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Purification and Characterization of Novel Glucanases from *Trichoderma harzianum* ETS 323

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Trichoderma harzianum ETS 323 secretes two glucanases, a 23.5 kDa endoglucanase (EG Th1) and a 61 kDa exoglucanase (ExG Th1). They were identified by their hydrolysis products and were purified to homogeneity. The optimal temperature and pH for both EG Th1 (7.3-fold purification, 5.0% yield) and ExG Th1 (33.7-fold purification, 0.15% yield) were 50 °C and pH 4.5, respectively. The kinetic parameters of EG Th1 (K_m = 23 mg mL^{-1}, V_{max} = 294 μM min^{-1}, specific activity = 7.4 U mg^{-1}) and ExG Th1 (K_m = 85 mg mL^{-1}, V_{max} = 385 μM min^{-1}, specific activity = 24.6 U mg^{-1}) toward carboxymethyl cellulose were determined. Both enzymes favored CMC and maintained 100% activity for 10 days at 38 °C. KCl, $MgCl_2$, $HgCl_2$, and $FeCl_3$ showed approximately 30% inhibition against EG Th1 but not ExG Th1. They catalyzed transglycosylation of glucose in the presence of cellobiose, but ExG Th1 exhibited better activity and higher product diversity.

KEYWORDS: Cellulose; glucanases; *Trichoderma harzianum* ETS 323

INTRODUCTION

Cellulose, a polymer of glucose units linked by β-1,4 glycosidic linkages, is one of the most abundant carbohydrate polymers in nature. Plant cell walls are rich in cellulose, which makes up 35−50% of the plant's dry weight. Cellulases represent an important group of enzymes because of their potential use in various biotechnological processes, including biomass conversions, production of fuel ethanol and other commodity products from cellulosic agricultural residues, and release of aromatic compounds (*1*). Recently, cellulose has gained the attention of bioindustries as the alarming concern over energy conservation rises. Saccharification and fermentation with appropriate enzymes can hydrolyze cellulose into simple units of glucose and produce ethanol (*2*, *3*). Cellulolytic enzymes have various commercial applications in the feed, food, textile, alcohol production, detergent manufacturing, and paper industries.

Celluloses can be hydrolyzed into glucose units by a combination of three enzymes: endoglucanases, exoglucanases, and β-glucosidases (*4*). Exoglucanases degrade crystalline cellulose from the nonreducing or reducing ends, producing cellobiose. Endoglucanases degrade amorphous cellulose randomly and produce cellooligosaccharides of various lengths. Cellobiose and cellooligosaccharides are degraded by β-glucosidase to produce fermentable glucose and shorter length cellooligosaccharides (*5*). Because the rate-limiting step in enzymatic cellulose depolymerization by exoglucanases and endoglucanases is the formation of nonfermentable cellooligosaccharide intermediates, the discovery and incorporation of improved cellulases with transglycosylation activity is likely a straightforward approach for reducing costs (*4*, *6*).

Trichoderma is a soil-borne filamentous fungus used as a biological control agent against a wide range of economically important aerial and soilborne plant pathogens, especially *Botrytis*, *Fusarium*, *Pythium*, and *Rhizoctonia* (*7*). The effectiveness of *Trichoderma* in biological control is mainly attributed to the secretion of cell wall-degrading enzymes such as β-1,4-glucanases; chitinases; β-1,3 glucanases; β-1,6 glucanases; proteases; and xylanases (*7*, *8*). The purpose of the present study was to purify and characterize glucanases from *T. harzianum* ETS 323 for use in industrial and biocontrol applications.

MATERIALS AND METHODS

All chemicals were purchased from Riedel-de Haën (Seelze, Germany), J. T. Baker (Phillipsburg, NJ), or Sigma (St. Louis, MO) unless otherwise noted.

Culture Conditions. *T. harzianum* ETS 323 was maintained on potato dextrose agar plates (Difco, Detroit, MI). Conidia were transferred aseptically to an incubation medium containing 1.4 g L^{-1} $(NH_4)_2SO_4$, 2.0 g L^{-1} KH_2PO_4, 6.9 g L^{-1} NaH_2PO_4, 0.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 10 g L^{-1} dextrose, 1.0 g L^{-1} peptone, and 0.3 g L^{-1} urea (pH 5.0). The medium was maintained at 22 °C and shaken at 180 rpm for 48 h. The mycelia pellet was transferred aseptically to an enzyme production medium containing 1.36 g L^{-1} KH_2PO_4, 0.5 g L^{-1} Na_2CO_3 H_2O, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.0 g L^{-1} peptone, 0.005 g L^{-1} $FeCl_3$, 0.0014 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.002 g L^{-1} $CaCl_2 \cdot 2H_2O$, and 10 g L^{-1} cellulose (pH 6.0). After 48 h of incubation at 22 °C and 180 rpm (*9*), the cultural medium was filtered through a Whatman no. 1 filter paper (Whatman, Clifton, NJ) and kept at 4 °C until use.

