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Characterization of a Thermostable Extracellular β-Glucosidase with Activities of Exoglucanase and Transglycosylation from *Paecilomyces thermophila*

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The purification and characterization of a novel extracellular β-glucosidase from *Paecilomyces thermophila* J18 was studied. The β-glucosidase was purified to 105-fold apparent homogeneity with a recovery yield of 21.7% by DEAE 52 and Sephacryl S-200 chromatographies. Its molecular masses were 116 and 197 kDa when detected by SDS-PAGE and gel filtration, respectively. It was a homodimeric glycoprotein with a carbohydrate content of 82.3%. The purified enzyme exhibited an optimal activity at 75 °C and pH 6.2. It was stable up to 65 °C and in the pH range of 5.0–8.5. The enzyme exhibited a broad substrate specificity and significantly hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), cellobiose, gentiobiose, sophorose, amygdalin, salicin, daidzin, and genistin. Moreover, it displayed substantial activity on β-glucans such as laminarin and lichenan, indicating that the enzyme has some exoglucanase activity. The rate of glucose released by the purified enzyme from cellooligosaccharides with a degree of polymerization (DP) ranging between 2 and 5 decreased with increasing chain length. Glucose and glucono-δ-lactone inhibited the β-glucosidase competitively with K_i values of 73 and 0.49 mM, respectively. The β-glucosidase hydrolyzed *p*NPG, cellobiose, gentiobiose, sophorose, salicin, and amygdalin, exhibiting apparent K_m values of 0.26, 0.65, 0.77, 1.06, 1.39, and 1.45 mM, respectively. Besides, the enzyme showed transglycosylation activity, producing oligosaccharides with higher DP than the substrates when cellooligosaccharides were hydrolyzed. These properties make this β-glucosidase useful for various biotechnological applications.

KEYWORDS: Characterization; exoglucanase; β-glucosidase; *Paecilomyces thermophila*; purification; thermostable; transglycosylation

INTRODUCTION

Cellulose is the most abundant and renewable biopolymer on earth. The enzymatic hydrolysis of cellulose with cellulases takes place fundamentally via two consecutive reactions: endo-1,4-β-D-glucanases (EC 3.2.1.4) and exo-1,4-β-D-glucanases (EC 3.2.1.91) are responsible for degrading the cellulose into cellobiose and β-glucosidases (EC 3.2.1.21) and then act upon the cellobiose to free glucose molecules. This last stage is of great importance in the efficient hydrolysis of cellulose (*1*). β-1,4-Glucosidases are an important component of the cellulose-degrading enzymes, mainly catalyzing the hydrolysis of the β-1,4-glycosidic linkage in various disaccharides, oligosaccharides, and alkyl- and aryl-β-D-glucosides. They have been grouped into three classes on the basis of substrate specificity: (i) aryl-β-glucosidases; (ii) true cellobiases; and (iii) broad substrate specificity enzymes, which are active on a variety of substrates. Most of the β-glucosidases characterized so far are placed in the last category (*2*).

Recently, β-glucosidases have been the focus of much research because of their important roles in a variety fundamental biological processes, namely, the biological conversion of cellulose to glucose, the release of aromatic compounds from flavorless glucosidic precursors or detoxification of cyanogenic glycosides, and the synthesis of useful β-glucosides (*1–4*). Detoxification of cyanogenic glycosides may be facilitated by the use of specific cyanogen-hydrolyzing β-glucosidases (*5, 6*). The synthesis of oligosaccharides, alkyl glucosidases, and glycoconjugates by the transglycosylation activity of β-glucosidase is another area of commercial interest (*2, 7*).

In industrial saccharification, thermostability is a highly desirable enzymatic property, because higher temperatures increase both reaction rates and hydrolytic efficiency (*8, 9*). To improve the process of saccharification of lignocellulosic materials, the search for β-glucosidases insensitive to product inhibition, and of high thermostability, has increased recently (*2, 8, 10, 11*). Fungi are known to be good producers of β-glucosidases. In comparison with mesophilic fungi, relatively little

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attention has been given to thermophilic fungi, such as *Chaetomium thermophilum* var. *coprophilum* (*9*), *Humicola grisea* var. *thermoidea* (*10, 12*), *Scytalidium thermophilum* (*11*), *Thermomyces lanuginosus* (*13*), *Thermoascus aurantiacus* (*14*), and *Talaromyces thermophilus* (*15*), as potential sources of β-glucosidases.

The newly isolated thermophilic fungus *Paecilomyces thermophila* J18 is known as a good source of xylanase (*16*). Further investigations of the secreted relevant enzymes showed that the strain could produce an extracellular β-glucosidase (data not shown). Hence, the objective of the present study was to purify and characterize the β-glucosidase secreted by the strain, which exhibited a remarkable thermostablity, broad substrate specificity, high glucose tolerance, and transglycosylation activity. This is the first report on the purification and characterization of a thermostable β-glucosidase from *P. thermophila*.

