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ORIGINAL ARTICLE

Purification and Characterization of a Novel β-1,3/1,4-glucanase from *Sistotrema brinkmannii* HQ717718

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Abstract A highly efficient extracellular β-1,3/1,4-glucanase was purified from the culture broth of *Sistotrema brinkmannii* HQ717718. The molecular mass of β-1,3/1,4-glucanase was respectively determined to be 83 and 166 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography, indicating that the enzyme is a dimer. The optimum activity of β-1,3/1,4-glucanase against Avicel was observed at pH 4.0 and 65°C. Under the same conditions, V_{max}, K_m, and k_{cat} values for Avicel were 136.5 U · mg^{-1} of protein, 3.8 mM, and 211 s^{-1}, respectively. Furthermore, the DNA sequence of gene coding the enzyme showed a significant homology with hydrolases from the glycoside hydrolase family 55. Although β-1,3/1,4-glucanases have been purified and characterized from several other sources, *S. brinkmannii* β-1,3/1,4-glucanase is distinct from other β-1,3/1,4-glucanases due to its high catalytic efficiency toward Avicel and broad substrate specificity.

Keywords β-1,3/1,4-glucanase · catalytic efficiency · glycoside hydrolase · *Sistotrema brinkmannii* · purification

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Introduction

Lignocellulose is the major structural component of plants and is comprised of lignin, hemicellulose, and cellulose. The development of enzyme mixtures for lignocellulose depolymerization is one of the principal challenges in the economical production of biofuels from plant biomasses. In the enzyme mixture for depolymerization of lignocellulosic biomass, β-1,3/1,4-glucanases hydrolyzes β-glucan, which is the major cellulose and hemicellulose component in plants and grasses (Carpita, 1996).

A variety of glycoside hydrolases that recognize glucosyl residues in β-linked glycans have been characterized. Furthermore, many bacterial β-1,3/1,4-glucanases have been identified, purified, and characterized (Erfle et al., 1988; Gosalbes et al., 1991; Louw et al., 1993; Planas, 2000; Teng et al., 2007). Moreover, several fungal β-1,3/1,4-glucanases, including those from *Achlya ambisexualis* (Loprete and Hill, 2002), *Aspergillus japonicus* (Grishutin et al., 2006), *Cochliobolus carbonum* (Gorlach et al., 1998), *Melanocarpus* sp. (Kaur et al., 2007), *Orpinomyces joyonii* (Ye et al., 2001), *Paecilomyces thermophila* (Yang et al., 2008), *Rhizopus microspores* var. *microsporus* (Celestino et al., 2006), *Talaromyces emersonii* (McCarthy et al., 2003), and *Rhizomucor miehei* (Yanbin et al., 2012) have been characterized to date. All of the enzymes, except for that produced by *T. emersonii,* showed little or no activity toward the β-1,3-glucan, laminarin. To achieve high hydrolysis efficiency of β-1,3/1,4-glucans, discovery of additional efficient enzymes with different properties is necessary. However, to our knowledge, no enzyme with exclusive β-1,3/1,4-glucanase activity has been identified in fungus, which has high activity toward Avicel. In the present study, we isolated a fungal strain showing high β-1,3/1,4-glucanase activity. We further isolated the enzyme displaying the activity and cloned its gene. From the sequence and functional analyses, it is likely that the enzyme is a β-1,3/1,4-glucanase belonging to a glycoside hydrolase subfamily 55. Thus,

molecular mass, optimum pH and temperature, substrate specificity, and kinetic parameters of the enzyme were characterized.

Materials and Methods

Screening and isolation of the microorganism. The soil samples collected from Sorak Mountain (Korea) by the capillary tube method were diluted in sterile dilution solution (0.9% saline), and aliquots were spread on potato dextrose agar (PDA) plates, and were incubated for 3 days. Initial screening of β-1,3/1,4-glucanase producing fungi was carried out in agar plates containing 0.5% Avicel. Based on the zone of clearance observed from zymogram gels, which contained the substrate 4-methylumbelliferyl β-D-cellobioside (MUC), strains were selected for the analysis of β-1,3/1,4-glucanase activity. Different colonies were inoculated into 3 mL of the growth medium containing peptone 8 g · L^{-1}, yeast extract 2 g · L^{-1}, KH_2PO_4 5 g · L^{-1}, K_2HPO_4 5 g · L^{-1}, $MgSO_4$ · $7H_2O$ 3 g · L^{-1}, thiamine·HCl 0.005 g · L^{-1}, and Avicel 20 g · L^{-1} (Sigma, USA), and cultured by agitation at 200 rpm for 5 days at 28°C (Lee et al., 2011b).

Identification of the microorganism. Fatty acid composition was analyzed by gas chromatography (Agilent 6890N, USA), and the isolated strain was identified using the Microbial IDentification Inc. database. For the sequence analysis, the ITS1-5.8 S-ITS2 rDNA region of the fungus was amplified by PCR using primer set pITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and pITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The 604-bp amplicon, thus obtained, was cloned and sequenced. The partial gene sequence was initially analyzed using BLAST (blastn) tool on the NCBI website (http://www.ncbi.nlm.nih.gov), and the corresponding sequences were downloaded. The phylogenetic tree was constructed by neighbor-joining method (Saitou and Nei, 1987) using the MEGA4 package (Tamura et al., 2007). The sequences were submitted to GenBank (Accession No.: HQ717718 [*Sistotrema brinkmannii*]). Pairwise evolutionary distances and phylogenetic tree were constructed using the MegAlign software (DNA Star, USA). The identified strain *S. brinkmannii* was deposited at the Korean Culture Center of Microorganisms with the accession number 11246P.