Enzyme Purification. All purification steps were carried out at 4 °C. The extracellular proteins were precipitated from the suspension by slowly

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adding 80% (w/v) ammonium sulfate to the filtrate and stirring overnight. The protein precipitate was collected by centrifugation at 10000*g* for 30 min. The pellet was resuspended in water and dialyzed against 10 mM Tris-HCl (pH 8.3) overnight. After dialysis, the total protein preparation was further purified using a Superdex 75 prep grade (1.6 cm ×100 mm) column previously equilibrated with 10 mM Tris-HCl (pH 8.3) and 150 mM NaCl at a flow rate of 1 mL min^{-1}. The eluting buffer contained 10 mM Tris-HCl (pH 8.3) and 150 mM NaCl. Fractions containing glucanase activity were pooled by precipitation with 80% cold acetone and then resuspended in a small volume of water. Two fractions, one with endoglucanase (1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4) and another with exoglucanase (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.74) activity, were collected and named EG Th1 and ExG Th1, respectively. Both enzymes were further purified by HPLC (Agilent HPLC series 1100; Agilent Technologies, Palo Alto, CA) using a SynChropak Q (250 mm × 4.6 mm) ion exchange column (Eichrom Technologies, Darien, IL). The column was washed with 10 mM Tris-HCl (pH 8.3) for 15 min and eluted with a linear gradient elution from 0 mM NaCl with an increment of 20 mM/min for 30 min at a flow rate of 0.5 mL min^{-1}. The purification step was repeated for ExG Th1 at pH 5. ExG Th1 was further purified in an Agilent Zorbax Bio Series GF-250 column, at a flow rate of 0.5 mL min^{-1}, eluting with 10 mM Tris-HCl (pH 5.0) containing 150 mM NaCl. The purity and molecular mass of EG Th1 and ExG Th1 were determined using 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (*10*) and staining with Coomassie brilliant blue R-250 (*11*). Low molecular mass markers (Bio-Rad, Hercules, CA) used were bovine serum albumin, 66 kDa; chicken egg ovalbumin, 45 kDa; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine erythrocytes carbonic anhydrase, 29 kDa; bovine pancreas trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; bovine milk α-lactalbumin, 1.42 kDa; and bovine lung aprotinin, 6.5 kDa. High molecular mass markers (Bio-Rad) used were myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; fructose-6-phosphate kinase, 84 kDa; bovine serum albumin, 66 kDa; glutamic dehydrogenase, 54 kDa; chicken egg ovalbumin, 45 kDa; and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 36 kDa.

Enzyme Activity Assay. Twenty microliters of enzyme solution was mixed with 100 μL of 1% carboxymethyl cellulose (CMC) in 100 mM sodium acetate buffer (pH 4.8) in a 96-well plate and incubated at 50 °C for 1 h. One hundred microliters of 3,5-dinitrosalicyclic acid (DNS) reagent (*12*) was then added, and the OD_{570} was determined using an enzyme-linked immunosorbent assay (ELISA) plate reader (model 68; Bio-RadA). One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 μmol of reducing sugar per minute under the assay conditions.

Enzyme Characterization. The protein concentration was determined using the bicinchoninic acid (BCA) method (*13*) according to the manufacturer's instructions using bovine serum albumin (BSA) as a standard. The pH stability of the purified enzyme (2 μg of EG Th1 or ExG Th1) was assayed in various pH buffers (Tris-HCl buffer, pH 8.0−10; phosphate buffer, pH 6.0−pH 7.5; or acetate buffer, pH 3.0−5.5) for 24 h. Optimal temperature of the purified enzyme (2 μg of EG Th1 or ExG Th1) was determined by measuring the activity at different temperatures (from 20 to 90 °C) for 1 h. The thermostability of the purified enzyme (2 μg of EG Th1 or ExG Th1) was determined following incubation at different temperatures for 4 h. Subsequently, 1% CMC substrate was added to the preincubated enzyme, and the residual activity was assayed at 50 °C for 1 h. To evaluate the effects of different metal ions, the enzyme activity of the purified enzyme (2 μg of EG Th1 or ExG Th1) was determined in the presence of 1 mM of the indicated metal ion ($CaCl_2$, $MnCl_2$, $MgCl_2$, $HgCl_2$, $CoCl_2$, $CuCl_2$, $BaCl_2$, $FeCl_3$, KCl, EDTA 2Na, or NaCl) in acetate buffer (pH 4.5) at 50 °C for 1 h.

K_m and V_{max} values of each purified enzyme (2 μg of EG Th1 or ExG Th1) were derived from Lineweaver−Burk plots by hydrolysis of CMC from 0.1 to 4 mM in acetate buffer (0.1 M, pH 4.5) at 50 °C for 1 h.

Transglycosylation activity was investigated by mixing 2 μg of purified enzyme (EG Th1 or ExG Th1) with 500 mM cellobiose and 500 mM glucose at 38 °C. The reaction was stopped by boiling, and an aliquot (0.5 μL) was analyzed by thin layer chromatography (TLC) on a silica plate using 1-propanol and water (5:2, v/v) as the eluant. The sugars were visualized using 20% sulfuric acid dissolved in ethanol as the spraying reagent and heating at 150 °C for 10 min.

