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Purification and Characterization of a New Endoglucanase from *Aspergillus aculeatus*

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Endoglucanase has been isolated from *Aspergillus aculeatus.* The purified enzyme showed a single band and had a molecular weight of 45 000 Da as indicated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, with a specific activity of 1.4 units/mg. The purified enzyme was identified as endoglucanase, showing a high specific activity toward CM-cellulose and low specific activity toward Avicel. The activity of the isolated enzyme was optimum at a pH of 5.0 and temperature of 40 °C, respectively. The isoelectric point of the enzyme was 4.3. T_m was found to be 57 °C. The treatment of the endoglucanase with diethylpyrocarbonate resulted in the modification of the histidine residues present in the enzyme, with a concomitant loss of 70% of the original enzymatic activity. However, carbodiimide completely inactivated the endoglucanase. The results show that the enzyme is able to sustain 50% of its activity even when heated at 90 °C for a period of 5 h. Endoglucanase can be used in the controlled hydrolysis of cellulose and other cellulose-rich foods. It can be used in the development of targeted functional foods from agrimaterials for value addition in the food chain.

KEYWORDS: ***Aspergillus aculeatus*; activity; CM-cellulose; carbodiimide; endoglucanase**

INTRODUCTION

Cellulose is the most abundant renewable resource on earth, accounting for about half of the organic material in the biosphere. Cellulose is biodegradable by a variety of microbes by the activities of many different cellulolytic enzymes. The treatment of cellulose has drawn the attention of not only biotechnologists interested in the hydrolysis of this abundant bioresource but also has importance in fundamental research.

It is composed of unbranched polymers of glucose residues linked by β-1,4-glycosidic bonds with the adjacent glucan chains packed together by hydrogen bonding, resulting in a crystalline structure (*1*). Microbial cellulases have attracted considerable interest in research and commercial importance because of their enormous potential applications in biotechnology and industry (*1*, *2*). Many cellulolytic microorganisms, especially fungi, are efficient producers of very active cellulases.

Aspergillus species are efficient producers of cellulolytic enzymes, which comprise three types of enzymes acting synergistically (*3*, *4*); Endo-1,4-β-glucanase (EC 3.2.1.4) is characterized by its activity toward substituted cellulose derivatives, such as carboxymethylcellulose (CMC), and exo-1,4-β-glucanase (EC 3.2.1.91) hydrolyzes microcrystalline cellulose (Avicel), producing cellooligosaccharides and cellobiose, respectively. These products are converted to glucose by β-glucosidase (EC 3.2.1.21), thereby reducing cellulose inhibition of endo-1,4-β-glucanase and exo-1,4-β-glucanase; their activity on cellulose is synergistic (*5*). Progress on the mechanism of action, active-site structure, and three-dimensional structures have led to the prediction of their role in cellulose hydrolysis and their wide range of potential applications (*6*−*8*).

Endoglucanases preferentially cleave the internal glycosidic bonds of cellulose chains and act synergistically with cellobiohydrolase and β-glucosidase during the solubilization of crystalline cellulose. Also, endoglucanases play a key role in increasing the yield of fruit juices, beer filtration, and oil extraction, improving the nutritive quality of bakery products and animal feed, and enhancing the brightness, smoothness, and over all quality of cellulosic garments (*9*). Hence, there is a need for a wide range of endoglucanases with varying pH and temperature optima, stability, and substrate specifications. This paper describes the purification and biochemical characterization of endoglucanase from *Aspergillus aculeatus*.

MATERIALS AND METHODS

Viscozyme, a commercial cellulase preparation of *A. aculeatus*, was obtained from Novozymes, Bangalore, India. Avicel PH 101, CMC (medium viscosity), *p*-nitrophenyl-β-D-glucopyranoside (pNPG), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-bromosuccinimide (NBS), and a cellulose nitrate dialysis membrane having a 10 kDa molecular weight cut-off (MWCO) were purchased from Sigma-Aldrich, Co., St. Louis, MO. The Diafuro YM10 membrane was purchased from Amicon Co., Beverly, MA. Chromatography media were purchased from

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Amersham Pharmacia Biotech, Ltd., U.K. All of the chemicals used were of analytical grade.

Purification of Cellulase. A 25 mL crude cellulase solution [diluted 1:5 (v/v) with 0.02 M sodium acetate buffer at pH 5.0, where 5 mL of crude enzyme was made to 25 mL] was centrifuged for 20 min at 8000*g* to remove the solid and desalted on G-25 column equilibrated with 0.02 M sodium acetate buffer at pH 5.0. Ammonium sulfate (80% saturation) was slowly added to the crude cellulase with constant stirring. After the addition of the required amount of ammonium sulfate, the mixture was stirred for 1 h, kept at 4 °C overnight, and then centrifuged at 8000*g* for 15 min. The supernatant was dialyzed against the buffer overnight with three changes.

