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ISOLATION, PURIFICATION, AND SOME OF THE PROPERTIES OF HEMICELLULASE FROM *Fusarium* Sp.

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ABSTRACT

An extracellular hemicellulase from a soil fungus (*Fusarium* sp.) grown on a medium containing groundnut hemicellulose B was purified 76-fold by ammonium sulphate fractionation, chromatography on DEAE-cellulose, and gel filtration on Sephadex G-100. It was found to be homogeneous by disc-gel electrophoresis at pH 8. It showed optimal activity at pH 5.6 and 37°. It was observed that groundnut and sesame hemicellulose B were degraded considerably (~80 and 58%, respectively) by the purified hemicellulase, whereas glucomannan and xylan from groundnut were comparatively poorly hydrolysed (~30–40%).

INTRODUCTION

Hemicellulases catalysing the hydrolysis of hemicelluloses have been found in bacteria, fungi, insects, and plant materials. Very recently, Dekker and Richards[1] have reviewed in detail the production, purification, properties, and mode of action of hemicellulases from such different origins as bacteria, fungi, and plant materials. Among the microbial hemicellulases, the most extensively studied are those of fungal origin, for example, *Aspergillus niger*[2], *A. wenti*[3], *Neurospora* sp.[4], *Penicillum janthinellum*[5], *and Trichoderma viridie*[6]. There are some preliminary reports on the hemicellulase from *Fusarium* sp.[7–9]. Our studies on the carbohydrates of groundnuts led us to look for hemicellulases active on groundnut hemicelluloses. The present communication describes the studies on such a hemicellulase from *Fusarium* sp.

MATERIALS AND METHODS

The microorganisms capable of degrading groundnut hemicellulose B were isolated from soil by the enrichment-culture technique. The culture media for growth of the fungal organism was used as described by Davis and Mingioli[10], with some modifications. The medium contained mineral salts and either groundnut hemicellulose

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B, groundnut flour, protein-free groundnut residue, or starch- and protein-free residue. The salts were (g/L): KCl, 0.5; $MgSO_4 \cdot 7\ H_2O$, 0.5; $(NH_4)_2HPO_4$, 2.5; $NaHPO_4$, 0.5; $CaCl_2 \cdot H_2O$, 0.01; $FeSO_4 \cdot 7\ H_2O$, 0.01; $ZnSO_4 \cdot 7\ H_2O$, 0.002; the solution was adjusted to pH 5.6 and contained 3% (w/v) of hemicellulose B or groundnut residues. The fungus was purified by us and characterized as *Fusarium* sp.[11] by our colleagues in the Microbiology Discipline of this institution. The cultures were maintained at 3-monthly transfer on potato–dextrose agar slants at 4°.

Substrates. — The polysaccharide substrates used, groundnut hemicellulose B[12], glucomannan and xylan from groundnut[13], and hemicellulose B from sesame[14] (*Sesamum indicum*) were local isolates. Commercial xylan was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Chromatographic supports. — DEAE-Cellulose and Sephadex G-100 were purchased from Koch–Light Labs, Colnbrook, England and Pharmacia Fine Chemicals, Uppsala, Sweden, respectively.

Analytical methods. — (*a*) *Protein concentration.* Protein content was determined by the method of Lowry *et al.*[21] with crystalline bovine serum albumin as standard.

(*b*) *Paper chromatography.* Paper-chromatographic detection and characterization of sugars in the enzymic hydrolysates was performed as described by Tharanathan *et al.*[12].

(*c*) *Disc-gel electrophoresis of the enzyme.* Electrophoresis was performed according to the method of Ornstein and Davis[22], with 7.5% gels in Tris-glycine buffer, pH 8.0 (10mM).