MATERIALS AND METHODS

Materials. The *p*-nitrophenyl (*p*NP) substrates *p*NP-β-D-glucopyranoside (*p*NPG), *p*NP-α-D-glucopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-xylopyranoside, *p*NP-α-D-xylopyranoside, *p*NP-β-fucopyranoside, *p*NP-α-mannopyranoside, *p*NP-β-mannopyranoside, and *p*NP-β-D-galactopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO). Birchwood xylan, carboxymethylcellulose (CMC, low viscosity), laminarin, lichenan, β-1,3-glucan, cellobiose, gentiobiose, sophorose, salicin, and amygdalin were also obtained from Sigma. Cellooligosaccharides (cellotriose to cellopentaose) were prepared according to the method of Miller (*17*). Daidzin, genistin, and rutin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sephacryl S-200 HR was from Pharmacia (Pharmacia, Uppsala, Sweden). DEAE 52 (Preswollen Microgranular Anion Exchange Celluloses) was from Whatman (Whatman Inc., Fairfield, NJ). Corncobs were obtained locally and were chopped by a chopper into small pieces and ground in a hammer mill. The ground materials were then separated into 0.45 mm (40 mesh) particles and were used in media. All other chemicals used were analytical grade reagents unless otherwise stated.

Fungal Strain and Growth Conditions. *P. thermophila* J18 was isolated by Yang et al. (*16*) and was deposited (under no. AS3.6885) at the Center for Culture Collection of Microorganisms of China. Stock cultures were maintained on potato dextrose agar (PDA) at 4 °C and were transferred every 6–7 weeks. PDA was prepared as follows: The thinly sliced, peeled white potatoes (20 g) were boiled for 30 min and filtered through cheesecloth. After the addition of 2.0 g of glucose and 2.0 g of agar, the total solution volume was made up to 100 mL and autoclaved at 121 °C for 20 min. PDA plates were incubated at 50 °C for 4–5 days and stored at 4 °C until use.

For β-glucosidase production, the optimized medium of culture contained (g L^{-1}) corncob powder (40 mesh), 55; yeast extract, 10; tryptone, 10; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4$, 0.3; KH_2PO_4, 0.3; and Tween-80, 5. A piece (1 cm^2) of growing 4–5-day-old culture was used to inoculate the medium (80 mL) in 250 mL flasks. Triplicate cultures were shaken at 200 rpm for 5 days at 50 °C. After the cultures had been centrifuged at 12000*g* for 10 min, the supernatant was used as crude enzyme.

Enzyme Assay and Protein Determination. β-Glucosidase activity was determined at 50 °C with 5.0 mM *p*NPG as substrate in 50 mM phosphate buffer (pH 6.0). After 10 min of incubation, the reaction was stopped by adding 3 volumes of sodium tetraborate saturated solution, and the absorbance was read at 405 nm (*10*). The reported extinction coefficient for *p*-nitrophenol is 18.4 cm^{-1} mM^{-1} (Sigma). A molar extinction coefficient (pH 6.0) of 1819 cm^{-1} M^{-1} for *p*NP at 405 nm was used. One unit of β-glucosidase activity was defined as the amount that produced 1 μmol of *p*NP per minute.

Protein concentrations were measured according to the Lowry method (*18*) with bovine serum albumin (BSA) as the standard. Specific activity was expressed as units per milligram of protein.

Purification of β-Glucosidase. All purification steps were carried out at 4 °C unless stated otherwise. The crude enzyme was dialyzed against 20 mM phosphate buffer (pH 7.2). Purification was done by ion exchange chromatography followed by gel filtration. The crude enzyme solution was applied to a DEAE 52 column (8 × 1.0 cm) pre-equilibrated with 20 mM phosphate buffer (pH 7.2). The bound proteins were eluted with a NaCl gradient (0.1–0.2 M) at a flow rate of 0.6 mL min^{-1}. The active fractions were combined and concentrated to 0.6 mL by ultrafilation using a 10 kDa membrane (Stirred Cell Model 8050, Millipore). The concentrated sample was loaded on a Sephacryl S-200 HR column (60 × 1.0 cm) and pre-equilibrated with 20 mM phosphate buffer (pH 7.2), and the proteins were eluted at a flow rate of 0.30 mL min^{-1}. This purification step yielded one β-glucosidase active fraction, and its homogeneity was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme solution contained purified β-glucosidase, which was stored at 4 °C and used for subsequent studies.

SDS-PAGE, Zymogram and Molecular Mass Determination. SDS-PAGE was performed using 7.5% (w/v) acrylamide in gels as described by Laemmli (*19*). Protein bands were visualized by Coomassie brilliant blue R-250 staining. The molecular mass standard used was the high molecular weight calibration kit for SDS electrophoresis (Sigma): myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), glutamic dehydrogenase (53 kDa). Samples were treated with sample buffer in 0.5% SDS and 1% β-mercaptoethanol and boiled for 5 min before application to the gel. Glycoprotein was detected by periodic acid−Schiff (PAS) staining of gels after the SDS-PAGE (*20*).

Activity staining was performed by incubating the SDS-PAGE gel with 50 mL of 0.26 μM 4-methylumbelliferyl β-glucoside at 50 °C for 0.5 h after the gel had been washed with 50 mM phosphate buffer at pH 6.0. The β-glucosidase activity was visualized under UV light. N-Terminal sequences of the purified β-glucosidase were determined using an automated Edman degradation using a PROCISE amino acid sequencer (Applied Biosystems) at the Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing.

For molecular mass determination by gel filtration, a Sephacryl S-200 HR column was equilibrated with 50 mM phosphate buffer containing 50 mM NaCl, pH 7.2. The column (120 × 1.6 cm) was calibrated with the standard proteins (Sigma): albumin bovine (67 kDa), phosphorylase *b* (97.2 kDa), alcohol dehydrogenase (150 kDa), and apoferritin horse spleen (445 kDa), each at 10 mg mL^{-1}. The flow rate for elution was 0.1 mL min^{-1}.

