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Purification and properties of an extracellular endoglucanase from *Myceliophthora thermophila* D-14 (ATCC 48104)

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An extracellular endoglucanase (1,4-β-glucanohydrolase, EC 3.2.1.4) produced by *Myceliophthora thermophila* D-14 (ATCC 48104) has been purified to homogeneity by ammonium sulphate precipitation and two consecutive ion-exchange chromatographic steps on DEAE-Sephadex A-50 columns. The enzyme was purified 13·8-fold and was homogeneous by analytical PAGE and SDS-PAGE. It has a high apparent M_r, of about 100000. The pH and temperature optima for its activity were 4·8 and 65 °C respectively. The K_m of the purified enzyme for CMC (sodium salt) was 3 mg ml^{-1}. The enzyme displayed low activity toward salicin and *p*-nitrophenyl β-D-glucoside. The activity was enhanced in the presence of Na^+, K^+ and Ca^{2+} but effectively inhibited by Hg^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} and NH_4^+. Inhibition studies indicated that the enzyme may be a metalloprotein and/or that it requires metal ions for its optimum activity.

Introduction

There have been a number of reports on the microbial hydrolysis of cellulose and, especially in the last decade, many papers have appeared on cellulolysis and cellulases. Cellulase is the key enzyme of potential use for the bioconversion of cellulosic materials to simple sugars, which can serve as feedstock for production of different chemicals and fuels. Cellulases from various cellulolytic microorganisms have been studied, but considerable attention has been paid only to those from efficient producers, such as *Trichoderma reesei* (Mandels, 1982), *Clostridium thermocellum* (Ng & Zeikus, 1981), *Thermomonospora* sp. (Moreira *et al.*, 1981), *Cellulomonas* sp. (Langsford *et al.*, 1984), *Bacillus subtilis* (Au & Chan, 1987). The cellulases from thermophiles, with the ability to operate at temperatures at or above 55 °C, provide some advantages, including an increased reaction rate and a stable enzyme system (Rosenberg, 1975). Moreover, the elevated growth temperature of the thermophiles, and the high operating temperature and lower pH requirement of the cellulases from these microbes, restrict the growth of contaminating organisms (Romanelli *et al.*, 1975; Eriksen & Goksoyr, 1974).

Myceliophthora thermophila D-14, a thermophilic fungus possessing a unique cellulase system, was isolated in this laboratory and its cellulolytic activity reported (Sen *et al.*, 1981, 1982, 1983). It produces all three basic cellulase components in extracellular culture filtrates in appreciable amount (Sen *et al.*, 1982; Roy *et al.*, 1990). Because cellulase activity is associated with an enzyme complex, it was considered necessary to examine the properties of the purified cellulase components of this organism. The thermostable property of the enzyme (up to 65–70 °C) and occurrence of a relatively greater amount of the β-glucosidase component also deserved special attention. This paper describes the purification and partial characterization of a major endoglucanase (EC 3.2.1.4) of *M. thermophila*, which has not previously been reported.

Methods

Chemicals. Carboxymethylcellulose (CMC; sodium salt), *p*-nitrophenyl β-D-glucoside (PNPG), salicin, BSA, Coomassie brilliant blue R, M_r marker proteins, α-cellulose and ammonium persulphate were obtained from Sigma. Sephadex G-200 (superfine), DEAE-Sephadex A-50 and blue dextran were purchased from Pharmacia. 3,5-Dinitrosalicylic acid and ammonium sulphate were products of E. Merck. D-Glucose and acrylamide were obtained from BDH. Bisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were purchased from Koch-Light and SRL (India), respectively. All other chemicals were obtained from commercial sources and were of analytical grade.

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Abbreviations: PCMB, *p*-chloromercuribenzoate; PNPG, *p*-nitrophenyl β-D-glucoside.

Organism and culture conditions. The thermophilic fungus *Myceliophthora thermophila* D-14, isolated from city waste (Sen *et al.*, 1981) was used. It was maintained on Czapek–Dox agar slants with 1% (w/v) glucose and 1% (w/v) CMC at 45 °C. Growth from such slants was resuspended in 0·9% NaCl containing 1% (w/v) Tween 80 and used for inoculation of experimental media. The organism was grown under optimal culture conditions on a synthetic medium containing, g l^{-1}: $NaNO_3$, 3·0; KCl, 0·5; KH_2PO_4, 1·0; $FeSO_4.7H_2O$, 0·01; $MgSO_4.7H_2O$, 0·5; crystalline cellulose, 10·0. The pH of the medium was adjusted to 5·5 before sterilization (Roy *et al.*, 1988, 1989). Erlenmeyer flasks (250 ml) containing 100 ml of the above medium were inoculated with 1·0 ml of spore suspension (10^6 spores ml^{-1}) and incubated at 50 °C on a rotary shaker (160 r.p.m., 4 cm eccentric throw) for 11 d. After growth, the mycelia were harvested by centrifugation (6000 *g*, 30 min, 4 °C) and the supernatants were used as the source of enzyme.

