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Characterization and application of an acidophilic and thermostable β-glucosidase from *Thermofilum pendens*

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The gene encoding a β-glucosidase from the archaeon *Thermofilum pendens* (Tpbgl) was cloned and expressed in *Escherichia coli*. The purified recombinant enzyme had a molecular mass of 77.8 kDa and released glucose or mannose from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), cellobiose, mannobiose, and genistin. Peak Tpbgl activity was detected at 90°C, and 50% activity remained after incubation for 60 min at 95°C. The optimal pH for *p*NPG hydrolysis was 3.5. When the enzyme was incubated with *p*NPG in the presence of ethanol and propanol, the glucose moiety was transferred to acceptor alcohols. Tpbgl is the archaeal β-glucosidase from glucoside hydrolase family 3 and found to be most heat stable under extremely acidic conditions (pH 3.5). The kinetic parameters revealed that Tpbgl had the highest catalytic efficiency toward *p*NPG (k_{cat}/K_m = 3.05) with strong substrate affinity for such natural substrates as cellobiose (K_m = 0.149) and mannobiose (K_m = 0.147). Genistin solubilized in 10–40% DMSO was hydrolyzed to genistein with nearly 99% conversion, indicating that high concentrations of the water-insoluble isoflavone glycoside can be treated by the enzyme. Our results indicate that Tpbgl has great potential in cellulose saccharification and the glucoside hydrolysis of natural compounds.

[**Key words:** β-Glucosidase; *Thermofilum pendens*; Cellulose; Glucoside; Archaeon]

β-Glucosidase (EC 3.2.1.21), which catalyzes the hydrolysis of β-glucosidic bonds in polysaccharides, releasing glucose from the non-reducing end, is involved in the synthesis and degradation of cellulosic compositions in plants, animals, and bacteria. β-Glucosidase has substrate specificity for the hydrolysis of short-chain cello-oligosaccharides and cellobiose resulting from the synergistic action of endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91) into glucose (1). The efficient conversion of lignocellulose to fermentable sugars has been recognized as the major bottleneck for the economical production of biofuels and feedstock chemicals from almost infinite renewable resources (2). Recently, several new cellulases, including cellobiohydrolase, endoglucanase, and β-glucosidase from bacteria and fungi, have been cloned and used to improve the saccharification of cellulose.

Thermostable β-glucosidases could facilitate the recycling of β-glucosidases in the bioconversion of cellulosic biomasses (3) and promote the rapid hydrolysis of quercetin glucosides in hot water (4). Other possible advantages to the use of thermostable β-glucosidases include higher substrate concentrations, increased product formation, and decreased microbial contamination (5). Thus, new sources of thermostable β-glucosidases are particularly attractive for biotechnological applications.

Hyperthermophiles have developed enzymes with the unique structure–function properties of high thermostability and optimal activity at temperatures above 70°C (6). Thermostable β-glucosidases from hyperthermophiles, including the genera *Sulfolobus* (7,8), *Pyrococcus* (9,10), and *Thermotoga* (11–13) have been extensively studied by overexpression in *Escherichia coli*, and their optimal temperatures for activity are between 85°C and 105°C, with an optimal pH between 5 and 7.

The archaeon *Thermofilum pendens* is an anaerobic, sulfur-dependent, hyperthermophile first isolated from a solfatara in Iceland (14) and lately also found at sites with temperatures ranging from 67°C to 93°C and pH values ranging from 2.8 to 7.6. Genomic sequencing of *T. pendens* has revealed several genes that might be related to the hydrolysis of cellulose and transportation (15).

In this study, we cloned a gene (Tpen_1494) encoding a β-glucosidase (Tpbgl) from *T. pendens* Hrk 5, expressed it in *E. coli*, characterized the biochemical properties of the gene product, and studied the kinetics of the expressed enzyme.

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MATERIALS AND METHODS

Chemicals *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG) was purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Genistin, cellobiose, and mannobiose were purchased from Sigma (St. Louis, MO, USA). All restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, and PrimeSTAR® HS DNA Polymerase were supplied by Takara Bio (Shiga, Japan). Water was prepared using a double-distilled water system (BSZ-2; Botonyc, Shanghai, China). All other chemicals used were of reagent grade and were purchased from Sinopharm Chemical (Shanghai, China).

Cloning and expression of the gene encoding Tpbgl The gene encoding Tpbgl (Tpen_1494) was amplified from *T. pendens* Hrk 5 (DSM 2475) genomic DNA (kindly provided by Dr. Claudia Reich, University of Illinois at Urbana-Champaign) by PCR using PrimeSTAR® HS DNA Polymerase at an annealing temperature of 62°C. Tpbgl gene-specific primers flanking the 5′ and 3′ ends of Tpen_1494 were designed based on the complete genome sequence of chromosome of *T. pendens* Hrk 5, NC_008698. The forward (Tpbgl-*Nde*I, 5′-GGGCATATGAGGGGAAGGGGCCCTG-3′) and reverse primers (Tpbgl-*Hind*III, 5′-CCGAAGCTTTCAGGGCGCGAATCTCA-3′) contained *Nde*I and *Hind*III restriction sites (underlined), respectively. The amplified fragment (2.1 kb) was digested with *Nde*I and *Hind*III and ligated into the expression vector p6xHis119 with a *BLMA* promoter (16) to create p6xHisTpbgl.