Table 1. Purification of EG Th1 from *T. harzianum* ETS 323

purification step	total volume (mL)	total protein (mg)	total activity (U)	specific activity[a] (U/mg)	purification (fold)	yield (%)
ammonium sulfate	10	20.3	20.75	1	1	100
Superdex 75 prep grade	20	9.6	12.33	1.2	1.2	59
SynChropak Q	1.5	0.14	1.02	7.4	7.3	5

[a] CMCase activity.

The 10-day stability of the purified enzyme (EG Th1 or ExG Th1) was determined by placing the enzyme in acetate buffer (pH 4.5) in a 38 °C water bath for 10 days. An aliquot of 20 μL (5 μM) was removed each day, and the enzyme activity was tested as described above.

Enzyme Activity Staining. Enzyme activity staining was performed according to the method described by Schwarz et al. (*14*), but with slight modification. Briefly, the enzyme mixtures were boiled at 100 °C for 2 min and separated in 10% SDS-PAGE containing 0.1% CMC. After electrophoresis, the gel was soaked in 1% Triton X-100 solution for 1 h. The solution was changed three times every 20 min. The gel was then transferred to 100 mM acetate buffer (pH 4.8) and maintained at 4 °C for 1 h and then at 50 °C for 24 h. The gel was then stained with 0.1% Congo red for 1 h. Finally, the gel was washed with 1 M NaCl for 10 min.

Purified Protein Sequence Determination. The sequences of both enzymes were determined by LC-MS/MS as described previously (*15*). Briefly, in-gel digested proteins were analyzed using a nanoscale capillary LC-MS/MS with an Ultimate capillary LC system, Switchos valve switching unit, and Famos autosampler (LC Packings, San Francisco, CA) coupled to a quadrupole time-of-flight mass spectrometer (QSTAR XL; Applied Biosystem/MDS Sciex, Foster City, CA) equipped with a nanospray ionization source. Subsequently, MS/MS data were subjected to search algorithms against the Swiss-Prot protein sequence database using Mascot software (Matrix Science, London, U.K.).

Analysis of Hydrolysis Products from Cellooligosaccharides. The hydrolysis products, which varied in their degree of polymerization (DP), were determined separately by TLC. Two micrograms of purified enzyme (EG Th1 or ExG Th1) was mixed with 1 mL of 1 mg mL^{-1} glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), or cellopentaose (G5) in 100 mM sodium acetate buffer (pH 4.8) and incubated at 50 °C for 24 h. The reactions were stopped by heating the reaction mixtures at 100 °C for 10 min. The mixtures were then analyzed by TLC with 1-propanol and water (5:2, v/v) as the mobile phase and 20% H_2SO_4 in ethanol as the spraying agent.

RESULTS AND DISCUSSION

Enzyme Purification. After purification, EG Th1 achieved a 7.3-fold purification with a specific activity of 7.4 U mg^{-1} and a 5% yield compared to the original ammonium sulfate protein extract. On the other hand, purified ExG Th1 had a 33.7-fold purification with a specific activity of 24.6 U mg^{-1} and yield of 0.15% compared to the original ammonium sulfate protein extract (**Tables 1** and **2**). Through the successive purification steps, EG Th1 and ExG Th1 were purified to homogeneity (**Figure 1A**,**C**) with activities to substrate CMC presented corresponding to the purified enzyme on zymogram (**Figure 1B**,**D**). Furthermore, enzyme activity staining of the SDS-PAGE product for EG Th1 and ExG Th1 denoted a single band for each, which indicated the homogeneity of the purified enzyme samples.

From the Comprehensive Enzyme Information System (BRENDA http://www.brenda-enzymes.info/index.php4), the specific activity (μmol/min/mg) of endoglucanase (EC 3.2.1.4) was from 0.018 (*Thermobifida fusca*) to 450 (*Bacillus* sp.), and that of *Trichoderma viride* was 0.258. Another category of endoglucanase, EC 3.2.1.91, had specific activity from 0.00188 (*Paenibacillus* sp.) to 340 (*Clostridium thermocellum*). The specific activity of exoglucanase (EC 3.2.1.74) was reported from 0.0011 (*Irpex lacteus*) to 177 (*Clostridium cellulovorans*). It should be noted that the tested substrate of each value was very diverse.

Table 2. Purification of ExG Th1 from *T. harzianum* ETS 323

purification step	total volume (mL)	total protein (mg)	total activity (U)	specific activity[a] (U/mg)	purification (fold)	yield (%)
ammonium sulfate	50	98.45	71.91	0.730	1	100
Superdex 75 prep grade	15	7.158	10.24	1.431	1.9	14.2
SynChropak Q (pH 8.3)	5	1.48	2.415	1.631	2.2	3.3
SynChropak Q (pH 5.0)	3	0.036	0.332	9.283	12.7	0.4
Zorbax Bio series GF-250	0.5	0.00456	0.112	24.6	33.7	0.15

[a] CMCase activity.

