Characterization of Cellobiohydrolase from a Newly Isolated Strain of *Agaricus arvencis*

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A highly efficient cellobiohydrolase (CBH)-secreting basidiomycetous fungus, *Agaricus arvensis* KMJ623, was isolated and identified based on its morphological features and sequence analysis of internal transcribed spacer rDNA. An extracellular CBH was purified to homogeneity from *A. arvencis* culture supernatant using sequential chromatography. The relative molecular mass of *A. arvencis* CBH was determined to be 65 kDa by SDS–PAGE and 130 kDa by size-exclusion chromatography, indicating that the enzyme is a dimer. *A. arvencis* CBH showed a catalytic efficiency (k_{cat}/K_m) of 31.8 mM^{-1} s^{-1} for *p*-nitrophenyl-β-D-cellobioside, the highest level seen for CBH-producing microorganisms. Its internal amino acid sequences showed significant homology with CBHs from glycoside hydrolase family 7. Although CBHs have been purified and characterized from other sources, *A. arvencis* CBH is distinguished from other CBHs by its high catalytic efficiency.

Keywords: Catalytic efficiency, cellobiohydrolase, enzyme production, *Agaricus arvensis*, glycoside hydrolase

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.* [25]. 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) [2]. EG and CBH act cooperatively or synergistically in depolymerizing cellulose to cellobiose and short oligosaccharides, which are converted by BGL to glucose [4]. CBHs are most efficient on highly ordered crystalline cellulose and cleave mainly cellobiose from the opposite ends of the glucose chains, whereas EGs act more randomly in the middle of the chains, probably in the more amorphous regions of cellulose [30].

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 are important components in the multienzyme cellulase complexes [29] 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 several glycoside hydrolase families (GH5, GH6, GH7, and GH48) according to amino acid sequence similarity [9, 11−13, 18]. Of these families, only GH7 is thought to be exclusively fungal, and this family contains CBHIs and EGs from both ascomycete and basidiomycete fungi [7]. Potent cellulolytic fungi generally produce two different CBHs: CBHI and CBHII. These two types of enzymes, which are classified based on sequence identity, have extended tunnel-shaped active sites and can achieve complete, although slow, solubilization of cellulose crystals even without help of EGs [29].

White-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 [15]. In the present study, a potent CBH-producing fungus, *Agaricus arvencis*, was isolated and identified. Under optimized conditions, *A. arvencis* produced CBH with a specific

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activity of 3.0 U/mg-protein. Here, we purified a novel CBH from the isolated *A. arvencis* to 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

Isolation of Microorganism

The soil samples collected from Sorak Mountain (South Korea) by the capillary tube method were diluted in sterile dilution solution (0.9% saline), aliquots were spread on potato dextrose agar plate, and the plates were incubated for 3 days. Initial screening of CBH-producing fungi was carried out in agar plates containing 0.5% CMC. Based on the zone of clearance observed by Congo red staining, strains were selected to analyze CBH activity. The different colonies were inoculated into 3 ml of the growth medium containing (g/l) peptone (8), yeast extract (2), KH_2PO_4 (5), K_2HPO_4 (5), $MgSO_4 \cdot 7H_2O$ (3), thiamine·HCl (0.005), and microcrystalline cellulose (20) (Sigma), and cultivated at 28°C with agitation at 200 rpm for 5 days. The CBH activity of the culture broth was analyzed using *p*-nitrophenyl-D-cellobiopyranoside (pNPC; Sigma) as described previously [25]. One unit of pNPC-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 µmol of *p*-nitrophenol per minute. After analyses, the strain with the highest CBH activity was selected.

Identification of Microorganism

The Fatty acid composition was analyzed by gas chromatography (Agilent 6890N, CA, USA) and the identification of the isolated strain was determined using the MIDI database. For the sequence analysis, the ITS1-5.8 S-ITS2 rDNA region of the fungus was amplified by PCR using the primer set pITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and pITS4 (5'-TCCTCCGCTTATTGATATGC-3') [32]. The 604 bp amplicon thus obtained was cloned and sequenced. The sequences were proofread, edited, and merged into composite sequences using the PHYDIT program (version 3.1).

Culture Conditions

For flask culture, the mycelia of *A. arvencis* were inoculated into 100 ml of potato dextrose broth. Precultures (100 ml) were inoculated into 4 l of cellulolytic medium in a 7 l fermenter. This culture media contained (g/l) tryptone (40), KH_2PO_4 (20), K_2HPO_4 (20), $MgSO_4 \cdot 7H_2O$ (12), thiamine·HCl (0.005), and Rice straw (40). The CBH production was investigated after 7 days of cultivation. The dry cell weight (DCW) was estimated from a calibration curve derived from the relationship between absorbance at 600 nm and dry cell weight.