Enzyme assays. — (*a*) *Hemicellulase activity.* This enzyme was assayed according to the method of Dekker and Richards[15] as modified by Wankhede *et al.*[16]. The assay mixture contained suitably diluted enzyme and a solution (0.5%, w/v) of groundnut hemicellulose B in 50mM sodium acetate buffer, pH 5.6 (2 mL). The mixture was incubated for 30 min at 37°. The reducing sugars released were estimated by the method of Nelson[17], with D-xylose as the standard. One unit of hemicellulase activity was defined as the amount of enzyme that liberates one μmol of D-xylose per min under the experimental conditions, and the specific activity was the number of units per mg of protein.

(*b*) *CM-Cellulase activity.* This activity was assayed by the method of Pettersson *et al.*[18]. The assay system contained 1 mL of CM-cellulose solution (1% w/v), 0.5 mL of 0.2M acetic acid–sodium hydroxide buffer, pH 5.4, and 2 mL of culture broth (~8 mg portein) as enzyme source, and was incubated for 1 h at 37°. The reaction was stopped by the addition of 2 mL of Nelson reagent and the reducing sugars released were determined.

(*c*) *Proteolytic activity.* This activity was assayed according to the method of Kunitz[19], with 1% casein solution.

(*d*) *Amylase activity.* This analysis was performed according to the procedure of Manners and Marshall[20], with a 1% solution of starch as substrate.

TABLE I

HEMICELLULASE, CELLULASE, AMYLASE, AND PROTEASE ACTIVITIES IN CULTURE BROTH

Substrates	*Hemicellulase activity (units/mL)*	*CM-cellulase activity (units/mL)*	*Amylase activity (units/mL)*	*Protease activity (units/mL)*
Groundnut hemicellulose B	0.280	0.06		0.008
Groundnut flour	0.008	0.07	0.006	0.006
Protein-free groundnut residue	0.015	0.08	0.005	0.007
Starch- and protein-free groundnut residue	0.021	0.07	0.002	0.005

RESULTS

Preparation of the crude enzyme. — The fungus was grown in submerged liquid culture on mineral salt and groundnut hemicellulose B or groundnut residue medium as described in Materials and Methods. Starter cultures were prepared by static growth for 4 days at 37° on the foregoing medium, which had been inoculated from a potato–dextrose agar slant. Six Erlenmayer flasks (1 L capacity), each containing 500 mL of the medium, were sterilized (15 min at 120°), inoculated from the starter cultures, and then agitated in a New Brunswick Gyrotary incubator shaker (100 r.p.m., at 37° ±2) for 60 h. The medium was then centrifuged at 10,000*g* for 30 min at 5°. The resultant centrifugate was designated as hemicellulase crude extract.

Effect of different growth substrates on formation of enzyme. — Activities of hemicellulase, cellulase, amylase, and protease in the culture broth of fungus grown on different substrates are shown in Table I. The results revealed that maximal production of hemicellulase was obtained at 60 h, with hemicellulose B as substrate. It was also observed that β-D-xylosidase activity could not be detected when methyl β-D-xyloside and *p*-nitrophenyl β-D-xyloside were used as substrates. The protease and cellulase activities disappeared on storing the enzyme extract for 48 h below 0°, but hemicellulase activity was not affected.

Purification of the enzyme. — The culture was grown in a medium containing groundnut hemicellulose B as already described, and the culture broth was processed further. Unless otherwise stated, all subsequent steps involved in enzyme purification were conducted at 5–10°.

(*a*) *Precipitation by ammonium sulphate.* The enzyme extract (3 L) was brought to 60% saturation with respect to ammonium sulphate by slow addition of powdered ammonium sulphate with gentle stirring. The resultant solution was kept for 5 h and centrifuged at 8000*g* for 10 min. The supernatant solution was brought to 80% saturation by addition of ammonium sulphate with stirring, and the mixture was kept overnight. The precipitate was collected by centrifugation at 8000*g* for 20 min, dissolved in 10 mL of 50mM sodium acetate buffer, pH 5.6, and dialysed against the foregoing buffer overnight.