Ion-Exchange Chromatography on DEAE-Sephadex A-50. The dialyzed fraction was loaded onto the pre-equilibrated DEAE-Sephadex A-50 column (50 × 1.9 cm). The protein was eluted with 0.02 M sodium acetate buffer at pH 5.0 containing a linear gradient of sodium chloride from 0 to 0.5 M at a flow rate of 25 mL/h at 4 °C, and 2 mL fractions were collected. The pooled fraction, which showed maximum enzyme activity, was concentrated by an ultrafiltration membrane to its minimum volume and desalted on a G-25 column pre-equilibrated with sodium acetate buffer at pH 5.0 and 4 °C. Protein absorbance was measured at 280 nm. The protein was determined by Lowry's method (*10*).

Assay of Avicelase. The reaction mixture consisted of 0.9 mL of 1% Avicel suspension in 0.02 M sodium acetate buffer at pH 5.0 and 0.1 mL of the enzyme solution. Incubation took place at 37 °C for 1 h with shaking. Reducing sugar was analyzed by the method of Nelson−Somogyi (*11*), using glucose as the standard (*4*).

Assay of Carboxymethyl Cellulase (CMCase). The reaction mixture consisted of 0.9 mL of 0.5% CMC in 0.02 M sodium acetate buffer at pH 5.0 and 0.1 mL of the enzyme solution. After incubation at 37 °C for 1 h, the reaction was stopped and reducing sugars were determined by the addition of the Nelson−Somogyi reagent. Absorbance was measured at 540 nm (*4*).

Assay for β-Glucosidase. The assay was performed by the addition of 0.1 mL of the enzyme solution to 0.9 mL of 0.1% pNPG in 0.02 M sodium acetate buffer at pH 5.0 and incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 mL of 0.5 M glycine buffer at pH 9.0 containing 0.002 M ethylenediaminetetraacetic acid (EDTA). The concentration of *p*-nitrophenol was measured at 400 nm, using an absorption coefficient of 13 700 M^{-1} cm^{-1} (*4*).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Slab gel electrophoresis on a 12% gel using the discontinuous buffer system of Laemmli (*12*) was done to analyze enzyme purity. The gel was stained for protein by the silver-staining method. Bovine serum albumin (MW of 68 000), ovalbumin (MW of 45 000), and chymotrypsinogen (MW of 25 000) were used as standard markers.

pH and Temperature Optima Determination. The pH and temperature optima of endoglucanase were determined by measuring the activity as described above using sodium acetate buffer (pH 3−5.5), phosphate buffer (pH 6.0−8.0), and borate buffer (pH 9.0) and in the temperature range of 25−80 °C, respectively.

Fluorescence Studies. Fluorescence emission spectral measurements were done using a Shimadzu RF-5000 automatic recording spectrofluorimeter at different temperatures ranging from 20 to 80 °C. The enzyme was incubated for 15 min at each temperature and then scanned. The temperature of the cell was maintained by circulating the water through the thermo-jacketed holder from a circulating water bath. The protein was excited at 280 nm and emission-scanned from 300 to 400 nm (*13*). The bandwidths for excitation and emission monochromators were fixed at 5 and 10 nm, respectively.

Thermal Stability Studies. Activity loss as a function of the temperature was followed in acetate buffer (0.02 M, pH 5.0). The enzyme was incubated for 30 min at different temperatures in the range of 20−90 °C. After cooling, the residual activity was measured (40 °C) by transferring an aliquot to the assay mixture. The activity of the unincubated enzyme was taken as 100%.

Thermal Transition Temperature. T_m was determined using a Cary100 Bio UV−vis spectrophotometer, M/s Varian, Austria. Measurements were done at different temperatures in the range of 20−90 °C, with 1 °C/min increments in the temperature, and the spectrum was recorded at 287 nm. The apparent thermal transition temperature (apparent T_m) was calculated by monitoring the progress of denaturation followed by changes in the absorbance or van't Hoff plot (*14*).

Determination of the Degree of Randomness by the Viscometric Method. The activity of endoglucanase on CMC was measured viscometrically at 37 °C after 1 h of incubation using 0.5% substrate. The ratio of the activity of endoglucanase on CMC measured viscometrically to the activity on CMC measured by the increase of reducing sugars is an indication of the degree of randomness in cellulose hydrolysis (*15*).

