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Comparison of Three Thermostable β-Glucosidases for Application in the Hydrolysis of Soybean Isoflavone Glycosides

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ABSTRACT: A novel thermostable β-glucosidase (Te-BglA) from *Thermoanaerobacter ethanolicus* JW200 was cloned, characterized and compared for its activity against isoflavone glycosides with two β-glucosidases (Tm-BglA, Tm-BglB) from *Thermotoga maritima*. Te-BglA exhibited maximum hydrolytic activity toward *p*NP-β-D-glucopyranoside (*p*NPG) at 80 °C and pH 7.0, was stable for a pH range of 4.6−7.8 and at 65 °C for 3 h, and had the lowest K_m for the natural glycoside salicin and the highest relative substrate specificity $(k_{cat}/K_m)^{(salicin)}/(k_{cat}/K_m)^{(pNPG)}$ among the three enzymes. It converted isoflavone glycosides, including malonyl glycosides, in soybean flour to their aglycons more efficiently than Tm-BglA and Tm-BglB. After 3 h of incubation at 65 °C, Te-BglA produced complete hydrolysis of four isoflavone glycosides (namely, daidzin, genistin and their malonylated forms), exhibiting higher productivity of genistein and daidzein than the other two β-glucosidases. Our results suggest that Te-BglA is preferable to Tm-BglA and Tm-BglB, but all three enzymes have great potential applications in converting isoflavone glycosides into their aglycons.

KEYWORDS: thermostable β-glucosidase, hydrolysis, soybean flour, isoflavone glycosides

INTRODUCTION

Isoflavones, which are abundant in soybeans, reportedly have various health benefits related to their estrogenic activities, including reduced risk of cardiovascular disease, lower rates of prostate, breast and colon cancers and improved bone health.[1] Isoflavones in soybeans exist predominantly in glucoside forms and are rarely found in aglycon forms.[2] The content and composition of isoflavones vary in soybean-based foods, depending on processing techniques such as heat treatment, defoaming, soaking in water, enzymatic hydrolysis and microbial fermentation.[3−5] Numerous studies have demonstrated that isoflavone aglycons are superior to isoflavone glycosides in various bioactivities, due to their effective absorption into the human body.[2,6,7] Therefore, there is much interest in increasing the amounts of isoflavone aglycons in soy products and converting isoflavone glycosides into their aglycons.

The formation of free aglycons should be associated with the isoflavone conjugates hydrolyzing β-glucosidase.[8,9] The abilities of many bacterial and fungal β-glucosidase for converting isoflavone glycosides into the aglycons have been extensively studied in recent years,[10−13] due to their importance in the preparation of isoflavone aglycons. Tsangalis et al. reported that β-glucosidase from *Bifidobacteria*in soy milk was capable of converting glycosides to their aglycons.[14] Ming et al.[15] partially purified and characterized β-glucosidase from soybean that could hydrolyze soybean isoflavone conjugates, and the temperature optimum was 30 °C. Liu et al.[16] reported that an isoflavone conjugate hydrolyzing β-glucosidase (ICHG) was purified from endophytic bacterium, *Pseudomonas* ZD-8, and the temperature optimum was 40 °C. Kuo et al.[17] reported that two β-glucosidases from *Bacillus subtilis* had high specific activity toward soybean isoflavones. Suzuki et al.[18] reported one β-glucosidase (GmICHG) from the roots of soybean seedling with high specificity toward soybean isoflavone conjugates, and the temperature optimum was 40 °C. Xie et al.[19] reported that they isolated one enzyme from an *Absidia* sp. R from Liquor Qu that could hydrolyze soybean isoflavone conjugates, and the temperature optimum was 50 °C. Two β-glucosidases from the legumes *Dalbergia cochinchinensis* and *Dalbergia nigrescens* were compared for their ability to hydrolyze soybean isoflavone conjugates from soybean.[8] Recently, comparison of two β-glucosidases from a thermophilic fungus, *Paecilomyces thermophila* J18, and almond was also reported.[20] However, there are few reports on β-glucosidase from the thermophilic bacteria with preferential activity toward isoflavone glucosides.[8,9] The advantages of thermostable enzymes in industrial processes include reduced risk of contamination, increased substrate solubility, higher stability of enzymes against denaturing agents and proteolytic enzyme, and reduced cost of external cooling.[21] Therefore, in view of the commercial importance of β-glucosidase enzymes, there is still a great need for finding better β-glucosidases than those currently tested. High activity and thermostability of β-glucosidase should attract considerable attention as characteristics of an efficient enzyme.

Thermoanaerobacter ethanolicus JW200 grows optimally 58−78 °C and has two different exoacting, β-specific glycosyl hydrolases, including a β-xylosidase/α-arabinofranosidase[22] and one β-glucosidase (Te-BglA). *Thermatoga maritima* is of considerable interest because of the hyperthermostability of its enzymes, which includes two different β-glucosidases (Tm-BglA and Tm-BglB).[23,24]

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In our previous study, *T. maritima* Tm-BglA was found to have the ability to hydrolyze isoflavone glucosides and their malonylated forms, and potential to increase the isoflavone aglycons in soybean flour.[13,25] Although the Tm-BglB had been reported previously, there have been no reports regarding its applications in the hydrolysis of isoflavone glycosides. To get highly active and efficient isoflavone conjugates hydrolyzing β-glucosidase, we selected three thermostable β-glucosidases from two thermophilic bacterias to evaluate their potentials as catalysts.

