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ARTICLE

Purification and Properties of Endoglucanase from a Sugar Cane Bagasse Hydrolyzing Strain, *Aspergillus glaucus* XC9

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An endoglucanase (EG) from *Aspergillus glaucus* XC9 grown on 0.3% sugar cane bagasse as a carbon source was purified from the culture filtrate using ammonium sulfate, an anion exchange DEAE Sepharose fast flow column, and a Sephadex G-100 column, with a purification fold of 21.5 and a recovery of 22.3%. The ideal time for EG production is on the fourth day at 30 °C using bagasse as a substrate. Results obtained indicate that the enzyme was a monomer protein, and the molecular weight was determined to be 31 kDa. The optimum pH and temperature of EG for the hydrolysis of carboxymethylcellulose sodium (CMC-Na) were pH 4.0 and 50 °C, respectively. EG was stable over the pH range from 3.5 to 7.5 and at temperatures below 55 °C. Kinetic behavior of EG in the hydrolysis of CMC-Na followed Michaelis−Menten kinetics with constant K_m of 5.0 mg/mL at pH 4.0 and 50 °C. The enzyme activity was stimulated by Fe^{2+} and Mn^{2+} but inhibited by Cd^{2+}, Pb^{2+}, and Cu^{2+}. The EDC chemical modification suggested that at least one carboxyl group probably acted as a proton donor in the enzyme active site.

KEYWORDS: *Aspergillus* species; cellulase; endoglucanase; purification and properties; chemical modification

INTRODUCTION

Cellulose, a major polysaccharide constituent of plant cell walls, is a β-1,4-linked linear polymer of 8000−12000 glucose units. A cellulosic enzyme system consists of three major components: endoglucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase (BG, EC 3.2.1.21). EG acts in random fashion, cleaving β-linked bonds within the cellulose molecule; CBH removes cellobiose units from the nonreducing ends of the cellulose chain, and BG degrades cellobiose and cellooligosaccharides to glucose.

Hemicellulose is the second most abundant polysaccharide in nature and consists of shorter chains of 500−3000 sugar units. Besides glucose, sugar monomers in hemicellulose can include xylose, mannose, galactose, rhamnose, and arabinose. Various agricultural residues, such as corn stover, wheat straw, rice straw, and sugar cane bagasse, contain about 20−40% hemicellulose. Xylanase (EC 3.2.1.8) breaks down hemicellulose.

Cellulolytic microorganisms produce a complex array of glycosyl hydrolases during growth on cellulosic substrates. Filamentous fungi, such as *Trichoderma* species, include all of the components of a multienzyme system with different specificities and modes of action. The cellulase system from *Trichoderma reesei* has been the most widely studied among the cellulolytic fungi. Cellulase production has been also described for some *Aspergillus* species (*1*−*3*). In our previous work (*4*), a cellulase-producing fungus, *Aspergillus glaucus* XC9, had been isolated from mildew maize cob, and different conditions including nitrogen source, initial pH, temperature, and surface active agent for the production of cellulase had been studied. This strain was used to hydrolyze sugar cane bagasse in this paper. Bagasse is a major cellulosic waste material in southern China. Sugars from appropriate saccharification or hydrolysis of this cellulosic biomass can be used for the production of ethanol as energy and fuel sources, single cell protein, feedstock, and industrially important chemicals (*5*). Thus, this research work is meaningful in the conversion and utilization of renewable biomass and has good social and economical significance. The aim of the present work is to investigate cellulase production, purification, and characterization of an endoglucanase produced from *A. glaucus* XC9.

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MATERIALS AND METHODS

Reagents. Carboxymethylcellulose sodium (CMC-Na) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China); xylan, salicin, *p*-nitrophenyl-β-D-cellobioside (*p*NPC), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), Sigmacell-101, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma (St. Louis, MO); DEAE Sepharose Fast Flow and Sephadex G-100 were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were local products of analytical grade.

