Molecular cloning and characterization of a novel β-glucosidase with high hydrolyzing ability for soybean isoflavone glycosides and glucose-tolerance from soil metagenomic library

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HIGHLIGHTS

► A novel β-glucosidase (Bgl1269) was obtained from a soil metagenomic library.
► Bgl1269 had high hydrolyzing ability for soybean isoflavone glycosides.
► Bgl1269 exhibited a very high glucose-tolerance (Ki = 4.28 M).
► Bgl1269 is a good candidate in the production of soybean isoflavone aglycones.

ABSTRACT

A novel β-glucosidase (Bgl1269) was identified from a metagenomic library of mangrove soil by activity-based functional screening. Sequence analysis revealed that Bgl1269 encodes a protein of 422 amino acids. After being overexpressed in Escherichia coli and purified, the enzymatic properties of Bgl1269 were investigated. The recombinant enzyme displayed a pH optimum of 6.0 and a temperature optimum of 40 °C, and the addition of most common metal ions (1 or 10 mM) increased the enzymatic activity evidently. In addition, the enzyme showed high hydrolyzing ability for soybean isoflavone glycosides, and 0.8 unit of enzyme could completely converted daidzin and genistin (0.5 mg/mL) to daidzein and genistein at 40 °C for 0.5 h. Interestingly, Bgl1269 also exhibited a very high glucose-tolerance, with the highest inhibition constant Ki (4.28 M) among β-glucosidases reported so far. These properties make it a good candidate in the production of soybean isoflavone aglycones after further study.

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1. Introduction

Isoflavones are diphenolic secondary metabolites of plants, and have the highest content in soybeans. Many pharmacological effects of isoflavones have been investigated, and results revealed that isoflavones could prevent certain cancers (Ravindranath et al., 2004), lower the risk of cardiovascular diseases (Goodman-Gruen and Kritz-Silverstein, 2001), and improve bone health (Cotter and Cashman, 2003). Furthermore, numerous studies have indicated that the biological effects of isoflavones are mainly from their aglycones, such as daidzein and genistein instead of their glycosides form. However, most of isoflavones in natural food materials exist as glycosylated form and are difficult to absorb in the intestines (Izumi et al., 2000). Therefore, to obtain good absorb-ability and pharmacological effects, glycosidic forms of isoflavones need to be converted to isoflavone aglycones.

β-Glucosidases (β-D-glucoside glucohydrolase, EC3.2.1.21), which catalyze the hydrolysis of β-glucosidic linkages of various oligosaccharides and glycosides to form glucose and a shorter/debranched oligosaccharide, have attracted considerable interests in recent years due to their important roles in various biotechnological processes, such as hydrolysis of glucosides of isoflavones (Matsuura and Obata, 1993), the production of fuel ethanol from agricultural residues (Coughlan, 1985), the release of aromatic compounds from flavorless precursor, and improvement of the organoleptic properties in fruit juices (Gueguen et al., 1996). Among abovementioned biotechnological processes, hydrolysis of glucosides of isoflavones is an application with highly commercial value. Recently, many researchers have investigated the transformation of isofalvone glycosides to aglycones using β-glucosidases from the microorganisms Aspergillus oryzae (Horri et al., 2009), Escherichia coli (Ismail and Hayes, 2005), Bacillus subtilis (Kuo and Lee, 2008), Thermotoga maritime (Xue et al., 2009),
Paecilomyces thermophila (Yang et al., 2009). However, the hydrolysis ability of these β-glucosidases was still not satisfying, and their activity and conversion efficiency of glucosidic isoflavones were further reduced due to their sensitive to glucose inhibition. Furthermore, all these enzymes were from cultured microorganisms, and little attention had been paid to β-glucosidases from unculturable microorganisms, which account for over 99% of microorganisms in the environment (Amann et al., 1995). It is imaginable that there is a great deal of industry-potential β-glucosidases for the hydrolysis of glucosides of isoflavones in the unculturable microorganisms of environment.