In this study, we report the cloning and characterization of a novel thermostable Te-BglA from *T. ethanolicus* JW200, and comparison of three thermostable β-glucosidases (Tm-BglA, Tm-BglB and Te-BglA) for soybean glycoside hydrolysis, in order to find a better catalyst for converting isoflavone glycosides into their aglycons. Their hydrolysis of the artificial substrate *p*NP-glucopyranoside (*p*NPG) and the natural glycoside salicin, pH profiles, and thermal stabilities also are compared.

MATERIALS AND METHODS

Materials. In the present study, soybeans were purchased from Yan Zhi Fang Co. (Hefei, China). The *p*-nitrophenyl (*p*NP) glycoside substrate *p*NP-β-D-glucopyranoside (*p*NPG) was purchased from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile were purchased from Fisher Scientific (Hanover Park, IL). Isoflavone standards of daidzin (Din), daidzein, genistin (Gin), and genistein were purchased from Sigma Chemical Co. (St. Louis, MO); malonyl genistin (MGin) and malonyl daidzin (MDin) were purchased from Wako Chemical Co. (Wako Pure Chemical Industries, Ltd., Japan). *Thermoanaerobacter ethanolicus* JW200 (ATCC31550) and *Thermotoga maritima* (ATCC-43589) were gifts from Dr. J. Wiegel (University of Georgia, USA) and Dr. Weilan Shao (Nanjing Normal University, China). *T. ethanolicus* JW200 and *T. maritima* cells used for isolation of genomic DNA were grown in serum bottles containing 100 mL of prereduced clostridial media at 60 °C,[22] and anaerobically at 80 °C in modified Luria−Bertani (LB) medium,[26] respectively. The recombinant plasmid pET-20b-*Tm-bglA* previously described was used in preparing purified Tm-BglA.[13,25] *Escherichia coli* JM109 (DE3) (Promega, Madison, WI) was used as host for the expression of *Tm-bglA*, *Tm-bglB* and *Te-bglA* via the T7 RNA polymerase expression system with pET-20b plasmids (Novagen). Cells were cultured at 37 °C in LB supplemented with 100 μg of ampicillin/mL. All other chemicals used were analytical grade reagents unless otherwise stated.

DNA Manipulation. Routine DNA manipulations were carried out essentially as described.[27] Plasmid DNA and PCR products were purified using QIAGEN plasmid kit and PCR purification kit (Germany). PCR reactions were performed in a PE Applied Biosystems 9700 thermal cycler (Foster City, CA) using standard reaction conditions. DNA modifying enzymes and polymerases were purchased from TAKARA (Dailian, China).

Construction of Recombinant Plasmid. Based on the DNA sequence of the β-glucosidase B (TM0025) from *T. maritima*, two synthetic oligodeoxyribonucleotides P1 (5′-CCCCATATGATGGAAAGGATCGATGAAA-3′) and P2 (5′-CCGCTCGAGTGGTTTGAATCTCTTCTC-3′) were used as primers. The PCR primers P3 (5′-GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATG ATAAAATTTCCAAAAGA-3′) and P4 (5′-CCCCTCGAGCTCAATAGAATTTTTCTGT-3′) were designed and synthesized based on the *bglA* gene (ZP_00777761) sequence from *Thermoanaerobacter pseudethanolicus* ATCC 33223, and used to amplify the *Te-bglA* gene from *T. ethanolicus* JW200 genomic DNA. PCR amplification 30 cycles with *Pyrobest* DNA polymerase (TAKARA, China) were carried out in a 50 μL reaction containing 0.2 mM dNTPs each, 20−35 pmol of each primer and 0.2 μg of chromosomal DNA template. The *Tm-bglB* gene was ligated into pET-20b at the *Nde*I and *Xho*I restriction sites, resulting in the recombinant plasmid pET-20b-*Tm-bglB*, while the *Te-bglA* gene was ligated into pET-20b at *Xba*I and *Xho*I restriction sites, resulting in the recombinant plasmid pET-20b-*Te-bglA*. These expression plasmids were sequenced and characterized by restriction analysis. Oligonucleotide primer synthesis was performed by Sangon (Shanghai, China).

Purification of Recombinant Enzymes. All *E. coli* JM109 (DE3) containing pET-20b-*Tm-bglA*, pET-20b-*Tm-bglB* and pET-20b-*Te-bglA* were grown in LB with ampicillin at 37 °C to OD_{600} of 0.8, and incubated further with isopropylthio-β-galactoside (IPTG, 0.8 mM) for 10 h. The cells were harvested by centrifugation, and washed twice with water, resuspended in 20 mL of 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris−HCl buffer (pH 7.9), and fragmentated by three passes through a French press. The cell extracts were heat-treated (70 °C, 20 min), then cooled in an ice bath, and centrifuged (9600*g*, 4 °C, 30 min). The resulting supernatants were purified by Ni-affinity chromatography (Novagen) to homogeneity as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) using 10% polyacrylamide running gels with 4% polyacrylamide stacking gels.

Protein Determination and Enzyme Assay. Total protein concentration was determined by the Bradford method,[28] using bovine serum albumin (BSA, Sigma) as a standard. Enzyme activity was quantified by *p*-nitrophenol (*p*NP) release from *p*NPG. The reaction mixture contained 10 μL of suitably diluted enzyme, 170 μL of 0.1 M potassium phthalate buffer (PPB, pH 7.0 for Tm-BglA and Te-BglA; pH 5.8 for Tm-BglB) and 20 μL of substrate solution (20 mM *p*NPG). After incubation at 65 °C for 5 min, the reaction was stopped by adding 0.6 mL of 1 M Na_2CO_3, and the A_{405} was read. A standard curve was prepared using *p*NP. One unit was determined as the amount of enzyme producing 1 μmol of *p*NP/min. All reactions were done in triplicate.