Neutral Sugar Content Determination and Monosaccahride Composition. An aliquot (50 μL) of the purified β-glucosidase was mixed with 100 μL of 5% sulfuric acid and was hydrolyzed at 100 °C for 2 h. After cooling, 550 μL of distilled water was added; 0.1 g of $BaCO_3$ was used for sedimentation. The resulting solution was dried under vacuum and was redissolved in 0.2 mL of distilled water before sugar analysis. Estimation of the carbohydrate content of the protein samples was done by using the phenol−sulfuric acid method (*21*), with D-glucose as standard. The resulting monosaccharides were analyzed by high-performance anion exchange chromatography (HPAEC), combined with pulsed amperometric detection. Hydrolysates (25 μL) were applied onto a Dionex BioLC system (model 2500) fitted with a Dionex CarboPac PA10 analytical column (4 × 250 mm) (Dionex Corp.) combined with a CarboPac PA10 precolumn (4 × 50 mm).

Characterization of Purified β-Glucosidase. Investigation to find the optimum pH for β-glucosidase activity was carried out in five different buffers (50 mM) at pH 2.5–11.0: citrate buffer for pH 2.5–5.5; MES buffer for pH 5.2−7.2; MOPS buffer for pH 6.2–8.2; Tris-HCl buffer for pH 7.0–9.0; glycine for pH 8.5–11.0. To determine the pH stability of the enzyme, the purified β-glucosidase was incubated in different buffers as mentioned above at 50 °C for 30 min, and then the remaining activities of these treated enzymes were measured according to the standard assay procedure.

To determine the optimum temperature, the enzyme was preincubated for 3 min and measured by assaying its activity at different temperatures (30–100 °C) in 50 mM MES buffer (pH 6.2). For thermostability determination, the purified β-glucosidase in 50 mM MES buffer (pH 6.2) was incubated at different temperatures for 30 min in the absence of substrate. After cooling of the treated enzymes on ice for 30 min,

the residual β-glucosidase activities were measured according to the standard assay method.

The effects of various metal ions and reagents at 4 mM on the β-glucosidase activities were determined by preincubating the enzyme with the individual reagent in 50 mM MES buffer (pH 6.2) at 22 °C for 30 min. Activities were then measured at 50 °C in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was taken as 100%. The results presented are the average of three trials.

Inhibition by Glucose and Glucono-δ-lactone. The inhibition pattern from glucose and glucono-δ-lactone was determined in the presence of 0–150 mM glucose and 0–1 mM glucono-δ-lactone in the enzyme assay at pH 6.2 and 50 °C for the purified β-glucosidase. The inhibition constants were determined from the corresponding Lineweaver–Burk plots. For glucono-δ-lactone, assays were performed using the freshly prepared solution, because it is susceptible to breakdown in water. All assays were performed in triplicate. The K_i values given are the averages of three independent experiments.

Specificity of Purified β-Glucosidase and Kinetic Parameters. The specificity was determined using *p*NP-glycosides, disaccharides, oligosaccharides, and polysaccharides. Activities toward *p*NP derivatives were measured by the rate of *p*NP formed during hydrolysis from 5 mM of the substrates in 50 mM MES buffer (pH 6.2) at 50 °C for 10 min and detected by spectrophotometry at 405 nm. Hydrolysis of disaccharides and oligosaccharides (10 mg mL^{-1}) was monitored for 10 min at 50 °C by the release of glucose in 50 mM MES buffer (pH 6.2). Glucose released was estimated using the glucose oxidase method (Beijing BHKT Clinical Reagent Co., Ltd.). β-Glucosidase activity on polysaccharides was determined using 1% (w/v) of substrate in 50 mM MES buffer (pH 6.2) at 50 °C for 10 min and by measuring the reducing sugars according to the dinitrosalicylic acid (DNS) method (*22*). Enzyme units were defined as the amount that produced 1 μmol of *p*NP, glucose, or reducing sugar per minute. However, 1 unit of activity toward gluco-disaccharides is the amount of enzyme that forms 2 μmol of glucose per minute from cellobiose, sophorose, or gentiobiose. Blank experiments without enzyme were performed, and background controls were subtracted. All of the experiments were carried out in triplicate.

For the kinetic experiments, initial hydrolysis rates were determined at six different concentrations ranging from approximately 0.5 to 2.0 times the K_m values. Various concentrations for each substrate were prepared in 50 mM MES buffer (pH 6.2) and incubated with the purified β-glucosidase at 50 °C for 5 min (except 2 min for *p*NPG). K_m and k_{cat} and their standard errors were calculated using the nonlinear regression analysis program "Grafit".

Hydrolysis of Cellooligosaccharides by the Purified β-Glucosidase. The hydrolysis of cellooligosaccharides was evaluated by incubating 1% (w/v) of individual cellooligosaccharide with 0.2 unit mL^{-1} of the purified β-glucosidase in 50 mM MES (pH 6.2) at 50 °C for 6 h. Samples were withdrawn at different time intervals. The samples was boiled for 5 min. Glucose released was determined using the glucose oxidase method. Products of enzymatic hydrolyses were analyzed qualitatively by TLC on Kieselgel 60 plates (Merck) with a butan-1-ol-acetic acid/water (2:1:1, v/v) solvent system. The plates were developed with one run followed by heating for a few minutes at 130 °C in an oven after the plates had been sprayed with a methanol/sulfuric acid mixture (95:5, v/v).