Soil is an important source of many useful biocatalysts since microbial diversity per one gram of soil is in the range of a million species by computing estimates (Curtis and Sloan, 2005). However, only less than 1% of the microorganisms in soil can be cultured using conventional methods (Amann et al., 1995). To discover novel biocatalysts from unculturable microorganisms in the environment, a new strategy that involves the direct cloning of the total microbial genome (metagenome) from environmental samples into a cultivable bacterium, such as E. coli, has been developed recently (Handelsman et al., 1998). Metagenomic approach has been successfully employed in the isolation and identification of enzymes with novel biocatalytic activities or secondary metabolites from the unculturable organisms of various environmental samples (Banik and Brady, 2010), and several β-glucosidases obtained via metagenomic strategies have been reported (Bao et al., 2012; Fang et al., 2010; Feng et al., 2009; Jiang et al., 2011;). Nevertheless, the hydrolysis ability of these enzymes for isoflavone glucosides was not investigated. Therefore, screening novel β-glucosidases with high conversion efficiency for glycosylated form of soybean isoflavones and good enzymatic properties (such as high glucose-tolerance) from soil by metagenomic approach is still urgently in demand.

In the present study, a metagenomic library from soil samples of Shenzhen Mangrove Reserve was constructed for the screening novel β-glucosidases. Finally, one glucose-tolerant β-glucosidase with high hydrolyzing ability for isoflavone glucosides was identified and expressed in E. coli. Furthermore, the enzymatic properties of the enzyme were also investigated after purification. This novel enzyme displayed some excellent enzymatic properties, and thus was considered as a good candidate of β-glucosidases in the production of soybean isoflavone aglycones after further study.

2. Methods

2.1. Chemicals and reagents

Esculin, ferric ammonium citrate, β-Nitrophenyl-β-glucopyranoside (pNPG), and isoflavone standards (daidzin and genistin) were purchased from Sigma (St. Lois, MO, USA). All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

2.2. Microbial strains, vectors and cultural conditions

E. coli DH5α (Invitrogen, Carlsbad, CA, USA) was used as the host for gene cloning, and E. coli BL21 (DE3) (Novagen, Madison, WI, USA) was used for protein expression. The pUC118 (TaKaRa, Dalian, China) and pET-32a (+) (Novagen) were used to construct metagenomic libraries and express the target protein, respectively. E. coli transformants were grown at 37 °C in Luria–Bertani (LB) broth with ampicillin (100 μg/mL).

2.3. Isolation and partial purification of soybean isoflavone conjugates

Isoflavone conjugates were prepared as follows: Soybean seeds (50 g) were ground to a fine powder with a coffee grinder, and the powder was extracted with 100 mL of chloroform for 30 min with stirring. The slurry was filtered and washed two times with chloroform. The defatted soybean flour was extracted twice with 50 mL of distilled water. The aqueous extract (100 mL) was clarified by using a C18 PrepSep column. The crude aqueous preparation was passed through the column, and the column was washed successively with water and 1 mL of 20% acetonitrile to release bound daidzin, genistin, genistein, and daidzein. The above soybean extracts was regarded as partially purified soybean isoflavones, and was used for the component analysis of isoflavones by high-performance liquid chromatography (HPLC) and hydrolyzing experiments by Bgl1269.

2.4. DNA manipulation

Conventional DNA manipulations were carried out according to standard techniques or manufacturer’s recommendations. Restriction enzymes, T4 DNA ligase and PrimeSTAR HS DNA polymerase were purchased from TaKaRa. Each enzyme was used according to the recommendations of the manufacturer. Plasmids were prepared from E. coli DH5α by using a QIAquick Gel Extraction Kit according to the manufacturer’s instructions (QIAGEN, Hilden, Germany). DNA fragments were isolated from agarose gels by using a QIAquick Gel Extraction Kit (QIAGEN). Electroporation was performed with a Gene-Pulser II electroporation apparatus (Bio-Rad, Hercules, CA, USA).

