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THE ENDO-(1→4)-β-D-GLUCANASE SYSTEM OF *Penicillium pinophilum* CELLULASE: ISOLATION, PURIFICATION, AND CHARACTERIZATION OF FIVE MAJOR ENDOGLUCANASE COMPONENTS

K. MAHALINGESHWARA BHAT, SHEILA I. MCCRAE, AND THOMAS M. WOOD*
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB (Great Britain)
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ABSTRACT

Five major endo-(1→4)-β-D-glucanases (I–V) have been isolated from a cellulase preparation of *P. pinophilum*. The pI values for I–V were 7.4, 4.8, 4.1, 3.7, and 4.0, respectively, and the respective molecular weights were 25,000, 39,000, 62,500, 54,000, and 44,500, when determined by SDS-gel electrophoresis. Endoglucanase V was optimally active at 65–70° and I–IV were most active at 50–60°. The pH optima of I and III–V were in the range 4.0–5.0. Antiserum prepared to I reacted only with I; II antiserum reacted only with II. Endoglucanases I and V were more random in their attack on CM-cellulose and H_3PO_4-swollen cotton cellulose, and showed no activity against cello-oligosaccharides containing less than five D-glucose residues, whereas III and IV were active against all the cello-oligosaccharides tested and acted in a less random manner, and II was intermediate in its catalytic action. III was adsorbed completely on both Avicel PH101 and H_3PO_4-swollen cellulose, whereas IV was not adsorbed. The endoglucanases I–V have distinct roles in the digestion of cellulose.

INTRODUCTION

The extracellular cellulases of certain cellulolytic micro-organisms are multi-component systems comprising[1–3] endo-(1→4)-β-D-glucanase [(1→4)-β-D-glucan glucanohydrolase, EC 3.2.1.4], exo-(1→4)-β-D-glucanase [(1→4)-β-D-glucan cellobiohydrolase, EC 3.2.1.91], and β-D-glucosidase (cellobiase or β-D-glucoside glucohydrolase, EC 3.2.1.21). However, the nature of the synergistic interactions that result in solubilisation of cellulose is not known.

There is controversy about the origin, nature, and involvement of the endoglucanases found in cell-free culture filtrates of most cellulolytic micro-organisms, but particularly the fungi. The study of the endoglucanase system of *Penicillium pinophilum* (Hedgecock) (syn. *Penicilium funiculosum* Thom), now reported, was undertaken with this problem in mind.

*Author for correspondence.

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The extracellular cellulase of *P. pinophilum,* which has been studied extensively[4–9], can solubilize crystalline cellulose in the form of the cotton fibre and has commercial potential for the conversion of cellulose into D-glucose[5,8]. Realisation of this potential depends on an understanding of how the enzymes interact. Two cellobiohydrolases have been isolated and their synergistic action studied[6,7], and a glucohydrolase, which did not appear to act synergistically with the endoglucanase system, has been described[8].

The heterogeneity of the endoglucanase system of *P. pinophilum* is not artefactual. Each endoglucanase in the stationary culture phase also appeared in the early phase of growth and did not seem to be affected significantly by the proteolytic activity in the culture filtrate[10]. Thus, each endoglucanase appears to have a specific role to play in the solubilization of cellulose and the data now reported support this contention.

EXPERIMENTAL

Materials and methods. — *Penicillium pinophilum* IMI 87160ii was obtained from the Commonwealth Mycological Institute (Kew, Surrey), Avicel (PH101) was purchased from Honeywell and Stein, Cellofas B (CM-cellulose) was a gift from I.C.I. Ltd., larch-wood xylan, laminarin, lichenin, and CM-pachyman were purchased from Sigma, Ampholine Carrier ampholytes of low and high pI range, standard marker proteins, and Ultrogels AcA 54 and AcA 202 were supplied by LKB Instruments, and DEAE-Sephadex A-50 was purchased from Pharmacia.

Partial fractionation of P. pinophilum *cellulase.* — *P. pinophilum* was cultured[10] in a Microgen 16-L stirred tank fermentor (SF-16) (New Brunswick Scientific Co.) for 10 days at 29°, using a modified Mandels and Weber salts medium[11] (10 L) containing 6% of Avicel PH101 as the carbon source. The culture broth (5.96 L) was then filtered through a glass-fibre disc (porosity 1.6 μm) and concentrated ~6-fold by ultrafiltration (at 25–30 p.s.i.) using an Amcon cell (650 mL) fitted with a PM-10 membrane (step 1). The protein was precipitated by adding $(NH_4)_2SO_4$ to 80% saturation at 0° and collected by centrifugation at 25,000 r.p.m. for 25 min (step 2). The precipitate was dissolved in 0.01M ammonium acetate buffer (46 mL, pH 5.0), desalted in two 23-mL batches on a column (5 × 41.5 cm) of Ultrogel AcA-202 equilibrated with 0.01M ammonium acetate buffer (pH 5.0), and freeze-dried (step 3). Details of the recovery of protein and enzyme are given in Table I.

Fractions containing the desired activities after column chromatography were combined, concentrated by ultrafiltration using a PM-10 membrane (10,000 mol. wt. cut off) in a 50-mL Amicon flow cell, and then desalted on a column of Bio-gel P-2, and the desired fractions were combined and freeze-dried.

H_3PO_4-Swollen absorbent cotton. — Prepared as described[12], this substrate was used to study the effect of various endoglucanases on the d.p. of cellulose chains.

TABLE I

PURIFICATION OF THE ENDOGLUCANASE COMPONENTS FOUND IN *Penicillium pinophilum* CELLULASE

