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Purification and Characterization of Thermostable β-Glucosidase from the Brown-Rot Basidiomycete *Fomitopsis palustris* Grown on Microcrystalline Cellulose

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An extracellular β-glucosidase was purified 154-fold to electrophoretic homogeneity from the brown-rot basidiomycete *Fomitopsis palustris* grown on 2.0% microcrystalline cellulose. SDS-polyacrylamide gel electrophoresis gel gave a single protein band and the molecular mass of purified enzyme was estimated to be approximately 138 kDa. The amino acid sequences of the proteolytic fragments determined by nano-LC-MS/MS suggested that the protein has high homology with fungal β-glucosidases that belong to glycosyl hydrolase family 3. The K_ms for *p*-nitorophenyl-β-D-glucoside (*p*-NPG) and cellobiose hydrolyses were 0.117 and 4.81 mM, and the K_{cat} values were 721 and 101.8 per sec, respectively. The enzyme was competitively inhibited by both glucose (K_i= 0.35 mM) and gluconolactone (K_i= 0.008 mM), when *p*-NPG was used as substrate. The optimal activity of the purified β-glucosidase was observed at pH 4.5 and 70°C. The *F. palustris* protein exhibited half-lives of 97 h at 55°C and 15 h at 65°C, indicating some degree of thermostability. The enzyme has high activity against *p*-NPG and cellobiose but has very little or no activity against *p*-nitrophenyl-β-lactoside, *p*-nitrophenyl-β-xyloside, *p*-nitrophenyl-α-arabinofuranoside, xylan, and carboxymethyl cellulose. Thus, our results revealed that the β-glucosidase from *F. palustris* can be classified as an aryl-β-glucosidase with cellobiase activity.

Keywords: β-glucosidase, brown-rot fungus, *Fomitopsis palustris*, purification, microcrystalline cellulose

Lignocellulose materials are the most abundant and renewable biomasses available on earth. Recently, the production of ethanol from lignocellulosics has received much attention due to immense potential for conversion of renewable biomaterials into biofuels and chemicals. Cellulose, which is a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bonds, is the main constituent of wood tissue. The enzyme system for the conversion of cellulose to glucose involves at least three types of cellulases such as endo-1,4-β-glucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase (BGL, EC 3.2.1.21) (Eriksson *et al*., 1990). The enzymatic degradation of cellulose to form glucose starts with exoglucanases and endoglucanases. CBH which acts as exoglucanase releases cellobiose as the main product. Various EGs act randomly along the cellulose chains, thus produce cellulose fragments to generate new sites on which CBH reacts to produce cellobiose. BGL then hydrolyzes cellobiose, which is a strong inhibitor of both EG and CBH (Woodward *et al*., 1982). Thus, it does not only produce glucose from cellobiose but also reduces cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently (Saha *et al*., 1994). Product inhibition and thermal inactivation of BGL constitute two major barriers to the development of enzymatic hydrolysis of cellulose as a commercial process (Woodward *et al*., 1982).

Cellulolytic enzymes from soft-rot and white-rot fungi have been extensively studied in some model organisms such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (Claeyssens *et al*., 1989; Eriksson *et al*., 1990; Uzcategui *et al*., 1991). Recently, the genome sequence of the white-rot fungus *P. chrysosporium* strain RP8 was revealed (Martinez *et al*., 2004) and the genomic information greatly facilitated the identification of the extracellular proteins produced during the degradation of cellulose by the wood-rot fungi. However, there is little information available on the mechanism of hydrolysis of cellulose by brown-rot fungi to date.

We have previously reported that the brown-rot basidiomycete *Fomitopsis palustris* produced all of the known cellulase components necessary for the degradation of lignocellulosic biomass (Yoon and Kim, 2005). There is an increasing demand for the development of BGL in the conversion of cellulose to glucose for the subsequent production of fuel ethanol (Saha *et al*., 1994). In this paper, we report the purification and characterization of an extracellular BGL from *F. palustris* grown on microcrystalline cellulose to increase our knowledge of the cellulolytic enzyme systems of brown-rot basidiomycetes.