β-Glucosidase activity toward the available natural glycoside salicin (Sigma) was determined by assaying the amount of glucose released. The reaction mixture (0.1 mL) containing 1 mM salicin, 50 mM PPB, and appropriately diluted enzyme in the same buffer was incubated at the respective temperature for 10 min, and was stopped by adding 0.75 mL of 3,5-dinitrosalicylic acid.[29] The reaction mixture was then boiled for 5 min and cooled in an ice water bath, and adding 1.075 mL of ddH_2O, at last the A_{520} was then read.[25] Enzyme activities are reported in international units (U), the amount of enzyme that releases 1 μmol of glucose in a minute.

Kinetic Parameters. For determinations of the kinetic parameters (K_m, V_{max}, k_{cat}/K_m), the purified enzymes were assayed at substrate concentrations ranging from 1.0 to 10 mM for salicin, and from 0.2 to 2.0 mM for *p*NPG at their optimal conditions. Kinetic parameters (K_m and V_{max}) were determined by the Eadie−Hofstee representation of the Michaelis−Menten model. The specificity constant, k_{cat}/K_m, was calculated to determine the substrate specificity of each enzyme. To calculate the catalytic constant, apparent k_{cat}, the amount of protein was divided by the subunit molecular mass of 52,338 Da for Tm-BglA, 52,639 Da for Te-BglA or 81,846 Da for Tm-BglB. Each experiment was done in duplicate. The standard error was recorded to be <2%.

HPLC Analysis. HPLC analysis was used to measure the amounts of daidzein, genistein, daidzin (Din), genistin (Gin), malonylgenistin (MGin), and malonyldaidzin (MDin) to quantify changes in isoflavone content of crude extracts treated with the three recombinant β-glucosidases. Separation and quantification of isoflavones were achieved with an Agilent HC-C 18 (4.6 × 250 mm, 5 μm) reverse phase column on an HP series 1100 HPLC (Agilent Corp., Palo Alto, CA) with the UV detector set at a wavelength of 260 nm in a manner similar to that Chuankayan et al. described for the separation of soybean isoflavones.[8,9] A linear HPLC gradient was employed. Solvent A was 0.1% phosphoric acid in water, and solvent B was acetonitrile. Solvent B was increased

