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Identification and Functional Analysis of a Gene Encoding β-Glucosidase from the Brown-Rot Basidiomycete *Fomitopsis palustris*

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The brown-rot basidiomycete *Fomitopsis palustris* is known to degrade crystalline cellulose (Avicel) and produce three major cellulases, exoglucanases, endoglucanases, and β-glucosidases. A novel β-glucosidase designated as Cel3A was identified from *F. palustris* grown at the expense of Avicel. The deduced amino acid sequence of Cel3A showed high homology with those of other fungal β-glucosidases that belong to glycosyl hydrolase (GH) family 3. The sequence analysis also indicated that Cel3A contains the N- and C-terminal domains of GH family 3 and Asp-209 was conserved as a catalytic nucleophile. The cloned gene was successfully expressed in the yeast *Pichia pastori*s and the recombinant protein exhibited β-glucosidase activity with cellobiose and some degree of thermostability. Considering the size and sequence of the protein, the β-glucosidase identified in this study is different from the protein purified directly from *F. palustris* in the previous study. Our results suggest that the fungus possesses at least two β-glucosidase genes.

***Keywords*:** β-glucosidase, *F. palustris*, brown-rot fungus, glycosyl hydrolase, *P. pastoris*

Cellulose, a linear polymer of D-glucose units linked by 1,4-β-D-glucosidic bond, is the main constituent of wood tissue and the most abundant renewable biomass available on earth. The enzymatic saccharification of cellulosic materials to glucose involves the synergistic action of three different types of cellulases (Lynd *et al*., 2002): (i) endo-1,4-β-glucanases (EC 3.2.1.4), (ii) cellobiohydrolases (EC 3.2.1.91), and (iii) β-glucosidases (EC 3.2.1.21). A number of microorganisms produce various cellulases (Rabinovich *et al*., 2002), which belong to some families of glycosyl hydrolase (GH). The classification of cellulases is based on amino acid sequence similarities, the folding of an enzyme and the structural features of catalytic domains (Henrissat and Davies, 1997; Lynd *et al*., 2002).

Cellulolytic enzymes from white-rot fungi have been exceedingly studied in *Phanerochaete chrysosporium* (Martinez *et al*., 2004; Wymelenberg *et al*., 2005) and *Trichoderma reesei* (Mach and Zeilinger, 2003; Ouyang *et al*., 2006), and are powerful materials in biofuel industry. However, the *T. reesei* produces β-glucosidase at low levels compared to other fungi such as *Aspergillus* spp. Furthermore, the β-glucosidases of *T. reesei* are subject to product (glucose) inhibition, whereas those of *Aspergillus* spp. are more glucose-tolerant (Riou *et al*., 1998). The cellulase preperations from *T. reesei*, supplemented with the *Aspergillus* β-glucosidase, are often considered for cellulose saccharification on an industrial scale (Lynd *et al*., 2002).

There are a few reports on cellulolytic enzymes from brown-rot fungi (Kerem *et al*., 1999; Yoon and Kim, 2005). Importantly, the enzymes from brown-rot fungi did not degrade lignin but they selectively depolymerize cellulose in wood (Cohen *et al*., 2005). Both *Fomitopsis palustris* and *Fomitopsis pinicola* (Yoon and Kim, 2005; Yoon *et al*., 2008a) were known to have three kinds of cellulases, and especially the presence of processive endoglucanase possessing both endoglucanase and cellobiohydrolase activities was reported (Yoon *et al*., 2007). Although the enzymatic characteristics of purified endoglucanase and β-glucosidase from *F. palustris* have been reported in previous studies (Yoon *et al*., 2007, 2008b), only limited information is available on the genes, including each of glycosyl hydrolase (GH) families 12 and 15 (Yoon *et al*., 2006; Song *et al*., 2008). In this paper, we present the isolation and sequence analysis of a gene encoding β-glucosidase from *F. palustris* that belongs to GH family 3. The expression of the gene in *Pichia pastoris* and enzymatic properties of functionally expressed protein are also discussed.