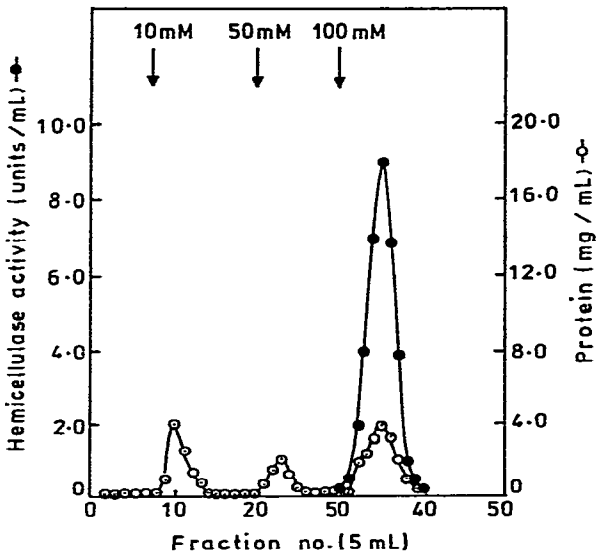

Fig. 1. Elution profile of extracellular hemicellulases on DEAE-cellulose. (●———●) hemicellulase activity; (⊙———⊙) protein.

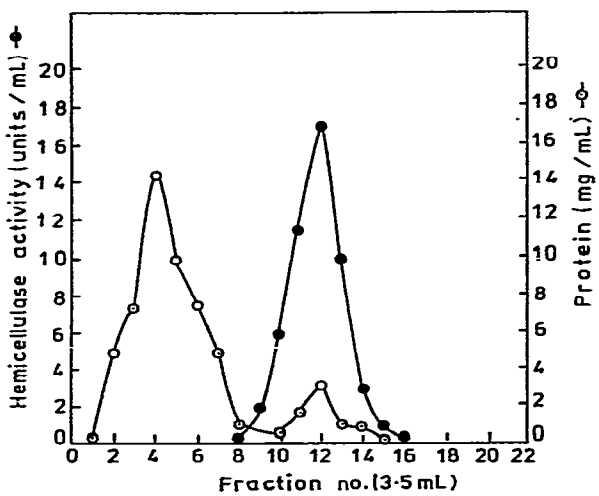

Fig. 2. Elution profile of extracellular hemicellulases on Sephadex G-100. (●———●) hemicellulase activity; (⊙———⊙) protein.

(*b*) *Chromatography on DEAE-cellulose.* The enzyme obtained from the foregoing step was applied to a column (3.5 × 60 cm) of DEAE-cellulose pre-equilibrated with 5mM sodium acetate buffer, pH 5.6, that was eluted stepwise with increasing concentrations (10, 50, and 100mM) of the buffer. Fractions (5 mL) were collected at the rate of 25 mL/h. The elution pattern is graphically depicted in Fig. 1.

(*c*) *Gel-permeation chromatography on Sephadex G-100.* The enzyme solution

TABLE II

PURIFICATION OF HEMICELLULASE OF *Fusarium* Sp.

Purification step	*Total units*	*Total recovery (%)*	*Specific activity (units/mg protein)*	*Purification fold*
Culture broth	845	100	0.056	1
Ammonium sulphate fractionation (60–80%)	830	98	0.69	12
DEAE-Cellulose chromatography	800	94.6	1.01	18
Sephadex G-100	700	82.7	4.25	76

(12.5 mL) was applied to a column (2.5 × 55 cm) of Sephadex G-100 pre-equilibrated with 50mM sodium acetate buffer, pH 5.6. The column was eluted with same buffer at 5°. Fractions (3 mL) were collected at the rate of 20 mL/h. The appropriate fractions (9–15) were pooled and dialysed against the same buffer. The elution profiles of protein and enzyme activity are shown in Fig. 2. The results of purification experiments are summarised in Table II.

