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Purification and Characterization of an Extracellular β-Glucosidase Produced by *Phoma* sp. KCTC11825BP Isolated from Rotten Mandarin Peel

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A β-glucosidase from *Phoma* sp. KCTC11825BP isolated from rotten mandarin peel was purified 8.5-fold with a specific activity of 84.5 U/mg protein. The purified enzyme had a molecular mass of 440 kDa with a subunit of 110 kDa. The partial amino acid sequence of the purified β-glucosidase evidenced high homology with the fungal β-glucosidases belonging to glycosyl hydrolase family 3. Its optimal activity was detected at pH 4.5 and 60°C, and the enzyme had a half-life of 53 h at 60°C. The K_m values for *p*-nitrophenyl-β-D-glucopyranoside and cellobiose were 0.3 mM and 3.2 mM, respectively. The enzyme was competitively inhibited by both glucose (K_i=1.7 mM) and glucono-δ-lactone (K_i=0.1 mM) when *p*NPG was used as the substrate. Its activity was inhibited by 41% by 10 mM Cu^{2+} and stimulated by 20% by 10 mM Mg^{2+}.

Keywords: *Phoma* sp., β-glucosidase, identification, purification, characterization

Mandarins are a popular fruit on Jeju Island, Korea. In 2008, approximately 500,000 tons of mandarin was produced and broadly employed as a fresh food, and as a raw material for juice and other processed foods. However, mandarin peels are one of the major agricultural wastes in Jeju Island and more than 38,000 tons is produced annually. About 70% of this waste is recycled into useful resources such as animal feed and oriental medical materials, and the rest is dumped into the ocean. Therefore, the utilization of these remaining agricultural wastes could prove beneficial in this district, and research into the microbial degradation of mandarin peels is expected to facilitate a huge reduction in waste.

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Cellulose and pectin are the principal components of mandarin peel [7]. In particular, cellulose, a linear polymer of D-glucose units linked by β-1,4-glucosidic bonds, is a renewable carbon source that may be the key to long-term solutions to energy, chemical, and food resource problems [15]. The most useful technology thus far devised for the utilization of cellulose is its biological conversion to alcohol *via* cellooligosaccharides; however, the depolymerization of cellulose into cellooligosaccharides with commercial enzymes remains an expensive proposition [21]. The enzymatic hydrolysis for the conversion of cellulose to the fermentable monomeric sugar, glucose, involves the synergistic activity of three types of cellulases: endoglucanase (EG, E.C. 3.2.1.4), cellobiohydrolase (CBH, E.C. 3.2.1.91), and β-glucosidase (BGL, E.C. 3.2.1.21). In particular, BGL catalyzes the hydrolysis of cellobiose and performs an important role in the process of cellulose saccharification by removing cellobiose, which is known to be a strong inhibitor of CBH and EG [3].

The ascomycete *Phoma* sp. KCTC11825BP was recently isolated from rotten mandarin peel, and has been shown to evidence significant activity in degrading mandarin peels. In this paper, for the first time, we describe the purification and characterization of an extracellular BGL from this fungus.

Materials and Methods

Isolation of Microorganism and Growth Conditions

The fungal strains on a sample of rotten mandarin peel were plated on potato dextrose agar (PDA; Difco) plates, and incubated for 7 days at 28°C. After two subsequent transfers on the same plate, the isolates were maintained purely at 28°C. For cellulase production, the isolate was cultivated in cellulolytic medium containing (w/v), 0.05% $(NH_4)_2SO_4$, 0.05% L-asparagine, 0.05% KCl, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 0.05% yeast extract, and 0.5%

dextrose with 5% cellulose powder. The culture was then cultivated at 28ºC and pH 4.5 under aerobic conditions for 14 days. The culture supernatant was used to purify the cellulolytic enzyme.

