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Purification and characterization of a cellulase from *Catharanthus roseus* stems

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Abstract

Cellulase was purified to homogeneity from stems of *Catharanthus roseus* (periwinkle), by anion exchange, gel filtration and affinity chromatography. The gel filtration on Sephacryl S-200 indicated M_r 96 kDa, diffusion coefficient 5.4×10^{-7} cm^2/s, Stokes' radius 40×10^{-8} cm and frictional ratio of 1.37. The subunit M_r was 25 kDa by SDS-PAGE. The purified enzyme contained 7% carbohydrate. The K_m of the enzyme was 0.44 mg/ml for CM-cellulose. The enzyme exhibited optimum activity between 30 and 35° and at pH 5.2. The enzyme was strongly inhibited by Hg^{2+} and Teepol, but cellobiose had no effect. Reaction product analysis suggests endo-nature of the purified enzyme. The most striking feature of *Catharanthus* cellulase is its ability to hydrolyze suspensions of crystalline (cellulose powder and filter paper) and partially (phospho-, alkali-) swollen cellulose, although at rates lower than CM-cellulose.

Keywords: *Catharanthus roseus*; *Apocynaceae*; Periwinkle; Enzyme purification; Cellulase; β-endo-1; 4-glucanase; Cellulose

1. Introduction

Cellulase from higher plants have been closely related to numerous physiological aspects of plant growth (Kemmerer & Tucker, 1994), including cell-wall loosening in the primary wall (Hayashi, Wong & Maclachlan, 1984; Nakamura & Hayashi, 1993). Cellulase catalyzes the degradation of cell-wall cellulose and is believed to facilitate the penetration of the parasite *Cuscuta reflexa* haustoria into the angiosperm host (*Lantana camara*) stem (Nagar, Singh & Sanwal, 1984). In order to determine the possible role of cellulase in host-parasite interaction, the enzyme is to be characterized. In an earlier communication (Chatterjee, Chauhan & Sanwal, 1997), cellulase from *C. reflexa* was characterized. The present study is concerned with the purification and characterization of cellulase [EC 3.2.1.4. 1,4-(1 : 3;1 : 4)-β-D-glucan 4-glucanohydrolase] from stems of the host plant *Catharanthus roseus* (periwinkle). Purification and properties of cellulases from abscission zone of leaves (Wang, Craker & Mao, 1994), anther (Sexton, del Campillo, Duncan & Lewis, 1990), cotyledons (Lew & Lewis, 1974), epicotyl (Byrne, Christou, Verma & Maclachlan, 1975), fruits (Hatfield & Nevins, 1986) and seedlings (Hayashi & Ohsumi, 1994) have been reported.

2. Results and discussion

2.1. Purification of enzyme

Cellulase was purified from stems of *C. roseus*, employing techniques of anion exchange, molecular sieve and affinity chromatography. The summary of the purification of cellulase is given in Table 1. A four- to five-fold purification was achieved at the step of DEAE-cellulose chromatography, which works by both anion exchange and affinity separation. The use of gel filtration through Sephacryl S-200 and Sephadex G-200 was also very effective resulting in three- to seven-fold purification. Affinity chromatography on Con A-Sepharose resulted in over two-fold purifi-

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Table 1
Purification of cellulase from *Catharanthus* stems

Fraction	Total activity (nkat RA[a])		Total protein (mg)	Specific activity (nkat RA[a] /mg protein)	
$(NH_4)_2SO_4$ (25–80%)	33.5	158.1	132.0	0.25	1.19
DEAE-cellulose	23.2	148.9	23.9	0.97	6.23
Sephacryl S-200	11.2	94.4	4.0	2.80	23.60
Sephadex G-200	6.5	83.3	1.9	3.33	42.72
ConA-Sepharose	4.8	59.1	0.67	7.16	88.21

[a] RA = Relative activity, expressed in kilo units.