Fungus and Culture Conditions. In our previous work, a cellulase-producing fungus was isolated from mildew maize cob and was identified

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by 18S rRNA gene sequence analyses and morphological characteristics as *A. glaucus* (*4*). The strain was named *A. glaucus* XC9. The fungus was cultured in a modified medium of Mandels and Sternburg (MS medium) (*6*) containing, per liter, 3 g of sugar cane bagasse, 1.4 g of $(NH_4)_2SO_4$, 0.3 g of urea, 2.0 g of KH_2PO_4, 0.3 g of $CaCl_2$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.75 g of tryptone, 5 mg of $FeSO_4 \cdot 7H_2O$, 1.4 mg of $ZnSO_4$, 1.6 mg of $MnSO_4 \cdot H_2O$, and 2 mL of Tween 80. The culture medium (200 mL) in 500 mL shake flasks was inoculated with 2% (v/v) overnight-grown culture, and the cultivation was performed at 30 °C with rotatory shaking at 180 rpm. Finally, the culture was centrifuged at 8000*g* for 20 min, and the supernatant was collected for enzyme purification.

Pretreatment of Agricultural Residues. The lignocellulosic materials, namely, sugar cane bagasse, grass, rice straw, and wheat stalks, were all obtained locally. They were first dried and chopped into small pieces by a chopper, then ground into smaller particles in a hammer mill, and finally separated by a 0.45 mm (40 mesh) sieve as described by Gao (*3*). Sugar cane bagasse was composed of approximately 28.4% cellulose, 27.5% hemicellulose, 19.7% lignin, and 8.4% ash according to the analytical procedure recommended by Yang (*7*).

Purification of Endoglucanase. A calculated amount of solid ammonium sulfate was added into the supernatant to achieve 90% saturation for precipitation of the enzyme. The precipitate was redissolved in a small amount of 50 mM NaAc buffer (pH 5.0) and then dialyzed against the same buffer until no ammonium sulfate could be detected by $BaCl_2$. Then the redissolved enzyme solution (about 50 mL) was sealed in a dialytic bag covered with polyethylene glycol 20000 (PEG-20000). PEG-20000 absorbed water and concentrated the solution within the dialysis bag. The concentrated enzyme solution (about 5 mL) was applied onto an anion exchange DEAE Sepharose Fast Flow column (15 × 650 mm). Equilibration and elution were performed first with 50 mM Tris-HCl (pH 7.8) to remove unbound proteins and then with a linear salt gradient from 0 to 0.5 M NaCl. The active fractions were combined and applied onto a Sephadex G-100 column (15 × 650 mm). Elution of the enzyme was carried out with 50 mM NaAc buffer (pH 5.0). The active fractions were pooled. The final preparation was applied to native polyacrylamide gel electrophoresis (native-PAGE) for the assay of homogeneity.

Determination of Molecular Weight. The whole molecular weight of EG was determined by using the methods of gel filtration on Sephadex G-100. Insulin (12.7 kDa), trypsin (25.0 kDa), albumin egg (45.0 kDa), and bovine serum albumin (68.0 kDa) were used as standard proteins. The molecular weight of the subunit was determined by SDS-PAGE. SDS-PAGE was done on a discontinuous horizontal thick layer with a stacking gel of 5% acrylamide and a separating gel of 12.5% acrylamide. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

Assay of Cellulase and Xylanase. EG activity (determined as CMCase) of the enzymes was measured by incubating 50 μL of the sample with 250 μL of 1% CMC-Na in 700 μL of 50 mM NaAc buffer (pH 4.0) at 50 °C for 30 min (*8*). The amount of reducing sugar produced was measured by the 3,5-dinitrosalicylic (DNS) reagent method (*9*).

Filter paper activity (FPA) was assayed by incubating 1 mL of the suitably diluted enzyme solution with 50 mM NaAc buffer (pH 4.0) containing one strip of Whatman no. 1 filter paper (50 mg, 1 × 6 cm). The reaction mixture was incubated at 50 °C for 30 min in a serological water bath. Then the reaction mixture was centrifuged after inactivation by adding 3 mL of DNS reagent, and reducing sugars were estimated in the supernatant using the DNS method (*9*).