Identification of Isolated Strain

For genomic DNA preparations, the strain was grown on a 2% PDA plate overlaid with sterile cellophane sheets and incubated for 7 days at room temperature. DNA was extracted from the hyphae of the isolate using an AccuPrep Genomic DNA extraction kit (Bioneer, Daejeon, Korea). The ITS region (ITS) of the nuclear ribosomal DNA operon was amplified using the primer pair ITS5 (5'-GGAAGTAAA AGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTATATGATATGC-3') [20]. PCR reaction mixtures containing AccuPrep PCR premix (Bioneer), 5−50 ng DNA, and 5−10 pmol each primer in a total volume of 20 µl were subjected to the following protocol: 5 min initial denaturation at 95°C, followed by 40 cycles of denaturation (95°C for 30 min), annealing (48°C for 30 s), and extension (72°C for 80 s). Final extension was conducted at 72°C for 7 min. PCR products were subjected to electrophoresis in 1% agarose gel containing EtBr and visualized *via* UV illumination. The PCR products were purified using an AccuPrep PCR Purification kit (Bioneer). Sequencing was conducted at Macrogen (Seoul, Korea). For the phylogenetic analysis, sequences from the isolate were aligned with sequences acquired from BLAST searches of GenBank. Sequences were aligned with ClustalW version 1.83. The resultant multiple alignments were manually corrected using PHYDIT version 3.2. The phylogenetic tree was constructed *via* the neighbor-joining method using MEGA version 4.0 [9].

Enzyme Assay and Protein Determination

BGL activity was assayed in a 1 ml reaction volume containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as substrate and 0.4 U/mg enzyme in 100 mM sodium acetate buffer (pH 4.5). After 30 min of incubation at 60°C, the reaction was stopped by adding 100 µl of 2 M Na_2CO_3, and the amount of *p*-nitrophenol released was monitored at 405 nm. One unit of BGL activity was defined as the amount necessary to produce 1 µmol of *p*-nitrophenol per minute. The protein concentration in the enzyme solution was determined *via* the Bradford method [1], using bovine serum albumin as the standard.

Enzyme Purification

The supernatant solution (2 l) was filtered through a filter paper and concentrated using a stirred ultrafiltration cell equipped with a 10 kDa cutoff polyethenesulfone membrane and dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.0). The concentrated solution was loaded on a DEAE Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden) equilibrated with the same buffer. The bound BGL eluted with a step gradient of 0 to 500 mM NaCl was prepared in 20 mM Tris-HCl buffer (pH 7.0) at a flow rate of 5.0 ml/min. Fractions containing BGL activity were collected, concentrated, and dialyzed against 50 mM sodium acetate buffer (pH 5.0) containing 0.15 M NaCl. The dialyzed enzyme was further purified *via* fast protein liquid chromatography (FPLC) on a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). Elution was conducted with the same buffer at a flow rate of 2.0 ml/min and the activity fractions were concentrated and dialyzed in 20 mM sodium acetate buffer (pH 4.5). The dialysate was then applied to a MonoQ ion-exchange column 5/50 GL (GE Healthcare) equilibrated with the same buffer, and subsequently eluted with 20 mM sodium acetate buffer (pH 4.5) containing 0.5 M NaCl. The active fractions were pooled, concentrated, and used as a purified enzyme for subsequent studies.

Determination of Molecular Mass

To determine the subunit molecular mass of the purified enzyme, SDS−PAGE was carried out as described by Laemmli [10] on 12% gel. All protein bands were stained with Coomassie Brilliant Blue and destained with an aqueous mixture of 12% (v/v) methanol and 8% (v/v) acetic acid. The molecular mass of the native enzyme was determined *via* gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative-grade column (GE Healthcare). The enzyme solution was applied to the column and eluted with 50 mM sodium acetate buffer (pH 4.5) containing 0.5 M NaCl at a flow rate of 1 ml/min. The column was then calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), and albumin (66 kDa) as reference proteins (Sigma, St. Louis, MO, USA), and the molecular mass of the native enzyme was calculated *via* comparison with the migration length of the reference proteins.

Determination of Partial Amino Acid Sequence

The purified enzymes were submitted for internal amino acid sequencing of selected peptides that were reduced, alkylated, and then digested with 12.5 ng/ml trypsin. The digested peptides were analyzed by nano-LC−MS/MS using a fused silica microcapillary C18 column (particle size, 5 µm; inner diameter, 75 µm; length, 100 mm) at Probiond (Seoul, Korea). To identify the peptide sequences, homology searches were carried out using the MS data analysis program SEQUEST (ThermoFinnigan, San Jose, CA, USA), against the fungi protein database obtained from the NCBI protein sequence database. Spectra matching well with known sequences from fungal species were selected and validated with the *de novo* sequencing program, Mascot Distiller 2.1.0 (Matrix Science, Boston, MA, USA).