Isoelectric Focusing. Analytical thin-layer gel isoelectric focusing was performed in the pH range of 3.5−9.5. After electrofocusing, the gel was fixed in solution containing a methanol/acetic acid solution. Later, it was silver-stained. The pI of endoglucanase was determined using the plot of relative mobility of standard protein markers versus their pI.

Free Cysteine and Disulfide Estimation. Free cysteine and disulfide were determined by assaying for sulfydryl content using ′DTNB. The reaction mixture contained 0.1 M Tris-glycine buffer at pH 8.0 having 0.01 M EDTA, and 50 μL of DTNB (10 mM) was added to the reaction mixture and kept for 15 min before measuring the absorbance at 412 nm according to the procedure of Ellman (*16*).

End-Product Analysis by High-Pressure Liquid Chromatography (HPLC). To identify the purified enzyme showing CM-cellulase activity, it was incubated in 0.5% CMC at 37 °C for 1 h in sodium acetate buffer at pH 5.0. The enzyme was inactivated by placing the tubes in a boiling water bath for 10 min. The products were analyzed (50 μL) by HPLC using an Aminex (0.25 × 5 cm) column with a light-scattering detector and acetonitrile/water (60:40) as the solvent with a flow rate of 1 mL/min.

Circular Dichroism (CD) Spectra. CD spectra were obtained using a Jasco J-810 automatic recording spectropolarimeter fitted with a 250 W xenon lamp. The sample was analyzed in 0.1 cm optical path length cells in the far-UV (below 250 nm) region. The secondary structure of endoglucanase was analyzed using the computer program of Yang et al. (*17*), which calculates the structural component ratio of secondary structures for the protein, by the least-squares method. The reported CD values were the average of at least three independent runs. Before the spectrum was recorded, all samples were previously centrifuged and filtered through Millipore filters (0.45 μm pore diameter).

Amino Acid Analysis. The endoglucanase was hydrolyzed in sealed evacuated tubes with 6 N HCl for 24 h at 110 °C. Amino acid analysis was performed according to the method of Bidlingmeyer et al. (*18*) using a Waters Associates Pico-Tag amino acid analysis system.

Spectrophotometric Titration of Endoglucanase with NBS. To 1 mL of endoglucanase in 0.02 M sodium acetate buffer at pH 5.0, 10 μL of 10 mM NBS solution was added to the sample and reference cuvette having buffer. The decrease in the absorbance was measured at 280 nm upon the addition of NBS in a stepwise manner until further addition of NBS lead to an increase in the absorbance (*19*). This was measured using a Shimadzu 1601 UV−vis spectrophotometer.

Fast Protein Liquid Chromatography (FPLC) of the Isolated Protein. Isolated protein was loaded to a Superdex-75 gel-filtration column pre-equilibrated with 0.02 M sodium acetate buffer at pH 5.0.

Carbohydrate Estimation. The total neutral carbohydrate was estimated by the phenol−sulfuric acid method using glucose as the standard according to Dubois et al. (*20*).

Chemical Modification of Endoglucanase. The effect of various specific reagents, such as dithiotreitol (DTT), NBS, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDAC), and diethyl pyrocarbonate (DEPC), on the endoglucanase activity was studied.

RESULTS AND DISCUSSION

Isolation and Purification of *A. aculeatus* Endoglucanase. The desalted fractions obtained from G-25 were pooled and subjected to partial purification using 80% ammonium sulfate precipitation. As a result, the total protein content was reduced by about 72% and the specific activity was increased by 100%, indicating 2-fold purification (**Table 1**). Subsequent to am-

Table 1. Purification Steps of Endoglucanase from Crude Cellulase from *A. aculeatus*

purification step	volume (mL)	protein (mg/mL)	total protein (mg)	activity (units/mL)	total activity (units)	specific activity (units/mg of protein)	yield (%)	purification factor
crude	25	6.28	157	2.18	54.40	0.35	100	1
$(NH_4)_2SO_4$	20	2.19	43.8	1.73	34.66	0.80	64	2.2
DEAE-Sephadex	20	0.5	10	0.69	13.83	1.40	25	4.0

