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Purification and Characterization of a Thermostable Cellobiohydrolase from *Fomitopsis pinicola*

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A screening for cellobiohydrolase (CBH) activity was performed and *Fomitopsis pinicola* KMJ812 was selected for further characterization as it produced a high level of CBH activity. An extracellular CBH was purified to homogeneity by sequential chromatography of *F. pinicola* culture supernatants. The molecular mass of the *F. pinicola* CBH was determined to be 64 kDa by SDS–PAGE and by size-exclusion chromatography, indicating that the enzyme is a monomer. The *F. pinicola* CBH showed a $t_{1/2}$ value of 42 h at 70°C and catalytic efficiency of 15.8 mM^{-1} s^{-1} (k_{cat}/K_m) for *p*-nitrophenyl-β-D-cellobioside, one of the highest levels seen for CBH-producing microorganisms. Its internal amino acid sequences showed a significant homology with hydrolases from glycoside hydrolase family 7. Although CBHs have been purified and characterized from other sources, the *F. pinicola* CBH is distinguished from other CBHs by its high catalytic efficiency and thermostability.

Keywords: Cellobiohydrolase, enzyme production, *Fomitopsis pinicola*, glycoside hydrolase, purification, stability

Cellulose, a main component of plant cell wall, represents the most abundant renewable biomass available on Earth. It is a linear biopolymer composed of 100–10,000 β-D-glucopyranosyl units linked by β-1,4-D-glucosidic bonds. Microbial cellulases catalyzing the hydrolysis of plant polysaccharides are industrially important enzymes used to saccharify industrial and agricultural cellulose-containing residues, treat cellulose pulp wastes in the paper industry, enhance the extraction of fermentable substances in the beer brewing and alcohol fermentation industries, *etc*. [33]. The cellulose degradation requires a multienzymatic system composed of three activities: endo-1,4-β-glucanase (EG, E.C. 3.2.1.4), cellobiohydrolase (CBH, E.C. 3.2.1.91), and β-glucosidase (BGL, E.C. 3.2.1.21) [3]. EG and CBH act cooperatively and synergistically in depolymerizing cellulose to cellobiose and short oligosaccharides, which are converted by BGL to glucose [6]. CBHs are most efficient on highly ordered crystalline cellulose and cleave mainly cellobiose from the opposite ends of the glucose chains, whereas EGs act randomly in the middle of the chains, probably in the more amorphous regions of cellulose [37].

In the process of cellulose hydrolysis, enzyme production is still the most crucial and costly step. Filamentous fungi are the major source of cellulases and hemicellulases. Cellobiohydrolases (CBHs) are important components in the multienzyme cellulase complexes [36] and they display an exo-type of attack on polymeric substrates, and the major product of their action on cellulose is cellobiose. They are classified into three glycoside hydrolase families (GH6, GH7, and GH48) according to amino acid sequence similarity [14, 16–18, 24]. Of these families, only GH7 is thought to be exclusively of fungal origin, and this family contains CBH I cellobiohydrolases and EG I endoglucanases from both ascomycete and basidiomycete fungi [10]. Potent cellulolytic fungi generally produce two different CBHs, CBH I and CBH II. These two types of enzymes, which are classified based on sequence identity, have extended tunnel-shaped active sites and can achieve, although slowly, complete solubilization of cellulose crystals even without help of endoglucanases [36].

Brown-rot fungi primarily utilize the cellulose and hemicellulose components of wood biomass and then rapidly depolymerize the cellulose without removing the surrounding lignin that normally prevents microbial attack [19]. In the present study, a potent CBH-producing fungus, *Fomitopsis pinicola*, was screened. Under optimized conditions, *F. pinicola* produced CBH with a 4.3 U/mg-protein specific activity. Here, we purified a novel CBH from the isolated *F. pinicola* KMJ812 (KACC 93064P) to

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homogeneity and characterized its physiological and kinetic parameters. The properties of the enzyme, including its substrate specificity, molecular form, inhibition by product cellobiose, and partial amino acid sequence revealed that this enzyme is a CBH, a member of GH7.

Materials and Methods

Screening of CBH-Producing Strain

All fungal strains were obtained from the Korean Agricultural Culture Collection (KACC). Initial screening of CBH-producing fungi was carried out on agar plates containing 10 mM 4-methylumbelliferyl cellobioside. Based on the fluorescence observed, 15 strains were inoculated into 3 ml of the growth medium containing (g/l) peptone (8), yeast extract (2), KH_2PO_4 (5), $K_2H\ PO_4$ (5), $MgSO_4{\cdot}7H_2O$ (3), Thiamine·HCl (0.005), and microcrystalline cellulose (Sigma, MO, USA), and cultivated at 28°C with agitation at 200 rpm for 7 days. The CBH activity of the culture broth was analyzed using *p*-nitrophenyl-β-D-cellobioside (pNPC; Sigma, MO, USA) as described previously [8]. After analyses, the strain with the highest CBH activity was selected.