Enzyme Assay

CBH activity was assayed using pNPC (Sigma) as the 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. The amount of *p*-nitrophenol released was measured at 415 nm after addition of 2 M Na_2CO_3 to the reaction mixtures [5]. One unit of pNPC-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 µmol 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 was measured by the the Bradford method [3], using bovine serum albumin as a standard. Protein in the column effluents was monitored by measuring the absorbance at 280 nm. All chromatographic separations were performed using a Biologic FPLC system (Bio-Rad, USA).

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: Hydroxyapatite chromatography. The dialyzed enzyme solution was loaded on a hydroxyapatite column (1.0×10 cm; Amersham Biosciences) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.0). The enzyme was eluted with a 150 min linear gradient of 0−0.5 M sodium phosphate buffer at a flow rate of 0.5 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 with ultrafiltration for further purification.

Step 3: Superdex-200 gel filtration chromatography. The concentrated enzyme solution was loaded on a HiPrep 16/60 Superdex-200 column (1.0 cm×120 cm; Amersham Biosciences) 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 with an 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 3.0−4.5), sodium acetate (100 mM, pH 4.5−6.0), phosphate (100 mM, pH 6.0−8.0). To determine the optimal temperature, the enzyme was incubated in sodium acetate buffer (100 mM, pH 5) for 15 min at different temperatures: from 30 to 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 h), 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 by Laemmli [16]. Protein bands were visualized with Coomassie brilliant blue R-250 (Sigma). The molecular mass of the purified enzyme was determined by size-exclusion chromatography using a Superdex-200pg (Amersham Pharmacia Biotech, Uppsala, Sweden) column attached to a Biologic FPLC system (Bio-Rad). The enzyme was eluted with 50 mM phosphate buffer with 150 mM NaCl (pH 7.0) at a flow rate of 1 ml/min.

Substrate Specificity

The substrate specificity of CBH was determined by using *p*-nitrophenyl-β-D-cellobioside (pNPC), *o*-nitrophenyl-β-D-cellobioside (oNPC), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *o*-nitrophenyl-β-D-glucopyranoside (oNPG), *p*NP-β-galactopyranoside (pNPgal), *p*NP-β-D-mannopyranoside (pNPM), *p*NP-β-D-lactopyranoside (pNPL), cellobiose, cellotriose, and cellotetraose as substrates at 10 mM concentration. CBH activities on polysaccharides carboxymethylcellulose (CMC) 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 [22]. The activities on polysaccharides were estimated by measuring the reducing sugars released by the DNS method [23] using glucose 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 5.0, at 50°C with pNPC at concentrations ranging from 0.5 to 20 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 and reagents at 1 mM on CBH activity were determined by preincubating the enzyme with the individual reagents in 10 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

The purified protein was resolved by SDS−PAGE and then electroblotted onto a polyvinylidene trifluoride membrane (Bio-Rad). 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 (12.5% polyacrylamide), and the separated peptides were transferred to a polyvinylidene trifluoride membrane by electroblotting. Peptide bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 (Sigma). 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.

RESULTS

Strain Isolation and Identification

Among the 137 fungal strains that were screened for the cellulase activity, 19 strains were selected based on the clearance zone that was observed using the Congo red staining method [26]. Out of these 19 strains, an efficient
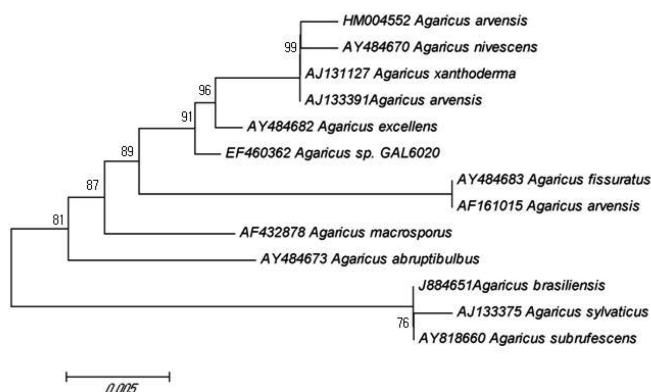

Fig. 1. The phylogenetic dendrogram for *A. arvensis* KMJ623.
The phylogenetic dendrogram for *A. arvensis* KMJ623 (HM004552) and related strains based on the ITS rDNA sequence. Numbers following the names of the strains are accession numbers of published sequences.