RESULTS AND DISCUSSION

β-Glucosidase Production by *P. thermophila*. The effect of various carbon sources (Avicel, CMC, cellulose, corncob, wheat bran, wheat straw, or sugar cane bagasse) on the production of β-glucosidase activity by *P. thermophila* J18 was evaluated using *p*NPG as substrate (data not shown). The stain produced the highest extracellular aryl β-glucosidase activities (0.41 unit mL^{-1}) in corncob-grown medium. Thus, corncob was used as the main carbon source for enzyme production in the optimizing experiments. It produced the highest β-glucosidase activity (1.27 units mL^{-1}) for 5 days in the optimized medium (data not shown). It is interesting that *P. thermophila* J18 can produce β-glucosidase using low-cost lignocellulosic materials (i.e., corncob). Also, *T. aurantiacus* produced relatively high levels of β-glucosidase when grown on corncob (*14*).

Table 1. Summary of β-Glucosidase Purification from *Paecilomyces thermophila*

purifn step	total activity[a] (units)	protein[b] (mg)	specific activity (units mg^{-1})	purifn factor (-fold)	recovery (%)
crude enzyme	260	340	0.77	1	100
DEAE 52	143	20.1	7.1	9.3	55
Sephacryl S-200	56.4	0.7	80.6	105	21.7

[a] Activity was measured in 50 mM phosphate buffer (pH 6.0) at 50 °C using 5 mM *p*NPG as substrate. [b] The protein was measured by using the Lowry method (*18*), with BSA as the standard.

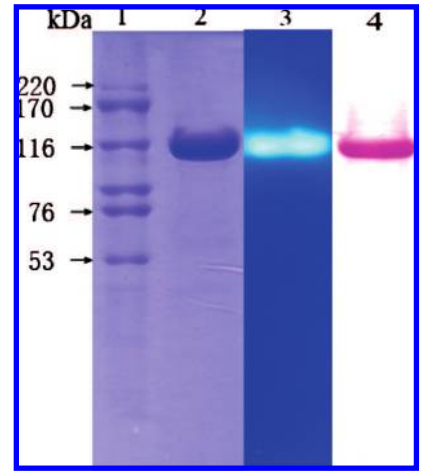

Figure 1. SDS-PAGE, zymogram analysis, and periodic acid−Schiff staining of the purified β-glucosidase from *P. thermophila*: lane 1, high molecular weight calibration kit; lane 2, SDS-PAGE of the purified β-glucosidase; lane 3, zymogram analysis with 4-methylumbelliferyl β-glucoside; lane 4, periodic acid−Schiff staining of the β-glucosidase.

Purification of β-Glucosidase from *P. thermophila*. A summary of the β-glucosidase purification is presented in **Table 1**. β-Glucosidase was purified to 105-fold apparent homogeneity by ion exchange and gel filtration chromatographies with a recovery yield of 21.7% (**Table 1** and **Figure 1**). The molecular mass of the purified enzyme estimated by SDS-PAGE analysis was approximately 116 kDa (**Figure 1**). Zymogram analysis showed that a single fluorescent band on activity-stained gel had the same mobility as that of a single stained protein. The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was approximately 197 kDa (data not shown). Its glycoprotein character was indicated by positive periodic acid−Schiff staining. The carbohydrate content of the purified enzyme was estimated to be 82.3% using the method of Dubois et al. (*21*). The monosaccharide composition (molar ratio percent) was found to mainly consist of galactose (51.32%), glucose (25.50%), arabinose (14.40%), xylose (6.12%), fucose (1.58%), and rhamnose (1.09%) detected by the high-performance anion exchange chromatography (HPAEC) analysis (data not shown). Isoelectric focusing (IEF-PAGE) of the purified β-glucosidase indicated a p*I* of about 4.4 (data not shown). Besides, direct N-terminal sequencing of the β-glucosidase by Edman degradation was not possible, because the N terminus was blocked. After removal of *N*-acetylserine or *N*-acetylthreanine according to the method of Wellner et al. (*23*), the first 10 residues at the N terminus were H_2N−?−V−I−Q−L−N−?−?−T−I.

A simple and efficient two-step purification protocol, consisting of anion exchange and gel filtration chromatography, resulted in an apparently homogeneous enzyme preparation in a yield of 21.7% (**Table 1**). The purified β-glucosidase was homogeneous with a denatured molecular mass of 116 kDa (**Figure 1**). Nevertheless, the gel filtration showed that the native enzyme had a molecular mass of 197 kDa. Hence, it is assumed that the native β-glucosidase is a homodimer. The high molecular mass of this β-glucosidase is in agreement with those of many extracellular β-glucosidases characterized from other fungal sources (*13, 14, 23, 24, 26*). The enzymes exhibiting subunit molecular mass in the range 40–60 kDa are generally monomeric, whereas large subunit enzymes (70–120 kDa) are observed to be multimeric (*6, 11–15, 23, 30*). The β-glucosidase purified from *T. lanuginosus*-SSBP consisted of two identical subunits with a native molecular mass of 200 kDa (*13*). A thermostable β-glucosidase purified from *T. aurantiacus* was a homotrimer with a monomer molecular mass of 120 kDa (*14*). The apparent monomer molecular mass of β-glucosidase (116 kDa) in this study (**Figure 1**) is much higher than the values of 43 kDa β-glucosidase from *C. thermophilum* var. *coprophilum* (*9*) and 50 kDa β-glucosidase from *T. thermophilus* CBS 236.58 (*15*).

