JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biochemical Characterization of a Novel Thermostable β-1,3-1,4-Glucanase (Lichenase) from *Paecilomyces thermophila*

SHAOQING YANG,[†] YAN QIAOJUAN,[‡] ZHENGQIANG JIANG,*[,†] GUANGSEN FAN,[†] AND LAN WANG[†]

Department of Biotechnology, College of Food Science and Nutritional Engineering, and Bioresource Utilization Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

The purification and characterization of a novel extracellular β-1,3-1,4-glucanase from the thermophilic fungus *Paecilomyces thermophila* J18 were studied. The strain produced the maximum level of extracellular β-glucanase (135.6 U mL^{-1}) when grown in a medium containing corncob (5%, w/v) at 50 °C for 4 days. The crude enzyme solution was purified by 122.5-fold with an apparent homogeneity and a recovery yield of 8.9%. The purified enzyme showed as a single protein band on SDS-PAGE with a molecular mass of 38.6 kDa. The molecular masses were 34.6 kDa and 31692.9 Da when detected by gel filtration and mass spectrometry, respectively, suggesting that it is a monomeric protein. The enzyme was a glycoprotein with a carbohydrate content of 19.0% (w/w). Its N-terminal sequence of 10 amino acid residues was determined as $H_2N-A(?)GYVSNIVVN$. The purified enzyme was optimally active at pH 7.0 and 70 °C. It was stable within pH range 4.0−10.0 and up to 65 °C, respectively. Substrate specificity studies revealed that the enzyme is a true β-1,3-1,4-D-glucanase. The K_m values determined for barley β-D-glucan and lichenan were 2.46 and 1.82 mg mL^{-1}, respectively. The enzyme hydrolyzed barley β-D-glucan and lichenan to yield bisaccharide, trisaccharide, and tetrasaccharide as the main products. Circular dichroism studies indicated that the protein contains 28% α-helix, 24% β-sheet, and 48% random coil. Circular dichroism spectroscopy is also used to investigate the thermostability of the purified enzyme. This is the first report on the purification and characterization of a β-1,3-1,4-glucanase from *Paecilomyces* sp. These properties make the enzyme highly suitable for industrial applications.

KEYWORDS: Characterization; circular dichroism; β-glucanase; lichenase; *Paecilomyces thermophila*; purification; secondary structure; thermostable

INTRODUCTION

The major cell wall polysaccharide in the endosperm of cereals is a linear β-1,3-1,4-D-glucan (β-glucan), which accounts for up to 5.5% of the dry weight of grains (*1*). More specifically,the β-1,4-linked glucose chain is interrupted with β-1,3-linkages in the cereal β-glucan structure (*2*). Endoglucanases that depolymerize β-1,3-1,4-D-glucans can be grouped into four main categories: (a) specific β-1,3-1,4-D-glucanases or true lichenases (β-1,3-1,4-D-glucan-4-glucanohydrolase or lichenase; EC 3.2.1.73) that strictly cleave β-1,4-glycosidic linkages adjacent to a 3-O-substituted glucose residue but are inactive against β-1,4-glucans; (b) endo-β-1,4-D-glucanases (endocellulase; EC 3.2.1.4), which hydrolyze β-1,4-glycosidic bonds other than those targeted by lichenases; (c) β-1,3(4)-D-glucanases (EC 3.2.1.6) active on β-1,3-1,4-D-glucans and β-1,3-D-glucans; and (d) β-1,3-D-glucanases (β-1,3-glucan-3-glucanohydrolase or laminarinase; EC 3.2.1.39) (*3–7*). Among these, β-1,3-1,4-glucanases are important biotechnological aids in the brewing and animal feed stuff industries. The addition of exogenous β-1,3-1,4-glucanases can reduce the negative effects of barley β-glucan during mashing in the brewing industry and improve the β-glucan digestibility in poultry feed stuffs (*8–10*).

Most of the β-1,3-1,4-glucanases have been identified from many *Bacillus* species (*11–15*), some rumen bacteria (*9, 16, 17*), and a number of plant tissues (*3, 18*). Extracellular β-1,3-1,4-glucanases have been purified from several fungi such as *Orpinomyces* sp. (*19*), *Cochliobolus carbonum* (*20*), *Talaromyces emersonii* (*4*), *Aspergillus japonicus* (*21*), *Rhizopus microsporus* var. *microsporus* (*10*), and *Trichoderma koningii* (*22*). So far, little is known about fungal β-1,3-1,4-glucanases, their mode of action or specificity (*4, 6, 10, 23*). No previous literature reports could be found that describe the production, purification, or characterization of a β-glucan degrading enzyme from *Paecilomyces* sp. (*4, 10, 19, 24*).

* To whom correspondence should be addressed. Phone: +86 10 62737689. Fax: +86 10 82388508. E-mail: zhqjiang@cau.edu.cn.
[†] College of Food Science and Nutritional Engineering.
[‡] College of Engineering.