CBH-producing isolate, KMJ623, was selected for further studies based on its efficiency for producing the highest level of CBH activity. The ITS rDNA gene of the isolated fungus was sequenced, and the isolated strain exhibited the highest identity (99%) with *A. arvensis*. The phylogenetic relationships were inferred through the alignment and cladistic analysis of the homologous nucleotide sequences of the known microorganisms. The isolated fungus and *A. arvensis* belonged to the same branch with a 99% probability (Fig. 1). The isolated fungus was characterized by a cream-colored cap, free blackish gills, pale yellow pilei, and an umbonate center. The spores are elliptical and smooth with dimensions of 7−9×4.5−6 μm. Additionally, the isolated strain had the same cellular fatty acid composition (data not shown) as that of *A. arvensis* [1]. Based on the morphology, fatty acid composition, and rDNA gene sequence, the isolated strain was identified as a strain of *A. arvensis* and was named *A. arvensis* KMJ623. The sequence was submitted to the GenBank with Accession No. HM004552. Pairwise evolutionary distances and a phylogenetic tree were constructed with the MegAlign software (DNA Star, Madison, WI, USA). The identified strain *A. arvensis* KMJ623 was deposited at the Korean Culture Center of Microorganisms (KCCM, Accession No. 11246P).

Effects of Carbon and Nitrogen Sources on CBH Production

To select a suitable carbon source for CBH production, *A. arvensis* KMJ623 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 2.2 U/mg-protein in a flask culture.

Rice straw (20 g/l) was used as the carbon source. Among the various nitrogen sources (peptone, corn steep powder,
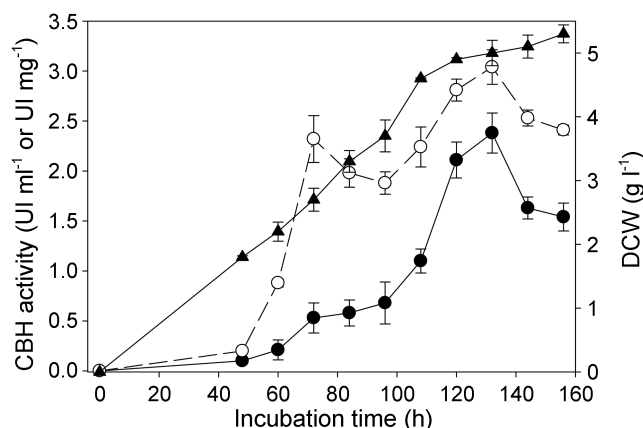
Fig. 2. Profiles of cell growth and cellobiohydrolase production by *A. arvencis* on rice straw.
DCW (g/l), filled triangle), total activity (U/ml, filled circle), specific activity (U/mg-protein, empty circle).

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 (3.0 U/mg-protein), followed by yeast extract, whereas other nitrogen sources were poor sources of nitrogen in the absence of pH control. The time course of CBH production by *A. arvensis* grown on rice straw, yeast extract, and peptone was studied (Fig. 2). A maximum specific activity of 3.0 U/mg-protein was obtained after 6 days of growth. Then the activity declined gradually.

Purification of a CBH from *A. arvensis* Culture

CBH was purified as described in the Materials and Methods section. Fractionation by ultrafiltration with a molecular mass cutoff of 30 kDa increased the specific activity about 1.1-fold, with 93.3% recovery of CBH activity. The active fractions were applied to a hydroxyapatite column, and CBH was eluted with approximately 0.5 M sodium phosphate buffer, resulting in 11.1-fold purification of CBH with a recovery of 15.4%. A subsequent gel filtration chromatography produced four peaks containing protein; the second peak showed CBH activity. These chromatography methods resulted in a 21.6-fold purification of CBH with a recovery of 1.1%. Analysis of the purified enzyme by gel electrophoresis in the presence of SDS (Fig. 3A, lane 4) revealed one band with a M_r of 64,000±5,000. Size-exclusion chromatography on a Superdex-200 prep grade column resulted in the elution of the enzyme activity as a symmetrical peak corresponding to a M_r of approximately 130,000 (Fig. 3B). These results indicate that the enzyme migrated as a dimer in gel filtration under the mild conditions used.