Effects of pH, Temperature, and Metal Ions

To evaluate the effects of pH on BGL activity, pH values were varied using 100 mM sodium acetate (pH 3−6), 100 mM phosphate (pH 6−8), and 100 mM Tris-HCl (pH 8−10) buffers . The optimum temperature for the hydrolysis of *p*NPG was measured by assaying its activity at different temperatures (30−90°C) in 100 mM sodium acetate buffer (pH 4.5). To test the thermostability, the enzymes were incubated at 30, 40, 50, and 60°C for varying time periods. A sample was withdrawn at each time interval and was assayed in 100 mM sodium acetate buffer (pH 4.5) at 60°C for 30 min. The half-lives of the enzyme were calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA, USA). The effects of various metal ions were measured *via* pre-incubation of the enzyme with 5 and 10 mM reagent, respectively. Activity was then assessed under optimal conditions, and the activity assayed in the absence of metal ions was expressed as 100%.

Substrate Specificity

Activity on *p*NP derivatives were determined as described above. Cellobiose-hydrolyzing activity was estimated by measuring the amount of glucose produced from cellobiose. The reaction mixture containing 0.4 U/mg enzyme and 1 mM cellobiose was incubated in 100 mM sodium acetate buffer (pH 4.5) at 60°C for 30 min. Glucose concentrations were measured with a Glucose CII-Test Wako (Wako Pure Chemistry, Osaka, Japan) and detected at 505 nm. BGL activity on CMC, xylan,

and Avicel were determined using 1% (w/v) of substrate in 100 mM sodium acetate buffer (pH 4.5), and by measuring the reducing sugar *via* the dinitrosalicylic acid (DNS) method.

Kinetic Parameters and Inhibition Constants

Various concentrations of *p*NPG (from 0.05 mM to 1 mM) and cellobiose (from 0.05 mM to 1 mM) were used to determine the kinetic parameters (K_m and k_{cat}) of the purified enzyme. The reactions were performed in 100 mM sodium acetate buffer (pH 4.5) at 60°C. The inhibition constant (K_i) for glucose and glucono-δ-lactone was determined in the presence of 0–10 mM glucose and 0–1 mM glucono-δ-lactone at pH 4.5 and 60°C with *p*NPG as substrate. The enzyme kinetic parameters, K_m and k_{cat} values, were defined *via* fitting to the Michaelis–Menten equation.

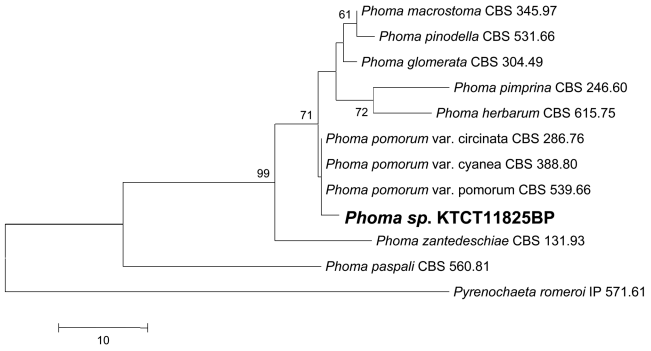

Fig. 1. The phylogenetic tree of KCTC11825BP and related fungal species based on the ITS regions.

Neighbor-joining tree based on ITS sequences showing the position of *Phoma* sp. KCTC11825BP among reference species (CBS collection) in genus *Phoma*. Bootstrap values for 1,000 replications of >50% are indicated above the nodes. The trees generated were evaluated *via* bootstrap analysis using 1,000 replicates. *Pyrenochaeta romeroi* (DQ836802) was used as an outgroup taxon of the concatenated tree of the ITS region.

RESULTS AND DISCUSSION

Identification of Isolation Strain

A BGL-producing fungal strain was isolated from a sample of rotten mandarin peel. The phylogenetic tree of the isolated fungus and related fungal species based on the ITS regions is provided in Fig. 1. It can be clearly seen that the isolated fungus was included in the genus *Phoma*, and was closely related to the species *Phoma pomorum*, showing the highest sequence similarities with *P. pomorum* var. *circinata* CBS 286.76 (99.4%), *P. pomorum* var. *cyanea* CBS 388.80 (99.6%), and *P. pomorum* var. *pomorum* CBS 539.66 (99.6%). *P. pomorum* is one of the most common fungal species presenting a biological risk to plants. It causes mildew and brown lesions on the leaves of various fruit plants, such as apple, cherry, and pear trees, and strawberry plants [13]. *P. pomorum* is also well-known as a fungus that is being developed as a biocontrol agent for hounds tongue disease [2]. Because the isolated strain was phylogenetically so similar to the ascomycetous fungus *P. pomorum* group, it was regarded as a variety of this species; however, additional experiments will be required to accurately assign a varietal designation.

