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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

Yanbin Tang,[†] Shaoqing Yang,[†] Qiaojuan Yan,[‡] Peng Zhou,[†] Jian Cui,[‡] and Zhengqiang Jiang*[,†]

[†]Department of Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

[‡]Bioresource Utilization Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

ABSTRACT: Production, purification, and characterization of a novel β-1,3−1,4-glucanase (lichenase) from thermophilic *Rhizomucor miehei* CAU432 were investigated. High-level extracellular β-1,3−1,4-glucanase production of 6230 U/mL was obtained when oat flour (3%, w/v) was used as a carbon source at 50 °C. The crude enzyme was purified to homogeneity with a specific activity of 28818 U/mg. The molecular weight of purified enzyme was estimated to be 35.4 kDa and 33.7 kDa by SDS−PAGE and gel filtration, respectively. The optimal pH and temperature of the enzyme were pH 5.5 and 60 °C, respectively. The K_m values of purified β-1,3−1,4-glucanase for barley β-glucan and lichenan were 2.0 mM and 1.4 mM, respectively. Furthermore, the gene (*RmLic16A*) encoding the β-1,3−1,4-glucanase was cloned and its deduced amino acid sequence showed the highest identity (50%) to characterized β-1,3−1,4-glucanase from *Paecilomyces thermophila*. The high-level production and biochemical properties of the enzyme enable its potential industrial applications.

KEYWORDS: *characterization, gene cloning, purification, β-1,3−1,4-glucanase, Rhizomucor miehei*

INTRODUCTION

β-Glucans, a kind of linear polysaccharides, are principal components of the cell wall in the endosperm of cereal endosperm. They account for up to 5.5% of the dry weight of grains and 75% of the carbohydrate in barley endosperm.[1,2] β-1,3−1,4-Glucanases (1,3; 1,4-β-glucan-4-glucanohydrolase or lichenase; EC 3.2.1.73) are important hydrolytic enzymes catalyzing the cleavage of β-1,4 glycosidic linkages strictly adjacent to a 3-*O*-substituted glucopyranose unit in mixed-linked β-glucans, which have been widely used in brewing and poultry feed industries. In the brewing industry, β-1,3−1,4-glucanases can be used to hydrolyze β-glucan, in order to enhance the extraction yield and increase the filtration rate, and clarify beer and juices.[3] In the poultry industry, β-1,3−1,4-glucanases can improve the digestibility of feedstuff by degrading the barley β-glucan, which is the antinutrient factor in poultry feedstuff.[4,5]

β-1,3−1,4-Glucanases are widely distributed in various bacteria,[2,4,6] fungi,[7,8] and plants.[3] So far, many β-1,3−1,4-glucanases have been purified and characterized.[8−10] Compared to mesophilic fungi, thermophilic fungi possess more advantages, in that the β-1,3−1,4-glucanases produced by thermophilic fungi usually have higher reaction temperature and excellent thermostability.[11] However, only a few β-1,3−1,4-glucanases have been purified and characterized from thermophilic fungi to date, such as *Talaromyces emersonii*[7] and *Paecilomyces thermophila*.[12] The commercial β-1,3−1,4-glucanases currently marketed may not be ideally suitable for brewing and poultry industries due to their properties. Usually, malting (50−70 °C) or feed pelleting processes (65−90 °C) require enzymes with good thermostability. Therefore, thermostable β-1,3−1,4-glucanases will be highly desirable owing to their stability at high operation temperatures.[7,8,11,12]

The thermophilic fungus *Rhizomucor miehei* is known as an excellent industrial enzyme producer, especially for aspartic protease[13] and lipase production.[14] In addition, several other enzymes from this species have been purified and characterized, such as β-1,3(4)-glucanase,[15] β-glucosidase,[16] and xylanase.[17] However, there is no report on the β-1,3−1,4-glucanase production from this species. *Rhizomucor miehei* CAU432 as a newly isolated thermophilic fungus was found to secrete a large amount of β-1,3−1,4-glucanase in submerged culture (data not shown). Hence, the objective of the present study was to produce, purify, and characterize the β-1,3−1,4-glucanase from *R. miehei* CAU432.

MATERIALS AND METHODS

Materials. TRIzol (Invitrogen, Carlsbad, US) and Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany) were used for total RNA extraction and mRNA purification. SMARTer RACE cDNA Amplification was purchased from Clontech (Palo Alto, California, USA). Restriction endonucleases and T4 DNA ligase were purchased from England Biolabs (Ipswich, Massachusetts, USA). DNA polymerase Ex Taq and pMD18-T simple vector system were purchased from TaKaRa (Dalian, China). *Escherichia coli* strain JM109 (Bomaide, Beijing, China) was used for propagation of plasmids. Barley β-glucan, lichenan (from *Cetraria islandica*), laminarin (from *Laminaria digitata*), pullulan (from *Aureobasidium pullulans*), birchwood xylan, locust bean gum (LBG), soluble starch, carboxymethylcellulose (CMC) cellobiose, and *p*-nitrophenyl derivates were obtained from Sigma Chemical Company (St. Louis, Missouri, USA). β-1,3-Glucan (from *Euglena fracilis*) was obtained from Fluka Chemical Co. (Steinheim, Germany). DEAE 52 was purchased from Whatman Company

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(Whatman Inc., Fairfield, New Jersey, USA). Q-Sepharose Fast Flow was the product of Pharmacia Company (Pharmacia, Uppsala, Sweden). All other chemicals used were of analytical grade unless otherwise stated.

Fungal Strain and Growth Conditions. *R. miehei* CAU432 used in this study was deposited in China General Microbiological Culture Collection Center (CGMCC, accession NO. 4967). It was maintained on potato dextrose-agar (PDA) plate at 4 °C and transferred every 4−5 weeks.