Culture Conditions

For flask culture, the mycelia of *F. pinicola* KMJ812 were inoculated into 100 ml of potato dextrose broth. Precultures (5 ml) were inoculated into 200 ml of cellulolytic medium in a 1-l fermenter. The medium was the same as the growth medium in the screening step. The effect of carbon or nitrogen source on CBH production was investigated after 17 days of cultivation in flasks containing medium composed of 50 g/l of carbon sources and various nitrogen sources. The concentration of nitrogen source was adjusted to the same content of nitrogen using the Kjeldahl method [5]. The medium used for CBH production was the same as the growth medium except that rice straw was used instead of crystalline cellulose.

Enzyme Assay

The CBH activity was assayed using pNPC (Sigma, MO, USA) as substrate. The enzymatic reaction mixtures (1 ml) containing 100 µl of enzyme solution and 10 mM pNPC (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 15 min at 50°C [8]. The amount of *p*-nitrophenol released was measured at 405 nm after addition of 2 M Na_2CO_3 to the reaction mixtures [8]. One unit of pNPC-hydrolyzing activity was defined as the amount of enzyme equivalent to release the of 1 mmol of *p*-nitrophenol per minute.

Purification of Cellobiohydrolase

All procedures were performed at 4°C, and 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT) was used in the purification procedures unless otherwise stated. Protein estimations were performed by the Bradford method [4] using bovine serum albumin as a standard. Proteins in the column effluents were monitored by measuring the absorbance at 280 nm. All chromatographic separations were performed using an ÄKTA FPLC system (ÄKTA, Sweden).

Step 1: Preparation of crude enzyme. Cells from the culture broth were harvested by centrifugation at 10,000 ×*g* for 30 min. After washing with 20 mM sodium acetate buffer (pH 5.0), the washes and supernatants were combined, concentrated, and desalted by ultrafiltration through a polyether sulfone membrane (30 kDa cutoff) in a stirred cell (Amicon, Beverly, MA, USA).

Step 2: DEAE Sepharose chromatography. The dialyzed enzyme solution was loaded on a DEAE Sepharose Fast Flow column (1.6×10 cm; Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM sodium acetate buffer (pH 5.0), and proteins were eluted with a 180-min linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 1 ml each were collected and assayed for CBH activity. Active fractions were pooled, dialyzed against the same buffer, and concentrated by ultrafiltration for further purification.

Step 3: Sephacryl gel filtration chromatography. The concentrated enzyme solution was loaded on a HiPrep 16/60 Sephacryl S-300 HR column (1.0 cm×120 cm; Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM sodium acetate buffer containing 100 mM NaCl at pH 5.0, and proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled, dialyzed against the same buffer, and concentrated by ultrafiltration.

Step 4: MonoQ ion-exchange chromatography. The enzyme was further purified with a MonoQ ion-exchange column 5/50 GL (1.0×10 cm; Amersham Biosciences, Uppsala, Sweden) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with a 180-min linear gradient of 0−0.5 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The combined active fractions were pooled, concentrated, and dialyzed against the same buffer and concentrated with a Centricon (Millipore, Bedford, MA, USA) ultrafiltration device with a molecular mass cutoff of 30 kDa, and then used as a purified enzyme in the following experiments.

Determination of pH and Temperature Optima

The optimal pH of CBH activity was determined by incubating the purified enzyme at 50°C for 15 min in different buffers: citrate (100 mM, pH 3−4.5), sodium acetate (100 mM, pH 4.5−5.5), and phosphate (100 mM, pH 5.5−8). To determine the optimal temperature, the enzyme was incubated in sodium acetate buffer (100 mM, pH 5) for 15 min at different temperatures (30−80°C). To determine the thermostability of CBH activity, the purified enzyme was incubated at different temperatures (30°C−70°C) in the absence of substrate. After keeping them for certain periods of time (0−25 days), the residual CBH activity was determined as described above.