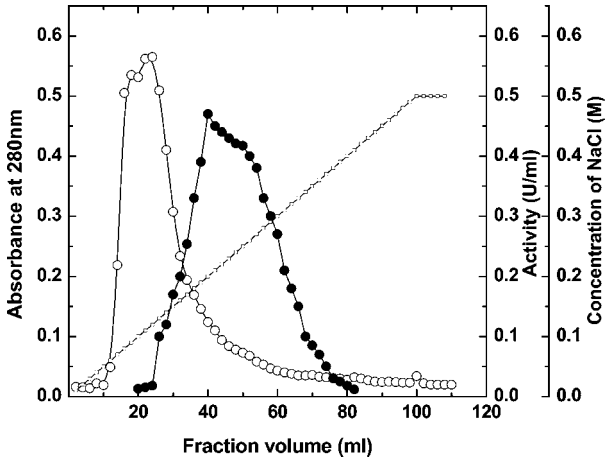

Figure 1. DEAE-Sephadex A-50 column chromatography of the ammonium sulfate fraction. The concentrated ammonium sulfate fraction was applied to the pre-equilibrated column in 0.02 M sodium acetate buffer at pH 5.0. The column was eluted with a linear gradient of NaCl in the same buffer. The flow rate was 25 mL/h, and the fraction volume was 2 mL. (●) activity, (○) absorbance at 280 nm, and (□) NaCl gradient of 0–0.5 M.

Table 2. Specific Activities of Endoglucanase Purified from *A. aculeatus*, Using CMC, Avicel, and pNPG as Substrates

substrate	specific activity (units/mg of protein)
CMC	1.4
Avicel	0.047
pNPG	0.037

monium sulfate precipitation, the supernatant of the same (having CMC activity) was dialyzed using a cellulose nitrate dialysis membrane versus 0.02 M sodium acetate buffer at pH 5.0 and then loaded onto a DEAE-Sephadex A-50 ion-exchange column equilibrated in 0.02 M sodium acetate buffer at pH 5.0. The eluent used was 0.02 M sodium acetate buffer at pH 5.0 with a sodium chloride gradient (**Figure 1**). Subsequent to DEAE-Sephadex A-50 chromatography purification, the total protein content of the ion-exchange fraction was reduced by 94%, but the specific activity was increased by about 4-fold compared to the crude. The peak obtained containing cellulase activity was typical endoglucanase, showing a high specific activity toward CM-cellulose and low specific activity toward Avicel (**Table 2**). The fractions were pooled and applied to a G-25 column equilibrated in 0.02 M sodium acetate buffer at pH 5.0; the peak obtained was pooled and concentrated. The enzyme was pure as confirmed by SDS−PAGE (inset in **Figure 2**).

Biochemical Characterization of Endoglucanase. Different purification fractions were analyzed for their homogeneity by SDS−PAGE. The crude extract showed numerous bands; the number of bands was reduced after the ammonium sulfate precipitation step. The DEAE-Sephadex fraction showed a single band, indicating the purity of the isolated enzyme preparation (inset in **Figure 2**). The molecular weight of the purified

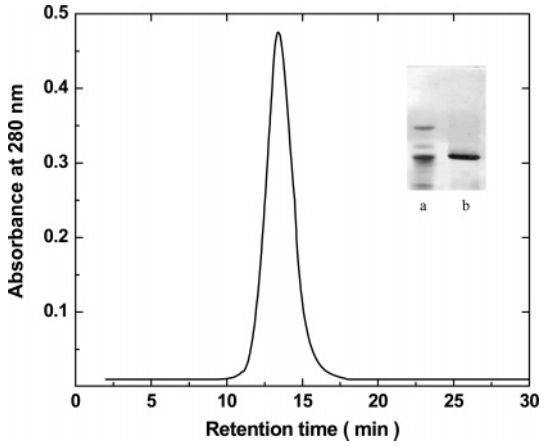

Figure 2. FPLC gel-filtration profile with 0.02 M sodium acetate buffer at pH 5.0. (Inset) SDS–PAGE of endoglucanase purified from commercial cellulase derived from *A. aculeatus.* The gel had a final acrylamide concentration of 12%. Proteins were stained by silver staining. (a) Molecular-weight markers: bovine serum albumin (68 000), ovalbumin (45 000), and chymotrypsinogen (25 000). (b) Endoglucanase.

endoglucanase was estimated from the results of the SDS−PAGE (inset in **Figure 2**). The purified enzyme had an apparent MW of ∼45 000 Da. Araujo et al. (*21*) reported that cellulases produced by *Aspergillus terreus* were resolved in SDS−PAGE into four components, with a molecular weight range of 16 000−90 000 Da. The homogeneity of the enzyme was confirmed with a single peak with FPLC using gel filtration (**Figure 2**). The isolated enzyme was identified as a typical endoglucanase, showing high activity toward CMC (**Table 2**); the specific activity toward Avicel and pNPG was 30 times lower. The release of glucose and cellobiose as the end products during incubation with CMC (**Figure 3**) confirmed it as endoglucanase.

The ratio of the activity of endoglucanase on CM-cellulose measured viscometrically to the activity on CM-cellulose measured by the increase of reducing sugars is an indication of the degree of randomness in cellulose hydrolysis. The degree of randomness for the isolated enzyme was found to be 0.527, which is in accordance with endoglucanase (*4*).