Homogeneity of the enzyme. — The enzyme (~200 μg of protein) obtained from the Sephadex G-100 step was subjected to disc-gel electrophoresis at pH 8.0. It showed a single band, indicating homogeneity.

Properties of purified hemicellulase. — *(a) pH Optimum.* The pH optimum of the hemicellulase was determined by incubation of enzyme solution (0.05 mL) and 1% groundnut hemicellulose B solution (0.5 mL) in buffer solutions (acetate buffer, pH 3.7–5.6; phosphate buffer pH 5.7–8.0) for 30 min at 37°. The enzyme had optimal activity at pH 5.6.

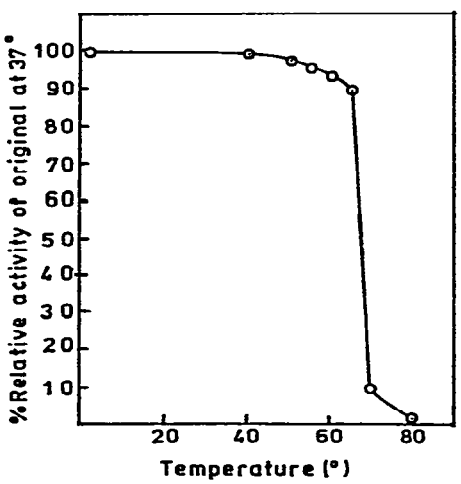

Fig. 3. Temperature stability of extracellular hemicellulases.

TABLE III

KINETIC STUDIES OF HEMICELLULASE

Substrates	V_{max}[a]	K_m *(mg/mL)*
Groundnut hemicellulose B	60.0	4.8
Groundnut xylan	69.5	5.3
Sesame hemicellulose B	65.0	6.2

[a]μMol of D-xylose/min/mg of protein.

TABLE IV

ACTION OF HEMICELLULASE ON DIFFERENT POLYSACCHARIDES

Substrates	*Specific activity*	*Sugars detected in enzyme hydrolysates*[a,b]
Groundnut hemicellulose B	0.288	Xyl_1 (+), Xyl_2 (+++), Xyl_3 (++), tr. (Glc, U_1, U_2)
Sesame hemicellulose B	0.215	Xyl_1 (+), Xyl_2 (+++), Xyl_3 (++), tr. (U_1, U_2)
Groundnut xylan	0.104	Xyl_1 (+), Xyl_2 (+++), Xyl_3 (++), U_1 (++)
Groundnut glucomannan	0.08	Glc (+), tr. (U_1, U_2)
Xylan (Sigma Chemical Co., U.S.A.)	0.07	Xyl_1 (+), Xyl_2 (+++), Xyl_3 (++), tr. (U_1, U_2, U_3)

[a]Xyl_1 denotes xylose; Xyl_n, xylo-oligosaccharides; Glc, glucose; tr., trace; U, denotes unidentified higher oligosaccharides. [b]Relative sugar proportions, as evaluated by the visual-score method on the paper chromatogram, indicated by (+), (++), or (+++).

(*b*) *Temperature optimum.* Incubating the hemicellulase (0.05 mL), 50mM sodium acetate buffer (pH 5.6, 0.5 mL), and groundnut hemicellulose B solution (1% w/v, 0.5 mL) at different temperatures for 30 min established that the optimal temperature for the hemicellulase was 37°.

(*c*) *Thermal stability of the hemicellulase.* The enzyme solution (0.05 mL) was incubated at different temperatures for 30 min, cooled to room temperature, and the activity determined. The results are presented in Fig. 3. A 20% decrease in activity was observed when the enzyme was heated at 55°, but the activity later remained almost constant up to 15 min. At 65°, then was considerable diminution in activity, and the activity was lost when the enzyme was heated for 1 min at 80°.