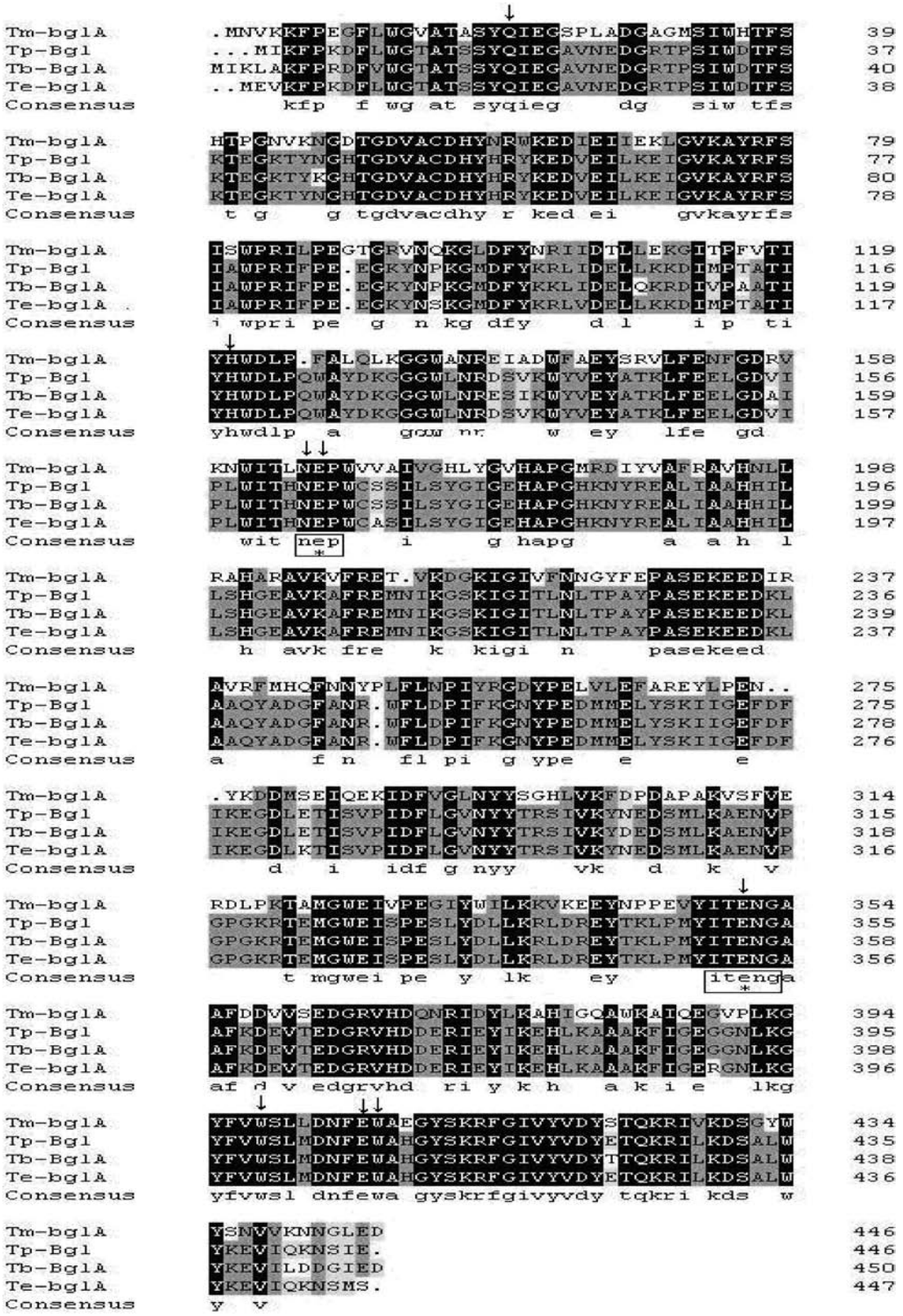

Figure 1. Comparison of the amino acid sequences of Te-BglA to those of other thermophilic bacteria β-glucosidases. Abbreviations: Te, *Thermoanaerobacter ethanolicus* JW200 (GU391423); Tp, *Thermoanaerobacter pseudethanolicus* ATCC 33223 (ZP_00777761); Tb, *Thermoanaerobacter brockii* (Z56279.1); Tm, *Thermotoga maritima* (X74163). The conserved and similar amino acids in these β-glucosidases are indicated by solid and gray boxes, respectively. The conserved motifs surrounding the nucleophile are boxed, while the respective catalytic residue is underlined by an asterisk. Sugar-binding amino acid residues that are highly conserved are indicated by arrowheads.

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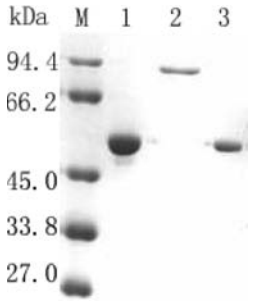

Figure 2. SDS−PAGE of purified enzymes: lane M, the molecular mass standards (M); lane 1,Tm-BglA; lane 2, Tm-BglB; lane 3, Te-BglA.

from 15 to 35% over 45 min. The solvent flow rate was 0.8 mL/min. Peaks of soy isoflavone glucosides (Gin, Din, MGin, and MDin) and aglycons (genistein and daidzein) were identified by matching retention times with isoflavonoid standards. Relative amounts were calculated from relative peak areas, because all peaks were well within the linear range of the instrument.

Hydrolysis of Soybean Flour. First, soybean flour (SF) was extracted with three volumes of *n*-hexane by stirring for 30 min at room temperature for defatting, precipitated by centrifugation at 9600*g* for 20 min and air-dried. To compare the hydrolysis efficiencies of the three β-glucosidases toward the isoflavonoid glycosides in SF, 10% (w/v) SF was incubated with the purified Tm-BglA, Tm-BglB and Te-BglA in 0.1 M PPB buffer (pH 7.0 for Tm-BglA and Te-BglA, pH 5.8 for Tm-BglB) in a thermostatically controlled incubator at 65 °C. The enzyme concentration for each purified enzyme was 50 U per gram SF (activity was determined with *p*NPG as substrate at 65 °C). The reactions were stopped by ice water cooling before analysis.[13] The hydrolyzed solutions were centrifuged at 13000*g* for 20 min to collect the supernatants and pellets, respectively. The pellets were then extracted with 80% methanol at 30 °C for 2 h, and the methanol extract was collected by centrifugation. The reaction supernatant were dried by speed vacuum and then resuspended in 80% methanol. The methanol extracts were collected by centrifugation and filtered through a 0.45 μm filter for the quantification of isoflavones using HPLC. A control reaction of crude extract without enzyme at 65 °C was set up in the same manner in 0.1 M PPB buffer of pH 7.0 and pH 5.8, respectively. Each assay was done in duplicate, and 20 μL of each sample was injected for HPLC analysis. The percent enzymatic hydrolysis of the three β-glucosidases was evaluated by the following equation:

$$\text{hydrolysis (\%)} = 100 - 100 \times (\text{isoflavone content in enzyme-hydrolyzed samples} / \text{isoflavone content in control samples without enzyme})$$

The amounts of isoflavone aglycons produced from SF were expressed as mg per 100 g of sample.

DNA Sequence Analysis. DNA sequencing was performed by Sangon (Shanghai, China), and the sequences were analyzed with Dnaman, version 6.0, of the sequence analysis software package (Lynnon Biosoft, USA). The homology was analyzed in GenBank with the BLAST program.

Nucleotide Sequence Accession Number. The nucleotide sequence of the β-glucosidase gene *bglA* from *T. ethanolicus* JW200 was deposited in the GenBank database under accession number GU391423.