Culture conditions. *S. brinkmannii* HQ717718 was used for β-1,3/1,4-glucanase production. The fungal strain was sub-cultured every 3 weeks, streaked on plates, and stored at 4°C. A 500-mL flask containing 50 mL of potato dextrose broth (PDB) was used for seeding the culture. After 4–5 days of incubation, 5 mL of this pre-culture was inoculated into 50 mL of the standard medium, which contained peptone 8 g · L^{-1}, yeast extract 2 g · L^{-1}, KH_2PO_4 5 g · L^{-1}, K_2HPO_4 5 g · L^{-1}, $MgSO_4$ $7H_2O$ 3 g · L^{-1}, thiamin hydrochloride 0.02 g · L^{-1}, and Avicel 20 g · L^{-1}, and the pH was adjusted to 5.0. The inoculated flasks were shaken at 150 rpm at 25°C.

Enzyme Assay. β-1,3/1,4-Glucanase activity was assayed using *p*-nitrophenyl-β-D-cellobioside (*p*NPC, Sigma, USA), laminarin (Sigma), and Avicel (Fluka) as the substrates. The enzymatic reaction mixtures (1 mL) containing 100 μL of enzyme solution and 10 mM *p*NPC 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 after the addition of 2 M Na_2CO_3 to the reaction mixture. One unit of *p*NPC-hydrolyzing activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute. Avicel and laminarin were diluted to the appropriate concentrations using 0.1 M sodium acetate buffer (Kanokratana et al., 2008; Lee et al., 2011a), and β-1,3/1,4-glucanase activity was measured by dinitrosalicylic acid method or GOD–POD method (Miller, 1959). Specific activity of the purified β-1,3/1,4-glucanase for 1% Avicel or laminarin was determined in buffer at pH 5.0 by incubation for 1 h at 50°C. To detect β-1,3/1,4-glucanase activity in chromatography fractions, the hydrolysis assay was carried out using 1% (w/v) Avicel or laminarin as the substrate in a total volume of 100 μL buffer containing 100 mM sodium phosphate (pH 5.0). The reaction was stopped by boiling the reaction mixture for 10 min. Free glucose was measured as described above.

Purification of β-1,3/1,4-glucanase. All purification procedures were performed at 4°C by using 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol (DTT), unless otherwise stated. Protein estimations were performed by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard. All chromatographic separations were performed using a BioRad Fast protein liquid chromatography ensemble was incubated system (BioRad, USA). Proteins in the column effluents were monitored by measuring the absorbance at 280 nm.

Cells from the culture broth were harvested by centrifugation at 10,000 × *g* for 30 min. After washing the pellet with 20 mM sodium acetate buffer (pH 5.0), the washes and supernatant were combined, concentrated, and desalted by ultrafiltration through a polyether sulfone membrane (30-kDa cutoff) in a stirred cell (Amicon, USA). The dialyzed enzyme solution was loaded on a hydroxyapatite fast flow column (1.6 × 10 cm; Amersham Biosciences) equilibrated with 0.01 M sodium phosphate buffer (pH 5.0), and the proteins were eluted using a 180-min linear gradient of 0.01–0.5 M of the same buffer at a flow rate of 1.0 ml·min^{-1}. Fractions of 1 mL each were collected and assayed for β-1,3/1,4-glucanase activity. Active fractions were pooled, dialyzed against the same buffer, and concentrated by ultrafiltration for further purification. The concentrated enzyme solution was loaded on a HiPrep 16/60 Sephacryl S-300 HR column (1.0 cm × 120 cm; Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer at pH 5.0, and the proteins were eluted using the same buffer at a flow rate of 0.5 mL · min^{-1}. Active fractions were pooled, dialyzed against the same buffer, and concentrated by ultrafiltration.

Determination of pH and temperature optima. The optimal pH of β-1,3/1,4-glucanase activity was determined by incubating the purified enzyme at 50°C for 20 min in different buffers: citrate (100 mM, pH 3–4), sodium acetate (100 mM, pH 4–6), and

phosphate (100 mM, pH 6–8). To determine the optimal temperature, the enzyme was incubated in sodium acetate buffer (100 mM, pH 5.0) for 15 min at different temperatures, from 40 to 80°C. To determine the thermostability of β-1,3/1,4-glucanase activity, the purified enzyme was incubated at different temperatures (40, 50, 60, 70, and 80°C) in the absence of a substrate. After incubation for various time periods (0–72 h), the residual β-1,3/1,4-glucanase activity was determined as described above.

Determination of molecular mass. For determination of the molecular mass of the enzyme, sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli et al. (1970) using 10% gels. Protein bands were visualized after staining the gels with Coomassie brilliant blue R-250 (Sigma). Native-PAGE was performed using 10% polyacrylamide gels without SDS. The ensemble was incubated for 30 min at 50°C for MUCase activity. MUC hydrolysis by the β-1,3/1,4-glucanase released fluorescent methylumbelliferone, which is visualized under UV light.

