JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Hydrolysis of Isoflavone Glycosides by a Thermostable β-Glucosidase from *Pyrococcus furiosus*

Soo-Jin Yeom, Bi-Na Kim, Yeong-Su Kim, and Deok-Kun Oh*

Department of Bioscience and Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

ABSTRACT: The recombinant β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* was purified with a specific activity of 330 U/mg for genistin by His-trap chromatography. The specific activity of the purified enzyme followed the order genistin > daidzin > glycitin> malonyl glycitin > malonyl daidzin > malonyl genistin. The hydrolytic activity for genistin was highest at pH 6.0 and 95 °C with a half-life of 59 h, a K_m of 0.5 mM, and a k_{cat} of 6050 1/s. The enzyme completely hydrolyzed 1.0 mM genistin, daidzin, and glycitin within 100, 140, and 180 min, respectively. The soybean flour extract at 7.5% (w/v) contained 1.0 mM genistin, 0.9 mM daidzin, and 0.3 mM glycitin. Genistin, daidzin, and glycitin in the soybean flour extract were completely hydrolyzed after 60, 75, and 120 min, respectively. Of the reported β-glucosidases, *P. furiosus* β-glucosidase exhibited the highest thermostability, k_{cat}, k_{cat}/K_m, yield, and productivity for hydrolyzing genistin. These results suggest that this enzyme may be useful for the industrial hydrolysis of isoflavone glycosides.

KEYWORDS: *β-glucosidase, Pyrococcus furiosus, isoflavone hydrolysis, genistein, daidzein, glycitein, soybean flour extract*

INTRODUCTION

Isoflavones are the structural homologues of human estrogens and exist as glycosides most frequently in leguminous plants, particularly soybean.[1,2] Isoflavones have recently attracted much attention due to their effects against osteoporosis, cancer, cardiovascular disease, and menopausal symptoms.[3−5] Many studies have focused on the hydrolysis of isoflavone glycosides, as isoflavone aglycones possess higher pharmaceutical activity than isoflavone glycosides.[6−8] Isoflavone aglycones are absorbed more quickly in humans than their glycosides.[7] However, isoflavone aglycones exist at low concentrations or are absent in leguminous plants.

The isoflavone aglycones daidzein, genistein, glycitein, malonyl daidzein, malonyl genistein, and malonyl glycitein are produced by removing sugar moieties from the isoflavone glycosides daidzin, genistin, glycitin, malonyl daidzin, malonyl genistin, and malonyl glycitin, respectively. These isoflavone aglycones have been obtained by hydrolysis reactions of microorganisms, including *Lactobacillus paraplantarum, Enterococcus durans, Streptococcus salivarius, Weissella confuse,*[9] *Bacillus subtilis,*[10] and *Rhizopus oligosporus.*[11] However, microbial hydrolysis exhibits poor productivity because of long fermentation time. Thus, enzymatic hydrolysis has been proposed as a potentially useful method because of its high specificity, yield, and productivity. Enzymatic hydrolysis has been achieved using microbial β-glucosidases from *Aspergillus oryzae,*[6] *B. subtilis,*[12] *Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus casei,*[13] *Dictyoglomus turgidum,*[14] *Escherichia coli,*[15] *Paecilomyces thermophila,*[16] *Pseudomonas* sp.,[17] *Sulfolobus solfataricus,*[18] *Thermoanaerobacter ethanolicus,*[19] and *Thermotoga maritima*[20] and plant β-glucosidases from *Dalbergia cochinchinensis, Dalbergia nigrescens,*[21] *Glycine max* (soybean), and commercial almond.[22] Recently, thermostable β-glucosidases from *D. turgidum,*[14] *S. solfataricus,*[18] and *T. maritima*[20] exhibited high hydrolytic activities for isoflavone aglycones. However, the hydrolysis of isoflavone glycosides by these enzymes is inefficient in terms of the conversion yield and productivity. Because the enzyme reaction velocity is increased at higher temperatures, more thermostable enzyme is required for the effective hydrolysis of isoflavone glycosides. β-Glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus* showed a remarkable thermostability with a half-life of 85 h at 100 °C and maximum activity at 100 °C for *p*-nitrophenyl (*p*NP)-β-D-glucopyranoside.[23] Thus, the enzyme would be highly useful for the hydrolysis of isoflavone glycosides.

In this study, the optimal pH and temperature for the conversion of genistin to genistein were determined using β-glucosidase from *P. furiosus*, and the hydrolytic activity and kinetic parameters of the enzyme for isoflavone glycosides were investigated. Moreover, hydrolysis of isoflavone glycosides from a reagent and a soybean flour extract was attempted.

MATERIALS AND METHODS

Materials. The isoflavone standards, including genistin, daidzin, glycitin, malonyl glycitin, malonyl daidzin, malonyl genistin, genistein, daidzein, glycitein malonyl glycitein, malonyl daidzein, and malonyl genistein, were purchased from Sigma (St. Louis, MO). HPLC grade acetonitrile and acetic acid were purchased from Fisher Scientific (Hanover Park, IL). All of the restriction enzymes were purchased from New England Biolabs (Hertfordshire, U.K.). All other regents were purchased from Sigma.