Enzyme assay. Endoglucanase (CMCase) activity was assayed according to Stewart & Leatherwood (1976). Appropriately diluted enzyme solution and 25 mg CMC in a final volume of 2 ml, adjusted with 50 mM-sodium acetate buffer, pH 4·8, were incubated at 65 °C for 30 min. The reaction was stopped by addition of 0·5 ml 3,5-dinitrosalicylic acid reagent (Miller, 1959), and A_{540} was measured in a Shimadzu UV 240 spectrophotometer. The enzyme activities were obtained from a calibration curve prepared following the same procedure using D-glucose as standard.

One unit (U) of enzyme activity was defined as the amount of enzyme that catalysed the liberation of reducing sugar equivalent to 1·0 μmol D-glucose min^{-1} under the assay conditions. Specific activity was expressed as units (mg protein)$^{-1}$.

Protein concentrations were determined by the Lowry method, except for the enzyme pools obtained during purification, when A_{280} was used. BSA was used as the standard for both methods.

Purification procedure. All procedures were done at 0–4 °C unless otherwise indicated. Extracellular endoglucanase was purified from freeze-dried supernatant of broth cultures of *M. thermophila* D-14, which had been stored at −20 °C.

(*a*) *Ammonium sulphate precipitation.* Finely powdered ammonium sulphate was added to the culture supernatant. The endoglucanase activity was associated with the fraction precipitated at 50–80% saturation. Precipitated proteins were pelleted by centrifugation (12000 *g*), dissolved in 50 mM-Tris/HCl buffer pH 7·4, and dialysed against the same buffer.

(*b*) *DEAE-Sephadex A-50 chromatography.* The dialysed material was applied to a DEAE-Sephadex A-50 column (2·2 × 42 cm) equilibrated with 50 mM-Tris/HCl buffer pH 7·4. The column was washed with 2 bed volumes of buffer and the retained proteins were eluted with a linear KCl gradient (500 ml; 0–500 mM) in the same buffer at a flow rate of 20 ml h^{-1}. The endoglucanase activity in each 5 ml fraction was determined. The active fractions were pooled, concentrated and dialysed extensively against the same buffer.

(*c*) *Rechromatography on DEAE-Sephadex A-50.* Dialysed material from the first ion-exchange chromatography step was rechromatographed on a column (2·2 × 36 cm) of the same matrix, equilibrated with 50 mM-Tris/HCl buffer pH 7·4. The column was eluted with a linear KCl gradient (500 ml; 0–400 mM) in the same buffer at a flow rate of 16 ml h^{-1} and fractions of 4 ml were collected. The active fractions were pooled, concentrated, dialysed extensively against the same buffer and stored at −20 °C.

Electrophoresis. Non-denaturing PAGE and SDS-PAGE were used to ascertain the degree of protein purity and for estimation of subunit M_r. Analytical non-denaturing PAGE was done in tubes in 7·5% (w/v) polyacrylamide with Tris/glycine buffer pH 8·3, by the method of Davis (1964). SDS-PAGE was done on slabs (15 × 10 × 0·2 cm) composed of 4% (w/v) stacking gels (pH 6·8) and 10% (w/v) resolving gels (pH 8·8) containing 0·1% (w/v) SDS by the method of Laemmli (1970). In both cases gels were stained for protein with Coomassie brilliant blue R and destained by repeated washing in a solution containing 7% (v/v) acetic acid and 5% (v/v) methanol. To determine the subunit M_r of the enzyme by SDS-PAGE, gels were run as above in the presence of the following marker proteins: β-galactosidase (M_r 116000), BSA (66000), ovalbumin (45000), trypsinogen (24000) and β-lactoglobulin (18400). Staining with periodic acid/Schiff reagent was used for the detection of glycoproteins according to the Pharmacia manual *PAGE Laboratory Techniques* (1980).