2.5. DNA extraction from soil samples

The topsoil samples (5–10 cm depth) were collected from Shenzhen Mangrove Reserve of Shenzhen city (22° 31’ 26” N, 114° 0’ 47” E), Guangdong province of China. Samples were stored at –80 °C until the DNA extraction was performed. Extraction of the total genomic DNA from soil samples was performed using QIAamp DNA stool Mini kit according to the recommendations of suppliers (QIAGEN).

2.6. Construction of metagenomic libraries and screening for β-glucosidase gene

The metagenomic library was constructed from environmental DNA isolated from soil samples of Shenzhen Mangrove Reserve. DNA fragments (3 to 10 kb) obtained after partial HindIII digestion were inserted into the pUC118 vector, which had been previously digested with HindIII and dephosphorylated with calf intestine alkaline phosphatase (CIAP). E. coli DH5α was transformed via electroporation with the library and cultured on LB agar plates containing 0.2% esculin (w/v), 0.05% ferric ammonium citrate (w/v), and 100 μg/mL ampicillin at 37 °C overnight. Those colonies forming clear black halos were selected as positive colonies (Eberhart et al., 1964). Then, positive clones were used to prepare plasmid DNA and sequenced on ABI 377 DNA sequencer.

2.7. Analysis of β-glucosidase gene

The open reading frame search from DNA sequences was carried out using ORF-finder (NCBI) (http://www.ncbi.nlm.nih.gov), and database homology search was performed with BLAST program provided by NCBI. Furthermore, the multiple amino acid sequence alignment and the conserved patterns of discrete amino acid sequences of Bgl1269 and known the most homologous
2.8. Cloning and overexpression of the β-glucosidase gene in E. coli and purification of the recombinant protein

The putative β-glucosidase activity gene was amplified from the pUC118-bgl1269 plasmid by using the primers introduced BamHI and HindIII restriction sites for cloning to the pET-32a (+). The following primers were used: bgl1269-F (5′-CGCGGATCCATGGAATGG CAATTCCCCACAAT-3′) and bgl1269-R (5′-CCC AAGCTTCTTGGAG CTAACATTTCTGTTGACGT-3′). The underlined sequences represent the recognition sites of restriction enzymes BamHI and HindIII, respectively. The PCR product was digested with BamHI and HindIII and then inserted into EcoRI/HindIII-treated expression vector pET-32a (+) and transformed into E. coli BL21 (DE3), and positive transformants formed black halos on LB plates containing esculin and ferric ammonium citrate. Then the E. coli BL21 strain harboring the recombinant plasmid was grown in a 250 mL flask containing 50 mL of LB (100 μg/mL ampicillin) until the cell concentration (OD600) reached to 1.3. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to final concentration of 1.2 mM, and the culture was incubated at 25 °C for 16 h with shaking at 200 rpm. Cells were then collected by centrifugation (6000 g, 10 min) at 4 °C and suspended in binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris–HCl, pH 7.9). The cells were disrupted by sonication, and the supernatant was collected by centrifugation at 13,000 g for 10 min at 4 °C. The sample was loaded onto a Ni–NTA His Bind column (Novagen) pre-equilibrated with binding buffer. Then the column was washed with binding buffer and washing buffer (0.5 M NaCl, 60 mM imidazole, 20 mM Tris–HCl, pH 7.9). Finally, the bound protein was eluted with eluting buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The molecular mass of the denatured recombinant protein and the purity of the recombinant protein was determined using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were stained with Coomassie brilliant blue G-250. Protein marker (TaKaRa) was used to estimate the molecular weight of the enzyme subunit.