Materials and Methods

Strain and culture conditions

The brown-rot fungus *Fomitopsis palustris* FFPRI 0507 (Berkeley et Curtis) Murill was used for this study. For precultivation, the *F. palustris* mycelia were incubated on PDA (Potato Dextrose Agar, Conda, USA) plates at 28°C for 7 days. The 10 agar-mycelium plugs with 5 mm diameter were punched out and inoculated into 100 ml of PDB (Potato Dextrose Broth, Conda) media at 28°C for 7 days with shaking at 200 rpm. Finally, precultures were incubated into 100 ml of Avicel liquid media at 28°C for 2 to 4 weeks. The liquid medium contained 2% (w/v) Avicel (Fluka, Switzerland) as a main carbon source, 0.8% (w/v) peptone, 0.5% (w/v) KH_2PO_4, 0.5% (w/v) K_2HPO_4, 0.3% (w/v) $MgSO_4 \cdot 7H_2O$, 0.2% (w/v) yeast extract and 5 ppm thiamine-HCl.

mRNA extraction and cDNA synthesis

The *F. palustris* mycelia were recovered by filtration and transferred to an RNase-free bowl. Cell pellets were rapidly frozen by liquid nitrogen and crushed by pestle. Total RNA was isolated from mycelia using a total RNA Extraction kit (RNA-spin, iNtRON Biotechnology, Korea). Polyadenylated RNA was isolated from total RNA using an

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mRNA purification kit (Oligotex mRNA Mini kit, QIAGEN, Germany). cDNA was synthesized using Maxime RT-premix (iNtRON Biotechnology, Korea) from polyadenylated RNA. The above steps were performed according to the manufacturer's instructions. The cDNA synthesized from 2, 3, and 4 weeks-grown mycelia was used to search the cellulase genes.

PCR amplification and cloning of β-glucosidase gene from *F. palustris*

To isolate the β-glucosidase genes from *F. palustris*, the degenerate oligonucleotide primers (FP-F, FP-R) were designed on the basis of the highly conserved amino acid sequences obtained by multiple sequence alignment of fungal β-glucosidases (Table 1). The cDNA synthesized from 2, 3, and 4 weeks-grown mycelia was used as templates and PCR was performed using a PCR premix (Bioneer, Korea) as following steps: initial denaturation at 94°C for 10 min, 30 cycles of extension at 94°C for 30 sec, at 55°C for 30 sec, and at 72°C for 30 sec, and final extension at 72°C for 10 min. The amplified DNA fragments were cloned into T&A cloning vectors and the cloned partial fragments were sequenced and analyzed by Solgent (Korea). Rapid amplification of cDNA ends (RACE) was performed to obtain full-length cDNA sequences. RACE-PCR was performed with 5′ and 3′-RACE specific primers using the CapFishing kit (Seegene, Korea). Reaction conditions for RACE-PCR were same as above except that the annealing temperature used was 56°C to 66°C at 2°C intervals. The fragments obtained from the 5′ and 3′-RACE were cloned into T&A cloning vector and then sequenced as described above.

Comparative sequence analysis with other fungal β-glucosidases was carried out using Jalview version 2.4 program (Clamp *et al*., 2004). To predict the protein domain families, the amino acid sequences were scanned by using Pfam version 24.0 program (http://www.sanger.ac.uk/Software/Pfam/).

Heterologous expression of β-glucosidase gene in *Pichia pastoris*

The specific primers were synthesized to construct recombinant plasmids containing the coding region of β-glucosidase gene. The forward primer (BGL-F) was designed by introducing *Eco*RI restriction site and initiation codon (ATG) located at the beginning of potential translation initiation region. The reverse primer (BGL-R) was designed by introducing *Xba*I restriction site (Table 1). The full-length cDNA fragment cloned into T&A cloning vector was digested with *Eco*RI and *Xba*I restriction enzymes and subcloned into same restriction sites of pPICZαC expression vector.

The resulting plasmid pPICZαC-cel3A was linearized by digesting with *Pme*I and transformed into *Pichia pastoris* GS115 using the *Pichia* EasyComp™ Transformation kit (Invitrogen, USA) according to the manufacture's instructions. Yeast cells transformed with plasmid were screened on YPDS/Zeocin plates containing 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol and zeocin (100 μg/ml). A single colony harboring pPICZαC-cel3A was inoculated into BMGY medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 1% (v/v) glycerol, 1.34% (w/v) yeast nitrogen base without amino acid, 4×10^{-5}% (w/v) biotin in 100 mM potassium phosphate buffer (pH 6.0), and the culture was incubated at 30°C with shaking at 200 rpm. The culture was harvested by centrifugation at 5,000×g for 10 min and transferred to fresh BMMY medium consisting of the same composition as BMGY medium, except that 0.5% (v/v) methanol was added instead of glycerol for induction. For continuous induction, methanol (0.5%, v/v) was added every 24 h.