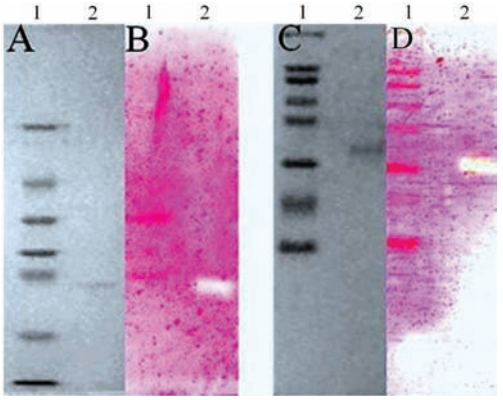

Figure 1. 10% SDS-PAGE analysis (**A**, **C**; stained with Coomassie brilliant blue R-250) and activity stain (**B**, **D**; stained with 0.1% Congo red) of purified EG Th1 (**A**, **B**) and ExG Th1 (**C**, **D**) from *T. harzianum* ETS 323. Lanes: 1, low molecular mass markers (**A**, **B**) and high molecular mass markers (**C**, **D**); 2, purified EG Th1 (**A**, **B**) and ExG Th1 (**C**, **D**).

On the basis of 10% SDS-PAGE analysis, the molecular masses of EG Th1 and ExG Th1 were 23.5 and 61 kDa, respectively (**Figure 1A**,**C**). The molecular masses of these two purified enzymes were unique when checked against the BRENDA enzyme database. The molecular masses of known endoglucanases and exoglucanases vary immensely. The largest endoglucanase is a 252 kDa enzyme from *Bursaphelenchus xylophilus*, and the smallest is a 13 kDa enzyme from *Trichoderma koningii* (*16*, *17*). A 230 kDa enzyme from *Ruminococcus flavefaciens* and a 22 kDa enzyme from *Thermotoga* sp. are the largest and smallest exoglucanases reported, respectively. Although endoglucanse and exoglucanase activity was previously reported in *T. harzianum*, the enzymes studied in the previous studies were not completely purified and their molecular masses were not determined for stringent comparison (*18*, *19*). The amino acid sequences of both purified glucanases were determined via LC-MS/MS. The partial sequence of EG Th1, NFFNYLR, was matched to the endo-β-1,4-glucanase of *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) having an M_r of 25143(*20*) that accorded well with the 23.5 kDa of EG Th1. Further details on the total amino acid sequence will be deduced from the ongoing cDNA cloning experiments. However, no significant match was found for ExG Th1, but further cloning of this enzyme is currently being performed in our laboratory.

Substrate Specificity and Catalytic Properties. To determine the identity of the purified enzymes, the hydrolysis products were assessed by TLC following incubation of each purified enzyme with various cellooligosaccharide substrates in 100 mM sodium acetate buffer (pH 4.8) at 50 °C for 24 h (**Figure 2**). EG Th1 hydrolyzed G5 (cellopentaose) to G4 (cellotetraose), G3 (cellotriose), G2 (cellobiose), and G1 (glucose); hydrolyzed G4 to G3, G1, and poorly to G2; and poorly hydrolyzed G3 and G2 to G2 and G1, respectively (**Figure 2A**). On the other hand, ExG Th1 effectively hydrolyzed G5, G4, and G3 to G2 and G1, but did not hydrolyze G2 (**Figure 2B**). Endoglucanases randomly cleave internal glycosidic bonds within an unbroken glucan chain to produce cellooligosacharides of diverse DP, whereas exoglucanases cleave cellobiose dimers from the end of glucan chains and therefore hydrolyze cellooligosaccharides to cellobiose and tag end glucose (*21*). On the basis of these results, we concluded that EG Th1 was an endogucanase and ExG Th1 an exoglucanase.

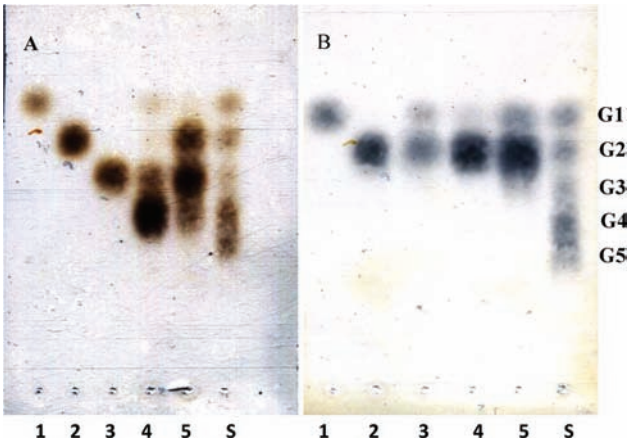

Figure 2. TLC analysis of the hydrolysis products obtained when various substrates were incubated with purified EG Th1 (**A**) or ExG Th1 (**B**) in 100 mM sodium acetate buffer (pH 4.8) at 50 °C for 24 h. Substrates: lane 1, glucose; lane 2, cellobiose; lane 3, cellotriose; lane 4, cellotetraose; lane 5, cellopentaose. Lane S is G1−G5 standards including G1 (glucose), G2 (cellobiose), G3 (cellotriose), G4 (cellotetraose), and G5 (cellopentaose).

