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Purification, properties and factors affecting the activity of *Trichoderma viride* cellulase

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Cellulases produced by *Trichoderma viride* grown on wheat bran were fractionated by ammonium sulphate (50%) and purified by chromatography on DEAE-Sephadex A50 followed by CM-Sephadex C50. The overall purification method showed a protein reduction of 99·8% of the original protein content of the culture filtrate. On the other hand the specific activity was increased about 228-fold. Amino acid analysis of the purified enzyme showed that glycine was the predominant one followed by glutamic acid, serine, alanine and leucine. Cysteine and tyrosine were the least common amino acids.

Electrophoresis by Disc-PAGE showed that the purified enzyme was homogeneous since it gave only one distinctive band. The enzyme had a MW of 58 000 as indicated by SDS-PAGE. The isoelectric point of the enzyme was 5·7. The cellulolytic activity was directly proportional to the enzyme concentration and was substrate-dependent. The maximum enzyme activity was found at pH 5 when incubated at 45°C for 30 min.

INTRODUCTION

In recent years, attention has been paid to decreasing the cost of enzymic hydrolysis of cellulosic plant materials (Ganbarov & Muraclov, 1989; Macris *et al.*, 1989). Only true cellulolytic organisms, possessing exoglucanase activity, can hydrolyse native cellulose, e.g. *Aspergillus terreus* (Garg & Neelakantan, 1981); *Trichoderma Koningii* (Wood, 1968); *T. reesei* (Erickson, 1980; Henri *et al.*, 1984; Mosrati *et al.*, 1988; Otter *et al.*, 1989), *T. viride* (Ostrikova & Konovalova, 1981; Witkowska *et al.*, 1989).

A variety of chromatographic and electrophoretic methods have been used to separate the enzymes of the cellulase complexes. Studies have been reported involving adsorption to chromatography on either cotton fibre (Ogawa & Toyama, 1972) or avicel (Li *et al.*, 1965); ion-exchange chromatography on DEAE-Sephadex (Erikson & Pettersson, 1975) or dextran gels (Selby & Maitland, 1967; Wood, 1968). Also, electrophoresis (Araujo *et al.*, 1986; Ogawa *et al.*, 1986) and isoelectric focusing (Wood & McCrae, 1972) have been used in several studies. The aim of the present investigation is to use a combination of these methods to achieve separation of the cellulase components from a cellulosic waste by *Trichoderma viride*. Some characteristics of the purified enzyme have also been investigated.

MATERIALS AND METHODS

Microorganism

The fungus used throughout this study, namely *Trichoderma viride* was kindly provided by the Microbiological Resource Centre (MIRCEN), Ain Shams University, Cairo, Egypt.

Raw material (cellulosic waste)

The cellulosic waste tested throughout this work comprises wheat bran which was kindly provided by the North Alexandria Flour Mills Company, Alexandria.

Media and cultivation

The stock cultures of *T. viride* were maintained on glucose–peptone agar slants. They were stored at room temperature with transfers at monthly intervals. Cultivation was achieved in 250 ml Erlenmeyer flasks, each containing 50 ml medium with the following composition (g/litre): $NaNO_3$, 2; K_2HPO_4, 1; $MgSO_4 . 7H_2O$, 0·5; KCl, 0·5; $FeSO_4 . 7H_2O$, 0·01. The carbon source was wheat bran (40 g/litre). The flasks were inoculated after autoclaving with 2% spore suspension and surface cultures were performed at 30°C for 7 days.

Table 1. Purification steps of cellulase from culture filtrate of *T. viride*

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Cellulase activity (U/ml)	Total cellulase activity (U)	Specific activity (U/mg prot)	Yield (%)	Purification factor
Crude	1 000	3·8	3 800	2·97	2 970	0·78	100	1
$(NH_4)_2SO_4$	100	15	1 500	23·50	2 350	1·56	79	2
DEAE Sephadex	100	1	100	20·10	2 010	20·10	67	25·8
CM Sephadex	100	0·09	9	16·00	1 600	177·77	54	227·9

$$\text{Specific activity} = \frac{\text{Activity U/ml}}{\text{Protein mg/ml}}.$$

Preparation of the cell free extract

At the end of the incubation period, the surface fungal mats were picked from the culture medium using forceps. The residual wastes were separated by filtration through a sintered glass filter and the filtrate was then spun at 4000 rev/min for 20 min in a cooling centrifuge. The clear supernatant was considered as a source of enzyme and was analysed for its protein content and enzymic activity.