PAGE and Molecular Mass Determination

For the determination of subunit molecular mass, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed as described previously [22], with 10% gels. Protein bands were visualized by staining with Coomassie brilliant blue R-250 (Sigma, MO, USA). The molecular mass of the purified enzyme was determined by size-exclusion chromatography using a Superose 12 (Amersham Pharmacia Biotech, Uppsala, Sweden) column attached to an ÄCTA FPLC system (GE Health Care). The enzyme was eluted with 20 mM sodium acetate (pH 5.0) at a flow rate of 0.5 ml/min.

Substrate Specificity

The substrate specificity of CBH was determined by using pNPC, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*NP-β-galactopyranoside (pNPgal), *p*NP-β-D-mannopyranoside (pNPM), *p*NP-β-D-xylopyranoside (pNPX), *p*NP-β-D-lactopyranoside (pNPL), cellobiose, cellotriose,

cellotetraose, and cellopentaose as substrates at 10 mM concentration. CBH activities on polysaccharides carboxymethylcellulose (CMC), xylan, lichenan, laminarin, and avicel (1%) were also tested. The *p*-nitrophenol released was determined under standard enzyme assay conditions. The activities on oligosaccharides were estimated by assaying the amount of released glucose using the GOD-POD method [27]. The enzyme activity on polysaccharides was estimated by measuring released reducing sugars by the DNS method [29] using glucose or xylose as standards.

Determination of Kinetic Parameters and Inhibition Constants

The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for CBH by incubating in 100 mM sodium acetate buffer (pH 5) at 50°C with pNPC at concentrations ranging from 0.5 to 50 mM. Inhibition of CBH by cellobiose was determined in the presence of pNPC as the substrate. Values for K_m, V_{max}, and K_i were determined from Lineweaver–Burk plots using standard linear regression techniques.

Effects of Metals and Reagents

The effects of various metal ions ($BaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, $NiCl_2$, or $CoCl_2$) and reagents (2-mercaptoethanol and cysteine) at 1 mM on CBH activity were determined by preincubating the enzyme with the individual reagents in 20 mM sodium acetate buffer (pH 5.0) at 30°C for 30 min. Activities were then measured at 50°C for 15 min in the presence of the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was recorded as 100%.

Internal Amino Acid Sequence of CBH

Protein cleavage for peptide mapping was carried out at 37°C for 4 h with 100 ng of endoproteinase Asp-N or endoproteinase Lys-C or trypsin (Promega, Madison, WI, USA) to digest 20 μg of purified enzyme in 50 μl of 100 mM $(NH_4)_2CO_3$ (pH 8.5). The resulting peptide fragments were separated by SDS–PAGE (15% polyacrylamide), and the separated peptides were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Peptide bands were visualized by 0.1% Coomassie brilliant blue R-250 staining in 40% methanol. The partial amino acid sequence was determined by Edman degradation with an automatic protein sequencer (model 491A; Applied Biosystems, Division of Perkin-Elmer) at The National Instrumentation Center for Environmental Management (Seoul, Korea). The partial amino acid sequence was used to identify analogous proteins through a BLAST search of the nonredundant protein database from the National Center for Biotechnology Information. A protein domain similarity search was performed using the CDD (Conserved Domain Database) at http://www.ncbi.nlm.nih.gov/Structure/cdd.

RESULTS

Screening and Selection of CBH-Producing Strain

Among 112 fungal strains screened for CBH activity, 15 strains were selected based on the fluorescence observed when the agar plates containing 10 mM of MUC were exposed under UV. Out of 15 strains, an efficient CBH-producing *Fomitopsis pinicola* KMJ812 was selected for further study.

Optimization of Carbon and Nitrogen Sources for CBH Production

To select a suitable carbon source for CBH production, *F. pinicola* KMJ812 was cultivated in media with yeast extract (10 g/l) and varied carbon sources (cellulose, rice straw, wheat bran, xylan, Avicel, CMC, cellobiose, glucose, maltose, lactose, sucrose, or wood fiber). Among the carbon sources tested, rice straw was found to be the best carbon source for CBH production, leading to a CBH-specific activity of 4.1 U/mg-protein in a flask culture (Table 1).

Since the mechanisms that govern the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis, the effect of inorganic and organic nitrogen sources on CBH synthesis was also studied (Table 1). Rice straw (20 g/l) was used as a carbon source. Among the various nitrogen sources (peptone, corn steep powder, yeast extract, urea, ammonium sulfate, potassium nitrate, and sodium nitrate), a combination of yeast extract (5 g/l) and peptone (5 g/l) favored maximum CBH production (4.6 U/mg-protein), followed by yeast extract, whereas the other nitrogen sources were poor sources of nitrogen in the absence of pH control. The time

Table 1. Effects of various carbon and nitrogen sources on CBH production.

