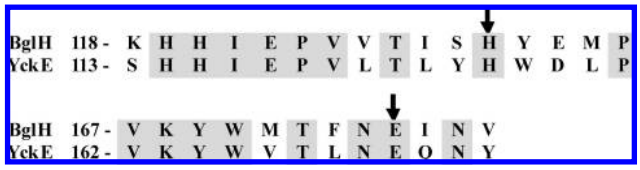

Figure 1. Putative catalytic regions of BglH and YckE. The arrows indicate the conserved catalytic residues.

Table 1. Purification of Recombinant BglH and YckE Expressed in *E. coli* M15[a]

name	purification step	total protein (mg)	total activity (U)	specific activity (U/mg)	purification (fold)	yield (%)
BglH	crude extract[b]	358.0	623.0	1.74	1.0	100.0
	His-binding column[c]	170.8	450.8	2.64	1.5	72.4
YckE	crude extract[b]	421.4	573.2	1.36	1.0	100.0
	His-binding column[c]	168.3	333.0	1.98	1.5	58.1

[a] One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol per minute. [b] The recombinant strain was grown in LB medium with 25 kanamycin μg/mL and 100 amplicillin μg/mL at 37 °C to OD_{600} 1.0 and was incubated further with IPTG at a final concentration of 0.05 mM for 12 h at 25 °C. The cells of 700 mL cultures were harvested by centrifugation at 10 000*g* for 10 min at 4 °C, resuspended in 50 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then disrupted by sonication. [c] The resulting supernatants were loaded onto an Ni-NTA affinity column equilibrated with the binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), and the bound proteins were eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fractions exhibiting enzyme activity were pooled and dialyzed against 100 mM sodium phosphate buffer, pH 6.0.

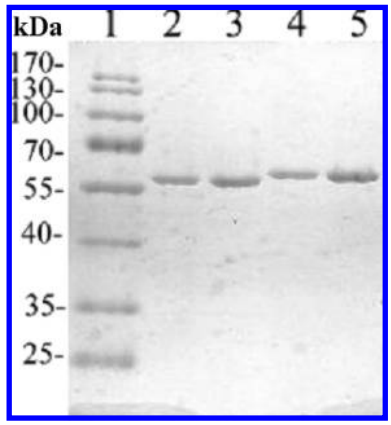

Figure 2. SDS-PAGE analysis of purified recombinant BglH and YckE: BglH (1 or 2 μg; lanes 2 and 3), YckE (1 or 2 μg; lanes 4 and 5), and molecular markers (lane 1) stained with Coomassie Blue.

MATERIALS AND METHODS

Chemicals and Reagents. Genistein, daidzein, genistein-7-*O*-glucoside (genistin), daidzein-7-*O*-glucoside (daidzin), *p*-nitrophenol, *p*-nitrophenyl-β-D-glucoside (*p*NPG), *o*-nitrophenyl-β-D-galactopyranoside (*p*NPGal), *p*-nitrophenyl-β-D-fructopyranoside (*p*NPF), and *p*-nitrophenyl-β-D-cellobioside were purchased from Sigma Chemical Co. (St. Louis, MO). Liquid chromatography grade acetonitrile and reagent grade absolute alcohol were purchased from Merck (Darmstadt, Germany). Nutrient broth (NB) and Bacto-agar were purchased from Difco (Detroit, MI). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from MDBio (Taipei, Taiwan).

Bacterial Strains, Plasmids, and Media. A strain of *B. subtilis* natto NTU-18 with high β-glucosidase activity (with *p*NPG as the substrate) was previously obtained from commercial natto product (*25*). The strain was maintained on nutrient broth (NB) (Difco, Detroit, MI) slants at 4 °C. *Escherichia coli* JM109 (Yeastern Biotech, Taiwan) containing the pGEM-T Easy vector (Promega, USA) was used for plasmid preparation and gene cloning, and the *E. coli* M15 strain (QIAGEN, Hilden, Germany) containing the pQE-30Xa vector (QIAGEN, Hilden, Germany) was used to express 6 × His-tagged recombinant proteins. All *E. coli* cells containing plasmids were grown aerobically in Luria–Bertani (LB) medium (DIFCO, USA) or on LB agar plates at 37 °C, supplemented with ampicillin (100 μg/mL) and (or) kanamycin (25 μg/mL) when necessary.