Determination of protein

The protein content of the chromatographic fractions was determined by monitoring at 280 nm.

Determination of cellulase activity

Enzyme assay was determined in terms in carboxy methyl cellulase (CMCase) activity according to the modified technique described by Miller (1959). The released reducing sugars (as glucose) were determined by the dinitrosalicylic methods (Meyer *et al.*, 1948) and measured colorimetrically at 540 nm. A calibration curve was constructed using standard glucose solutions of different concentrations. The enzyme unit of CM-Case was expressed as mmol glucose produced/min/ml of the enzyme solution.

Enzyme fractionation and purification

Ammonium sulphate (50% saturation) was slowly added to the culture filtrate (3 : 1, v/v). The mixture was stirred and kept at 4°C overnight then centrifuged at 8000 rev/min under cooling for 20 min. The supernatant was discarded and the precipitated enzyme was washed with acetone and then dried to an acetone-free preparation. The precipitate was dissolved in 0·02 M acetate buffer (pH 6·3) and dialysed overnight against 0·02 M phosphate buffer (pH 7·2) and purified by the following two successive chromatographic steps.

A. Ion-exchange chromatography on DEAE-Sephadex A50

The dialysed ammonium sulphate fraction was carefully layered on the top of a pre-equilibrated DEAE-Sephadex column (50 × 2·5 cm) and the proteins were eluted with 600 ml 0·02 M phosphate buffer (pH 7·2) containing a linear gradient of sodium chloride (from 0·0 to 0·5 M) at a flow rate of 1 ml/min at 4°C. Fractions (5 ml) were collected.

The pooled fraction which showed maximum enzyme activity was concentrated to its minimum volume and dialysed against 0·02 M phosphate buffer (pH 6·5) at 4°C.

B. Chromatography on CM-Sephadex C50

The concentrated and dialysed DEAE-Sephadex pooled sample was applied to a pre-equilibrated column (50 ×

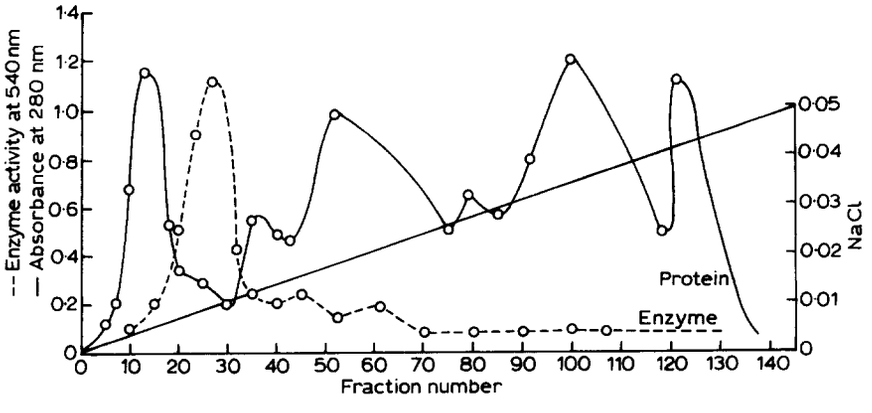


Fig. 1. DEAE—Sephadex A 50 column chromatography of the ammonium sulphate fraction.

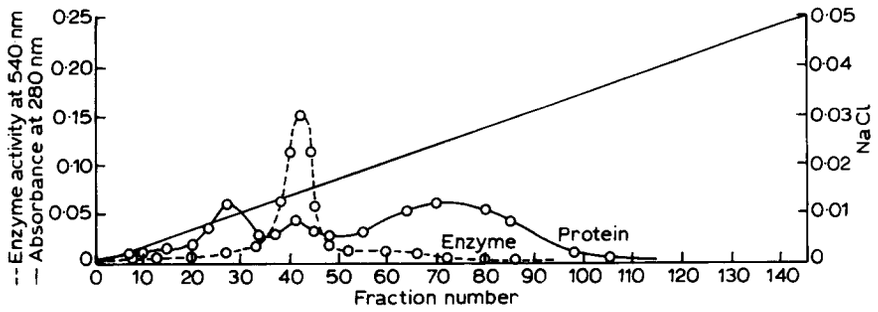


Fig. 2. CM—Sephadex G50 of DEAE—Sephadex fraction.

