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Biochemical Characterization of an Extracellular β-Glucosidase from the Fungus, *Penicillium italicum*, Isolated from Rotten Citrus Peel

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A β-glucosidase from *Penicillium italicum* was purified with a specific activity of 61.8 U/mg, using a chromatography system. The native form of the enzyme was an 88.5-kDa tetramer with a molecular mass of 354 kDa. Optimum activity was observed at pH 4.5 and 60°C, and the half-lives were 1,737, 330, 34, and 1 hr at 50, 55, 60, and 65°C, respectively. Its activity was inhibited by 47% by 5 mM Ni^{2+}. The enzyme exhibited hydrolytic activity for *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu), *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xyloside, and cellobiose, however, no activity was observed for *p*-nitrophenyl-β-D-lactopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, carboxymetyl cellulose, xylan, and cellulose, indicating that the enzyme was a β-glucosidase. The k_{cat}/K_m (s^{-1} mM^{-1}) values for *p*NP-Glu and cellobiose were 15,770.4 mM and 6,361.4 mM, respectively. These values were the highest reported for β-glucosidases. Non-competitive inhibition of the enzyme by both glucose (K_i = 8.9 mM) and glucono-δ-lactone (K_i = 11.3 mM) was observed when *p*NP-Glu was used as the substrate. This is the first report of non-competitive inhibition of β-glucosidase by glucose and glucono-δ-lactone.

KEYWORDS : β-Glucosidase, Cellulolytic fungi, Characterization, Identification, Purification

Introduction

Citrus fruits are popular on Jeju Island, Korea. In 2008, approximately 500,000 t of citrus were produced and were broadly employed as a fresh food, and as a raw material for juice and other processed foods. However, citrus peels are one of the major agricultural wastes on Jeju Island and more than 38,000 t are produced annually.

Approximately 70% of this waste is recycled into useful resources, including animal feed and oriental medical materials, and the rest is dumped into the ocean [1]. Citrus peels are rich in pectin, cellulose, lignin, and hemicellulose [2]. In particular, cellulose consists of a simple chemical structure (β-1,4 linked glucose homopolymer), and is a major cell-wall constituent in higher plants; it is a linear polysaccharide consisting of β-1,4 linked D-glucose residues. Enzymatic hydrolysis for conversion of cellulose to the fermentable monomeric sugar, glucose, involves the synergistic activity of three types of cellulases: endo-1,4-β-D-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). These enzymes provide a renewable carbon source (glucose) from cellulose, which may be the key to long-term solutions to energy, chemical, and food resource problems [3]. β-Glucosidase catalyzes hydrolysis of aryl-glucosides, alkyl-glucosides, cellobiose, and cellooligosaccharides, and plays a role in saccharification of cellulose by removal of cellobiose [4]. These products of β-glucosidase reaction have many potential applications in the pharmaceutical, cosmetic, and detergent industries [5]. Due to their potential use in various biotechnological processes, including biomass degradation [6], production of fuel ethanol from cellulosic agricultural residues [7], release of aromatic compounds in the flacour industry [8], and synthesis of useful β-glucosides [9], β-glucosidase represents an important group of enzymes. Therefore, the availability of β-glucosidase insensitive to inhibition by glucose and cellulose will have a significant impact on the enzymatic conversion of cellulosic biomass to glucose. In this study, we isolated a *P. italicum* strain that produces a β-glucosidase from rotten citrus peel. The β-glucosidase was purified and its biochemical properties, including optimum pH and temperature, metal ions, substrate specificity, and enzyme kinetics were investigated.