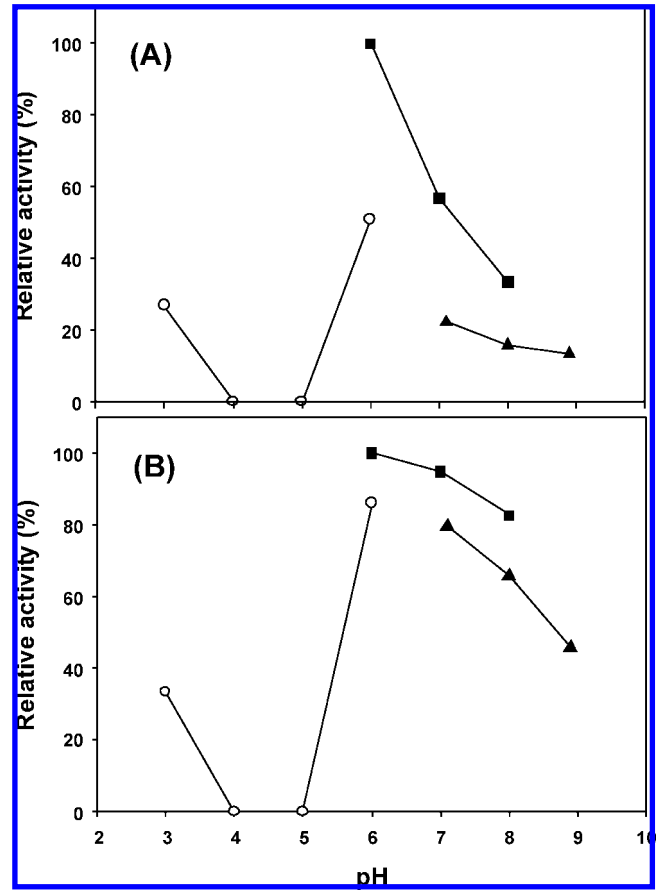

Figure 3. Effect of pH on the activity of recombinant BglH and YckE: (**A**) BglH, (**B**) YckE. The buffers used were the following: pH 3.0–6.0, 100 mM citric acid−citrate buffer (○); pH 6.0–8.0, 100 mM sodium phosphate buffer (■); pH 7.0–9.0, 50 mM Tris-HCl buffer (▲). The optimal pH was determined at 37 °C for 10 min in the 100 mM citric acid−citrate buffer at pH 3.0–6.0, 100 mM sodium phosphate buffer at pH 6.0–8.0, or Tris-HCl buffer at pH 7.0–9.0 with *p*NPG as a substrate.

Construction of the Expression System. Genomic DNA of *B. subtilis* natto NTU-18 was isolated according to Sambrook et al. (*28*) and used as the polymerase chain reaction (PCR) template. To amplify the DNA fragment encoding *bglH*, PCR was performed using the forward primer *bglH*-F (5′GCA**GGATCC**ATGAGTTCAAATGAAAAACGATTTCC-AGAAGG) and reverse primer *bglH*-R (5′CTA**CTGCAG**TCAGA-GACTCTCTCCGTTTGTGGCG), which introduce BamHI and PstI sites, respectively, at the 5′ and 3′-termini of the *bglH* gene. To amplify the DNA fragment encoding *yckE*, PCR was performed using the forward primer *yckE*-F (5′CTG**AGGCCT**ATGATCCACCAGCATCCAGAATC) and reverse primer *yckE*-R (5′CTA**GGATCC**TTATAAACTTTCTC-CGTTTCTCTTG), which introduce StuI and BamHI sites, respectively, at the 5′ and 3′-termini of the *yckE* gene (the enzyme sites are shown in bold). Amplification was carried out in a thermo Px2 thermal cycler machine using the conditions of 5 min of denaturation at 95 °C, 30 cycles for 1 min at 94 °C and 1 min at 55 °C, and a final extension phase at 72 °C for 1 min and 40 s. The PCR products were ligated into the pGEM-T Easy Vector System (Promega, USA), the ligation products were transformed into *E. coli* strain JM109, and the recovered plasmids were confirmed as correct by restriction analysis and DNA sequencing. The

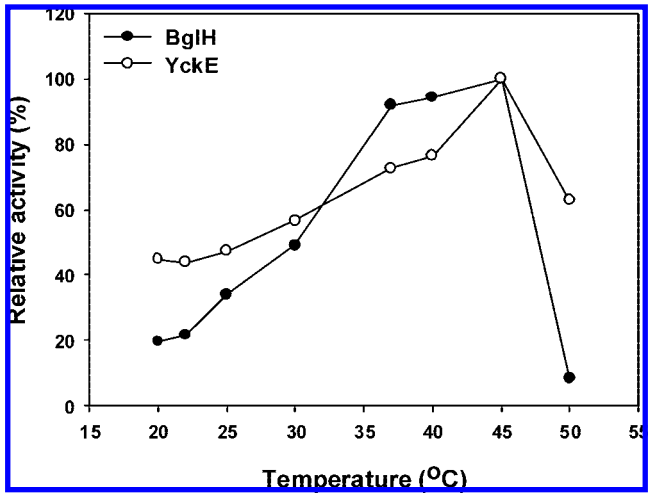

Figure 4. Effect of temperature on the activity of recombinant BglH and YckE: BglH (●), YckE (○). The optimal temperature for the enzyme activity was determined by assay at 20−50 °C in 100 mM sodium phosphate buffer, pH 6.0, with *p*NPG as a substrate.

nucleotide and amino acid sequences were compared to the sequences in the GeneBank at the National Center for Biotechnology Information (Bethesda, MD) using the BLAST network server. Plasmid DNA in the clones was extracted using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), digested appropriately, and ligated into pQE30-Xa (Qiagen, Hilden, Germany) which provides high-level expression in *E. coli* of proteins containing a 6 × His affinity tag at the N-terminus of the protein; then, the ligation products were transformed into *E. coli* strain M15, and the recovered plasmids were confirmed as correct by restriction analysis and DNA sequencing. The resulting plasmids were named pQE-30Xa-BglH and pQE-30Xa-YckE, respectively. DNA manipulations, including digestion with restriction enzymes, purification of DNA fragments, ligation with T4 ligase, and transformation, were performed as described by Sambrook et al. (*28*).

Expression and Purification of the Recombinant β-Glucosidases. *E. coli* M15 harboring the recombinant plasmid pQE-30Xa-BglH or pQE-30Xa-YckE were grown to an A_{600} of 1.0 in LB medium supplemented with 25 μg/mL kanamycin and 100 μg/mL ampicillin at 37 °C, then induced to produce a target protein by adding IPTG at a final concentration of 0.05 mM, followed by incubation for 12 h at 25 °C. All subsequent steps were at 4 °C. The cells were harvested by centrifugation, washed twice with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the cell pellet resuspended in the same buffer and lysed by sonication. The cell lysate was centrifuged at 10 000*g* for 20 min to remove cell debris, and the supernatant was applied to an Ni-NTA affinity column (Qiagen, Hilden, Germany) equilibrated with 1 × binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant proteins were eluted with Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). Fractions containing enzyme activity were pooled and dialyzed against 100 mM sodium phosphate buffer, pH 6.0, and the enzyme preparation was stored at −80 °C. The protein concentration was determined using a Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Electrophoresis Analyses and Protein Assay. The apparent molecular weights of the purified enzymes were determined using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie Brilliant Blue R-250.