RESULTS

Sequence Analysis, Expression and Purification. Sequence analysis of the *Te-bglA* gene from the *T. ethanolicus* JW200 revealed that its open reading frame encodes a protein (accession number, GU391423) of 447 amino acids (Figure 1). A predicted molecular weight of 52,639 Da was calculated for *Te-BglA* and was in agreement with the SDS−PAGE analysis of the recombinant protein (Figure 2). As expected from the analysis of the *Te-bglA* sequence, the translated *Te-bglA* sequence has the highest similarity with that of the β-glucosidase gene *bglA* of *Thermoanaerobacter pseudethanolicus* ATCC 33223 (98% identity) (ZP_00777761.1)[30] and the β-glucosidase gene *bglA* of *Thermoanaerobacter brockii* (95% identity) (Z56279.1),[31] but shares 53% identity with Tm-BglA (X74163) from *T. maritima*.[23] It contains the highly conserved peptide motifs Asn-Glu-Pro (residues 164−166) and Ile-Thr-Glu-Asn-Gly (residues 351−355) (Figure 1), in which the Glu (E165, E353) residues are typical catalytic residues of GH1 enzymes.[18] Sugar-binding amino acid residues that are highly conserved among all GH1 enzymes could also be identified in the translated *Te-bglA* sequence: Gln-19, His-119, Asn-164, Glu-165, Glu-353, Trp-400, Glu-407, and Trp-408. These results show that Te-BglA is a thermophilic bacteria member of the GH1 family.

The three recombinant enzymes were purified to gel electrophoretic homogeneity by Ni-affinity chromatography after heat treatment (Figure 2), and had apparent molecular masses of 52 kDa for Tm-BglA and Te-BglA and 82 kDa for Tm-BglB on SDS−PAGE, which were in accordance with the theoretically calculated molecular mass, respectively.

Biochemical and Kinetic Parameters. The biochemical properties of Te-BglA, Tm-BglA and Tm-BglB were evaluated. The temperature dependence of the three enzyme activities was determined with *p*NPG and salicin as substrates (Figure 3). The optimal temperatures using *p*NPG as a substrate were 90 °C for Tm-BglA, and 80 °C for Tm-BglB and Te-BglA (Figure 3A); those for Tm-BglA, Tm-BglB and Te-BglA using salicin as a substrate were 85 °C, 90 °C and 75 °C, respectively (Figure 3C). The optimal temperature of GH1 Tm-BglA and Te-BglA for *p*NPG was 5 °C higher than that for salicin, and that of GH3 Tm-BglB for *p*NPG was 10 °C lower than that for salicin. Analysis of enzyme activity using the substrate *p*NPG demonstrated that the three purified enzymes were most stable at 65 °C for 3 h (Figure 3E); when incubated for 5 h, Tm-BglB and Tm-BglA retained more than 80% of their activity, but Te-BglA lost 90% of its activity.

Next, the effects of pH on the three enzyme activities on the substrates, *p*NPG and salicin, were determined at 37 °C in various buffers ranging from pH 3.8−8.2 (Figure 3B,D). The optimal pH with *p*NPG as a substrate was 6.2 for Tm-BglA, 4.2 for Tm-BglB, and 7.0 for Te-BglA, and that using salicin as a substrate was 7.0 for Tm-BglA, 5.8 for Tm-BglB, and 7.0 for Te-BglA, indicating that the optimum pH for Tm-BglA and Tm-BglB differed with the different substrates. Analysis of enzyme activity with the substrate *p*NPG showed that pH stability for Te-BglA and Tm-BglA was higher than that of Tm-BglB over the pH range from 5.4 to 8.2, and the three enzymes were stable over a pH range of 4.6 to 7.8 (Figure 3F).

Concerning kinetic properties, the Michaelis constants for the three enzymes were measured with *p*NPG and salicin as substrates. The apparent K_m value of Tm-BglB for *p*NPG was lower than those of Tm-BglA and Te-BglA (Table 1). Its k_{cat}/K_m value was the highest due to a minimum in the K_m value and a maximum in the k_{cat} value compared to those of Tm-BglA and Te-BglA, indicating that Tm-BglB had the most efficient *p*NPG hydrolysis. For the hydrolysis of salicin, the K_m value of Te-BglA was 4.7 ± 0.04 mM, which was significantly lower than those of Tm-BglA and Tm-BglB; thus, its k_{cat}/K_m value was higher than