For β-1,3−1,4-glucanase production, a piece (1 cm^2) of agar from 3-day-old mycelia was inoculated into the basal culture medium (50 mL) containing (g/L) 30 oat flour, 10 soybean peptone, 0.3 $MgSO_4 \cdot 7H_2O$, 5 KH_2PO_4, and 0.3 $CaCl_2$ in 250 mL flasks. Triplicate cultures were cultivated at 50 °C for 4 days in a shaker with a rotation speed of 200 rpm. Then the cultures were centrifuged at 10000*g* for 10 min, and the supernatant was used as crude enzyme for subsequent enzyme purification. For fungal mycelium production, *R. miehei* CAU432 was grown in the above medium at 50 °C for 3 days, and then the mycelia were collected by centrifugation (5000*g*, 10 min) and washed twice with sterile water at 4 °C.

Enzyme Assay and Protein Quantification. The enzyme assay was performed according to the method of Bailey et al.[18] A reaction mixture containing 0.9 mL of 0.5% (w/v) barley β-glucan and 0.1 mL of suitably diluted enzyme in 50 mM citrate buffer (pH 6.0) was incubated at 60 °C for 10 min. The enzyme activity was quantified by measuring the released reducing sugars using the dinitrosalicyclic acid (DNS) method.[19] One unit of β-1,3−1,4-glucanase activity was defined as the amount of enzyme that produces 1 μmol reducing sugar per minute under the above conditions. Glucose was used as the standard.

Protein concentrations were measured by the Lowry method[20] with BSA (bovine serum albumin) as the standard.

Purification of β-1,3−1,4-Glucanase. The crude enzyme was subjected to 40−60% ammonium sulfate saturation and stirred at 4 °C for 30 min. The precipitated protein was collected and dialyzed against 20 mM, pH 7.5 phosphate buffer overnight. Dialyzed enzyme (20 mL) was then applied to a DEAE 52 ion-exchange column (5 cm ×1.5 cm), which was pre-equilibrated with 20 mM phosphate buffer (pH 7.5). The bound β-glucanase was eluted with a gradient of 0−300 mM NaCl solution at a flow rate of 1.0 mL/min. The active fractions were combined and concentrated by ultrafiltration using a 10 kDa membrane (Stirred Cell Model 8050, Millipore). The concentrated solution was applied to a Q-Sepharose Fast Flow column (10 cm ×1.5 cm) equilibrated with 20 mM, pH 7.0 phosphate buffer. The absorbed proteins were first washed with 150 mM NaCl solution, and then the bound β-glucanase was eluted with a gradient of 150−250 mM NaCl solution at a flow rate of 1.0 mL/min. The homogeneity of the eluted enzyme was checked by SDS−PAGE.

SDS−PAGE and Molecular Weight Determination. SDS−PAGE was performed using 12.5% (w/v) separation gels as described by Laemmli.[21] Protein bands were visualized by Coomassie brilliant blue R-250 staining. The standards used for molecular weight calibration were phosphorylase b (97.2 kDa), albumin (66.4 kDa), ovalbumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa).

For molecular mass determination by gel filtration, a Superdex 75 column (1.0 cm × 40.0 cm) was equilibrated with 20 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. The column was calibrated with the standard proteins (Sigma): phosphorylase b (97.2 kDa), fetuin from fetal calf serum (68.0 kDa), albumin from chicken egg white (45.0 kDa), α-chymotrypsinogen a (25.7 kDa), and cytochrome *c* (13.0 kDa), each at 2 mg/mL. The flow rate for elution was 0.3 mL/min.

Glycoprotein Detection and Carbohydrate Content Determination. Glycoprotein was detected by periodic acid Schiff (PAS) staining on the SDS−PAGE gel.[22] Carbohydrate content of the purified β-1,3−1,4-glucanase was measured by the phenol sulphuric acid method,[23] using glucose as the standard.

Internal Peptide Sequence Analysis. To determine the partial amino acid sequence, the purified β-1,3−1,4-glucanase was subjected to SDS−PAGE and stained with Coomassie brilliant blue R-250. The protein band was excised from the gel and submitted to the National Center of Biomedical Analysis (Beijing, China). The sample was analyzed for internal peptide amino acid sequences, using high performance liquid chromatography−electrospray tandem mass spectrometry (HPLC-ESI-MS/MS). Mass spectral sequencing was performed using a Q-TOF II mass analyzer (Q-TOF2) (Micromass Ltd., Manchester, UK). MS/MS spectra were transformed using MaxEnt3 (Masslynx, Micromass), and amino acid sequences were interpreted manually using PepSeq (Biolynx, Micromass).

Characterization of the Purified β-1,3−1,4-Glucanase. The optimal pH of the purified β-1,3−1,4-glucanase was tested by measuring the enzyme activity in different buffers of 50 mM concentration with pH ranging from 3.0 to 11.0. The buffers used were citrate buffer (pH 3.0−6.0), MOPS buffer (pH 6.5−8.0), CHES buffer (pH 8.0−10.0), and CAPS buffer (pH 10.0−11.0). To determine the pH stability of the enzyme, the purified β-1,3−1,4-glucanase was incubated in different buffers as mentioned above at 50 °C for 30 min, and then the residual activities were measured.

The optimal temperature of the purified β-1,3−1,4-glucanase was determined by assaying the enzyme activity at different temperatures (30 °C−100 °C) in 50 mM citrate buffer (pH 5.5). The thermostability of the enzyme was determined by measuring the residual activity after the enzyme was incubated at different temperatures (30 °C−100 °C) for 30 min. For denaturing half-life determination, the enzyme was incubated at 60, 70, 75, 80, and 85 °C for 4 h, and then the activity of aliquots withdrawn at different time intervals was measured according to the standard assay.

The effect of metal ions and reagents on β-1,3−1,4-glucanase activity was determined by measuring the residual activities after the enzyme was treated in 50 mM, pH 5.5 citrate buffer in the presence of 4 mM individual reagents at 22 °C for 30 min. The activity in the absence of metal ions or reagents was taken as 100%. The results presented are the average of three trials.