Table 1. Oligonucleotide primers used in this study

Primer name	Sequence[a] of sense and antisense (5′→3′)	Restriction site
Degenerate primer		
FP-F	GCNACRCACTTAATCGGBTAY GACCAR	
FP-R	GACYCTCTGCCASACVCARTC RTCSGC	
Specific primer		
BGL-F	*GAATTC*AATGGGACGTTGCGC AGGCAACGC	*Eco*RI
BGL-R	*TCTAGA*CCCACCCTCACTAGCT TTTGGGAC	*Xba*I

[a] R=(A/G), S=(G/C), Y=(C/T), B=(G/C/T), V=(A/G/C), N=(A/G/C/T). The restriction sites that were incorporated into the primers are italic.

Protein purification

The culture supernatant under inducing conditions was obtained by centrifugation 10,000×g for 30 min at 4°C and concentrated by using a ultrafiltration cell (Millipore Corp., USA) with a polyethersulfone membrane (10 kDa cut-off). The resulting concentrate was then subject to Ni-NTA chromatography (GE Healthcare, UK) according to the manufacturer's instructions. The purified protein was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme assay and characterization

The reaction mixtures containing the enzyme extract solution and 1 mM *p*-nitrophenyl β-D-glucopyranoside (*p*-NPG) (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 10 min at 50°C in water bath. The amount of *p*-nitrophenol released was measured at 405 nm. One unit of *p*-NPG hydrolyzing activity was defined as the amount of enzyme catalyzing the release of 1 μmol *p*-nitrophenol equivalent per min. Protein concentration of enzyme mixture was measured with a Protein Assay kit (Bio-Rad Laboratories, USA) based on the Bradford method. The optimum temperature of enzyme activity was assayed at various temperatures (20 to 90°C) using *p*-NPG as substrate. The pH optimum of enzyme activity was measured using sodium acetate buffer (pH 4-7) and Tris-HCl buffer (pH 6-8). The enzyme stability against heat inactivation was determined by incubating the enzyme solution at temperatures 45°C, 55°C, and 65°C in 100 mM sodium acetate buffer (pH 5.0). Substrate specificity against cellobiose, carboxymethyl cellulose and xylan was determined by determining the reducing sugars as described by the Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952).

Results and Discussion

Isolation and sequence analysis of β-glucosidase gene from *F. palustris*

A β-glucosidase gene from *F. palustris* was isolated by PCR using degenerate oligonucleotide primers designed on the basis of conserved regions of amino acid sequences which were aligned with the aid of other known fungal cellulase genes (Table. 1). The amino acid sequence deduced from the nucleotide sequence of an amplified DNA fragment (1,200 bp) obtained by PCR showed a significant similarity (53%) to that of a glycosyl hydrolase from *Ustilago maydis*. A full-length cDNA fragment was obtained by 5'- and 3'-RACE-PCR and the deduced amino acid sequence of the cDNA fragment contained an open reading frame of 649 amino acid residues. However, the upstream amino acid sequence (boxed in Fig. 1)