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Materials and Methods

Isolation of microorganism and growth conditions. Fungal strains on a sample of rotten citrus peel were plated on potato dextrose agar (PDA; Difco, Detroit, MI, USA) plates, and incubated for seven 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 0.05% (w/v) $(NH_4)_2SO_4$, 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 28°C 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 preparation of genomic DNA, the strain was grown on a 2% PDA plate overlaid with sterile cellophane sheets and incubated for seven days at room temperature. DNA was extracted from the hyphae of the isolate using an AccuPrep Genomic DNA extraction kit (Bioneer, Daejeon, Korea). The internal transcribed spacer region (ITS) of the nuclear ribosomal DNA operon was amplified using the primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTATATGATATGC-3') [10]. PCR reaction mixtures containing AccuPrep PCR premix (Bioneer), 5~50 ng DNA, and 5 pmol of each primer in a total volume of 20 μL were subjected to the following protocol: 5 min initial denaturation at 95°C, followed by 30 cycles of denaturation (95°C for 30 sec), annealing (48°C for 30 sec), and extension (72°C for 30 sec). Final extension was performed at 72°C for 7 min. PCR products were subjected to electrophoresis in 1% agarose gel containing EtBr and visualized via UV illumination. An AccuPrep PCR Purification kit (Bioneer) was used for purification of PCR products. Sequencing was performed at Macrogen (Seoul, Korea). For the phylogenetic analysis, sequences were proofread and compared to those in the GenBank database using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences of ITS from this study were aligned with those obtained from GenBank using MAFTT v.6.864 [11] and manually edited using MacClade 4.08 [12]. Character based maximum parsimony (MP) analysis was performed using Paup* software ver. 4.0 b10 [13] using a heuristic search with 10 random addition sequences. MP bootstrap proportions and neighbor joining bootstrap proportions (1,000 replications) were used for assessment of tree reliability. Tree diagrams were viewed with TreeView [14] and redrawn for publication using Adobe Illustrator CS5.

Enzyme assay and protein determination. Unless otherwise stated, the reaction was performed at 60°C for 30 min in 50 mM citrate/phosphate buffer (pH 4.5) containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu) and 0.05 U/mL enzyme, and the activity was determined by release of *p*-nitrophenol. Absorbance was measured at 405 nm after quenching the reactions by addition of 200 mM Na_2CO_3. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*NP per min at 60°C and pH 4.5. The Bradford method was used for determination of protein concentration in the enzyme solution [15], 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 7.5). The concentrated solution was loaded onto a HiPrep DEAE FF16/10 column (GE Healthcare, Uppsala, Sweden) equilibrated with the same buffer. Bound β-glucosidase eluted with a step gradient of 0 to 500 mM NaCl was prepared in 20 mM Tris-HCl buffer (pH 7.5) at a flow rate of 1.0 mL/min. Fractions exhibiting β-glucosidase activity were collected, concentrated, and dialyzed against 50 mM Na_2HPO_4 buffer (pH 7.2) containing 150 mM NaCl. The dialyzed enzyme was further purified via fast protein liquid chromatography on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare). Elution was performed using the same buffer at a flow rate of 1.0 mL/min and the active 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. SDS-PAGE was performed under denaturing conditions for examination of the subunit molecular mass of β-glucosidase, using the proteins of a pre-stained ladder (Bio-Rad Laboratories, Hercules, CA, USA) as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of the native enzyme was determined by gel filtration chromatography using a HiLoad 16/600 Superdex 200 pg column (GE Healthcare). The enzyme solution was applied to the column and eluted with 50 mM Na_2HPO_4 buffer (pH 7.2) containing 150 mM NaCl at a flow rate of 1 mL/min. The column was calibrated with thyroglobulin (669 kDa), albumin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), and albumin (66 kDa) as reference proteins and the molecular mass of the native enzyme was calculated by comparison with the migration length of reference proteins.

Effects of pH, temperature, and metal ions. To evaluate the effects of pH on β-glucosidase activity, pH values were varied using 50 mM citrate/phosphate buffer (pH 3.0~6.5). The optimum temperature for hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu) was measured by assay of its activity at different temperatures (35~75°C) in 50 mM citrate/phosphate buffer (pH 4.5). To test the thermostability, the enzymes were incubated at 50, 55, 60, and 65°C for varying time periods. A sample was withdrawn at each time interval and was assayed using 50 mM citrate/phosphate buffer (pH 4.5) at 60°C for 30 min. Sigma Plot ver. 12.0 software (Systat Software, San Jose, CA, USA) was used for calculation of the half-lives of the enzyme. The effects of various metal ions were measured via pre-incubation of the enzyme with 1 and 5 mM reagent, respectively. Assessment of activity was then performed under optimal conditions, and the activity assayed in the absence of metal ions was expressed as 100%.