Substrate Specificity and Kinetic Parameters of the Purified β-1,3−1,4-Glucanase. The substrate specificity of the purified β-1,3−1,4-glucanase was determined in 50 mM citrate buffer (pH 5.5) at 60 °C for 10 min using different polysaccharides (barley β-glucan, lichenan, laminarin, pullulan, birchwood xylan, soluble starch, LBG, and CMC) and *p*NP (*p*-nitrophenyl)-glycosides (*p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-fucopyranoside, *p*NP-β-mannopyranoside, and *p*NP-β-D-galactopyranoside) as substrates. The concentrations of polysaccharides and *p*NP-glycosides were 0.5% (w/v) and 5 mM, respectively. The released reducing sugars were measured by the DNS method.[19] The *p*-nitrophenol formed was determined by spectrophotometry at 405 nm. The enzyme unit was defined as the amount of enzyme that produces 1 μmol of reducing sugar or *p*-nitrophenol per minute under the above conditions.

The kinetic parameters of the purified β-1,3−1,4-glucanase toward barley β-glucan or lichenan were determined by measuring the enzyme activities with six different substrate concentrations in 50 mM cirate buffer (pH 5.5) at 60 °C for 5 min. K_m and k_{cat} values were calculated using the Grafit program.

Hydrolytic Properties of the Purified β-1,3−1,4-Glucanase. In order to analyze the hydrolytic properties of β-1,3−1,4-glucanase, 10 U of purified β-1,3−1,4-glucanase was incubated with 10 mg of barley β-glucan and lichenan in 1 mL of 50 mM citrate buffer (pH 5.5) at 50 °C for 6 h. Aliquots were periodically withdrawn and analyzed by thin-layer chromatography (TLC) on Kieselgel 60 plates (Merck) with a butan-1-ol−acetic acid−water (2:1:1, v/v/v) solvent system.[12] The plates were developed with one run followed by heating for a few minutes at 130 °C in an oven after spraying the plates with a methanol−sulfuric acid mixture (95:5, v/v). Glucose, cellobiose, cellotriose, and cellotetraose were used as the standards.

Cloning of the Full-Length β-Glucanase cDNA and Its Sequence Analysis. DNA manipulations were performed according to Sambrook and Russell.[24] Genomic DNA of *R. miehei* was isolated with Fungal DNA Midi Kit (Omega Bioteck, Doraville, US). The total RNA was isolated with the Trizol reagent (Invitrogen, Carlsbad, US), and mRNA was purified using Oligotex mRNA Midi Kit (Qiagen, Germany). *R. miehei* CAU432 cDNA was synthesized using PrimeScript First Strand cDNA Synthesis Kit (Takara Bio, INC., Otsu, Shiga, Japan) and used as the template for subsequent polymerase

chain reaction (PCR) amplification. To clone the β-1,3−1,4-glucanase gene, degenerate primers RmLic16ACP1 and RmLic16ACP2 (Table 1)

Table 1. Oligonucleotide Primers Used in This Work

primers	primer sequence (5′→3′)[a]
RmLic16ACP1	CAACGGCGAGATCGAYRTNATHGA
RmLic16ACP2	CGGCCCAGTCGCCRCARAANGT
RmLic16AGSP1	GGAAATCGGCCTTGGGAGTA
RmLic16ANGSP1	GGTCGCCAACACCGCAACCT
RmLic16AGSP2	GCGACCCCTCCAGCAACTCT
RmLic16ANGSP2	GATATTAGCAGTGGCAATCCTGATC
RmLic16ADNAF	ATGCGCTTCACATCTCTTCTCG
RmLic16ADNAR	TTATTGCTGGTAGACCTTAAGGTAG

[a]Y = C/T, R = A/G, H = A/C/T, and N = A/T/C/G.

were designed based on the conserved sequences (GEIDIIE and TFCGDWAG) of other known fungal β-glucanases using the CODEHOP algorithm.[25]

The full-length cDNA sequence of the β-1,3−1,4-glucanase was obtained by 5′ and 3′ RACE (rapid amplification of cDNA ends) using SMART RACE cDNA Amplification Kit in accordance with the manufacturer's instructions. PCR was performed with the following primer pairs (Table 1): RmLic16AGSP1 and Universal Primer A Mix for (UPM) first PCR, Nested Universal Primer A (NUP) and RmLic16ANGSP1 for 5′ RACE, and RmLic16AGSP2 and UPM for first PCR, NUP A, and RmLic16ANGSP2 for 3′ RACE. The obtained PCR products were purified, cloned and sequenced. To amplify this region from the *R. miehei* genomic DNA, PCR was performed using the specific primers RmLic16ADNAF and RmLic16ADNAR (Table 1). The β-1,3−1,4-glucanase cDNA sequence from *R. miehei* CAU432 was deposited in the GenBank nucleotide sequence database under accession number JQ088103.

Nucleotide and deduced amino acid sequences were analyzed using the ExPASy Proteomics tools (http://www.expasy.ch/tools/). Database homology searches of nucleotide sequences obtained were carried out using BLAST in GenBank at the NCBI. The amino acid sequences were aligned using the ClustalW program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalW/). The signal peptide was analyzed by the Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP). *N*-Glycosylation sites were predicted using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

RESULTS AND DISCUSSION

Production of β-1,3−1,4-Glucanase from *R. miehei* CAU432. The fermentation conditions for β-1,3−1,4-glucanase production by *R. miehei* CAU432 were optimized by single-factor experiment. The highest β-1,3−1,4-glucanase activity of 6230 U/mL was obtained on the fifth day when 3.0% (w/v) oat flour was used as the main carbon source under the optimized conditions (Figure 1A). Zymogram and SDS−PAGE (Figure 1B) analysis showed that the major protein in the crude sample was a β-glucanase with a molecular weight of 35.4 kDa.

The newly isolated thermophilic fungus *R. miehei* CAU432 is an excellent microbial β-1,3−1,4-glucanase producer. To date, 6230 U/mL of β-1,3−1,4-glucanase produced by *R. miehei* CAU432 reported in this study is the highest level of β-1,3−1,4-glucanase production by microorganisms in a shake flask. The enzyme activity is much higher than those of β-1,3−1,4-glucanases from other microorganisms such as *Paecilomyces thermophila* (135.6 U/mL),[12] *Talaromyces emersonii* CBS814.70 (20.3 U/mL),[7] and *Bacillus subtilis* (251 U/mL).[5] Thus, *R. miehei* CAU432 exhibits high potential for β-1,3−1,4-glucanase production in an industrial scale.