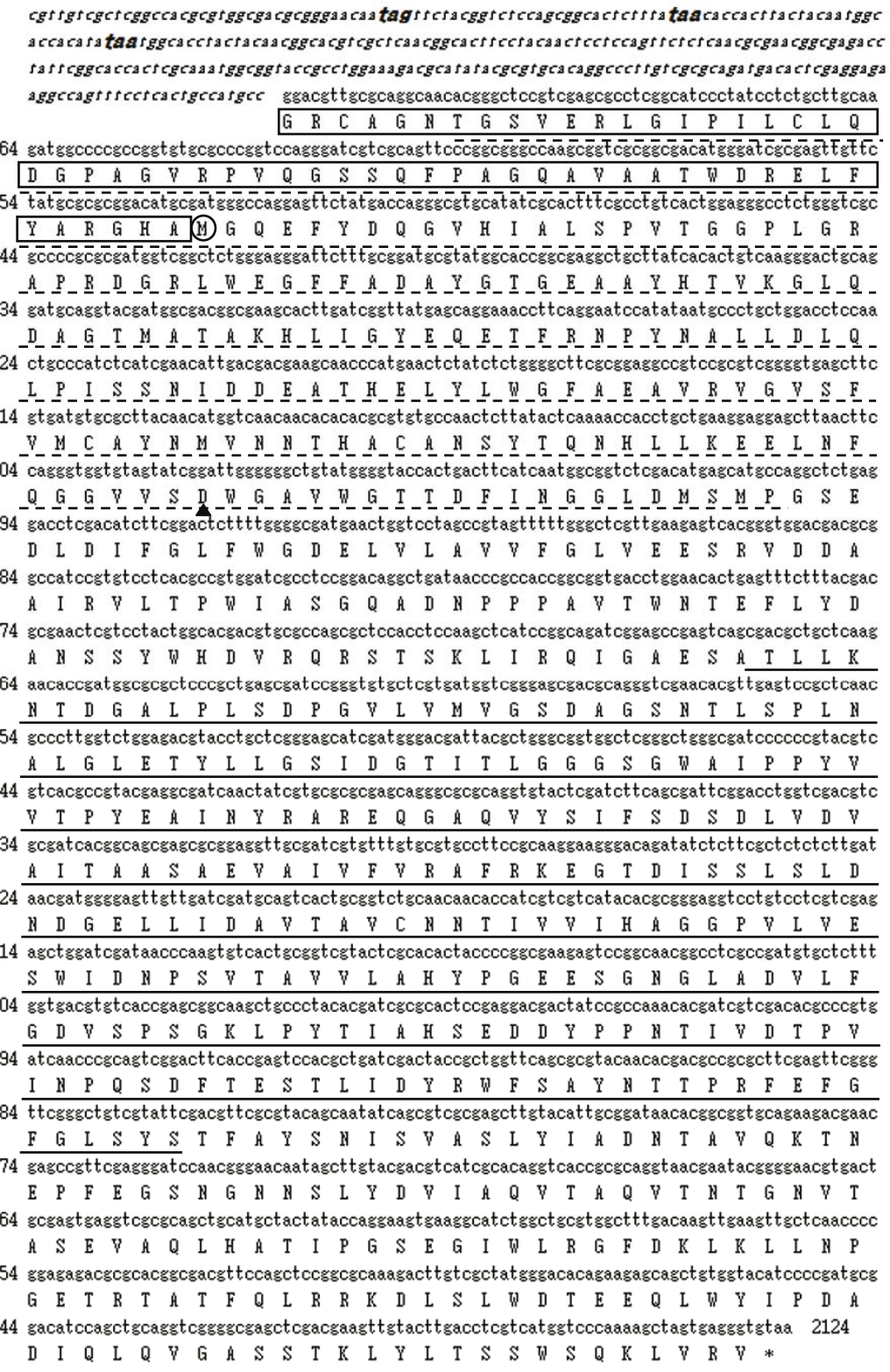

Fig. 1. The nucleotide sequence and deduced amino acid sequence of β-glucosidase from *F. palustris*. Three italic and bold characters indicate stop codon near 5′ region of cDNA sequence. The first methionine appearing in the deduced amino acid sequence was circled. The boxed amino acid sequence prior to the first methionine is the region which shows high sequence similarity with those of β-glucosidases. The nucleotide sequence in italic shows no homology with any other proteins. The N-terminal domain of GH family 3 is dotted underlined and the C-termianl domain is solid underlined. The closed triangle indicates a catalytic residue in the conserved motif (SDW) among members of GH family 3.