Materials and Methods

Microorganism and culture conditions

The brown-rot basidiomycete *F. palustris* FFPRI 0507

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(Berkeley et Curtis) Murill used for the experiment originated from Foresty and Forest Products Research Institute (FFPRI) of Japan. The mycelia of *F. palustris*, which had been incubated on a potato dextrose agar plate at 28°C for 7 days, were punched out and inoculated into 100 ml of potato dextrose broth in a 500 ml Erlenmeyer flask. After the plugs were incubated in this medium at 28°C for 7 days on a rotary shaker at 105 rpm, 5 ml of *F. palustris* precultures were aseptically inoculated into 5 L of cellulolytic medium in a 10 L bioreactor. This culture medium contained 0.8% (w/v) peptone, 0.2% (w/v) yeast extract, 0.5% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.3% (w/v) $MgSO_4 \cdot 7H_2O$, and 2% (w/v) microcrystalline cellulose (Avicel, Fluka, Switzerland). Avicel was used as a carbon source for the cultivation of this fungus. The culture in a 10 L bioreactor was incubated at room temperature for 14 days.

Enzyme purification

The culture solution (5 L) was filtered through filter paper (Toyo Roshi Kaisha, Japan), and concentrated with a stirred ultrafiltration cell (model 8400; Millipore Corp.) equipped with a PM 10 membrane (Millipore Corp., USA) under nitrogen pressure of 4.0 kg/f/cm^2 and dialyzed against 20 mM sodium acetate buffer (pH 5.0). The crude extract (30 ml) was loaded onto a Toyopearl DEAE-650S (Tosoh, Japan) column (2.0× 20 cm), which had been equilibrated with the same buffer. The dialyzed enzyme solution was eluted with a step gradient of 0 mM, 200 mM, 300 mM, 400 mM, and 500 mM NaCl in a volume of 1,000 ml. The fractions with the enzyme activity were pooled, concentrated by ultrafiltration, and dialyzed against 50 mM sodium acetate (pH 5.0) containing 0.15 M NaCl. The dialyzate (4 ml) was applied to a Sephacryl 300-S HR (Amersham Biosciences, USA) column (1.6×60 cm), which had been equilibrated with 50 mM sodium acetate (pH 5.0) containing 0.15 M NaCl, and then eluted with the buffer at a flow rate of 0.5 ml/min. The active fractions were concentrated by ultrafiltration and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1.5 M $(NH_4)_2SO_4$. The dialyzed enzyme solution (2.0 ml) was then loaded onto a Resource PHE (Amersham Biosciences, USA) column (1.0×1.0 cm) equilibrated with the same buffer containing 1.5 M $(NH_4)_2SO_4$. The active fractions were eluted with a linear gradient from 1.5 to 0 M of $(NH_4)_2SO_4$ in the buffer at a flow rate of 1.0 ml/min. Fractions containing the β-glucosidase activity were concentrated with an Ultrafree-0.5 centrifugal filter (Millipore Corp., USA), and the purity was checked by sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the purified enzyme was calculated with the following molecular mass standards: myosin (220 kDa), β-galactosidase (115 kDa), bovine serum albumin (96 kDa), ovalbumin (51 kDa), carbonic anhydrase (37 kDa), soybean trypsin inhibitor (30 kDa), and lysozyme (20 kDa).

Determination of amino acid sequences by LC-MS/MS analysis

The purified enzyme were submitted for internal amino acid sequencing of selected peptides that were digested by 10 ng/ml sequencing grade modified trypsin, chymotrypsin and Glu-C (Promega, USA) in 50 mM NH_4HCO_4 buffer (pH 8.0) at 37°C for 15 h as described (Shevchenko *et al*., 1996). The digested peptides were analyzed by nano-LC-MS/MS with a fused silica microcapillary C18 column (particle size; 5 μm, inner diameter; 75 μm, length; 100 mm) at Probiond, Co. Ltd. (Korea).