Substrate specificity. Substrate specificity of *S. brinkmannii* β-1,3/1,4-glucanase was determined by using *p*NPC, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*NP-β-D-lactopyranoside (*p*NPL), *p*-nitrophenyl-β-D-mannopyranoside, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose as substrates at 10 mM. Enzyme activities on the polysaccharides carboxy methylcellulose (CMC), xylan, laminarin, and Avicel (1%) were also tested. The *p*-nitrophenol released was determined under standard enzyme assay conditions. The enzyme activities on the oligosaccharides were estimated by assaying the amount of released glucose using Glucose oxidase-Peroxidase method (Lin et al., 1999), and those on polysaccharides were estimated by measuring released reducing sugars by dinitrosalicylic acid method (Miller, 1959) using glucose as a standard.

Determination of kinetic parameters. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for β-1,3/1,4-glucanase by incubating the enzyme in 100 mM sodium acetate buffer (pH 4.0, 65°C) with Laminarin at concentrations ranging from 0.5 to 100 mg · mL^{-1} and Avicel at concentrations ranging from 0.5 to 100 mg · mL^{-1}. Values for K_m, and V_{max} were determined from Lineweaver–Burk plots using standard linear regression techniques.

Effects of metals and reagents. The effects of various metal ions and chemicals on the β-1,3/1,4-glucanase activity were determined by pre-incubating the enzyme with the individual reagents in 100 mM sodium acetate buffer (pH 5.0) at 30°C for 30 min. Activities were then measured at 65°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 set as 100%.

Cloning of β-1,3/1,4-glucanase gene from *S. brinkmannii*. The degenerate primers BGF and BGR (Supplementary Table S1) were designed according to the conserved amino acids sequences: regions I (NQNNFFRTVRNF) and II (RNVKDFGAKGDGVTD DT). Degenerate PCR was performed using the cDNA of *S. brinkmannii* as the template. A 339-bp PCR fragment was obtained and cloned to pGEM-T Easy Vector (Promega, USA) for sequencing. Subsequent DNA sequencing further confirmed that the 339-bp PCR fragment contained specific sequence of β-1,3/1,4-glucanase gene. To obtain the complete structural gene encoding β-1,3/1,4-glucanase, thermal asymmetric interlaced (TAIL)-PCR (Jeya et al., 2010) was performed to amplify the 5 and 3 flanking sequences of the known partial sequence 900-bp of β-1,3/1,4-glucanase gene using the cDNA of *S. brinkmannii* as the template. Three interlaced specific primers, namely SPR1, SPR2, SPR3 and SPF1, SPF2, and SPF3 were used for flanking the 5 and 3 regions, respectively (Table S1). Five random degenerate primes (RP), AD1, AD2, AD3, AD4, and AD5 (Table S1), were used for flanking both the 5 and 3 regions. The TAIL-PCR product was cloned to pGEM-T Easy Vector (Promega) for DNA sequencing. After obtaining the 5- and 3'-flanking sequences of the known 950-bp β-1,3/1,4-glucanase gene, high fidelity PCR was performed to amplify the complete structural gene using the cDNA of *S. brinkmannii* as the template and BGS.br-F and BGS.br-R as the specific primers (sequence shown in Table S1). Finally, the PCR product, 2301-bp complete structural gene encoding β-1,3/1,4-glucanase was cloned using pGEM-T Easy Vector (Promega) for DNA sequencing and designated as *S. brinkmannii* β-1,3/1,4-glucanase.

Isolation and sequencing of cDNAs. Total RNA was extracted by using QIAGEN RNeasy Plant (QIAGEN, Italy) by following the manufacturer's instructions. According to the known 5 and 3-end sequences of the β-1,3/1,4-glucanase structural gene, primer BGS.br-F was designed to match the start codon ATG region and primer BGS.br-R was designed to match the sequence immediately downstream of the stop codon (Table S1). Using BGS.br-F and BGS.br-R as the specific primers, reverse transcription-polymerase chain reaction was then performed to amplify the full-length cDNA with PrimeSTAR™ HS DNA Polymerase (Takara, Japan). The amplified cDNA fragments were then eluted and cloned, and their identities were confirmed by sequencing. The nucleotide and amino acid sequences were analyzed by using the BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST). The molecular mass of the mature protein was predicted using Vector NTI 10.0 software. Multiple alignments of protein sequences were performed using the ClustalW program (http://www.ebi.ac.uk/clustalW/).

