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

Ah-Reum Park¹, Joo Hee Hong¹, Jae-Jin Kim² and Jeong-Jun Yoon*²

¹Green Materials Technology Center, Chungcheong Regional Division, Korea Institute of Industrial Technology (KITECH), Cheonan 331-825, Korea
²Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

(Received July 31, 2012. Revised August 24, 2012. Accepted August 27, 2012)

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 Ni²⁺. The enzyme exhibited hydrolytic activity for p-nitrophenyl-β-D-glucopyranoside (pNP-Glu), β-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, carboxymethyl cellulose, xylan, and cellulose, indicating that the enzyme was a β-glucosidase. The k₁/Km (s⁻¹ mM⁻¹) values for pNP-Glu and cellobiose were 15,770.4 mM⁻¹ and 6,361.4 mM⁻¹, respectively. These values were the highest reported for β-glucosidases. Non-competitive inhibition of the enzyme by both glucose (Kᵢ = 8.9 mM) and glucono-5-lactone (Kᵢ = 11.3 mM) was observed when pNP-Glu was used as the substrate. This is the first report of non-competitive inhibition of β-glucosidase by glucose and glucono-5-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 β-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.

*Corresponding author <E-mail : jjyoon@kitech.re.kr>

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
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) (NH₄)₂SO₄, 0.05% L-asparagine, 0.05% KCl, 0.1% KH₂PO₄, 0.02% MgSO₄·7H₂O, 0.01% CaCl₂, 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'-CTTGGTGTACTTATAGGAGTAA-3') and ITS4 (5'-TCTCCGCTATATGATATGC-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 MAFFT 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 (pNP-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 Na₂CO₃. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of pNP 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 polyethylenesulfone membrane and dissolved in a small volume of 20 mM Tris-HCl buffer (pH 7.5). The concentrated solution was loaded onto a HiLoad 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 Na₂HPO₄ 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 Na₂HPO₄ 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 (pNP-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 pNP-Glu, p-nitrophenyl-β-D-cellobioside (pNP-Cell), p-nitrophenyl-β-D-lactopyranoside (pNP-Lac), p-nitrophenyl-β-D-xyloside (pNP-Xyl), and p-nitrophenyl-β-D-galactopyranoside (pNP-Gal), cellobiose, carboxymethyl 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 under optimal conditions, and the activity assayed in the absence of metal ions was expressed as 100%.

Kinetic parameters and inhibition constants. Various concentrations of pNP-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 \( V_{max} \) (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) 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 pNP-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 pNP-Glu as a 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 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 *Penicillium 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

| 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 (pNP-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 pNP-Glu and 0.05 U/mL enzyme for 30 min. Data represent the means of three experiments and error bars represent standard deviation.
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.

In 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, Zn²⁺ at 5 mM stimulated the enzyme to 109% of relative activity, whereas β-glucosidase activity was strongly inhibited by Ni²⁺ (47%) when applied at 5 mM. Mn²⁺, Mg²⁺, Fe²⁺, Co²⁺, Ca²⁺, and Ba²⁺ 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 pNP-Glu and cellobiose, whereas minimal activity was observed for pNP-Cel, pNP-Xyl, and no activity was observed for pNP-Lac, pNP-Gal, CMC, Xylan, and Cellulose, indicating that the enzyme is a β-glucosidase with narrow substrate specificity. The highest activity was observed with pNP-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 pNP-Glu and cellobiose only (Table 3).

**Kinetics.** The kinetic parameters of the purified enzyme for pNP-Glu and cellobiose are shown in Table 4. The Lineweaver-Burk plots indicated that the *Kₚ* for pNP-Glu and cellobiose were 0.11 mM and 0.41 mM, and the *kₐ* values were 1,745.5 s⁻¹ and 2,640.9 s⁻¹, respectively. The catalytic efficiency (*kₐ/Kₚ*) values for hydrolysis of pNP-Glu and cellobiose were calculated as 15,770.4 and 6,361.4 s⁻¹ M⁻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 *Kₚ* value of 0.11 mM for pNP-Glu. In comparison, the *Kₚ* values for pNP-Glu from other fungi ranged from 0.09 to 21.7 mM. The *Kₚ* value for β-glucosidase from *P. italicum* was similar to that of *P. brasiliianum* [22], and differs from other β-glucosidases in that it possesses a significantly higher catalytic efficiency (pNP-Glu and cellobiose were calculated as 15,770.4 and 6,361.4 s⁻¹ M⁻¹) than β-glucosidases purified from other