Identification of the Partial Peptide Fragment

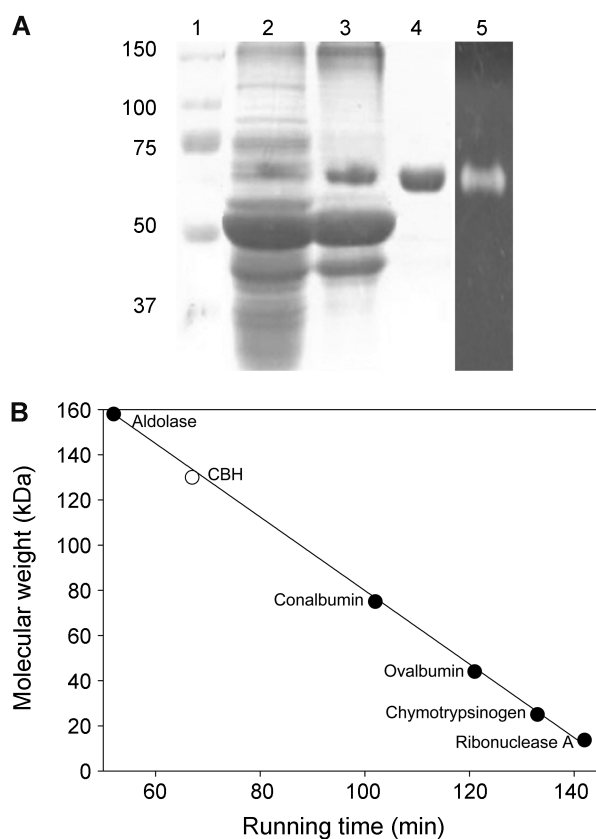The pure enzyme (1.5 μg) was separated by 10% SDS–PAGE and blotted onto a polyvinylidene fluoride (PVDF)

Fig. 3. PAGE and determination of molecular mass of CBH purified from the *A. arvencis*.
A. PAGE of CBH purified from *A. arvencis*. Lane 1, molecular marker; lane 2, cell extract; lane 3, hydroxyapatite fraction; lane 4, gel filtration fraction; lane 5, zymogram activity staining. **B**. Determination of native molecular mass of *A. arvencis* CBH by gel filtration chromatography on a Superdex-200 prep grade column. The column was calibrated with standard molecular weight proteins such as aldolase (168 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

membrane. Automated 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. The fragments were sequenced on an automatic protein sequencer and found to contain CGLNGALYF, KYGTGYCD and DIKFINGEAN.

Optimum pH and Temperature

The optimum pH for the CBH was 4.0, with 94% and 92% of the maximum activity appearing at pH 3.5 and 3.0, respectively (Fig. 4A). An acidic pH optimum and maximal activity at about pH 4.0–4.5 are common features of similar CBH enzymes isolated from diverse microbial systems [20]. The optimum temperature for the hydrolysis reaction was 65°C with 76% and 58% of the maximum activity observed at 60 and 70°C, respectively (Fig. 4B).
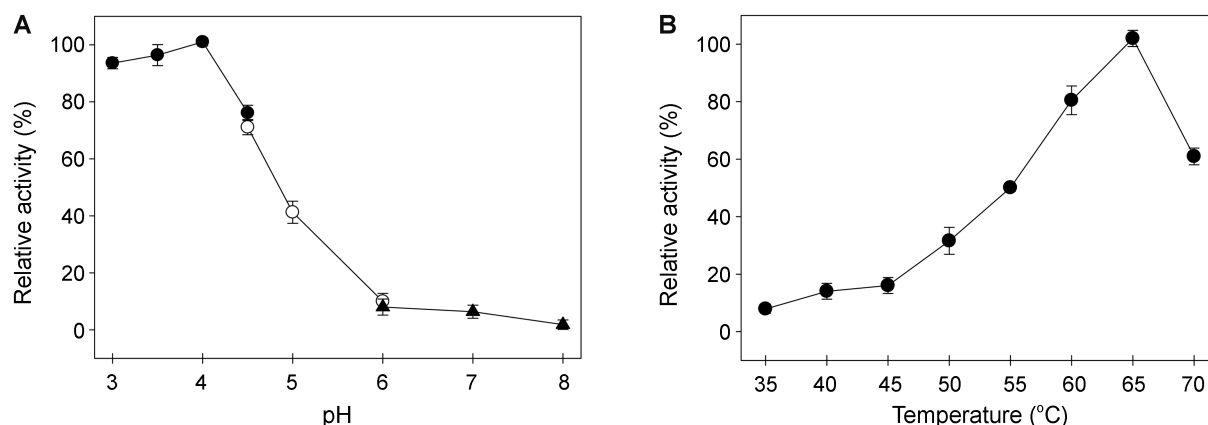
Fig. 4. Effects of pH and temperature on the activity of *A. arvencis* CBH.
A. Effect of pH on the activity of purified CBH from *A. arvencis*. The enzyme activity was assayed by the standard assay method by changing the buffer to obtain the desired pHs. The buffers used were citrate (pH 3.0 to 4.5), sodium acetate (pH 4.5 to 6.0), and phosphate (pH 6.0 to 8.0). **B**. Effect of temperature on the activity of purified CBH from *A. arvencis*. 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%.