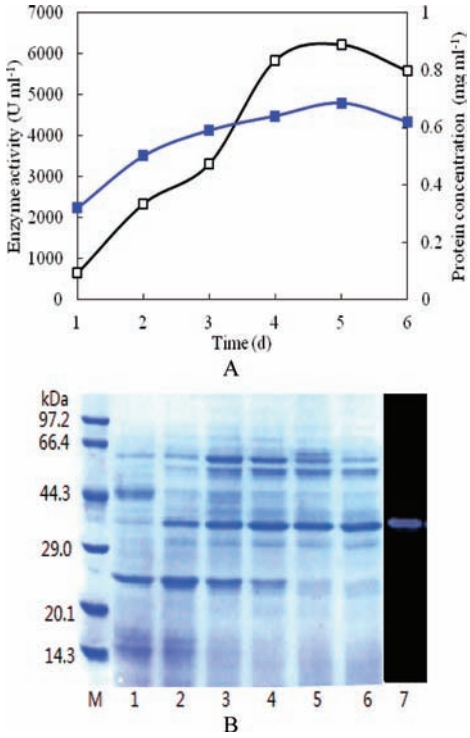

Figure 1. Time course (A) and SDS−PAGE (B) analysis of the β-1,3−1,4-glucanase produced by *Rhizomucor miehei* CAU432. (□), protein concentration; (■), β-1,3−1,4-glucanase activity. Lane M, low molecular weight calibration kit; lanes 1−6, SDS−PAGE profile of crude culture supernatants at 1−6 days, respectively; lane 7, zymogram of the crude supernatant after 4 days of fermentation.

Purification of β-1,3−1,4-Glucanase. The β-1,3−1,4-glucanase was purified to apparent homogeneity with a recovery yield of 7.1% (Table 2). The specific activity of the

Table 2. Purification of β-1,3-1,4-Glucanase from *Rhizomucor miehei* CAU432

purification step	total activity (U)[a]	protein (mg)[b]	specific activity (U/mg)	purification factor (-fold)	recovery (%)
culture supernatant	72,4125	170	4,259.5	1	100
$(NH_4)_2SO_4$ precipitation	392,809	42	9,341.1	2.2	54.9
DEAE 52	167,997	11.1	15,179.3	3.6	23.2
Q-Sepharose Fast Flow	51,969	1.8	28,871.7	6.8	7.1

[a]Activity was measured in 50 mM citrate buffer (pH 6.0) at 60 °C using barley β-D-glucan as the substrate. [b]The protein was measured by the Lowry method,[20] using BSA as the standard.

enzyme was increased by 6.8-fold from 4260 U/mg to 28872 U/mg. The purified enzyme migrated as a single band on SDS−PAGE with an apparent molecular mass of 35.4 kDa (Figure 2). The native molecular mass of the enzyme was estimated to be 33.7 kDa by gel filtration chromatography on a Superdex-75 column, indicating that it is a monomer.

Generally, the molecular masses of β-1,3−1,4-glucanases from fungi tend to be larger than those from *Bacilli*.[7] Most of the fungal β-1,3−1,4-glucanases are monomeric proteins, having a molecular mass in the range of 27 to 41 kDa. In contrast, a thermostable β-1,3−1,4-glucanase from *Laetiporus sulphureus* var. *miniatus* is a dimer of 52 kDa.[10] The molecular mass (35.4 kDa) of the purified

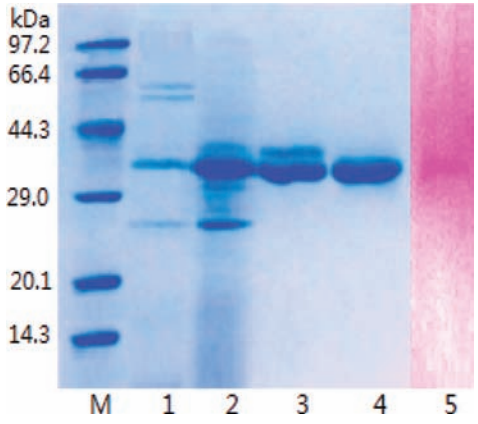

Figure 2. Purification of the β-1,3−1,4-glucanase from *Rhizomucor miehei* CAU432. Lane M, low molecular weight calibration kit; lane 1, crude enzyme; lane 2, after ammonium sulfate precipitation (40−60%); lane 3, after DEAE 52; lane 4, after Q-Sepharose Fast Flow; lane 5, periodic acid Schiff staining of the purified β-1,3−1,4-glucanase.

β-1,3−1,4-glucanase in the present study is smaller than that of the β-1,3−1,4-glucanases from *T. emersonii* CBS 814.70 (40.7 kDa),[7] *Paecilomyces thermophila* (38.6 kDa),[12] and *Rhizopus microsporus* var. *microspores* (36.5 kDa)[8] but larger than that of the β-1,3−1,4-glucanases from some fungi such as *Orpinomyces* sp. (27 kDa),[26] *Cochliobolus carbonum* (29.5 kDa),[27] and *Aspergillus japonicas* (28 kDa).[28] Periodic acid-Schiff reagent staining on SDS−PAGE showed that the enzyme was a glycoprotein (Figure 2). The total carbohydrate content of the enzyme was about 26.0% (w/w). Most fungal β-glucanases have been reported to be glycoproteins.[7,12,29] The carbohydrate content of the enzyme in the present study is much lower than that of the enzyme from *Talaromyces emersonii* CBS 814.70 (77%)[7] but higher than that of other fungal β-1,3−1,4-glucanases from *Paecilomyces thermophila* (19%)[12] and *Thermoascus aurantiacus* (5.2%).[29]