Cloning and Gene Expression. The genomic DNA from *P. furiosus* DSMZ 3638 (Microbank, Daejeon, Korea), *E. coli* ER2566 (New Englands Biolabs), and pET24a(+) plasmid (Novagen, Madison, WI) were used as the source of the β-glucosidase gene, host cells, and expression vector. The gene encoding the β-glycosidase was amplified by polymerase chain reaction (PCR) using the genomic DNA isolated from *P. furiosus* as template. The sequence of the oligonucleotide primers used for gene cloning was based on the DNA

Received: October 31, 2011
Revised: January 16, 2012
Accepted: January 18, 2012
Published: January 18, 2012

sequence of *P. furiosus* β-glucosidase, *celB* gene (GenBank accession no. AAC25555). Forward (5′-GGATCCATGAAGTTCTCCAAAAAAC-3′) and reverse primers (5′-GTCGACCTTTCTTGTAACAAATT-3′) were designed to introduce the *Bam*HI and *Sal*I restriction sites (underline), respectively, and were synthesized by Bioneer (Daejon, Korea). The amplified DNA fragment obtained by PCR was purified and inserted into the pET24a(+) vector digested with the same restriction enzymes. *E. coli* ER2566 strain was transformed with the ligation mixture using an electroporator (MicroPulser, Bio-Rad, Hercules, CA) and plated on LB agar containing 25 μg/mL of kanamycin. A kanamycin-resistant colony was selected, and plasmid DNA from the transformant was isolated with a plasmid purification kit (Promega, Madison, WI). DNA sequencing was conducted using a DNA analyzer (ABI Prism 3730xl, Perkin-Elmer, Waltham, MA). Gene expression was evaluated by both SDS-PAGE and enzyme activity.

Culture Conditions. *P. furiosus* was grown anaerobically at 90 °C on a complex medium containing starch as the carbon and energy source.[24] *E. coli* cells containing the β-glucosidase/pET24a(+) gene were cultivated in a 2000 mL flask containing 500 mL of Luria−Bertani (LB) medium and 20 μg/mL kanamycin at 37 °C with shaking at 200 rpm. When the optical density of bacteria reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce the expression of β-glucosidase, and then the culture was incubated with shaking at 150 rpm at 16 °C for 4 h.

Enzyme Preparation. The grown cells were harvested and disrupted by sonication on ice for 2 min in 50 mM phosphate buffer (pH 7.0) containing 300 mM NaCl with the addition of 1 mg/mL lysozyme. The unbroken cells and cell debris were removed by centrifugation at 13000*g* for 20 min at 4 °C, and the supernatant obtained was used as a crude extract. The enzyme solution of the supernatant was applied to a His-trap affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer (pH 7.0). The bound protein was then eluted at 4 °C with the same buffer containing 250 mM imidazole at a flow rate of 1 mL/min. The active fractions were collected and dialyzed at 4 °C for 16 h against 50 mM citrate/phosphate buffer (pH 6.0). The resultant solution was used as a purified enzyme. The purification step using the column was conducted using a fast protein liquid chromatography system (Bio-Rad) in a cold room at 4 °C.

Hydrolytic Activity. The hydrolytic reactions of *P. furiosus* β-glucosidase were performed in 50 mM citrate/phosphate buffer (pH 6.0) containing 0.017 U/mL enzyme and 1.0 mM *p*−nitrophenyl-(*p*NP)-β-D-glucopyranoside or 0.2 mM isoflavone glycoside genistin, daidzin, glycitin, malonyl genistin, malonyl daidzin, or malonyl glycitin for 5 min at 95 °C. The activity for *p*NP-β-D-glucopyranoside was determined by release of *p*NP. The absorbance at 415 nm was measured after the reactions had been quenched by the addition of 200 mM Na_2CO_3. One unit (U) of enzyme activity used for the hydrolysis of *p*NP-β-D-glucopyranoside was defined as the amount of enzyme required to liberate 1 μmol of *p*NP per minute at 95 °C and pH 6.0. The activity for isoflavone glycoside was determined by release of aglycon isoflavone. One unit of enzyme activity used for the hydrolysis of isoflavone glycosides was defined as the amount of enzyme required to liberate 1 μmol of genistein from genistin as a substrate per minute at 95 °C and pH 6.0. The specific activity (U/mg) was defined as the produced amount of NP or isoflavone aglycone as a product per enzyme amount per unit of reaction time. The productivity (mM/h) was defined as the increase in the concentration of isoflavone aglycone as a product per unit of reaction time.

Kinetic Parameters. Various concentrations of *p*NP-β-D-glucopyranoside (from 0.1 to 3 mM), genistin (from 0.1 to 1.5 mM), daidzin (from 0.1 to 2.5 mM), and glycitin (from 0.05 to 2.5 mM) were used to determine the kinetic parameters of the enzyme. The reactions were performed in citrate/phosphate buffer (pH 6.0) at 95 °C for 5 min. The amounts of isoflavone aglycones were detected by HPLC assay. K_m (mM) and k_{cat} (1/s) were determined by the Lineweaver−Burk plot from the Michaelis−Menten equation. To calculate the catalytic constant, k_{cat}, the amount of protein was divided by the subunit molecular mass of 221952 Da. Protein concentrations were determined according to the Bradford method using bovine serum albumin as a standard protein.[25]

Effects of Metal Ions, pH, and Temperature. To investigate the effect of metal ions on enzyme activity, the enzyme assay was carried out after treatment with 1 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C for 1 h or after the addition of 1 mM of each metal ion such as Co^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, or Ca^{2+}. The reactions were performed in 50 mM citrate/phosphate buffer (pH 6.0) containing each metal ion at 95 °C.

To evaluate the effects of pH and temperature on β-glucosidase activity, the temperatures were varied from 75 to 98 °C at pH 6.0, and the pH values were varied from 4.5 to 7.0 using 50 mM citrate/phosphate buffer (pH 4.5−7.0) at 95 °C. The effect of temperature on enzyme stability was monitored as a function of incubation time by applying the enzyme solution at six different temperatures (70, 75, 80, 85, 90, and 95 °C) in 50 mM citrate/phosphate buffer (pH 6.0). Samples were withdrawn at time intervals and then assayed in 50 mM citrate/phosphate buffer (pH 6.0) containing 0.2 mM genistin at 95 °C for 5 min. The half-life of the enzyme was calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA).