Purification and Molecular Mass of β-Glucosidase

The BGL from *Phoma* sp. KCTC11825BP was purified 8.5-fold with a 34.3% yield and a final specific activity of 84.5 U/mg with *p*NPG as substrate (Table 1). Proteins obtained at each purification step were analyzed by SDS–PAGE and the final purified enzyme showed a single band with a molecular mass of approximately 110 kDa (Fig. 2A). The native enzyme existed as a tetramer with a molecular mass of 440 kDa (Fig. 2B). This BGL had a higher molecular mass and was more multimeric than the well-known BGL in nature [6, 22].

Identification of the Partial Peptide Fragment

The partial amino acid fragments of the purified protein by LC–MS/MS were determined as GIQDAGVIA, AGTGSIM, and IMAAYYLVGR. BLAST searches showed that the purified protein has a high homology with β-1,4-glucosidases of glycosyl hydrolase family 3 (GH family 3) from *Phaeosphaeria nodorum* (Accession No. AAT95381) and *Aspergillus aculeatus* (Accession No. BAA10968).

Optimum pH and Temperature

Maximal enzyme activity was noted at pH 4.5. At pH 4.0 and 5.0, the activity was approximately 97% and 81% of the maximum, respectively (Fig. 3A). No activity was observed at pH values below 3.0 or above 7.0. In general, fungal BGLs evidence optimal pH values in a range between 4.0 and 6.5 [11, 19, 24]. The optimal temperature for activity was detected at 65°C with 99% and 22% of the maximum

Table 1. Purification of the β-glucosidase from *Phoma* sp. KCTC11825BP.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	77.1	767.9	10.0	100.0	1.0
Concentration (10 kDa cutoff)	41.5	701.8	16.9	91.4	1.7
DEAE Sepharose Fast Flow	18.4	522.4	28.4	68.0	2.8
Sephacryl S-300 HR	6.1	329.1	54.1	42.9	5.4
Mono Q 5/50 GL	3.1	263.5	84.5	34.3	8.5

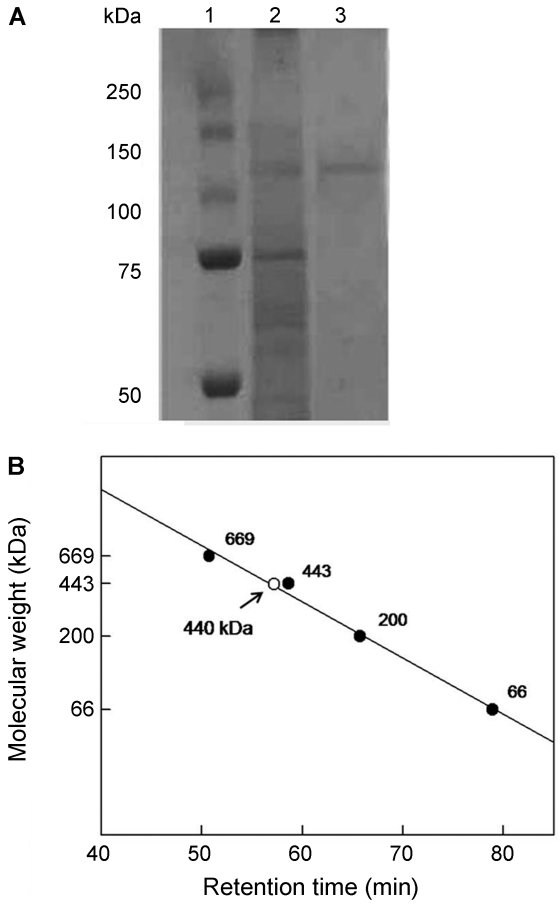

Fig. 2. Determination of molecular mass of purified BGL from *Phoma* sp. KCTC11825BP.
A. SDS–PAGE analysis of the purified enzyme. Lane 1, Protein marker; lane 2, crude extract; lane 3, Mono Q 5/50 GL product. **B**. Molecular mass of the native enzyme by gel filtration chromatography on a Sephacryl S-300 HR 16/60 preparative-grade column.