Substrate specificity. The substrate specificity of β-glucosidase was determined using *p*NP-Glu, *p*-nitrophenyl-β-D-cellobioside (*p*NP-Cel), *p*-nitrophenyl-β-D-lactopyranoside (*p*NP-Lac), *p*-nitrophenyl-β-D-xyloside (*p*NP-Xyl), and *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-Gal), cellobiose, carboxymetyl cellulose (CMC), xylan, and cellulose. Reactions for aryl-glycosides were performed in 50 mM citrate/phosphate buffer (pH 4.5) containing 1 mM aryl-glycoside and 0.05 U/mL enzyme at 60°C for 30 min, and the activity was determined by release of *p*-nitrophenol. The reactions for saccharides were performed in 50 mM citrate/phosphate buffer (pH 4.5) containing 1 mM cellobiose and 0.13 U/mL enzyme at 60°C for 30 min, and the activity was determined by the increased in the amount of glucose. An Aminex HPX-87H column (Bio-Rad Laboratories), operated by an HPLC system (YL9100; Younglin, Anyang, Korea) equipped with a YL9170 RI detector was used for analysis of glucose concentration. The column was eluted at 60°C with water at a flow rate of 0.5 mL/min. β-Glucosidase activity on CMC, xylan, and cellulose was determined using 1% (w/v) of substrate in 50 mM citrate/phosphate buffer (pH 4.5), and by measurement of the reducing sugar using the dinitrosalicylic acid method.

Kinetic parameters and inhibition constants. Various concentrations of *p*NP-Glu (from 0.05 to 0.8 mM) and cellobiose (from 0.2 to 2 mM) were used for determination of kinetic parameters of the enzyme. The enzyme kinetic parameters, K_m (mM) and k_{cat} (s^{-1}) values were determined by fitting to the Michaelis-Menten equation. The reactions were performed in 50 mM citrate/phosphate buffer (pH 4.5) at 60°C. The inhibition constant (K_i) for glucose and glucono-δ-lactone was determined by fitting to the Lineweaver-Burk plot in the presence of 0 to 6 mM glucose and 0 to 0.8 mM glucono-δ-lactone at pH 4.5 and 60°C with *p*NP-Glu as a substrate.

Results and Discussion

Identification of isolation strain. A β-glucosidase producing fungal strain was isolated from a sample of rotten citrus peel. The phylogenetic tree of isolated fungus and related fungal species based on the ITS regions is shown in Fig. 1. The aligned dataset was composed of 31 taxa. It comprised 538 characters, of which 354 sites were constant, 76 sites variable, and 108 sites parsimony informative; tree length was 543 steps with consistency index = 0.4972 and retention index = 0.5653. Our sequence was a 100% match with previously reported sequences of *Penicillium italicum* with high bootstrap supports (Fig. 1). It can be clearly seen that the isolated fungus was included in the genus *Penicillium*, and was closely related to the species *P. italicum*, showing the highest sequence similarities with *P. italicum* ATCC 48114 100% and *P. italicum* 346P 100%. In this study, for the first time, we studied β-glucosidase from *P. italicum*.

Enzyme purification and molecular mass of β-glucosidase. β-Glucosidase from *P. italicum* was purified 6.6-fold with a 60.1% yield and a final specific activity of 61.8 U/mg with *p*NP-Glu as a substrate (Table 1). Proteins