Preparation of Soybean Flour Extract. The extraction method for soybean flour was used on the basis of the extraction method for soy flour.[20] The extraction conditions were optimized by varying soybean flour concentration (10−25%, w/v), methanol concentration (50−100%, v/v), temperature (60−90 °C), and extraction time (1−5 h). The highest amount of isoflavones was obtained after extraction with 6 volumes of 80% methanol by stirring for 3 h at 80 °C for 3 g of soybean flour. Thus, these conditions were used for extracting soybean flour.

Time Courses for the Hydrolysis of Genistin, Daidzin, and Glycitin. The hydrolysis of the isoflavone glycosides genistin, daidzin, and glycitin from a reagent and a soybean flour extract was investigated using *P. furiosus* β-glucosidase. The hydrolytic reactions were performed at 95 °C in 50 mM citrate/phosphate buffer (pH 6.0) containing 1.65 U/mL enzyme and 1 mM reagent grade genistin, daidzin, or glycitin for 120, 160, or 200 min, respectively, and 33 U/mL enzyme and 7.5% (w/v) soybean flour extract for 160 min.

Analytical Methods. Isoflavones were assayed using the isoflavone standards and an HPLC system (Agilent 1100, Santa Clara, CA) equipped with a UV detector at 254 nm and a C18 column (50 × 4.6 mm, YMC, Kyoto, Japan). The column eluted at 35 °C with a gradient of solvent A (3% acetic acid in water) and solvent B (3% acetic acid in mixture of water and acetonitrile of 50:50 (v/v)) from 75:25 to 40:60 for 12 min and then from 40:60 to 75:25 for 3 min. The flow rate was 1.5 mL/min. The substrates daidzin, glycitin, genistin, malonyl daidzin, malonyl glycitin, and malonyl genistin were detected with retention times of 3.4, 3.8, 4.3, 4.6, 4.8, and 5.1 min, respectively. The products daidzein, glycitein, genistein, malonyl daidzein, malonyl glycitein, and malonyl genistein were detected with retention times of 5.4, 5.8, 7.2, 7.6, 8.0, and 8.6 min, respectively. The isoflavones in the reaction samples formed from different substrates and soybean flour extract were identified as the same retention times with the isoflavone standards. The amounts of isoflavones in soybean flour extract were determined using linear calibration curves relating the peak areas to the concentrations of isoflavone standards.

RESULTS

Gene Cloning and Purification, Molecular Mass Determination of *P. furiosus* β-Glucosidase. A gene encoding *P. furiosus* β-glucosidase, with the same sequence as reported in GenBank (accession no. AAC25555), was cloned and expressed in *E. coli*. The enzyme was purified from crude extract obtained from harvested cells as a soluble protein by His-trap affinity chromatography. The β-glucosidase was purified with a purification of 7.2-fold, a yield of 62%, and a specific activity of 330 U/mg for genistin. After IPTG induction, the specific activity of *P. furiosus* β-glucosidase was constant at approximately

330 U/mg regardless of incubation time. The molecular mass of the expressed protein analyzed by SDS-PAGE was about 56 kDa (Figure 1), consistent with the calculated value of

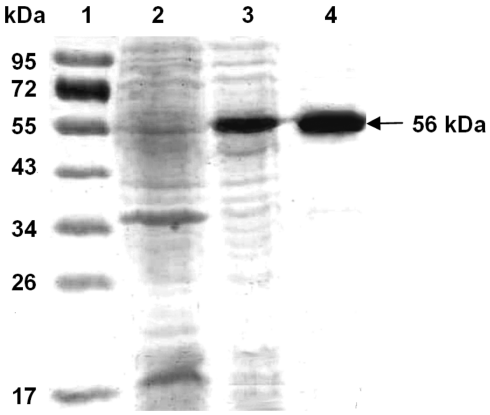

Figure 1. SDS-PAGE analysis of each purification step. Lanes: 1, marker proteins; 2, cell debris; 3, crude extract; 4, supernatant after His-trap affinity chromatography.

55488 Da based on the 472 amino acid residues plus 6 histidine residues (data not shown). β-Glucosidase from *P. furiosus* was reported as a tetramer with a total molecular mass of approximately 230 kDa.[23]

Effects of Metal Ion, pH, and Temperature on the Activity of *P. furiosus* β-Glucosidase. No isoflavone aglycones were formed when reactions were conducted without enzyme or with grown *E. coli* ER2566 cells that did not harbor the β-glucosidase gene from *P. furiosus*. The effect of metal ions such as Co^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, or Ca^{2+} on the hydrolytic activity of genistin by *P. furiosus* β-glucosidase was assessed. The enzyme was not activated by monovalent or divalent cations and was not inhibited by EDTA. Therefore, the enzyme was determined as metal-independent (data not shown).

The hydrolytic activity of genistin to genistein by *P. furiosus* β-glucosidase was examined in a pH range from 4.5 to 7.0 (Figure 2A). Maximal activity was observed at pH 6.0. The activity was about 80% of the maximum at pH 5.0 and 7.0. The temperature was varied from 75 to 98 °C at pH 6.0 to investigate its effect on the hydrolytic activity of genistin to genistein by the enzyme (Figure 2B). The activity was maximal at 95 °C. The thermal stability of *P. furiosus* β-glucosidase for genistin was examined at temperatures ranging from 70 to 95 °C (Figure 3). Thermal inactivation of the enzyme followed first-order kinetics with half-lives of 1020, 476, 307, 181, 96, and 59 h at 70, 75, 80, 85, 90, and 95 °C, respectively.