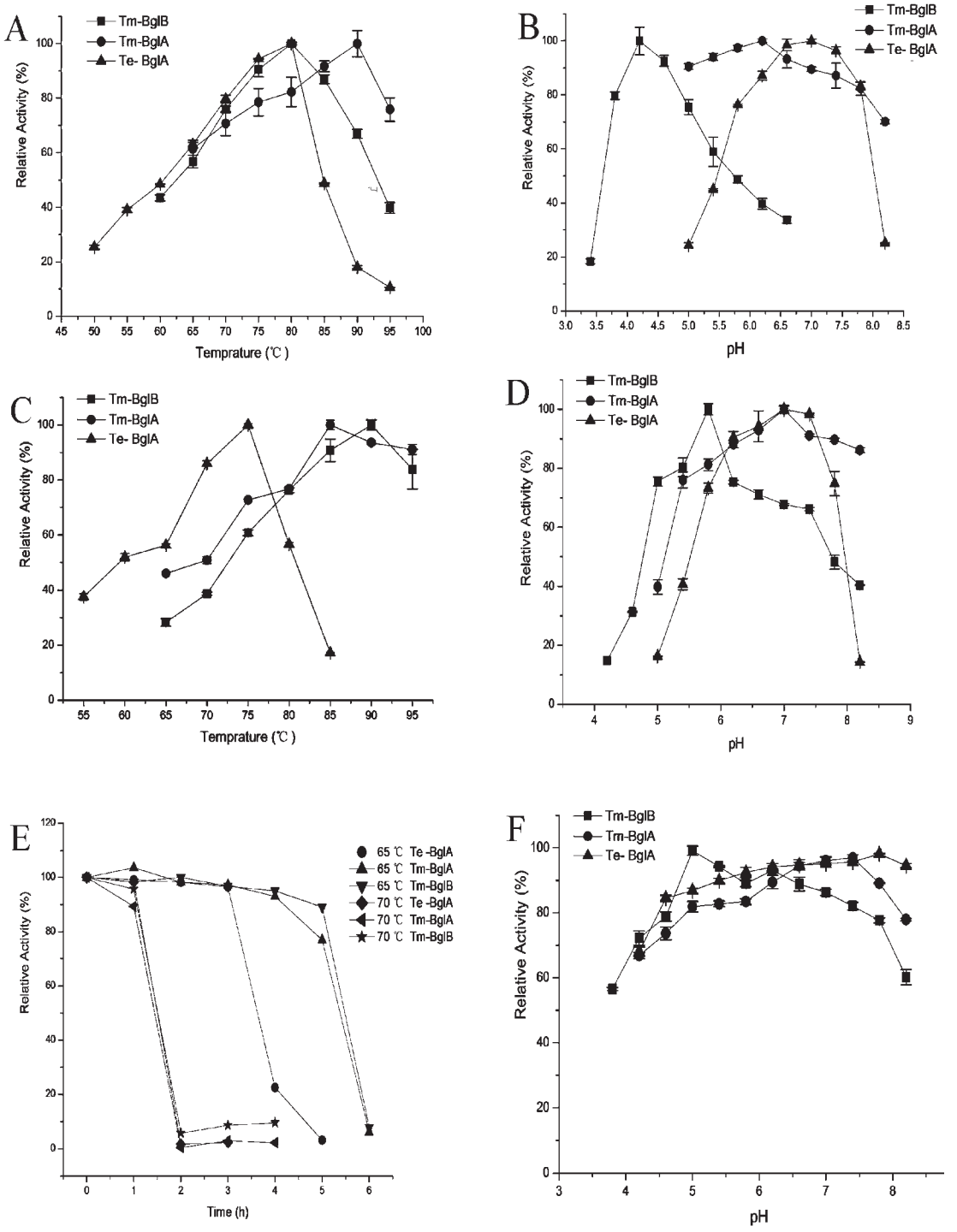

Figure 3. The optimal temperature (A, C) and pH (B, D) indicated the three recombinant β-glucosidases' activity against *p*NPG (A, B) and salicin (C, D) as the substrate. The optimal pH was determined in 0.1 M PIB from pH 3.4 to 8.2 at 80 °C. Optimal temperatures were detemined at the enzymes' optimal pH values, aliquots of purified proteins were incubated at various temperatures (50 to 95 °C), and β-glucosidase activities were assayed as described in Materials and Methods. The highest residual activity was defined as 100%. The thermostability (E) and pH stability (F) profiles of the three recombinant β-glucosidases with *p*NPG as substrate. The purified enzyme in its optimal pH was preincubated at 65 and 70 °C for different times in the absence of substrate, respectively, and these enzyme activities were then assayed as previously indicated in optimal conditions after cooling in an ice water bath. For pH stability experiments, the purified enzymes were preincubated in 0.1 M PIB from pH 3.8 to 8.2 for 1 h at 37 °C; then aliquots were transferred in standard reaction mixture to determine the amount of remaining activity. The activity determined prior to the preincubation was taken as 100%.

that of Tm-BglA, and was almost the same as that of Tm-BglB. It appeared that Te-BglA and Tm-BglB had higher catalytic efficiency for salicin hydrolysis than Tm-BglA.

Isoflavone Aglycon Production from Soy Flour. Because Din, Gin, MDin, and MGin were the predominant isoflavones in the soybean flour, the hydrolysis of these four isoflavone

Table 1. Kinetic Parameters of the Three Recombinant β-Glucosidases

substrate	enzyme	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}·s^{-1})
*p*NPG	Tm-BglA	0.63 ± 0.01	307.3 ± 0.7	268.1 ± 0.6	4.3 ± 0.08 × 10^5
	Tm-BglB	0.22 ± 0.01	1134.0 ± 1.4	1547.6 ± 2.9	7.2 ± 0.2 × 10^6
	Te-BglA	0.79 ± 0.01	67.0 ± 0.7	58.8 ± 0.6	7.5 ± 0.1 × 10^4
salicin	Tm-BglA	12.0 ± 0.3	217.5 ± 2.6	189.7 ± 2.3	1.6 ± 0.06 × 10^4
	Tm-BglB	9.8 ± 0.1	191.4 ± 2.5	261.1 ± 3.5	2.6 ± 0.01 × 10^4
	Te-BglA	4.7 ± 0.04	125.8 ± 3.5	110.4 ± 3.0	2.3 ± 0.04 × 10^4

The kinetic parameters were determined at their optimal pH and temperature for the substrate concentrations ranging from 1.0 to 10 mM for salicin, and from 0.2 to 2.0 mM for *p*NPG, using the standard assay as described in Materials and Methods.