Gel-filtration chromatography. The native M_r of the purified enzyme was estimated by gel-filtration chromatography through a column (1·6 × 75 cm) of Sephadex G-200 (superfine) by the method of Andrews (1964) with 50 mM-Tris/HCl buffer pH 7·4, containing 100 mM-KCl. The column was calibrated with Blue Dextran and standard M_r marker proteins (catalase, M_r 232000; aldolase, 158000; BSA, 132000). The elution volume (V_e) was taken to be the sum of fractions up to and including the peak fraction of the respective protein. The void volume (V_0) was assumed to be the elution volume of Blue Dextran. A plot of V_e/V_0 against log M_r was used to determine the native M_r of the endoglucanase.

Results

A typical purification is summarized in Table 1. Purification of one major endoglucanase from the culture supernatant was done as described in Methods. Ammonium sulphate precipitation (50–80% saturation) of the

Table 1. *Purification of endoglucanase from culture filtrate of M. thermophila D-14*

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein)$^{-1}$]	Yield (%)	Purification (-fold)
Culture filtrate	283·60	460·0	1·62	100·0	1·0
Ammonium sulphate precipitation (50–80% saturation)	122·60	392·8	3·20	85·4	2·3
DEAE-Sephadex A-50 chromatography (I)	5·36	80·9	15·10	17·6	9·3
DEAE-Sephadex A-50 chromatography (II)	1·69	37·7	22·32	8·2	13·8

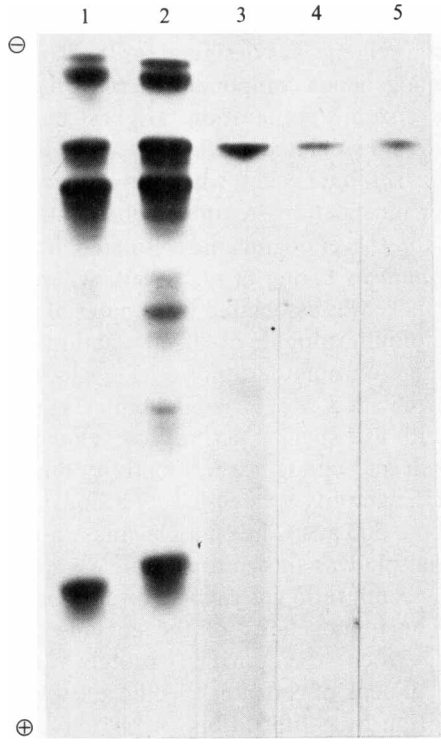

Fig. 1. PAGE of endoglucanase preparations at different steps of purification. Tracks: 1, crude enzyme (130–140 μg), 2, 50–80% saturation ammonium sulphate fraction (130–140 μg); 3, pooled active fractions of first DEAE-Sephadex A-50 step (22 μg); 4 and 5, pooled active fractions from second DEAE-Sephadex A-50 step (15 and 10 μg).

crude enzyme preparation resulted in recovery of 85% of the total activity, and 57% of the total protein was eliminated. The salt-fractionated enzyme preparation was chromatographed on a DEAE-Sephadex A-50 column. The enzyme eluted from the column at approximately 300 mM-KCl. The second DEAE-Sephadex A-50 column step was beneficial in removing trace contaminants. Yield was about 8·2% of the original total activity and the enzyme was purified approximately 13·8-fold. Homogeneity was determined by non-denaturing PAGE and SDS-PAGE (Fig. 1).

To determine whether the enzyme is a glycoprotein, its sugar content was estimated by the phenol/sulphuric acid method (Dubois *et al.*, 1956). The enzyme protein contained no detectable sugar residues. This was further confirmed by periodic acid/Schiff staining.

The M_r of the purified enzyme was 98000 as determined by gel-filtration chromatography through a calibrated column of Sephadex G-200 (superfine), where the enzyme eluted in a single symmetrical peak. A single band corresponding to an M_r fairly close to this value (100000) was also obtained by SDS-PAGE.

The optimum pH and temperature for activity of the enzyme under standard assay conditions were 4·8 and 65 °C respectively. The purified endoglucanase was quite stable for 60 min at temperatures up to 70 °C but it was denatured above this temperature. At 4 °C the enzyme was stable over a very broad pH range (3·5–7·0), but at room temperature (28 °C) it was stable only in a narrow acidic pH range (4·5–5·5).

Purified endoglucanase (180 μg ml^{-1}) in 50 mM-sodium acetate buffer pH 4·8 was stable for at least 6 months when stored at −20 °C. At a concentration of 25 μg ml^{-1} it could be kept at 4 °C for several days without any apparent loss of activity, but at room temperature (28 °C) the half-life of the enzyme was about 7 d.