The purified β-1,3−1,4-glucanase from *R. miehei* CAU432 exhibited a remarkable specific activity (28817.7 U/mg) against barley β-glucan, which is much higher than that of the β-1,3−1,4-glucanases from other microorganisms including bacteria with the specificity activity ranging from 1.57 to 10800 U/mg, such as *Fibrobacter succinogenes* (10800 U/mg),[30] *Bacillus subtilis* GN156 (555.1 U/mg),[31] and *Bacillus licheniformis* (1280 U/mg),[32] and fungi such as *Rhizopus microsporus* var. *microspores* (12.6 U/mg),[8] *Laetiporus sulphureus* var. *miniatus* (29 U/mg),[10] *Paecilomyces thermophila* (12300 U/mg),[12] and *Orpinomyces* sp. (3659 U/mg).[26] The outstanding specific activity of the β-1,3−1,4-glucanase from *R. miehei* CAU432 renders it potentially suitable for use in food and poultry industries.

Identification of the Purified β-1,3−1,4-Glucanase. β-1,3−1,4-Glucanase was subjected to SDS−PAGE, and the purified band was excised and submitted for the identification of partial amino acid sequences, as analyzed by Q-TOF2. Amino acid sequences of six internal peptide fragments were obtained. The number of amino acids for each peptide, I, II, III, IV, V, and VI, were 12, 9, 17, 15, 8 and 19, respectively. All six internal peptides were subjected to the NCBI-BLAST database. A comparison of the amino acid sequences of peptide fragments derived from *R. miehei* CAU432 with other known β-glucanases are shown in Table 3. A sequence alignment of peptide V showed a high degree of identity (100%) with some β-glucanases from *Trichophyton equinum*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Trichophyton verrucosum*, *Arthroderma gypseum*, *Arthroderma benhamiae*, and *Arthroderma otae*. Peptide I showed 80% and 73% identity with the β-glucanases from *Aspergillus niger* and *Aspergillus clavatus*, respectively. However, peptides II, III, IV, and VI did not show any sequence similarities with other β-glucanases. Thus, it is probably a novel β-1,3−1,4-glucanase.

Characterization of the Purified β-1,3−1,4-Glucanase. The purified β-1,3−1,4-glucanase displayed an optimal activity at pH 5.5 (Figure 3A). It retained more than 90% of its activity after being treated in various buffers with a pH range of 5−7.5 (Figure 3B) at 60 °C for 30 min. The optimal temperature of the purified β-1,3−1,4-glucanase was determined to be 60 °C in 50 mM pH 5.5 citrate buffer (Figure 4A). The enzyme exhibited good thermostability since almost no activity was lost when it was incubated at 60 °C for 30 min (Figure 4B). The denaturing half-lives of the enzyme at 60, 70, 75, 80, and 85 °C were 225, 101, 64, 53, and 27 min, respectively (Figure 4C).

The optimal pH (pH 5.5) of β-1,3−1,4-glucanase from *R. miehei* CAU432 is within the pH range of the β-1,3−1,4-glucanases from most other fungi (pH 4.0−7.0), such as *Orpinomyces* sp.,[26] *Cochliobolus carbonum*,[27] *Talaromyces emersonii*,[7] *Paecilomyces thermophila*,[12] and *Rhizopus microspores* var. *microspores*.[8] However, the optimal pH value is lower than

Table 3. Comparison of Amino Acid Sequences of Internal Peptides from β-1,3-1,4-Glucanase of *Rhizomucor miehei* CAU432 with Other Fungal β-Glucanases

sources	position[a]	sequence[b]	identity (%)	accession no.
peptide I		TYNSGLFLLDVK		
Aspergillus niger	432	TYNHGLFILDLE	80	CAK36992.1
Aspergillus clavatus	388	TYHSGLFLFDII	73	EAW10763.1
peptide II[c]		TLDTQLPGR		
peptide III[c]		LSRRMLAVG		
		ATGVVPVK		
peptide IV[c]		SVMTNVTP		
		NLTLLGR		
peptide V[c]		PDGRPSVR		
Trichophyton equinum	348	PDGRPSVR	100	EGE04671.1
Arthroderma otae	354	PDGRPSVR	100	EEQ29013.1
peptide VI		ALFTGRLLAVGGGTLVMK		

[a]Position is the first amino acid of the peptide sequence. [b]Identical amino acids are underlined. [c]Amino acid sequences of peptides II, III, IV, and VI displayed no similarities with other reported β-glucanases.

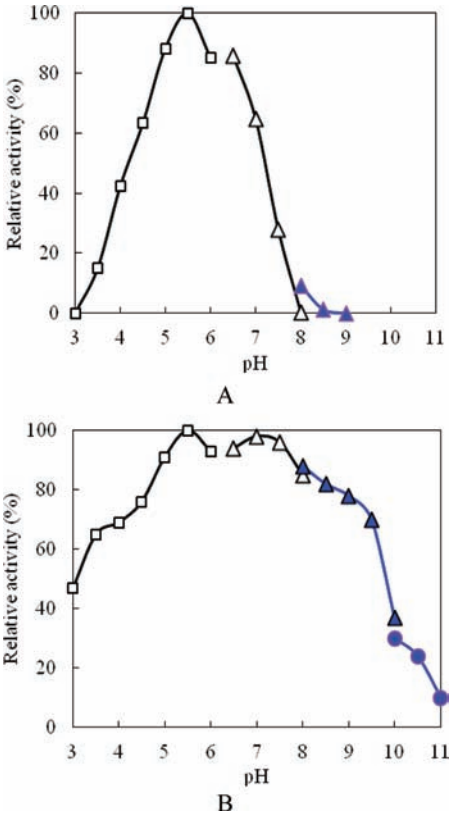

Figure 3. Optimal pH (A) and pH stability (B) of the purified β-1,3−1,4-glucanase from *Rhizomucor miehei* CAU432. Buffers used were citrate (□), MOPS (△), CHES (▲), and CAPS (●).