10.1021/jf800303b CCC: $40.75
Published on Web 06/11/2008

The industrial use of thermostable enzymes has attracted the interest of many research groups during the past few years (*5, 23, 25*). The newly isolated thermophilic fungus *Paecilomyces thermophila* J18 is known as a good source of xylanase (*26*). Previous work from our laboratory reported the purification and characterization of a thermostable xylanase and β-glucosidase from this organism (*24, 27*). Further, it secreted an extracellular β-1,3-1,4-glucanase (data not shown). Hence, we describe the purification and biochemical properties of a β-1,3-1,4-glucanase from the strain, which is characterized by its noteworthy thermostability and selective preference for β-1,3-1,4-glucans in the present study.

MATERIALS AND METHODS

Materials. Barley β-D-glucan, lichenan (from *Cetraria islandica*), laminarin (from *Laminaria digitata*), pullulan (from *Aureobasidium pullulans*), cellulose, birchwood xylan, carboxymethylcellulose (CMC, sodium salt, low viscosity), cellobiose, and gentibiose were obtained from Sigma Chemical Company (St. Louis, MO). DEAE 52 (preswollen microgranular anion exchange celluloses) was from Whatman (Whatman Inc., Fairfield, NJ). Q-Sepharose Fast Flow was from Pharmacia (Pharmacia, Uppsala, Sweden). The *p*-nitrophenyl (*p*NP) substrates *p*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-fucopyranoside, *p*NP-β-mannopyranoside, and *p*NP-β-D-galactopyranoside were also purchased from Sigma. β-1,3-Glucan (from *Euglena fracilis*) and hydroxyethyl-cellulose were purchased from Fluka Chemical Co. (Steinheim, Germany). Avicel was obtained from Merck Ltd. (Darmstadt, Germany). All other chemicals used were analytical grade reagents unless otherwise stated.

Fungal Strain and Growth Conditions. *Paecilomyces thermophila* J18 was used in this investigation (*25*). Stock cultures were maintained on potato dextrose-agar (PDA) at 4 °C and were transferred every 6−7 weeks. PDA plates were incubated at 50 °C for 4−5 days and stored at 4 °C until use.

For β-glucanase production, the basal medium of culture contained (g L^{-1}) the following: corncob (40−60 mesh), 50; tryptone, 10; yeast extract, 10; $MgSO_4 \cdot 7H_2O$, 0.3; $FeSO_4$, 0.3; $CaCl_2$, 0.3. A piece (1 cm^2) of growing 4−5 day-old culture of the strain was used to inoculate the growth medium (60 mL) adjusted to pH 5.0 in 250 mL Erlenmeyer flasks. Triplicate cultures were shaken at 200 rpm for 5 days at 50 °C. After the cultures were centrifuged at 12 000*g* for 10 min, the supernatant was used as a crude enzyme for subsequent enzyme purification studies.

Enzyme Assay and Protein Determination. All enzyme assays, unless otherwise stated, were carried out at 50 °C for 10 min in 50 mM MES buffer, pH 6.5, in a reaction volume of 200 μL with a final substrate concentration of 0.25% (w/v). The hydrolysis of barley β-glucan was quantified by measuring reducing sugars released by the dinitrosalicyclic acid (DNS) method of Miller (*28*). The absorbance of the reaction mixture was determined at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars per minute under the above conditions. Protein concentrations were measured by the Lowry method (*29*) with bovine serum albumin (BSA) as the standard. The specific activity was expressed as units per milligram of protein.

Purification of β-Glucanase. The crude supernatant was subjected to 40−60% ammonium sulfate saturation. The precipitated protein was collected by centrifugation (10 000*g*) and dissolved in 20 mM phosphate buffer (pH 7.2). The crude enzyme was dialyzed against 20 mM phosphate buffer (pH 7.2). Further purification was performed by two steps of ion-exchange chromatographies. A 50 mL solution was applied to a DEAE 52 column (8 cm × 1.0 cm) pre-equilibrated with 20 mM phosphate buffer (pH 7.2). The bound β-glucanase was eluted with a gradient of 100−200 mM NaCl at a flow rate of 0.8 mL min^{-1}. The active fractions were combined and concentrated to 0.6 mL by ultrafilation using a 10 kDa membrane (Stirred Cell Model 8050, Millipore). The concentrated solution was applied to a Q-Sepharose Fast Flow column (8 cm × 1.0 cm) equilibrated with 25 mM phosphate buffer (pH 6.5). The unbound fractions with high β-glucanase activity were pooled. Its homogeneity was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

Estimation of the carbohydrate content of the purified enzyme was performed by the phenol−sulfuric acid method (*30*), with D-glucose as the standard. N-terminal sequences of the purified β-glucanase were determined using an automated Edman degradation using a PROCISE amino acid sequencer (Applied Biosystems, CA) at Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

SDS-PAGE, Zymogram, and Molecular Mass Determination. SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels, as described by Laemmli (*31*). Protein bands were visualized by coomassie brilliant blue R-250 staining. The standard used was the low molecular weight calibration kit for SDS electrophoresis (Amersham): phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Glycoprotein was detected by Periodic acid-Schiff staining of gels after the SDS-PAGE (*32*).