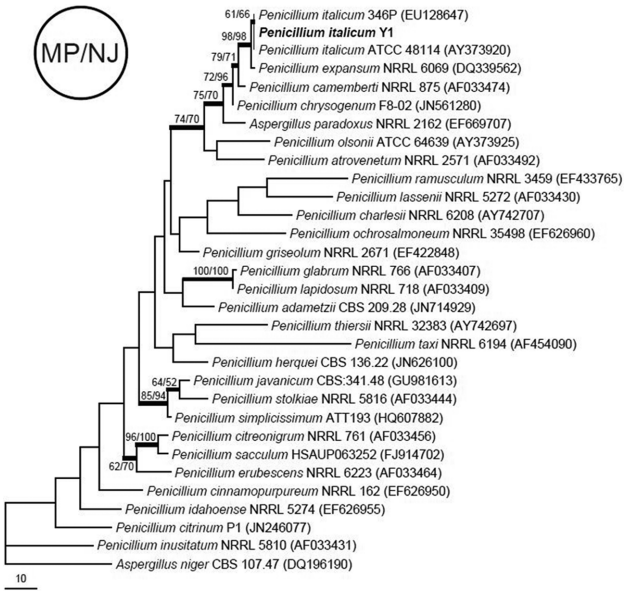

Fig. 1. One of the two most parsimonious trees of *Penicillium italicum* and its allied species based on the internal transcribed spacer region (ITS) sequences. *Aspergillus niger* (DQ196190) was used as an out group. Branches maintained in maximum parsimony (MP) and neighbor joining (NJ) analyses are indicated by bold lines. Numbers above or below the branches of the tree are bootstrap proportions (MPBP/NJBP).

Table 1. Purification of *Penicillium italicum* β-glucosidase

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	3,146.4	29,520.5	9.4	100.0	1.0
Concentration (10 kDa cutoff)	1,256.3	25,845.8	20.6	87.5	2.2
HiPrep DEAE FF 16/10	827.4	23,991.2	29.0	81.3	3.1
HiLoad 16/600 Superdex 200 pg	370.3	19,423.6	52.4	65.8	5.6
Mono Q 5/50 GL	287.0	17,732.5	61.8	60.1	6.6

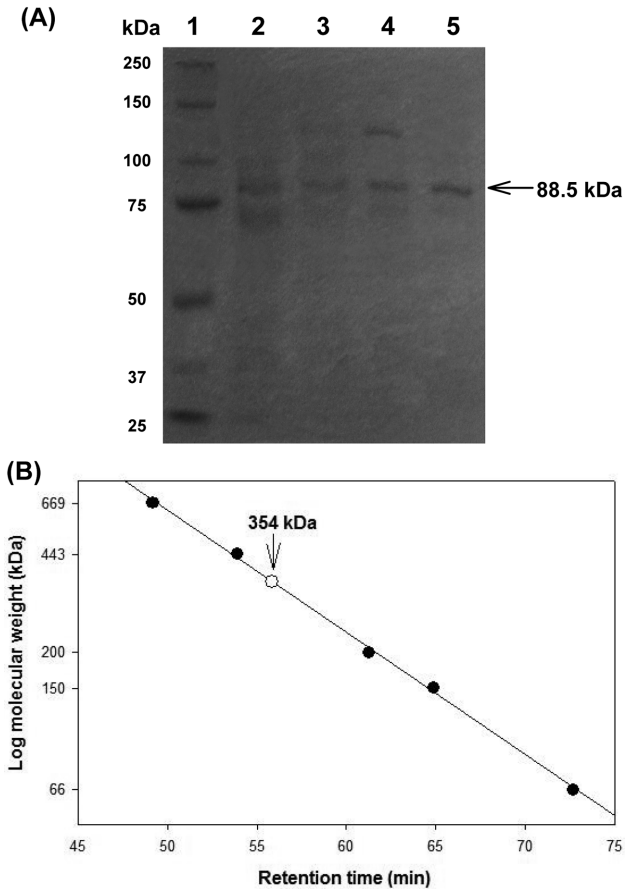

Fig. 2. Determination of molecular mass of purified β-glucosidase from *Penicillium italicum*. A, SDS-PAGE analysis of each purification step. Lane 1, marker proteins; lane 2, crude extract; lane 3, HiPrep DEAE FF 16/10 column product; lane 4, HiLoad 16/600 Superdex 200 pg column product; lane 5, Mono Q 5/50 GL column product (purified enzyme); B, Determination of molecular mass of *P. italicum* β-glucosidase by gel-filtration chromatography.