Kinetic studies of the purified endoglucanase showed that the K_m for CMC was 3 mg ml^{-1}, as determined from a Lineweaver–Burk plot.

Table 2. *Substrate specificity of endoglucanase from M. thermophila D-14*

Substrate	Concn	Linkage	Endoglucanase activity [U (mg protein)$^{-1}$]
CMC	12·5 mg ml^{-1}	β-1,4-	22·3
α-Cellulose	12·5 mg ml^{-1}	β-1,4-	1·6
Solka-Floc	12·5 mg ml^{-1}	β-1,4-	3·1
Filter paper	12·5 mg ml^{-1}	β-1,4-	7·8
Cellulose powder (microcrystalline)	12·5 mg ml^{-1}	β-1,4-	2·6
Cotton	12·5 mg ml^{-1}	β-1,4-	1·1
Starch	12·5 mg ml^{-1}	α-1,4-	0·0
PNPG	10·0 mM	β-1,4-	0·0
Salicin	10·0 mM	β-1,4-	0·0

Table 3. *Effect of cations on the endoglucanase from M. thermophila D-14*

Cation	Concn (mM)	Relative activity (%)*
Control	–	100·0
NH_4^+ [$(NH_4)_2SO_4$]	0·75	10·0
K^+ (KCl)	150·0	116·2
Na^+ (NaCl)	150·0	112·1
Ag^+ ($AgNO_3$)	0·075	60·2
Fe^{2+} ($FeSO_4$)	0·75	15·0
Cu^{2+} ($CuSO_4$)	0·75	62·1
Zn^{2+} ($ZnSO_4$)	0·75	50·0
Mg^{2+} ($MgSO_4$)	0·75	63·3
Mn^{2+} ($MnSO_4$)	0·75	52·1
Co^{2+} ($CoCl_2$)	0·75	86·3
Ca^{2+} ($CaCl_2$)	7·5	110·0
Hg^{2+} ($HgCl_2$)	0·75	38·0
Ba^{2+} ($BaCl_2$)	7·5	100·0

* Enzyme activity in absence of added substance (control) was taken as 100%, and was equivalent to 0·7 U ml^{-1}.

Table 4. *Effect of various reagents on the activity of endoglucanase from M. thermophila D-14*

Compound	Concn	Relative activity (%)*
Control	–	100·0
EDTA	7·5 mM	24·0
Sucrose	2·0% (w/v)	110·2
Glycerol	7·5% (w/v)	118·6
Methanol	22·5% (v/v)	118·2
Ethanol	22·5% (v/v)	120·0
Propan-2-ol	22·5% (v/v)	147·1
2-Mercaptoethanol	4·0 mM	174·3
DTT	5·0 mM	128·0
Glutathione (reduced)	5·0 mM	160·0
Cysteine	7·5 mM	109·4
Glycine	7·5 mM	93·1
Valine	7·5 mM	92·5
Iodoacetamide	7·5 mM	60·8
PCMB	0·1 mM	56·3
Glucose 1-phosphate	2·0% (w/v)	100·0
ATP	3·0 mM	199·2
Urea	2·6 mM	58·4
SDS	0·075% (w/v)	3·2
Tween 80	0·075% (w/v)	100·0
Triton X-100	0·075% (w/v)	95·7
BSA	7·5 mg ml^{-1}	109·2

* See Table 3.

The ability of the purified enzyme to use a number of substrates was tested (Table 2). The enzyme displayed highest activity towards CMC and significantly lower activity towards filter paper and Solka-Floc, followed by α-cellulose, cellulose powder and cotton. The purified enzyme did not exhibit detectable β-glucosidase or amylolytic activity.

Significant inactivation of the enzyme was observed with NH_4^+, Fe^{2+}, Cu^{2+} and Hg^{2+}. K^+, Na^+ and Ca^{2+} enhanced the enzyme activity (Table 3).

Among the various reagents tested (Table 4), thiols and alcohols stimulated enzyme activity but the thiol-group-binding agents (iodoacetamide, PCMB), urea, SDS and EDTA were inhibitory.

Discussion

The cellulases of true cellulolytic organisms are now well-established as multicomponent enzyme systems. The components include 1,4-β-D-glucan glucanohydrolase, 1,4-β-D-glucan cellobiohydrolase and β-glucosidase (Wood & McCrea, 1978; Ramasamy & Verachtert, 1980; Funaguma & Hara, 1988).