Step	*Purification steps*		*Total volume (mL)*	*Total protein (mg)*	*Total endoglucanase activity (I.U., μmol/min)*	*Specific activity (I.U./mg of protein)*	*Recovery of activity (%)*
	Crude culture filtrate		5956	3097	19,893	6.4	100
1	Concentration by ultrafiltration		1100	2900	18,920	6.5	95.1
2	$(NH_4)_2SO_4$ precipitation		46	2502	12,021	4.8	60.4
3	Desalting and freeze-drying		20.5	1980	10,820	5.4	54.4
4	Fractionation on DEAE-Sephadex						
	Fraction A (endo I and II)		198	313	1803	5.7	9.3
	Fraction B (endo III)		408	485	1149	2.3	5.9
	Fraction C (endo IV)		273	122	999	8.1	5.1
	Fraction D (endo V)		552	443	2158	4.9	10.8
5	Separation of I and II by chromatofocusing:						
	Endoglucanase I		41.5	23	358	15.5	1.8
	Endoglucanase II		43.8	67	315	4.7	1.6
	Purification of I						
6	Chromatofocusing		27.7	9.3	203	21.8	1.0
	Purification of II						
7	Chromatofocusing		32.2	12.3	190	15.0	0.9
8	Gel filtration		18.0	8.2	140	17.0	0.7
9	Isoelectric focusing		6.8	5.4	85	15.9	0.4
	Purification of III						
10	Chromatofocusing	(a)	45.0	40.0	420	10.5	2.1
11		(b)	60.0	31.5	427	13.5	2.1
12	Isoelectric focusing	(a)	28.0	16.0	299	18.7	1.5
13		(b)	8.8	3.2	88	27.7	0.4
	Purification of IV						
14	Chromatofocusing	(a)	49.0	15.0	330	20.0	1.6
15		(b)	50.0	8.0	150	18.8	0.7
16	Isoelectric focusing		4.0	2.0	66	32.9	0.3
	Purification of V						
17	Chromatofocusing	(a)	121.0	24.3	328	13.5	1.6
18		(b)	40.0	8.0	149	10.6	0.7
19	Isoelectric focusing		4.0	1.7	33	19.1	0.2

Cadoxen solution. — The solution, which contained ethylenediamine (280 g) and cadmium oxide (100 g) in distilled water (720 mL), was prepared as described[13] and was kept at 4°.

Cello-oligosaccharides. — These were prepared[14] by acetolysis of dewaxed cotton and isolated by gradient elution (aqueous 0→35% ethanol) from a column (40 × 3.5 cm) of charcoal (BDH; acid-washed)–Celite (1:1).

Isoelectric focusing. — The procedure was that described[15], using an LKB column (110 mL) at 4–5° and a sucrose density gradient containing 1% of Ampholine carrier ampholytes of the required pH. A solution of the sample in the minimum volume of light gradient solution (*i.e.*, the solution containing Ampholine; see LKB Application Note 8100) was applied to the column through a gradient mixer after the column was half-filled.

Analytical methods. — D-Glucose was determined by the D-glucose oxidase method[16], reducing sugars by the method of Somogyi–Nelson[17], and total carbohydrate by the phenol–H_2SO_4 method[18]. Soluble protein was determined (*a*) in cell-free culture filtrates by precipitation with aqueous 5% trichloroacetic acid (3 mL), collection by centrifugation, dissolution in M NaOH (1 mL), and application of the method of Lowry *et al.*[19]; (*b*) in fractions collected from some column effluents and in other samples directly by the method of Lowry *et al.*[19]. The method of Bradford[20] was used when the reagents interfered with the Folin reagent. Each reagent was calibrated, using bovine serum albumin as standard.

Enzyme assays. — *(a) CM-Cellulase (carboxymethylcellulase) activity.* This activity was measured either viscometrically[13] or by the reducing sugar method[13].

(b) Xylanase activity. This activity was measured using aqueous 0.1% larchwood-xylan as substrate. The mixture, which consisted of substrate (1.5 mL; prepared as described[21]) and diluted enzyme (0.5 mL), was incubated for 15 min at 50°. The reducing sugar was then determined. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of xylose equivalent per min.

(c) Activity towards p-nitrophenyl β-D-glucopyranoside. The incubation mixture contained 0.2M sodium acetate buffer (0.5 mL, pH 4.0), 5mM *p*-nitrophenyl β-D-glucopyranoside (0.5 mL), and diluted enzyme (1 mL). After incubation for 30 min at 50°, 0.4M glycine–NaOH buffer (2.0 mL, pH 10.8) was added and the liberated *p*-nitrophenol was determined spectrophotometrically at 430 nm. One unit of activity is defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per min.

(d) Swelling activity. The swelling factor (S-factor) was measured as described[13].

(e) Activity towards cello-oligosaccharides. Incubation mixtures (0.5 mL) consisting of 10mM cello-oligosaccharides (0.125 mL), 0.1M sodium acetate buffer (0.25 mL, pH 5.0), and diluted enzyme (0.125 mL, 2.5 μg of protein) were incubated at 50° for 30 min and then assayed for reducing sugar. Activity towards cellohexaose was studied by using 1 mg of substrate in a 0.2-mL incubation volume.

(f) Activity towards soluble polysaccharides. A mixture (2 mL) consisting of

aqueous 1% substrate (1.0 mL), 0.2M sodium acetate buffer (0.5 mL, pH 5.0), and diluted enzyme (0.5 mL, 5 μg of protein) was incubated at 50° for 15 min. Aliquots (25 μL) were then used for determination of reducing sugar and glucose.

(g) Activity towards insoluble cellulose. Each incubation mixture (5 mL), which was 0.02% with respect to NaN_3, contained substrate (20 mg) suspended in distilled water (2 mL), 0.2M sodium acetate buffer (2.5 mL, pH 5.0), and diluted enzyme (0.5 mL, 5 μg of protein). The mixture was incubated for 24 h at 40°, and total sugar and reducing sugar in the supernatant solution were then determined.

(h) Fractionation of the products of hydrolysis of H_3PO_4-swollen cellulose. The supernatant solutions of the reaction mixtures from (*g*) were deionized using Amberlite IR-120 (H^+) and IRA-400 (HCO_3^-) resins. The eluent and washings were combined and freeze-dried, and a solution of the residue in distilled water (0.2 mL) was filter-centrifuged, using nylon-66 filters (pore size, 0.2 μm). A portion (20 μL) of each sample was then analysed by h.p.l.c., using a column (25 × 0.46 cm) packed with apex silica (5 μ) coated with amine modifier *in situ,* and elution with acetonitrile–water (67:33) containng 0.01% of amine modifier I at 1.5 mL/min. The cello-oligosaccharides were quantified on the basis of peak heights.

(i) Effect of endoglucanases on the d.p. of H_3PO_4-swollen cellulose. The change in d.p. in relation to total sugar released from H_3PO_4-swollen cellulose was studied[13] using 5.0 μg of protein.

Enzyme purity and characterization. — (a) Determination of molecular weights. (i) Gel filtration. An LKB h.p.l.c. unit was used with a column (7.5 × 600 mm) of TSK G-2000 SW equilibrated with 0.05M phosphate buffer (pH 7.0) containing 0.15M sodium chloride. The standard proteins used were cytochrome C (mol. wt. 12,384), ribonuclease (13,700), chymotrypsinogen (24,000), carbonic anhydrase (29,000), β-lactoglobulin (36,000), ovalbumin (45,000) and catalase (60,000).