Purification of recombinant Tpbgl *E. coli* MC1061 (*F*⁻, *araD139*, *recA13*, Δ*(araABC-leu)7696*, *galU*, *galK lacX74*, *rpsL*, *thi*, *hsdR2*, and *mcrB*) cells transformed with p6xHisTpbgl were cultured for 20 h at 250 rpm and 37°C in six flasks containing 250 mL of Luria-Bertani broth (1% (w/v) Oxoid tryptone (Basingstoke, Hampshire, UK), 0.5% (w/v) Oxoid yeast extract, and 0.5% (w/v) NaCl) supplemented with ampicillin (100 μg/mL). The cells were then collected by centrifugation at 7000 ×*g* and 4°C for 20 min, resuspended in 150 mL of binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 5 mM imidazole), and sonicated 3 × 50 mL × 4 times for 3 min each in an ice bath using a JY92-II sonicator at an output power of 800 W (Scientz Biotechnology, Ningbo, China). A total of 110 mL of cell-free supernatant, collected by centrifugation at 12,000 ×*g* and 4°C for 30 min, was then heated at 70°C for 20 min to remove all thermolabile proteins. The crude enzyme was further purified using Ni Sepharose™ 6 Fast Flow packed in a XK 26/20 column with a bed height of 10 cm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) using an ÄKTApurifier system (GE Healthcare Bio-Sciences AB) as described previously (16). The active fractions in elution buffer (20 mM sodium phosphate, pH 7.4, 500 mM NaCl, and 500 mM imidazole) were dialyzed against 20 mM sodium phosphate buffer (pH 7.4). The protein concentration was determined according to the Bradford method using bovine serum albumin as a standard (17). The purity and molecular weight of the protein were analyzed by discontinuous SDS-PAGE (18).

Enzyme assay The activities of Tpbgl determined by the *p*NPG method was assayed in a total of 1 mL of 50 mM sodium acetate buffer at pH 3.5 containing 1 mM *p*NPG and enzyme solution. After incubation of the substrate solution at 90°C for 5 min, the reaction was initiated by the addition of 10 μL of the diluted enzyme solution and continued for 20 min at 90°C; it was terminated by the addition of 1 mL of 100 mM NaOH. The resulting color was detected by a spectrophotometer (UV2201; Shimadzu, Kyoto, Japan) at 410 nm. By the *p*NPG method, 1 U of enzyme activity is defined as the amount of enzyme required to produce 1 μmol of *p*NP per min under the above assay conditions (8).

Effects of pH, temperature, metal ions, and organic solvents on Tpbgl activity To determine the optimal pH and temperature for Tpbgl, an enzyme activity assay was done using the *p*NPG method. The relative activity of the enzyme against *p*NPG was examined at various pH values ranging from 2.5 to 6.0 using 50 mM sodium acetate buffer. The optimal temperature was determined by examining Tpbgl activity in 50 mM sodium acetate buffer (pH 3.5) at temperatures ranging from 60°C to 100°C in a water bath.

The pH stability of the enzyme was evaluated by incubation of 50 U/mL Tpbgl for 1 or 2 h at 90°C and pH 3.0–6.0. After cooling at room temperature, the remaining activity was determined by the *p*NPG method.

The thermostability of the enzyme was evaluated by incubation of 50 U/mL Tpbgl at 85°C, 90°C and 95°C for up to 120 min. After cooling at room temperature, the remaining activity was determined by the *p*NPG method.

The influence of 5 mM metal ions and organic solvents was investigated by the incubation of 50 U/mL Tpbgl at 90°C. Enzyme activity was determined after 1 h of pre-incubation using the *p*NPG method.

Kinetic parameters of Tpbgl as determined by isothermal titration calorimetry (ITC) Calorimetric assays were carried out with ITC_{200} system instruments (MicroCal Inc., Piscataway, NJ, USA). Reaction cells (200 μL) were filled with degassed solutions and equilibrated at 70°C. The stirring speed was 400 rpm, the thermal power was recorded every 2 s, and the instrument feedback was 10–50%. Enzyme reaction rates were determined by measuring the change in instrument thermal power supplied to the sample cell after addition of the substrate or enzyme through a sterile injection syringe.

Effects of DMSO on the hydrolysis of genistin by Tpbgl To assess the enzymatic hydrolysis of β-glucoside compounds in water-miscible organic solvents, 2–50 mM of genistin was dissolved in 10–50% DMSO in sodium acetate buffer (pH 4.0) as shown in Table 3 and then preincubated at 90°C for 3 min. The reactions were initiated by adding 80 μL of Tpbgl (866.22 U/mL) and then run at 90°C for 1 h.