To identify the peptide sequences, homology search was carried out using a MS data analysis program, SEQUEST (ThermoFinnigan, USA) against fungi protein database obtained from NCBI protein sequence database. Well matched spectra with known sequences from fungal species were selected and validated with *de novo* sequencing program, Mascot distiller 2.1.0 (Matrix Science, USA).

Enzyme assays and protein determination

BGL activity was assayed using *p*-NPG (Sigma) as substrate. The enzymatic reaction mixtures (1 ml) containing 100 μl of extracellular solution and 1 mM *p*-NPG (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 10 min at 50°C. The amount of *p*-nitrophenol released was measured at A_{405} (ε_{405}=17.0/mM/cm) after addition of Na_2CO_3 to the reaction mixtures. One unit of *p*-NPG-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 μmol of *p*-nitrophenol per min. Cellobiose-hydrolyzing activity was assayed in 100 mM sodium acetate buffer (pH 5.0) by monitoring the amount of glucose produced from cellobiose. The enzyme solution (5 μl) was incubated with 10 mM cellobiose in a final volume of 50 μl for 10 min at 50°C and the concentration of glucose was estimated by a Glucose CII-Test Wako (Wako Pure Chemistry, Japan). One unit of cellobiose-hydrolyzing activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the assay conditions. The protein concentration in the enzyme solution was measured by the method of Bradford (Bradford, 1976).

Determination of kinetic parameters

The kinetic parameters (K_m and K_{cat}) of hydrolysis of *p*-NPG and cellobiose under the action of purified enzyme were determined at pH 5.0 and 50°C where the substrate concentration varied in ranges of 0.1∼8.0 mM and 1∼80 mM, respectively. The kinetic parameters were calculated and determined with DeltaGraph 5.0 (SPSS Inc.) and KaleidaGraph 3.6J (Synergy Software) software. Product inhibition constants (K_i) for gluconolactone and glucose were determined by the Michaelis-Menten equation allowing for competitive product inhibition with *p*-NPG as substrate.

Influence of temperature and pH on the β-glucosidase activity

The effect of temperature on BGL was analyzed by measuring the enzyme activity at various temperatures (10 to 90°C) in the 100 mM sodium acetate buffer (pH 5.0). The pH optimum of the BGL activity was determined by measuring the residual activity at various pHs (3.0 to 8.0) at 50°C in the 100 mM sodium acetate buffer. Stability against heat inactivation was determined by incubating the enzyme solution at the specified temperature and assaying remaining activity at various times using the method as described above.

Table 1. Summary of purification of β-glucosidase from *F. palustris*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Crude extract	255	317	1.24	1
Ultrafiltration (10 kDa cut-off)	166	253	1.53	1.2
Toyopearl DEAE-650S	86.2	243	2.80	2.3
Sephacryl S-300HR	1.2	99.8	83.2	67
Resource PHE	0.15	28.6	191	154

Results and Discussion

Purification of BGL from *F. palustris*

Previous studies reported that the brown-rot basidiomycete *F. palustris* produced BGL along with both endo-glucanases and exo-glucanases, when the fungus was grown on a liquid culture containing 2.0% (w/v) microcrystalline cellulose (Avicel) as a carbon source (Yoon and Kim, 2005). An extracellular BGL was purified to homogeneity from the culture filtrates of *F. palustris* grown on 2.0% Avicel as a carbon source. A summary of purification steps of the BGL from *F. palustris* is presented in Table 1. The BGL was purified 154-fold with a specific activity of 191 U/mg. SDS-PAGE gel gave a single protein band and the molecular mass of the purified protein was estimated to be approximately 138 kDa (Fig. 1). The molecular size is similar to the β-glucosidase isolated from brown-rot basidiomycete *Laetiporus sulphureus* (132 kDa; Lee *et al*., 2007). The molecular masses of β-glucosidases from basidiomycetes widely ranged from 50 to 256 kDa (Cai *et al*., 1998; Lo *et al*., 1988; Magalhães *et al*., 2006).