Substrate Specificity of *P. furiosus* β-Glucosidase for Isoflavone Glycosides. The specific activity of *P. furiosus* β-glucosidase was investigated using isoflavone glycosides (Table 1). The specific activity of the enzyme followed the order genistin > daidzin > glycitin > malonyl glycitin > malonyl daidzin > malonyl genistin. The specific activity for genistin was 2.8-fold lower than that for *p*NP-β-D-glucopyranoside. The Michaelis–Menten constants (K_m), turnover numbers (k_{cat}), and catalytic efficiencies (k_{cat}/K_m) for genistin, daidzin, and glycitin are presented in Table 1. The orders of the k_{cat} and k_{cat}/K_m values of *P. furiosus* β-glycosidase (genistin > daidzin > glycitin) were the same as those observed for specific activity. However, the substrate affinity of the enzyme followed the order glycitin > daidzin > genistin.

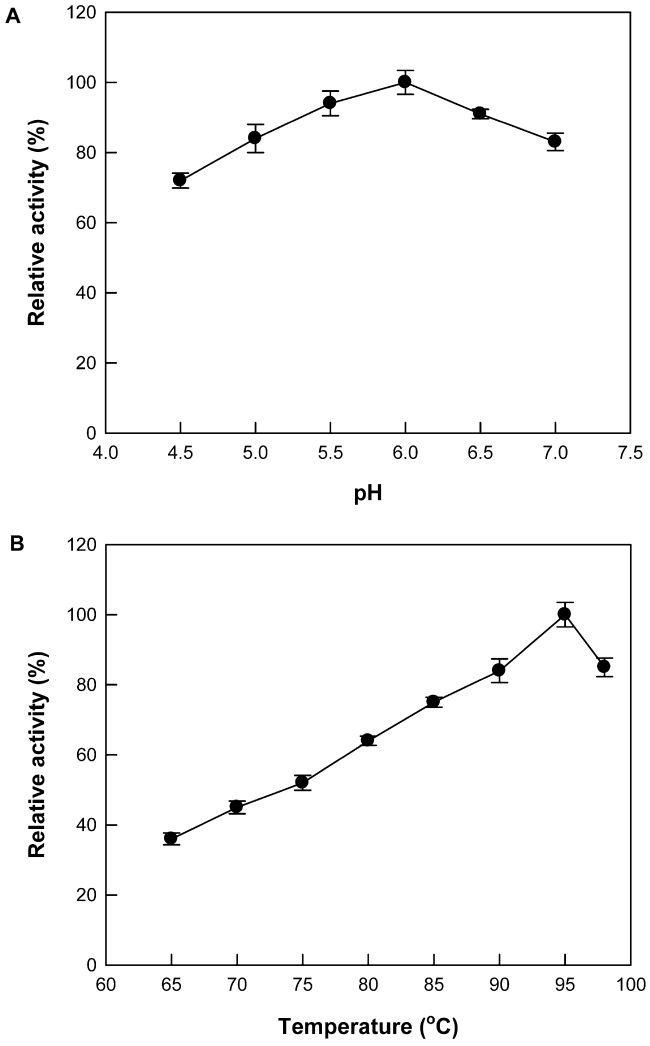

Figure 2. Effects of (A) pH and (B) temperature on the activity of *P. furiosus* β-glucosidase. The reactions for pH experiments were performed with 0.2 mM genistin and 0.017 U/mL enzyme at 95 °C for 5 min. The reactions were performed in 50 mM citrate/phosphate buffer (pH 6.0) containing 0.2 mM genistin and 0.017 U/mL enzyme for 5 min. Data represent the mean of three experiments, and error bars represent the standard deviation.

Hydrolysis of Isoflavone Glycosides from a Reagent and a Soybean Flour Extract to Isoflavone Aglycones. The hydrolysis of isoflavone glycosides to isoflavone aglycones was investigated at pH 6.0 and 95 °C using *P. furiosus* β-glucosidase. The enzyme completely hydrolyzed 1.0 mM genistin, daidzin, and glycitin to 1.0 mM genistein, daidzein, and glycitein, within 100, 140, and 180 min, with no byproducts. The productivities corresponded to 0.60, 0.43, and 0.33 mM/h, respectively (Figure 4). The concentration of soybean flour extract was adjusted to 7.5% (w/v) to obtain 1.0 mM genistin. The soybean flour extract at 7.5% (w/v) contained 3.0 mM total isoflavones, which consisted of 1.03 mM genistin, 0.86 mM daidzin, 0.25 mM glycitin, 0.39 mM genistein, 0.34 mM daidzein, and 0.13 mM glycitein. Genistin, daidzin, and glycitin in the soybean flour extract were completely hydrolyzed to genistein, daidzein, and glycitein, within 60, 75, and 120 min, corresponding to productivities of 1.00, 0.80, and 0.50 mM/h, respectively (Figure 5).

dx.doi.org/10.1021/jf204432g | J. Agric.Food Chem. 2012, 60, 1535–1541

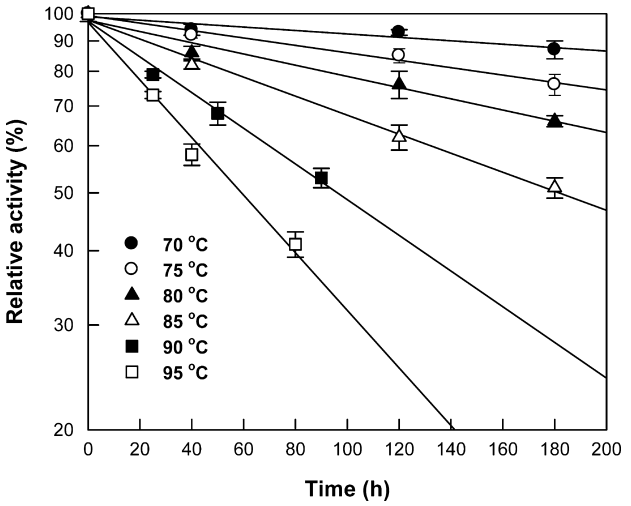

Figure 3. Thermal inactivation of the activity of *P. furiosus* β-glucosidase. The enzymes were incubated at 70, 75, 80, 85, 90, and 95 °C for various times. A sample was withdrawn at each time interval, and the relative activity was determined. Data represent the mean of three experiments, and error bars represent the standard deviation.