It is generally agreed that it is difficult to obtain a single cellulase component in a homogeneous state (Ng & Zeikus, 1981). *M. thermophila* D-14, like other cellulolytic organisms, produces a mixture of hydrolytic enzymes in the culture filtrate. In the present investigation the purification scheme was designed for the isolation of one major homogeneous component of endoglucanase from the crude enzyme preparation. Highest endoglucanase activity was associated with the 50–80% saturation ammonium sulphate pellet, which was collected and used for further purification. A similar observation has been reported for the endoglucanase isolated from *Thermoascus aurantiacus* (Tong *et al.*, 1980), where the major endoglucanase was associated with material precipitated with ammonium sulphate at 30–40% saturation. During ion-exchange chromatography on DEAE-Sephadex A-50 at pH 7·4 with 50 mM-Tris/HCl buffer, endoglucanase activity with low specific activity was eluted during the buffer wash and endoglucanase activity with relatively high specific activity was eluted by a linear gradient of KCl at about 300 mM. The endoglucanase activity of the purified sample was shown to comprise a single protein species by its migration as a single protein band on SDS-PAGE. The homogeneity of the enzyme protein was confirmed when the purified protein eluted as a symmetrical peak of constant specific activity during M_r determination by gel filtration.

The endoglucanase of *M. thermophila* D-14 has a higher M_r than other purified endoglucanases. Generally, endoglucanases are low-M_r proteins, e.g. *Trichoderma viride*, M_r 37000, 52000 and 49000 (Shoemaker & Brown, 1978); *Sporotrichum pulverulentum*, M_r 32000, 36700, 28300, 37500 and 37000 (Eriksson & Pettersson, 1975); and *Bacillus subtilis*, M_r 23000 (Au & Chan, 1987). However, a high-M_r endoglucanase was reported in *Clostridium thermocellum* (M_r 94000) (Ng & Zeikus, 1981) and in *Thermomonospora curvata* (M_r 114000) (Lupo & Stutzenberger, 1988). The results from gel filtration and SDS-PAGE both indicated that the endoglucanase from *M. thermophila* D-14 was likely to be a single peptide protein. This is in agreement with the fact that extracellular enzymes are generally composed of one peptide (Pugsley & Schwartz, 1985).

The purified endoglucanase from *M. thermophila* D-14 is not a glycoprotein, as also shown by Eriksson & Pettersson (1975) for the enzyme from *Sporotrichum*.

Cellulolytic enzymes are generally thermostable, with high temperature optima (Tong *et al.*, 1980; Au & Chan, 1987). The endoglucanase from *M. thermophila* D-14 has a temperature optimum of 65 °C for activity and is stable up to 70 °C for 60 min. The pH optimum of cellulolytic enzymes is usually between pH 4·0 and pH 6·0 (Tong *et al.*, 1980). The pH optimum of the endoglucanase from *M. thermophila* D-14 was 4·8 in 50 mM-sodium acetate buffer and the enzyme was stable over a broad range of pH (4·0–7·0).

The K_m (3 mg ml^{-1}) for CMC of purified endoglucanase from *M. thermophila* D-14 is similar to that of the

enzyme from *Bacillus subtilis* (4 mg l^{-1}) (Au & Chan, 1987) and higher than that of the enzyme from *Thermoascus aurantiacus* (1·9 mg ml^{-1}) (Tong *et al.*, 1980).

The effects of ions on enzyme activity (Table 3) may be due to changes in electrostatic bonding, which would change the tertiary structure of the enzyme. Enhancement of enzyme activity in the presence of thiol compounds, e.g. cysteine, glutathione (reduced) and DTT, and inhibition of activity by thiol-group-blocking agents such as PCMB and iodoacetamide, suggests the possible involvement of thiol groups in the active site of the enzyme. The inhibition of activity may be explained by destabilization caused by alteration in protein structure brought about by disruption of disulphide bridges. Similar observations were reported for the enzyme from *Acetivibrio cellulolyticus* (Mackenzie & Bilous, 1982), *Humicola lanuginosa* (Olutiola, 1982) and *Bacillus subtilis* (Au & Chan, 1987). Inhibition of enzyme activity in the presence of EDTA may be due to the chelation of certain metal ions required for activation and/or stabilization of the enzyme.

Lastly, it should be emphasized that the work described so far represents the purification and properties of a major endoglucanase component, and that this does not represent the total endoglucanase activity of *M. thermophila* D-14.

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