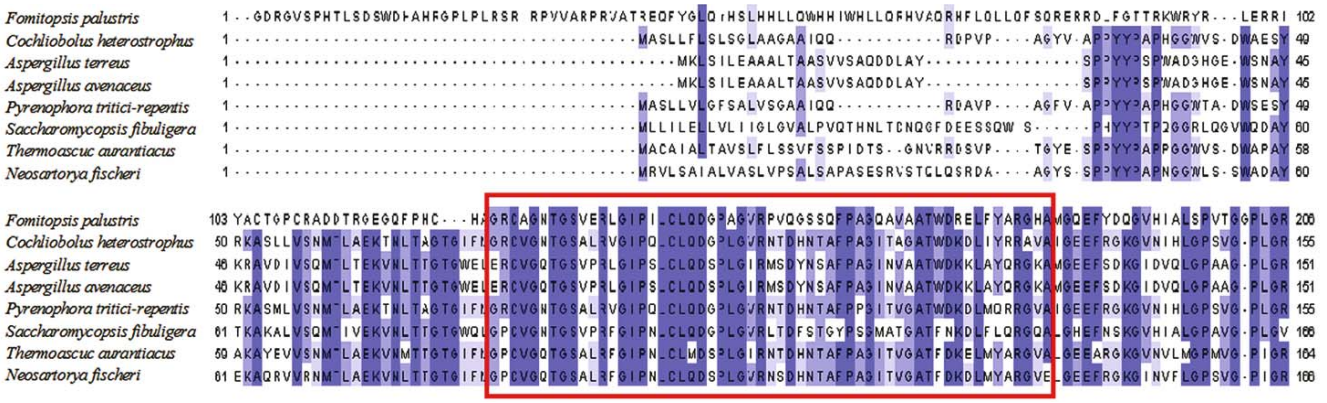

Fig. 2. Alignment of the deduced amino acid sequence of β-glucosidase from *F. palustris* with those of other fungal enzymes. Multiple sequence alignment was performed using Jalview 2.4 program. The box indicates the highly conserved region of GH family 3 domain prior to the first methionine. Data for other fungal enzymes were obtained from the GenBank database; *Fomitopsis palustris* (this study), *Cochliobolus heterostrophus* (AAB84005), *Aspergillus terreus* (XP_001212225), *Aspergillus avenaceus* (AAX39011), *Pyrenophora tritici-repentis* (XP_001937375), *Saccharomycopsis fibuliger* (P22507), *Thermoascuc aurantiacus* (ABX56927), *Neosartorya fischeri* (EAW16089).

prior to the first methionine located in the N-terminal region was highly homologous with other fungal β-glucosidases (Fig. 2). Therefore, the translation initiation site is likely present between the last stop codon (TAA) and the boxed sequence. When the whole upstream sequence prior to the first methionine was amplified using genomic DNA, the nucleotide sequence also corresponded with that of cDNA (data not shown). Therefore, the putative sequence encoding the β-glucosidase was determined by introducing the initiation codon ATG in front of the boxed sequence.

Comparison of the putative β-glucosidase sequence with those of other proteins by BLASTX search showed sequence similarities with GH family 3 enzymes from *Ustilago maydis* (47%), *Penicillium chrysogenum* (44%), *Aspergillus oryzae* (44%), and *Nectria haematococca* (43%). Analysis of the deduced amino acid sequence using the Pfam version 24.0 program (http://www.sanger.ac.uk/Software/Pfam/) indicated that the sequence contained the N- and C- terminal domains that belong to GH family 3, but the signal peptide was not found in the sequence (Fig. 1). Aspartic acid indicated by the closed triangle in Fig. 1 was also shown to be conserved at the catalytic site like other GH family 3 proteins (Steenbakkers *et al*., 2003; Joo *et al*., 2009).

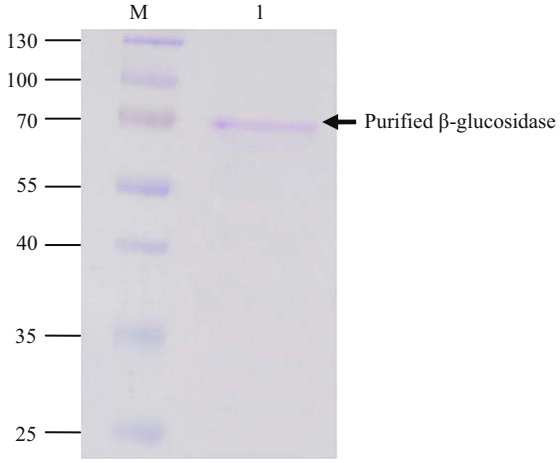

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified enzyme. Lanes: M, protein molecular markers; 1, 0.5 μg of purified enzyme