Carbon source (20 g/l)	CBH activity (U/mg-protein)	Total protein (mg)	pH
Cellulose	4.0±0.27	4.0±0.22	3.92
Glucose	0.4±0.04	0.9±0.11	2.57
Lactose	2.8±0.13	2.3±0.23	2.95
Maltose	1.4±0.13	3.3±0.14	2.72
Cellobiose	0.8±0.11	3.1±0.32	2.63
CMC	2.2±0.21	2.7±0.18	3.90
Xylan	1.3±0.07	4.8±0.11	3.99
Rice straw	4.1±0.32	5.3±0.32	3.71
Avicel	2.9±0.10	3.4±0.12	3.83
Sucrose	3.7±0.28	3.5±0.21	3.36
Softwood powder	1.2±0.04	3.03±0.31	5.67
Hardwood powder	3.9±0.21	4.55±0.12	3.77
Nitrogen sources (10 g/l)	**CBH activity (U/mg-protein)**	**Total protein (mg)**	**pH**
Yeast extract+peptone	4.3±0.34	5.5±0.42	3.92
Yeast extract	4.1±0.32	5.3±0.32	3.71
Peptone	1.8±0.10	3.7±0.15	3.95
Corn steep powder	0.9±0.06	2.4±0.17	4.01
Urea	1.7±0.11	2.9±0.24	7.48
$(NH_4)_2SO_4$	1.5±0.08	1.1±0.04	5.05
KNO_3	0.1±0.01	0.6±0.02	4.86
$NaNO_3$	0.3±0.02	0.9±0.07	3.37

All flasks were incubated at 25°C and 150 rpm for 15 days. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

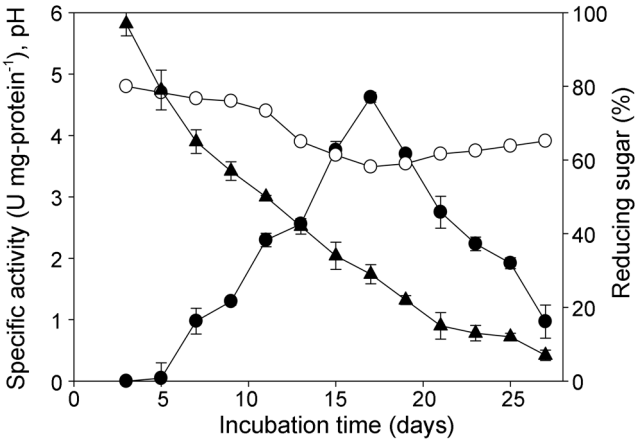

Fig. 1. Time course of cellobiohydrolase production by *F. pinicola* KMJ812 on rice straw.
Filled circles, specific activity; empty circles, pH; filled triangles, residual reducing sugar level.

course of CBH production by *F. pinicola* grown on rice straw, yeast extract, and peptone was studied (Fig. 1). CBH activity increased up to 17 days, after which it decreased gradually.

Purification of a Cellobiohydrolase

CBH was purified as described in the Materials and Methods section, and the results are summarized in Table 2. Fractionation by ultrafiltration increased the specific activity about 2-fold, with 32% recovery of CBH activity. The active fractions were applied to a DEAE Sepharose column, and CBH was eluted with approximately 0.1 M NaCl. A subsequent gel filtration step produced three protein peaks of which the second peak showed CBH activity. FPLC elution of a MonoQ ion-exchange chromatography column with 0.5 M NaCl produced an active CBH protein peak. These chromatography methods resulted in a 7-fold purification of CBH with a recovery of 0.1%. Analysis of the purified enzyme by gel electrophoresis in the presence of SDS (Fig. 2A, lane 4) revealed one band with an M_r of 64,000±1,000. Size-exclusion chromatography on a Sephacryl S-300 high resolution column resulted in the elution of the enzyme activity as a symmetrical peak corresponding to an M_r of