Enzyme Assay. The β-glucosidase assay was modified from the work of Choi et al. (*26*). The activity of the purified β-glucosidases was estimated by mixing 50 μL of enzyme with 50 μL of 5 mM *p*NPG in 100 mM sodium phosphate buffer, pH 6.0, incubating the mixture for 10 min at 37 °C, adding 100 μL of 2 M Na_2CO_3 to stop the reaction, measuring the absorbance at 405 nm, and comparing it to a standard curve prepared by measuring the A_{405} of various concentrations of *p*-nitrophenol. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute.

Effect of Temperature on Enzyme Activity and Thermostability of the Enzymes. The optimal temperature for enzyme activity was determined over a temperature range of 20–50 °C using the standard assay conditions. Thermostability was tested by incubating aliquots of enzyme at different temperatures (30, 37, 45, and 50 °C) for different times and, then, assaying the remaining activity under the standard conditions.

Effect of pH on Enzyme Activity. To determine the optimum pH for the enzyme, activities were measured over a pH range of 3.0–9.0 in increments of 1.0 pH unit using the standard assay conditions. The buffers used were a 100 mM citric acid−citrate buffer (pH 3.0–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), and 50 mM Tris-HCl buffer (pH 7.0–9.0).

Substrate Specificity and Kinetic Studies. Substrate specificity was determined by incubating the enzyme at 37 °C for 10 min in 100 mM sodium phosphate buffer, pH 6.0, containing a 0–2.5 mM final concentration of different nitrophenyl substrates or isoflavone glucosides and the Michaelis–Menten kinetic parameters for the purified enzyme determined from substrate saturation assays. The substrates used were *p*NPG, *p*NPGal, *p*NPF, *p*-nitrophenyl-β-D-cellobioside, daidzin, and genistin. The values for the maximum velocity and half-saturation coefficient (K_m) were determined by plotting the substrate concentration vs the initial velocity for each reaction and subjecting the data to nonlinear regression analysis. Kinetic analyses by curve fitting were performed using SigmaPlot software. Values shown are the mean of duplicate experiments with each substrate, and the variation about the mean was below 5%.

HPLC Analyses. HPLC analysis of isoflavones was based on the work of Wang and Murphy (*29*). The HPLC system consisted of a Shimadzu SCL-10Avp system controller, two Shimadzu LC-10ATvp liquid chromatograph pumps, and a Shimadzu SPD-M10Avp photodiode array detector. A Develosil ODS-5 column (250 mm with 4.6 mm packing) was used. The solutions used to generate the gradient were 0.1% (v/v) glacial acetic acid in H_2O (solvent A) and 80% (v/v) acetonitrile (Merck, Darmstadt, Germany) in solvent A (solvent B). Following injection of 20 μL of sample, solvent B was increased linearly from 15% to 70% over 30 min and, then, returned to 15% for 10 min The solvent flow rate was 1.0 mL/min. Elution was monitored by UV absorbance at 262 nm, and the spectral data from 190 to 800 nm of the peaks were recorded to confirm their identity. Peak areas were integrated for quantification. Daidzin, genistin, daidzein, and genistein were identified by comparison with the HPLC retention time and UV spectra of the authentic compounds. The isoflavone concentration of the samples was calculated using calibration curves prepared using various concentrations of the isoflavone standards.

Statistical Analysis. The data of the significantly altered expression in each enzyme assay mean value (from triplicate experiments) with the corresponding mean value of the control and the test expression change using student's *t* test. P values < 0.05 were considered statistically significant.

RESULTS

Gene Cloning of Two β-Glucosidases from *B. subtilis* natto. The *B. subtilis* natto NTU-18 strain was chosen for this β-glucosidase gene cloning study because it efficiently hydrolyzes isoflavone glucosides into isoflavone aglycones (*25*), and this reaction is known to be catalyzed by β-glycosidase (*16*). Two β-glycosidase genes (*bglH* and *yckE*) in *B. subtilis* 168 were identified by searching the NCBI genome database and were amplified from the genome of *B. subtilis* natto NTU-18 by PCR (*30*). The sizes of these two genes were 1410 base pairs (*bglH*) and 1434 base pairs (*yckE*). The BglH contained 470 amino acids, with a calculated molecular weight of 53 kDa. And, the YckE contained 478 amino acids, with a calculated molecular weight of 54 kDa. Alignment of the two nucleotide sequences with the NCBI database, the nucleotide sequence of *bglH* showed 98% and 78% homology with the gene of *B. subtilis* 168 and *B. licheniformis*, respectively, and the nucleotide sequence of *yckE* showed 98% and 78% homology with the