(ii) SDS-gel electrophoresis. An LKB 2001 vertical Electrophoresis Unit was used[22]. The marker proteins were bovine albumin (mol. wt. 66,000), egg albumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase from bovine erythrocytes (29,000), trypsinogen from bovine pancreas (24,000), trypsin inhibitor from soybean (22,500), and L-lactalbumin from bovine milk (14,200). A plot of log mol. wt. of the markers against mobility was linear.

(b) Amino acid analysis. Samples, each containing 0.1 mg of purified enzyme, were hydrolysed in 6M HCl at 110° for 18 h and then analysed in a Chromaspek Amino Acid Analyser. Cysteine and methionine were determined as cysteic acid and methionine sulphone, respectively, after oxidation with performic acid.

(c) Isoelectric focusing. The thin-layer 4.5% polyacrylamide gels contained ampholytes covering the pH ranges 3.5–5.0 and 3.5–10.0 in the ratio of 2:1. Gels were stained for protein using 0.005% Coomassie Blue in methanol–acetic acid–water (10:1:9), and destaining was effected with methanol–acetic acid–water (3.5:1:8).

(d) Preparation of antibody against endoglucanases I and II. The method was

as described[18], except that three initial injections into rabbits were given at intervals of 2 weeks followed by two injections at intervals of 3 weeks. Blood samples (20–25 mL) were collected 2 weeks after the fifth injection.

(e) Ouchterlony double immunodiffusion[23]. 1% Ionagar in 1:1 0.28M NaCl/100mM Tris HCl buffer (pH 7.4) was used. Immunodiffusion and staining were performed as described[18].

(f) Immunoelectrophoresis–immunodiffusion. Immunoelectrophoresis of crude *P. pinophilum* cellulase (100 μg of protein) or purified endoglucanases (10 μg of protein) was performed in aqueous 1.5% agar gel at 12°, using an LKB 217 Multiphore (Application Note 85). The antibody prepared against either endoglucanase I (1.6 mg of protein) or II (3.6 mg of protein), diluted (1:1) with 0.3M potassium phosphate buffer (pH 8.0), was applied to the lanes cut alongside the wells containing the enzymes. Diffusion was allowed to proceed overnight in a humid incubator at room temperature. The gel was partially dried, washed twice with M NaCl to remove the unprecipitated antibody, then stained with 0.5% Coomassie Blue R 250 in ethanol–acetic acid–water (9:2:9). Destaining was done after 30 min with ethanol–acetic acid–water (9:2:9).

(g) Adsorption of endoglucanases to Avicel PH101 or H_3PO_4-swollen cellulose. Avicel PH101 or H_3PO_4-swollen cellulose (20 mg) in 50mM sodium acetate buffer (2.0 mL, pH 5.0) was incubated with a solution of endoglucanase (0.5 mL, 50 μg of protein) for 1 h at 0°. The unadsorbed CM-cellulase activity in the supernatant solution was measured enzymically using the reducing sugar method, and unadsorbed protein was determined by measuring the absorbance at 280 nm.

RESULTS

Isolation and purification of the endoglucanases I–V. (a) Fractionation on DEAE-Sephadex (step 4). A solution of the partially purified cell-free culture filtrate (1.98 g of protein) from steps 1–3 in 60mM sodium acetate buffer (20.5 mL, pH 4.8) was applied to a column (3.2 × 55 cm) of DEAE-Sephadex A-50 equilibrated with 60mM sodium acetate buffer (pH 4.0), and eluted at 22 mL/h with the same buffer until 270 fractions (5.0 mL) had been collected. The column was then eluted with a gradient formed by the progressive addition of 0.1M NaOH–acetic acid buffer (700 mL, pH 3.8) to 0.06M NaOH–acetic acid buffer (700 mL, pH 4.8). Fractions 45–96 (*A*), 97–135, 136–186, 195–276 (*B*), 277–312, 318–348 (*C*), and 352–420 (*D*) were combined appropriately. Only fractions *A–D*, which were associated with >90% of the endoglucanase activity eluted from the column (Table I), were processed further.

Separation of endoglucanases I and II by chromatofocusing (step 5). — Fraction *A* (step 4) yielded two xylanase, one β-D-glucosidase, and two endoglucanase components on chromatofocusing (Fig. 1). Endoglucanases I and II constituted 26% of the total endoglucanase activity (Table I) and were eluted at pH 7.1 and 6.0, respectively.

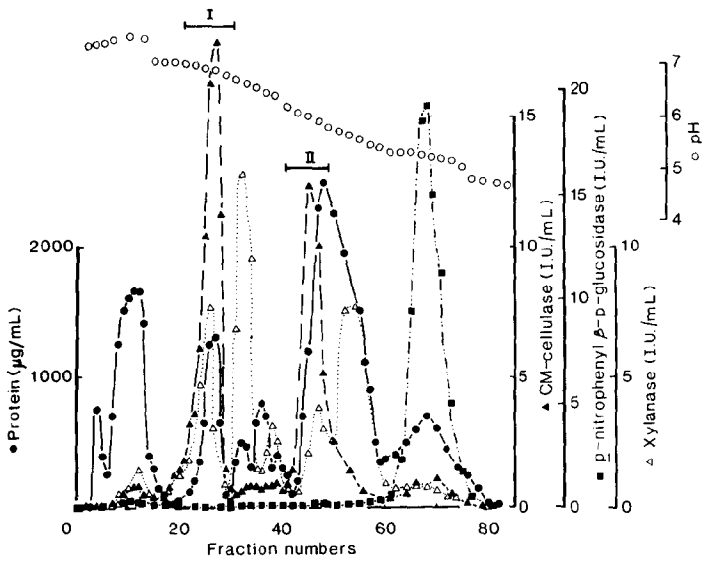

Fig. 1. Chromatofocusing of endoglucanases I and II. Fraction A (step 4, 198 mL) was concentrated using a PM-10 membrane in an Amicon cell, desalted on a Bio-gel P-2 column, and freeze-dried. A portion (300 mg) was dissolved in Polybuffer-74 (diluted 10-fold and adjusted to pH 4.5 with HCl), applied to a column (30 × 1.5 cm) of PBE-94 equilibrated with imidazole–HCl buffer (pH 7.4), and eluted (15 mL/h) with the diluted Polybuffer-74. Fractions (5 mL) were assayed for CM-cellulase (▲), xylanase (△), *p*-nitrophenyl β-D-glucosidase (■), and protein (●); pH (○).

The specific activities of endoglucanases I (fractions 20–28) and II (fractions 42–58) were 15.5 and 4.7, respectively (Table I).