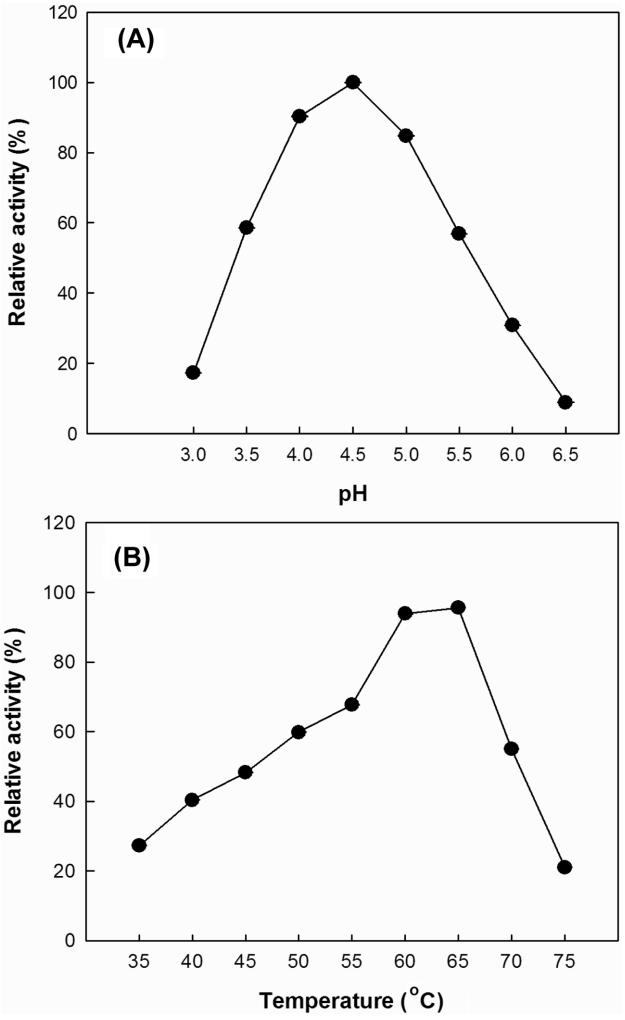

Fig. 3. Effects of temperature and pH on the activity of *Penicillium italicum* β-glucosidase. A, pH effect. The reactions were performed in 50 mM citrate/phosphate buffer containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glu) and 0.05 U/mL enzyme at 60°C for 30 min; B, Temperature effect. The reactions were performed in 50 mM citrate/phosphate buffer (pH 4.5) containing 1 mM *p*NP-Glu and 0.05 U/mL enzyme for 30 min. Data represent the means of three experiments and error bars represent standard deviation.

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 88.5 kDa (Fig. 2A). The native enzyme existed as a tetramer with a molecular mass of 354 kDa (Fig. 2B).

Effects of pH, temperature, and metal ions. The activity of β-glucosidase from *P. italicum* was examined over a pH range of 3.0 to 6.5 at 60°C. Maximum activity was observed at pH 4.5 (Fig. 3A). At pH 3.5 and 5.5, the activity was approximately 60% of the maximum. In

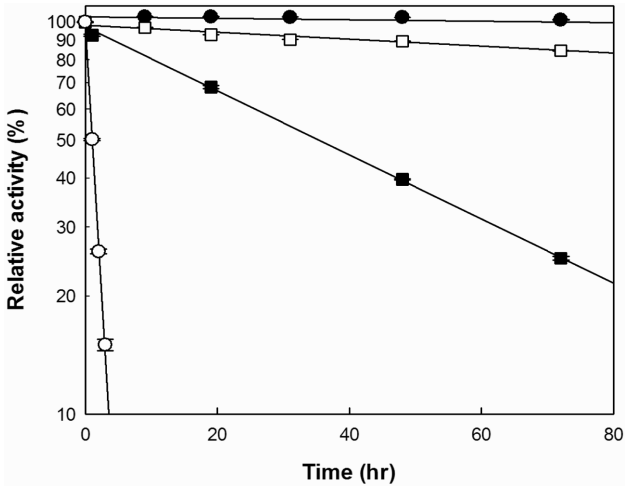

Fig. 4. Thermal inactivation of *Penicillium italicum* β-glucosidase. The enzymes were incubated at 50 (●), 55 (□), 60 (■), and 65°C (○) for varying periods of time. A sample was withdrawn at each time interval and the relative activity was determined. Data represent the means of three experiments and error bars represent standard deviation.