Xylanase activity was determined under similar conditions as described above, except that a 1% xylan solution was used as substrate in place of CMC-Na.

β-Glucosidase (BG) activity was estimated using *p*NPG as substrate. The total of assay mixture (1 mL) consisting of 250 μL of *p*NPG (1 mM), 50 μL of enzyme, and 700 μL of 50 mM NaAc buffer (pH 4.0) was incubated at 50 °C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after development of the color with 2 mL of 2 M sodium carbonate.

One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate 1 mmol of glucose, xylose, or *p*-nitrophenol from the appropriate substrates per minute per milliliter of crude filtrate under assay condition.

Optimal pH and pH Stability. The optimum pH was determined by measuring the enzyme activities as described above at the different pH values (pH 2.5−8.0) at 50 °C. The pH stability of the enzyme was monitored by incubating the enzyme in different pH buffers (pH 2.5−9.0) for 4 h at 4 °C. Then 50 μL of treated enzyme was assayed at the optimum pH and the optimum temperature.

Optimal Temperature and Thermal Stability. The optimum temperature of the enzyme was determined by measuring the activity as described above at various temperatures (30−70 °C) at pH 4.0. The thermal stability of the enzyme was monitored by incubating the enzyme at various temperatures (30−70 °C) for 1 h. Then 50 μL of treated enzyme was assayed at the optimum pH and temperature.

Effects of Metal Ions on the Enzyme Activity. Experiments were conducted in the standard assay system described above with different concentrations of various metal ion compounds.

Chemical Modification of the Enzyme Active Site by EDC. Purified EG was incubated with freshly prepared 100 mM tetramethylenediamine (TEMED)-HCl buffer (pH 4.5) containing different concentrations of EDC. After being reacted for 30 min at room temperature, the residual activity of enzyme samples was determined according to the standard assay method.

Determination of the carboxylates with EDC is performed after kinetics analysis of the inactivation process during the labeling and by using different concentrations of modifier (*10*). The inactivation reaction of the biocatalyst can be represented by

$$E + nI \xrightarrow{k_1} EI_n$$

where E is the free enzyme, I the modifier, n the number of moles of modifier, and EI_n the enzyme−modifier complex. The rate of the inactivation reaction is described by the equation $v = -d[E]/dt = k_1[E][I]^n$.

As in most situations, when the modifier concentration greatly exceeds that of the biocatalyst, pseudo-first-order kinetics is observed. For the pseudo-first-order rate, k, $k = k_1[I]n$, and after taking the logarithms, one has $\lg k = \lg k_1 + n \lg[I]$. If $t_{1/2}$ is the half-life of the enzyme, a plot of $\lg(1/t_{1/2})$ versus lg[I] should give a straight line with a slope equal to n, where n is equal to the number of molecules of modifier reacting with each active unit of the enzyme to produce an inactive EI complex.

Determination of the Protein Concentration. The protein concentration was measured by using the Coomassie Brilliant Blue G-250 reagent, with bovine serum albumin (BSA) as the standard (*11*).

Statistical Analysis. Statistical analysis was carried out according to the method described by Kenney and Keeping (*12*). The values are the average of three independent experiments.

RESULTS AND DISCUSSION

Time Course of Enzyme Production Using Bagasse. Cellulase production was studied at 30 °C for 8 days in liquid MS medium containing 0.3% sugar cane bagasse as substrate (**Figure 1**). EG (determined as CMCase) production started from the second day and reached a maximum on the fourth day (33.5 U/mL). From the sixth day onward, a marked decrease in EG activity was observed. The maximum FPA (1.2 U/mL) and BGL activity (0.24 U/mL) were observed on the sixth day. Xylanase activity peaked on the third day (16.3 U/mL). According to the results, the ideal time for the EG production is on the fourth day, because after this period a decrease in productivity was observed.