Purification of endoglucanase I by chromatofocusing (step 6). — Fractions 20–28 from Fig. 1 were combined, concentrated, desalted, and freeze-dried. A solution of the residue in diluted Polybuffer-96 (4.5 mL diluted 10-fold and adjusted to pH 6.0 with HCl) was applied to a column (30 × 1.5 cm) of PBE-94 equilibrated with 25mM imidazole–acetic acid buffer (pH 7.6), and eluted with diluted Polybuffer-96 (500 mL, 15 mL/h). Endoglucanase I (fractions 21–26) was eluted at pH 7.4, was well separated from contaminating proteins, and gave a single protein band (Coomassie Blue stain) on isoelectric focusing in polyacrylamide gel (Fig. 2) and in SDS-gels. The specific activity of endoglucanase I was now 21.8 (Table I).

Purification of endoglucanase II. — (a) By chromatofocusing (step 7). Fractions 42–50 (step 5, Fig. 1) were combined, concentrated by ultrafiltration, desalted on a column of Bio-gel P-2, and freeze-dried. A solution of the residue in the minimum volume of Polybuffer-74 (previously diluted 10-fold and brought to pH 5.2 with HCl) was applied to a column (30 × 1.5 cm) of PBE-94 equilibrated with 25mM histidine–HCl buffer (pH 6.5), and then eluted with the Polybuffer-74 at 15 mL/h. A single peak of CM-cellulase activity (215–245 mL) was obtained.

(b) By gel filtration (step 8). The CM-cellulase component from (*a*) was concentrated in an Amicon cell using a PM-10 membrane, desalted on a column of

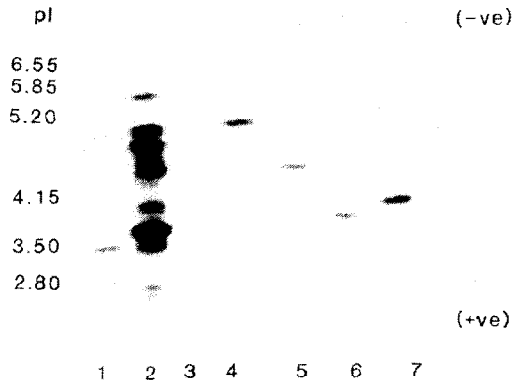

Fig. 2. Isoelectric focusing of the purified endoglucanases in a thin-layer polyacrylamide gel. Lane 1: pepsinogen (pI 2.80), amyloglucosidase (3.50), D-glucose oxidase (4.15), soyabean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), and human carbonic anhydrase B (6.55); lane 2: *P. pinophilum* crude cellulase; lane 3: endoglucanase I; lane 4: endoglucanase II; lane 5: endoglucanase III; lane 6: endoglucanase IV; lane 7: endoglucanase V; 15 μg of protein were applied to each lane.

Bio-gel P-2, and freeze-dried. A solution of 12.3 mg of the resulting protein in 0.1M sodium acetate buffer (2.0 mL, pH 5.0) was applied to a column (91 × 1.6 cm) of Ultrogel AcA-54 equilibrated with above buffer, and eluted with the same buffer at 10 mL/h to yield a more purified endoglucanase II (124–142 mL).

(c) By isoelectric focusing (step 9). The solution of endoglucanase II from (*b*) was concentrated, desalted, and freeze-dried. The residue was dissolved in a "light gradient solution" and applied to an LKB electrofocusing column (110 mL) which contained, in a sucrose density gradient, 1% of a mixture of narrow (3.5–5.0) and broad (3.5–10.0) pH-range ampholytes in the ratio of 2:1. Electrofocusing was performed at 4° for 18 h at 1,600 V and 8 mA. The column was emptied at a flow rate of 110 mL/h using distilled water. Purified endoglucanase II (64–76 mL) showed only one protein band (Coomassie Blue stain) on isoelectric focusing in a polyacrylamide gel (Fig. 2) and on SDS-gel electrophoresis.

Purification of endoglucanase III. — (a) By chromatofocusing (steps 10 and 11). — Fraction B (step 4, 408 mL) was concentrated using a PM-10 membrane in an Amicon cell, desalted on a column of Biogel P-2, and freeze-dried. A solution of the residue (485 mg) in the minimum volume of Polybuffer-74 (diluted 10-fold and adjusted to pH 4.0 with HCl) was applied to a column (30 × 1.5 cm) of PBE-94 equilibrated with 25mM piperazine–HCl buffer (pH 5.5) and eluted with Polybuffer-74 (500 mL, pH 4.0). Fractions rich in CM-cellulase activity (350–390 mL) were collected and re-chromatographed on the same column after preparation of the sample as indicated in step 10.

(b) By isoelectric focusing (steps 12 and 13). The solution from step 11 was concentrated using a PM-10 membrane, desalted on a column of Biogel P-2, and freeze-dried. The product was focused in three equal portions in a stable pH

gradient, using conditions similar to those in step 9, except that 1% of ampholytes covering the pH range 3.5–5 were used.

Fractions containing endoglucanase activity were combined and concentrated, and the isoelectric focusing was repeated using conditions identical to those used in step 9 to give endoglucanase III as a single symmetrical peak in the pH region 4.0–4.1. The final endoglucanase III (3.2 mg of protein) showed only one protein band on isoelectric focusing gel (Fig. 2) and SDS-polyacrylamide gel electrophoresis after staining with Coomassie Blue, and had a specific activity of 27.7 (Table I). A minor component (endoglucanase IIIa) constituted 6–7% of the total endoglucanase activity (step 4) and was not studied further.

Purification of endoglucanase IV by chromatofocusing (steps 14 and 15). — Fraction C (step 4) contained ~15% of the total endoglucanase activity and 66.4% of the β-D-glucosidase activity (Table I). Chromatofocusing was carried out twice (steps 14 and 15) using a pH gradient covering the range 5.0–4.0 (see Fig. 3). The two major β-D-glucosidase components (fractions 25–30 and 60–70) and the minor β-D-glucosidase component (fractions 3–10) were well separated from the major, endoglucanase component (fractions 75–85), which, however, were still not pure as shown by isoelectric focusing. Fractions 77–85 (154–170 mL) were combined, concentrated, desalted, and freeze-dried, and the product was applied to a column (110 mL) containing a stabilised pH gradient covering the range 3.5–5.0. Electrofocusing (step 16) was carried out as in step 9. The major endoglucanase peak (46–54 mL) was pure by isoelectric focusing (Fig. 2) and SDS-gel electrophoresis, and the specific activity was 32.9.