cation. Chromatographic profiles of proteins resolved from the $(NH_4)_2SO_4$ fraction of the initial extract of *Catharanthus* stem are shown in Fig. 1a-c. An over-all purification of the enzyme by about 30-fold on the basis of reducing sugar assay of cellulase activity with 14% recovery and about 75-fold on viscometric assay with 37% recovery from $(NH_4)_2SO_4$ fraction was achieved. The low recovery and fold-purification of the enzyme on the basis of reducing sugar may be due to apparent high activity of the initial fraction, because of the presence of β-glucosidases. The purified *Catharanthus* enzyme appears to be homogeneous on the basis of PAGE and SDS-PAGE. The gel showed only one protein band on silver staining (Fig. 1 d). The purified *Catharanthus* enzyme had specific activity much more than the enzyme from kidney bean cotyledons (Lew & Lewis, 1974) and coleus leaf abscission zone (Wang et al., 1994) and had similar activity to that of the enzyme from *Lathyrus odoratus* anther (Sexton et al., 1990).

2.2. Characterization of the purified enzyme

2.2.1. Carbohydrate content

The carbohydrate content (glucose equivalent) of the dialysed purified enzyme was about 7% of the enzyme. The ratio of protein to carbohydrate was 13.5 : 1. *Catharanthus* enzyme appears to be a glycoprotein as observed for cellulases from avocado fruit (Bennett & Christoffersen, 1986) and pepper (Ferrarese et al., 1995). None of the glucanases from pea epicotyl (Maclachlan, 1988) or purified pI 9.5 cellulase from *Phaseolus vulgaris* (Koehler, Lewis, Shannon & Durbin, 1981) contain carbohydrate moiety.

2.2.2. Molecular weight

The M_r of *Catharanthus* cellulase was found 96 kDa as determined by gel filtration through Sephacryl S-200 (Fig. 2). This value is high as compared to cellulase from other plant sources. The observed high M_r of the enzyme may be due to the association of the enzyme with carbohydrate as well as due to its polymeric nature. Generally M_r of cellulase from plant sources is reported in the range of 40–70 kDa (Nakamura & Hayashi, 1993; Wang et al., 1994; Sexton et al., 1990; Byrne et al., 1975; Hatfield & Nevins, 1986; Hayashi & Ohsumi, 1994; Koehler et al., 1981; Truelsen & Wyndaele, 1991). The SDS-PAGE electrophoresis of *Catharanthus* cellulase shows subunit M_r of 25 kDa (Fig. 1 d). It, therefore, appears that *Catharanthus* cellulase is a homotetramer. In contrast to this observation, Byrne et al. (1975) found no change in M_r of auxin-treated pea epicotyl cellulase even under strong dissociating conditions. Nakamura and Hayashi (1993) found M_r of purified endoglucanase from suspension-cultured poplar cells as 40 kDa by gel filtration and 50 kDa by SDS-PAGE, thus showing no subunit structure. Wang et al. (1994) suggested a monomeric structure for coleus cellulase.

2.2.3. Hydrodynamic properties

Stokes' radius of the purified cellulase was found 40×10^{-8} cm from gel filtration data. The diffusion coefficient of the *Catharanthus* cellulase was determined as 5.4×10^{-7} cm^2/s, close to the value of 4.72×10^{-7} cm^2/s for cellulase from pea epicotyl (Byrne et al., 1975) and 5.15×10^{-7} cm^2/s for *Cuscuta* cellulase (Chatterjee et al., 1997). The fractional ratio of *Catharanthus* enzyme was 1.37 compared to 0.92 and 1.61 for buffer-soluble and buffer-insoluble cellulases from pea epicotyl (Byrne et al., 1975) and 1.27 for *Cuscuta* enzyme (Chatterjee et al., 1997). For globular proteins the frictional ratio greater than unity are generally observed (Creighton, 1984). These results, when compared to the values obtained for standard proteins, suggest a globular conformation of *Catharanthus* cellulase.

2.2.4. Stability of enzyme

The enzyme was stable for at least 15 days when stored at 0–4°. The purified enzyme was stable when heated for 5 min at 50°, but lost complete activity at 60°.

2.2.5. Enzyme and time linearity

Cellulase exhibited enzyme linearity up to 25 μg protein under the standard assay conditions (37°, 6 h). The rate of enzyme reaction increased linearity up to 6 h with CM-cellulose as the substrate.

2.2.6. pH-activity relationship

The enzyme exhibited optimum activity at pH 5.2 when 0.02 M citrate buffer in the pH range 4.0–8.0 and 0.02 M succinate buffer in the range 5.0–6.0 were employed.