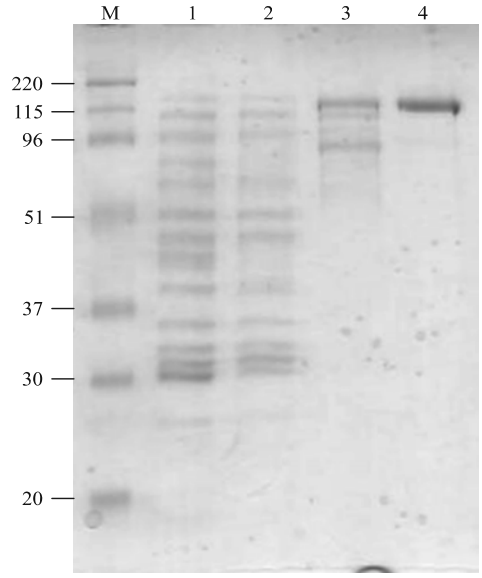

Fig. 1. SDS-PAGE of β-glucosidase from each purification step. Lane M, molecular standard proteins; 1, crude extract; 2, Toyopearl DEAE650S step; 3, Sephacryl S-300HR step; 4, Resource PHE step.

Identification of the purified protein by analysis of peptide sequences

Partial amino acid sequences determined by LC-MS/MS were analyzed to identify the protein. A proteolytic fragment was determined as LPYTIAK. BLAST search of the amino acid sequence indicated that the protein has high homology with that of C-terminal domain of glycosyl hydrolase family 3 from fungi such as *Hypocrea jecorina* (accesion number AAP57760), *Neosartorya fischeri* NRRL 181 (accesion number XP_001261562), *Aspergillus fumigatus* Af293 (accesion number XP_748896), and *Cryptococcus neoformans var. neoformans* JEC21 (accesion number XP_569544).

Kinetic properties of BGL

The K_ms for *p*-NPG and cellobiose were 0.117 and 4.81 mM, respectively, and the K_{cat} values were 721 and 101.8/sec, respectively. The purified BGL has 41 times higher affinity towards *p*-NPG than cellobiose. The kinetic efficiency (K_{cat}/K_m) for the hydrolysis of *p*-NPG and cellobiose were calculated as 6.2×10^3 and 2.1×10/sec/mM, respectively. The ratios of K_{cat} and K_m (K_{cat}/K_m) for the hydrolysis of *p*-NPG and cellobiose were almost equal to that for β-glucosidase from *T. reesei* (Chen *et al*., 1992). Thus, these results revealed that the β-glucosidase from *F. palustris* can be classified as an aryl-BGL with cellobiase activity.

Inhibition study by glucose was performed with *p*-NPG as the substrates. Glucose and gluconolactone acted as competitive inhibitors of *p*-NPG hydrolysis, with inhibition constants (K_is) of 0.35 and 0.008 mM, respectively. Most microbial BGLs have glucose K_is ranging from as low as 0.5 mM to no more than 100 mM (Woodward *et al*., 1982; Chirico and Brown, 1987; Saha *et al*., 1994). However, the K_i values of β-glucosidases from *P. chrysosporium* (Lymar *et al*., 1995) and *F. palustris* (this study) were 0.27 and 0.35 mM, respectively, indicating that glucose had a greater effect on the BGLs from these fungi. The inhibition by glucose, which is a common characteristic of BGLs from several cellulolytic fungi (Chirico and Brown, 1987; Lymar *et al*., 1995), is an important obstacle for industrial utilization of this enzyme, that is, glucose produced during lignocellulosic saccharification strongly inhibits the BGL that catalyzes the hydrolysis of cellobiose to glucose (Cantarella *et al*., 2004). The glucose inhibition of BGL could be overcome by a simultaneous saccharification and fermentation (SSF) process (Abe and Takagi, 1991) that can rapidly converts glucose to ethanol by yeast.