those of most bacterial β-1,3−1,4-glucanases which exhibit pH optima around neutrality (pH 6.0−7.5).[4]

Generally, the optimal temperature of most reported β-1,3−1,4-glucanases range from 40 to 65 °C.[4,6,8,26] Though the enzyme in the present study exhibited optimal activity at 60 °C, which is a little lower than those of β-1,3−1,4-glucanases from *Paecilomyces thermophila*[12] and *Laetiporus sulphureus* var. *miniatus*,[10] it showed excellent thermostability at relatively high temperatures (80 °C, half-life, 53 min) when compared to that of other enzymes. Most of the fungal β-1,3−1,4-glucanases are unstable at 80 °C, such as the enzymes from *Rhizopus microsporus* var. *microspores* (<1 min),[8] *Paecilomyces thermophila* (13 min),[12] and *Talaromyces emersonii* CBS 814.70 (25 min).[7] However, the β-1,3−1,4-glucanases from *Laetiporus sulphureus* var. *miniatus* are stable at 80 °C, with a half-live of 60 min.[10] Thermostability is an important criterion of enzymes, especially for application in the brewing and feed industry. Malting (50 °C −70 °C) or feed pelleting processes (65 °C −90 °C) usually require enzymes that can endure extreme conditions, especially high operation temperature.[10] Thus, the β-1,3−1,4-glucanase from *R. miehei* CAU432 may be a desirable enzyme from an industrial point of view.

The effect of various cations and compounds at 4 mM on the activity of β-1,3−1,4-glucanase was tested (data not shown). The activity was strongly inhibited by Cr^{2+} (11%), Ag^{+} (52%), and Fe^{3+} (57%), and moderately inhibited by Mn^{2+} (67%), β-mercaptoethanol (73%), EDTA (74%), Cu^{2+} (78%), Sr^{2+} (83%), K^{+} (89%), Zn^{2+} (89%), and Ni^{2+} (90%). However, the enzyme activity was enhanced by SDS (109%) and Mg^{2+} (105%). The β-1,3−1,4-glucanase from *Laetiporus sulphureus* var. *miniatus*[10] shows similar sensitivity to the divalent metal ion copper and zinc. In addition, the purified enzyme was moderately inhibited by the metal

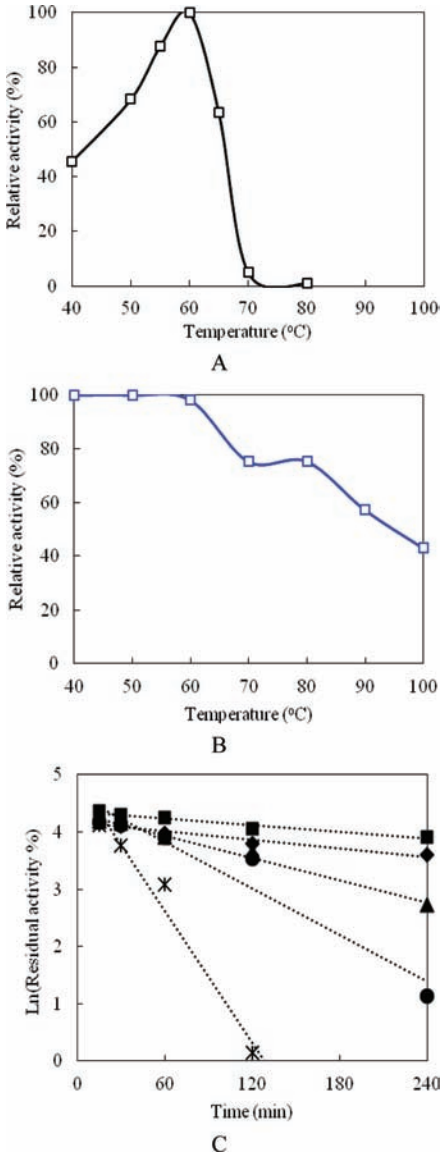

Figure 4. Optimal temperature (A), thermostability (B), and half-lives (C) of the purified β-1,3−1,4-glucanase from *Rhizomucor miehei* CAU432. For estimation of denaturing half-life, the enzyme was preincubated at 60 °C (■), 70 °C (♦), 75 °C (▲), 80 °C (●), and 85 °C (*) in 50 mM pH 5.5 citrate buffer for 4 h.

chelator, EDTA, and β-mercaptoethanol, indicating that metal ions are required and that disulfide linkages may be involved in catalytic activity.

Substrate Specificity and Kinetic Parameters of the Purified β-1,3−1,4-Glucanase. The specific activities of the purified β-1,3−1,4-glucanase toward different substrates were determined (Table 4). The highest activity of 33476 U/mg was observed with barley β-glucan as the substrate followed by lichenan (31888 U/mg, 95.2%). β-1,3−1,4-Glucanase showed very low activity toward laminarin (231 U/mg, 0.6%) and did not act on pullulan, birchwood xylan, soluble starch, LBG, CMC, *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-fucopyranoside, *p*NP-β-mannopyranoside, and *p*NP-β-D-galactopyranoside. Thus, the enzyme should be classified as a β-1,3−1,4-glucanase based on its substrate specificity. The K_m and k_{cat} values of the β-1,3−1,4-glucanase for barley β-glucan and lichenan were 2.0 mg/mL and

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Table 4. Substrate Specificity of the Purified β-1,3-1,4-Glucanase from *Rhizomucor miehei* CAU432[a]

substrate	major linkage type (ratio of linkage types)	specific activity (U/mg)[b]	relative activity (%)
barley β-D-glucan	β-1,3; β-1.4 (1:2.3−2.7)	33476 ± 1150	100
lichenan	β-1,3; β-1,4 (1:2)	31888 ± 1080	95.2
laminarin	β-1,3; β-1,6 (7:1)	213 ± 8	0.6

[a]The enzyme was inactive on birchwood xylan, CMC, pullulan, soluble starch, LBG, cellulose, Avicel, β-1,3-glucan, *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, and *p*NP-β-D-galactopyranoside. [b]Data represent the mean and standard deviation from three separate experiments.