2.2.7. Effect of temperature

The effect of temperature was studied in the temperature range 20–55°. The optimum activity was

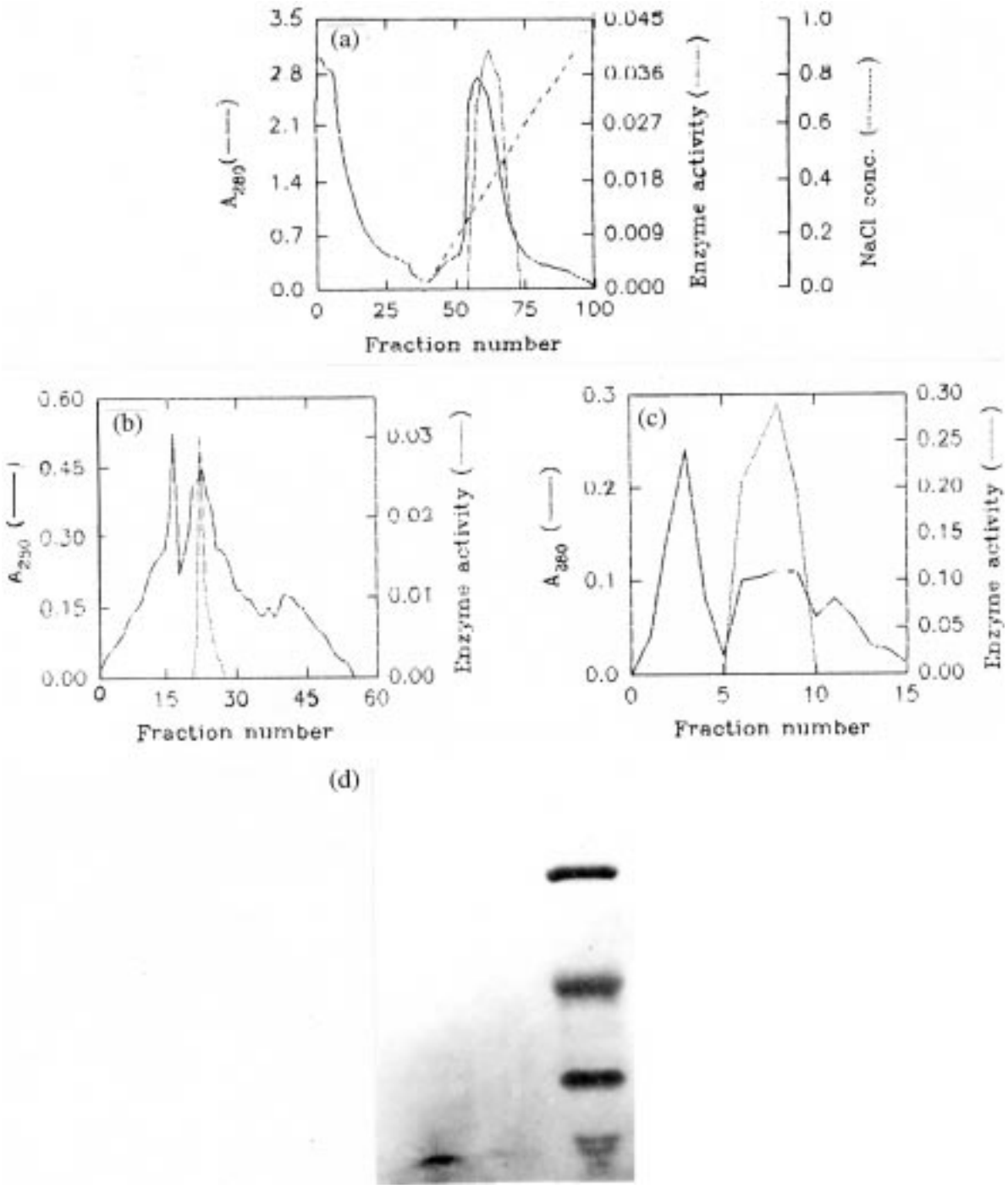

Fig. 1. Purification profile of cellulase from *Catharanthus* stem: (a) DEAE-cellulose; (b) Sephacryl S-200; (c) Con A-Sepharose; (d) SDS-PAGE of the purified enzyme.

observed between 30 and 35° (Fig. not shown); the activity at 45° was reduced by about 50% compared to the activity at 35°. Arrhenius plot of the data indicated energy of activation of 11.4 kcal/mole.