Most of the fungal β-glucosidases are glycoproteins (*8–11, 24, 30*). Especially, thermophilic fungi usually produce highly glycosylated β-glucosidases (*8–11*). The carbohydrate content of the purified β-glucosidase was estimated to be 82.3%, which is the highest value among the reported β-glucosidases as far as we know. It is much higher than those of β-glucosidases from *Talaromuces emersonii* [50%, (*8*)], *C. thermophilum* var. *coprophilum* [73%, (*9*)], *Humicola grisea* var. *thermoidea* [35%, (*10*)], and *Scytalidium thermophilum* [15%, (*11*)]. The p*I* value was estimated to be 4.4, which is similar to those of most microbial β-glucosidases, which are acidic proteins with p*I* values ranging from 3.5 to 5.5 (*15, 24, 28, 31–33*).

Effect of pH and Temperature on the Activity and Stability of β-Glucosidase. The purified β-glucosidase was most active at pH 6.2 (**Figure 2A**). It retained more than 90% of its activity at 50 °C for 30 min when tested in the pH range of 5.0−8.5 (**Figure 2B**). Most fungal β-glucosidases exhibit pH optima ranging from 4.0 to 6.5 (*2, 8, 24*). The optimal pH for the β-glucosidase was pH 6.2 (**Figure 3**), which is higher than most β-glucosidases from thermophilic fungi (*8, 12, 14*), such as pH 5.5 from *C. thermophilum* var. *coprophilum* (*9*) and pH 6.0 from *T. lanuginosus*-SSBP (*13*). Like some β-glucosidases isolated from thermophilic fungi, the β-glucosidase is also stable over a wide pH range (*8*).

The enzyme exhibited its optimal activity at 75 °C for 10 min assay (**Figure 3A**). It was fairly stable up to 65 °C for 30 min (**Figure 3B**) and retained 57.9% of its activity at 70 °C. In general, temperature optima for fungal β-glucosidases range from 40 to 50 °C (*2, 6, 8, 24, 25, 28*). Most thermophilic fungal β-glucosidases exhibit temperature optima ranging from 55 to 65 °C (*8–13, 15*). However, the β-glucosidase was optimally active at 75 °C (**Figure 3A**) compared to 70–75 °C from *T. emersonii* and *T. aurantiacus* (*8*). β-Glucosidases produced by thermophilic fungi are usually more thermostable than those of mesophilic fungi (*8–12, 28*). The thermostability of the purified enzyme is higher that those of *H. grisea* var. *thermoidea*, *T. lanuginosus*-SSBP, and *S. thermophilum* (*10–13*). Thus, its high optimal temperature and thermostability are attractive features for industrial applications.

The effect of various cations and compounds at 4 mM was tested on the activity of β-glucosidase (**Table 2**). β-Glucosidase activity was strongly inhibited by Hg^{2+} (3.5%) and Ag^{+} (20.1%). The enzyme was slightly inhibited by Ca^{2+} (97.9%), DTT (97.4%), EDTA (95.3%), β-mercaptoethanol (93.7%), and SDS (91.1%). However, it was activated by Zn^{2+} (142.1%), Fe^{2+} (136.5%), Ni^{2+} (125.5%), Co^{2+} (124.2%), Mn^{2+} (119.0%), Mg^{2+} (114.5%), Sr^{2+} (107.6%), and Cu^{2+} (107.2%). The chelating agent EDTA slightly affected β-glucosidase activity, indicating that the β-glucosidase is not a metalloprotein. Furthermore, DTT was not an inhibitor, suggesting that disulfide bonds are not essential for the enzyme. β-Glucosidases of microbial origin are usually inhibited markedly by Hg^{2+} and/or Ag^{+} (*9, 10, 24, 28*). Group-specific reagents (such as β-mercaptoethanol), EDTA, and the cations Mg^{2+}, Ca^{2+}, Al^{3+}, Co^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+} at 1.0 mM did not affect the activity (*9, 10*). It has been demonstrated that some fungal β-glucosidases are activated by several cations, including Ca^{2+}, Mg^{2+}, Co^{2+}, and/ or Mn^{2+} (*25, 28*).

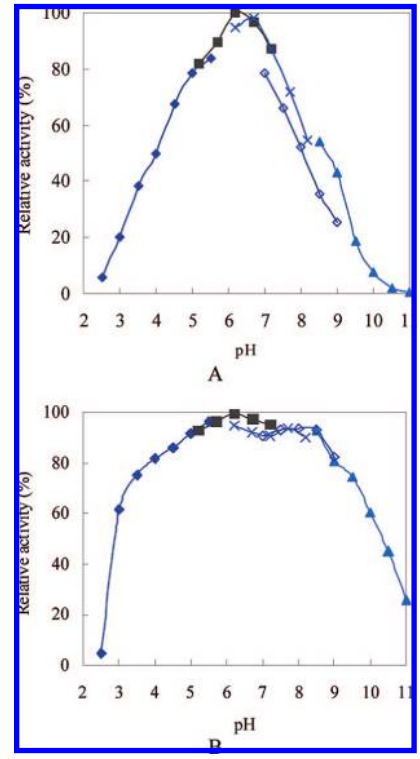

Figure 2. Optimal pH (**A**) and pH stability (**B**) of the purified β-glucosidase from *P. thermophila*. The influence of pH on β-glucosidase activity was determined at 50 °C using 50 mM concenrations of different buffers. The highest specific activity of 97.2 units mg^{-1} was taken as 100%. The remaining activities were measured after incubation for 30 min at 50 °C over various pH ranges. Buffers used: citrate (♦); MES (■), MOPS (×); Tris-HCl (◊); glycine (▲).