The location of β-glucanase activity in SDS-PAGE was determined by the zymogram, which was obtained by copolymerizing 0.2% barley β-glucan with 12.5% (w/v) polyacrylamide. Samples were treated with sample buffer in 0.5% SDS and 1% β-mercaptoethanol and boiled for 5 min before application to the gel. SDS-PAGE gels were washed with 25% (v/v) isopropanol in 20 mM phosphate buffer (pH 7.2) to remove SDS and β-mercaptoethanol. After a 20 min incubation period at 50 °C in a sealed chamber, the gel was stained with 0.05% (w/v) Congo red. Activity bands appeared as clear zones against a red background after destaining with 1 M NaCl. The addition of 0.1 M acetic acid caused the gel to turn blue, thereby enhancing the contrast between the activity bands (clear zones) and unhydrolyzed β-glucan (blue background).

For molecular mass determination by gel filtration, a Superdex 75 column (1.0 cm × 40.0 cm) was equilibrated with 50 mM phosphate buffer (pH 7.2) containing 50 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 kDa), each at 2 mg mL^{-1}. The flow rate for elution was 0.1 mL min^{-1}.

The purified β-glucanase (0.5 mg mL^{-1}) was analyzed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry with a autoflex Π mass spectrometer (Bruker Daltonik, Germany) in linear positive mode. The purified enzyme sample (10 μL) was mixed with 10 μL of a matrix solution of α-cyano-4-hydroxy-cinnamic acid (peptide/protein matrix kit) and applied to the MALDI plate.

Characterization of Purified β-glucanase. The optimum pH was determined by monitoring β-glucanase activity in six different buffers (50 mM) between pH 2.5 and 11.0: citrate buffer between pH 2.5 and 5.5; MES buffer between pH 5.0 and 7.0; phosphate buffer between pH 6.0 and 8.0; MOPS buffer between pH 6.0 and 8.5; CHES between pH 8.0 and 11.0; and CAPS between pH 9.0 and 11.0. To determine the pH stability of the enzyme, the purified β-glucanase was incubated in different buffers as mentioned above at 50 °C for 30 min, and then the remaining activities of these treated enzymes were measured by the standard assay procedure.

The effect of temperature on the enzyme activity was carried out at different temperatures (30−100 °C) in 50 mM MES buffer (pH 7.0). For thermostability determination, the purified β-glucanase in 50 mM MES buffer (pH 7.0) was incubated at different temperatures for 30 min. The thermostability of the enzyme was further studied at 60, 65, 70, 75, and 80 °C by incubating the enzyme for 2 h in 50 mM MES buffer (pH 7.0). Aliquots were withdrawn at different time intervals. After cooling the treated enzymes on ice for 30 min, the residual β-glucanase activities were measured according to the standard assay method.

The effects of various metal ions and reagents at 4 mM on the β-glucanase activities were determined by preincubating the enzyme with the individual reagent in 50 mM MES buffer (pH 7.0) at 22 °C for 30 min. Activities were then measured at 50 °C in the presence of

the metal ions or reagents. The activity assayed in the absence of metal ions or reagents was taken as 100%. The results presented are the average of three trials.

Specificity of Purified β-Glucanase and Kinetic Parameters. The specificity was determined using *p*NP-glycosides and polysaccharides. Activities toward *p*-nitrophenyl derivatives were measured by the rate of *p*-nitrophenol formed during hydrolysis from 5 mM of the substrates in 50 mM MES buffer (pH 7.0) at 50 °C for 10 min and detected by spectrophotometry at 405 nm. The β-glucanase activity on polysaccharides was determined using 1% (w/v) of substrate in 50 mM MES buffer (pH 7.0) at 70 °C for 10 min and by measuring the reducing sugars by the DNS method (*28*). Enzyme units were defined as the amount that produced 1 μmol of *p*-nitrophenol or reducing sugar per minute.

For a preliminary investigation of kinetic parameters, nine different concentrations for each substrate were prepared in 50 mM MES buffer (pH 7.0) and incubated with the purified β-glucanase at 50 °C for 5 min. The substrate concentrations were varied in the range 0.5−15 mg mL^{-1}. After five preliminary experiments, six different concentrations ranging from approximately 0.5 to 2.0 times the K_m values were ultimately used to determine initial hydrolysis rates. K_m, k_{cat}, and their respective standard errors were calculated using the nonlinear regression analysis program Grafit (*33*).

Hydrolysis Properties of Purified β-Glucanase. For the analysis of barley β-glucan or lichenan degradation products, 10 U of purified β-glucanase was incubated with 10 mg of each substrate in 1 mL of 50 mM MES buffer (pH 7.0) at 50 °C. Aliquots were periodically withdrawn, and the reaction was stopped by placing the samples in boiling water for 5 min. Products of enzymatic hydrolyses (5 μL) were analyzed qualitatively by thin-layer chromatography (TLC) on Kieselgel 60 plates (Merck) with a butan-1-ol−acetic acid−water (2:1:1, v/v) solvent system (*24*). 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, cellotetraose, and cellopentaose were used as the standards.