These two properties are generally accepted to distinguish endoglucanase from exoglucanase and β-glucosidase. Schoemaker reported two other endoglucanases with different degrees of randomness in their action on CMC, Endo III and Endo IV (*22*, *23*). The less random type of enzyme referred to by these authors as Endo III was similar to the isolated enzyme.

Because it gave less decrease in viscosity compared to the increase of reducing sugars during incubation with CMC and has the same molecular weight and pI of Endo II (45 000) and Endo V (pI 4.4), respectively, isolated by Beldman et al. (*4*), it was identified as endoglucanase. The isoelectric focusing of the purified enzyme on polyacrylamide gel having a pH range of 3.5−9.5 showed a sharp band, confirming the purity of the enzyme. The pI of the purified endolgucanase was 4.3. The carbohydrate content of the pure endoglucanase was around 25 ± 2. The carbohydrate content of the endoglucanase is

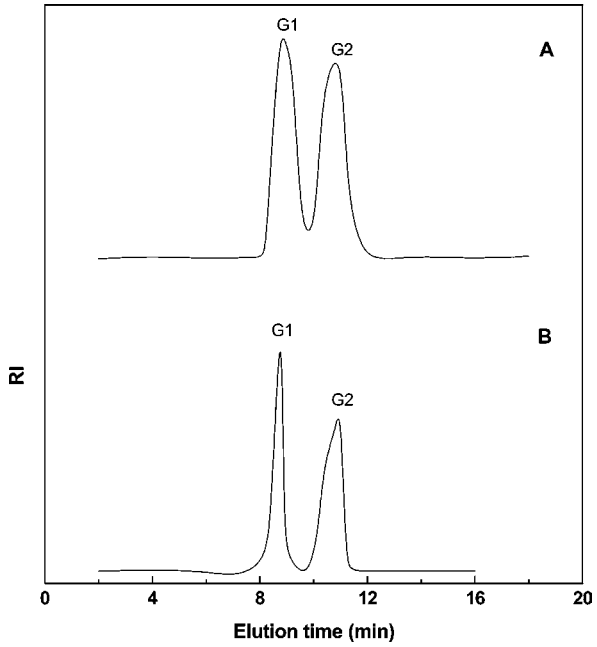

Figure 3. HPLC analysis of products released from CMC. Incubation took place in 0.02 M sodium acetate buffer at pH 5.0 and 37 °C with endoglucanase for 1 h. (A) Sample after incubating for 1 h. (B) Standards: G1, glucose; G2, cellobiose; RI, refractive index.

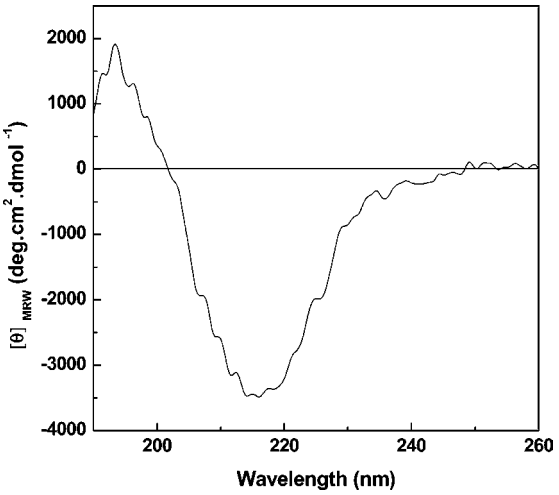

Figure 4. CD spectra of endoglucanase. The enzyme was taken in 0.02 M sodium acetate buffer at pH 5.0 and 25 °C, with a protein concentration of 0.25 mg/mL. The samples were filtered, and their spectra were recorded as indicated in the Materials and Methods.

significantly higher than that reported by Berghem et al. (*24*). The enzyme had an optimum pH of 5.0. The optimum temperature was 40 °C. Lineweaver−Burk analysis showed that K_m was 0.06 g/100 mL and V_{max} was 0.08 unit (mg of protein)$^{-1}$ min^{-1}.