---

**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 ± 0.1             | 100 ± 0.1             |
| Mn²⁺       | 100 ± 0.1             | 98 ± 0.3              |
| Mg²⁺       | 98 ± 0.3              | 98 ± 0.3              |
| Fe²⁺       | 101 ± 0.4             | 102 ± 0.6             |
| Zn²⁺       | 101 ± 0.1             | 109 ± 0.7             |
| Co²⁺       | 102 ± 0.2             | 99 ± 0.2              |
| Ca²⁺       | 96 ± 0.4              | 91 ± 0.4              |
| Cu²⁺       | 100 ± 0.3             | 97 ± 0.2              |
| Ba²⁺       | 90 ± 0.2              | 96 ± 0.7              |
| Ni²⁺       | 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-cellobiose | βGlc | 11.1 ± 0.8            |
| p-Nitrophenyl-β-D-lactopyranoside | βGlc | ND                    |
| p-Nitrophenyl-β-D-xylloside | βXyl | 7.0 ± 0.2             |
| p-Nitrophenyl-β-D-galactopyranoside | βGal | ND                    |

Saccharides

| Cellulose (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, carboxymethyl cellulose.
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 pNP-Glu. According to the Lineweaver-Burk plots, glucose and glucono-δ-lactone were non-competitive inhibitors (Fig. 5A and 5B), with inhibition constants ($K_i$) of 8.9 and 11.3, respectively.

**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) |
|-------------|------------|----------------------|-----------------------------------|----------|----------------|------------|
| pNP-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.
pNP-Glu, *p*-nitrophenyl-β-D-glucopyranoside; Glu, α-glucose; GL, α-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$^{-1}$) | $K_i$ (mM) | Reference |
|-------------------------|----------|----------------------|-----------------|---------|------------|----------------------|------------|-----------|
| Aspergillus niger       | 330      | Tetramer             | 70              | 4.6-5.3 | 1.1        | 66.7                 |            | [16]      |
| Candida peltata         | 43       | Monomer              | 50              | 3.5     | 3.3        | NR                   |            | [17]      |
| Ceriporiopsis subvermispora | 110     | NR                   | 60              | 5       | 1.5        | NR                   |            | [18]      |
| Daldinia eschscholtzi   | 64       | Monomer              | 50              | 1.5     | 2.3        | NR                   |            | [19]      |
| Fomitopsis pinicola     | 105      | Monomer              | 50              | 1.8     | 8.9        | NR                   |            | [20]      |
| Melanocarpus sp.        | 92       | Monomer              | 60              | 3.3     | 66.7       | NR                   |            | [21]      |
| Penicillium brasiliannum| 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              | 5.1     | 5.1        | NR                   |            | [24]      |
| Penicillium verruculosum| 116     | NR                   | 60              | 5       | 0.44       | 160                  |            | [25]      |
| Phanerochaeta 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              | 4.5     | 1.8        | 96                   |            | [27]      |
| Stachybotrys sp.        | 75       | Monomer              | 50              | 5       | 0.27       | NR                   |            | [28]      |
| Xylaria regalis         | 85       | NR                   | 50              | 1.7     | 5.0        | NR                   |            | [29]      |

Data represent the means of three separate experiments.
Kinetic parameters of β-glucosidases are shown for pNP-Glu.
pNP-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 pNP-Glu. According to the Lineweaver-Burk plots, glucose and glucono-δ-lactone were non-competitive inhibitors (Fig. 5A and 5B), with inhibition constants ($K_i$) of 8.9 and 11.3, respectively.

![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.](image-url)
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.

**Acknowledgements**

This work was supported by a grant from Korea Institute of Industrial Technology, Republic of Korea (JA-12-0001).

**References**

1. Choi JY, Park AR, Kim YJ, Kim JJ, Cha CJ, Yoon JJ. Purification and characterization of an extracellular beta-glucosidase produced by *Phoma* sp. KCTC11825BP isolated from rotten mandarin peel. J Microbiol Biotechnol 2011;21:503-8.

2. Mamma D, Kourtoglou E, Christakopoulos P. Fungal multicenzyme production on industrial by-products of the citrus-processing industry. Bioresour Technol 2008;99:2373-83.

3. Murai T, Ueda M, Kawaguchi T, Arai M, Tanaka A. Assimilation of cellobioigosacharides by a cell surface-engineered yeast expressing beta-glucosidase and carboxymethylcellulase from *Aspergillus niger*. Appl Environ Microbiol 1998;64:4857-61.

4. Shewale JG. Beta-Glucosidase: its role in cellulase synthesis and hydrolysis of cellulose. Int J Biochem 1982;14:435-43.

5. Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnol Adv 2000;18:355-83.