Inhibition by Glucose and Glucono-δ-lactone. The effect of glucose and glucono-δ-lactone on the hydrolysis of *p*NPG by β-glucosidase was studied (data not shown). The enzyme was competitively inhibited by glucose and glucono-δ-lactone with K_i values of 72 and 0.49 mM for β-glucosidase, respectively.

β-Glucosidase is frequently a rate-limiting factor during enzymatic hydrolysis of cellulose and is very sensitive to glucose inhibition (*25, 28, 29*). Most of the microbial β-glucosidases reported to date are competitively inhibited by glucose and exhibit K_i values ranging from as low as 0.2 mM to no more than 100 mM (*12–14, 24, 25, 29, 30, 32*). However, several β-glucosidases from a few fungi and yeasts show high glucose tolerance with K_i values of more than 100 mM (*26–28*). In addition, β-glucosidase from *S. thermophilum* was stimulated by glucose at concentrations varying from 50 to 200 mM (*11*).

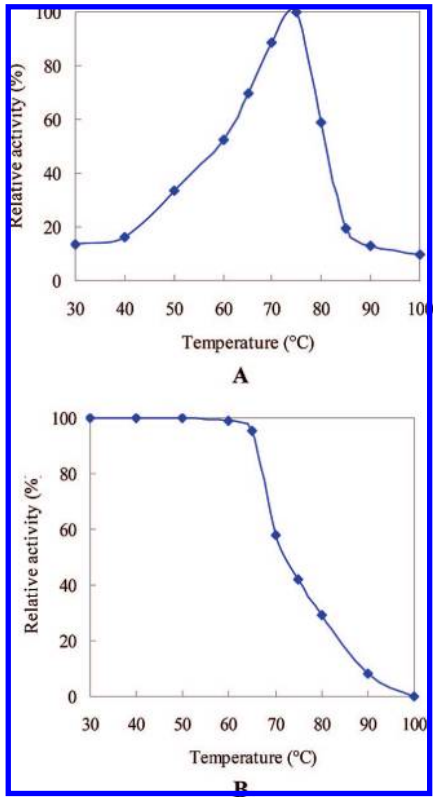

Figure 3. Optimal temperature (**A**) and thermostability (**B**) of the purified β-glucosidase from *P. thermophila*. The optimal temperature was measured at different temperatures. The highest specific activity of 240 units mg^{-1} was taken as 100%. For determination of thermostability, the residual activities of the treated enzymes were measured after 30 min of preincubation at different temperatures at pH 6.2.

Table 2. Effect of Various Reagents on the Enzyme Activity of Purified β-Gluosidase

reagent	specific activity (units mg^{-1})	relative activity[a] (%) (4 mM)
control	97.2 ± 1.5	100
Fe^{2+}	132.6 ± 3.3	136.5
Fe^{3+}	96.0 ± 2.7	98.8
Cu^{2+}	104.2 ± 2.8	107.2
Ag^{+}	19.5 ± 0.8	20.1
Ca^{2+}	95.2 ± 1.8	97.9
Mg^{2+}	111.3 ± 2.7	114.5
Mn^{2+}	115.7 ± 2.3	119.0
Co^{2+}	120.7 ± 2.8	124.2
Zn^{2+}	138.1 ± 4.2	142.1
Hg^{2+}	3.4 ± 0.1	3.5
Ni^{2+}	122 ± 3.1	125.5
Sr^{2+}	104.6 ± 1.9	107.6
DTT	94.7 ± 2.1	97.4
SDS	88.5 ± 2.5	91.1
β-mercaptoethanol	91.1 ± 3.0	93.7
EDTA	92.6 ± 2.6	95.3

[a] Values are means ± SD of three different experiments.

The β-glucosidase is tolerant to glucose inhibition with a K_i of 73 mM for *p*NPG. Therefore, this indicates that the enzyme is comparatively tolerant to glucose.

Glucono-δ-lactone, as a transition state analogue, was by far the most potent inhibitor for the microbial β-glucosidases (*32*). Like other fungal β-glucosidases, the enzyme was competitively inhibited by glucono-δ-lactone (*14, 24, 28*). Glucono-δ-lactone is the effective inhibitor of the β-glucosidase activity with a K_i value of 0.49 mM in this study, indicating that the enzyme is relatively tolerant to glucono-δ-lactone. K_i values reported for glucono-δ-lactone are in the range of 3–30 μM (*14, 24, 32*) with the exception of the β-glucosidase from *Aspergillus oryzae*, which exhibited a K_i value of 12.5 mM (*28*).