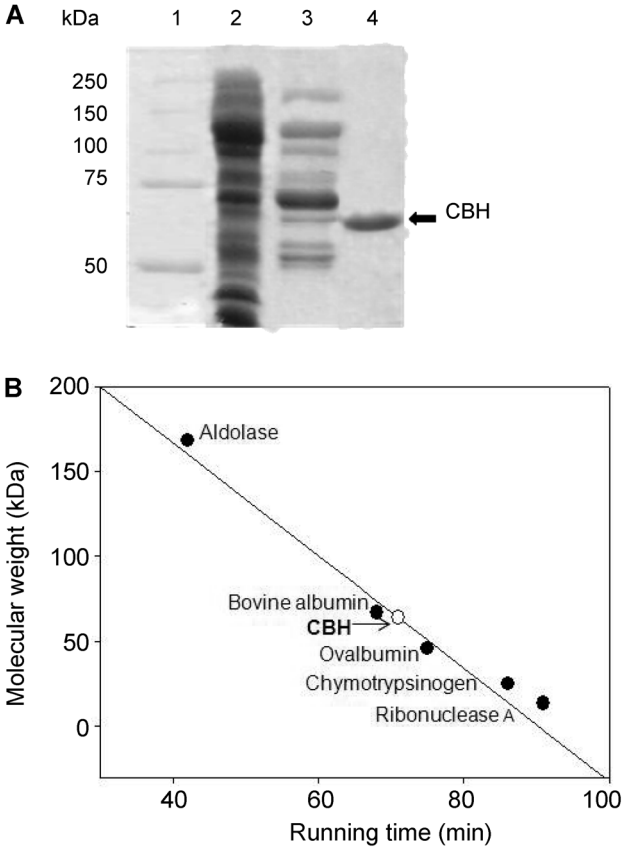

Fig. 2. PAGE and determination of molecular mass of CBH purified from the *F. pinicola*.
A. PAGE of CBH purified from *F. pinicola*. Lane 1, molecular marker; lane 2, cell extract; lane 3, DEAE ion-exchange fraction; lane 4, SDS–PAGE of MonoQ ion-exchange fraction. **B**. Determination of native molecular mass of *F. pinicola* CBH by gel filtration chromatography on a Sephacryl S-300 high-resolution column. The column was calibrated with standard molecular mass proteins such as aldose (168 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

approximately 64,000 (Fig. 2B). These results indicate that the enzyme migrates as a monomer in gel filtration under the mild conditions used, and thus may also be present active as a monomer in solution.

Identification of the Partial Peptide Fragment

The pure enzyme (1.5 μg) was separated by 10% SDS–PAGE and blotted onto a PVDF membrane. Automated

Table 2. Purification of CBH from the culture broth of *F. pinicola* KMJ812.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg-protein)	Yield (%)	Purification fold
Cell extract	1,200	4,590	4	100	1
Ultrafiltration (PES30)	383	2,240	6	32	1.6
Salting out	195	1,760	9	16	2.3
DEAE ion exchange chromatography	76	988	13	6	3.4
Gel filtration-chromatography	10	149	15	1	3.9
MonoQ ion-exchange chromatography	1.3	34	26	0.1	6.8

Edman degradation of the enzyme protein was unsuccessful, implying that the N-terminal of the enzyme was blocked. The CBH was partially digested with trypsin, endoproteinase Asp-N, and endoproteinase Lys-C, and then separated by 12.5% SDS–PAGE and blotted onto a PVDF membrane. Three fragments, a Lys-C fragment (LYS), an Asp-N fragment (ASP), and a trypsin fragment (TRY), were sequenced on an automatic protein sequencer. The LYS fragment contained a RFLEQYDK segment. The TRY and ASP fragments contained GGLEAMGESLDR and TYLMQD, respectively.

Optimum pH and Temperature

The optimum pH for the CBH was 5.0, with 90% and 93% of the maximum activity appearing at pH 4.5 and 5.5, respectively (Fig. 3A). An acidic pH optimum and maximal activity at about pH 4.5 are common features of similar CBH enzymes isolated from diverse microbial systems [25]. The isoelectric point, pI, was determined to be 5.1, which is typical for extracellular CBHs [1]. The optimum temperature for the hydrolysis reaction was 50°C with 80% and 95% of the maximum activity at 45 and 55°C, respectively (Fig. 3B).