Purification of endoglucanase V. — *(a) By chromatofocusing (steps 17 and 18).* Fraction D (step 4, 550 mL) was concentrated, desalted, and freeze-dried, and

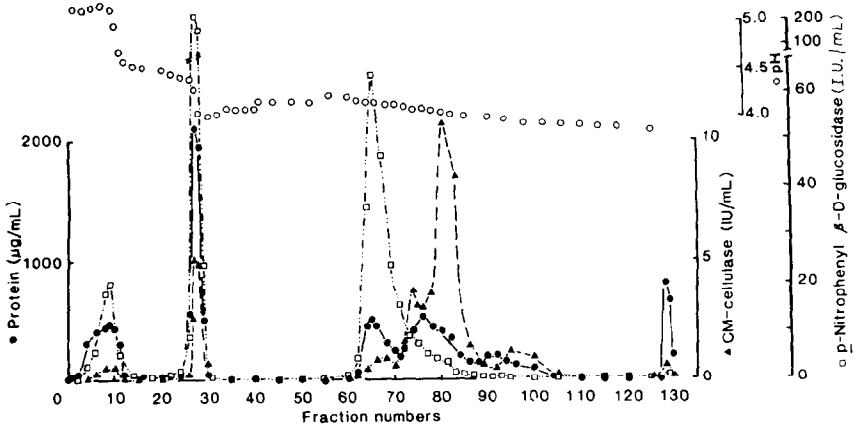

Fig. 3. Chromatofocusing of endoglucanase IV. Fraction C (step 4, 120 mg) was dissolved in the minimum volume of Polybuffer-74 (diluted 10-fold and adjusted to pH 4.0 using HCl), and chromatofocused on a column (30 × 1.5 cm) of PBE-94 equilibrated with 25mM piperazine–HCl buffer (pH 5.2). The column was eluted with the diluted Polybuffer-74 (500 mL). Fractions (2 mL) were assayed for CM-cellulase (▲) and *p*-nitrophenyl β-D-glucosidase (□); pH (○).

divided into three batches (130 mg of each). A solution of each batch in Polybuffer-74 (5 mL, diluted 10-fold and adjusted to pH 4.0) was applied to a column (30 × 1.5 cm) of PBE-94 equilibrated with 25mM piperazine–HCl buffer (pH 5.2) and eluted with Polybuffer-74 HCl (pH 4.0) (step 17). Endoglucanase V, which was eluted in the region 126–150 mL, was concentrated, desalted, and freeze-dried, and the residue was re-chromatographed using the conditions in step 17.

(b) By isoelectric focusing (step 19). The product from step 18 was concentrated, desalted, and freeze-dried, the residue (8.0 mg) was dissolved in "light gradient solution", and the solution was applied to an LKB electrofocusing column (110 mL) containing 1% of narrow (3.5–5.0) pH range ampholytes in a sucrose density gradient. The electrofocusing conditions were identical to those used in step 9. Endoglucanase V, which focused in the pH region 3.9–4.0, had a specific activity of 19.1 and showed only one protein band on isoelectric focusing (Fig. 2) and SDS-gel electrophoresis.

Physico-chemical properties of endoglucanases. — Endoglucanases I and II showed broad pH optima, and III–V were optimally active around pH 5.0. Each was stable at pH <6.0, but the stability decreased markedly at higher pH.

Endoglucanases I–IV showed temperature optima at 50–55°, and V at 65–70°. The activities fell sharply above these optimum temperatures.

As shown in Table II, endoglucanase I was a basic protein with a pI of 7.4, whereas II–V showed pI values between 3.7–4.8. Of the five endoglucanases, those of lowest molecular weight (I and II) showed different molecular weights on gel filtration and SDS-gel electrophoresis.

The affinity of endoglucanase II for CM-cellulose (K_m, 25 mg/mL) was significantly lower than those (K_m, 3.2–4.8 mg/mL) of I, III, or IV, and twelve times lower than that (K_m, 2.0 mg/mL) of V.

The amino acid composition of endoglucanases I–V is presented in Table III. They were rich in aspartic acid, threonine, glutamic acid, glycine, and alanine; II had a low content of serine, and I and IV had high contents of glutamic acid. I and IV had lower contents of alanine than the other enzymes. Endoglucanases I–V

TABLE II

PROPERTIES OF THE ENDOGLUCANASES I–V[a]

Property	*I*	*II*	*III*	*IV*	*V*
pH Optimum	4.0–5.0	3.0–3.4	5.0	5.0	4.8–5.2
Temperature optimum (°)	50–55	55–60	55	50–55	65–70
pI value	7.4	4.8	4.1	3.7	4.0
M_r value					
Gel filtration	10,500	23,000	61,000	54,000	44,000
SDS-gel electrophoresis	25,000	39,000	62,500	54,000	44,500
K_m (mg/mL)	4.5	25.0	3.2	4.8	2.0
V_{max} (μmol/min)	12.5	33.3	22.2	23.8	11.8

[a]Where appropriate, CM-cellulose was the substrate.

TABLE III

AMINO ACID COMPOSITION (MOL%) AND CARBOHYDRATE CONTENT OF THE ENDOGLUCANASES I–V

Amino acid	*I*	*II*	*III*	*IV*	*V*
Asp	10.0	14.9	10.6	11.5	11.4
Thr	8.9	9.7	13.0	6.3	9.4
Ser	20.8	9.9	16.4	11.2	14.5
Glu	11.1	8.7	6.2	11.7	6.2
Pro	1.9	3.8	3.5	6.0	3.2
Gly	12.6	10.8	16.0	12.3	16.9
Ala	7.0	11.9	10.8	8.6	13.3
Cys	1.9	1.3	3.6	1.0	2.5
Val	3.1	4.5	3.0	4.8	2.6
Met	0.4	0.6	1.1	—	1.2
Ile	2.2	1.6	1.8	2.8	1.6
Leu	5.6	5.8	3.7	6.7	3.0
Tyr	6.7	3.4	2.7	3.7	2.3
Phe	2.4	3.5	1.4	3.7	3.3
His	1.5	4.0	2.8	2.4	3.5
Lys	3.9	3.8	3.4	3.1	3.6
Arg	—	1.8	—	4.2	1.5
Carbohydrate content (%)	7.9	9.7	12.2	10.9	18.2

were glycoproteins with carbohydrate contents (phenol/H_2SO_4 method) ranging from 7.9–18.2%.

Rabbit antisera were prepared against endoglucanases I and II. On two-dimensional agar gel electrophoresis–immunodiffusion, the crude cellulase preparation of *P. pinophilum* contained only one protein component which reacted with endoglucanase I antibodies and only one which reacted with endoglucanase II antibodies. Furthermore, purified I cross-reacted only with I antiserum (Fig. 4), and II only with II antiserum.