The stability of purified CBH was studied at various temperatures from 40°C to 75°C. According to the thermostability assay, the purified *A. arvencis* CBH lost ~50% activity when incubated at 60°C for 25 min. About 90% activity was lost after 10 min incubation at 70°C. At temperatures over 50°C, the CBH activity sharply decreased depending on the incubation time. The enzyme showed $t_{1/2}$ values of 120 min, 65 min, 23 min, and 12 min at 40°C, 50°C, 60°C, and 70°C, respectively.

Substrate Specificity and Kinetic Parameters of *A. arvencis* CBH

The activities of *A. arvencis* CBH with various substrates are shown in Table 1. Whereas oNPC, pNPL, Avicel,

lichenan (β-1,3-1,4-glucan), and laminarin (β-1,3-glucan) showed an activity of 30.2%, 19.8%, 11.4%, 9.2%, and 3.1%, respectively, other compounds did not serve as substrates. In comparison with barley β-glucan, the *A. arvencis* CBH showed little activity for β-1,3-glucan. Initial velocities were determined in the standard assay mixture at pH 4. All the substrates tested had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear. Fig. 5 shows 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 2.27 mM and V_{max} of 67.8 μmol min^{-1} mg-protein^{-1}. *A. arvencis* CBH showed a

Table 1. Substrate specificity of CBH purified from *A. arvencis*.

Substrate (10 mM)	CBH activity (U/mg-protein)	Relative activity (%)
p-Nitrophenyl-β-D-glucopyranoside	0.05	0.11
o-Nitrophenyl-β-D-glucopyranoside	0.02	0.05
p-Nitrophenyl-β-D-galactopyranoside	0.11	0.26
p-Nitrophenyl-β-D-cellobioside	41.7	100.0
o-Nitrophenyl-β-D-cellobioside	12.8	30.2
p-Nitrophenyl-β-D-mannopyranoside	0.27	0.64
p-Nitrophenyl-β-D-lactopyranoside	8.27	19.8
p-Nitrophenyl-β-D-xylopyranoside	-	-
Cellobiose	0.05	0.12
Cellotriose	0.56	1.34
Cellotetraose	0.72	1.73
1% Avicel	4.77	11.4
1% CMC	1.18	2.83
1% Laminarin	1.31	3.14
1% Lichenan	3.84	9.21
1% Xylan	-	-

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%.
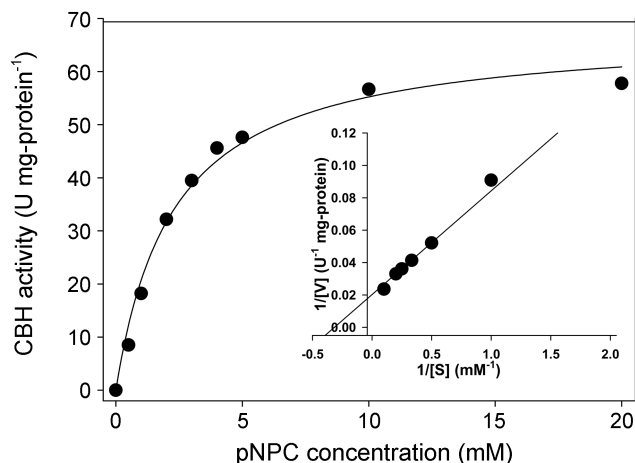
Fig. 5. Effects of substrate concentration on the activity of *A. arvencis* CBH.

The CBH activity of the enzyme 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%.

catalytic efficiency (k_{cat}/K_m) of 31.8 mM^{-1} s^{-1} for pNPC. Product inhibition studies under nonsaturating conditions showed that cellobiose inhibited CBH competitively, with a K_i value of 21.1 mM (Fig. 6).

Effects of Metal Ions and Various Compounds

The CBH activity was measured in the presence of metal ions or various other compounds. The CBH activity was stimulated (approximately 30–40%) by $CaCl_2$, $CuCl_2$, $MgCl_2$ (each at 1 mM concentration), and it was neither inhibited nor activated by EDTA, $CoCl_2$, $HgCl_2$, $MnCl_2$, glycerol, and L-cysteine at concentrations ranging from 1 to 10 mM. In contrast, Zn^{2+} ions caused significant inhibition (50%) that appeared to be competitive with respect to the substrate pNPC. The addition of 0.5 mM β-mercaptoethanol, a sulfhydryl compound, to the reaction mixture increased the CBH activity by approximately 20%.