Circular Dichroism (CD) Measurements. Circular dichroism spectra were collected on a Jasco J-815 spectropolarimeter equipped with a temperature control Peltier system (JASCO International Co., Ltd., Japan). Far-UV measurements were performed with a protein concentration of 0.2 mg mL^{-1} in 5 mM phosphate (pH 7.0), in 1 mm path length cells. The secondary structure stability of the enzyme was monitored between 25 and 95 °C. Thermal-denaturation experiments were performed at constant heating rates of 1 °C min^{-1}, allowing temperature equilibration for 5 s before recording each spectrum. Blank spectra of buffer without protein obtained at identical conditions were subtracted. Spectra of the purified enzyme resulted from the accumulation of 5 scans that were subsequently averaged. Results are expressed as molar residue ellipticity (θ, deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight assuming average weight of 31692.9 Da for the enzyme. To estimate secondary structure, the database of CD spectra of proteins supplied with K2D program was used (*34*).

RESULTS AND DISCUSSION

Production of β-Glucanase from *Paecilomyces thermophila*. The production of β-glucanase by *Paecilomyces thermophila* J18 was studied in shake cultures using 3% (w/v) of several carbon sources such as corncob, wheat straw, wheat bran, barley β-D-glucan, lichenan, and CMC. The highest titer of β-glucanase (28.8 U mL^{-1}) was produced in a medium containing 3% corncob as the carbon source. Thus, corncob was selected as the main carbon source for further optimization of β-glucanase production. The result of a single-factor experiment revealed that 5.0% corncob, initial pH 5.0, and cultivation temperature of 50 °C were the optimal conditions. A maximum β-glucanase activity of 135.6 U mL^{-1} was obtained after 4 days of fermentation. SDS-PAGE (**Figure 1A**) and zymogram analysis (**Figure 1B**) of the crude supernatants showed a single clear band of β-glucanase activity corresponding to a minor protein band of 38 kDa at the incubation period of 3−5 days; it was subsequently identified as β-glucanase (i.e., β-1,3-1,4-glucanase or lichenase).

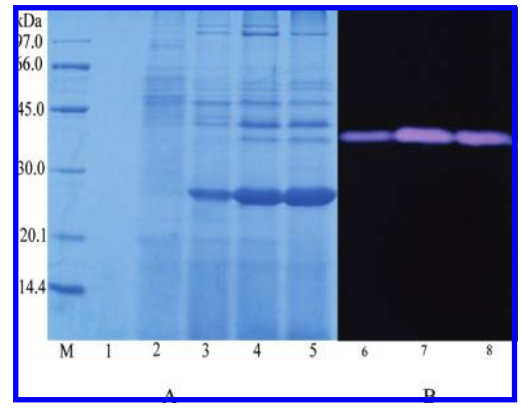

Figure 1. SDS-PAGE (**A**) and zymogram (**B**) of the crude enzyme produced by *Paecilomyces thermophila* J18. Lanes are as designated as follows: Lane M, low molecular weight calibration kit; lanes 1−5, SDS-PAGE profile of crude culture supernatants after 1−5 days, respectively; lanes 6−8, zymogram of crude culture supernatants after 3−5 days of fermentation.

Purification of β-Glucanase from *Paecilomyces thermophila*. The summary of the β-glucanase purification is presented in **Table 1**. The β-glucanase was purified by 122.5-fold with an apparent homogeneity and a recovery yield of 8.9% (**Table 1** and **Figure 2**). The purified enzyme migrated as a single band during SDS-PAGE with an apparent molecular mass of 38.6 kDa (**Figure 2**). The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Superdex 75 was around 34.6 kDa. This value is a little higher than that (31692.893 Da) determined by mass spectrometry analysis for this enzyme (data not shown). Isoelectric focusing (IEF-PAGE) of the purified β-glucanase indicated a p*I* of about 4.2 (data not shown).

The purified enzyme band stained with positive Periodic acid-Schiff indicated that it is a glycoprotein. As quantitatively determined by the phenol−sulfuric acid method (*30*), the total carbohydrate content of the purified enzyme was 19.0% (w/w). In the previous report, the xylanase and β-glucosidase from *P. thermophila* J18 were also glycolsylated (*24, 27*). The carbohydrate content of this enzyme is much lower than that (77%, w/w) of *Talaromyces emersonii* CBS 814.70 β-1,3-1,4-glucanase (*4*).

β-1,3,1-4-Glucanases with similar values of molecular mass have been purified from *Rhizopus microsporus* var. *microsporus* (36.5 kDa (*10*)) and *T. emersonii* CBS 814.70 (40.7 kDa (*4*)). In contrast, this value tends to be larger than those from the mesophilic fungi such as *Orpinomyces* sp. (27 kDa (*19*)), *Cochliobolus carbonum* (29.5 kDa (*20*)), *Aspergillus japonicus* (28 kDa (*21*)), and *Trichoderma koningii* (26.5 kDa (*22*)). *Bacillus* β-1-3,1-4-glucanases are usually monodomain proteins with molecular masses of 25−30 kDa (*12, 14, 15*). A high molecular weight lichenase (64.5 kDa) secreted by *Thermomonospora* sp. exhibits bifunctional activity toward lichenan and xylan (*35*). Besides, the purified enzyme exhibits a high specific activity, which was much higher than those of most of the reported β-1-3,1-4-glucanases (*4, 15, 16, 18, 21*).