2.9. Enzymatic activity analysis of β-glucosidase

β-Glucosidase activity was assayed in micro-titre plates using pNPB as substrate (Parry et al., 2001). The reaction mixture (100 μl) containing 25 μl of appropriately diluted enzyme, 25 μl of pNPB (10 mM) and 50 μl of sodium citrate buffer (50 mM, pH 6.0) was incubated at 40 °C for 10 min in dark. The reaction was stopped by addition of 50 μl of 1 M Na2CO3 and the developed color was read at 405 nm using ELISA reader. The intensity of developed color was quantified using standard curve of p-nitrophenol. One unit of enzymatic activity (U) was defined as that the amount of enzyme that produced 1 μmol of p-nitrophenol per minute in the reaction mixture under these analysis conditions.

2.10. Effect of pH and temperature on enzyme activity

The optimal pH of the enzyme was measured using pNPB as a substrate at 40 °C and pH range of 3.0–11.0. The buffers used for the measurement were listed as below: 50 mM citrate–citrate sodium buffer (pH 3.0–6.0), 50 mM phosphate buffer (pH 7.0–8.0), 50 mM glycine–sodium hydroxide buffer (pH 9.0–11.0). The pH stability was investigated under standard assay conditions after incubation of the purified enzyme for 30 min at 25 °C in the above buffer systems in the absence of substrate. In the same way, the optimum temperature was determined by measuring enzymatic activity at pH 6.0 in the temperature range of 20–60 °C. Temperature stability was measured by analyzing residual activity after incubation of aliquots of enzyme for 30 min at different temperatures.

2.11. Effect of metal ions and chemical reagents on enzyme activity

After pre-incubating the enzyme solutions containing each individual metal ions or chemical reagents at pH 6.0 and 50 mM Tris–HCl buffer at 25 °C for 30 min, substrate pNPG (10 mM) was then added, and the enzyme activity was measured under standard conditions. The relative activity was defined as the relative value to the activity of control without metal ions and chemical reagents.

2.12. Determination of kinetic parameters

Michaelis–Menten kinetic parameters for activity of purified enzyme were determined from substrate saturation assays by using pNPB ranging from 0.5–2.5 μM as substrate. Values for the maximum velocity (Vmax) and half-saturation coefficient (Km) were determined by plotting the substrate concentration vs. the initial velocity of each reaction and subjecting the data to nonlinear regression analysis. Kinetic analyses by curve fitting were performed with the SigmaPlot software (Systat Software, Chicago, IL, USA). Furthermore, the glucose inhibition constant (Ki) was determined by fitting to Dixon plots using enzyme solution and various concentrations of glucose solutions (1.3, 1.6, 2.0, 2.3, 2.6, 3.0, 3.3, 3.6 M) with two concentrations of pNPB (5 and 7 mM) as substrate (Dixon, 1953). The mixture solutions (25 μl) containing 1 μl of enzyme solution and 24 μl of various concentrations of glucose solutions were firstly incubated at 25 °C for 1 h. Then, each pre-incubation mixture solution was mixed with 25 μl of pNPB (5 or 7 mM) and 50 μl of sodium citrate buffer (50 mM, pH 6.0), which contain same concentration of glucose. The absorbency (the reaction velocity) of reaction mixture was measured at 405 nm, and the Kf value was analyzed by plotting the reciprocal of the reaction velocity and glucose concentration at each pNPB concentration.

2.13. HPLC analysis

HPLC analysis was used to measure the amounts of daidzin and genistin of samples, and thus investigated the hydrolyzing ability of Bgl1269 to soybean isoflavone extracts and commercial products of daidzin and genistin. HPLC analyses were performed on a Diamonsil C18 column (5 μm, 250 mm × 4.6 mm) using an acetonitrile: phosphate buffer (100 mM, pH 3.0; 70:30, v/v). The solvent flow rate was 0.8 mL/min and the absorption was measured at 260 nm. The instrument used was an HP1100 HPLC system. To examine the changes of daidzin and genistin content, 40 μl of partially purified soybean extracts and commercial products of daidzin and genistin (0.5 mg/mL, Sigma) were respectively incubated with 400 μl of Bgl1269 (2 U/mL) for 30 min at 40 °C, and then 500 μl of methanol containing 1000 ppm benzoic acid was added to stop the reaction. The reaction mixture was centrifuged to remove insoluble substances, and the supernatant was used to the analysis of daidzin and genistin content by HPLC. A same procedure was used to analyze the hydrolyzing ability of Bgl1269 to commercial products of daidzin and genistin.