Biochemical Parameters of EG Th1 and ExG Th1. The pH optima (pH_{opt}) of various cellulases purified to date are reported to be between pH 2.5 and 10, whereas the temperature optima (T_{opt}) are reported to be between 25 and 105 °C (*22*−*26*). The pH_{opt} and T_{opt} of both EG Th1 (**Figure 3A**,**C**, circles) and ExG Th1 (**Figure 3B**,**D**, circles) were 4.5 and 50 °C, respectively. EG Th 1 retained 100% activity between pH 5 and 6, but this dropped significantly beyond this range (**Figure 3A**, squares), whereas ExG Th1 retained almost 100% activity throughout the tested pH range (**Figure 3B**, squares). On the other hand, the temperature stability of EG Th1 decreased profoundly to ≤20% of relative activity at 50 °C and higher, but ExG Th1 retained the same tendency.

The presence of Hg^{2+}, Fe^{3+}, Mg^{2+}, and Mn^{2+} is reported to inhibit the activity of two endoglucanases isolated from *Bacillus* strains, CH43 and HR68 (*27*). Likewise, the ability of EG Th1 to hydrolyze CMC in 100 mM sodium acetate buffer (pH 4.8) at 50 °C was strongly inhibited by the presence of 1 mM Fe^{3+}, Hg^{2+}, or Mg^{2+} (32−36% inhibition, **Table 3**). This may indicate that thiol groups are involved in the active catalytic site. However, under similar conditions, the activity of ExG Th1 was only slightly inhibited by 1 mM Hg^{2+} or Fe^{3+} (1−2% inhibition). EG Th1 was also inhibited by KCl, EDTA, and $CaCl_2$. On the other hand, the activities of both EG Th1 and ExG Th1 were stimulated greatly (189.7 and 183.5%, respectively) by Mn^{2+} and increased slightly by the addition of Co^{2+} or Cu^{2+}.

The enzyme kinetic parameters of EG Th1 (**Figure 4A**) and ExG Th1 (**Figure 4B**) were determined by Lineweaver−Burk plots. It was observed that the intercepts of the *Y*-axis gave a value of 0.003 μM min^{-1} for EG Th1 and 0.002 μM min^{-1} for ExG Th1 and the *X*-axis intercepts were −0.043 mg mL^{-1} for EG Th1 and −0.012 mg mL^{-1} for ExG Th1. Therefore, the corresponding V_{max} was calculated to be 294 μM min^{-1} for EG Th1 and

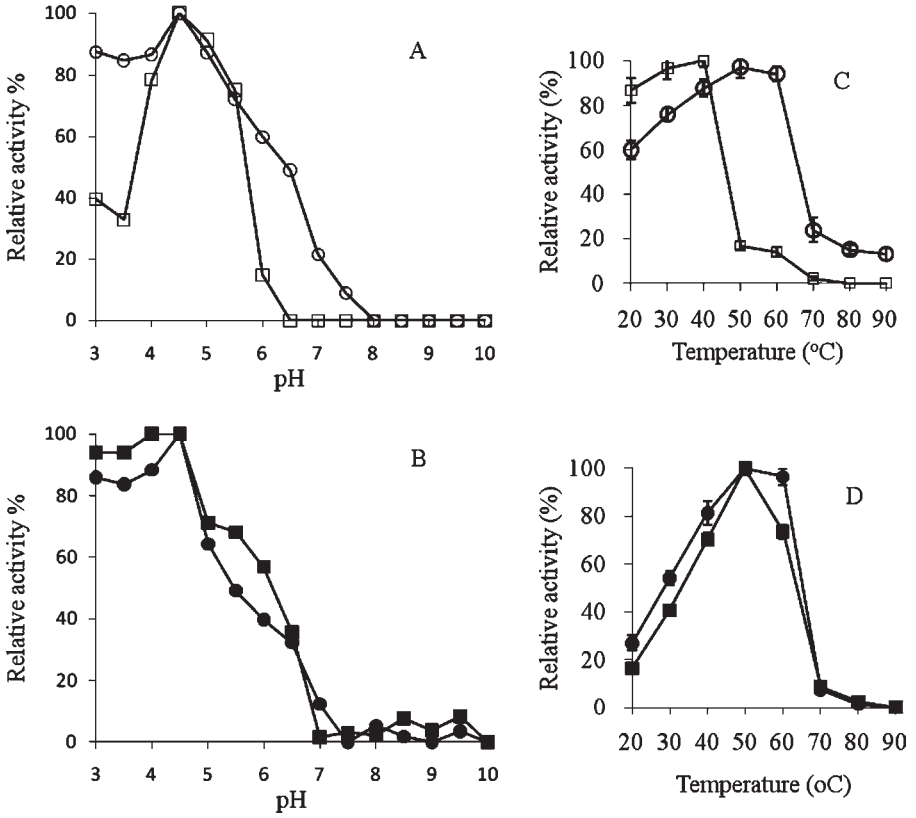

Figure 3. pH and temperature profiles of purified EG Th1 (**A**, **C**) and ExG Th1 (**B**, **D**). Optimal pH/temperature (circles) and pH/temperature stability (squares) were investigated as described under Materials and Methods.