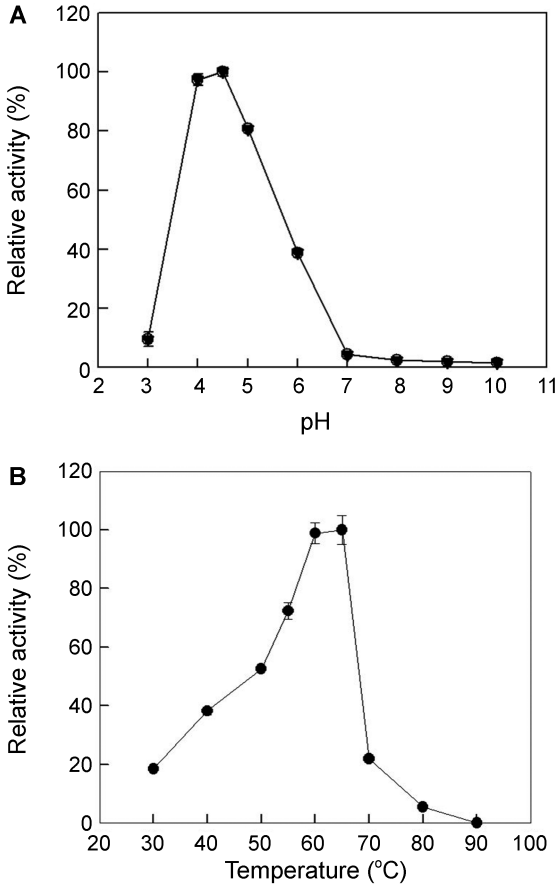

Fig. 3. Effects of pH (**A**) and temperature (**B**) on the activity of the purified BGL from *Phoma* sp. KCTC11825BP.
Values represent the mean of three replicate determinations; error bars indicate standard deviation.

activity being observed at 60 and 70°C, respectively (Fig. 3B). The half-lives of the purified enzyme for incubation at pH 4.5 were 3,629, 2,776, and 53 h at 40, 50, and 60°C, respectively (Fig. 4). The optimal temperature range of 40°C–65°C is a common feature of BGL enzymes isolated from diverse fungal strains [4, 5, 16]. However, the BGL from *Phoma* sp. KCTC11825BP was found to have higher thermostability than that reported for the majority of other fungal BGLs [14, 17, 18]. *Trichoderma viride* BGL has been found to be stable at 60°C; however, its half-life is 2.3 h, compared with the 53 h observed in our experiments.

Kinetics

The kinetic parameters of the purified enzyme for *p*NPG and cellobiose are shown in Table 2. The Lineweaver–Burk plots indicated that the K_m for *p*NPG and cellobiose were 0.3 mM and 3.2 mM, and the k_{cat} values were 0.5 s^{-1} and 699.4 s^{-1}, respectively. The kinetic efficiency (k_{cat}/K_m) values for hydrolysis of *p*NPG and cellobiose were calculated as 1.6 and 215.8 s^{-1} mM^{-1}, respectively. The purified BGL had a 10-fold higher affinity for *p*NPG than cellobiose. The effects of glucose and glucono-δ-lactone were detected with *p*NPG used as a substrate. Glucose and glucono-δ-lactone inhibited BGL competitively, with inhibition constants (K_i) of 1.7 mM and 0.1 mM, respectively, thereby indicating

Table 2. Kinetic characteristics of the β-glucosidase from *Phoma* sp. KCTC11825BP.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m(s^{-1}/mM^{-1})	Compound	Inhibition pattern	K_i (mM)
*p*NPG	0.3	0.5	1.6	Glucose	Competitive	1.7
Cellobiose	3.2	699.4	215.8	Glucono-δ-lactone	Competitive	0.1

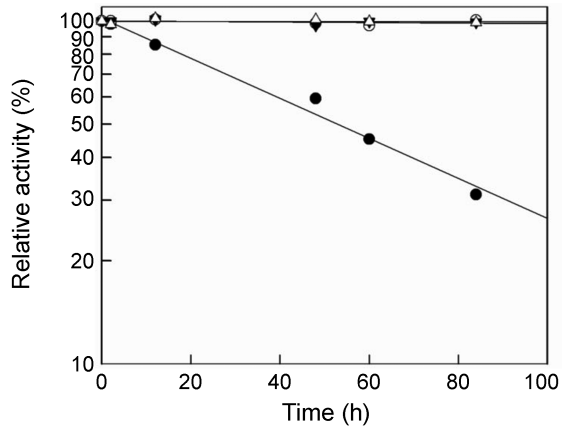

Fig. 4. The thermostability of the purified BGL from *Phoma* sp. KCTC11825BP BGL at temperatures of 30°C (△), 40°C (▲), 50°C (○), and 60°C (●).