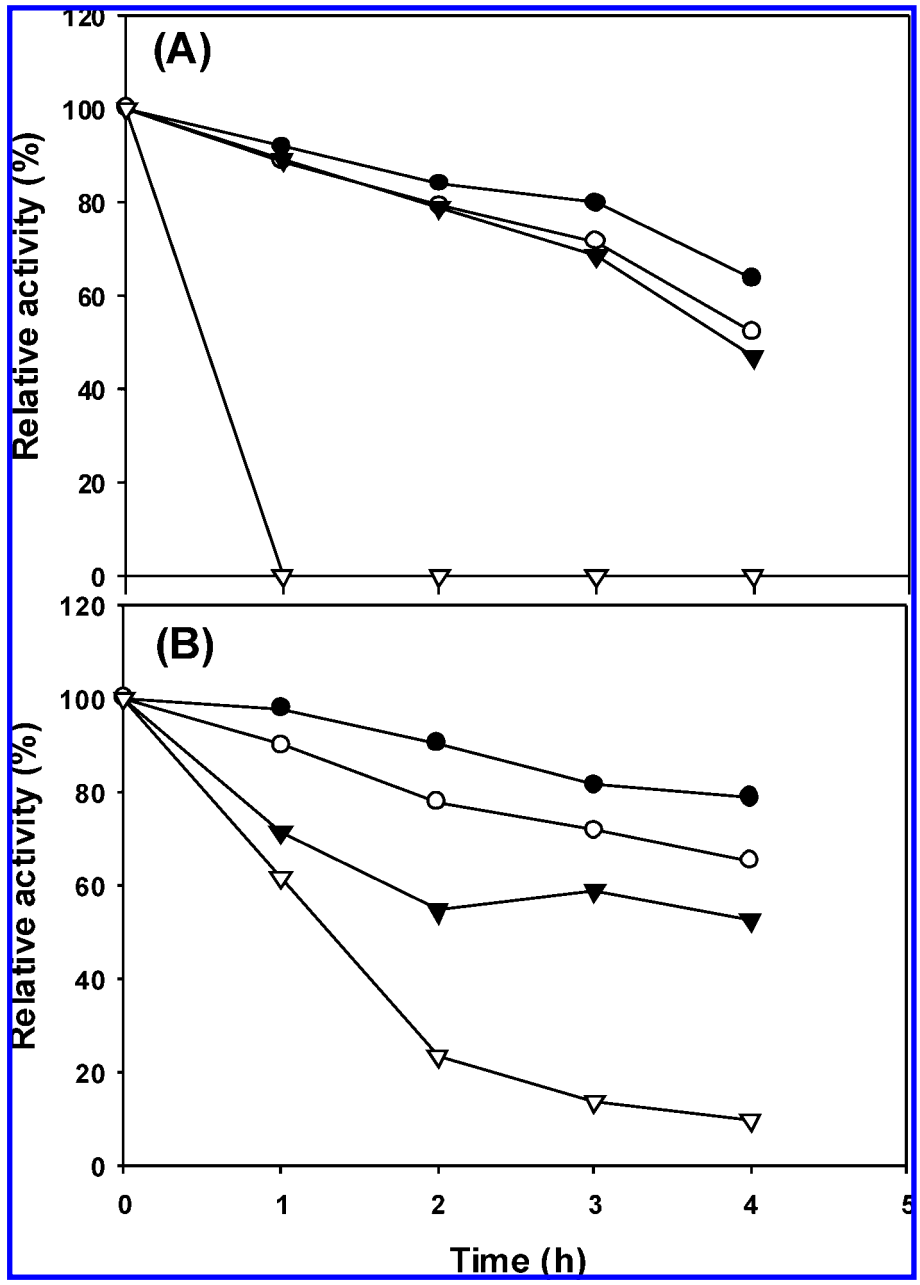

Figure 5. Thermal stability of recombinant BglH and YckE: (**A**) BglH, (**B**) YckE. The purified enzyme in 100 mM sodium phosphate buffer, pH 6.0, was preincubated for various times at 30 (●), 37 (○), 45 (▼), or 50 °C (▽) in the absence of substrate and, then, was assayed under standard conditions with *p*NPG as a substrate. The activity of the enzyme without preincubation is taken as 100%.

gene of *B. subtilis* 168 and *B. amyloliquefaciens*, respectively. The deduced amino acid sequence of BglH and YckE were used to compare with other amino acid sequences deposed in the NCBI database. The BglH exhibits the highest identity score with *B. subtilis* 168 BglH (99% identity), followed by *B. licheniformis* BglH (87%) and *B. amyloliquefaciens* BglH (85%). In the case of YckE, the highest score was also obtained with *B. subtilis* 168 YckE (98% identity), followed by *B. licheniformis* YckE (86%) and *B. amyloliquefaciens* YckE (84%). By comparison of *bglH* and *yckE* in *B. subtilis* natto NTU-18, it showed 56.2% similarity in nucleotide sequence and 43.7% identical in amino acid sequences. For the found catalytic domain of BglH and YckE, we using the SIM-Alignment tool to compare the amino acid sequence of the putative catalytic domain with other β-glucosidases. BglH and YckE were found to contain histidine and glutamate residues in the catalytic domain (**Figure 1**).

Overexpression and Purification of the Two Recombinant β-Glucosidases. To investigate the biochemical properties of BglH and YckE, we expressed the six-histidine N terminal-tagged proteins in *E. coli* M15. Bacteria were transformed with the expression vector and induced with IPTG which expressed the histidine-tagged protein. After treatment of the His-binding column, recombinant BglH was purified 1.5-fold with a 72.4% yield and YckE was purified 1.5-fold with a 58.1% yield (**Table 1**). The apparent molecular weights, determined by SDS-PAGE (12.5% gel), were about 57 and 58 kDa, respectively (**Figure 2**). Using the pQE30-Xa expression system, the N-terminal of the expression protein contains a six-histidine tag and a factor Xa recognition site, resulting in a 4 kDa increase in the molecular weight of the expressed protein. Thus, the actual molecular weight of BglH was 53 kDa and that of YckE was 54 kDa, corresponding to the expected molecular weights.