DISCUSSION

The hydrolytic activity of *P. furiosus* β-glucosidase for genistin was maximal at pH 6.0 and 95 °C (Figure 2). The hydrolytic activities of the enzyme for *p*NP-β-D-glucopyranoside[23] and ginsenoside Rd[26] were maximal at pH 5.0 and 100 °C and at pH 5.5 and 95 °C, respectively. The optimal pH values of β-glucosidases from *A. oryzae*,[27] the genus *Dalbergia*,[21] *D. turgidum*,[14] *P. thermophila*,[16] *Pseudomonas* sp.,[17] *S. solfataricus*,[28] and *T. maritima*[20] for hydrolyzing isoflavone glycosides were 5.0, 5.5, 5.0 6.2, 6.0, 5.5, and 5.0, respectively. The maximal activities of β-glucosidases from *A. oryzae*,[27] *D. nigrescens*,[21] *D. turgidum*,[14] *P. thermophila*,[16] *Pseudomonas* sp.,[17] *S. solfataricus*,[28] and *T. maritima*[20] for hydrolyzing isoflavone glycosides were observed at 55, 65, 85, 75, 40, 90, and 70 °C, respectively. These results suggest that temperature for the hydrolysis of genistin to genistein is the highest among β-glucosidases.

The half-lives of *P. furiosus* β-glucosidase for genistin at 75, 80, 85, 90, and 95 °C were 476, 307, 181, 96, and 59 h, respectively (Figure 3). The half-lives of the enzyme for *p*NP-β-D-glucopyranoside at 100 and 105 °C were 85 and 13 h, respectively,[23] and for ginsenoside Rd at 75, 80, 85, 90, and 95 °C were 572, 333, 172, 118, and 68 h, respectively.[26] These results indicate that the thermostability of *P. furiosus* β-glucosidase for substrate follows the order *p*NP-β-D-glucopyranoside > ginsenoside Rd > genistin. β-Glucosidase from *Pseudomonas* sp. for genistin showed 44% relative activity at 50 °C after 30 min.[17] The half-life of *P. thermophila* β-glucosidase was approximately 0.3 h at 70 °C.[16] The half-life of *T. maritima* β-glucosidase (Tm-BglB) was 1.5 h at 70 °C; those of *D. turgidum* β-glucosidase for genistin at 75, 80, 85, and 90 °C were 14, 9, 6, and 0.3 h, respectively;[14] and those of *S. solfataricus* β-glucosidase for daidzin at 75, 80, 85, 90, and 95 °C were 80, 51, 31, 18, and 10 h, respectively.[28] This enzyme exhibited the previous highest thermostability of the reported β-glucosidases for the hydrolysis of isoflavone glycosides. The half-lives of β-glucosidase from the hyperthermophile *P. furiosus* at 75, 80, 85, 90, and 95 °C were approximately 3−6-fold higher than those from *S. solfataricus*. Thus, *P. furiosus* β-glucosidase is the most thermostable β-glucosidase reported to date for the hydrolysis of isoflavone glycosides.

The specific activity of β-glucosidase from *P. furiosus* followed the order genistin > daidzin > glycitin > malonyl glycitin > malonyl daidzin > malonyl genistin (Table 1), those from *T. maritima*,[20] *Pseudomonas* sp.,[17] *D. nigrescens*, and *D. cochinchinensis*[21] followed the order genistin > daidzin > malonyl genistin > malonyl daidzin, and those from *P. thermophila* and *D. turgidum* followed the order genistin > daidzin > glycitin.[14,16] However, *S. solfataricus* β-glucosidase followed the order daidzin > glycitin > genistin > malonyl daidzin > malonyl glycitin > malonyl genistin. These results indicate that β-glucosidases except *D. turgidum* β-glucosidase exhibit the highest activity for genistin among isoflavone glycosides. The kinetic parameters of the β-glucosidases for the substrate genistin are summarized in Table 2. Previously, the highest reported k_{cat} for genistin was 3660 1/s for a β-glucosidase from *D. turgidum*,[14] and the highest reported k_{cat}/K_m was 10500 1/(mM s) for a β-glucosidase from *D. nigrescens*.[21] Notably, the k_{cat} and k_{cat}/K_m of *P. furiosus* β-glucosidase are 1.7- and 1.2-fold higher than the previously highest values, respectively. These results suggest that the k_{cat} and k_{cat}/K_m of *P. furiosus* β-glucosidase are the highest yet reported and that this enzyme is a very efficient producer of isoflavone aglycones.