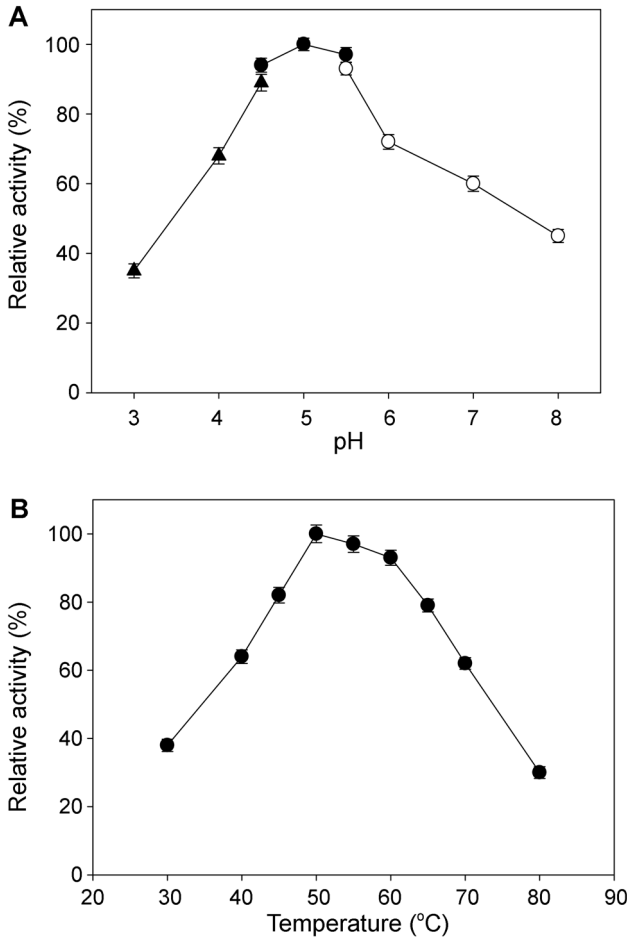

Fig. 3. Effects of pH and temperature on the activity of *F. pinicola* CBH.

A. Effect of pH on the activity of purified CBH from *F. pinicola* KMJ812. The enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pHs. The buffers used were citrate (pH 3.0 to 4.5), sodium acetate (pH 4.5 to 5.5), and phosphate (pH 5.5 to 8.0). **B**. Effect of temperature on the activity of purified CBH from *F. pinicola* KMJ812. The enzyme activity was assayed at various temperatures by the standard assay method. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

Thermostability of *F. pinicola* CBH

The stability of purified CBH was studied at various temperatures from 40°C to 80°C. According to the thermostability assay, the purified *F. pinicola* CBH was highly stable and maintained ~90% activity when incubated at 60°C for 24 h. About 90% activity was maintained after 8 h of incubation at 70°C. At temperatures over 80°C, the CBH activity sharply decreased depending on the incubation time. The enzyme showed $t_{1/2}$ values of 552 h, 144 h, 96 h, and 42 h at 40°C, 50°C, 60°C, and 70°C, respectively (Fig. 4).

Substrate Specificity and Kinetic Parameters of *F. pinicola* CBH

The activities of *F. pinicola* CBH with various substrates are shown in Table 3. Whereas pNPL, Avicel, and lichenan showed an activity of 42.1%, 29.6%, and 33.8%, respectively, the other compounds did not serve as substrates. *F. pinicola* CBH was almost completely inactive with polymeric cellulose and xylan, but had a high preference for pNPC.

Initial velocities were determined in the standard assay mixture at pH 5. All the substrates tested had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear. The concentration of pNPC varied from

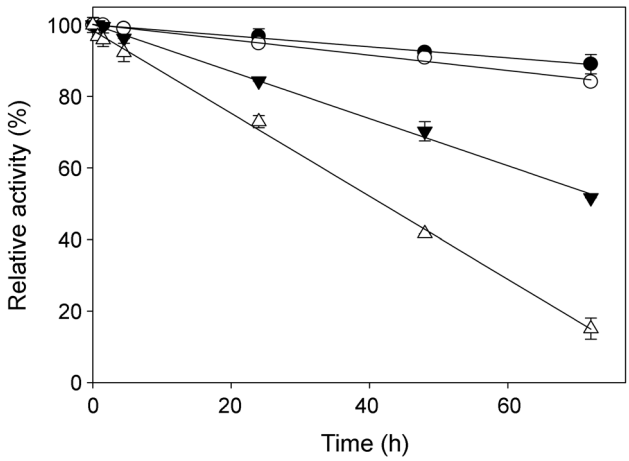

Fig. 4. Thermal inactivation of *F. pinicola* CBH.

The enzymes were incubated at 40°C (filled circles), 50°C (empty circles), 60°C (filled triangles), and 70°C (empty triangles) for varying periods of time. Samples were withdrawn at each time interval and relative activities were determined.

Table 3. Substrate specificity of CBH purified from *F. pinicola* KMJ812.