Table 3. Substrate Specificity of the Purified β-Glucosidase

substrate	specific activity (units mg^{-1})	relative activity[a] (%)
*p*NP-β-glucopyranoside	97.2 ± 1.5	100
cellobiose	49.1 ± 1.3	50.5
cellotriose	43.3 ± 1.2	44.6
cellotetraose	31.2 ± 0.8	32.1
cellopentaose	29.6 ± 0.9	30.5
gentiobiose	44.8 ± 1.0	46.1
sophorose	47.3 ± 1.1	48.7
genistin	46.4 ± 1.6	47.7
daidzin	43.3 ± 1.5	44.6
amygdalin	39.6 ± 1.4	40.7
salicin	32.7 ± 1.2	33.6
laminarin	20.8 ± 0.5	21.4
lichenan	16.2 ± 0.3	16.7

[a] The activity for *p*NPG was defined as 100%. The enzyme exhibited very little activity on *p*NP-α-L-arabinofuranoside (0.7%) and *p*NP-α-D-xylopyranoside (0.6%). It was inactive on maltose, lactose, xylobiose, sucrose, trehalose, raffinose, Avicel, CMC, filter paper, birchwood xylan, β-1,3-glucan, rutin, starch, pullulan, and locust bean gum.

Substrate Specificity and Kinetic Parameters of the Purified β-Glucosidase. Relative rates of hydrolysis of various substrates by the purified β-glucosidase were studied (**Table 3**). The highest activity was observed with *p*NPG followed by cellobiose (50.5%) and sophorose (48.7%). The purified enzyme had very little activity on *p*NP-α-L-arabinofuranoside (0.7%) and *p*NP-α-D-xylopyranoside (0.6%). No detectable activity toward *p*NP-α-glucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-galactopyranoside, and *p*NP-β-fucopyranoside was observed. It was inactive on maltose, lactose, xylobiose, sucrose, trehalose, and raffinose. However, the enzyme was able to attack cellooligosaccharides with a degree of polymerization of 2−5 units (**Table 3**). The order of decreasing activity against cellooligosaccharides shown by the β-glucosidase was G2 > G3 > G4 > G5. Moreover, genistin, daidzin, amygdalin, and salicin were hydrolyzed at 47.7, 44.6, 40.7, and 33.6% of that of *p*NPG. The enzyme did not act toward Avicel, CMC, filter paper, birchwood xylan, β-1,3-glucan, rutin, starch, pullulan, and locust bean gum. Unexpectedly, it hydrolyzed laminarin and lichenan at 21.4 and 16.7% of that of *p*NPG, respectively.

A broad substrate specificity was shown for the β-glucosidase to hydrolyze aryl β-D-glucosides (*p*NPG and salicin), alkyl β-D-glucosides (amygdalin), and cellooligosaccharides (**Table 3**). Similarly, β-glucosidases of thermophilic fungi and other microbial sources are also characterized by a broad substrate specificity (*2, 6, 7, 10–12, 14, 24, 25, 28, 29*). The preferred substrates for β-glucosidase are *p*NPG and cellobiose, and this type of β-glucosidase is most common in cellulolytic microbes (*2*). Also, β-glucosidase hydrolyzed sophorose and gentiobiose effectively. Several β-glucosidases reported possess the ability to hydrolyze sophorose (*6, 25, 28, 36*). Comparatively, some β-glucosidases are capable of hydrolyzing β-1,6-linked diglucosides and gentiobiose (*7, 13, 14, 24, 25, 28, 29*). Besides, the purified enzyme exhibited very low activity on *p*NP-α-L-arabinofuranoside and *p*NP-α-D-xylopyranoside. A number of β-glucosidases from various biological sources are accompanied by β-galactosidase and/or β-xylosidase activity (*10, 11, 24, 25, 27*). Interestingly, the β-glucosidase hydrolyzes genistin, daidzin, amygdalin, and salicin with comparable rates of hydrolysis. Its ability to hydrolyze amygdalin is interesting as only a few β-glucosidases, especially plant β-glucosidases, have been reported

Table 4. Kinetic Parameters for the Purified β-Glucosidase[a]

substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mg^{-1} s^{-1} mL)
*p*NP-β-glucopyranoside	780.3 ± 28.9	0.27 ± 0.01	13.0	48.2
cellobiose	272.1 ± 12.5	0.65 ± 0.03	4.5	7.0
gentiobiose	251.3 ± 9.8	0.77 ± 0.03	4.2	5.4
sophorose	221.4 ± 9.1	1.06 ± 0.08	3.7	3.5
amygdalin	475.4 ± 19.7	1.45 ± 0.06	7.9	5.5
salicin	426.3 ± 21.5	1.39 ± 0.07	7.1	5.1

[a] Enzymatic reactions were carried out for 5 min (except 2 min for *p*NPG) at 50 °C in 50 mM MES buffer (pH 6.2).

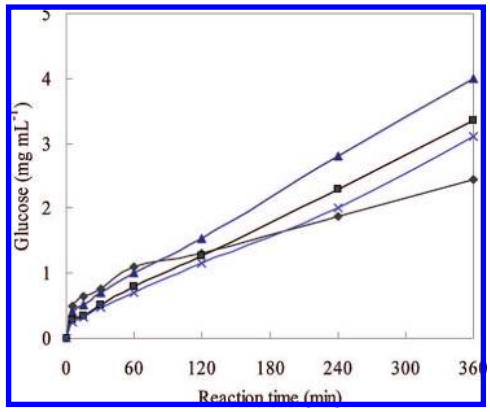

Figure 4. Glucose released from cellooligosaccharides by the purified β-glucosidase. Cellooligosaccharides: ♦, cellobiose; ▲, cellotriose; ■, cellotetraose; ×, cellopentaose. Each cellooligosaccharide (10 mg mL^{-1}) was incubated with 0.2 unit of β-glucosidase for 6 h at 50 °C, and glucose released was estimated using the glucose oxidase method.

to possess this activity (*5, 6*). It is worth noting that the enzyme is able to hydrolyze soybean isoflavone glycosides, namely, daidzin and genistin, which exhibits potential application to increase free isoflavones in soy products (*3, 4, 29*). Polymers are not usually substrates for β-glucosidases (*2, 8*). Surprisingly, the purified β-glucosidase can hydrolyze long glucans, such as laminarin (a soluble polysaccharide with a β-1,3-glucosidic linkage) and lichenan (β-1,3 and β-1,4 linkages), and in this respect resembles an exoglucanase. Several β-glucosidases have been found to hydrolyze laminarin and/or lichenan (*7, 8, 28, 29, 33*).