The conversion of genistin to genistein by β-glucosidases is summarized in Table 3. The productivity of genistein using 1.0 mM genistin as a single substrate and 1.65 U/mL enzyme by β-glucosidase from *P. furiosus* was 0.60 mM/h (Figure 4A), whereas the productivity of genistein using 7.5% (w/v) soybean flour extract with 1.0 mM genistin as a mixture and 33 U/mL enzyme was 1.00 mM/h (Figure 5). Although the productivity of genistein for the single substrate was 1.7-fold lower than that for the mixture, the specific productivity of genistein for the single substrate (364 mmol U^{1-} h^{-1}) was 12-fold higher than that for the mixture (30.3 mmol U^{1-} h^{-1}). The previously highest reported mole yield was 98% for a β-glucosidase from *E. coli* using reagent grade genistin, whereas the previously highest reported productivity was 0.11 mM/h for a β-glucosidase from *A. oryzae* using soybean flour extract. β-Glucosidase from *P. furiosus* completely hydrolyzed genistin from the reagent and

Table 1. Hydrolytic Activity and Kinetic Parameters of *P. furiosus* β-Glucosidase[a]

substrate	product	specific activity (U/mg)	K_m (mM)	k_{cat} (s)	k_{cat}/K_m (1/(mM s))
*p*NP-β-D-glucopyranoside	*p*NP	912 ± 14			
genistin	genistein	330 ± 12	0.50 ± 0.09	6050 ± 52	12100 ± 390
daidzin	daidzein	180 ± 5.5	0.42 ± 0.02	1880 ± 23	4480 ± 108
glycitin	glycitein	75 ± 1.5	0.33 ± 0.02	610 ± 28	1840 ± 130
malonyl glycitin	malonyl glycitein	46 ± 1.6			
malonyl daidzin	malonyl daidzein	37 ± 0.9			
malonyl genistin	malonyl genistein	5 ± 0.1			

[a]Data are expressed as the mean of three experiments ± standard deviation.

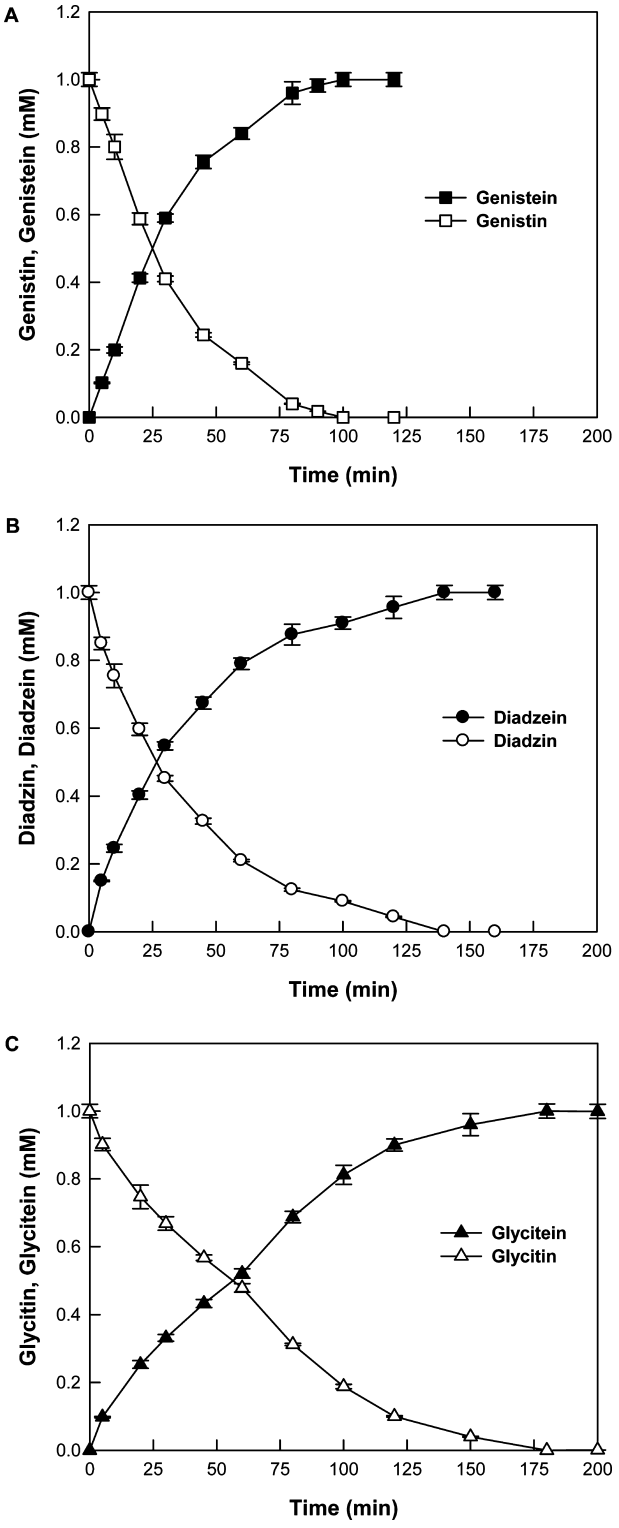

Figure 4. Conversion of the reagent grade isoflavone glycosides to isoflavone aglycones by *P. furiosus* β-glucosidase, respectively: (A) conversion of genistin to genistein; (B) conversion of daidzin to daidzein; (C) conversion of glycitin to glycitein. The reactions were performed in 50 mM citrate/phosphate buffer (pH 6.0) containing 1.0 mM reagent grade isoflavone glycoside and 1.65 U/mL enzyme at 95 °C for 120−200 min. Data represent the mean of three experiments, and error bars represent the standard deviation.

soybean flour extract to genistein with a conversion mole yield of 100% (Figures 4A and 5). The productivity obtained using