The secondary structure of endoglucanase in solution was determined using far-UV CD. A typical CD spectrum at 25 °C is shown in **Figure 4**. It exhibits a broad negative peak at 217 nm and a characteristic positive absorption band starting from ∼207 nm, and its relatively weak magnitude (mean residue ellipticity = −3300° cm^2/dmol) is consistent with a predominance of β-sheet structure (β sheet ∼ 60%, and α helix ∼ 14%) in endoglucanase, which is true with other endoglucanases as well as with a negligible contribution from α helices. The role of several functional groups for the activity of endoglucanase was elucidated, employing chemical reagents with specific

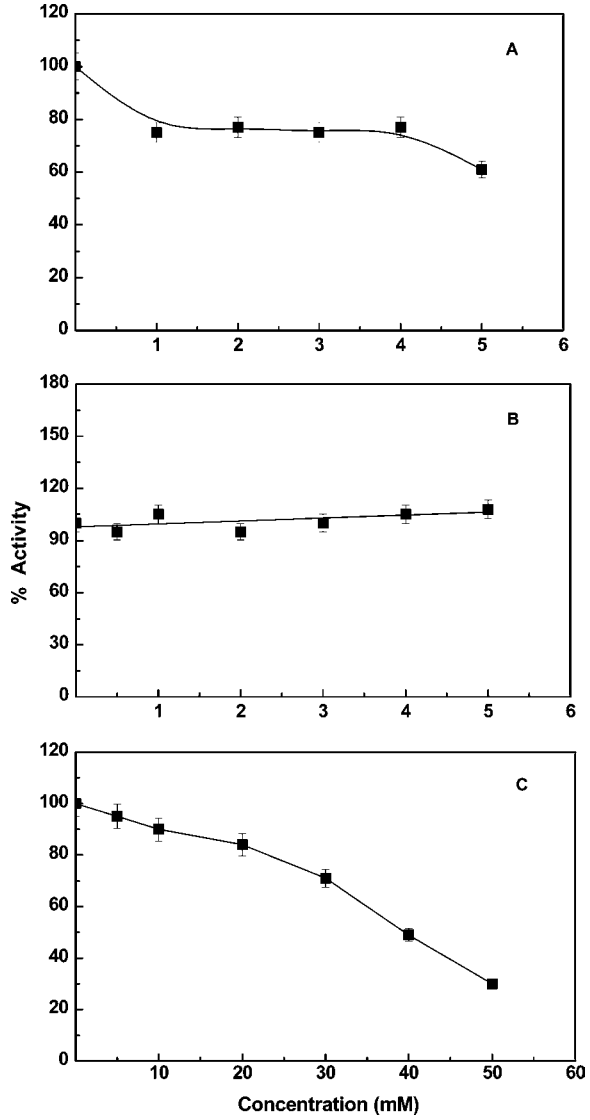

Figure 5. Effect of chemical modification on endoglucanase activity. The enzyme in 0.02 M sodium acetate buffer at pH 5.0 was treated with different concentrations of A–C for 2 h at 37 °C. From these samples, the residual activity was determined by a reducing sugar assay with CM-cellulose as the substrate. (A) DTT, (B) *N*-bromosuccinamide, and (C) DEPC.

reactivity. In the presence of 5 mM DTT, endoglucanase activity was reduced to 60% (**Figure 5A**) compared to the control, indicating that disulfide bonds play an important role at the catalytic site.

NBS is a potent oxidizing agent; a specific reagent for the modification of tryptophan did not have any effect on the activity of endoglucanase (**Figure 5B**), showing that tryptophan is not involved in the catalytic activity. It has been shown by Clarke (*25*) that modification of the tryptophan residue with NBS led to concomitant inactivation of the enzyme, showing that tryptophan is involved in the binding of the substrate and is also a part of the catalytic site. In the present study, it is shown that NBS did not have any effect on the endoglucanase activity, indicating the noninvolvement of tryptophan in the catalytic activity (**Table 3**).

The conversion of histidyl residues of the enzyme was achieved by treatment with different concentrations of DEPC, in 0.02 M sodium acetate buffer at pH 5.0 and 37 °C for 2 h. The residual activity was determined with CM-cellulose as the substrate expressed as a percentage of an appropriate control.

Table 3. Amino Acid Composition of Endoglucanase

amino acid	gram %	amino acid	gram %	amino acid	gram %
Asp	17	Thr	11	Cys	1
Glu	6	Ala	5	Ile	3
Ser	14	Pro	3	Leu	6
Gly	8	Tyr	4	Phe	4
His	1	Val	5	Lys	4
Arg	2	Met	1	Trp[a]	5

[a] Determined by the NBS method.

Carboxymethylation of the histidyl residues of the enzyme showed a 70% loss in the activity (**Figure 5C**), suggesting that these residues participated mechanistically and also in the maintenance of the conformation necessary for the active enzyme. It may be playing a role in the binding of the substrate to the enzyme and proper orientation of the catalytic site of the enzyme for its hydrolysis.