Effect of temperature and pH on enzyme activity and stability

The activity of the purified enzyme towards *p*-NPG was de-

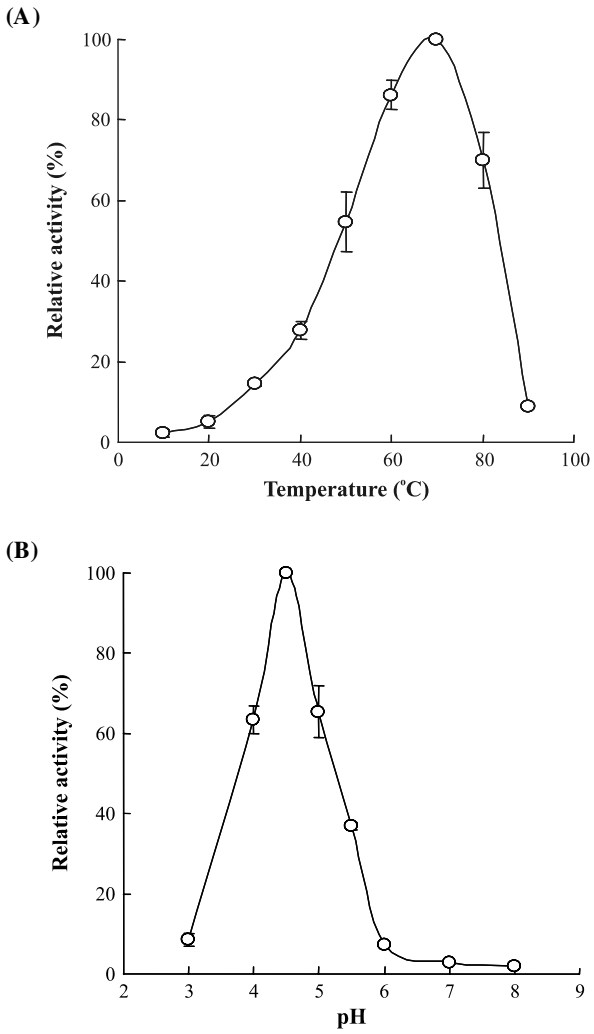

Fig. 2. Effect of temperature (A) and pH (B) on the activity of the purified β-glucosidase from *F. palustris*. The enzyme solution in 100 mM sodium acetate buffer (pH 5.0) was incubated for 10 min at various temperatures (A) and pH values (B). The residual enzyme activity was assayed as described in 'Materials and Methods'.

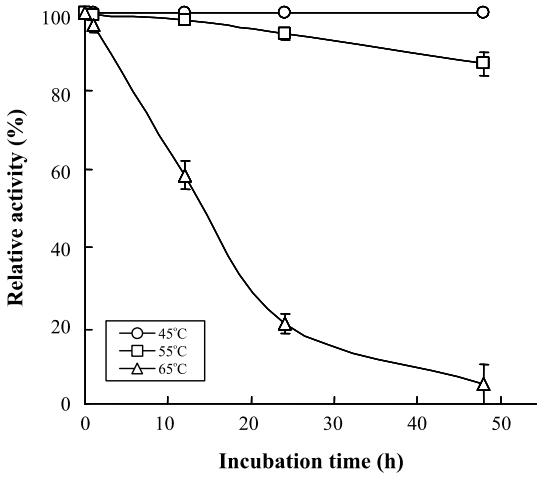

Fig. 3. Thermal inactivation of the purified β-glucosidase from *F. palustris*. Temperature stability was determined by incubating the enzyme at the specified temperature for 50 h. The enzyme activity was assayed as described in 'Materials and Methods'.

termined at a pH range of 3.0 to 8.0 at 50°C. The optimal activity of the BGL from *F. palustris* was observed at pH 4.5 (Fig. 2). No activity was observed below pH 3.0 and above pH 7.0.