The first 10 residues at the N terminus were H_2N−A(?) GYVSNIVVN. This sequence was scanned by using the BLAST program. The N-terminal sequence of 10 amino acid residues showed a high degree of identity with the N-terminal sequences of the hypothetical protein AN3511.2 from *Aspergillus nidulans*

Table 1. Summary of β-1,3-1,4-Glucanase Purification from *Paecilomyces thermophila* J18

purification step	total activity (U)[a]	protein (mg)[b]	specific activity (U mg^{-1})	purification factor (-fold)	recovery (%)
crude culture supernatant	54000	1060	50.9	1	100
ammonium sulfate precipitation (40−60%)	30672	23.8	1288.7	25.3	56.8
DEAE 52	10584	2.75	3848.7	75.6	19.6
Q-Sepharose Fast Flow	4806	0.77	6241.6	122.5	8.9

[a] Activity was measured in 50 mM MES buffer (pH 6.5) at 50 °C using barley β-glucan as the substrate. [b] The protein was measured by the Lowry method (*29*), using BSA as the standard.

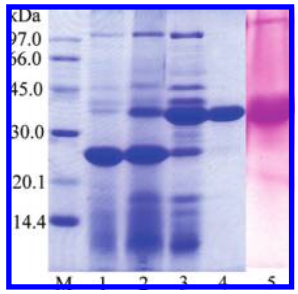

Figure 2. SDS-PAGE of proteins during purification of β-1,3-1,4-glucanase. Lanes are as designated as follows: Lane M, molecular mass standard proteins; lane 1, crude culture supernatant; lane 2, ammonium sulfate precipitation (40−60%); lane 3, after DEAE 52; lane 4, after Q-Sepharose Fast Flow; lane 5, Periodic acid-Schiff staining of the purified β-1,3-1,4-glucanase.

FGSC A4 (88%; accession no. EAA59072.1) and the putative endo-1,4-β-glucanase (77%; accession no. EAW17250.1) from *Neosartorya fischeri* NRRL181.

Effect of pH and Temperature on the Activity and Stability of β-Glucanase. The purified β-glucanase was most active at pH 7.0 (**Figure 3A**). It retained more than 90% of its activity at 50 °C for 30 min when tested in the pH range 4.0−10.0 (**Figure 3B**). The enzyme exhibited its optimal activity at 70 °C for a 10 min assay (**Figure 4A**). It was fairly stable up to 65 °C for a 30 min incubation (**Figure 4B**) and had 80.7% of its activity after a 30 min incubation at 70 °C. The rates of thermal inactivation of the purified β-1,3-1,4-glucanase were further investigated in the temperature range between 60 and 80 °C (**Figure 4C**). The plot of the log of the percent of remaining activity versus time is linear at the temperature range tested, indicating the first-order inactivation kinetics for the purified β-1,3-1,4-glucanase. The first-order rate constants for thermal inactivation at 70, 75, and 80 °C for the enzyme were calculated as 0.004, 0.015, and 0.055 min^{-1}, respectively. Almost no activity loss (5.5%) was observed at 60 °C by incubation at pH 7.0 for 2 h. At 60 °C, the enzyme had a half-life of 598 min. The enzyme was stable up to 65 °C with a half-life of 413 min. It was thermostable with half-lives of 174 and 47 min at 70 and 75 °C, respectively, but only 13 min at 80 °C. Therefore, the enzyme showed good thermostability.

The pH optimum is similar to that observed for most bacterial β-1-3,1-4-glucanases with pH optimum around neutrality (pH 6.0−7.5) (*11, 13, 14, 16*). However, it is higher (optima pH 4.0−6.0) than those characterized from rice (*Oryza sativa* L.) bran (*18*), *Orpinomyces* sp. (*19*), *Cochliobolus carbonum* (*20*), *Talaromyces emersonii* (*4*), and *Rhizopus microsporus* var. *microsporus* (*10*).

In general, temperature optima for most reported β-1-3,1-4-glucanases range from 40 to 65 °C (*10, 12, 14–17, 19, 20*). A β-1,3-1,4-glucanase (lichenase) from *Clostridium thermocellum* was reported to have an optimal temperature of 80 °C (*36*). Thermostability is an important criterion of enzymes, especially for cellulases and hemicellulases, with enormous potential