DISCUSSION

The present study reveals a unique *A. arvencis* CBH showing the highest CBH activity ever reported. 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 *A. arvencis*. Table 2 shows a comparison of the properties of various CBHs from a number of different sources. The purified CBH showed maximum activity at a pH and temperature of 4–4.5 and 65°C, respectively, which are similar to those of other fungal CBHs [21]. In comparison, the K_m values of the purified CBHs for pNPC from other fungi ranged
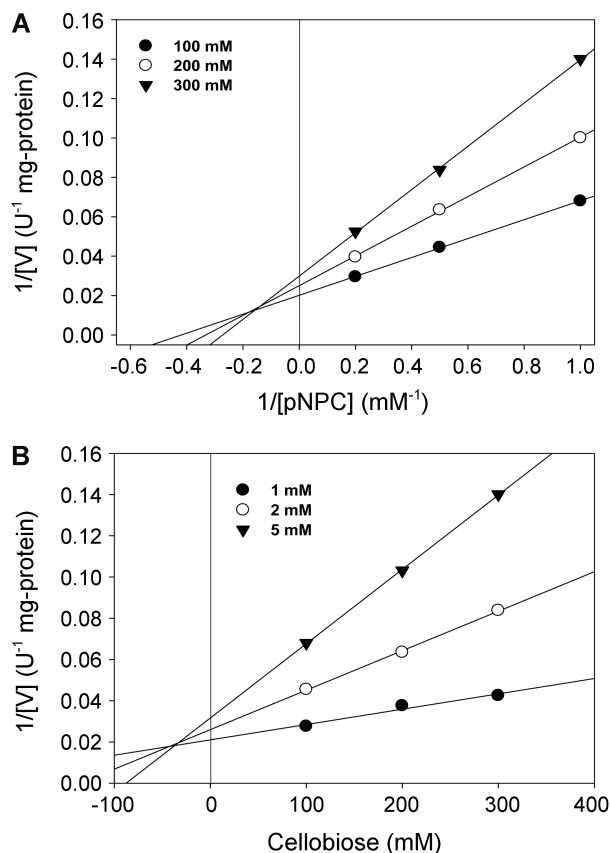

Fig. 6. Graphical analysis of the inhibition of *A. arvencis* CBH by cellobiose.

A. Lineweaver–Burk plot of initial velocity versus various fixed substrate concentrations showing inhibitory effects of cellobiose [100 mM (●), 200 mM (○), and 300 mM (▲)] on pNPC hydrolysis by *A. arvencis* CBH. **B**. The secondary plot for competitive inhibition with pNPC [1 mM (●), 2 mM (○), and 5 mM (▼)] is shown. The product binds to CBH with a K_i of 21 mM.

from 0.58 to 6.8 mM. Thus, the K_m value (2.27 mM) of *A. arvencis* CBH was in agreement with those recorded for CBHs from other fungi. However, the catalytic efficiency value (k_{cat}/K_m=31.8 mM^{-1}s^{-1}) of *A. arvencis* CBH was significantly higher than other CBHs from *Trametes versicolor* (0.053 mM^{-1}s^{-1}), *Talaromyces emersonii* (4.82 mM^{-1}s^{-1}), *Penicillium janthinellum* (0.08 mM^{-1}s^{-1}), *Penicillium occitanis* (1.09 mM^{-1}s^{-1}), *etc.* [6, 8, 10, 15, 17, 19, 21, 26, 28, 31].

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. The peptide fragments of *A. arvencis* CBH, CGLNGALYF and DIKFINGEAN, are identical to those of the CBHs from *Polyporus arcularius* and *Schizophyllum commune* belonging to GH7, respectively [7, 14, 25]. The evidence from enzymology and bioinformatics experiments strongly suggests that *A. arvencis* CBH should be classified as a member of GH7. In a simultaneous saccharification and fermentation (SSF)

Table 2. Properties of CBHs from various sources.