2·5 cm) of CM-Sephadex C50. The protein samples were eluted with 0·02 M sodium phosphate (pH 6·5) containing a linear gradient of 0·0–0·05 M NaCl, at a flow rate of 1 ml/min and fractions (6 ml) were collected.

The previously obtained enzyme preparations, namely ammonium sulphate, DEAE-Sephadex and CM-sephadex fractions were subjected to protein determination at 280 nm and to cellulase activity measurement.

Amino acid analysis

The amino acid composition was determined in the acid hydrolysate of the purified enzyme using a Beckmann Amino Acid Analyser (Model 119 GK) according to the method described by Speckman *et al.* (1958).

Electrophoresis and isoelectric focusing

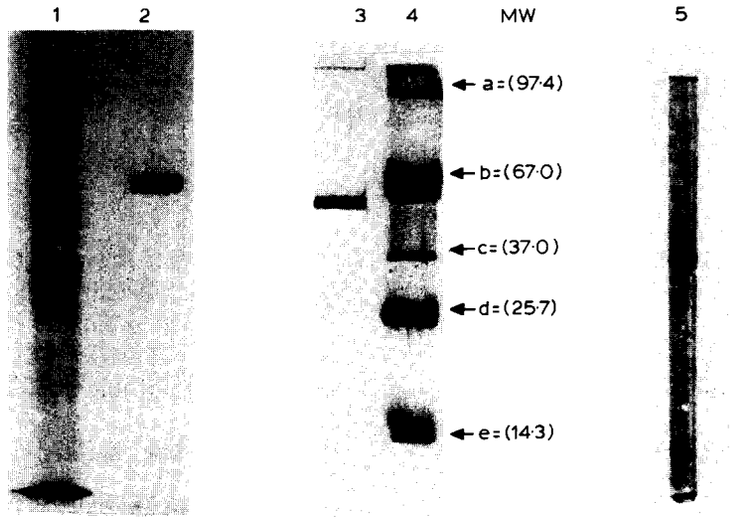
Discontinuous polyacrylamide gel electrophoresis (Disc. PAGE) was performed using a POOMA-PHOR apparatus (Labor Müller, Germany) according to the method of Stegemann *et al.* (1987). The separation gel was prepared from 15% acrylamine in Tris-HCl buffer (pH 8·8) whereas the stacking gel was made by 5% acrylamide in Tris-HCl buffer (pH 6·8). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 6% gel in Tris- borate buffer (pH 7·1) containing 0·01% SDS according to the method of Koenig *et al.* (1970) and Stegemann (1979).

Polyacrylamide gel isoelectric focusing (PAGIF) was performed in cylindrical 5% gel containing 1% ampholytes (Servalyte T pH 4–9). Gel staining and destaining were carried out according to the method of Stegemann *et al.* (1987).

RESULTS AND DISCUSSION

I. Isolation and purification of *T. viride* cellulase

The crude culture filtrate was subjected to partial purification using 50% ammonium sulphate. As a result of this step, the total protein content was reduced by about 60% and the specific activity was increased by 100%, indicating a two-fold purification factor (Table 1).



A = Discontinuous polyacrylamide gel electrophoresis
B = Sodium dodecyl sulphate polyacrylamide gel electrophoresis
C = Polyacrylamide gel iso electric focussing
1 = The crude enzyme extract
2,3,5 = The purified enzyme
4 = Marker protein for MW measurment

Fig. 3. Electrophoretic properties of *T. viride* cellulase.