Functional expression of the β-glucosidase gene in *Pichia pastoris*

The putative coding sequence of β-glucosidase gene determined as described in the above sequence analysis was inserted into the vector pPICZαC to generate an in-frame fusion of the leader peptide of the yeast α-mating factor and Cel3A. The resulting recombinant plasmid was transformed into *P. pastoris* and induced for expression of Cel3A. The β-glucosidase activity was detected in the culture supernatant during the induction period, showing the highest activity at 72-96 h. The enzyme activity was 5.6 folds higher than that from the clone containing the pPICZαC vector only. When the culture supernatant was concentrated and subject to Ni-NTA chromatography, the β-glucosidase was highly purified with a specific activity of 29.4 U per mg protein. SDS-PAGE analysis showed that the molecular mass of purified protein was estimated to be approximately 70 kDa (Fig. 3), which corresponds to the molecular mass calculated from the putative coding sequence. Native molecular mass estimated by gel filtration chromatography indicated that the β-glucosidase is a monomeric protein. The monomeric structure is common in

Table 2. Purification of the recombinant β-glucosidase by Ni-NTA chromatography

	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	78.54	83.25	1.06	1	100
Purified enzyme	0.32	9.42	29.43	27.76	11.31

[a] Enzyme activity was assayed using *p*-NPG as substrate. One unit is defined as the amount of enzyme catalyzing the release of 1 μmol of *p*-nitrophenol equivalent per min.

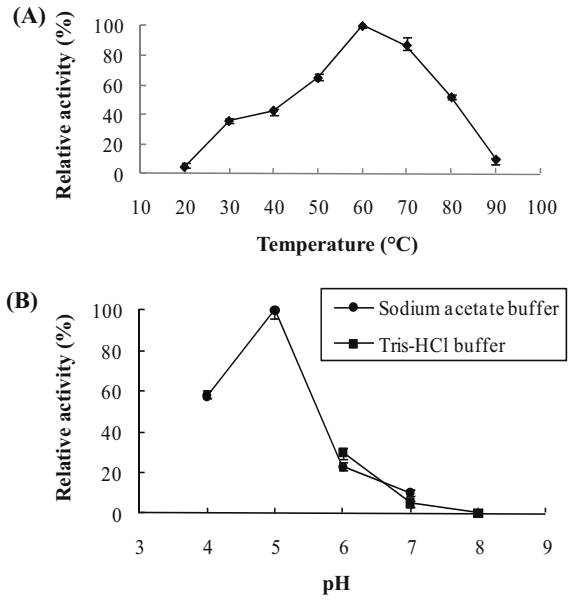

Fig. 4. Effects of temperature (A) and pH (B) on the activity of the purified β-glucosidase. The activity of purified enzyme toward *p*-NPG was determined at a temperature range of 20°C to 90°C and at a pH range of 4 to 8.

fungal β-glucosidases, while some fungal enzymes are dimeric or trimeric (Dashtban *et al*., 2009).

Characterization of the purified recombinant β-glucosidase

The effects of temperature and pH on the enzyme activity towards *p*-NPG are shown in Fig. 4. The optimum temperature of the purified β-glucosidase was observed at 60°C and the enzyme activity was maintained over 80% at 50°C to 70°C (Fig. 4A). The optimum pH of the purified enzyme was detected at pH 5.0. The enzyme activity was significantly low at pH values higher than 5.0, while the activity maintained better at acid conditions (Fig. 4B). These results coincide with those from many fungal β-glucosidases, whereas various alkaline cellulases were known in many bacterial strains (Ito, 1997). As shown in Fig. 5, the *F. palustris* β-glucosidase displayed half-lives of 140.5 h at 55°C and at 15.7 h 65°C, indicating some degree of thermostability. The specificity of purified β-glucosidase against various substrates is shown in Table 3. The enzyme showed high activity towards *p*-NPG (29.4 U/mg) and cellobiose (23.0 U/mg) but very little or no activity against *p*-nitrophenyl-β-D-cellobiose, *p*-nitrophenyl-β-D-lactoside, carboxymethyl cellulose and xylan, indicating the typical features of many microbial β-glucosidases.

Table 3. Substrate specificity of the purified β-glucosidase

Substrates	Relative activity[a] (%)
p-NPG	100
p-Nitrophenyl-β-D-cellobiose	0.45
p-Nitrophenyl-β-D-lactoside	ND
Cellobiose	78.3
Carboxymethyl cellulose	<0.01
Xylan	ND

ND: not detected
[a] Relative activity was presented as a percentage of the activity (29.43 U/mg) for *p*-NPG as substrate.