The participation of specific carboxyl groups in the active site of the enzyme was indicated by EDAC modification of endoglucanase. The involvement of carboxyl groups in the mechanistic pathway was investigated using water-soluble carbodiimide i.e. EDAC in sodium acetate buffer at pH 5.0. The initial experiment pertained to the incubation of endoglucanase for 3 h at 37 °C with different concentrations of carbodiimide (**Figure 6a**). In another experiment, it was incubated for different time intervals at the concentration of EDAC that caused maximum inactivation of the enzyme activity. The treatment of the enzyme with EDAC (80−100 mM) in 0.02 M sodium acetate buffer at pH 5.0 for 3 h lead to the complete inactivation of the endoglucanase.

The water-soluble EDAC showed complete inactivation of the enzyme. EDAC has been successfully employed for the inactivation of yeast enolase (*26*) and the identification of specific carboxyl residues involved in the binding of cytochrome *c* to cytochrome oxidase (*27*, *28*). In the present study, the kinetics of the reaction with endoglucanase indicated that the essential carboxyl group of the enzyme is modified during inactivation. Semilogarithmic plots of the residual activity as a function of the time of inactivation for the various concentrations of EDAC (**Figure 6b**) were linear and obey pseudo-first-order kinetics. A plot of the apparent rate of inactivation determined from the slope of log residual activity versus time as a function of the EDAC concentration (**Figure 6c**) revealed that the inactivation is first-order with respect to the carbodiimide concentration (*29*).

Thermal stability of the endoglucanase was studied by incubating it for 30 min at different temperatures ranging from 20 to 90 °C. At 90 °C, the enzyme sustained 50% of its activity after 30 min of incubation (**Figure 7a**). To further check the stability of the enzyme, the incubation time was increased to 5 h; the enzyme was able to maintain its 50% activity with no further loss. The apparent thermal transition temperature (T_m) using the UV spectrophotometer was found to be 57 °C. Similar results were obtained by fluorescence data, where the fraction unfolded was measured after incubating the enzyme for 15 min at different temperatures ranging between 20 and 80 °C (**Figure 7b**). The results show that the unfolding of the molecule does not have much effect on its activity because the enzyme still retains 50% of its activity at 90 °C, where the maximum unfolding of the molecule was observed.

From the data described above, an endoglucanase from *A. aculeatus* having a high specific activity on CMC has been isolated and characterized. Chemical modification studies have shown that carboxyl groups are involved in the mechanism of action of the endoglucanase and histidine has an important role in the binding site of the enzyme. Endoglucanase is able to retain 50% of its original activity at 90 °C. Disulfide bridges are known