2.2.8. *Effect of sulfhydryl binding agent and sulfhydryl agents*

The sulfhydryl binding agent, *p*-CMB produced over 70% inhibition tested in 5 mM concentration, but iodoacetate did not inhibit the enzyme activity. The sulfhydryl agent 2-ME, cysteine-HCl and DTT, tested in 10 mM concentration inhibited the reaction by 17, 11 and 50%, respectively. *Catharanthus* cellulase resembles cellulases from kidney beans and pea epicotyl in being inhibited by *p*-CMB, but differs from *Cuscuta* cellulase in not being stimulated by thiol agents.

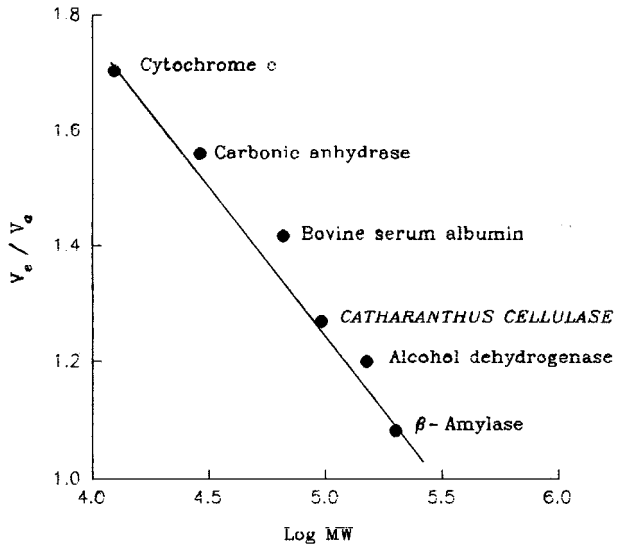

Fig. 2. Estimation of molecular weight of cellulase from *Catharanthus* on Sephacryl S-200 column (1.6 × 95 cm).

2.2.9. *Effect of metal ions and EDTA*

Effect of metal ions was tested in 5 mM concentration. Sodium and potassium ions added as chlorides slightly (15%) stimulated activity, whereas NH_4^+ ions slightly (10%) inhibited the activity. Ba^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} did not significantly affect the enzyme activity, whereas Fe^{3+} slightly (15%) activated the reaction and Cu^{2+} and Mn^{2+} inhibited the activity by about 30%. Hg^{2+} powerfully (95%) inhibited the enzyme activity. EDTA produced 40% inhibition of the enzyme activity in 5 mM concentration. The inhibition of EDTA could not be reversed by Ca^{2+}.

2.2.10. *Effect of some compounds*

NaF, tested in 0.2 M concentration, had no effect on *Catharanthus* cellulase, but NaN_3 in 7.5 mM concentration inhibited cellulase activity by 35%. Cellobiose and sucrose, tested in 5 mM concentration, had no effect on cellulase activity. Cellobiose has been reported as an end product inhibitor of kidney bean (Lew & Lewis, 1974) and pea epicotyl (Wong, Fischer & Maclachlan, 1977) cellulases. In contrast to *Cuscuta* cellulase (Chatterjee et al., 1997), aromatic amino acids L-phenylalanine, L-tyrosine and L-tryptophan, tested in 1 mM concentration, had no marked inhibition on cellulase activity. The non-ionic detergent, Triton X-100 and the cationic detergent, CTAB, did not alter significantly cellulase activity but, Teepol, the sulfonic deter-