glycosides and the isoflavone aglycons produced after reactions with or without enzyme at different times were analyzed by HPLC, in order to evaluate the ability of three β-glucosidases for hydrolyzing soybean isoflavone glycosides. As shown in Tables 2A and 3, the hydrolysis of Din and Gin for Te-BglA and Din by Tm-BglA and Tm-BglB was complete after reaction for 10 min, however, 10.5% and 6.6% of Gin remained in the reactions containing Tm-BglA and Tm-BglB, respectively. Simultaneously, Te-BglA, Tm-BglB and Tm-BglA were found to hydrolyze 59.5% and 81.4%, 37.4% and 75.9%, and 17.8% and 18.6% of the hydrolysis of MDin and MGin, respectively (Table 3), indicating that Te-BglA had the most efficient malonyl glycoside hydrolysis. After 3 h of incubation, the remaining MDin and MGin were completely hydrolyzed by Te-BglA, however, 6.2% of MDin and 12.2% MGin remained in the reaction with Tm-BglB, and 3.7% of Gin, 26.5% of MDin and 14.5% of MGin remained in the reaction with Tm-BglA (Table 2A). It appeared that Te-BglA hydrolyzed both conjugated (MGin and MDin) and nonconjugated (Gin and Din) glycosides in SF more efficiently than Tm-BglB and Tm-BgA.

Subsequently, the production of aglycon isoflavones with the three recombinant β-glucosidases was evaluated (Table 2B). In 10 min reaction containing Te-BglA and Tm-BglB, the amount of two isoflavone aglycons increased rapidly to 68.1 (20.4 ± 1.8 mg of daidzein, 47.7 ± 0.2 mg of genistein) and 54.2 (13.8 ± 0.1 mg of daidzein, 41.4 ± 0.1 mg of genistein) mg/100 g, respectively, and achieved 82.8% and 75.2% of their amounts in 3 h reaction, respectively. In contrast, Tm-BglA produced 39.4 mg (12.0 ± 0.2 mg of daidzein, 27.4 ± 0.4 mg of genistein) of two isoflavone aglycons per 100 g of SF after 10 min, which hold only 59.2% of their amounts in 3 h reaction. The hydrolysis of Te-BglA and Tm-BglB resulted in increases of 0.7-fold and 0.2-fold in daidzein and 0.7-fold and 0.5-fold in genistein within 10 min of incubation, respectively. It was exhibited that Te-BglA had faster conversions from isoflavone glucosides to aglycons and higher yield of two aglycon isoflavones than Tm-BglA and Tm-BglB.

DISCUSSION

For effective conversion of isoflavone glycosides into their aglycons with high productivity and low cost, three recombinant thermostable β-glucosidases were targeted. The thermostable enzymes exhibit high hydrolysis yields due to their high initial velocities, contamination prevention abilities, high substrate solubility, and increased half-lives.[32] In this study, to get a better catalyst for converting isoflavone glycosides into the aglycons, we reported the cloning of a novel thermostable β-glucosidase (Te-BglA) from *T. ethanolicus* JW200 and comparison of three thermostable β-glucosidases (Tm-BglA, Tm-BglB and Te-BglA) for soybean glycoside hydrolysis and their physiochemical properties. Properties corresponding optimal pH and pH stability ranges for Te-BglA and Tm-BglA were found to be higher than those of Tm-BglB, indicating that the pK_a values of Tm-BglA and Te-BglA were higher than that of Tm-BglB. The optimum pH of Tm-BglA and Tm-BglB differed with the different substrates, a possible explanation being that the interactions of Tm-BglA and Tm-BglB with different substrates influence the pK_a values of the glutamic acid proton donor.[33,34] The optimal temperatures of Tm-BglA and Tm-BglB were higher than those of Te-BglA, but their temperature optimum differed with the different substrates. The explanation for this observation is the possibility that the interactions of the three β-glucosidases with different substrates influence their conformational forms with thermostability properties. Despite a previous study that showed that the thermal stability of recombinant enzyme was affected by fusing his-tag, resulting in an obvious enzyme inactivation in the temperature range 65−95 °C,[25] the three recombinant β-glucosidases that had six extra histidines added at the C-terminal of the enzyme in this study, with temperature optimum over 75 °C, exhibited high thermostability at 65 °C, which makes them highly suitable for industrial biological processes. As demonstrated by the kinetic results of three enzymes, Tm-BglB exhibited the lowest K_m for *p*NPG and the most efficient *p*NPG hydrolysis, but Te-BglA showed the lowest K_m for natural glycoside salicin and the highest relative substrate specificity $(k_{cat}/K_m)^{(salicin)}/(k_{cat}/K_m)^{(pNPG)}$. Moreover, Te-BglA and Tm-BglB had higher catalytic efficiency for salicin hydrolysis than Tm-BglA, exhibiting a good liberation of glucose from the natural glycoside salicin.

The three recombinant β-glucosidases were evaluated for hydrolysis of isoflavone glycosides and isoflavone aglycons produced from SF. Of three enzymes, the conversions from isoflavone glucosides to their aglycons for Te-BglA and Tm-BglB were found to be higher than that for the Tm-BglA. However, the hydrolyses of Din, MDin and MGin for Te-BglA were higher than those of Tm-BglB and Tm-BglA, resulting in complete hydrolysis of four isoflavone glycosides (Din, Gin, MDin and MGin) after 3 h of incubation. Furthermore, Te-BglA increased the isoflavone aglycons from SF more rapidly than Tm-BglB and Tm-BgA and produced the highest yield of each isoflavone aglycon. It is known that the predominant soybean isoflavone forms are malonyl glycosides, which made up about 70% of the total soybean isoflavone in soybean.[20,35] Most β-glucosides are not effective in hydrolyzing the conjugated isoflavones (malonyl and acetyl glycosides) to their aglycon.[10,18,20] In our study, three thermostable β-glucosides could hydrolyze isoflavone glucosides, including their malonylated forms, but Te-BglA converted malonyl glycosides to