Screening of Cellulose Production Using Different Agricultural Residues. Table 1 presents the effects of various agricultural residues on cellulase production by *A. glaucus*. Four agricultural residues, namely, sugar cane bagasse, grass, rice straw, and wheat stalk, with reference to three pure cellulose powders, namely, cellobiose, CMC-Na, and Sigmacell-101, were used to induce cellulase production. All of these carbon sources were used at a concentration of 0.3% in a shaken flask at 30 °C for 3 days. Flasks containing pure cellulose powders yielded higher activities in both FPA and BGL than agricultural residues due to their high accessibility for microorganisms. No significant differences were observed in CMCase when using different substrates (**Table 1**). Compared with *Penicillium janthinellum*, *Penicillium echinulatum*,

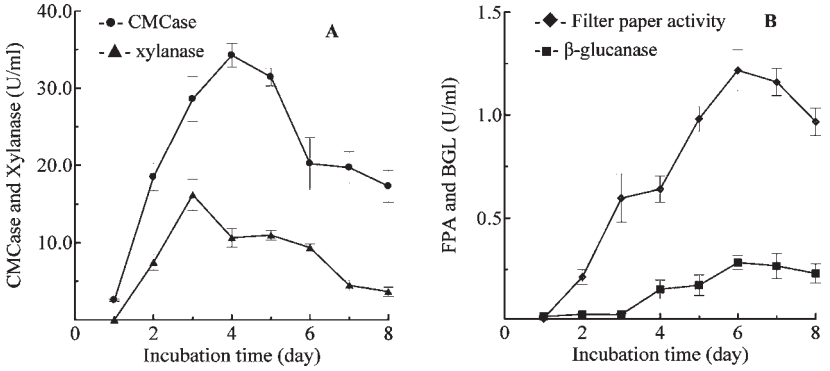

Figure 1. Time course of enzyme production by *A. glaucus* XC9 using sugar cane bagasse as carbon source. Each value is the mean of three replicates ± SD.

Table 1. Cellulase Production of *A. glaucus* XC9 from Various Agricultural Residues and Pure Cellulose Powders after Growth in Shaken Flasks at 30 °C for 3 Days[a]

substrate	CMCase (U/mL)	FPA (U/mL)	BGL (U/mL)
baggase	28.2	0.60	0.07
grass powder	31.5	4.70	0.28
rice straw	28.0	1.40	0.81
wheat stalk	22.4	0.76	0.10
cellobiose	43.1	13.4	4.82
CMCNa	38.7	10.1	5.70
Sigmacell-101	24.3	12.0	2.2

[a] The values are the average of three independent experiments.

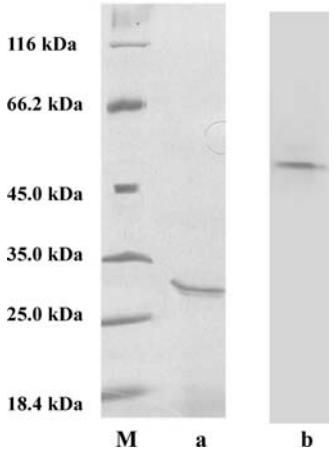

Figure 2. Native-PAGE and SDS-PAGE of EG produced by *A. glaucus* XC9. Lanes: M, protein markers; a, SDS-PAGE of EG; b, native-PAGE of EG.

and *Trichoderma viride* (*13*, *14*), *A. glaucus* possesses an equivalent ability to produce cellulase by using sugar cane bagasse without alkali pretreatment.

Purification of EG. An extracellular EG from the culture filtrates of *A. glaucus* (EG) grown on 0.3% sugar cane bagasse was purified by 90% $NH_4(SO_4)_2$, an anion exchange DEAE Sepharose Fast Flow (SFF) column, and a Sephadex G-100 column. The final preparation was determined to be homogeneous by native-PAGE (**Figure 2**, lane b). The specific activity of the purified EG was determined to be 11.8 U/mg, with a purification fold of 21.5 and a recovery of 22.3%. The purification steps are summarized in **Table 2**.