2.14. Effect of glucose on enzyme activity

The inhibitory effect of glucose on enzyme activity was investigated according to the method reported by Uchima et al. (2012). In brief, 24 μl of various concentrations of glucose solutions (0.1, 0.3, 0.6, 1.0, 1.3, 1.6, 2.0, 2.3, 2.6, 3.0, 3.3, and 3.6 M) were pre-incubated with 1 μl of enzyme solution for 1 h at room temperature, respectively. Then, each pre-incubation mixture was mixed with 25 μl of pNPB (10 mM) and 50 μl of sodium citrate buffer
(50 mM, pH 6.0), which contain same concentration of glucose. The residual enzyme activity of reaction mixture was measured under standard conditions. The relative activity was defined as the relative value to the activity of the control without glucose.

2.15. Nucleotide sequence accession number

The nucleotide sequence data reported here have been submitted to the nucleotide sequence databases (GenBank) under accession number (J95756).

3. Results and discussion

3.1. Construction of a metagenomic library and screening for clones with β-glucosidase activity

A metagenomic library was constructed using DNA isolated from soil samples of Shenzhen Mangrove Reserve for obtaining clones with β-glucosidase activity. Restriction analysis of 20 randomly selected clones indicated that 90% of clones contained different inserts of 3 to 10 kb in size, with an average size of 5 kb. The results of restriction analysis suggested that a metagenomic library with high quality and diversity was constructed. Out of about 30,000 clones, one positive clone showing a brown-black halo was identified. The plasmid of this clone was extracted and an insert of about 6 kb was sequenced. An ORF-finder and BlastX analysis revealed that presence of an open reading frame of 1269 bp which encodes a glycoside hydrolase family 1 (GH family 1) protein (Bgl1269) of 422 amino acids. A protein blast (Blastp) search in the databases of NCBI indicated that the protein has the highest similarity (74%) with the β-glucosidase from *Paenibacillus mucilaginosus* 3016. Multiple sequence alignments of Bgl1269 with nine most homologous β-glucosidases and flavonoid β-glucosidases from GH family 1 allowed the identification of the active site (Fig. 1). Amino acid sequence comparisons revealed that Bgl1269 shared many conserved amino acid residues with other known β-glucosidases from GH family 1 (Fig. 1, the arrowhead denoted the active site).

3.2. Overexpression and purification of recombinant enzyme

The *bgl1269* gene was inserted into the expression vector pET-32a (+) and expressed in *E. coli* BL21 (DE3) and induced with 1.2 mM IPTG at 25 °C. The cells were harvested and disrupted by sonication in ice-water bath. The cell lysates were found fully clear, and no inclusion bodies were formed, which suggested that the recombinant enzyme was highly soluble. The recombinant enzyme was purified by Ni-NTA chromatography, and then cell debris and supernatant from cell lysates as well as purified protein were applied to SDS–PAGE (Fig. 2) together to determine the molecular mass of recombinant protein. The calculated molecular weight of Bgl1269 is about 48 kDa, and the molecular weight of an N-terminal fusion of 156 amino acids is about 18 kDa corresponding to thioredoxin tag (Trx Tag), polyhistidine tag (His Tag), S tag epitope (S tag), and a unique thrombin cleavage site (thrombin), and thus the total molecular weight of the recombinant protein should be about 66 kDa. In Fig. 2, a clear protein band of approximately 66 kDa was observed (Lane 3), which was in accordance with the predicted molecular weight. Next, the purified recombinant protein was used to investigate its biochemical properties.