Microorganism	M_r (kDa)	Quaternary structure	K_m (mM)	Opt. pH	Opt. temp (°C)	k_{cat}/K_m (mM^{-1}s^{-1})	Specific activity (U/mg)	Reference
Duchomitus squalens Ex-1	39	Monomer	NR	5.0	60	NR	13.6	[27]
Irpex lacteus Ex-1	53	NR	NR	5.0	50	NR	33.2	[10]
Irpex lacteus Ex-2	56	NR	NR	5.0	50	NR	34.0	[10]
Chrysosporium lucknowense CBH IA	65	Monomer	NR	4.5	NR	NR	0.02	[8]
Chrysosporium lucknowense CBH IB	60	Monomer	NR	NR	NR	NR	0.02	[8]
Chrysosporium lucknowense CBH IIA	43	Monomer	NR	5.5	65	NR	0.08[a]	[8]
Trametes versicolor	55	Monomer	0.58	5.0	40	0.53	1.0	[17]
Penicillium occitanis CBH I	60	NR	1	4.0−5.0	60	1.09	1.09	[21]
Penicillium occitanis CBHII	55	NR	5	4.0−5.0	65	0.05	0.03	[21]
Talaromyces emersonii CBHIA	66	Monomer	2.1	3.6	78	4.82	7.7	[31]
Chaetomium thermophilus CT2	67	Monomer	0.95	5.0	65	NR	NR	[19]
Penicillium janthinellum	57	Monomer	0.82	5.0	50	0.08	0.07	[15]
Coniophora puteana CBH I	52	NR	6.8	5.0	40	0.06	0.46	[28]
Coniophora puteana CBH II	50	NR	4.3	5.0	40	0.08	0.4	[28]
Agaricus arvencis	65	Dimer	2.3	4.0	65	31.8	41.7	This work

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

process, the ideal CBH should be one that is stable and that is active at low pH [6]. Since *A. arvencis* CBH is stable at low pH 3.0−4.0, showing only 5~8% of CBH activity loss, it appears to be a good candidate for enhancing enzymatic hydrolysis and fermentation in SSF processing.

In conclusion, a potent CBH from *A. arvencis* culture was purified and characterized. *A. arvencis* CBH possesses the highest catalytic efficiency among the CBHs reported. The successful purification and characterization of CBH produced by *A. arvencis* allows us to characterize a novel CBH and now sets the stage for more detailed investigations of this CBH, such as cloning of the full-length gene followed by protein engineering studies.

Acknowledgment

REFERENCES

1. Barros, L., P. Baptista, D. M. Correia, S. Casal, B. Oliveira, and I. C. F. R. Ferreira. 2007. Fatty acid and sugar compositions and nutritional value of five wild edible mushrooms. *Food Chem.* **105:** 140−145.
2. Bayer, E. A., H. Chanzy, R. Lamed, and Y. Shoham. 1998. Cellulose, cellulases and cellulosomes. *Curr. Opin. Struct. Biol.* **8:** 548−557.
3. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248−254.
4. Bukhtojarov, F. E., B. B. Ustinov, T. N. Salanovich, A. I. Antonov, A. V. Gusakov, O. N. Okunev, and A. P. Sinitsyn. 2004. Cellulase complex of the fungus *Chrysosporium lucknowense*: Isolation and characterization of endoglucanases and cellobiohydrolases. *Biochemistry* **69:** 542−551.
5. Deshpande, M. V., K. E. Eriksson, and L. G. Pettersson. 1984. An assay for selective determination of exo-1,4-b-glucanases in a mixture of cellulolytic enzymes. *Anal. Biochem.* **138:** 481−487.
6. Doran-Peterson, J., A. Jangid, S. K. Brandon, E. DeCrescenzo-Henriksen, B. Dien, and L. O. Ingram. 2009. Simultaneous saccharification and fermentation and partial saccharification and co-fermentation of lignocellulosic biomass for ethanol production. *Methods Mol. Biol.* **581:** 263−280.
7. Edwards, I. P., R. A. Upchurch, and R. Z. Donald. 2008. A classification of glycosyl hydrolases based on amino acid sequence similarity. *Appl. Environ. Microbiol. 74.* **74:** 3481−3489.
8. Gusakov, A. V., A. P. Sinitsyn, T. N. Salanovich, F. E. Bukhtojarov, A. V. Markov, B. B. Ustinov, C. Van Zeijl, P. Punt, and R. Burlingame. 2005. Purification, cloning and characterization of two forms of thermostable and highly active cellobiohydrolase I (Cel7A) produced by the industrial strain of *Chrysosporium lucknowense. Enzyme Microb. Technol.* **36:** 57−69.
9. Haakana, H., A. Miettinen-Oinonen, V. Joutsjoki, A. Mantyla, P. Suominen, and J. Vehmaanpera. 2004. Cloning of cellulose genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei. Enzyme Microb. Technol.* **34:** 159−167.
10. Hamada, N., K. Ishikawa, N. Fuse, R. Kodaira, M. Shimosaka, Y. Amano, T. Kanda, and M. Okazaki. 1999. Purification,

characterization and gene analysis of exo-cellulase II (Ex-2) from the white rot basidiomycete *Irpex lacteus*. *J. Biosci. Bioeng.* **87:** 442−451.

11. Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochem. J.* **280:** 309−316.

12. Hong, J., H. Tamaki, K. Yamamoto, and H. Kumagai. 2003. Cloning of a gene encoding thermostable cellobiohydrolase from *Thermoascus aurantiacus* and its expression in yeast. *Appl. Microbiol. Biotechnol.* **63:** 42−50.