Table 2. Effect of Metal Salts on the Activity of Recombinant BglH and YckE[a]

substance	relative activity (%)	
	BglH	YckE
control	100	100
$FeSO_4$	36.8	90.5
NaF	76.8	93.4
$MnCl_2$	99.5	85.6
LiCl	76.8	92.7
$MgCl_2$	84.3	95.5
$FeCl_3$	100	81.9
$CuCl_2$	56.8	84.1
$CdCl_2$	26.6	80.7
$CaCl_2$	77.9	92.6

[a] The compound was added into the reaction mixture at the final concentration of 1 mM. The purified enzymes were incubated with each reagent for 10 min before the addition of the *p*NPG substrate solution to initiate the enzyme reaction. The reaction was determined at 37 °C for 10 min in 100 mM sodium phosphate buffer, pH 6.0. The activity without the added reagent was taken as 100%.

Biochemical Characterization of the Two Recombinant β-Glucosidases. To determine the optimal pH for recombinant BglH and YckE, we measured the enzyme activity at 37 °C and various pH values (pH 3.0–9.0), using *p*NPG as substrate. As shown in **Figure 3**, both enzymes showed the highest activity at pH 6.0, but YckE was active over a wider pH range. Meanwhile, precipitation was observed for both the recombinant BglH and YckE treated at pH 4 and 5, and no enzyme activity was detected in this pH range. To determine the optimal temperature, enzyme activity was determined over the range of 20–50 °C. As shown in **Figure 4**, in the temperature range of 35–45 °C, recombinant BglH exhibited more than 95% of its maximal activity, with a sharp decrease above 45 °C, while YckE exhibited about 70–100% of its maximal activity over the same range. To examine the thermal stability of recombinant BglH and YckE, we incubated the enzymes at different temperatures (30, 37, 45, and 50 °C) and measured the residual activity under standard assay conditions. As shown in **Figure 5**, recombinant BglH and YckE were fairly stable up to 45 °C, but the thermal stability profiles of the two recombinant enzymes were very different. Recombinant BglH retained 80% of its activity when incubated at 45 °C for 2 h, but was completely inactivated by incubation at 50 °C for 1 h, whereas recombinant YckE retained 60% of its activity when incubated at 45 °C for 2 h or at 50 °C for 1 h.

The effects of different metal ions (Na^+, Li^+, Fe^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Cd^{2+}, Ca^{2+}, and Fe^{3+}) on enzyme activity were investigated by addition of the test ions to the reaction mixture at a final concentration of 1 mM. The activity was then measured under standard assay conditions and expressed as a percentage of the activity obtained in the absence of the added ion. As shown in **Table 2**, recombinant BglH was inhibited 73%, 63%, and 43% by 1.0 mM Cd^{2+}, Fe^{2+}, or Cu^{2+}, respectively, while other divalent metal ions resulted in 0–23% inhibition, whereas YckE was inhibited by less than 20% by any of the divalent metal ions.

Kinetic Properties of the Two Recombinant β-Glucosidases. To determine the kinetic properties, the enzyme activity of the two recombinant β-glucosidases was assayed by monitoring the hydrolysis of β-glucosides over different substrates and a range of concentrations of these substrates. The substrate specificities of the two recombinant β-glucosidases are summarized in **Table 3**. Recombinant BglH showed a lower Michaelis constant (K_m) and higher k_{cat} for daidzin and genistin than for the other substrates. Comparison of the K_m and k_{cat} values revealed that BglH had about a 16-fold higher affinity for genistin than for *p*-NPG and hydrolyzed the former substrate about 3-fold faster than the latter. The specificity constant of BglH for the isoflavone conjugates was higher than that for the chromogenic substrate *p*-NPG. The catalytic efficiency (k_{cat}/K_m) of the enzyme for different substrates was considered a measurement of the enzyme's specificity (specificity constant). The catalytic efficiency (k_{cat}/K_m) values of recombinant BglH for genistin, daidzin, *p*-NPG, *p*-NPGal, and *p*-NPF were 147.20, 49.19, 3.01, 1.76, and 0.90 1/(mM s), respectively. Among the substrates we tested, recombinant BglH showed the highest catalytic efficiency toward genistin. These results indicated that genistin was clearly the preferred substrate. The catalytic efficiency (k_{cat}/K_m) values of recombinant YckE for genistin, daidzin, *p*-NPG, *p*-NPGal, and *p*-NPF were 19.45, 17.93, 2.44, 1.23 and 306.57 1/(mM s), respectively. The preferred substrate for recombinant YckE was *p*NPF, the catalytic efficiency for *p*NPF being about 150 times that for *p*NPG and 16 times that for genistin. Recombinant YckE also had isoflavone glucoside deglycosylation activity, but its main activity was against *p*NPF. To compare these two recombinant β-glucosidases, YckE had lower K_m values for daidzin and genistin, but BglH's k_{cat} values for daidzin and genistin were 20 times higher; the catalytic efficiency of recombinant BglH for genistin was about 7.7 times higher than that of YckE. BglH therefore hydrolyzed soybean isoflavone glucosides more efficiently than YckE.