3.3. Effect of pH and temperature on catalytic activity and stability of Bgl1269

The enzymatic activity of recombinant Bgl1269 was measured at various pH values (pH 3.0–11.0) using pNPG as substrate. The pH–activity profile of the enzyme was bell-shaped. The enzyme displayed high activity (more than 80%) at pH values between 6.0 and 8.0 with a pH optimum of 6.0. Moreover, the enzyme was found to be very stable in the range of pH 6.0–9.0. In addition, the optimal temperature of recombinant Bgl1269 was 40 °C, and the enzyme showed high thermostability under 40 °C. However, its stability was drastically reduced over 40 °C, and the remained activity was only 20% of maximum activity after pre-incubation of the enzyme for 30 min at 45 °C. Because most applications of β-glucosidases require higher temperature (50 °C or above), interests in identifying novel β-glucosidases with high optimal temperature and thermostability have been increasing in the last decade. Recently, several thermostable β-glucosidases have been reported (Dotsenko et al., 2012; Krisch et al., 2012; Yan et al., 2012). Comparing to Bgl1269, these enzymes displayed higher optimal temperature (65 °C–70 °C) and better thermostability. However, one of important drawbacks of these enzymes is their low glucose tolerance, with a *k* value of 0.60 mM (Dotsenko et al., 2012) and 8 mM (Krisch et al., 2012), respectively. Therefore, these galactosidases are not still enough satisfying for biotechnological application. To increase the application potential of Bgl1269, the immobilization and direct evolution work of improving its optimal temperature and thermostability is now under study in this laboratory.

3.4. Effect of metal ions and chemical reagents on enzymatic activity

The effect of different metal ions and chemical reagents on the activity of recombinant enzyme was investigated by using pNPG as substrate (Table 1). The results revealed that the addition of most metal ions (1 or 10 mM) increased the enzymatic activity evidently. However, the addition of Ag⁺ and Hg⁺ (1 or 10 mM) increased the enzymatic activity. In addition, when added at 1 or 10 mM concentration, SDS completely inhibited the enzymatic activity. In addition, when added at 1 or 10 mM concentration, SDS completely inhibited the enzymatic activity.

3.5. Determination of kinetic parameters

The kinetic parameters of *V*<sub>max</sub> and *K*<sub>m</sub> of Bgl1269 were determined by plotting the substrate concentration vs the initial velocity of each reaction and subjecting the data to nonlinear regression analysis. Result showed that *K*<sub>m</sub> and *V*<sub>max</sub> of Bgl1269 were 0.228 mM and 0.798 μM/min, respectively.

3.6. Hydrolysis of soybean isoflavone glycosides by Bgl1269

Hydrolysis of soybean isoflavone glycosides is an important application of β-glucosidases in industry. The partially purified soybean isoflavone extracts containing glucosidic isoflavones (daidzin and genistin) and isoflavone aglycons (daidzein and genistein) were used to investigate the hydrolyzing ability of Bgl1269 for soybean isoflavone glycosides by HPLC. The peaks of daidzin and genistin were identified in the soybean flour extract by comparison with commercial standards. Results suggested that daidzin and genistin were the predominant isoflavones in the soybean flour extract. However, after 30 min of hydrolysis of Bgl1269, the peaks of daidzin and genistin were remarkably decreased, which suggested most of glycosidic soybean isoflavone were converted
to corresponding aglycones by the recombinant enzyme. To the best of our knowledge, it is the first report on the cloning and the characterization of a β-glucosidase gene with hydrolysis function of soybean isoflavone glycosides via metagenomic approach, and thus this study provides a new strategy and insight for identifying novel isoflavone-conjugates-hydrolyzing β-glucosidase, and also demonstrated the advantage of metagenomic approach for cloning novel glucosidases through function-based screening.