The Michaelis–Menten constants were determined for some preferred substrates (**Table 4**). The purified β-glucosidase is effectively active on *p*NPG, cellobiose, gentiobiose, sophorose, amygdalin, and salicin with K_m values of 0.26, 0.65, 0.77, 1.06, 1.45, and 1.39 mM, respectively. The kinetics of the hydrolysis of preferred substrates indicates that the enzyme is more specific for *p*NPG than for normal disaccharides such as cellobiose, gentiobiose, and sophorose. A broad range of K_m values for *p*NPG and cellobiose has been reported from different fungal sources. K_m values for *p*NPG and cellobiose are similar to those of some fungal β-glucosidases (*2, 8, 11, 12, 24*).

Hydrolysis of Cellooligosaccharides by the Purified β-Glucosidase. The release of glucose from various cellooligosaccharides (G2−G5) by purified β-glucosidase was monitored and followed by analyzing the reaction products by TLC (**Figures 4** and **5**). When cellooligosaccharides were hydrolyzed, the purified enzyme exhibited transglycosylation activity. Some oligosaccharides with a degree of polymerization 1 unit or more longer than the substrates were observed with the substrates tested (**Figure 5**).

The β-glucosidase hydrolyzes cellooligosaccharides up to G5 and seems to be more applicable to the final steps of cellulose

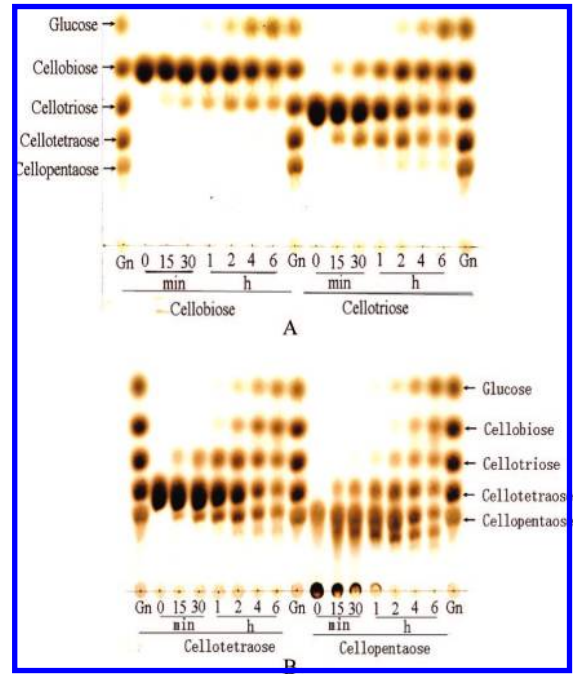

Figure 5. TLC analysis of hydrolysis products from cellooligosaccharides by the purified β-glucosidase: (**A**) cellobiose and cellotriose; (**B**) cellotetraose and cellopentaose. Incubation times (hours or minutes) and substrates are indicated. Lanes Gn represent a mixture of glucose to cellopentaose.

saccharification. A similar pattern has been observed in some β-glucosidases (*14, 24, 27, 28, 36*). The β-glucosidase appeared to hydrolyze cellooligosaccharides in a stepwise manner, releasing one glucose unit at a time. A similar pattern is also observed for β-glucosidase from *T. aurantiacus* (*14*). Simultaneously, TLC analysis of the hydrolysis products revealed that higher oligosaccharides than the substrates were observed with all substrates, consistent with transglycosylation activity, a reaction that is a widespread feature of β-glucosidases (*2, 7, 14, 15, 24, 30, 32, 34, 36*). It should be noted that longer oligosaccharides appear to give higher transglycosylation efficiency compared to cellobiose.

In conclusion, the purified β-glucosidase from *P. thermophila* was characterized as a dimeric glycoprotein with a carbohydrate content of 82.3%. The β-glucosidase was a thermostable enzyme with a temperature optimum of 75 °C and was also glucose tolerant. It was found to have a broad substrate specificity with exoglucanase activity. The rate of glucose released from cellooligosaccharides by the enzyme decreases with increasing chain length. Besides, the enzyme possesses transglycosylation activity. Therefore, the β-glucosidase shows great potential for several biotechnological applications.

ABBREVIATIONS USED

BSA, bovine serum albumin; CMC, carboxymethylcellulose; DNS, dinitrosalicylic acid; DP, degree of polymerization; EDTA, ethylenediaminetetracetic acid; G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; HPAEC, high-performance anion exchange chromatography; MES, 2-(*N*-morpholino)ethane sulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PDA, potato dextrose agar; *p*NP, *p*-nitrophenyl; *p*NPG, *p*NP-β-D-glucopyranoside; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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