Substrate (10 mM)	CBH activity (U/mg-protein)	Relative activity (%)
p-Nitrophenyl-β-D-glucopyranoside	0.03	0.11
p-Nitrophenyl-β-D-galactopyranoside	0.07	0.26
p-Nitrophenyl-β-D-cellobioside	26.0	100
p-Nitrophenyl-β-D-mannopyranoside	0.17	0.64
p-Nitrophenyl-β-D-xylopyranoside	0.17	0.64
p-Nitrophenyl-β-D-lactopyranoside	11.2	42.1
Cellobiose	0.03	0.11
Cellotriose	0	0
Cellotetraose	0	0
1% Xylan	0.3	1.13
1% Avicel	7.89	29.6
1% CMC	0.07	0.26
1% Lichenan	9.01	33.8
1% Laminarin	0.4	1.5

The purified enzyme was assayed in the standard assay condition with various compounds. Each value represents the mean of triplicate measurements and varied from the mean by not more than 10%.

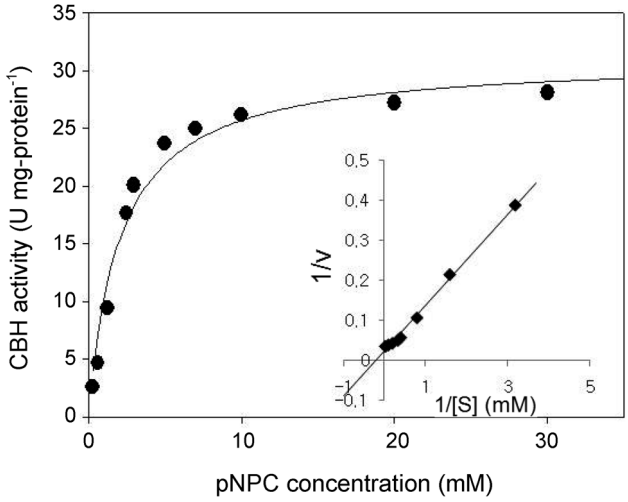

Fig. 5. Effects of substrate concentration on the activities of *F. pinicola* CBH.
The CBH activity of the enzyme (1 unit) was measured in the presence of the indicated concentrations of pNPC, at pH 5.0. The inset shows a Lineweaver–Burk plot of initial velocity versus various fixed pNPC concentrations. Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%.

0 to 50 mM. Fig. 5 shows the typical Michaelis–Menten-type kinetics for CBH activity with increasing pNPC concentrations. The Lineweaver–Burk plot (inset in Fig. 5) obtained for the conversion of pNPC under standard assay conditions showed a K_m of 2.1 mM and a V_{max} of 31.1 mmol/min/mg. Product inhibition studies under nonsaturating conditions showed that cellobiose inhibited CBH competitively, with a K_i value of 14.8 mM.

Effects of Metal Ions and Various Compounds

CBH activity was measured in the presence of metal ions or various other compounds (data not shown). The CBH activity was not stimulated by $BaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, $NiCl_2$, or $CoCl_2$ (each at 1 mM concentration), and it was neither inhibited nor activated by EDTA at concentrations ranging from 1 to 10 mM. In contrast, Hg^{2+} ions caused significant inhibition (60%) that appeared to be competitive with respect to the substrate pNPC. *N*-Ethylmaleimide (NEM) is often used to inactivate enzymes, presumably by reacting with the thiol group of cysteine residues. Incubation of purified CBH with 10 mM NEM resulted in an approximately 30% inhibition of CBH activity, suggesting that cysteine is at least partly responsible for CBH activity. The time required to reach half-maximal inhibition ($t_{1/2}$) with NEM was approximately 6.5 min.

Discussion

Although the purification and properties of CBH from several strains have been reported, this is the first report on the purification and characterization of CBH from *F. pinicola*, a basidomycete. The present study reveals a unique *F. pinicola* CBH showing one of the highest CBH activities ever reported. Table 4 shows a comparison of the properties of various CBHs from a number of different sources [2, 7, 13, 15, 21, 26, 31, 38]. In comparison, the K_m values of purified CBHs for pNPC from other fungi range from 0.58 to 6.8. Thus, the K_m value (2.1 mM) of *F. pinicola* CBH was in agreement with those recorded for CBHs from other fungi. However, the catalytic efficiency value (k_{cat}/K_m=15.8 $mM^{-1}s^{-1}$) of *F. pinicola* CBH was significantly higher than other CBHs from *Trichoderma reesei* (0.045 $mM^{-1}s^{-1}$), *Talaromyces emersonii* (2.52 $mM^{-1}s^{-1}$), *Chrysosporium lucknowense* (0.17 $mM^{-1}s^{-1}$), *Cellulomonas fimi* (8.0 $mM^{-1}s^{-1}$), *etc*. The extracellular CBH purified from *F. pinicola* was a monomer with a molecular mass of 64 kDa. The molecular mass of this CBH is in agreement with those of many extracellular CBHs characterized from other fungal sources.