Results and Discussion

Identification of the isolated strain. A fungal microorganism that produced high levels of β-1,3/1,4-glucanase was isolated from the soil. When the ITS rDNA gene of the isolated fungus was sequenced, the isolated strain showed highest identity with that of *S. brinkmannii*. Through alignment and cladistic analysis of homologous nucleotide sequences of known microorganisms, phylogenetic relationships could be determined, and the approximate phylogenetic position of the strain is shown in Fig. 1. The isolated fungus and *S. brinkmannii* belonged to the same branch with 99%

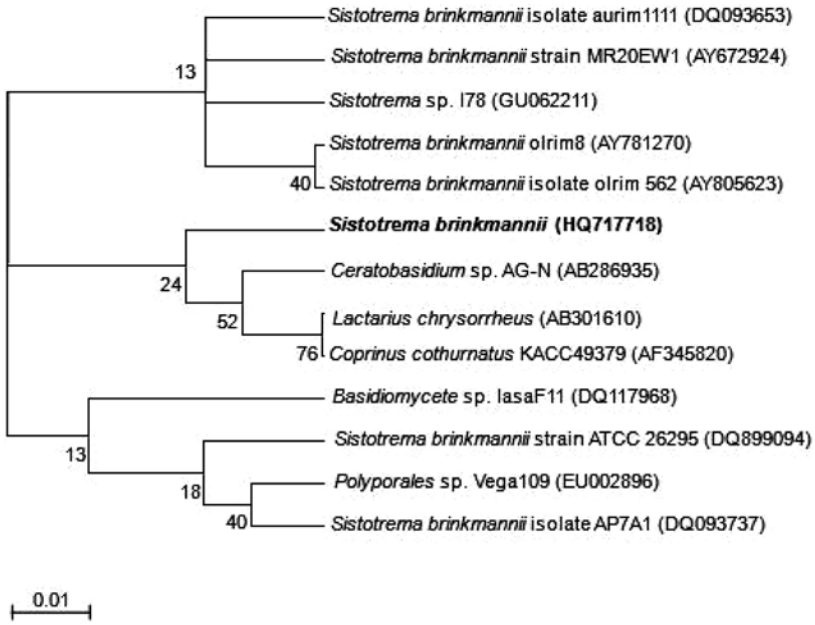

Fig. 1 The evolutionary history was inferred using the neighbor-joining method; the sequences were retrieved from the NCBI database, showing the phylogenetic relationships of *Sistotrema brinkmannii* HQ717718 and other fungal strains. Numbers at nodes shows the level of bootstrap support based on data for 1000 replication. Bar, 0.01 substitutions per nucleotide position, and numbers in parenthesis represent GenBank accession numbers.

Table 1 Purification of â-1,3/1,4-glucanase from the culture broth of *Sistotrema brinkmannii*

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold
Crude	480	1950	4.06	100	-
Ultrafiltration	205	4240	20.7	42.7	5.10
Hydroxyapatite	8.43	518	61.4	1.8	15.1
Gel filtration	0.52	57.4	110	0.1	27.2

probability. Based on its rDNA gene sequence, the isolated strain was identified as a strain of *S. brinkmannii* and was named *S. brinkmannii* HQ717718.

Purification of a β-1,3/1,4-glucanase from *S. brinkmannii.* β-1,3/1,4-Glucanase was purified as described in the Materials and Methods, and the results are summarized in Table 1. Fractionation by ultrafiltration increased the specific activity by about 5-fold with 47% recovery of β-1,3/1,4-glucanase activity. The active fractions were applied to a hydroxyapatite column, and the β-1,3/1,4-glucanase eluted at approximately 0.1 M NaCl. A subsequent gel filtration step produced two peaks containing proteins; the second peak showed β-1,3/1,4-glucanase activity. These chromatography methods resulted in a 27-fold purification of β-1,3/1,4-glucanase. The purified enzyme appeared as a single protein band on polyacrylamide gel electrophoresis both in the presence and absence of SDS (Fig. 2A-lane 4). The molecular mass of *S. brinkmannii* β-1,3/1,4-glucanase was ~85 kDa. Activity staining of the non-denaturing gel, showed a single band with the same mobility as that of the protein band (Fig. 2A-lane 5). 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 a M_r of approximately 170 kDa (Fig. 2B). These results indicate that the enzyme migrates as a dimer in gel filtration under the mild conditions used, and thus, may also be present as an active dimer in solution.

Optimum pH and temperature. The optimum pH of the purified β-1,3/1,4-glucanase was 4.0 (Fig. 3A). The optimum temperature for the hydrolysis reaction was 65°C (Fig. 3B). The stability of the β-1,3/1,4-glucanase was tested in standard buffer. Preparations were stored at 70, 65, 60, and 50°C, and retained 50% of their initial activities after 1.2, 4, 10 h, and 2 days, respectively.

Substrate specificity. The activity of β-1,3/1,4-glucanase on various substrates under the standard assay conditions are shown in Table 2. The enzyme showed the highest activity for *p*NPG. The β-1,3/1,4-glucanase activity with *p*NPG was 360% of the activity with *p*NPC. The presence of the nitro group at the *ortho* position decreased the activity, as was observed with *O*-nitrophenyl-β-D-glucopyranoside. The enzyme also showed an activity with cello-oligosaccharides. CMC was practically non-hydrolyzable by β-1,3/1,4-glucanase. Other substrates such as β-1,3-glucan and xylan showed low activity.