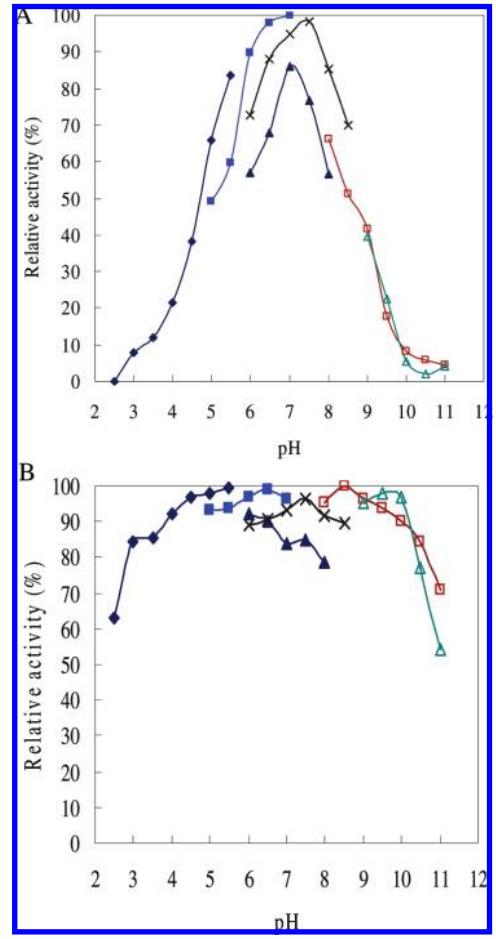

Figure 3. Optimal pH (**A**) and pH stability (**B**) of the purified β-1,3-1,4-glucanase from *Paecilomyces thermophila* J18. The influence of pH on β-glucanase activity was determined at 50 °C using 50 mM of different buffers: citrate (♦), MES (■), phosphate (▲), MOPS (×), CHES (□), CAPS (Δ). The remaining activity was measured after incubation for 30 min at 50 °C at different pH values.

applications in biotechnology and industry (*5, 23, 25*). The half-lives of the purified enzyme in the study at 60 and 80 °C were 598 and 13 min, respectively, which are comparable to the β-1-3,1-4-glucanase from *T. emersonii* CBS 814.70, which represents most thermostable fungal β-1−3,1-4-glucanases reported to date (*4*). The thermostability of the purified enzyme is higher than those of *Rhizopus microsporus* var. *microsporus*, *Bacteroides succinogenes*, and *Orpinomyces* strain PC-2 (*10, 16, 19*).

The effect of various cations and compounds at 4 mM was tested on the activity of β-glucanase (data not shown). Mn^{2+}, Cu^{2+}, and Hg^{2+} completely inhibited the enzyme activity, and SDS (88.2%), DTT (82.3%), β-mercaptoethanol (82.7%), and Na^{+} (80.3%) exhibited moderate inhibition, while β-glucanase activity was strongly inhibited by Ni^{2+} (11.5%), Zn^{2+} (11.6%), Fe^{2+} (14.9%), Fe^{3+} (13.5%), EDTA (43.4%), Na^{+} (47.3%), Sr^{2+} (49%), Mg^{2+} (52.9%), Ca^{2+} (55.5%), Ag^{+} (56.9%), and

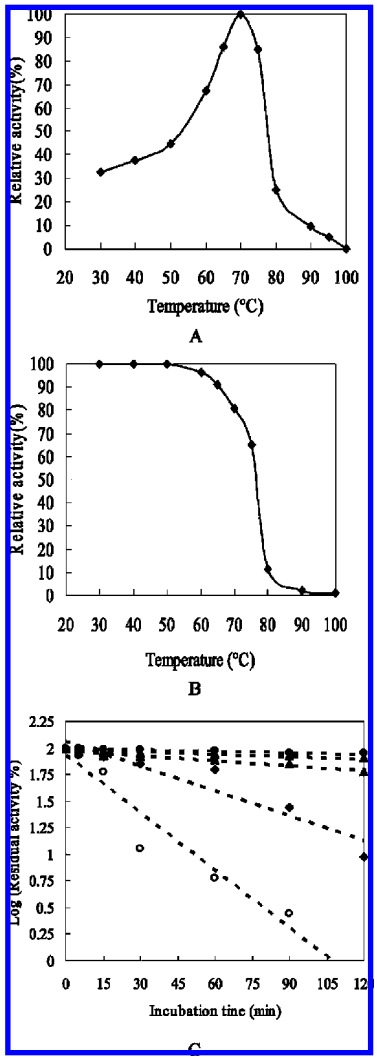

Figure 4. Optimal temperature (**A**), thermostability (**B**), and thermal inactivation (**C**) of the purified β-1,3-1,4-glucanase from *Paecilomyces thermophila* J18. To determine the optimal temperature, the activity was measured at different temperatures in 50 mM MES buffer (pH 7.0). For the thermostability, the residual activities of the treated β-glucanases were measured after a 30 min preincubation at different temperatures. Thermal inactivation of the purified β-1,3-1,4-glucanase was determined at 60 °C (●), 65 °C (■), 70 °C (▲), 75 °C (□), and 80 °C (○). 60 °C, $Y = -0.0005x + 1.9981$, $R^2 = 0.9712$; 65 °C, $Y = -0.0007x + 1.9881$, $R^2 = 0.9286$; 70 °C, $Y = -0.0016x + 1.9768$, $R^2 = 0.948$; 75 °C, $Y = -0.0077x + 2.0575$, $R^2 = 0.9036$; 80 °C, $Y = -0.018x + 1.9276$, $R^2 = 0.9239$.