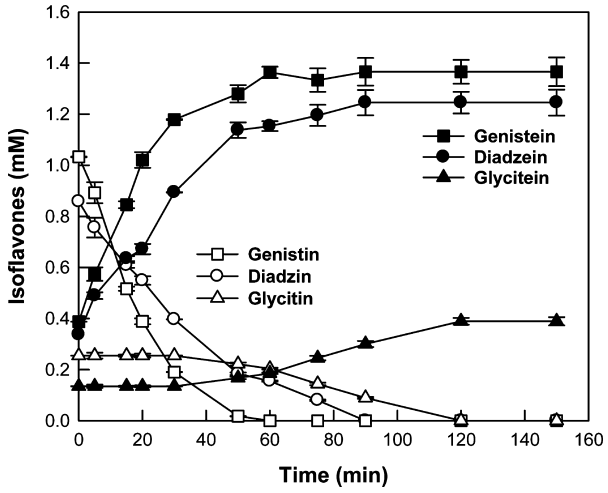

Figure 5. Conversion of genistin, daidzin, and glycitin in soybean flour extract to genistein, daidzein, and glycitein, respectively, by β-glucosidase from *P. furiosus*. The reactions were performed in 50 mM citrate/phosphate buffer (pH 6.0) containing 7.5% (w/v) soybean flour extract and 33 U/mL enzyme at 95 °C for 150 min. Data represent the mean of three experiments, and error bars represent the standard deviation.

Table 2. Hydrolytic Activity and Kinetic Parameters of β-Glucosidases for Genistin

strain	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM s))	temperature (°C)	ref
Bacillus natto	0.15	22	147	37	12
Dictyoglomus turgidum	0.83	3660	4440	85	14
Pseudomonas sp.	0.12	5.3	443	40	17
Sulfolobus solfataricus	0.30	932	3110	90	18
Thermotoga maritima	4.22	348	83	70	20
Dalbergia nigrescens	0.07	700	10500	30	21
Dalbergia cochinchinensis	0.13	208	1700	30	21
Glycine max	0.33	4.45	13	40	22
almond	11	0.65	0.06	40	22
Pyrococcus furiosus	0.48	2400	5000	65	this study
	0.50	6050	12100	95	this study

P. furiosus β-glucosidase was 9.1-fold higher than that achieved using *A. oryzae* β-glucosidase. Thus, *P. furiosus* β-glucosidase converted genistin to genistein with the highest productivity and yield yet reported.

The enzyme reactions for industrial biological processes have needed temperature optimum >65 °C to reduce the operation cost at high temperature. Thus, the k_{cat} of *P. furiosus* β-glucosidase at 65 °C was determined, and its k_{cat} is highest except that at 85 °C of *D. turgidum* β-glucosidase among β-glucosidases for the hydrolysis of isoflavone glycosides (Table 2). The k_{cat} of *P. furiosus* β-glucosidase at 85 °C was 4360 1/s, which was 1.3-fold higher than that (3360 1/s) at the same temperature of *D. turgidum* β-glucosidase. These results indicate that *P. furiosus* β-glucosidase is the most effective enzyme for the hydrolysis of isoflavone glycosides even though the enzyme reaction is performed at 65 °C for industrial biological process.

dx.doi.org/10.1021/jf204432g | J. Agric.Food Chem. 2012, 60, 1535−1541

Table 3. Conversion of Genistin to Genistein by β-Glucosidases

source	strain	genistin (mM)	genistein (mM)	mole yield (mM/mM)	productivity (mM/h)	ref
genistin	*Escherichia coli*	0.046	0.045	0.98	0.01	15
	Pyrococcus furiosus	1.00	1.00	1.00	0.60	this study
soybean milk	soybean	NR[a]	NR	0.17	NR	29
soybean flour	*Thermotoga maritima*	NR	0.38[b]	0.94	NR	20
soybean flour extract	almond	NR	NR	0.79	NR	30
	Aspergillus oryzae	1.20	0.54	0.45	0.11	31
	Aspergillus oryzae	5.00	4.09	0.76	0.08	6
	Paecilomyces thermophila	NR	NR	0.95	NR	16
	Pyrococcus furiosus	1.00	1.00	1.00	1.00	this study

[a]NR, not reported. [b]Geinstein (mg)/soybean flour (g).

In conclusion, β-glucosidase from *P. furiosus* was found to be the most thermostable among β-glucosidases for the hydrolysis of isoflavone glycosides. This enzyme exhibited the highest k_{cat}, k_{cat}/K_m, productivity, and yield yet reported. These results should be useful in the soybean product industry.

AUTHOR INFORMATION

Corresponding Author

*Phone: (822) 454-3118. Fax: (822) 444-6176. E-mail: deokkun@konkuk.ac.kr.

Funding

This study was supported by the National Research of Korea (NRF) through the National Research Laboratory Program, Ministry of Education, Science and Technology (R0A-2007-000-20015-0) and the Next-Generation BioGreen 21 Program grant, Rural Development Administration, Republic of Korea.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Faranke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. K. Quantitation of phytoestrogens in legumes by HPLC. *J. Agric. Food Chem.* **1994**, *42*, 1905−1913.

(2) Halabalaki, M.; Alexi, X.; Aligiannis, N.; Lambrinidis, G.; Pratsinis, H.; Florentin, I.; Mitakou, S.; Mikros, E.; Skaltsounis, A. L.; Alexis, M. N. Estrogenic activity of isoflavonoids from *Onobrychis ebenoides*. *Planta Med.* **2006**, *72*, 488−493.

(3) Jacobsen, B. K.; Knutsen, S. F.; Fraser, G. E. Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). *Cancer Causes Control* **1998**, *9*, 553−557.

(4) Adlercreutz, H. Phytoestrogens and breast cancer. *J. Steroid. Biochem. Mol. Biol.* **2002**, *83*, 113−118.

(5) Omoni, A. O.; Aluko, R. E. Soybean foods and their benefits: potential mechanisms of action. *Nutr. Rev.* **2005**, *63*, 272−283.