Kinetics. Initial velocities were determined in the standard assay

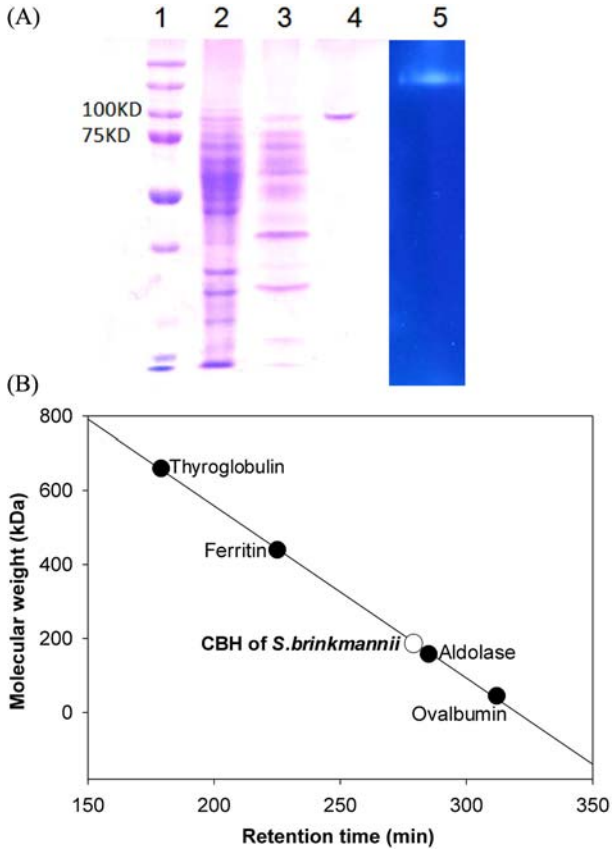

Fig. 2 Determination of the molecular mass of *Sistotrema brinkmannii* β-1,3/1,4-glucanase by SDS-PAGE and gel filtration chromatography. (A) Determination of subunit molecular mass by SDS-PAGE. Lane 1, Protein marker; Lane 2, Crude fraction; Lane 3, Hydroxyapatite ion exchange fraction; Lane 4, purified *S. brinkmannii* β-1,3/1,4-glucanase; and Lane 5, zymogram of purified β-1,3/1,4-glucanase. (B) Determination of native molecular mass of *S. brinkmannii* β-1,3/1,4-glucanase by gel filtration chromatography on Sephacryl S-300 high-resolution column from Amersham.

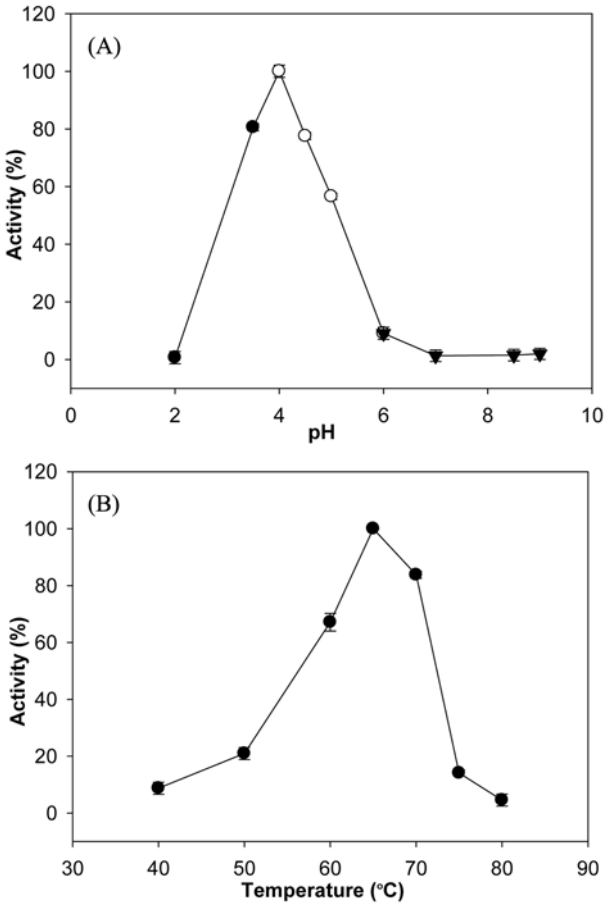

Fig. 3 (A) Effect of pH on the activity of purified *Sistotrema brinkmannii* β-1,3/1,4-glucanase. The enzyme activity was determined by the standard assay method by changing the buffer to obtain the desired pH. The buffers used were citrate (●), sodium acetate (○), and phosphate (▼). (B) Effect of temperature on the activity of purified *S. brinkmannii* β-1,3/1,4-glucanase. The enzyme was determined by the standard assay method at different temperatures ranging from 40 to 80°C.

mixture at pH 4.0. All the substrates tested had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear. Fig. 4 shows typical Michaelis-Menten-type kinetics for β-1,3/1,4-glucanase activity, with increasing laminarin and Avicel concentrations. The Lineweaver–Burk plot obtained for the conversion of laminarin under standard assay conditions showed a V_{max} of 48 U · mg^{-1} of protein, K_m of 5.5 mM, and k_{cat} of 149 s^{-1}. The V_{max}, K_m and k_{cat} of the enzyme for Avicel were 136 U · mg^{-1} of protein, 3.8 mM, and 423 s^{-1}, respectively. A comparison of kinetic parameters showed that *S. brinkmannii* β-1,3/1,4-glucanase has higher catalytic efficiency toward Avicel than those by other β-1,3/1,4-glucanases (Table 3).