Ag^+ (60.6%). The purified enzyme was highly sensitive to Cu^{2+}, Mn^{2+}, and Hg^{2+}. β-1,3-1,4-Glucanase produced by *R. microsporus* var. *microsporus* shows similar sensitivity to Cu^{2+} (*10*). On the contrary, it was activated by Co^{2+} (117%) and NBS (122.2%) in this study. *N*-bromosuccinimide (NBS) modification resulted in a significant increment of activity, suggesting that tryptophan residues are not involved in the enzyme active site.

Substrate Specificity and Kinetic Parameters of β-Glucanase. The purified β-glucanase was assayed for hydrolytic activity against a variety of substrates. The highest activity was observed with barley β-glucan (12 415 U mg^{-1}, 100%) followed by lichenan (6416 U mg^{-1}, 51.7%). β-Glucanase did not act toward Avicel, carboxymethylcellulose, cellulose, filter paper, β-1,3-glucan, hydroxyethyl-cellulose, laminarin, starch, pullulan, birchwood xylan, locust bean gum, cellobiose, and xylobiose.

Table 2. Kinetic Parameters for the Purified β-1,3-1,4-Glucanase[a]

substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mg mL^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (mg^{-1} s^{-1} mL)
barley β-glucan	9980.9 ± 300	2.46 ± 0.17	166.4	67.6
lichenan	4897.0 ± 382	1.82 ± 0.14	81.6	44.8

[a] Enzymatic reactions were carried out for 5 min at 50 °C in 50 mM MES buffer (pH 7.0).

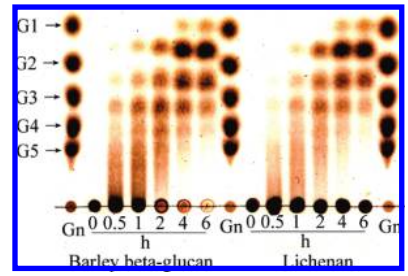

Figure 5. TLC analysis of products of barley β-glucan and lichenan hydrolysis by the purified β-1,3-1,4-glucanase. Substrates and incubation times (h) were indicated. Glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), and cellopentaose (G5) were used as the standards (Gn).

No activity was observed for *p*NP-β-glucopyranoside, *p*NP-β-xylopyranoside, *p*NP-β-fucopyranoside, *p*NP-β-galactopyranoside, and *p*NP-β-mannopyranoside. On the basis of the above, the enzyme may be classified as a true β-1,3-1,4-glucanase.

The enzyme exhibits strict preference for barley β-glucan and lichenan, which consist of mixed β-1,3-1,4-glycosidic linkages. No activity is detected against the other substrates tested. Most β-1,3-1,4-glucanases are also characterized by a strict substrate specificity (i.e., act on β-glucans with alternating β-1,3- and β-1,4-linkages but not on β-glucans containing only 1,3- or 1,4-glucosidic bonds *4, 10, 13, 14, 18, 19, 22, 36*). Several β-1,3-1,4-glucanases can hydrolyze laminarin or CMC at a low rate (*9, 17, 21*). In addition, some β-glucanases have an endohydrolytic mode of action with substrate specificity toward glucans that contain β-1,4 linkages, either alone (CMC) or as mixed linkage β-1,3-1,4-glucans (barley β-glucan and lichenan) (*5, 6, 37*).

The Michaelis−Menten constants were determined for barley β-glucan and lichenan (**Table 2**). The K_m and k_{cat} values were 2.46 mg mL^{-1} and 166.4 s^{-1} for barley β-glucan and 1.82 mg mL^{-1} and 81.6 s^{-1} for lichenan, respectively. A broad range of K_m values for barley β-glucan and lichenan has been reported from different sources (*10, 14, 16, 17*). K_m values of 1.2−1.5 mg mL^{-1} for barley β-glucan and 0.8−2 mg mL^{-1} for lichenan have been reported for the β-1,3-1,4-glucanases produced by the *Bacillus* species (*14*). The affinity constants for barley β-glucan and lichenan in this study are substantially lower than those of *Talaromyces emersonii* (*4*) and *Rhizopus microsporus* var. *microsporus* (*10*).

Hydrolysis Properties of the Purified β-Glucanase. The reaction products of the β-glucanase with barley β-glucan and lichenan were studied by TLC. Hydrolysis products formed during the action of the purified enzyme on barley β-glucan and lichenan are shown in **Figure 5**. When barley β-glucan and lichenan were used substrates, oligosaccharides were formed as the major products of hydrolysis after prolonged incubation with the enzyme, indicating that the enzyme may be classified an an endo-β-1,3-1,4-glucanase.