Catalytic properties of endoglucanases. — (a) Action on soluble polysaccharides. Table IV shows that endoglucanases I–V attacked (1→3,4)-β-D-glucans and CM-cellulose, but the activity was 2–3 times higher on the former. There was no activity towards larchwood xylan or (1→3)-β-D-glucans (laminarin, CM-pachyman).

Only endoglucanases I, III, and IV released glucose from both CM-cellulose and (1→3,4)-β-D-glucans.

(b) Action on insoluble celluloses. — Endoglucanases I–V attacked H_3PO_4-swollen cellulose, but only I showed significant activity on oat-straw α-cellulose (Table V). Their action was minimal on celluloses that contain a high proportion of crystalline regions (cotton, Avicel, Whatman No. 1 filter paper).

Endoglucanases III–V released significant amounts of glucose (data not shown in Table V) and other reducing sugars from H_3PO_4-swollen cellulose, but I and II released mostly reducing sugars other than glucose.

H.p.l.c. of the products of hydrolysis of H_3PO_4-swollen cellulose (Table VI)

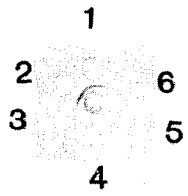

Fig. 4. Ouchterlony double immunodiffusion. Prior to diffusion, the central well contained endoglucanase I antiserum (1.6 mg), and wells 1–6 contained *P. pinophilum* crude cellulase (100 μg), I (10 μg), II (10 μg), III (10 μg), IV (10 μg), and V (10 μg), respectively.

showed that endoglucanases II–IV released glucose, cellobiose (major product), and cellotriose; V released a small amount of cellotetraose in addition to glucose, cellobiose, and cellotriose, and I gave mainly cellotriose and cellotetraose.

(c) Action on cello-oligosaccharides (Table VII). Cellobiose was not a substrate for endoglucanases I–V, but cellotriose and higher cello-oligosaccharides were hydrolysed by III and IV, cellotetraose was hydrolysed by II, cellopentaose was the smallest substrate for V, and cellohexaose was the smallest for I.

(d) Action on CM-cellulose. Because viscosity is related to chain length, the greater the distance from the chain ends in CM-cellulose that the enzyme attacks the greater will be the decrease in the viscosity of a solution per unit increase in reducing power.

The viscosity/reducing power relationship for the action of the endoglucanases on CM-cellulose is shown in Fig. 5. Thus, V and I, with gradients of 6.66×10^{-3}, 3.75×10^{-3}, respectively, can be classified as "more randomly-acting" enzymes[13,25], whereas II–V, with gradients of 1.66×10^{-3}, 0.73×10^{-3}, and 0.67×10^{-3}, respectively, are "less-randomly acting" enzymes.

(e) Effect of D-*glucose and cellobiose.* D-Glucose at 100mM had little effect on any of the endoglucanases, whereas 100mM cellobiose inhibited the CM-cellulase activity of II–V by 30–40% (Table VIII); K_i values of 140, 103, 95, and 137mM were obtained for II–V, respectively. 100mM Cellobiose did not affect the activity of I.

(f) Action on H_3PO_4-swollen cellulose. Endoglucanases I and V produced the most rapid change in d.p. of H_3PO_4-swollen cellulose (Fig. 6) and reduced the average chain-length from 1600 to 400 within 60 min whilst hydrolysing only 0.2% of substrate. In contrast, III and IV solubilized H_3PO_4-swollen cellulose to the extent of 1.5% whilst decreasing the average chain-length from 1600 to 600.

(g) Effect on the uptake of alkali by cotton fibre. Cotton fibres treated with cellulolytic enzymes showed an increase in the uptake of alkali compared to the control. This phenomenon has been called[24] the swelling factor (S-factor) activity and is one of the most sensitive measures of cellulase activity.

The data in Table IX show that all the endoglucanases effected some degree of swelling, and significant differences in the swelling factor activity were observed.

(h) Adsorption on to Avicel PH101 and H_3PO_4-swollen cellulose. Table X

TABLE IV

HYDROLYSIS OF SOLUBLE β-D-GLUCANS BY ENDOGLUCANASES I–V

Substrate	*I*		*II*		*III*		*IV*		*V*	
	Glucose (μg)	*Reducing sugar[a] (μg)*	*Glucose (μg)*	*Reducing sugar (μg)*	*Glucose (μg)*	*Reducing sugar (μg)*	*Glucose (μg)*	*Reducing sugar (μg)*	*Glucose (μg)*	*Reducing sugar (μg)*
CM-cellulose (1→4)	22	225	0	129	31	167	30	156	3	121
Larch-wood xylan (1→4)	0	58	0	33	0	14	0	23	0	14
Laminarin (1→3)	0	28	0	12	3	23	6	14	0	11
CM-pachyman (1→3)	0	0	0	6	0	0	0	0	0	0
Barley glucan (1→3,4)	16	808	0	466	41	351	40	309	0	106
Lichenin (1→3,4)	13	639	0	427	10	259	14	253	0	143
Oat glucan (1→3,4)	11	770	0	284	4	124	0	97	0	59

[a]Glucose equivalent.

TABLE V

ACTION OF THE ENDOGLUCANASES I–V ON INSOLUBLE CELLULOSES

Enzyme	*Assay*	*Substrates*				
		H_3PO_4-swollen cellulose	*Cotton*	*Avicel*	*Whatman No. 1 filter paper*	*α-Cellulose*
I	Total sugar[a] (μg)	1462	20	78	117	255
	Reducing sugar[a] (μg)	980	6	14	38	152
II	Total sugar (μg)	491	18	39	25	0
	Reducing sugar (μg)	303	12	4	8	0
III	Total sugar (μg)	1199	22	51	44	81
	Reducing sugar (μg)	937	3	6	0	24
IV	Total sugar (μg)	755	23	59	54	41
	Reducing sugar (μg)	594	12	4	2	4
V	Total sugar (μg)	523	18	39	35	14
	Reducing sugar (μg)	292	4	4	2	3

[a]Glucose equivalent.

TABLE VI

SOLUBLE SUGARS RELEASED[a] FROM H_3PO_4-SWOLLEN CELLULOSE BY THE ENDOGLUCANASES I–V DURING 24 h

Sugar	*I*	*II*	*III*	*IV*	*V*
Glucose	27	35	200	158	252
Cellobiose	170	529	1411	1208	1357
Cellotriose	278	459	203	281	340
Cellotetraose	317	—	—	—	69
Cellopentaose	14	—	—	—	—

[a]In μg: see Experimental.