general, optimal pH values for fungal β-glucosidases range between 3.5 and 6.0 (Table 5. The temperature on enzyme activity was investigated, and maximum activity was recorded at 65°C (Fig. 3B). At temperatures of 50 and 65°C, the activity was approximately 60% of the maximum. Thermostability was examined by measurement of the activity over time (Fig. 4). β-Glucosidase demonstrated first-order kinetics for thermal inactivation, and the half-lives of the enzyme were 1,737, 330, 34, and 1 hr at 50, 55, 60, and 65°C, respectively. The effects of various divalent ions at 1 mM and 5 mM were tested on the activity of β-glucosidase (Table 2). The enzyme was not activated by metal ions and was not inhibited by EDTA. However, Zn^{2+} at 5 mM stimulated the enzyme to 109%

Table 2. Effect of metal ions on the activity of the enzyme from *Penicillium italicum*

Metal ions	Relative activity (%)	
	1 mM	5 mM
None	100 ± 0.2	100 ± 0.1
EDTA	100 ± 0.2	100 ± 0.1
Mn^{2+}	100 ± 0.1	100 ± 0.5
Mg^{2+}	98 ± 0.3	98 ± 0.3
Fe^{2+}	101 ± 0.4	102 ± 0.6
Zn^{2+}	101 ± 0.1	109 ± 0.7
Co^{2+}	102 ± 0.2	99 ± 0.2
Ca^{2+}	96 ± 0.4	91 ± 0.4
Cu^{2+}	100 ± 0.3	97 ± 0.2
Ba^{2+}	90 ± 0.2	96 ± 0.7
Ni^{2+}	103 ± 0.1	47 ± 0.2

Data represent the means of three separate experiments.

Table 3. Substrate specificity for the β-glucosidase from *Penicillium italicum*

Substrate	Main linkage type	Relative activity (%)
Aryl-glycosides		
p-Nitrophenyl-β-D-glucopyranoside	βGlc	100 ± 1.2
p-Nitrophenyl-β-D-cellobioside	βGlc	11.1 ± 0.8
p-Nitrophenyl-β-D-lactopyranoside	βGlc	ND
p-Nitrophenyl-β-D-xyloside	βXyl	7.0 ± 0.2
p-Nitrophenyl-β-D-galactopyranoside	βGal	ND
Saccharides		
Cellobiose (1 mM)	(β-1,4)Glc	60.8 ± 0.9
CMC [1% (w/v)]	(β-1,4)Glc	ND
Xylan [1% (w/v)]	(β-1,4)Xyl	ND
Cellulose [1% (w/v)]	(β-1,4)Glc	ND

Data represent the means of three separate experiments.
ND, not determined in the methods used; CMC, carboxymetyl cellulose.

of relative activity, whereas β-glucosidase activity was strongly inhibited by Ni^{2+} (47%) when applied at 5 mM. Mn^{2+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, and Ba^{2+} did not have a significant influence on enzyme activity. Therefore, the enzyme was determined as metal-independent.

Substrate specificity. β-Glucosidase from *P. italicum* exhibited hydrolytic activity for *p*NP-Glu and cellobiose, whereas minimal activity was observed for *p*NP-Cel, *p*NP-Xyl, and no activity was observed for *p*NP-Lac, *p*NP-Gal, CMC, Xylan, and Cellulose, indicating that the enzyme is a β-glucosidase with narrow substrate specificity. The highest activity was observed with *p*NP-Glu (specific activity, 61.8 U/mg; 100%), followed by cellobiose (specific activity, 37.5 U/mg; 60.8%). These results indicated that this enzyme exhibits high specific activities for *p*NP-Glu and cellobiose only (Table 3).