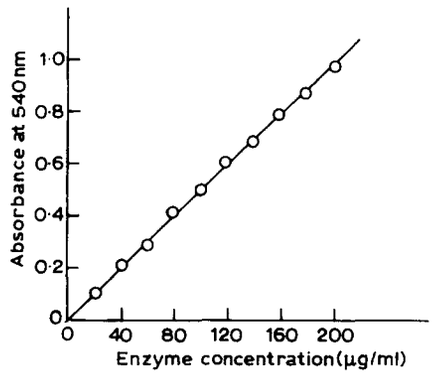


Fig. 4a. Effect of enzyme concentration of *T. viride* cellulase activity

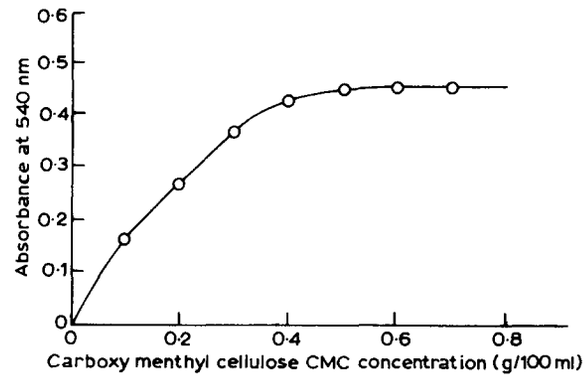


Fig. 4b. Effect of substrate concentration on *T. viride* cellulase activity.

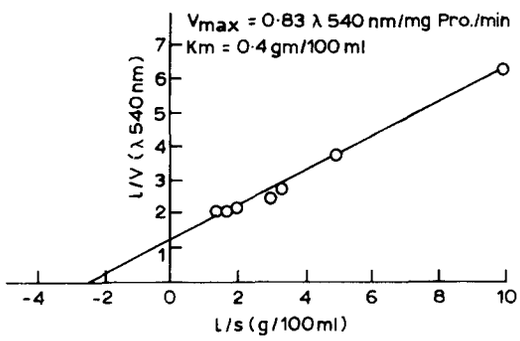


Fig. 4c. Lineweaver—Burk relationship between substrate concentration and enzyme activity.

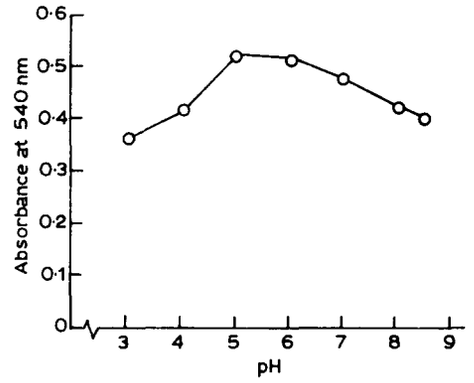


Fig. 4d. Effect on pH on the *T. viride* cellulase activity.

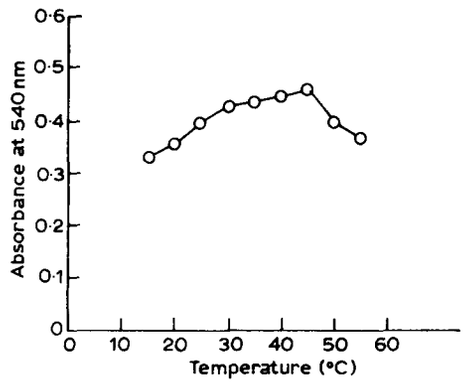


Fig. 4e. Effect of incubation temperature of *T. viride* cellulase activity.

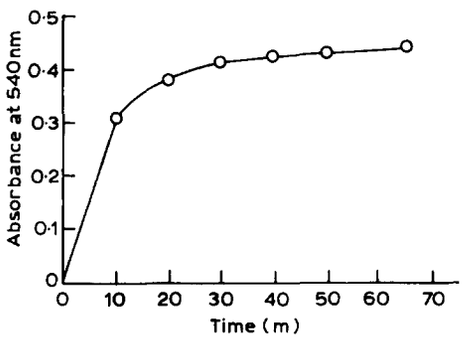


Fig. 4f. Effect of incubation time on *T. viride* cellulase activity.

DEAE-Sephadex A50 ion-exchange chromatography showed that the ammonium sulphate fraction contained different proteins but only one band for cellulolytic activity (Fig. 1). After the DEAE-Sephadex A50 chromatography, the total protein content was reduced by 93% but the specific activity was increase about 25·8-fold, compared with the crude preparation. Chromatography on DEAE-Sephadex has been used by many workers for purification of microbial cellulases such as *Aspergillus terreus* (Murao *et al.*, 1985; Araujo *et al.*, 1986) and *Trichoderma reesei* (Ogawa *et al.*, 1986; Norman and Woodward, 1989).