1145 s^{-1}, and 1.4 mg/mL and 664.3 s^{-1}, respectively (data not shown).

The β-1,3−1,4-glucanase from *R. miehei* CAU432 is similar to most other fungal β-1,3−1,4-glucanases, which exhibit strict substrate specificity toward barley β-glucan and lichenan while showing little or no activity toward laminarin such as the β-1,3−1,4-glucanases from *Cochliobolus carbonum*,[27] *Rhizopus microsporus* var. *microspores*,[8] *Aspergillus japonicas*,[28] *Melanocarpus* sp.,[9] and *Paecilomyces thermophila*.[12] However, it differs from the β-1,3−1,4-glucanase from *Laetiporus sulphureus* var. *miniatus*,[10] which exhibited activity toward laminarin, giving a relative activity of 15%. It is noteworthy that the ratio of laminarin hydrolyzing activity to β-1,3−1,4-glucanase activity of the β-1,3(4)-glucanase from *R. miehei* DSM 1330 is high (up to 33.2%),[15] while the present enzyme showed negligible activity toward laminarin (0.6%).

The K_m values of the purified enzyme for barley β-glucan and lichenan are similar to that of the β-1,3−1,4-glucanases produced by various *Bacillus* species[4] and the fungus *Paecilomyces thermophila*.[12] Meanwhile, the K_m of the purified enzyme for barley β-glucan is much lower than that of the β-1,3−1,4-glucanase from *Rhizopus microspores* var. *microspores* (22.39 mg/mL)[8] and slightly higher than those of the β-1,3−1,4-glucanases from *Laetiporus sulphureus* var. *miniatus*[10] and *Orpinomyces* sp.[26]

Hydrolysis Properties of the Purified β-1,3−1,4-Glucanase. The hydrolytic products of barley β-glucan and lichenan by the purified β-1,3−1,4-glucanase from *R. miehei* CAU432 were analyzed on TLC (Figure 5). When barley β-glucan was used as the substrate, the enzyme produced trisaccharide and tetrasaccharide as the predominant products as well as a series of other higher degree of polymerization (DP). Meanwhile, when lichenan was hydrolyzed, the hydrolysis product was mainly trisaccharide (Figure 5B). In addition, the releasing rate of reducing sugar during the hydrolysis did not proceed linearly with respect to time, which suggested an endotype of action mode. Thus, the enzyme is expected to be an endo-β-1,3−1,4-glucanase.

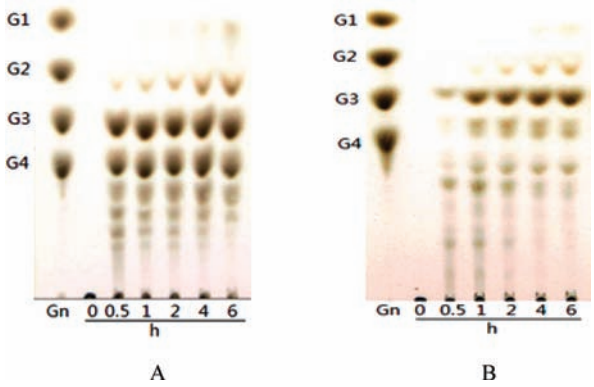

Figure 5. TLC analysis of hydrolytic products of barley β-D-glucan (A) and lichenan (B) by purified β-1,3−1,4-glucanase. Substrates and incubation times (h) are indicated. Glucose (G_1), cellobiose (G_2), cellotriose (G_3), and cellotetraose (G_4) were used as the standards (G_n).

Most endo-β-1,3−1,4-glucanases hydrolyze β-1,4 linkages adjacent to β-1,3 glycosidic bonds, yielding mainly disaccharide, trisaccharide, and tetrasaccharide.[3,12,26] The main hydrolysis product of barley β-glucan and lichenan by *R. miehei* β-1,3−1,4-glucanase was not disaccharide, which is different from other reported β-1,3−1,4-glucanases.[7,12,28]

Cloning of the Full-Length β-Glucanase cDNA and Sequence Analysis. A partial gene of *R. miehei* CAU432 was amplified by PCR using degenerate primers of RmLic16ACP1 and RmLic16ACP2 (Table 1). The 440-bp amplified fragment had the highest sequence identity (66%) with a glycosyl hydrolase (GH) family 16 β-glucanase gene from *Neosartorya fischeri* (XM001265138). The 5′ and 3′ RACE yielded 648-bp and 364-bp DNA fragments, respectively (data not shown). After sequence assembling, the putative full-length β-glucanase cDNA (*RmLic16A*) of 1131-bp was obtained with an open reading frame (ORF) of 942-bp (Figure 6). There are two introns of 70 and 69 bp, respectively, in the *RmLic16A* coding region. The full-length ORF of *RmLic16A* contains a sequence for a predicted signal peptide of 20 residues and a catalytic domain belonging to GH16 β-glucanase. The deduced 294-amino acid protein has a predicted molecular mass of 31,564 Da and a deduced p*I* of 3.90. The protein sequence contains two possible N-glycosylation sites at 72 (NKT) and 275 (NPT), respectively. Four internal peptide sequences of the enzyme (Table 3) completely corresponded to the deduced amino acid sequence from *RmLic16A*, supporting the idea that the cloned gene encodes the characterized β-1,3−1,4-glucanase from *R. miehei* CAU432. The deduced amino acid sequence of *RmLic16A* showed 50% identity to the characterized β-1,3−1,4-glucanase (ADK55597.1) from *Paecilomyces thermophila*, while displaying the higher identities to putative β-1,3(4)-glucanases from other fungi, such as *Rhizopus oryzae* (AAQ20798.1), *Aspergillus flavus* (EED55723.1), *Aspergillus fumigatus* (EAL88240.1), *Neosartorya fischeri* (EAW23242.1), *Aspergillus clavatus* (EAW08208.1), and *Talaromyces stipitatus* (EED16874.1) at 59%, 56%, 54%, 53%, 53%, and 52%, respectively. However, its eight Cys residues (Cys70, Cys132, Cys150, Cys163, Cys231, Cys248, Cys264, and Cys268) were present in RmLic16A, which might have a role in maintaining the stability of the β-1,3−1,4-glucanase by forming an S−S bridge in the molecule, and they were conserved in all the β-1,3(4)-glucanases, excepted two Cys residues from *Rhizomucor miehei* and *Rhizopus oryzae*, which are not at the same position with other β-glucanases.