Effects of metal ions and various compounds. Zn^{2+}, Hg^{2+}, and Cu^{2+} ions caused significant inhibition that appeared to be competitive with respect to the substrate *p*NPC. Addition of Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, glycerol, β-mercaptoethanol, and cysteine did not affect the activity, suggesting that thiol groups were not essential for catalytic activity (Yan and Lin, 1997). *N*-ethylmaleimide, which often inactivates enzymes presumably by reacting with the thiol group of cysteine residues, had no effect at 10 mM on the activity. This confirmed that thiol groups were not essential for promotion of catalytic activity.

Identification of the partial peptide fragment. The purified enzyme (1.5 μg) was separated by 10% SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) membrane. Automated Edman degradation of the enzyme protein was unsuccessful, implying that the *N*-terminal of the enzyme was blocked. The β-1,3/1,4-glucanase was partially digested with trypsin, separated on 12.5% SDS-PAGE, and blotted onto a PVDF membrane. The fragments sequenced on an automatic protein sequencer were found to contain the sequences NQNNFFRTVRNF and RNVKD FGAKGDGVTDDT, fragments that showed homology towards other reported β-1,3/1,4-glucanase, which were identical to the β-1,3/1,4-glucanase of *Lentinula edodes*, and *Phanerochaete*

Table 2 Activities of *S. brinkmannii* β-1,3/1,4-glucanase with different substrates. 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 15%

Substrate (10 mM)	Relative activity (%)	Specific activity (U mg^{-1})
*p*NP β-cellobiopyranoside	100	110
*p*NP β-glucopyranoside	360	397
*o*NP β-glucopyranoside	46.4	51.2
*p*NP mannopyranoside	2.1	2.32
*p*NP α-glucopyranoside	96	106
Laminarin 2% (β-1,3 linkage)	32.7	36.1
β-1,3-Glucan 1% (β-1,3 linkage)	7.8	8.6
Cellobiose (G2)	7.1	7.8
Cellotriose (G3)	26.6	29.3
Cellotetraose (G4)	35.6	39.3
Cellopentaose (G5)	40.3	44.5
Cellohexaose (G6)	53.2	58.7
CMC 2%	ND	ND
Xylan 2%	4.9	5.4
Avicel 2%	72.6	80.1

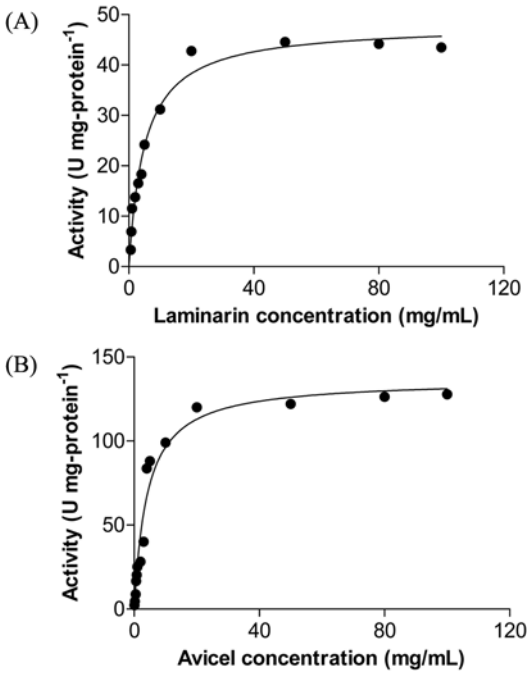

Fig. 4 (A) Effect of laminarin concentration on the activity of *Sistotrema brinkmannii* β-1,3/1,4-glucanase. (B) Effect of Avicel concentration on the activity of *S. brinkmannii* β-1,3/1,4-glucanase. Activity was measured in the presence of the indicated concentrations of laminarin/Avicel at pH 4.0. The inset shows Lineweaver-Burk plots of initial velocity versus various fixed substrate concentrations. Each value represents the mean of triplicate measurements and varied by not more than 15%.

chrysosporium belonging to GH55, respectively (Kawai et al., 2006; Sakamoto et al., 2009). Enzymology and bioinformatics experiments strongly suggest that *S. brinkmannii* β-1,3/1,4-glucanase should also be classified as a member of GH55.

Cloning of the full-length β-1,3/1,4-glucanase gene by TAIL-PCR. A full-length β-1,3/1,4-glucanase gene was cloned by TAIL-PCR, and a partial 339-bp amplicon was obtained by PCR using a degenerate primer pair, BGF and BGR, which were designed based on the peptide sequences conserved in β-1,3/1,4-glucanases. To amplify the DNA fragments upstream and downstream of β-1,3/1,4-glucanase gene, the specific primers (SPF1, 2, and 3; SPR1, 2, and 3) were designed based on the sequence of the partial β-1,3/1,4-glucanase. Tertiary PCR of 3 TAIL-PCR with SPF3 primer and RP1 random primer resulted in amplification of a 950-bp fragment. Similarly, tertiary PCR of 5 TAIL-PCR with SPR3 primer and RP2 random primer resulted in a 900-bp fragment. The PCR fragments were eluted using a gel elution kit and then both strands were sequenced. The *N*-terminal and *C*-terminal sequences partially overlapped with the already sequenced

Table 3 Properties of β-1,3/1,4-glucanases from various sources. Enzyme activity was assayed with Avicel