Kinetics. The kinetic parameters of the purified enzyme for *p*NP-Glu and cellobiose are shown in Table 4. The Lineweaver-Burk plots indicated that the K_m for *p*NP-Glu and cellobiose were 0.11 mM and 0.41 mM, and the k_{cat} values were 1,745.5 s^{-1} and 2,640.9 s^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) values for hydrolysis of *p*NP-Glu and cellobiose were calculated as 15,770.4 and 6,361.4 s^{-1} mM^{-1}, respectively. A comparison of the properties of various β-glucosidase from a number of different sources is shown in Table 5 [1, 16-29]. *P. italicum* β-glucosidase had a comparable K_m value of 0.11 mM for *p*NP-Glu. In comparison, the K_m values for *p*NP-Glu from other fungi ranged from 0.09 to 21.7 mM. The K_m value for β-glucosidase from *P. italicum* was similar to that of *P. brasilianum* [22], and differs from other β-glucosidases in that it possesses a significantly higher catalytic efficiency (*p*NP-Glu and cellobiose were calculated as 15,770.4 and 6,361.4 s^{-1} mM^{-1}) than β-glucosidases purified from other

Table 4. Kinetic parameters of *Penicillium italicum* β-glucosidase

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	Compound	Inhibition type	K_i (mM)
*p*NP-Glu	0.11 ± 0.01	1,745.5 ± 0.6	15,770.4 ± 2.8	*Glu*	Non-competitive	8.9
Cellobiose	0.41 ± 0.02	2,640.9 ± 0.2	6,361.4 ± 1.5	*GL*	Non-competitive	11.3

Data represent the means of three separate experiments.
*p*NP-Glu, *p*-nitrophenyl-β-D-glucopyranoside; *Glu*, D-glucose; *GL*, D-glucono-δ-lactone.

Table 5. Properties of β-glucosidases from various sources

Strain	Mr (kDa)	Quaternary structure	Opt. temp. (°C)	Opt. pH	K_m (mM)	k_{cat} (s^{-1})	Reference
Aspergillus niger	330	Tetramer	70	4.6~5.3	1.1	66.7	[16]
Candida peltata	43	Monomer	50	5	2.3	NR	[17]
Ceriporiopsis subvermispora	110	NR	60	3.5	3.3	NR	[18]
Daldinia eschscholzii	64	Monomer	50	5	1.5	NR	[19]
Fomitopsis pinicola	105	Monomer	50	4.5	1.8	2990	[20]
Melamocarpus sp.	92	Monomer	60	6	3.3	66.7	[21]
Penicillium brasilianum	115	NR	60	4-6	0.09	NR	[22]
Penicillium italicum	354	Tetramer	60	4.5	0.11	1,745	This study
Penicillium occitanis	98	NR	60	4.5	0.37	NR	[23]
Penicillium purpurogenum	110	Monomer	65	5	5.1	NR	[24]
Penicillium verruculosum	116	NR	60	5	0.44	160	[25]
Phanerochaete chrysosporium	114	NR	NR	4~5.2	1.0	NR	[26]
Phoma sp.	440	Tetramer	60	4.5	0.3	0.5	[1]
Piptoporus betulinus	36	Monomer	60	4	1.8	96	[27]
Stachybotrys sp.	75	Monomer	50	5	0.27	NR	[28]
Xylaria regalis	85	NR	50	5	1.7	NR	[29]

Data represent the means of three separate experiments.
Kinetic parameters of β-glucosidases are shown for *p*NP-Glu.
*p*NP-Glu, *p*-nitrophenyl-β-D-glucopyranoside; NR, not reported.

sources. This work demonstrates that, due to its hydrolytic enzyme, *P. italicum* is able to perform rapid cellobiose hydrolysis with relatively high catalytic efficiency. Lineweaver-Burk plots (1/V vs. 1/[S]) were used to investigate the effects of the inhibitors glucose and glucono-δ-lactone using *p*NP-Glu. According to the Lineweaver-Burk plots, glucose and glucono-δ-lactone were non-competitive inhibitors (Fig. 5A and 5B), with inhibition constants (K_i)