TABLE VII

HYDROLYSIS OF CELLO-OLIGOSACCHARIDES BY THE ENDOGLUCANASES I–V

Substrate	*I*	*II*	*III*	*IV*	*V*
	Reducing sugar released (μg)				
Cellobiose	0	0	0	0	0
Cellotriose	0	0	345	257	0
Cellotetraose	0	209	313	201	0
Cellopentaose	0	241	747	884	345
Cellohexaose	178	n.d.[a]	n.d.	n.d.	270

[a]Not determined.

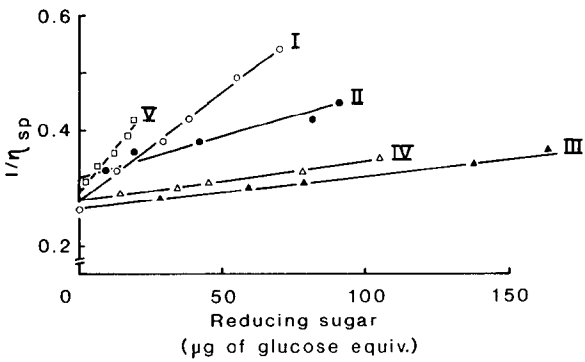

Fig. 5. Plot of increase in fluidity ($1/\eta_{sp}$) *versus* the release of reducing sugars for the hydrolysis of CM-cellulose by the endoglucanases I–V. A mixture consisting of 5.0 mL of aqueous 1% CM-cellulose (5 mL), 0.2M sodium acetate buffer (2.5 mL, pH 5.4), and diluted enzyme (2.5 mL, 1.0 μg of protein) was incubated at 30° in a viscometer (Ubbelohde-75ASTM-IP 2631). The time of outflow of the reaction mixture was measured after 10, 20, 30, 45, and 60 min, and the specific viscosity was calculated[12]. The reducing sugar assay was carried out using 2.0 mL of each mixture: ○, I; ●, II; ▲, III; △, IV; and □, V.

TABLE VIII

EFFECT OF D-GLUCOSE AND CELLOBIOSE ON THE ACTIVITIES OF THE ENDOGLUCANASES I–V

Endoglucanase[b]	*Inhibition of CM-cellulase activity*[a] *(%)*		
	D-*Glucose (100*mM)	*Cellobiose (100*mM)	*Cellobiose (100*mM)
I	0	0	0
II	5	35	12
III	0	30	3
IV	0	39	9
V	15	33	19

[a]Measured viscometrically as described[13]. [b]1 μg of protein (Folin–Lowry).

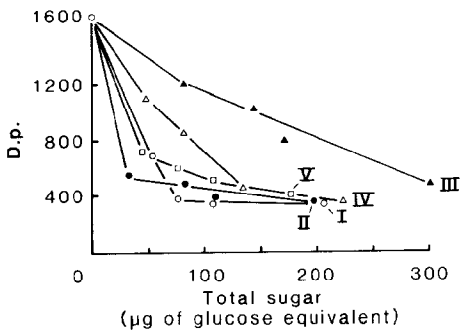

Fig. 6. Plot of the fall in the d.p. and the sugar released from H_3PO_4-swollen cellulose after incubation with the endoglucanases I–V. The d.p. was determined by viscometry after dissolution in cadoxen solution (see Experimental), and the total sugar was determined by the phenol–sulphuric acid method: ○, I; ●, II; ▲, III; △, IV; □, V.

TABLE IX

EFFECT OF THE ENDOGLUCANASES I–V ON THE UPTAKE OF ALKALI BY COTTON FIBRES[a]

Endoglucanase	*Increase in swollen cellulose weight (mg) by*	
	25 μg of protein[b]	*0.5 I.U. of CM-cellulase*
I	26	28
II	11	11
III	12	16
IV	7	15
V	7	10

[a]Assayed[24] using absorbent cotton. [b]Determined by the Folin–Lowry method.

TABLE X

ADSORPTION OF THE ENDOGLUCANASES I–V ON AVICEL PH101 AND H_3PO_4-SWOLLEN CELLULOSE

Endoglucanase[a]	*Adsorption (%)*	
	Avicel PH101	*H_3PO_4-swollen cellulose*
I	37	52
II	17	53
III	96	100
IV	6	0
V	61	81

[a]50 μg of protein (Folin/Lowry determination) was used in each assay (see Experimental).

shows that endoglucanase III was adsorbed completely on to both Avicel PH101 and H_3PO_4-swollen cellulose, but IV was not adsorbed, and I, II and V were adsorbed to various degrees.

DISCUSSION

Fractionation of the extracellular cellulase of *P. pinophilum* revealed several enzymes for which CM-cellulose was a substrate. Such enzymes are described commonly as endoglucanases but, since some purified cellobiohydrolases, isolated from the cellulase system of *T. reesei,* show appreciable activity against CM-cellulose, the definitions are becoming blurred.

By using several chromatographic techniques in 19 steps, some 8 endoglucanases were detected in *P. pinophilum* cellulase, but 90% of the total endoglucanase activity was associated with five major components (I–V) which were studied further. Studies of the physico-chemical properties, substrate specificity, kinetics of hydrolysis, and the mode of action on insoluble polysaccharides and soluble cello-oligosaccharides clearly differentiated the endoglucanases I–V,

established their biochemical uniqueness, and suggested that each might have a specific role to play in the hydrolysis of cellulose.

Antiserum prepared with purified endoglucanase I reacted only with I, and endoglucanase II antiserum reacted only with II. These results contrast with the findings of Niku-Paavola *et al.*[26], who concluded that the endoglucanases found in a culture filtrate of *T. reesei* mutant strain VTT-D-80133 existed as a series of immunologically related components that originated from a common ancestor. However, in a culture filtrate of strain QM 9414 of the same fungus, the presence of two immunologically unrelated endoglucanases was reported by Hakansson *et al.*[27]. Thus, the origin and nature of the multiplicity of endoglucanases in *T. reesei* cellulase preparations remain controversial. From the results presented here and previously[10], which showed that the endoglucanases of *P. pinophilum* appear in the early logarithmic growth phase and then increase in concentration with only minor alteration in the relative proportions, it seems that the multiplicity of endoglucanases is determined genetically.

Each of the endoglucanases I–V moved as a single component in SDS gel electrophoresis of the reduced enzyme, indicating single polypeptide chains. The endoglucanases I–V differed in this property from two of the three endoglucanases of *Sclerotium rolfsii*[28] and all four of the endoglucanases found in a culture filtrate of *Talaromyces emersonii*[29], each of which existed as two sub-units. In SDS gel electrophoresis, the apparent molecular weights of the endoglucanases of I–V ranged from 25,000 to 62,500, but those derived by gel filtration differed for I (25,000 and 10,500) and II (39,000 and 23,000). Similar observations have been made with endo-(1→4)-β-D-xylanase components of *T. koningii*[21] and the β-D-glucosidase of *T. reesei*[30]. It is possible that the anomalous behaviour on gel filtration is related to the shape of the enzyme.