(*d*) *Kinetic studies.* The kinetic values K_m and V_{max} were determined for a range of substrates in 50mM acetate buffer at pH 5.6. From the Lineweaver–Burk plots, the values for K_m and V_{max} from Michaelis–Menten kinetic studies were calculated and are shown in Table III.

TABLE V

THE EFFECT OF D-XYLOSE ON HEMICELLULASE ACTIVITY

Concentration of D-*xylose (mM)*	*Relative hemicellulase activity (%)*
0	100
2	98.2
4	91.8
8	85.6
10	68.7

Substrate specificity. — Groundnut hemicellulose B, xylan, and glucomannan from groundnut, sesame hemicellulose B, and xylan (Sigma Chemical Co.) were used as substrates in 0.5% (w/v) solution. The enzyme hydrolysates were deionized with Amberlite IRC-50 (H^+) and IR-45 (OH^-) resins. The sugars released were characterized by paper chromatography, and the results are presented in Table IV.

The effect of D-*xylose on the hydrolysis of groundnut hemicellulose B.* — Solutions of sodium acetate (50mM, pH 5.6, 0.5 mL) containing different concentrations of D-xylose (0–10mM) were incubated with the enzyme (0.05 mL) and hemicellulose B solution (1% w/v 0.5 mL) for 24 h at 37°. The results are presented in Table V.

DISCUSSION

The results of the present investigation show that proteolytic and amylolytic activities were present as minor constituents in the culture broth, but they were unstable or were removed during purification of the enzyme. The purified hemicellulase preparation contained no trace of these enzymes.

Hemicellulase of fungal origin (*Aspergillus niger*[2]) is generally active at pH 3.5–5.5 and stable over a wide range of pH (3–10). On the other hand, bacterial hemicellulases from *Bacillus subtilis*[23] and *Streptomyces xylophagus*[24] have somewhat higher pH optima and are stable over the pH range 5–7.3. However, the hemicellulase studied in the present investigation was active over the pH range 5.0–5.6, with optimal activity at pH ~5.6. Similar results were obtained by Gascoigne and Gascoigne[7] for the xylanases of *Fusarium roseum*, wherein they reported that the xylanases had a pH optimum at 6.2 and were stable over the pH range 5.2–6.3.

The fungal hemicellulases[1,24] seem to be most active at temperatures of 37–60°, and the bacterial enzyme also have an optimum temperature-range of 37–60°. In comparison, the enzyme investigated was fairly stable, even at 53°, but its activity was lost completely after 1 min at 80°. It thus resembled other fungal enzymes in its optimal pH, but it had a lower optimum temperature, between 34–37°. This result is in good agreement with those of Gascoigne and Gascoigne[7] for *Fusarium roseum*, and other reports[24].

Results of the present investigation revealed that groundnut hemicellulose B and black-sesame hemicellulose B were attacked to a considerable extent (80 and 58%, respectively), but the rate of hydrolysis of xylan and glucomannan was comparatively low (~30–40%). On the other hand, the xylan (Sigma Chem. Co., U.S.A.) was found to be a poor substrate (~12%). The enzyme catalyses the hydrolysis of xylan to xylose, xylobiose, and xylo-oligosaccharides. The production of xylo-mono-, di-, tri-, and higher oligosaccharides in the enzymic hydrolysates of different polysaccharides indicates that the enzyme acts on the polysaccharides in a random fashion, as does alpha amylase on starch. It was also observed that the presence of xylose, at 10mM concentration, suppressed the enzyme activity to a considerable extent (~30%). The foregoing findings are in good agreement with those of Dekker and Richards[15] for hemicellulase of *Ceratocystis paradoxa*. Similar results were also reported by Duncan *et al.*[25]. Tests for transpentosylation performed by incubation of xylose (5%) with hemicellulose or xylobiose or xylotriose gave negative results. Therefore, the enzyme preparation from *Fusarium* sp. is apparently a xylanase, probably a β-D-xylanase. It has been used in the laboratory-scale processing of groundnut flour for good recovery of protein[26].

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