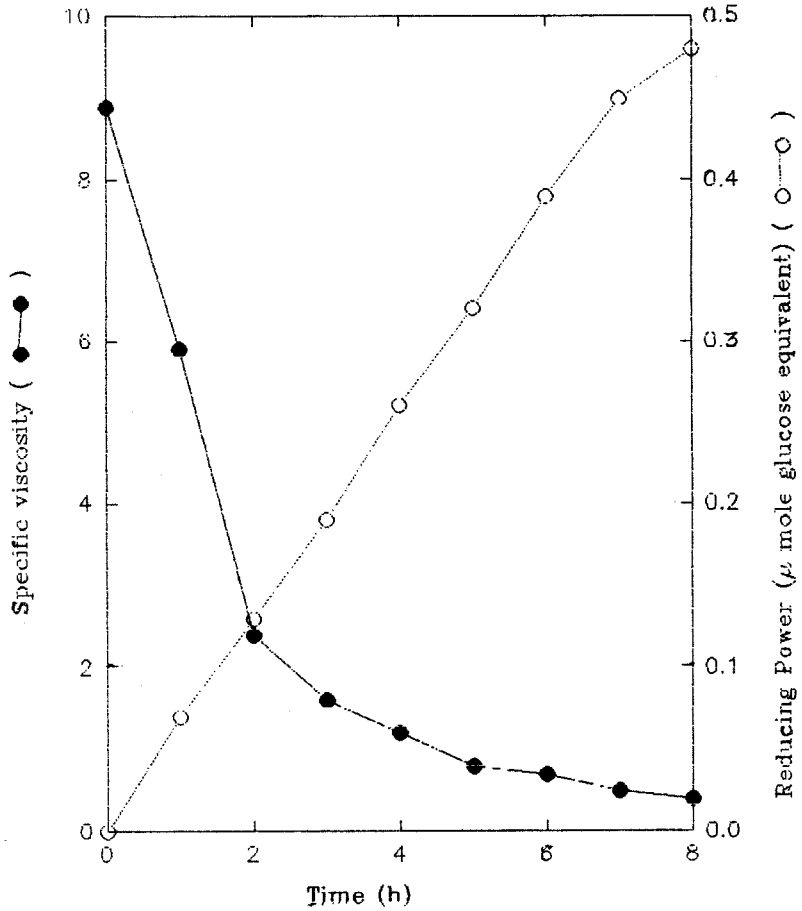

Fig. 3. Specific viscosity and reducing sugar released on incubation of CM-cellulose with purified cellulase.

Table 2
Substrate specificity of purified cellulase from *Catharanthus roseus*[a]

Substrate	Activity (%)
Carboxymethyl cellulose	100
Phosphoric acid-swollen cellulose	75
Alkali-swollen cellulose	85
Cellulose powder CF-12 (Whatman)	56
Filter paper (Whatman No. 1)	64
Cellobiose	0
Arabinogalactan	0
Xylan	0
Polygalacturonic acid	0
p-nitro phenyl-β-D-galactopyranoside	0
p-nitro phenyl-β-D-glucopyranoside	0

[a] Activity (%) indicates the release of reducing sugars from the substrates compared to CM-cellulose, after 16 h incubation in 1 ml solution that contained 0.5 ml of 1.5% substrate at pH 5.0 and enzyme at 37°.

gent tested in 0.1% concentration, strongly (80%) inhibited the activity.

2.2.11. Identification of reaction products

Paper chromatography of the reaction products revealed spots for reducing sugar near the origin, indicating release of longer chain oligosaccharides. No spot could be detected having migration near to glucose or cellobiose. The reaction products were also identified on the basis of viscosity measurement and determination of reducing sugar released during different time of enzyme assays. The enzyme very rapidly decreased the specific viscosity of CM-cellulose solution at the early stage of the reaction, whereas the reducing power of the incubation mixture increased at a low, but at constant rate (Fig. 3), indicating the endo-nature of *Catharanthus* cellulase.

2.2.12. Reaction rate-substrate concentration relationship

Kinetic studies were carried out using CM-cellulose in the range 0.015–0.75% (w/v). The substrate saturation curve was hyperbolic in nature. The K_m value calculated from the double reciprocal plot according to Lineweaver-Burk, was 0.44 mg/ml with V_{max} value of 8.9 nkat/mg protein.

2.2.13. Substrate specificity

Different polysaccharides were used as a substrate to assess the purity of the cellulase preparation in terms of contaminating hydrolytic activity. The substrate tested included larch arabinogalactan containing $(1 \rightarrow 3)$ β and $(1 \rightarrow 6)$ β-galactopyranosides with α-arabinofuranosidic branches, oat spelts xylan containing $(1 \rightarrow 4)$-β-xylopyranosidic linkages, polygalacturonic acid containing $(1 \rightarrow 4)$-D-galactouronopyranosidic linkages, *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-galactopyranoside. No hydrolytic activity against these substrates was detected in the purified enzyme preparation. The enzyme also did not utilize cellobiose (4-O-β-D-glucopyranosyl-D-glucose) as its substrate.