Molecular Weight (MW). The MW of the whole enzyme was 31.2 kDa according to the result from gel filtration on a Sephadex G-100 column (figure not shown). SDS-PAGE (**Figure 2**, lane a) indicated that the molecular weight of the subunit was about 31 kDa. Because the results obtained by gel filtration and

Table 2. Purification Steps and Folds of EG Produced by *A. glaucus* XC9

step	total protein (mg)	total activity (U)	specific activity (U/mg)	fold	recovery (%)
filtrate	624.3	341.1	0.55	1	100
$(NH_4)_2SO_4$	217.6	285.6	1.31	2.1	83.7
DEAE SFF	51.2	174.9	3.42	6.2	51.3
Sephadex G-100	6.5	76.1	11.8	21.5	22.3

Table 3. EG Characterizations from Some Fungi

fungus	MW (kDa)	optimum pH	optimum temperature (°C)	ref
A. niger	31	6.0	nr[a]	*15*
T. kongii	31	nr	nr	*16*
T. viride	38	4.5	nr	*17*
T. reesei	52	4.6	52	*19*, *20*
A. aculeatus	45	5	40	*21*
P. occitanis	28	3.5	50	*22*
A. glaucus	31	4.0	50	this work

[a] nr, not reported.

SDS-PAGE were the same, it can be concluded the enzyme was composed of one subunit. We found that EG from *A. glaucus* XC9 belongs to low molecular weight cellulase, similar to that of *Aspergillus niger* (31 kDa) (*15*), *Trichoderma koningii* (31 kDa) (*16*), and *Trichoderma viride* (38 kDa) (*17*) as shown in **Table 3**.

Kinetic Parameters and Substrate Specificity. The kinetics behavior of EG during the hydrolysis of CMC-Na was determined by varying the substrate concentration (CMC-Na) in the range of 2.6−9.6 mg/mL. The hydrolysis of CMC-Na by EG followed Michaelis−Menten kinetics. The kinetic parameters were obtained from a Lineweaver−Burk plot with K_m of 5.0 mg/mL and V_m of 44.2 μM/min (figure not shown).

To study the substrate specificity of EG, the hydrolytic activities of EG toward *p*NPC, salicin, crystalline cellulose (Sigmacell-101), and filter paper were determined. FPA is a relative measure of the overall cellulose-hydrolyzing capacity of microbial cellulase. The ability to degrade crystalline cellulose was commonly regarded as synonymous with exoglucanases. Salicin was usually used to determine the activity of β-glucosidase. Results revealed that the activity of EG toward CMC-Na was the highest. The relative activities of EG toward *p*NPC and filter paper were 87.2 and 78.3% of the activity toward CMC-Na, separately.

Generally, fungal endoglucanases show specificity toward various cellulosic substrates. In a few cases, endoglucanase can hydrolyze a broad range of substrates. For example, *T. reesei* exhibited hydrolytic activity toward β-glucan, carboxymethylcellulose, and methylumbelliferyl-β-D-cellobioside (*18*). Unlike *T. reesei* and some other *Aspergillus* strains (*15*, *18*, *19*), the

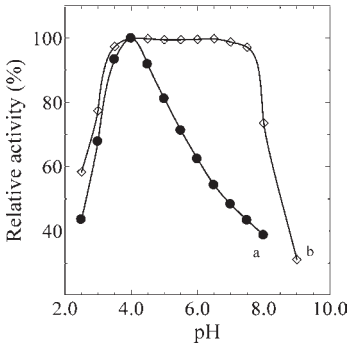

Figure 3. Effects of pH on EG activity (curve a), the enzyme activity was assayed at 50 °C. The pH stability of the EG (curve b), the enzyme was incubated at different pH buffers for 4 h at 4 °C, then 50 μL of the mixture was taken for activity assay at the optimum pH. The following buffers were used: Gly-HCl buffer (pH 2.0−4.0), NaAc buffer (pH 4.5−5.5), Na_2H-PO_4−NaH_2PO_4 buffer (pH 6.0−8.0), Gly-NaOH buffer (pH 8.5−9.0).