Table 3. Effects of Various Agents on EG Th1 and ExG Th1 Activities

	relative activity (%)	
agent[a]	EG Th1	ExG Th1
none	100	100
$MnCl_2$	190	184
$CoCl_2$	119	144
$CuCl_2$	116	111
$CaCl_2$	83	103
EDTA	80	107
KCl	74	104
$MgCl_2$	68	108
$HgCl_2$	65	99
$FeCl_3$	64	98

[a] The final concentration of the various agents was 1 mM.

384.6 μM min^{-1} for ExG Th1, and K_m was calculated to be 23 mg mL^{-1} for EG Th1 and 80 mg mL^{-1} for ExG Th1.

The substrate specificities of EG Th1 and ExG Th1 were determined by comparing their activities against CMC with their activities against various substrates such as xylan, laminarin, pustulan, and locust bean gum (**Figure 5**). CMC, a β-(1−4)-D-glucopyranose-based polymer, was the preferable substrate for both EG Th1 and ExG Th1. The activities of EG Th1 with xylan, laminarin, pustulan, and locust bean gum were 22.9, 7.79, 2.29, and 1.83%, respectively, compared to that of CMC, whereas the corresponding activities for ExG Th1 were 67.79, 16.1, 11, and 13.55%, respectively.

Cellulase activity is inhibited by cellobiose and, to a lesser extent, by glucose. Several methods have been developed to reduce this inhibition, including the use of high concentrations of enzymes, the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation, or the use of enzymes with transglycosylation activity. Apart from hydrolyzing the β-glycosidic bond linking carbohydrate residues in aryl-, amino-, or alkyl-β-D-glucosides, cyanogenic glucosides, and short-chain oligosaccharides and disaccharides, glycosidases catalyze the reverse of hydrolysis, that is, the synthesis of glycosyl bonds between different molecules (*28*, *29*). Transglycosylation by EG Th1 and ExG Th1 was analyzed by mixing 500 mM cellobiose with 2 μg of the purified enzyme at 38 °C and examining the mixture by TLC after 1, 3, 5, and 7 days. Interestingly, no transglycosylation was observed with EG Th1 or ExG Th1 using glucose as the only substrate (**Figure 6A**,**C**). However, EG Th1 and ExG Th1 showed transglycosylation activity with cellobiose as the substrate, producing other oligosaccharides (**Figure 6B**,**D**). After 5 days of incubation, EG Th1 had converted some cellobiose to glucose and cellooligosaccharides (**Figure 6B**). Meanwhile, ExG Th1 was much more active in transglycosylating cellobiose to cellooligosaccharides, even on the first day of the reaction (**Figure 6D**). The results indicated that both enzymes catalyzed transglycosylation only when glucose coexisted with cellobiose and not for glucose alone. Although glucanases are reported from different species of *Trichoderma,* including *T. reesei*, *T. viride*, *T. koningii*, and *T. harzianum*, EG Th1 and ExG Th1 are the first glucanases from *Trichoderma* to be reported with transglycosylation activity (*18*, *19*).

Successive stepwise saccharification and fermentation is possible with EG Th1 and ExG Th1, due to their transglycosylation activity (*3*). Applying cellulases for industrial purposes, such as bioethanol production, may require enzymes to be stable for prolonged periods. We have found that EG Th1 and ExG Th1 can both sustain their activities at approximately 100% at 38 °C (the simultaneous saccharification and fermentation-required temperature) for up to 10 days (**Figure 7**). This stability supports the potential for actual application of both of these *T. harzianum* glucanases over a long duration.