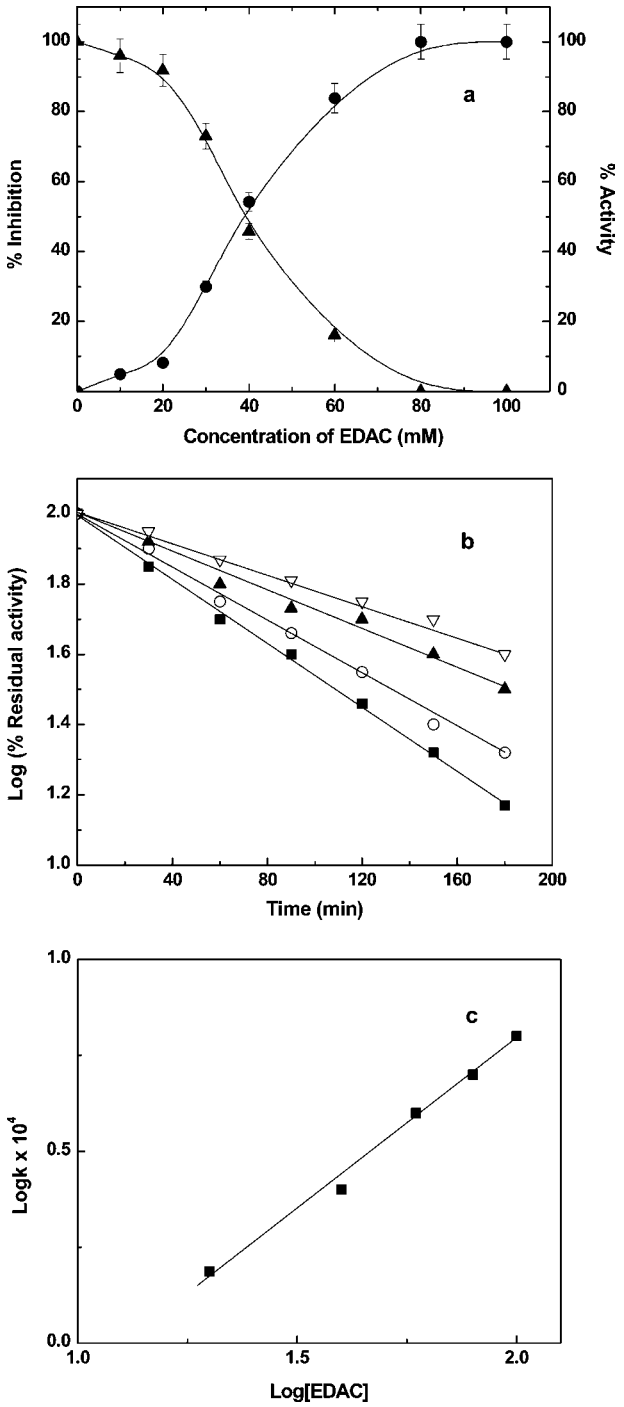

Figure 6. (a) Effect of EDAC on endoglucanase activity. The enzyme in 0.02 M sodium acetate buffer at pH 5.0 was treated with different concentrations of EDAC for 3 h at 37 °C. From these samples, the residual activity was determined by a reducing sugar assay with CM-cellulose as the substrate: (▲) activity; (●) inhibition. (b) Inactivation of endoglucanase by EDAC. The enzyme was treated with different concentrations of EDAC and 0.02 M sodium acetate buffer at pH 5.0 for 3 h at 37 °C. Final concentrations of EDAC were (■) 80 mM, (○) 60 mM, (▲) 40 mM, and (▽) 20 mM. (c) Apparent order of the reaction with respect to the reagent concentration. The pseudo-first-order rate constant (*k*) was calculated from the slope of the data.

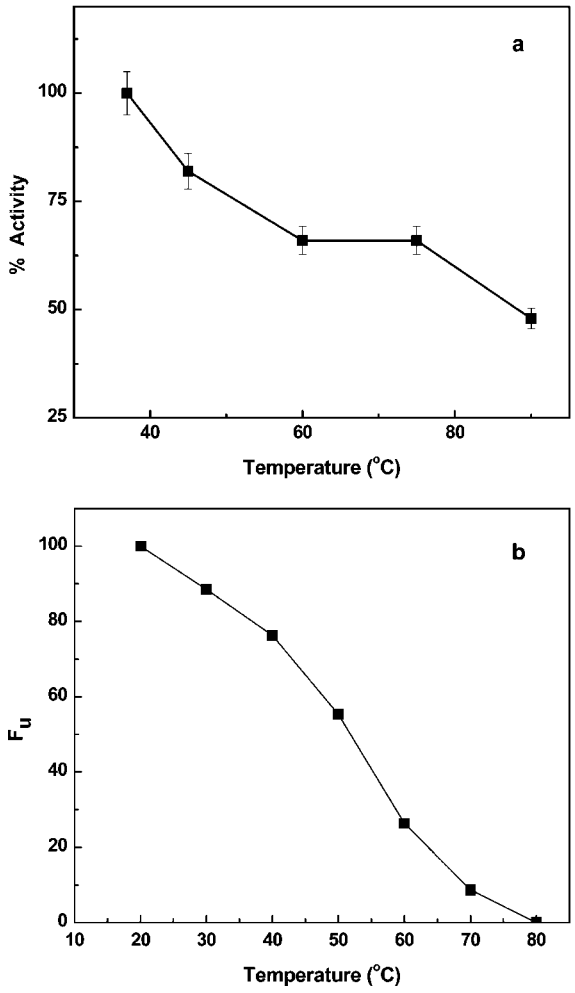

Figure 7. (a) Effect of the temperature on the stability of endoglucanase. Samples of the enzyme were incubated for 30 min at different temperatures, and their activities were then tested as described in the Materials and Methods. (b) Fractional change in the fluorescence intensity as a function of the temperature.

to be important in stabilizing proteins at high temperatures and are apparently important in the structural stability of the enzyme. It appears that the inaccessible disulfides have an important role in maintaining a stable active site, whereas reduction in the accessible disulfides had some effect on the activity. This may be due to the fact that the inaccessible disulfides may be located in the catalytic domain, while the accessible disulfides are in the binding domain. The stability and active conformation of the catalytic core of the Cellobiohydrolase I are reported to be maintained by its six disulfide bridges (*30*). These may likely have a role to play in the stability of the enzyme.

ABBRREVATIONS USED

CMC, carboxymethylcellulose; pNPG, *p*-nitrophenyl-β-D-glucopyranoside; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NBS, *N*-bromosuccinimide; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiotreitol; EDAC, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; DEPC; diethylpyrocarbonate; HPLC, high-pressure liquid chromatography; CMCase, carboxymethyl cellulase; CD, circular dichroism; MW, molecular weight; T_m, apparent thermal transition temperature; MWCO, molecular weight cut-off.

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