Hydrolysis of *p*-NPG by the purified BGL at pH 4.5 was the highest at a temperature of 70°C under the assay conditions used (Fig. 2). Thermostability of the purified BGL from *F. palustris* is shown in Fig. 3. The purified enzyme in 50 mM sodium acetate buffer (pH 5.0) was stable at up to 45°C after incubation for more than 50 h and retained about 87% activity after incubation for 50 h at 55°C. The enzyme exhibited half-lives of 97 h at 55°C and 15 h at 65°C, suggesting that the fungal enzyme is relatively thermostable. Moreover, the purified BGL from this fungus was stable at room temperature for more than 1 month (data not shown). This characteristic of the *F. palustris* BGL, therefore, may solve the problem of thermal inactivation of this enzyme during the enzymatic saccharification of lignocellu-

Table 2. Substrate specificity of the purified β-glucosidase from *F. palustris*

Substrates	Linkage of glycosyl group[a]	Relative initial rate of hydrolysis (%)[b]
Aryl-glycosides		
p-NPG	βGlc	100
p-Nitrophenyl-β-D-cellobioside (1 mM)	βGlc	22.4
p-Nitrophenyl-β-D-lactoside (1 mM)	βGlc	<0.01
p-Nitrophenyl-β-D-xyloside (1 mM)	βXyl	<0.01
p-Nitrophenyl-α-D-arabinofuranoside (1 mM)	αAra	0
Saccharides		
Cellobiose (10 mM)	(β-1,4)Glc	100
Carboxymethyl cellulose (1%, wt/vol)	(β-1,4)Glc	0
Xylan (1%, wt/vol)	(β-1,4)Xyl	0

[a] Glc, Glucopyranoside; Xyl, xylopyranoside; Ara, arabinopyranoside
[b] Values reported are averages from duplicate experiments for each substrate. Depending on the type of substrate, activity was determined by measuring the release of glucose (Somigyi-Nelson method) or of *p*-nitrophenol (405 nm).

losics as a commercial process.

Substrate specificity of BGL

The specificity of the purified BGL against various substrates is presented in Table 2. The enzyme hydrolyzed *p*-NPG and cellobiose effectively. The purified enzyme had very little or no activity on *p*-nitrophenyl-β-D-lactoside, *p*-nitrophenyl-β-D-xyloside, and *p*-nitrophenyl-α-L-arabinofuranoside. However, it hydrolyzed *p*-nitrophenyl-β-D-cellobioside with 22.4% of the level of *p*-NPG. hydrolysis, while it rapidly hydrolyzed cellooligosaccharides. These results indicated that the BGL from *F. palustris* has high specificity only for *p*-NPG and cellobiose. This type of BGL is most common in cellulolytic microbes (Woodward *et al*., 1982).

The brown-rot basidiomycete *F. palustris* secretes some cellulases that hydrolyze microcrystalline cellulose to produce cellobiose (Yoon and Kim, 2005). Although cellobiose is the main product of the hydrolysis of cellulose by cellulolytic enzymes, this hydrolysis is inhibited by cellobiose. The BGL plays a special role to solve the inhibition caused by cellobiose produced from the hydrolysis of cellulose. Especially, the BGL from *F. palustris* exhibits synergistic reaction with the other cellulases to increase the efficiency of glucose production from microcrystalline cellulose by converting cellobiose to glucose (Yoon *et al.,* 2007). The enzymatic properties of the BGL from *F. palustris* described to date indicate that it plays an important role in the enzymatic saccharification of lignocellulosic biomass to glucose. Studies on molecular cloning and functional analysis of the gene encoding BGL is currently in progress and would give a better understanding of the protein for the development of commercial process.

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