DISCUSSION

Two β-glucosidase genes (*bglH* and *yckE*) were cloned from *B. subtilis* natto NTU-18 and expressed in an *E. coli* system for enzyme characterization. β-Glucosidases BglH and YckE of *B. subtilis* natto showed high nucleotide sequence similarity and amino acid sequence identity with the relative genes of *B. subtilis* 168. β-Glucosidases belong to the cellulose family BG and form two subfamilies, BGA and BGB (*31*). The BGA subfamily contains mainly bacterial β-glucosidases with molecular weights of about 50 kDa. BGA subfamily β-glucosidases have a putative catalytic domain containing histidine and glutamate residues (*32, 33*) which has been suggested to be involved in the cleavage of β-glucosidic bonds. The sequence homology with the β-glucosidases of the BGA subfamily (*31–34*) seen using the SIM-Alignment tool suggests that BglH and YckE of *B. subtilis* natto should be classified as members of the BGA subfamily. Activity characterization revealed that the optimal pH values for recombinant BglH and YckE are similar to BglA β-glucosidase, an enzyme discovered in *Bacillus* sp. GL1, which acts on the gellan degradation, was classified as belonging to the BGA subfamily, and has its highest activity at pH 6.0 (*34*). This pH range is not unexpected, as most β-glucosidases from bacterial sources show a pH optimum in slightly acidic or neutral pH ranges (*35*). The recombinant BglH enzyme showed a significant difference in activity between citric acid−citrate buffer and sodium phosphate buffer at pH 6.0 and between sodium phosphate buffer and Tris-HCl buffer at pH 7.0. This phenomenon is similar to the result of the intracellular β-glucosidase of *B. circulan* subsp. *Alkalophilus* which showed that the buffers affected strongly the observed activity and the optimal pH was 6.0 in phosphate buffer (*36*). Inhibition of enzyme activity by Tris has been observed for several glucosidases and has been though to be attributed to changes in conformation or charge distribution (*37*). In addition, the enzymes were completely inactive at pH 4 and 5, while, at pH 3, both enzymes retained 35–40% activity (**Figure 3**). This means that the β-glucosidase activity of the recombinant

Table 3. Kinetic Properties of Recombinant BglH and YckE[a]

substrate	BglH[b]			YckE[c]		
	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM s))	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM s))
daidzein-7-*O*-glucoside (daidzin)	1.00	49.19	49.19	0.27	4.84	17.93
genistein-7-*O*-glucoside (genistin)	0.15	22.08	147.20	0.11	2.14	19.45
p-nitrophenyl-β-D-glucopyranoside	2.35	7.08	3.01	2.00	4.87	2.44
o-nitrophenyl-β-D-galactopyranoside	1.59	2.80	1.76	2.22	2.73	1.23
p-nitrophenyl-β-D-fructopyranoside	1.75	1.57	0.90	0.17	50.89	306.57
p-nitrophenyl-β-D-cellobioside	0	0	0	0	0	0

[a] Values shown are the mean of duplicate experiments with each substrate, and the variation about the mean was below 5%. [b] The purified recombinant BglH (1 mg/mL; 50 μL) was incubated with 50 μL of different nitrophenyl substrates (0–5 mM) at 37 °C for 10 min or isoflavone glucosides (0–5 mM) at 37 °C for 30 min in 100 mM sodium phosphate buffer, pH 6.0. To stop the reaction, to the mixture of enzyme and nitrophenyl substrates was added 100 μL of 2 M Na_2CO_3 buffer. Finally, the absorbance was measured at 405 nm and analyzed by HPLC after adding 100 μL EtOH. [c] The reaction condition of purified recombinant YckE with the above substrates other than *p*NPF was the same as BglH, and it took only 2 min for YckE to react with it.

enzymes of BglH and YckE were lost between pH 4 and 5. We used the ExPASy Proteomics tool to estimate the pI value of the two enzymes and found that the theoretical pI values of the two enzymes are both about pH 4.5. This suggests that this phenomenon was caused by conformation change of the two enzymes in this pH range. Both recombinant BglH and YckE showed that the activities were inhibited by divalent metal ions. Similarly, Hashimoto et al. (*34*) reported that divalent metal ions were 15–20% inhibited on BglA β-glucosidase from *Bacillus* sp. GL1.

In *B. subtilis*, the real function of YckE is still unknown. Meanwhile, the *bglH* gene has been tentatively identified as a β-glucosidase, but the activity of the corresponding gene product has not been directly demonstrated (*38–40*). In this study, the recombinant BglH showed the higher specificity constant for the isoflavone conjugates than that for the chromogenic substrate *p*-NPG. Similarly, the β-glucosidases purified from *Pseudomonas* ZD-8 (*41*), soybean [*Glycine max*] roots (*42*), and *Cicer arietinum* L (*43*) also showed a higher specificity constant for isoflavone conjugates over the generic chromogenic substrate, *p*-NPG. Setlow et al. (*44*) reported that, in *B. subtilis*, *bglH* is induced by β-glucosides and expressed during the late-exponential or stationary phase, while *yckE* is expressed at a low and constant level during growth, sporulation, and spore germination and is not induced by β-glucosides. In our previous study (*25*), the isoflavone glycoside-hydrolyzing β-glucosidases of *B. subtilis* natto were induced by isoflavone glycosides and expressed during the late-exponential phase in black soybean medium. According to these results, BglH may play a more important role than YckE in the deglycosylation of isoflavone glucosides in *B. subtilis* natto during fermentation.

In addition to being applied in cellulose degradation, β-glucosidases could also be used to hydrolyze the phenolic compounds (e.g., phloridzin, arbutin, and salicin) and phytoestrogen glucosides to improve their biological activity, with several uses in the field of medicine, in general biomedical research, and in the food industry (*45*). For example, the hydrolysis of phloridzin by β-glucosidase could liberate the aglycone moiety, which is a precursor of melanin, and the melanin is known to have the functions of reducing the risk of skin cancer and promoting a dark color of hair (*46*). Similarly, the deglycosylation of oleuropein by β-glucosidase could release a pharmacologically active compound hydroxytyrosol, which is used in the prevention of coronary heart disease and cancer (*47*). In order to effectively prepare the valuable drug-materials-free aglycones, it is necessary to isolate and screen new β-glucosidase producing microorganisms (*20*). In this study, we revealed the enzyme properties of recombinant BglH and YckE. Our data show the possibility that these β-glucosidases could be applied in the formation of aglycone of bioactive compounds. These aglycones then might alter the type of sugar attached to them via enzymes such as glycosyltrasferases, to change their bioactivity.