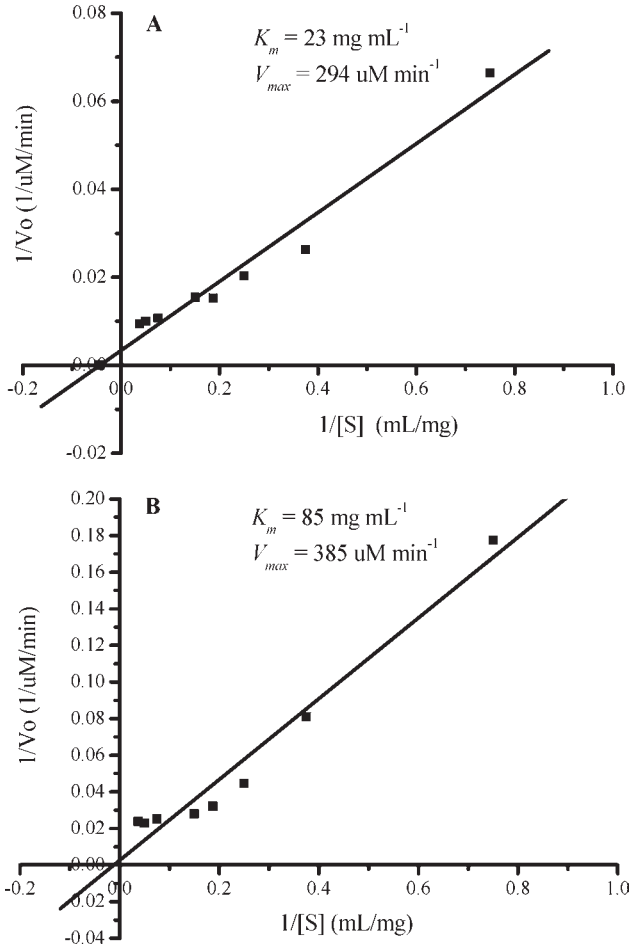

Figure 4. Lineweaver−Burk plot showing the $1/V_{max}$ and $-1/K_m$ intercepts for reciprocal initial velocities of EG Th1 (**A**) and ExG Th1 (**B**) against various substrate concentrations ranging from 0.1 to 4 mM at 50 °C.

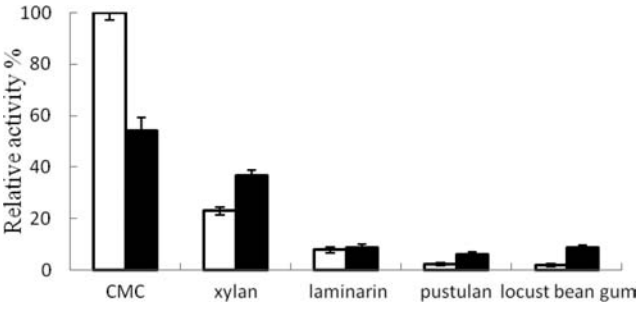

Figure 5. Substrate specificity of purified EG Th1 (white bars) and ExG Th1 (black bars).

In conclusion, *T. harzianum* ETS 323 secretes an endoglucanase (EG Th1) and an exoglucanase (ExG Th1) that apparently play roles in biocontrol mechanisms. The preferable substrate for both enzymes was (1,4)-D-glucopyranose-based CMC polymer. EG Th1 exhibited higher activity for hydrolyzing larger molecules, whereas ExG Th1 actively hydrolyzed all cellooligosaccharides tested. The two enzymes are new extracellular cellulases that were purified and characterized from *T. harzianum* for the first time. The enzyme characteristics of EG Th1 and ExG Th1 are sufficiently different from those of other glucanases for these enzymes to be considered as new enzymes or possibly proenzymes.

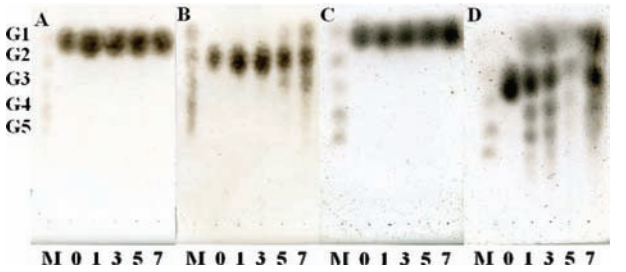

Figure 6. Transglycosylation activities of purified EG Th1 (**A**, **B**) and ExG Th1 (**C**, **D**) for 1, 3, 5, and 7 days. The substrates were glucose (**A**, **C**) and cellobiose (**B**, **D**). M represents a mixture of G1, G2, G3, G4, and G5 as a marker. Incubation periods were 0, 1, 3, 5, and 7 days.

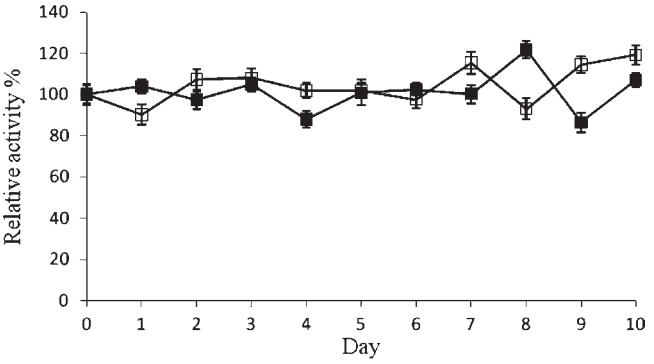

Figure 7. Enzyme stabilities of purified EG Th1 (□) and ExG Th1 (■) in acetate buffer (pH 4.5) at 38 °C for 10 days.