13. Jia, J., P. S. Dyer, and J. F. Buswell. 1999. Cloning of the CBHI and CBHII genes involved in cellulose utilization by the straw mushroom *Volvariella volvacea*. *Mol. Gen. Genet.* **261:** 985−993.

14. Kajisa, T., K. Igarahi, and M. Samejima. 2009. The genes encoding glycoside hydrolase family 6 and 7 cellulases from the brown-rot fungus *Coniphora puteana*. *J. Wood Sci.* **55:** 376−380.

15. Koch, A., C. T. Weigel, and G. Schulz. 1993. Cloning, sequencing, and heterologous expression of a cellulase-encoding cDNA (*cbh1*) from *Penicillium janthinellum*. *Gene* **124:** 57−65.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680−685.

17. Lahjouji, K., R. Storms, Z. Xiao, K. B. Joung, Y. Zheng, J. Powlowski, A. Tsang, and L. Varin. 2007. Biochemical and molecular characterization of a cellobiohydrolase from *Trametes versicolor*. *Appl. Microbiol. Biotechnol.* **75:** 337−346.

18. Lee, C. C., D. W. Wong, and G. H. Robertson. 2001. Cloning and characterization of two cellulase genes from *Lentinula edodes*. *FEMS Microbiol. Lett.* **205:** 355−360.

19. Li, Y. L., D. C. Li, and F. C. Teng. 2006. Purification and characterization of a cellobiohydrolase from the thermophilic fungus *Chaetomium thermophilus* CT2. *Wei Sheng Wu Xue Bao* **46:** 143−146.

20. Liete, R. S. R., E. Gomes, and R. Da-Silva. 2007. Characterization of β-glucosidases from a mesophilic *Aureobasidium pullulana* and thermophilic *Thermoascus aurantiacus*. *Process Biochem.* **42:** 1101−1106.

21. Limam, F., S. E. Chaabouni, R. Ghrir, and N. Marzouki. 1995. Two cellobiohydrolases of *Penicillium occitanis* mutant Pol 6: Purification and properties. *Enzyme Microb. Technol.* **17:** 340−346.

22. Lin, J., B. Pillay, and S. Singh. 1999. Purification and biochemical characterization of β-glucosidase from a thermophilic fungus, *Thermomyces lanuginosus*. *Biotechnol. Appl. Biochem.* **30:** 81−87.

23. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426−428.

24. Ohnishi, Y., M. Nagase, T. Ichiyanagi, Y. Kitamoto, and T. Aimi. 2007. Transcriptional regulation of two cellobiohydrolase encoding genes (*cel1* and *cel2*) from the wood-degrading basidiomycete *Polyporus arcularius*. *Appl. Microbiol. Biotechnol.* **76:** 1069−1078.

25. Percival Zhang, Y. H., M. E. Himmel, and J. R. Mielenz. 2006. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnol. Adv.* **24:** 452−481.

26. Wolfe, R. S., R. K. Thauer, and N. Pfennig. 1987. A capillary racetrack method for isolation of magnetotactic bacteria. *FEMS Microbiol. Lett.* **45:** 31−35.

27. Rouau, X. and E. Odier. 1986. Purification and properties of 2 enzymes from *Duchomitus squalens* which exhibit both cellobiohydrolase and xylanase activity. *Carbohydr. Res.* **145:** 279−292.

28. Schmidhalter, D. R. and G. Canevascini. 1993. Purification and characterization of two exo-cellobiohydrolases from the brown-rot fungus *Coniophora puteana* (Schum ex Fr) Karst. *Arch. Biochem. Biophys.* **300:** 551−558.

29. Teeri, T. T. 1997. Crystalline cellulose degradation: New insight into the function of cello-biohydrolases. *Trends Biotechnol.* **15:** 160−167.

30. Teeri, T. T., P. Lehtovaara, S. Kauppinen, I. Salovuori, and J. Knowles. 1987. Homologous domains in *Trichoderma reesei* cellulolytic enzymes: Gene sequence and expression of cellobiohydrolase II. *Gene* **51:** 43−52.

31. Tuohy, M. G., D. J. Walsh, P. G. Murray, M. Claeyssens, M. M. Cuffe, A. V. Savage, and M. P. Coughlan. 2002. Kinetic parameters and mode of action of the cellobiohydrolases produced by *Talaromyces emersonii*. *Biochim. Biophys. Acta* **1596:** 366−380.

32. White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315−322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). *PCR Protocols: A Guide to Methods and Applications* Academic Press, Inc., New York, NY.