In conclusion, the thermophilic fungus *R. miehei* CAU432 has the ability to secrete an outstanding amount of extracellular β-1,3−1,4-glucanase (6230 U/mL), and the purified β-1,3−1,4-glucanase exhibited a remarkably high specific activity of 28871.7 U/mg. The enzyme was a single subunit glycoprotein, with a molecular mass of 35.4 kDa. The optimal pH and temperature of the enzyme were pH 5.5 and 60 °C, respectively, and it exhibited good thermostability at high temperatures, up to 80 °C. Meanwhile, the purified enzyme displayed strict specificity for β-1,3−1,4-glucans. The action mode on barley β-glucan and lichenan revealed the endoacting nature of the purified β-1,3−1,4-glucanase. The properties of the enzyme make it a suitable candidate for industrial applications in food and poultry.

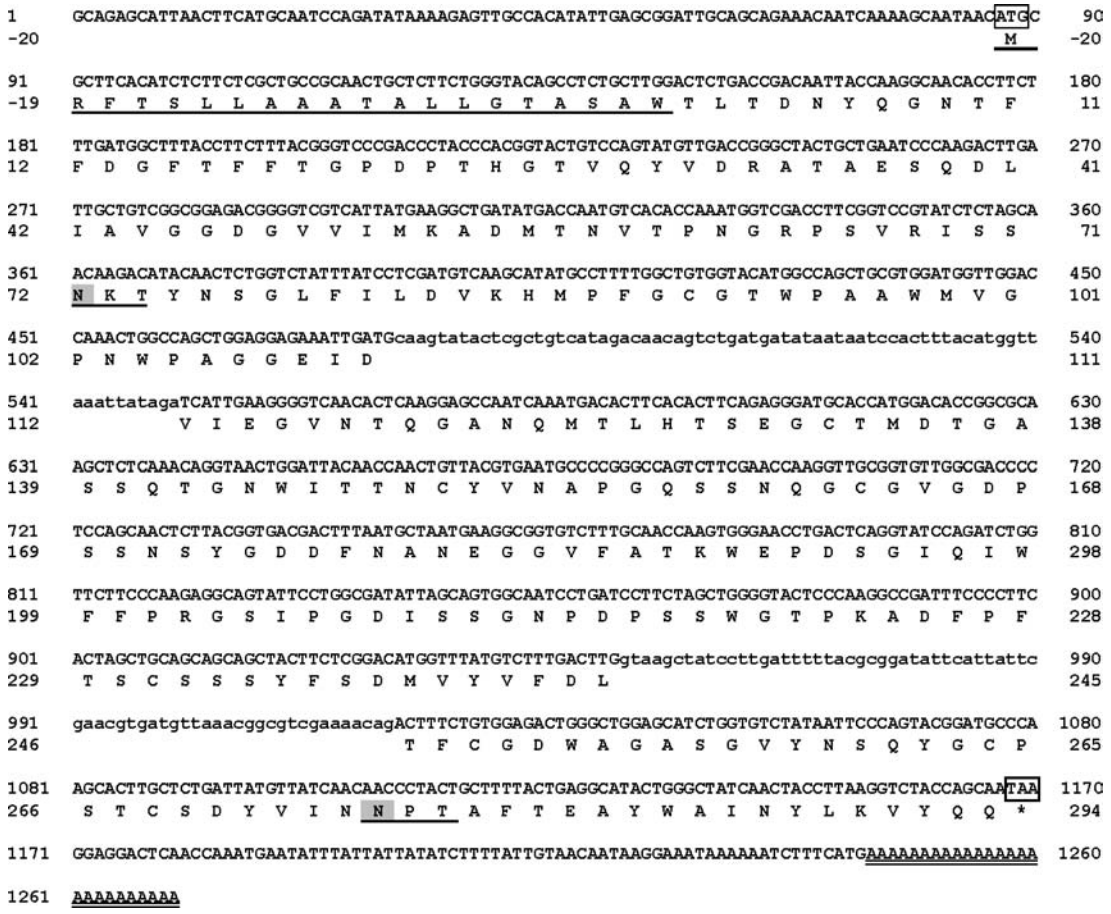

Figure 6. Full-length cDNA sequence and the deduced AA sequence of the RmLic16A. The RmLic16A mRNA has an open reading frame of 942-bp, which yields a 314-AA protein. The predicted AA sequence is presented in single letter code. The putative signal peptide is underlined. The translational initiation codon (ATG) and termination codon (TAA) are boxed. The asterisk indicates the stop codon. Two intron sequences are shown in lowercase letters and indicated by dotted underlining. The poly (A+) tail is double underlined. The nucleotide sequence reported here has been submitted to Genbank and has been given accession number JQ088103.

AUTHOR INFORMATION

Corresponding Author

*Post Box 294, China Agricultural University, No.17 Qinghua Donglu, Haidian District, Beijing 100083, China. Phone: +86 1062737689. Fax: +86 1082388508. E-mail: zhqjiang@cau.edu.cn

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

BSA, bovine serum albumin; CAPS, (cyclohexylamino)-1-propanesulhinic acid; CHES, 2-(cyclohexylamino) ethanesulfonic acid; CMC, carboxymethylcellulose; DNS, dinitrosalicylic acid; DP, degree of polymerization; EDTA, ethylenediaminetetracetic acid; LBG, locust bean gum; MOPS, 3-(*N*-morpholin)-propane sulfonic acid; RmLic16A, a glycosyl hydrolase (GH) family 16 β-1,3−1,4-glucanase from *Rhizomucor miehei* CAU432; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

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