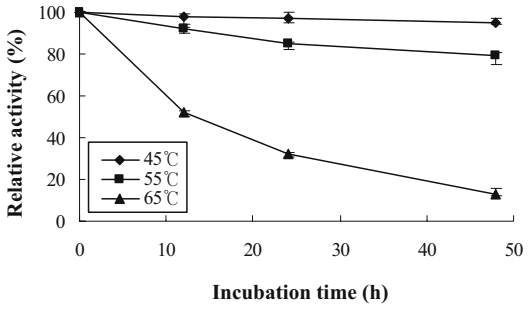

Fig. 5. Thermal inactivation of the purified β-glucosidase. The enzyme was incubated at various temperatures (◆, 45°C; ■, 55°C; ▲, 65°C) prior to assay.

In our previous study, an extracellular β-glucosidase was directly purified from *F. palustris* and characterized (Yoon *et al*., 2008b). The recombinant β-glucosidase from this study showed similar enzymatic properties with the directly purified protein, including pH and temperature optima, substrate specificity and thermostability. However, the subunit molecular mass of recombinant β-glucosidase was different from that of the protein purified directly from *F. palustris* (138 kDa). The previous study also reported a peptide sequence of LPYTIAK determined by LC-MS/MS analysis, which is found in the C-terminal domain of GH family 3. The deduced amino acid sequence of β-glucosidase revealed in this study also contained LPYTIAH. As shown in Fig. 6, the sequence alignment analysis indicated that the sequence of this region is well conserved among several fungal β-glucosidases and basic amino acids are common in the last residues as indicated by the arrow. In conclusion, these results suggest that *F. palustris* has at least two different β-glucosidase genes.

Glycosyl hydrolases are a widespread group of enzymes that hydrolyze the glycosidic bonds in glycosides. Based on the sequence similarities and predicted structures, GHs are

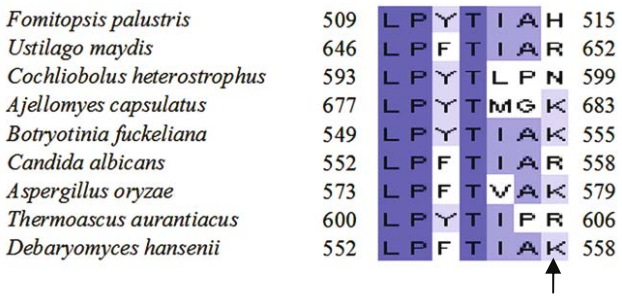

Fig. 6. Comparison of the partial amino acid sequence of β-glucosidase from *F. palustris* with those of other fungal β-glucosidases. The arrow indicates basic amino acid residues conserved at this region. Data for other fungal β-glucosidases were obtained from the GenBank database; *Fomitopsis palustris* (this study), *Ustilago maydis* (XP_760179), *Cochliobolus heterostrophus* (AAB82946), *Ajellomyes capsulatus* (EDN08586), *Botryotinia fuckeliana* (EDN20329), *Candida albicans* (XP_716473), *Aspergillus oryzae* (XM_001823575), *Thermoascus aurantiacus* (ABX56927), *Debaryomyces hansenii* (XP_459449).

classified into 113 families in the database: Carbohydrate Active enzymes, or CaZy (www.cazy.org) (Cantarel *et al*., 2009), and the catalytic mechanism and key catalytic residues are conserved in most of the GH families (Henrissat and Davies, 1997; Vuong and Wilson, 2010). Although the sequences of many microbial β-glucosidases that belong to GH family 3 have been reported, data from brown-rot fungi are scarce and the detailed structural information is limited in this group of proteins (Varghese *et al*., 1999; Pozzo *et al*., 2010). Previously, we reported the gene encoding endoglucanase that belongs to GH family 12 (Song *et al*., 2008). The present study first revealed the primary structure of another component of the cellulase system in the brown-rot fungus *F. palustris*. Although more efforts will be still needed for better understanding of the composition of cellulolytic systems, our results would be an additional asset as a potential source for industrial processes.

Acknowledgements

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