In addition, the hydrolyzing ability of Bgl1269 for commercial products of daidzin and genistin were also investigated by HPLC. Results revealed that daidzin and genistin (0.5 mg/mL) were completely hydrolyzed and converted to daidzein and genistein after incubation for 30 min with 0.8 unit of Bgl1269. Furthermore, the yields of two isoflavone aglycones were analyzed, and the productivity of daidzein and genistein were 0.60 and 0.58 mM h⁻¹, respectively. A comparison of conversion efficiency of glycosylated...
efficiency of genistin by Bgl1269 was nearly 100% (0.5 h), which was similar to that of those β-glucosidases from Soybean okara (1 h) (Chiu et al., 2010), P. thermophilia (4 h) (Yang et al., 2009), Pyrococcus furiosus (1.5 h) (Yeom et al., 2012), but much higher than that of β-glucosidase of A. oryzae (5 h) (Horri et al., 2009). In addition, the previously reported highest productivity of genistein was 0.60 mM h⁻¹, produced by a β-glucosidase from Pyrococcus furiosus, whereas the productivity of genistein (0.58 mM h⁻¹) by Bgl1269 was almost same with that. Considering its very high conversion efficiency of daidzin and genistin, second highest productivity for daidzein, as well as almost the highest productivity for genistin, Bgl1269 has good application potential in hydrolysis of soybean isoflavone glycosides after further study.

3.7. Inhibitory effect of glucose on Bgl1269

The competitive inhibition by glucose is a common characteristic of β-glucosidases that limits their use in enzymatic hydrolysis. The inhibitory effect of glucose on Bgl1269 was assayed with various concentrations of glucose (0.1 M to 3.6 M). Results showed that gluoses, whose concentrations was less than 1 M, did not notably affect the enzymatic activity, and even 3.6 M glucose only had a little inhibition to the enzymatic activity (Fig. 3A), which suggested Bgl1269 had very high tolerance to glucose. Interestingly, glucose also improved the thermostability of Bgl1269. When incubated at 45 °C for 60 min, Bgl1269 completely lost its activity. However, when 1.0, 1.6 or 2.0 M glucose was in existence, the residual activity of Bgl1269 was more than 70% (Fig. 3B). In addition, the inhibition constant (Kᵢ) of glucose to Bgl1269 was also investigated according to the intersection of the lines on Dixon plot analysis, and results indicated that the Kᵢ value of glucose to Bgl1269 was 4.28 M (Fig. 4). Most microbial β-glucosidases reported previously are strongly inhibited by glucose with Kᵢ values of 0.35–100 mM when assayed with pNPG as substrate (Dotsenko et al., 2012; Karnchanatat et al., 2007; Krish et al., 2012), although several β-glucosidases from microbes with high glucose-tolerance have been identified, such as β-glucosidase from A. oryzae (Kᵢ = 1.36 M) (Riou et al., 1998), Candida peltata (Kᵢ = 1.4 M) (Saha and Bothast, 1996), Aspergillus caespitosus (Kᵢ = 0.3 M) (Sonita et al., 2008). In this study, Bgl1269 showed very high tolerance to glucose inhibition, with Kᵢ value of 4.28 M, and its Kᵢ value is the highest among all of β-glucosidases reported previously. Bgl1269 with high tolerance to glucose could relieve the inhibition caused by the accumulation of glucose during the hydrolysis process of soybean isoflavone glycosides, and thus improved its enzymatic activity and conversion efficiency of glucosidic isoflavones. The feature of high tolerance to glucose makes Bgl1269 have obvious advantage in the production of soybean isoflavone glycone than those β-glucosidases which was sensitive to glucose. More importantly, high tolerance to glucose also makes Bgl1269 have great advantage in the hydrolysis of cellulosic biomass. During the process for the production of ethanol from cellulose, β-glucosidase is often inhibited by the end product glucose, and thus a β-glucosidase with high tolerance to glucose has additional advantages in conversion of cellulosic materials to fermentable sugars. It could relieve the inhibition of endoglucanase and cellobiohydrolase caused by the accumulation of cellobiose during the bioconversion process (Benoliel et al., 2010). Thus, Bgl1269 has also good application potential for the production of ethanol from cellulose after further study.