CBH 1A of *Talaromyces emersonii*, a moderately thermophilic fungus, showed a $t_{1/2}$ value of 0.57 h at 80°C [38]. *Trichoderma reesei* CBH, despite showing an optimum temperature of 70°C, had a $t_{1/2}$ at 65°C of less than 1 h [28]. The $t_{1/2}$ value (42 h at 70°C) of *F. pinicola* CBH was higher than other CBHs from *Chaetomium thermophilus* CT2 (1 h at 70°C), *Penicillium janthinellum* (0.67 h at 65 °C), *Trametes versicolor* (0.5 h at 60 °C), *etc*. Thus, *F. pinicola* CBH shows one of the highest thermostabilities among the CBHs isolated thus far from fungi, which suggests that it has potential industrial uses.

Table 4. Properties of CBHs from various sources.

Microorganism	M_r (kDa)	Quaternary structure	K_m (mM)	Opt. pH	Opt. temp (°C)	Half-life ($t_{1/2}$)	Specific activity (U/mg)	Reference
Dichomitus squalens Ex-1	39	Monomer	NR	5.0	60	NR	13.6	[34]
Irpex lacteus Ex-1	53	NR	NR	5.0	50	NR	33.2	[15]
Irpex lacteus Ex-2	56	NR	NR	5.0	50	NR	34.0	[15]
Chrysosporium lucknowense CBH IA	65	Monomer	NR	4.5	NR	5 h at 50°C (>90% retained)	0.02	[13]
Chrysosporium lucknowense CBH IB	60	Monomer	NR	NR	NR	5 h at 50°C (>90% retained)	0.02	[12]
Chrysosporium lucknowense CBH IIA	43	Monomer	NR	5.5	65	5 h at 50°C (>90% retained)	0.08[b]	[6]
Trametes versicolor	55	Monomer	0.58	5.0	40	0.5 h at 60°C	1.0	[23]
Penicillium occitanis CBH I	60	NR	1[a]	4.0–5.0	60	0.5 h at 60°C	1.09	[26]
Penicillium occitanis CBHII	55	NR	5[a]	4.0–5.0	65	0.5 h at 60°C	0.03	[26]
Talaromyces emersonii CBHIA	66	Monomer	2.1	3.6	78	0.57 h at 80°C	7.7	[38]
Chaetomium thermophilus CT2	67	Monomer	0.95	5.0	65	1 h at 70°C	NR	[28]
Penicillium janthinellum	57	Monomer	0.82	5.0	50	0.67 h at 65°C	0.07	[20]
Coniophora puteana CBH I	52	NR	6.8	5.0	40	NR	0.46	[35]
Coniophora puteana CBH II	50	NR	4.3	5.0	40	NR	0.4	[35]
Fomitopsis pinicola	64	Monomer	2.1	5.0	50	42 h at 70°C	26.0	This work

NR, not reported.
[a]Kinetic parameters of CBHs are shown for pNPC.
[b]Avicel as a substrate.

Based on amino acid sequence similarities, GHs have been classified into several families, with most CBHs belonging to either family 6, family 7, or family 48 [14, 16–18, 24]. Of these families, only GH7 is thought to be exclusively fungal, and this family contains CBH I cellobiohydrolases and EG I endoglucanases from both ascomycete and basidiomycete fungi [10]. The three-dimensional structures of GH7 CBH catalytic modules from mesophilic fungi *Trichoderma reesei* [9] and *Phanerochaete chrysosporium* [30], from a thermophilic fungus *Talaromyces emersonii* [11], and *Melanocarpus albomyces* [32] have been solved. The overall fold is a β-sandwich, where loops extending from the β-sandwich form an enclosed cellulose-binding tunnel. CBHs of *P. chrysosporium* have a GGLKQMGE sequence, similar to the *F. pinicola* fragment GGLEAMGE. The peptide fragments of *F. pinicola* CBH, RFLEQYDK and GGLEAMGESLDR, are identical to those of the *celA* (*Cellulomonas flavigena* DSM 20109) and *cel3A* (*Schizophyllum commune*) CBHs belonging to GH7, respectively.

In conclusion, a potent CBH-producing *F. pinicola* strain was identified. *F. pinicola* CBH possesses the highest thermostability and catalytic efficiency among the CBHs reported. The successful purification and characterization of CBH produced by *F. pinicola* allows us to characterize a novel CBH and now sets the stage for more detailed investigations of this enzyme, such as cloning of the full-length gene followed by protein engineering studies.

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