LITERATURE CITED

(1) Cappelletti, V.; Fioravanti, L.; Miodini, P.; Di Fronzo, G. Genistein blocks breast cancer cells in the G(2)M phase of the cell cycle. *J. Cell Biochem.* **2000**, *14*, 594–600.

(2) Miura, T.; Yuan, L.; Sun, B.; Fujii, H.; Yoshida, M.; Wakame, K.; Kosuna, K. Isoflavone aglycon produced by culture of soybean extracts with basidiomycetes and its anti-angiogenic activity. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2626–2631.

(3) Ravindranath, M. H.; Muthugounder, S.; Presser, N.; Viswanathan, S. Anticancer therapeutic potential of soy isoflavone, genistein. *Adv. Exp. Med. Biol.* **2004**, *546*, 121–165.

(4) Anthony, M. S.; Clarkson, T. B.; Hughes, C. L.; Morgan, T. M.; Burke, G. L. Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys. *J. Nutr.* **1996**, *126*, 43–50.

(5) Goodman-Gruen, D.; Kritz-Silverstein, D. Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women. *J. Nutr.* **2001**, *131*, 1202–1206.

(6) Cotter, A.; Cashman, K. D. Genistein appears to prevent early postmenopausal bone loss as effectively as hormone replacement therapy. *Nutr. Rev.* **2003**, *61*, 346–351.

(7) Weaver, C. M.; Cheong, J. M. Soy isoflavones and bone health: the relationship is still unclear. *J. Nutr.* **2005**, *135*, 1243–1247.

(8) Joannou, G. E.; Kelly, G. E.; Reeder, A. Y.; Waring, M.; Nelson, C. A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. *J. Steroid Biochem. Mol. Biol.* **1995**, *54*, 167–184.

(9) Kelly, G. E.; Joannou, G. E.; Reeder, A. Y.; Nelson, C.; Waring, M. A. The variable metabolic response to dietary isoflavones in humans. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 40–43.

(10) Kawakami, Y.; Tsurugasaki, W.; Nakamura, S.; Osada, K. Comparison of regulative functions between dietary soy isoflavones aglycone and glucoside on lipid metabolism in rats fed cholesterol. *J. Nutr. Biochem.* **2005**, *16*, 205–212.

(11) Hendrich, S. Bioavailability of isoflavones. *J. Chromatogr., B Analyt. Technol. Biomed. Life Sci.* **2002**, *777*, 203–210.

(12) Setchell, K. D.; Brown, N. M.; Zimmer-Nechemias, L.; Brashear, W. T.; Wolfe, B. E.; Kirschner, A. S.; Heubi, J. E. Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am. J. Clin. Nutr.* **2002**, *76*, 447–453.

(13) Brown, J. P. Hydrolysis of glycosides and esters. In *Role of the gut flora in toxicity and cancer*; Rowland, I. R., Ed.; Academic: San Diego, 1998; pp 109–144.

(14) Xu, X.; Harris, K. S.; Wang, H. J.; Murphy, P. A.; Hendrich, S. Bioavailability of soybean isoflavones depends upon gut microflora in women. *J. Nutr.* **1995**, *125*, 2307–2315.