Table 2. Comparison of Soybean Isoflavone Glycosides in the Hydrolyzed Samples and Isoflavone Aglycons Produced by the Three Recombinant β-Glucosidases[a]

		(A) rel amt of isoflavone (%):[b] isoflavone glycosides				(B) isoflavone aglycons (mg/100 g):[c] isoflavone aglycons	
enzyme	time	Din	Gin	MDin	MGin	daidzein	genistein
not treated		100	100	100	100	0.7	0.1
control (pH 5.8)	10 min	28.5 ± 0.5	67.0 ± 2.0	65.5 ± 0.5	76.5 ± 1.5	1.8 ± 0.05	3.4 ± 0.1
	3 h	40.0 ± 1.0	138.0 ± 4.0	59.0 ± 1.0	72.0 ± 1.0	3.2 ± 0.05	4.0 ± 0.1
control (pH 7.0)	10 min	57.5 ± 0.5	116.0 ± 1.0	68.0 ± 1.0	76.5 ± 1.5	0.6 ± 0.01	1.4 ± 0.05
	3 h	73.5 ± 0.5	123.0 ± 5.0	61.0 ± 2.0	71.5 ± 1.5	1.8 ± 0.07	2.1 ± 0.1
Tm-BglA	10 min	0.0	10.5 ± 0.5	55.5 ± 0.5	62.0 ± 1.0	12.0 ± 0.2	27.4 ± 0.4
	3 h	0.0	3.7 ± 0.1	26.5 ± 0.5	14.5 ± 0.5	17.7 ± 1.1	48.9 ± 0.4
Tm-BglB	10 min	0.0	6.6 ± 0.3	41.0 ± 1.0	18.5 ± 0.5	13.8 ± 0.1	41.4 ± 0.1
	3 h	0.0	0.0	6.2 ± 0.2	12.2 ± 0.05	23.4 ± 1.1	48.7 ± 0.9
Te-BglA	10 min	0.0	0.0	27.5 ± 0.5	14.2 ± 0.05	20.4 ± 1.8	47.7 ± 0.2
	3 h	0.0	0.0	0.0	0.0	26.2 ± 0.3	56.1 ± 1.1

[a] Soy flours were incubated in 0.1 M PPB (pH 5.8 or 7.0) at 65 °C for 10 min and 3 h, respectively. Samples that did not contain the enzyme were used as control, and the amounts at time 0 were set at 100%. Values represent the means ± standard deviation; $n = 2$ ($p < 0.05$). [b] Relative amounts (relative HPLC peak areas) at 10 min and at 3 h and with three enzymes were determined by HPLC, as described in Materials and Methods. [c] The isoflavone aglycon content is expressed as mg per 100 g of sample.

Table 3. Comparison of the Percent Hydrolysis of the Three Recombinant β-Glucosidases[a]

		percentage enzymatic hydrolysis (%): isoflavone glycosides			
enzyme	time	Din	Gin	MDin	MGin
Tm-BglA	10 min	100	91.0 ± 0.7	17.8 ± 0.4	18.6 ± 0.8
	3 h	100	97.0 ± 0.2	56.6 ± 0.5	79.7 ± 0.4
Tm-BglB	10 min	100	89.6 ± 0.3	37.4 ± 1.1	75.9 ± 0.3
	3 h	100	100	89.5 ± 0.5	83.0 ± 0.3
Te-BglA	10 min	100	100	59.5 ± 0.5	81.4 ± 0.5
	3 h	100	100	100	100

[a] Values represent the means ± standard deviation; $n = 2$.

their aglycons more efficiently than Tm-BglB and Tm-BgA. Therefore, *T. ethanolicus* JW200 β-glucosidase is preferable to the two β-glucosidases from *T. maritima* in enzymatic hydrolysis for converting isoflavone glycosides into their aglycons, and all three enzymes have great potential applications in food and medicine production. The three-dimensional structure of β-glucosidase A from *T. maritima* has been published.[36] Further work to improve isoflavone glycoside transformation via the structural modification of the enzymes by molecular modeling on the basis of the solved three-dimensional structures is in progress.

In conclusion, a novel thermostable β-glucosidase (Te-BglA) gene from *T.ethanolicus* JW200 was cloned and sequenced. It encodes a protein of 447 amino acids that belongs to the glycoside hydrolase family 1. The enzyme could more effectively hydrolyze isoflavone glucosides of soy flour to their aglycons than could two β-glucosidases from *T. maritima*. The study suggests that Te-BglA is preferable to Tm-BglA and Tm-BglB, and is a good candidate for use in industrial conversion of isoflavone glycosides into their aglycons.

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ABBREVIATIONS USED

BSA, bovine serum albumin; HPLC, high performance liquid chromatography; Din, daidzin; Gin, genistin; GH1, family 1 glycosyl hydrolases; GH3, family 3 glycosyl hydrolases; IPTG, isopropylthio-β-galactoside; MDin, malonyl daidzin; MGin, malonyl genistin; DMSO, dimethyl sulfoxide; *p*NP, *p*-nitrophenyl; *p*NPG, *p*NP-β-D-glucopyranoside; PPB, potassium phthalate buffer; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SF, soybean flour; Tm-BglA, β-glucosidase A from *Thermotoga maritima* MS8; Tm-BglB, β-glucosidase B from *Thermotoga maritima*; Te-BglA, β-glucosidase from *Thermoanaerobacter ethanolicus* JW200

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