(6) Kaya, M.; Ito, J.; Kotaka, A.; Matsumura, K.; Bando, H.; Sahara, H.; Ogino, C.; Shibasaki, S.; Kuroda, K.; Ueda, M.; Kondo, A.; Hata, Y. Isoflavone aglycones production from isoflavone glycosides by display of β-glucosidase from *Aspergillus oryzae* on yeast cell surface. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 51−60.

(7) 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.

(8) Izumi, T.; Piskula, M. K.; Osawa, S.; Obata, A.; Tobe, K.; Saito, M.; Kataoka, S.; Kubota, Y.; Kikuchi, M. Soy isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans. *J. Nutr.* **2000**, *130*, 1695−1699.

(9) Chun, J.; Kim, G. M.; Lee, K. W.; Choi, I. D.; Kwon, G. H.; Park, J. Y.; Jeong, S. J.; Kim, J. S.; Kim, J. H. Conversion of isoflavone glucosides to aglycones in soymilk by fermentation with lactic acid bacteria. *J. Food Sci.* **2007**, *72*, M39−M44.

(10) 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.

(11) Ibe, S.; Kumada, K.; Yoshida, M.; Onga, T. Production of natto which contains a high level of isoflavone aglycones. *J. Jpn. Soc. Food Sci.* **2001**, *48*, 27−34.

(12) Kuo, L. C.; Lee, K. T. Cloning, expression, and characterization of two beta-glucosidases from isoflavone glycoside-hydrolyzing *Bacillus subtilis* natto. *J. Agric. Food Chem.* **2008**, *56*, 119−125.

(13) Donkor, O. N.; Shah, N. P. Production of β-glucosidase and hydrolysis of isoflavone phytoestrogens by *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus casei* in soymilk. *J. Food Sci.* **2008**, *73*, M15−M20.

(14) Kim, Y. S.; Yeom, S. J.; Oh, D. K. Characterization of a GH3 family β-glucosidase from *Dictyoglomus turgidum* and its application to the hydrolysis of isoflavone glycosides in spent coffee grounds. *J. Agric. Food Chem.* **2011**, *59*, 11812−11818.

(15) Ismail, B.; Hayes, K. β-Glycosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, *53*, 4918−4924.

(16) Yang, S.; Wang, L.; Yan, Q.; Jiang, Z.; Li, L. Hydrolysis of soybean isoflavone glycosides by a thermostable β-glucosidase from *Paecilomyces thermophila*. *Food Chem.* **2009**, *115*, 1247−1252.

(17) 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.

(18) Kim, B. N.; Yeom, S. J.; Kim, Y. S.; Oh, D. K. Characterization of a β-glucosidase from *Sulfolobus solfataricus* for isoflavone glycosides. *Biotechnol. Lett.* **2012**, *34*, 125−129.

(19) Song, X.; Xue, Y.; Wang, Q.; Wu, X. Comparison of three thermostable β-glucosidases for application in the hydrolysis of soybean isoflavone glycosides. *J. Agric. Food Chem.* **2011**, *59*, 1954−1961.

(20) Xue, Y.; Yu, J.; Song, X. Hydrolysis of soy isoflavone glycosides by recombinant β-glucosidase from hyperthermophile *Thermotoga maritima*. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 1401−1408.

(21) Chuankhayan, P.; Rimlumduan, T.; Svasti, J.; Cairns, J. R. Hydrolysis of soybean isoflavonoid glycosides by *Dalbergia* β-glucosidases. *J. Agric. Food Chem.* **2007**, *55*, 2407−2412.

(22) 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.

(23) Kengen, S. W.; Luesink, E. J.; Stams, A. J.; Zehnder, A. J. Purification and characterization of an extremely thermostable β-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem.* **1993**, *213*, 305−312.

(24) Hansen, T.; Oehlmann, M.; Schonheit, P. Novel type of glucose-6-phosphate isomerase in the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* **2001**, *183*, 3428−3435.

(25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(26) Yoo, M. H.; Yeom, S. J.; Park, C. S.; Lee, K. W.; Oh, D. K. Production of aglycon protopanaxadiol via compound K by a thermostable β-glycosidase from *Pyrococcus furiosus*. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1019−1028.
(27) Ito, J.; Sahara, H.; Kaya, M.; Hata, Y.; Shibasaki, S.; Kawata, K.; Ishida, S.; Ogino, C.; Fukuda, H.; Kondo, A. Characterization of yeast cell surface displayed *Aspergillus oryzae* β-glucosidase 1 high hydrolytic activity for soybean isoflavone. *J. Mol. Catal. B: Enzym.* **2008**, *55*, 69−75.
(28) Kim, B. N.; Yeom, S. J.; Kim, Y. S.; Oh, D. K. Characterization of a β-glucosidase from *Sulfolobus solfataricus* for isoflavone glycosides. *Biotechnol. Lett.* **2011**, DOI: 10.1007/s10529-011-0739-9.
(29) Matsuura, M.; Obata, A. β-Glucosidases from soybeans hydrolyze daidzin and genistin. *J. Food Sci.* **1993**, *58*, 144−147.
(30) Wu, J.; Muir, A. D. Isoflavone during protease hydrolysis of defatted soybean meal. *Food Chem.* **2010**, *118*, 328−332.
(31) Horii, K.; Adachi, T.; Matsuda, T.; Tanaka, T.; Sahara, H.; Shibasaki, S.; Ogino, C.; Hata, Y.; Ueda, M.; Kondo, A. Improvement of isoflavone aglycones production using β-glucosidase secretory produced in recombinant *Aspergillus oryzae*. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 297−301.