Genistin and genistein were detected by HPLC (model 600S; Waters Corp., Milford, MA, USA) using a C18 column (Zorbax Eclipse XDB, 150 × 4.6 mm i.d., 3.5 μm particle size; Agilent, Santa Clara, CA, USA) and recorded at 254 nm with a UV detector. A gradient solvent system consisting of solvents A (0.1% formic acid) and B (100% acetonitrile) was used at a flow rate of 1.0 mL/min. Solvent B was increased gradually from 10 to 50% from 0 to 15 min. The concentrations of genistin and genistein were calculated from the genistin standard curve.

Sequence analysis of Tpbgl Sequence homology was investigated using Blastp provided by the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) (19,20). Sequence identities were calculated using EMBOSS Needle provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/psa/). Multiple alignments were done using ClustalX 2.1 (21). Phylip format tree outputs from the ClustalX analysis were visualized with TreeView 1.6.6 based on the distance matrix using the neighbor-joining method (22). Nucleotide and amino acid sequences of Tpen_1494 were retrieved from the submission, YP_920894.

RESULTS

Characteristics of the gene encoding Tpbgl A multiple alignment of the primary sequences revealed that the gene encoding Tpbgl is highly homologous to genes encoding GH3 β-glucosidases. The predicted amino acid sequence of Tpbgl showed 58.7% identity with that of *Thermotoga maritima* MSB8 β-glucosidase; TmbglB (12) and 58.2% with that of *Thermotoga neapolitana* β-glucosidase; TnbglB (11,23), but only 36.5% with that of *Clostridium thermocellum* β-glucosidase (24,25). On the other hand, Tpbgl possesses weak homology to the GH3 β-glucosidases of eukaryotic yeast and barley, showing 28.5% sequence identity with *Kluyveromyces marxianus* NBRC1777; KmbglI (26) and 21.1% with *Hordeum vulgare* subsp. Vulgare; HvExol (27). Tpbgl has much lower homologies (7.1–14.1% sequence identity) when compared to those of archaeal β-glucosidases from *Pyrococcus furiosus* (9,28), *Sulfolobus solfataricus* (7), *Pyrococcus horikoshii* (29), *Sulfolobus shibatae* (8), and *Thermococcus kodakarensis* (10) (Fig. 1), which are classified into glycosyl hydrolase family 1 (GH1). The open reading frame (Tpen_1494) consists of 2103 bp, corresponding to 701 amino acids with an estimated molecular mass of 77,652 Da. NCBI Blastp

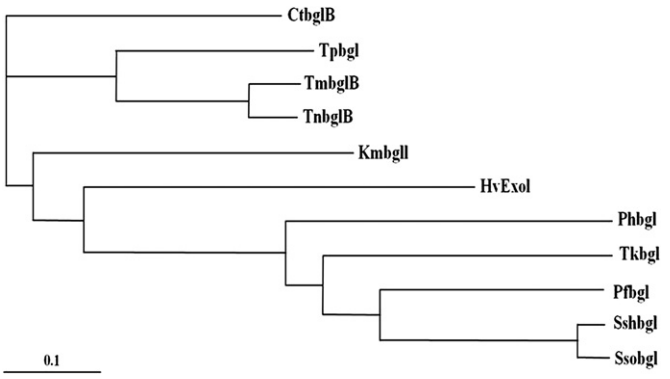

FIG. 1. Phylogenetic relationship between Tpbgl and related enzymes. Phylip formatted tree outputs from the ClustalX analysis were visualized with TreeView based on the distance matrix using the neighbor-joining method. The bar at the lower left corner indicates the substitution rate (substitution/site). The phylogram was built from the complete sequences of the following enzymes: Tpbgl, β-glucosidase from *T. pendens* Hrk 5 (gi: 4601403); KmbglI, β-glucosidase from *K. marxianus* NBRC1777 (gi: 268308673); HvExol, β-D-glucan exohydrolase isoenzyme from *H. vulgare* subsp. vulgare (gi: 4566505); TmbglB, β-glucosidase from *T. maritima* MSB8 (gi: 896841); TnbglB, β-glucosidase from *T. neapolitana* DSM 4359 (gi: 113015391); CtbglB, β-glucosidase from *C. thermocellum* ATCC 27405 (gi: 125713996); Pfbgl, β-glucosidase from *P. furiosus* DSM 3638 (gi: 18891981); Phbgl, β-glucosidase from *P. horikoshii* OT3 (gi: 14590274); Sshbgl, β-glucosidase from *S. shibatae* (gi: 1009227); Ssobgl, β-glucosidase from *S. solfataricus* P2 (gi: 152929); and Tkbgl, β-glucosidase from *T. kodakarensis* KOD1 (gi: 5041957).