LITERATURE CITED

(1) Himmel, M. E.; Ruth, M. F.; Wyman, C. E. Cellulase for commodity products from cellulosic biomass. *Curr. Opin. Biotechnol.* **1999**, *10*, 358−364.
(2) Lonnie, O.; Ingram, J. B. D. Conversion of cellulosic materials to ethanol. *FEMS Microbiol. Rev.* **1995**, *16*, 235−241.
(3) Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* **2002**, *83*, 1−11.
(4) Peralta-Yahya, P.; Carter, B. T.; Lin, H.; Tao, H.; Cornish, V. W. High-throughput selection for cellulase catalysts using chemical complementation. *J. Am. Chem. Soc.* **2008**, *130*, 17446−17452.
(5) Bhatia, Y.; Mishra, S.; Bisaria, V. S. Microbial β-glucosidases: cloning, properties, and applications. *Crit. Rev. Biotechnol.* **2002**, *22*, 375−407.
(6) Zhang, Y. H. P.; Himmel, M. E.; Mielenz, J. R. Outlook for cellulase improvement: screening and selection strategies. *Biotechnol. Adv.* **2006**, *24*, 452−481.
(7) Howell, C. R. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis.* **2003**, *87*, 4−10.
(8) Viterbo, A.; Ramot, O.; Chemin, L.; Chet, I. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie Van Leeuwenhoek* **2002**, *81*, 549−556.
(9) Kapat, A.; Zimand, G.; Elad, Y. Effect of two isolates of *Trichoderma harzianum* on the activity of hydrolytic enzymes produced by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol.* **1998**, *52*, 127−137.
(10) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(11) Maizel, J. V., Jr. Acrylamide-gel electrophorograms by mechanical fractionation: radioactive adenovirus proteins. *Science* **1966**, *151*, 988−990.
(12) Sengupta, S.; Jana, M. L.; Sengupta, D.; Naskar, A. K. A note on the estimation of microbial glycosidase activities by dinitrosalicylic acid reagent. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 732−735.
(13) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke,

N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **1985**, *150*, 76−85.
(14) Schwarz, W. H.; Bronnenmeier, K.; Grabnitz, F.; Staudenbauer, W. L. Activity staining of cellulases in polyacrylamide gels containing mixed linkage β-glucans. *Anal. Biochem.* **1987**, *164*, 72−77.
(15) Tseng, S. C.; Liu, S. Y.; Yang, H. H.; Lo, C. T.; Peng, K. C. Proteomic study of biocontrol mechanisms of *Trichoderma harzianum* ETS 323 in response to *Rhizoctonia solani*. *J. Agric. Food Chem.* **2008**, *56*, 6914−6922.
(16) Shibuya, H.; Kikuchi, T. Purification and characterization of recombinant endoglucanases from the pine wood nematode *Bursaphelenchus xylophilus*. *Biosci., Biotechnol., Biochem.* **2008**, *72*, 1325−1332.
(17) Wood, T. M.; McCrae, S. I. The cellulase of *Trichoderma koningii*. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone and in synergism with the cellobiohydrolase. *Biochem. J.* **1978**, *171*, 61−72.
(18) Chandra, M.; Kalra, A.; Sharma, P. K.; Sangwan, R. S. Cellulase production by six *Trichoderma* spp. fermented on medicinal plant processings. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 605−609.
(19) Sidhu, M. S.; Kalra, M. K.; Sandhu, D. K. Purification and characterization of cellulolytic enzymes from *Trichoderma harzianum*. *Folia Microbiol.* **1986**, *31*, 293−302.
(20) Okada, H.; Tada, K.; Sekiya, T.; Yokoyama, K.; Takahashi, A.; Tohda, H.; Kumagai, H.; Morikawa, Y. Molecular characterization and heterologous expression of the gene encoding a low-molecular-mass endoglucanase from *Trichoderma reesei* QM9414. *Appl. Environ. Microbiol.* **1998**, *64*, 555−563.
(21) White, A. R.; Brown, R. M. Enzymatic hydrolysis of cellulose: visual characterization of the process. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 1047−1051.
(22) Lachke, A. H.; Deshpande, M. V. *Sclerotium rolfsii*: status in cellulase research. *FEMS Microbiol. Rev.* **1988**, *4*, 177−194.
(23) Kim, J. Y.; Hur, S. H.; Hong, J. H. Purification and characterization of an alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. *Biotechnol. Lett.* **2005**, *27*, 313−316.
(24) Wood, T. M. Cellobiohydrolases of *Penicillium pinophilum*. *Methods Enzymol.* **1988**, *160*, 398−403.
(25) Anuradha, P.; Vijayalakshmi, K.; Umamaheswari, P.; Haritha, D. S.; Jyothsna, K. P. Production and characterization of three extracellular cellulases from *Bacillus* sp. involved in the degradation of sugarcane crop residue. *Asian J. Microbiol. Biotechnol. Environ. Sci.* **2007**, *9*, 267−271.
(26) Piens, K.; Stahlberg, J.; Nerinckx, W.; Teeri, T. T.; Claeyssens, M. Structure−reactivity studies of *Trichoderma reesei* cellobiohydrolase Cel7A. *Am. Chem. Soc. Symp. Ser.* **2004**, *No. 889*, 207−226.
(27) Mawadza, C.; Hatti-Kaul, R.; Zvauya, R.; Mattiasson, B. Purification and characterization of cellulases produced by two *Bacillus* strains. *J. Biotechnol.* **2000**, *83*, 177−187.
(28) Akiba, S.; Yamamoto, K.; Kumagai, H. Transglycosylation activity of the endo-β-1,4-glucanase from *Aspergillus niger* IFO31125 and its application. *J. Biosci. Bioeng.* **1999**, *87*, 576−580.
(29) Oikawa, T.; Tsukagawa, Y.; Chino, M.; Soda, K. Increased transglycosylation activity of *Rhodotorula glutinis* endo-β-glucanase in media containing organic solvent. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 1889−1892.

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