Most endo-β-1,3-1,4-glucanases (lichenase) cleave β-1,4 linkages adjacent to β-1,3 bonds in glucans, yielding chiefly

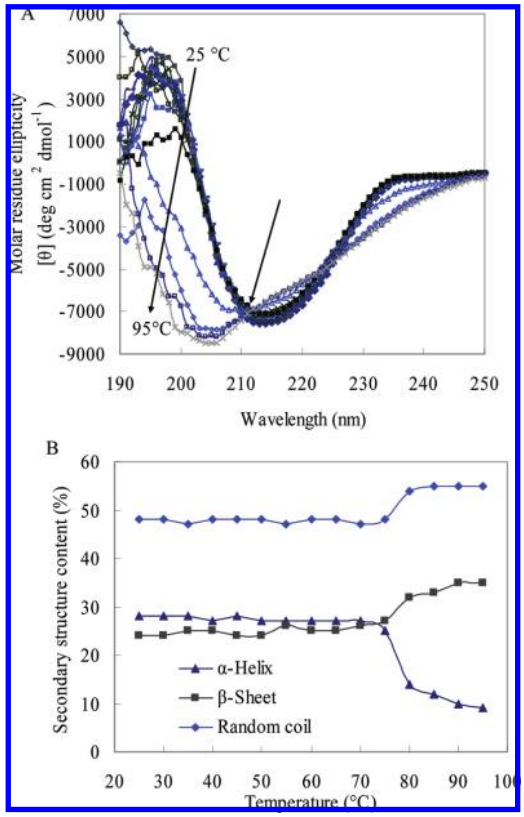

Figure 6. Far-UV CD (**A**) and the secondary structure content (**B**) of the purified β-1,3-1,4-glucanase as a function of temperature ranging from 25 to 95 °C. The spectra were recorded at temperatures from 25 °C (upper curve) to 95 °C (the lowest curve). The analysis was based on the far-UV CD spectra using the neural net program K2D.

cellobiosyltriose and cellotriosyltetraose (*2, 3, 16, 19*). In this study, the products of hydrolysis yielded a range of oligosaccharides, with bisaccharide, trisaccharide, and tetrasaccharide being the predominant oligosaccharide products. A bisaccharide was possibly laminaribiose migrating ahead of cellobiose. Similar results were reported by other researchers (*19, 21*). The hydrolysis patterns are similar to those described for β-1,3-1,4-glucanase from *Talaromyces emersonii* (*4*).

Secondary Structure Analysis of the Purified β-Glucanase. The far-UV CD spectrum of native glucanase exhibits one negative peak at 214 nm and a strong positive peak around 195 nm (**Figure 6A**). The overall shape of the spectrum is similar to that of many α/β proteins (i.e., proteins where there is some alternation of α-helix and β-sheet along the polypeptide chain). Usually for all α, all β, or $\alpha + \beta$ containing proteins there are two negative troughs at about 220 and 210 nm with different intensities corresponding to the extent of the secondary structural elements (*38, 39*). Secondary structure content was estimated from the far-UV CD spectra of the protein using the neural net program K2D (*34*). The spectral analysis revealed that native β-glucanase consists of 28% α-helix, 24% β-sheet, and 48% random coil (**Figure 6B**). As shown in **Figure 6**, changing the temperature between 25 and 95 °C affected the spectral profiles for the enzyme in 5 mM phosphate buffer (pH 7.0). The enzyme was denatured at temperatures between 75 and 80 °C. Almost no changes in the CD spectra characteristics of the enzyme were observed at temperatures up to 75 °C. Only at 80 °C, a clear shift in the spectrum was noticed. There was approximately an 11% decrease in α-helix, 5% increase in β-sheets, and 6% increase in unordered conformation when the temperature of the sample was increased from 75 to 80 °C. These results indicate that this enzyme is in fact thermostable and is a stable protein at pH 7.0 and up to 75 °C.

In conclusion, the thermophilic fungus *Paecilomyces thermophila* J18 has the ability to secrete an extracellular β-1,3-1,4-glucanase when grown in a corncob-based medium. A novel extracellular β-1,3-1,4-glucanase was purified to apparent homogeneity from the strain. The enzyme was a single subunit glycoprotein with molecular mass of 38.6 kDa and an estimated carbohydrate content of 19% (w/w). The purified enzyme displayed activity over broad ranges of pH and temperature, yielding respective optima values of pH 7.0 and 70 °C. The enzyme exhibited strict specificity for 1,3-1,4-β-D-glucans, with no detectable activity against 1,3- or 1,4-β-D-glucans. These properties make this β-glucanase a good candidate for use in industrial applications. Gene cloning and structural characterization are in progress.

ABBREVIATIONS USED

BSA, Bovine serum albumin; CAPS, (cyclohexylamino)-1-propanesulhinic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CMC, carboxymethylcellulose; DNS, dinitrosalicylic acid; EDTA, ethylenediaminetetracetic acid; MES, 2-(*N*-morpholino)ethane sulfonic acid; MOPS, 3-(*N*-morpholino)-propane sulfonic acid; PDA, potato dextrose-agar; *p*NP, *p*-nitrophenyl; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

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Received for review January 30, 2008. Revised manuscript received April 11, 2008. Accepted April 24, 2008. This work was financially supported by the Program for New Century Excellent Talents in University (NCET-05-0130) and the National Natural Science Foundation of China (Project No. 30500099).

JF800303B