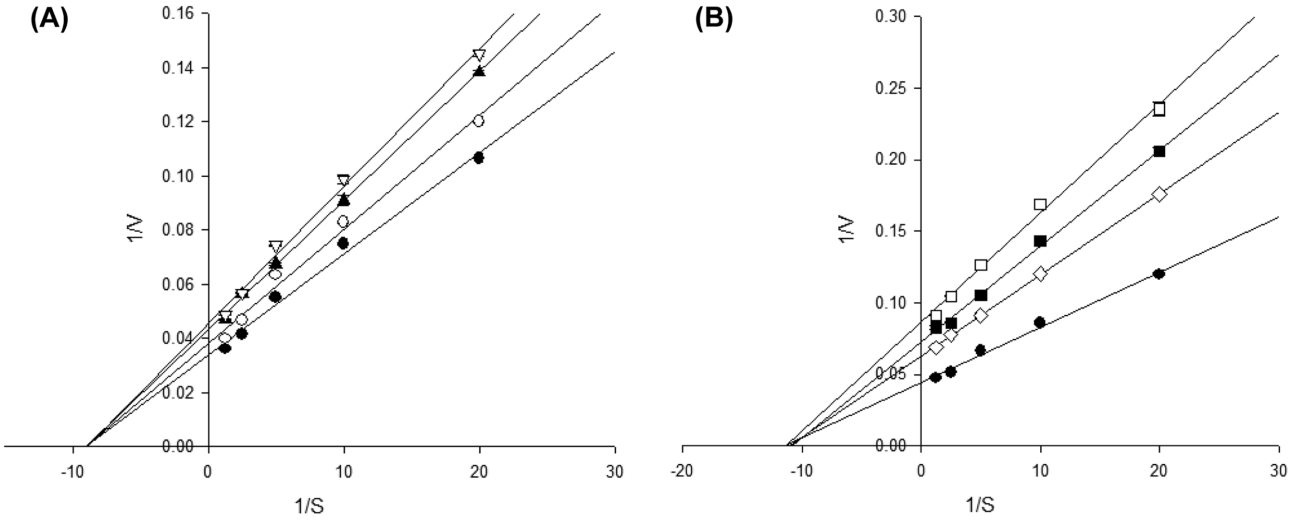

Fig. 5. Lineweaver-Burk plots of *Penicillium italicum* β-glucosidase for different concentrations of inhibitors. A, Glucose: The concentrations of glucose were 0.0 (●), 2.0 (○), 4.0 (▲), and 6.0 mM (▽); B, Glucono-δ-lactone: The concentrations of glucose were 0.0 (●), 0.2 (◇), 0.4 (■), and 0.8 mM (□). Data represent the means of three experiments and error bars represent standard deviation.

of 8.9 mM and 11.3 mM, respectively (Table 4), thereby indicating that glucose is a stronger inhibitor of β-glucosidase from *P. italicum* than glucono-δ-lactone. Glucose and glucono-δ-lactone were competitive inhibitors of all previously reported β-glucosidases [20, 24, 27]. This study reports on the first non-competitively inhibited β-glucosidases.

In conclusion, the results of this study, for the first time, identify the fungus isolated from rotten citrus peel as *P. italicum*, and demonstrate that this fungus produced a higher catalytic efficiency, compared with most other β-glucosidases. Inhibition of the enzyme by glucose and glucono-δ-lactone was non-competitive. The present study provides the first report of non-competitive inhibition of β-glucosidases. Competitive inhibitors compete with substrate for the active site of the enzyme, whereas binding of uncompetitive and non-competitive inhibitors occurs at sites other than the active site. Therefore, reduction of non-competitive inhibition by glucose and glucono-δ-lactone via immobilization or mutation of the enzyme may be possible. The enzymatic properties of β-glucosidase from *P. italicum* 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 β-glucosidase, such as molecular cloning and gene expression studies, are currently underway.

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