(15) Chiou, R. Y.; Cheng, S. L. Isoflavone transformation during soybean koji preparation and subsequent miso fermentation supplemented with ethanol and NaCl. *J. Agric. Food Chem.* **2001**, *49*, 3656–3660.
(16) Ibe, S.; Kumada, K.; Yoshiba, M.; Onga, T. Production of natto which contains a high level of isoflavone aglycons. *Nippon Shofuhin Kagaku Kogaku Kaishi* **2001**, *48*, 27–34.
(17) Toda, T.; Uesugi, T.; Hirai, K.; Nukaya, H.; Tauji, K.; Ishida, H. New 6-*O*-acyl isoflavone glycosides from soybeans fermented with Bacillus subtilis (natto). I. 6-*O*-succinylated isoflavone glycosides and their preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet. *Biol. Pharm. Bull.* **1999**, *22*, 1193–1201.
(18) Slavin, J. L.; Karr, S. C.; Hutchins, A. M.; Lampe, J. W. Influence of soybean processing, habitual diet, and soy dose on urinary isoflavonoid excretion. *Am. J. Clin. Nutr.* **1998**, *68*, 1492–1495.
(19) Tsangalis, D.; Ashton, J. F.; McGill, A. E.; Shah, N. P. Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase-producing bifidobacteria. *J. Food Sci.* **2002**, *67*, 3104–3113.
(20) Hessler, P. E.; Larsen, P. E.; Constantinou, A. I.; Schram, K. H.; Weber, J. M. Isolation of isoflavones from soy-based fermentations of the erythromycin-producing bacterium Saccharopolyspora erythraea. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 398–404.
(21) Esen, A. β-Glucosidase, overview. In *β-Glucosidase: Biochemistry and Molecular Biology*. Esen A., Ed.; American Chemical Society: Washinton, DC, 1993; pp 1–13.
(22) Ghosh, P.; Pamment, N. B.; Martin, W. R. B. Simultaneous saccharification and fermentation of cellulose: Effect of beta-D-glucosidase activity and ethanol inhibition of cellulases. *Enzyme Microb. Technol.* **1982**, *4*, 425–430.
(23) Lei, V.; Amoa-Awua, W. K.; Brimer, L. Degradation of cyanogenic glycosides by Lactobacillus plantarum strains from spontaneous cassava fermentation and other microorganisms. *Int. J. Food Microbiol.* **1999**, *53*, 169–184.
(24) Marotti, I.; Bonetti, A.; Biavati, B.; Catizone, P.; Dinelli, G. Biotransformation of common bean (Phaseolus vulgaris L.) flavonoid glycosides by bifidobacterium species from human intestinal origin. *J. Agric. Food Chem.* **2007**, *55*, 3913–3919.
(25) Kuo, L. C.; Cheng, W. Y.; Wu, R. Y.; Huang, C. J.; Lee, K. T. Hydrolysis of black soybean isoflavone glycosides by Bacillus subtilis natto. *Appl. Microbiol. Biotechnol.* **2006**, *73*, 314–320.
(26) Choi, Y. B.; Kim, K. S.; Rhee, J. S. Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria. *Biotechnol. Lett.* **2002**, *24*, 2113–2116.
(27) Matsuda, S.; Norimoto, F.; Matsumoto, Y.; Ohba, R.; Teramoto, Y.; Ohta, N.; Ueda, S. Solubilization of a novel isoflavone glycosidehydrolyzing β-glucosidase from Lactobacillus casei subsp. rhamnosus. *J. Ferment. Bioeng.* **1994**, *77*, 439–441.
(28) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning; a laboratory manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(29) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.
(30) Kunst, F.; Ogasawara, N.; Moszer, I.; Albertini, A. M.; Alloni, G.; Azevedo, V.; Bertero, M. G.; Bessières, P.; Bolotin, A.; Borchert, S.; Borriss, R.; Boursier, L.; Brans, A.; Braun, M.; Brignell, S. C.; Bron, S.; Brouillet, S.; Bruschi, C. V.; Caldwell, B.; Capuano, V.; Carter, N. M.; Choi, S. K.; Codani, J. J.; Connerton, I. F.; Danchin, A.; et al. The complete genome sequence of the gram-positive bacterium Bacillus subtilis. *Nature* **1997**, *390*, 249–256.
(31) Beguin, P. Molecular biology of cellulose degradation. *Annu. Rev. Microbiol.* **1990**, *44*, 219–248.
(32) Gräbnitz, F.; Rücknagel, K. P.; Seiss, M.; Staudenbauer, W. L. Nucleotide sequence of the Clostridium thermocellum bglB gene encoding thermostable beta-glucosidase B: homology to fungal beta-glucosidases. *Mol. Gen. Genet.* **1989**, *217*, 70–76.
(33) Baird, S. D.; Hefford, M. A.; Johnson, D. A.; Sung, W. L.; Yaguchi, M.; Seligy, V. L. The Glu residue in the conserved Asn-Glu-Pro sequence of two highly divergent endo-beta-1,4-glucanases is essential for enzymatic activity. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 1035–1039.
(34) Hashimoto, W.; Miki, H.; Nankai, H.; Sato, N.; Kawai, S.; Murata, K. Molecular cloning of two genes for β-D-glucosidase in Bacillus sp. GL1 and identification of one as a gellan-degrading enzyme. *Arch. Biochem. Biophys.* **1998**, *360*, 1–9.
(35) Gekas, V.; Lopez-Levia, M. H. Hydrolysis of lactose. *Process Biochem.* **1985**, *20*, 2–12.
(36) Paavilainen, S.; Hellman, J.; Korpela, T. Purification, characterization, gene cloning, and sequencing of a new beta-glucosidase from Bacillus circulans subsp. alkalophilus. *Appl. Environ. Microbiol.* **1993**, *59*, 927–32.
(37) Patchett, M. L.; Daniel, R. M.; Morgan, H. W. Purification and properties of a stable β-glucosidase from an extremely thermophilic anaerobic bacterium. *Biochem. J.* **1987**, *243*, 779–787.
(38) Krüger, S.; Hecker, M. Regulation of the putative bglPH operon for aryl-β-glucoside utilization in Bacillus subtilis. *J. Bacteriol.* **1995**, *177*, 5590–5597.
(39) Krüger, S.; Gertz, S.; Hecker, M. Transcriptional analysis of bglPH expression in Bacillus subtilis: evidence for two distinct pathways mediating catabolite repression. *J. Bacteriol.* **1996**, *178*, 2637–2644.
(40) Minami, Y.; Kanafuji, T.; Miura, K. Purification and characterization of a β-glucosidase from Polygonum tinctorium, which catalyzes preferentially the hydrolysis of indican. *Biosci. Biotechnol. Biochem.* **1996**, *60*, 147–149.
(41) Yang, L.; Ning, Z. S.; Shi, C. Z.; Chang, Z. Y.; Huan, L. Y. Purification and characterization of an isoflavone-conjugates-hydrolyzing β-glucosidase from endophytic bacterium. *J. Agric. Food Chem.* **2004**, *52*, 1940–1944.
(42) Hsieh, M. C.; Graham, T. L. Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **2001**, *58*, 995–1005.
(43) Hósel, W.; Barz, W. β-glucosidase from Cicer Arietinum L.: purification and properties of isoflavone 7-O-glucoside-specific β-glucosidases. *Eur. J. Biochem.* **1975**, *57*, 607–616.
(44) Setlow, B.; Cabrera-Hernandez, A.; Cabrera-Martinez, R. M.; Setlow, P. Identification of aryl-phospho-β-D-glucosidases in Bacillus subtilis. *Arch. Microbiol.* **2004**, *181*, 60–67.
(45) Bhatia, Y.; Mishra, S.; Bisaria, V. S. Micorbial β-glucosidasees: cloning, properties and applications. *Crit. Rev. Biotechnol.* **2002**, *22*, 375–407.
(46) Ridgway, T. J.; Tucker, G. A. Prospects for the production and use of new improved dietary oestrogens for cardioprotection. *Biochem. Soc. Trans.* **1997**, *25*, 59–63.
(47) Briante, R.; Patumi, M.; Febbraio, F.; Nucci, R. Production of highly purified hydroxytyrosol from Olea europaea leaf extract biotransformed by hyperthermophilic beta-glycosidase. *J. Biotechnol.* **2004**, *111*, 67–77.

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