4. Conclusions

A novel β-glucosidase was cloned using metagenomic DNA from mangrove soil combined with activity-based functional screening, and the recombinant Bgl1269 from E. coli expression system was
Table 2
A comparison of conversion efficiency of Bgl1269 for glycosylated soybean isoflavones (daidzin and genistin) and its productivity of isoflavone aglycones (daidzein and genistein) to that of other known β-glucosidases.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme origin</th>
<th>Conversion efficiency of daidzin (%)</th>
<th>Productivity of daidzein (mM h⁻¹)</th>
<th>Conversion efficiency of genistin (%)</th>
<th>Productivity of genistein (mM h⁻¹)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin and genistin</td>
<td>Unculturable microbes</td>
<td>100 (0.5 h)</td>
<td>0.60</td>
<td>~100 (0.5 h)</td>
<td>0.58</td>
<td>This study</td>
</tr>
<tr>
<td>Daidzin and genistin</td>
<td>Soybean okara</td>
<td>100 (1 h)</td>
<td>0.022</td>
<td>100 (1 h)</td>
<td>0.022</td>
<td>Chiou et al., 2010</td>
</tr>
<tr>
<td>Soybean flour extracts</td>
<td>Aspergillus oryzae</td>
<td>94 (5 h)</td>
<td>0.32</td>
<td>45% (5 h)</td>
<td>0.11</td>
<td>Horri et al., 2009</td>
</tr>
<tr>
<td>Daidzin</td>
<td>Escherichia coli</td>
<td>95 (4 h)</td>
<td>0.002</td>
<td>91 (4 h)</td>
<td>0.002</td>
<td>Ismail and Hayes, 2005</td>
</tr>
<tr>
<td>Daidzin</td>
<td>Sulfolobus solfataricus</td>
<td>100 (100 min)</td>
<td>1.0</td>
<td>NR</td>
<td>NR</td>
<td>Kim et al., 2012</td>
</tr>
<tr>
<td>Soybean flour extracts</td>
<td>Thermotoga maritima</td>
<td>86 (3 h)</td>
<td>NR</td>
<td>94 (3 h)</td>
<td>NR</td>
<td>Xue et al., 2009</td>
</tr>
<tr>
<td>Soybean flour extracts</td>
<td>Paecilomyces thermophila</td>
<td>98 (4 h)</td>
<td>NR</td>
<td>99.3 (4 h)</td>
<td>NR</td>
<td>Yang et al., 2009</td>
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<tr>
<td>Soybean flour extracts</td>
<td>Pyrococcus furiosus</td>
<td>100 (1.5 h)</td>
<td>0.57</td>
<td>100 (1.5 h)</td>
<td>0.60</td>
<td>Yeom et al., 2012</td>
</tr>
</tbody>
</table>

NR, not reported.

Fig. 3. Effect of various concentration of glucose on the activity (A) and thermostability (B) of recombinant Bgl1269 using pNPG as substrate. Several glucose concentrations used to investigate the effect of glucose on the thermostability of recombinant Bgl1269 were 0 M glucose (control, □), 0.1 M glucose (○), 1.0 M glucose (●), 1.6 M glucose (□), 2.0 M glucose (▲), 3.0 M glucose (●), and 3.6 M glucose (▼). Data points are the average of triplicate measurements, and error bars represent ± SD.

Fig. 4. Dixon plot of inhibitory effect of glucose on pNPG hydrolysis by purified Bgl1269. The pNPG concentrations used were 5 mM (□) and 7 mM (●).
References