that glucose is a stronger inhibitor of BGL from *Phoma* sp. KCTC11825BP than glucono-δ-lactone, as is the case with other cellulolytic fungi [8, 12]. These results demonstrated that the BGL from *Phoma* sp. KCTC11825BP could best be classified as an aryl-BGL with cellobiase activity.

Substrate Specificity

Relative rates of hydrolysis of the purified BGL from *Phoma* sp. KCTC11825BP against various substrates are shown in Table 3. The highest activity was observed with *p*NPG, followed by cellobiose (54.9%). The purified enzyme evidenced minimal or no activity on *p*-nitrophenyl-β-D-cellobiose, *p*-nitrophenyl-β-D-lactopyranoside, *p*-nitrophenyl-β-D-xyloside, and *p*-nitrophenyl-β-D-galactopyranoside, and no activities against cellulose, CMC, or xylan were observed. These results demonstrated that this enzyme evidences high specific activities only for *p*NPG and cellobiose. This type of BGL is most common in cellulolytic microbes [23].

Table 4. Results of purified BGL activity in the presence of various divalent ions added at 5 or 10 mM.

Metal ions	Relative activity (%)[a]	
	5 mM	10 mM
None	100±0.6	100±0.8
Mg^{2+}	119±1.4	120±1.5
Mn^{2+}	100±2.5	97±4.4
Fe^{2+}	91±0.1	91±0.8
Zn^{2+}	109±2.8	113±4.4
Co^{2+}	107±0.8	99±1.5
Ca^{2+}	105±4.8	97±2.5
Cu^{2+}	71±1.1	58±0.3

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

Effects of Metal Ions

The effects of various divalent ions at 5 or 10 mM were tested on the activity of BGL (Table 4). Mg^{2+} at 5 and 10 mM stimulated the enzyme to 120% of relative activity, whereas BGL activity was strongly inhibited by Cu^{2+} (58%) when applied at 10 mM. Mn^{2+}, Fe^{2+}, Co^{2+}, and Ca^{2+} did not significantly influence enzyme activity.

In conclusion, the results of this study, for the first time, identify the fungus isolated from rotten mandarin peel as *Phoma* sp. KCTC11825BP, and show that this fungus produced a thermostable β-glucosidase that hydrolyzes cellobiose to glucose. Although cellobiose is the principal product of the hydrolysis of cellulose by cellulolytic enzymes, this hydrolysis is inhibited by cellobiose. Thus, the BGL performs a special role against the inhibition caused by cellobiose produced from cellulose hydrolysis. The enzymatic properties of the BGL from *Phoma* sp. described thus far suggest that it may play a pivotal role in the enzymatic saccharification of cellulosic biomass to glucose. More detailed investigations of this BGL, such as molecular

Table 3. Relative initial rates of hydrolysis of various substrates by the purified β-glucosidase.

Substrates	Main linkage type	Relative activity (%)[a]
Aryl-glycosides		
p-Nitrophenyl-β-D-glucopyranoside (1 mM)	βGlc	100±3.1
p-Nitrophenyl-β-D-cellobioside (1 mM)	βGlc	3.8±0.3
p-Nitrophenyl-β-D-lactopyranoside (1 mM)	βGlc	ND[b]
p-Nitrophenyl-β-D-xyloside (1 mM)	βXyl	1.0±0.1
p-Nitrophenyl-β-D-galactopyranoside (1 mM)	βGal	ND[b]
Saccharides		
Cellobiose (1 mM)	(β-1,4)Glc	54.9±2.3
CMC [2% (w/v)]	(β-1,4)Glc	ND[b]
Xylan [2% (w/v)]	(β-1,4)Xyl	ND[b]
Cellulose [2% (w/v)]	(β-1,4)Glc	ND[b]

[a]Values are means±SD for three different experiments.
[b]ND, not determined.

cloning and expression studies, are currently under way. It is hoped that these studies will provide us with greater insights into this protein, and facilitate the development of a commercial process for its exploitation.

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