6. Coughlan MP. The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnol Genet Eng Rev 1985;3:39-109.

7. Bothast RJ, Saha BC. Ethanol production from agricultural biomass substrates. Adv Appl Microbiol 1997;44:261-86.

8. Guegguegn, Y. Chemardin P, Jaban G. Arnaud A, Galzy P. A very efficient beta-glucosidase catalyst for the hydrolysis of flavor precursors of wines and fruit juices. J Agric Food Chem 1996;44:2336-40.

9. Makropoulou M, Christakopoulos P, Tsitsimpikou C, Kekos D, Kolissis FN, Macris BJ. Factors affecting the specificity of beta-glucosidase from *Fusarium oxysporum* in enzymatic synthesis of alkyl-beta-D-glucosides. Int J Biol Macromol 1998;22:97-101.

10. White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR protocols: a guide to methods and applications. London: Academic Press; 1990. p. 315-22.

11. Katoh K, Toh H. Recent developments in the MAFFT multiple sequence alignment program. Brief Bioinform 2008;9:286-98.

12. Maddison DR, Maddison WP. MacClade 4: analysis of phylogeny and character evolution. Version 4.08. Sunderland: Sinauer Associates; 2005.

13. Swoford DL. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0 b10. Sunderland: Sinauer Associates; 2002.

14. Page RD. Tree View: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 1996;12:357-8.

15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

16. Rashid MH, Siddiqui KS. Purification and characterization of a beta-glucosidase from *Aspergillus niger*. Folia Microbiol (Praha) 1997;42:544-50.

17. Saha BC, Bothast RJ. Production, purification, and characterization of a highly glucose-tolerant novel beta-glucosidase from *Candida pelitata*. Appl Environ Microbiol 1996:62:3165-70.

18. Magalhaes PO, Ferraz A, Milagres AF. Enzymatic properties of two beta-glucosidases from *Ceriporiopsis subvermispora* produced in biopulping conditions. J Appl Microbiol 2006;101:480-6.

19. Karchanatat A, Petsom A, Sangvanich P, Piaphukiew J, Whalley AJ, Reynolds CD, Sihanont P. Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. FEMS Microbiol Lett 2007;270:162-70.

20. Joo AR, Jeya M, Lee KM, Sim WI, Kim JS, Kim IW, Kim YS, Oh DK, Gunasekaran P, Lee JK. Purification and characterization of a beta-1,4-glucosidase from a newly isolated strain of *Fomitopsis pinicola*. Appl Microbiol Biotechnol 2009;83:285-94.

21. Kaur J, Chadha BS, Kumar BA, Kaur GS, Saini HS. Purification and characterization of beta-glucosidase from *Melanocarpus* sp. MTCC 3922. Electron J Biotechnol 2007;10:260-70.

22. Krogh KB, Harris PV, Olsen CL, Johansen KS, Hojer-Pedersen J, Borjesson J, Olsson L. Characterization and kinetic analysis of a thermostable GH3 beta-glucosidase from *Penicillium brasiliianum*. Appl Microbiol Biotechnol 2010;
23. Bhiri F, Chaabouni SE, Limam F, Ghrir R, Marzouki N. Purification and biochemical characterization of extracellular beta-glucosidases from the hypercellulolytic Pol6 mutant of *Penicillium occitanis*. Appl Biochem Biotechnol 2008;149:169-82.

24. Jeya M, Joo AR, Lee KM, Tiwari MK, Lee KM, Kim SH, Lee JK. Characterization of beta-glucosidase from a strain of *Penicillium purpurogenum* KJS506. Appl Microbiol Biotechnol 2010;86:1473-84.

25. Korotkova OG, Semenova MV, Morozova VV, Zorov IN, Sokolova LM, Bubnova TM, Okunev ON, Sinitsyn AP. Isolation and properties of fungal beta-glucosidases. Biochemistry (Mosc) 2009;74:569-77.

26. Lymar ES, Li B, Renganathan V. Purification and characterization of a cellulose-binding beta-glucosidase from cellulose degrading cultures of *Phanerochaete chrysosporium*. Appl Environ Microbiol 1995;61:2976-80.

27. Valaskova V, Baldrian P. Degradation of cellulose and hemicelluloses by the brown rot fungus *Piptoporus betulinus*: production of extracellular enzymes and characterization of the major cellulases. Microbiology 2006;152(Pt 12):3613-22.

28. Amouri B, Gargouri A. Characterization of a novel beta-glucosidase from a *Stachybotrys* strain. Biochem Eng J 2006;32:191-7.

29. Wei DL, Kirimura K, Usami S, Lin TH. Purification and characterization of an extracellular beta-glucosidase from the wood-grown fungus *Xylaria regalis*. Curr Microbiol 1996;33:297-301.