The pooled fraction for the DEAE-Sephadex column was applied to cation-exchange chromatography on CM-Sephadex C50. The elution profile (Fig. 2) indicated that the enzyme molecule has only one cellulolytic activity band coinciding with one protein band. The protein content showed about 91% reduction and specific activity increased about 228 fold (Table 1).

Nothing was found in the literature indicating the use of combinations of DEAE Sephadex followed by CM-Sephadex for microbial cellulase purification.

II. Electrophoretic characterisation of the purified enzyme

Different purification fractions were examined for their homogeneity by Disc-PAGE. Although the crude extract showed numerous protein bands, the number of bands was reduced after the ammonium sulphate fractionation step. Further reduction in the number of bands took place after DEAE-Sephadex chromatography. The CM-Sephadex fraction showed only on distinctive band, indicating high purity of the isolated enzyme preparation (Fig. 3(A)).

Table 2. Amino acid constituents of the purified enzyme preparation of *T. viride* (g/100 g protein)

Amino acids	*T. viride* cellulase
Iso-leucine	4·13
Leucine	8·23
Lysine	6·15
Methionine	3·17
Cystine	0·88
Phenylalanine	3·51
Tyrosine	0·88
Threonine	3·17
Valine	3·35
Arginine	2·82
Histidine	4·90
Alanine	8·44
Aspartic acid	6·46
Glutamic acid	11·1
Glycine	21·0
Proline	2·93
Serine	8·90

In SDS-PAGE, the crude fraction also showed different protein bands but after DEAE-Sephadex, only three distinctive bands were seen, indicating that considerable amounts of protein were eliminated due to this purification step. After CM-Sephadex chromatography, the eluent showed only one protein band (i.e. one polypeptide chain) representing the cellulolytic activity. The relative electrophoretic mobility of the purified enzyme was found to be 0·29. By comparing this value with those of marker proteins of known molecular weight, it was found that the apparent MW of the purified enzyme was ~59 000 (Fig. 3(B)).

On focusing the purified enzyme in polyacrylamide gel of a pH range 4–9, it showed only one sharp band, confirming the purity of the enzyme. The isoelectric point of the enzyme was determined in the unstained gel by using a contact pH meter and was found to be 5·7 (Fig. 3(C)).

Jianhua & Shuzheno (1981) reported that the purified preparation of *Trichoderma Konignii* cellulase showed a single band of MW 58 000 as measured by SDS-PAGE. Its isoelectric point was 4·0. Araujo *et al.* (1986) reported that cellulases produced by *Aspergillus terreus* were resolved in SDS-PAGE into four components with a molecular weight range from 90 000 to 16 000. Ogawa *et al.* (1986) showed that the molecular weights of *Trichoderma reesei* cellulases were 48 000 and 38 000 as measured by SDS-PAGE.

III. Amino acid composition of the purified enzyme

The results in Table 2 indicate that *T. viride* cellulase is rich in glycine (21·0%) followed by glutamic acid, serine, alanine and leucine. Cysteine and tyrosine were the least common amino acids present in the enzyme molecule. Acidic amino acids (glutamic and aspartic) were found in higher proportions than basic ones, which explains the acidic isoelectric point of the enzyme.

Factors affecting the purified enzyme activity

Figure 4 shows that the enzyme activity was affected by various factors. The cellulolytic activity was directly proportional to enzyme concentration, indicating the dependence of CMC hydrolysis on the enzyme concentration (Fig. 4a). On the other hand, it was found that the hydrolytic activity of the enzyme was substrate-dependent. The rate of hydrolysis of CMC increased with substrate concentration up to a maximum of about 0·6 g/100 ml after which a plateau with saturation was attained (Fig. 4b). Lineweaver–Burk analysis showed that K_m was 0·4 g/100 ml and V_{max} was 0·83 (λ 540 nm)/mg protein/min (Fig. 4c). The optimum pH for the enzyme activity was found to be about 5 (Fig. 4d). This means that the enzyme shows the highest activity when it possesses a slight positive charge. The optimum temperature for the enzyme activity was 45°C (Fig. 4e). This relatively high optimum temperature makes the enzyme suitable for a wide range of uses and different applications. The enzyme activity reached its maximum rate after 30 min incubation (Fig. 4f), indicating that the enzyme had achieved the maximum amount of hydrolysis in quite a short time under these conditions.

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