Microorganism	Mr (kDa)	K_m (mM)	Opt. pH	Opt. temp. (°C)	V_{max} (U/mg)	Specific activity (U/mg)	Reference
Tenebrio molitor β-1,3/1,4-glucanase	50	ND	6	ND	ND	<4.4	(Genta et al., 2009)
Paenibacillus sp. β-1,3/1,4-glucanase	229	ND	ND	ND	ND	$<5.2\times10^{-3}$	(Cheng et al., 2009)
Clostridium thermocellum β-1,3/1,4-glucanase	148	ND	6	70	ND	3.63	(Fuchs et al., 2003)
Irpex lacteus Ex-1	53	NR	5	50	NR	20.9	(Hamada et al., 1999)
Irpex lacteus Ex-2	56	NR	5	50	NR	21.1	(Hamada et al., 1999)
Talaromyces emersonii CBH IA	66	2.1†	3.6	78	9,2†	7.7	(Tuohy et al., 2002)
Talaromyces emersonii CBH IB	56	0.8†	4.1	66	3.8†	2.4	(Tuohy et al., 2002)
Trichoderma reesei CBH I	66	0.22°	5.7	37	3.5°	0.04	(Shoemaker et al., 1983)
Fomitopsis pinicola CBH	64	NR	5	50	NR	7.98	(Shin et al., 2010)
Penicillium purpurogenum KJS506 CBH	60	NR	5	60	NR	6.5	(Lee et al., 2011a)
Sistotrema brinkmannii HQ717718	83	3.8	4	65	136.5	80.1	This study

°: *p*NPL as a substrate
†: *p*NPC as a substrate

internal fragment. Similarly, another round of TAIL-PCR was performed to amplify the full-length region of β-1,3/1,4-glucanase gene. Finally, PCR with BGS.br-F and BGS.br-R resulted in a 2301-bp amplicon, which was cloned and sequenced (supplementary Fig. S1). NCBI nucleotide BLAST results confirmed that the sequence belonged to the GH55 β-1,3/1,4-glucanases.

In conclusion, a new β-1,3/1,4-glucanase was purified and characterized from *S. brinkmannii*. *S. brinkmannii* β-1,3/1,4-glucanase released glucose from various β-glucans, including β-1,3- and β-1,4-linked oligosaccharides. Kinetic analysis showed that *S. brinkmannii* β-1,3/1,4-glucanase preferentially hydrolyzed Avicel. These results suggest that *S. brinkmannii* β-1,3/1,4-glucanase is a key enzyme that produces glucose from Avicel during the growth of *S. brinkmannii* on Avicel. *S. brinkmannii* β-1,3/1,4-glucanase can be discriminated from the other β-1,3/1,4-glucanases reported till due to its high catalytic efficiency toward Avicel, suggesting its potentially wide application.

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References

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**, 248–54.

Carpita NC (1996). Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol **47**, 445–76.

Celestino K, Cunha R, and Felix C (2006). Characterization of a β-glucanase produced by Rhizopus microsporus var. microsporus, and its potential for application in the brewing industry. BMC Biochem **7**, 1–9.

Cheng YM, Hong TY, Liu CC, and Meng MS (2009). Cloning and functional characterization of a complex endo-β-1,3-glucanase from *Paenibacillus* sp. Appl Microbiol Biot **81**, 1051–61.

Erfle JD, Teather RM, Wood PJ, and Irvin JE (1988). Purification and Properties of a 1,3-1,4-β-D-glucanase (lichenase, 1,3-1,4-β-D-glucan 4-glucanohydrolase, EC-3.2.1.73) from *Bacteroides succinogenes* cloned in *Escherichia coli*. Biochem J **255**, 833–41.

Fuchs KP, Zverlov VV, Velikodvorskaya GA, Lottspeich F, and Schwarz WH (2003). Lic16A of *Clostridium thermocellum*, a non-cellulosomal, highly complex endo-β-1,3-glucanase bound to the outer cell surface. Microbiol-Sgm **149**, 1021–31.

Genta FA, Bragatto I, Terra WR, and Ferreira C (2009). Purification, characterization and sequencing of the major β-1,3-glucanase from the midgut of *Tenebrio molitor larvae*. Insect Biochem Molec **39**, 861–74.

Gorlach JM, Van der Knaap E, and Walton JD (1998). Cloning and targeted disruption of MLG1, a gene encoding two of three extracellular mixed-linked glucanases of *Cochliobolus carbonum*. Appl Environ Microbiol **64**, 385–91.

Gosalbes MJ, Perezgonzalez JA, Gonzalez R, and Navarro A (1991). 2 β-Glycanase genes are clustered in *Bacillus polymyxa* - Molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-β-(1,3)-(1,4)-glucanase. J Bacteriol **173**, 7705–10.

Grishutin SG, Gusakov AV, Dzedzyulya EI, and Sinitsyn AP (2006). A lichenase-like family 12 endo-(1→4)-β-glucanase from *Aspergillus japonicus*: study of the substrate specificity and mode of action on beta-glucans in comparison with other glycoside hydrolases. Carbohyd Res **341**, 218–29.

Hamada N, Ishikawa K, Fuse N, Kodaira R, Shimosaka M, Amano Y et al. (1999). Purification, characterization and gene analysis of exo-cellulase II (Ex-2) from the white rot basidiomycete *Irpex lacteus*. J Biosci Bioeng **87**, 442–51.