Table 2 summarizes the results of the experiment in which the enzyme was tested on a wide variety of polymeric substrates by determining the release of reducing sugars. The enzyme specifically cleaved the 1,4-β-glucosyl linkages of carboxymethyl cellulose, phosphoric acid-swollen cellulose and alkali-swollen cellulose. Crystalline cellulose, such as insoluble cellulose powder and filter paper were also actively hydrolyzed by the purified *Catharanthus* cellulase preparation.

The most striking feature of *Catharanthus* enzyme is its ability to hydrolyze suspensions of crystalline or partially swollen cellulose, although activities are lower than towards CM-cellulose. Plant cellulases generally are not able to degrade crystalline cellulose (Wang et al., 1994; Fischer & Bennett, 1991). Durbin and Lewis (1988) have reported that purified pI 9.5 cellulase in bean had no activity towards β-1, 4 glucan, but that reducing sugar was observed in cellulase elution from a cellulose column suggesting hydrolysis of cellulose. Wong et al. (1977) reported hydrolysis of Whatman cellulose and swollen cellulose by pea cellulases. The *Catharanthus* enzyme was able to degrade crystalline cellulose from filter paper and thus resembles microbial cellulases (Fischer & Bennett, 1991). It is likely that *Catharanthus* cellulase may attack the crystalline region as well as the peripheral and integral non-crystalline region of cellulose microfibrils resulting in loss of cohesiveness within the fibril structure. O'Donoghue, Huber, Timpa, Erdos and Brecht (1994) studied the influence of avocado cellulase on the structural features of avocado cellulose and observed a downshift in the M_r of unbranched cell wall polymers in the range of 10^6–10^7 Da with cellulose fibrils appearing to lose cohesiveness in response to enzyme activity.

3. Experimental

3.1. Plant tissue

C. roseus (white flowers) were maintained in the greenhouse throughout the year or grown outdoors in the departmental garden. Stems were cut from plants which ranged in the age from 8 to 12 months.

3.2. Substrate preparation

CM-cellulose (Na-salt, high viscosity, Sigma) and cellulose powder (Whatman) were purified by precipitation from 20% (w/v) suspensions with 2 vols of

chilled EtOH at 4°. Phosphoric acid- and alkali-swollen cellulose were prepared according to Wood (1988). All substrates were prepared in 0.1 M NaOAc buffer, pH 5.0 to a final concentration of 1.5% (w/v).

3.3. Enzyme preparation

Freshly harvested *C. roseus* stems were washed with tap water followed by triple distilled water and dried with filter paper. The small pieces of 150 g tissue were homogenized in a Waring blender with 280 ml of cold freshly prepared homogenizing medium containing 0.001 M PMSF, 0.025 M $Na_2S_2O_5$ and 10% NaCl. The motor was operated at high speed for 2 min with four intervals. The homogenate was filtered through four layers of muslin and pH of the filtrate adjusted to 8.0 using chilled 1 N NaOH and brought to total 300 ml. The filtered homogenate was centrifuged at 15,000 *g* for 30 min to get a clear supernatant.