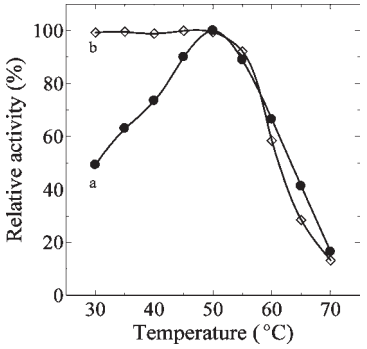

Figure 4. Effects of temperature on the EG activity (curve a), the enzyme activity was measured at various temperatures in 50 mM NaAc buffer (pH 4.0). Thermal stability of the EG (curve b), the enzyme was incubated at different temperature for 30 min in 50 mM NaAc buffer (pH 4.0), then 50 μL of the mixture was taken for activity assay at the optimum temperature.

purified endoglucanse from *A. glaucus* XC9 showed no activities of exoglucanase or β-glucosidase.

pH and Temperature Dependency. At 50 °C, the effect of pH on EG activity for the hydrolysis of CMC-Na was determined. The result showed that the optimal pH of EG was at pH 4.0 (curve a in **Figure 3**) and EG was stable at pH 3.5−7.5 (4 h at 4 °C) (curve b in **Figure 3**). This means that EG is fairly stable and highly active over a broad pH range.

At pH 4.0, EG activities were measured at various temperatures to determine the optimal temperature. The result showed that the optimal temperature for EG was 50 °C (curve a in **Figure 4**). EG was stable below 55 °C (curve b in **Figure 4**), whereas at 60 °C, only 60% activity was retained after incubation for 1 h.

The properties of some EGs from other fungal strains are shown in **Table 3**. The optimum pH of EG from *A. glaucus* was lower than that from *T. reesei* (pH 4.6) (*20*, *21*), *T. viride* (pH 4.5) (*17*), and *A. niger* (pH 6.0) (*15*).

Depending on the optimal temperature, enzymes can be classified as mesophilic (40−60 °C), thermophilic (50−80 °C), and hyperthermophilic (>80 °C) (*22*). For fungi, the optimal temperatures for EG activity are usually between 50 and 60 °C and stable up to 50−55 °C (**Table 3**). *T. reesei* is usually used in industrial cellulase production. The optimum temperature of EG from *A. glaucus* was similar to that of *T. reesei* (52 °C) (*19*, *20*), which suggested its potential industrial uses.

Effect of Metal Ions on EG Activity. Several metal ions and salts were assayed for their effects on enzyme activity on the hydrolysis of CMC-Na. The results are shown in **Table 4**. Na^+, K^+, Ba^{2+}, Ca^{2+}, Zn^{2+}, and Mg^{2+} have no effects on EG activity. The acid radicals SO_4^{2-}, Cl^-, and NO_3^- do not influence enzyme activity either. Fe^{2+} and Mn^{2+} activated the enzyme, whereas Cu^{2+}, Pb^{2+}, and Cd^{2+} showed various degrees of inhibitory effects.

Table 4. Effects of Metal Ions and Salts on EG Activity[a]

compound	relative activity (%)	compound	relative activity (%)
control	100	$CuCl_2$	49.8
NaCl	99.1	$FeCl_2$	133.3
$NaNO_3$	98.9	$Pb(NO_3)_2$	41.3
Na_2SO_4	99.5	$CdCl_2$	51.9
KCl	98.8	$BaCl_2$	101.2
$CaCl_2$	101.9	$MnCl_2$	130.0
$MgCl_2$	98.9	$ZnCl_2$	101.1

[a] The final concentration of each compound was 4 mM. Enzyme activities were determined in 50 mM NaAc buffer (pH 4.0) at 50 °C for 30 min.