Jeya M, Joo AR, Lee KM, Tiwari MK, Lee KM, Kim SH et al. (2010). Characterization of β-glucosidase from a strain of *Penicillium purpurogenum* KJS506. Appl Microbiol Biot **86**, 1473–84.

Kanokratana P, Chantasingh D, Champreda V, Tanapongpipat S, Pootanakit K, and Eurwilaichitr L (2008). Identification and expression of cellobiohydrolase (CBHI) gene from an endophytic fungus, *Fusicoccum* sp (BCC4124) in *Pichia pastoris*. Protein Expres Purif **58**, 148–53.

Kaur J, Chadha BS, Kumar BA, and Saini HS (2007). Purification and characterization of two endoglucanases from *Melanocarpus* sp MTCC 3922. Bioresource Technol **98**, 74–81.

Kawai R, Igarashi K, and Samejima M (2006). Gene cloning and heterologous expression of glycoside hydrolase family 55 β-1,3-glucanase from the basidiomycete *Phanerochaete chrysosporium*. Biotechnol Lett **28**, 365–71.

Laemmli UK and others (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–5.

Lee KM, Joo AR, Jeya M, Lee KM, Moon HJ, and Lee JK (2011a). Production and characterization of cellobiohydrolase from a novel strain of *Penicillium purpurogenum* KJS506. Appl Biochem Biotech **163**, 25–39.

Lee KM, Moon HJ, Kalyani D, Kim H, Kim IW, Jeya M et al. (2011b). Characterization of cellobiohydrolase from a newly isolated strain of *Agaricus arvencis*. J Microbiol Biotechnol **21**, 711–18.

Lin J, Pillay B, and Singh S (1999). Purification and biochemical characteristics of β-D-glucosidase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP. Biotechnol Appl Bioc **30**, 81–7.

Loprete DM and Hill TW (2002). Isolation and characterization of an endo-(1,4)-β-glucanase secreted by *Achlya ambisexualis*. Mycologia **94**, 903–11.

Louw ME, Reid SJ, and Watson TG (1993). Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4) β-glucanase-encoding gene from an alkalophilic *Bacillus brevis*. Appl Microbiol Biotechnol **38**, 507–13.

McCarthy T, Hanniffy O, Savage AV, and Tuohy MG (2003). Catalytic properties and mode of action of three endo-β-glucanases from *Talaromyces emersonii* on soluble β-1,4- and β-1,3;1,4-linked glucans. Int J Biol Macromol **33**, 141–8.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem **31**, 426–8.

Planas N (2000). Bacterial 1,3-1,4-β-glucanases: structure, function and protein engineering. Bba-Protein Struct M **1543**, 361–82.

Saitou N and Nei M (1987). The neighbor-joining method - a new method for reconstructing phylogenetic trees. Mol Biol Evol **4**, 406–25.

Sakamoto Y, Nakade K, and Sato T (2009). Characterization of the post-harvest changes in gene transcription in the gill of the Lentinula edodes fruiting body. Curr Genet **55**, 409–23.

Shin K, Kim YH, Jeya M, Lee JK, and Kim YS (2010). Purification and characterization of a thermostable cellobiohydrolase from *Fomitopsis pinicola*. J Microbiol Biotechn **20**, 1681–8.

Shoemaker S, Schweickart V, Ladner M, Gelfand D, Kwok S, Myambo K et al. (1983). Molecular cloning of exo-cellobiohydrolase-I derived from *Trichoderma reesei* strain-L27. Bio-Technol **1**, 691–6.

Tamura K, Dudley J, Nei M, and Kumar S (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol **24**, 1596–9.

Teng D, Fan Y, Yang YL, Tian ZG, Luo J, and Wang JH (2007). Codon optimization of *Bacillus licheniformis* β-1,3-1,4-glucanase gene and its expression in *Pichia pastoris*. Appl Microbiol Biot **74**, 1074–83.

Tuohy MG, Walsh DJ, Murray PG, Claeyssens M, Cuffe MM, Savage AV et al. (2002). Kinetic parameters and mode of action of the cellobiohydrolases produced by Talaromyces emersonii. Bba-Protein Struct M **1596**, 366–80.

Yan TR and Lin CL (1997). Purification and characterization of a glucose-tolerant β-glucosidase from *Aspergillus niger* CCRC 31494. Biosci

Biotechnol Biochem **61**, 965–70.
Yanbin T, Shaoqing Y, Qiaojuan Y, Peng Z, Jian C, and Zhengqiang J (2012). Purification and characterization of a novel β-1,3–1,4-glucanase (Lichenase) from thermophilic *Rhizomucor miehei* with high specific activity and its gene sequence. J Agr Food Chem **60** (9), 2354–61.
Yang SQ, Yan QJ, Jiang ZQ, Fan GS, and Wang L (2008). Biochemical characterization of a novel thermostable β-1,3-1,4-glucanase (Lichenase) from *Paecilomyces thermophila*. J Agr Food Chem **56**, 5345–51.
Ye XY, Ng TB, and Cheng KJ (2001). Purification and characterization of a cellulase from the ruminal fungus *Orpinomyces joyonii* cloned in *Escherichia coli*. Int J Biochem Cell B **33**, 87–94.