3.4. Purification of cellulase

The clear supernatant was fractionated with $(NH_4)_2SO_4$ (25–80%). The protein fr. so pptd, containing cellulase activity, was dialysed against 0.02 M Na-Pi buffer, pH 7 and loaded onto a DEAE-cellulose column (3.2 × 20 cm), previously equilibrated with the 0.02 M Na-Pi buffer, pH 7. After collecting the pass through, the column was washed with two bed vol of the same buffer. The adsorbed proteins on column were eluted using a linear NaCl gradient (0–1 M). Active frs. (0.3 M–0.4 M NaCl) were pooled and concentrated with sucrose. The enzyme preparation was then subjected to gel filtration through Sephacryl S-200 HR column (1.6 × 90 cm), which had been equilibrated with 0.02 M Na-Pi buffer, pH 7.0 containing 0.1 M NaCl and proteins eluted using Pi buffer containing 0.1 M NaCl and 20% glycerol. The cellulase-rich frs. were concentrated and applied on Sephadex G-200 column (1.6 × 90 cm) pre-equilibrated and eluted with the above buffer. Gel filtration resulted in a single peak of activity. Pooled active frs. were concentrated with sucrose and applied to Con A-Sepharose column (1 × 6.5 cm), which had been washed with 10 bed vol of the binding buffer (0.2 M Na-Pi buffer, pH 7.4 containing 0.5 M NaCl). Proteins were eluted employing 3 bed vol each of the above buffer, 10 mM α-D-methyl mannoside and 20 mM α-D-methyl mannoside, respectively. Cellulase activity was eluted in 10 mM α-methyl mannoside.

3.5. Enzyme assays

Enzyme activity was assayed by measuring the appearance of reducing end groups in a solution of CM-cellulose as described earlier (Chatterjee et al., 1997). The assay system consisted of 0.5 ml of 1.5% CM-cellulose (pH 5.0) and a suitable aliquot of enzyme and water to 1.0 ml. The reaction, initiated by the addition of enzyme was continued for 6 h at 37° and terminated by the addition of dinitro salicylic acid. One unit (n kat) of cellulase catalysed the liberation of 1 n mol of reducing group (glucose equivalent) in 1 s under conditions of the enzyme assay.

Cellulase activity was also determined by measuring the reduction in viscosity of a CM-cellulose solution. The assay mixture contained 0.5 ml of 1.5% CM-cellulose in 0.1 M acetate buffer, pH 5.0 and 0.5 ml of the enzyme solution. Drainage time through a calibrated portion of 500 µl pipette was used as a measure of viscosity. Viscosity was measured every 30 min for 3 h at room temperature (30°). Viscosity data were converted to intrinsic velocity and relative viscosity according to Almin, Eriksson, and Jansson (1967), as described in Durbin and Lewis (1988) for red kidney cellulase. The unit of the enzyme as Relative Activity (RA) was calculated as in Durbin and Lewis (1988).

3.6. Protein determination

Protein was determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951), with bovine serum albumin as a standard. The protein content in frs. obtained from column chromatography was also determined by measuring absorbance at 280 nm.

3.7. Carbohydrate content

The phenol sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) was used to determine carbohydrate in the purified enzyme, using a glucose standard which contained BSA at conc. comparable with protein in the test sample.

3.8. M_r determination

The M_r of cellulase was determined by gel filtration chromatography on a Sephacryl S-200-HR column (1.6 × 100 cm) by method in Whitaker (1963) and Andrews (1964) using cytochrome c (M_r 12,400), carbonic anhydrase (M_r 29,000), bovine serum albumin (M_r 66,000), alcohol dehydrogenase (M_r 150,000) and β-amylase (M_r 200,000) as reference proteins. SDS-polyacrylamide gel electrophoresis was carried out to determine subunit M_r according to the procedure of Laemmli (1970). The standard proteins used were myosin (S.U.M_r 205,000), α-galactosidase (S.U.M_r 116,000), phosphorylase-b (S.U.M_r 97,400), bovine serum albumin (S.U.M_r 66,000), egg albumin (S.U.M_r 45,000), and carbonic anhydrase (S.U.M_r 29,000). Silver staining was done as described in Blum, Beier, and Gross (1987).

3.10. Identification of reaction products

The reaction mixture at the end of 6 h incubation was heated at 100° for 10 min to stop the reaction. The undergraded substrate, CM-cellulose, was pptd. by addition of 5 vol of chilled 95% (v/v) EtOH. The ppt was centrifuged for 15 min at 12,000 *g* and the supernatant lyophilized. The lyophilized sample was dissolved in water and applied to a Whatman No. 1 chromatographic paper and developed in a solvent system *n*-BuOH–HOAc–H_2O (12 : 3 : 5) with glucose and cellobiose as standards. Reducing compounds were visualized with aniline pthalate reagent.

3.10. Hydrodynamic parameters

Hydrodynamic properties of the purified cellulase was estimated by gel filtration on a column of Sephacryl S-200 (1.6×95 cm) as described in Chatterjee et al. (1997).

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