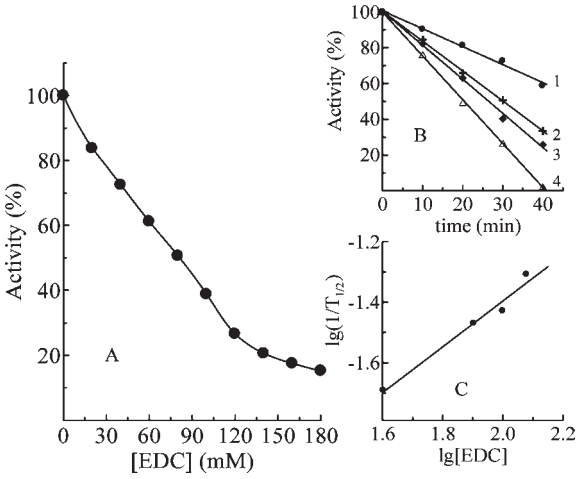

Figure 5. Inactivation of EG by EDC: (**A**) inactivation of EG by EDC modification; (**B**) rate of inactivation of EG by EDC determined by incubation in 50 mM, pH 4.5, TEMED-HCl, in the presence of different concentrations of EDC (the concentrations of EDC for curves 1−4 are 40, 80, 100, 120 mM, respectively); (**C**) determination of order of inactivation.

These results concurred with other work, reported elsewhere, that heavy metals inhibited endoglucanases, whereas Fe^{2+} can cause a slight activation of the enzyme (*19*, *23*−*26*). It seems that the heavy metals attack some certain groups at the active site of the enzyme, for example, the thiol groups, leading to the inactivation, whereas Fe^{2+} and Mn^{2+} could enhance the substrate binding affinity of the enzyme and stabilize the conformation of the catalytic site (*27*).

Modification of Carboxylates by EDC. The modification of EDC on the enzyme is shown in **Figure 5A**. The results showed that activity of the enzyme decreased with increasing concentrations of EDC. When the concentration of EDC was enhanced to 140 mM, the remaining activity of the enzyme was only 10%. It was indicated that carboxyl groups were essential for the activity of the enzyme. According to this analysis and from **Figure 5B**,**C**, it can be determined that the inactivation of EG by EDC follows pesudo-first-order kinetics and the number of the reactive carboxylates in EG equals 1 ($n \approx 0.7$).

EDC has been employed for the modification of protein carboxylates in several situations (*28*−*30*). This modifier reacts with the side chain of aspartic acid/glutamic acid residues as well as the C-terminal residue. Participation of carboxyl groups in the catalytic function had been reported in a variety of glycosyl hydrolases, and the carboxylate seemed to be an integral part of the catalytic site of these enzymes when acid/base catalysis was involved (*28*, *30*). In this research, one

carboxylate, which probably acts as a proton donor, was essential for the EG from *A. glaucus*. This result is similar to that for *T. viride* (*31*), but two carboxylates were identified to be essential in the active site of EG from *A. niger* (*28*).

In conclusion, compared with *P. janthinellum*, *P. echinulatum*, and *T. viride* (*13*, *14*), *A. glaucus* XC9 is a potential strain to produce cellulase using various agricultural residues including sugar cane bagasse. In southern China, the abundant sugar cane bagasse is a promising feedstock for industrial production of low-cost fuel ethanol. The EG from *A. glaucus* XC9 shares common characteristics with those from industrial cellulase-producing fungi, such as *A. niger*, *T. reesei*, and *T. viride* as compared in **Table 3**, suggesting its possible uses in industry.

ABBREVIATIONS USED

EG, endoglucanase; FPA, filter paper activity; BGL, β-glucanase; CMC-Na, carboxymethylcellulose sodium; CMCase, carboxylmethyl cellulase; *p*NPG, *p*-nitrophenyl-β-D-glucopyranoside; *p*NPC, *p*-nitrophenyl-β-D-cellobioside; K_m, Michaelis−Menten constant; PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; DNS, 3,5-dinitrosalicylic; NaAc buffer, sodium acetate buffer.

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