Endoglucanase II had a pH optimum (3.0–3.4) that was at least one unit less than those of I and III–V. Endoglucanase V had a high thermal stability. Most extracellular cellulases of fungi are glycoproteins[31] and the carbohydrate moiety is important for stability. Thus, the thermal stability of endoglucanase V may reflect the fact that the content of carbohydrate was higher than that of the other endoglucanases. Endoglucanase from *S. rolfsii*[28] has a pH optimum of 2.8–3.0, but for most other endoglucanases the optimum lies in the range 4–5.

Of the cello-oligosaccharides tested, only cellopentaose was a substrate for endoglucanase V, whereas cellohexaose was the shortest substrate for I. Hurst *et al.*[32] have reported an endoglucanase from *Aspergillus niger* that required a chain of five D-glucose residues, but, to our knowledge, no endoglucanase requiring more than five residues has been reported hitherto.

The action of endoglucanases I and V on CM-cellulose and H_3PO_4-swollen cellulose resulted in a rapid decrease in the d.p. per unit increase in reducing power, whereas III and IV produced only small changes in d.p. Thus, it appears that endoglucanases I and V act at points on the cellulose chain remote from the ends, whereas III and IV attack near the chain ends. The action of endoglucanase II is intermediate.

Endoglucanase I, which appeared on the basis of observations made with the cello-oligosaccharides to require six D-glucose residues for its active site, did not release any cellohexaose when acting on H_3PO_4-swollen cellulose. Instead, the main products were cellotetraose, cellotriose, and cellobiose together with small amounts of glucose and cellopentaose. These results may be due to the cellohexaose being hydrolysed immediately or to the absence of a relationship between attack on soluble cello-oligosaccharides and attack on insoluble cellulose.

The capacity of endoglucanase I to hydrolyse amorphous cellulose to give a mixture of cello-oligosaccharides, containing mainly cellotetraose and traces of cellopentaose, is unusual. The products released from H_3PO_4-swollen cellulose by *T. viride*[33] endoglucanases contained no oligomers higher than trisaccharide, although higher oligomers were obtained by transglycosylation. None of the endoglucanases I–V showed transferase activity at the concentrations of sugars found in the hydrolysates. Cellotetraose was found in traces in the hydrolysates of H_3PO_4-swollen cellulose by four endoglucanases isolated from *T. koningii*[13] cellulase, but the main product was cellobiose or glucose. Transglycosylation activity was also absent in *T. koningii*[13] endoglucanases.

Whereas the endoglucanases I–V were highly active on reactive, extensively or fully hydrated celluloses, and on short-chain cello-oligosaccharides, the capacity to produce soluble sugars from crystalline cellulase was minimal. Nevertheless, each endoglucanase could effect some changes in the cellulose microfibrils in cotton fibre, resulting in an increased capacity to absorb alkali (S-factor), and, thus, differed from the purified cellobiohydrolases I and II isolated from the same cellulase preparation[5,7]. These observations are consistent with some localised disaggregation either of the microfibrils, or the cellulose chains within these microfibrils, as a preliminary to hydrolysis of the cellulose chains.

Klyosov and his colleagues[34] have demonstrated the importance of adsorption of the endoglucanases on to the cellulose during solubilization. Endoglucanases that were adsorbed strongly preponderated in cellulase preparations that were highly active against crystalline cellulose[34]. Endoglucanases III and V, which were the most strongly adsorbed, did not act synergistically with either purified cellobiohydrolase I or II, and, in this respect, they were similar to I, II, and IV which were adsorbed to a lesser extent. However, when cellobiohydrolyses I and II were recombined, there was synergism[9] to a significant extent only with III or V. Further study of the enzymic interactions of reconstituted mixtures of the endoglucanases and cellobiohydrolases may throw new light on the complexities of the mechanism of cellulase action.

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REFERENCES

1 M. MANDELS, J. E. MEIDEIROS, R. E. ANDREOTTI, AND F. R. BISSET, *Biotechnol. Bioeng.*, 23 (1981) 2009–2026.
2 T. M. WOOD, *Biochem. J.*, 109 (1968) 217–227.
3 K.-E. ERIKSSON AND B. PETTERSSON, *Eur. J. Biochem.*, 51 (1975) 193–206.
4 T. M. WOOD AND S. I. MCCRAE, in T. K. GHOSE (Ed.), *Proc. Symp.: Bioconversion of Cellulolytic Substances into Energy, Chemicals and Microbial Protein*, IIT, Delhi, 1977, pp. 111–141.
5 T. M. WOOD, S. I. MCCRAE, AND C. C. MACFARLANE, *Biochem. J.*, 189 (1980) 51–65.
6 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 234 (1986) 93–99.
7 T. M. WOOD AND S. I. MCCRAE, *Carbohydr. Res.*, 148 (1986) 331–344.
8 T. M. WOOD AND S. I. MCCRAE, *Carbohydr. Res.*, 110 (1982) 291–303.
9 T. M. WOOD, S. I. MCCRAE, C. A. WILSON, K. M. BHAT, AND L. A. GOW, *FEMS Symp.*, 43 (1988) 31–52.
10 K. M. BHAT AND T. M. WOOD, *Biotechnol. Bioeng.*, in press.
11 M.. MANDELS AND J. WEBER, *Adv. Chem. Ser.*, 95 (1969) 391–414.
12 T. M. WOOD, *Biochem. J.*, 121 (1971) 353–362.
13 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 171 (1978) 61–72.
14 G. L. MILLER, J. DEAN, AND R. BLUM, *Arch. Biochem. Biophys.*, 91 (1960) 21–26.
15 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 128 (1972) 1183–1192.
16 J. B. LLOYD AND W. J. WHELAN, *Anal. Biochem.*, 30 (1969) 467–469.
17 N. NELSON, *J. Biol. Chem.*, 153 (1952) 376–380.
18 M. DUBOIS, K. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
19 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 376–380.
20 M. M. BRADFORD, *Anal. Chem.*, 72 (1976) 248–254.
21 T. M. WOOD AND S. I. MCCRAE, *Carbohydr. Res.*, 148 (1986) 321–330.
22 B. LUGTINBERG, J. MEIJERS, R. PETERS, P. H. VANDER, AND L. V. ALPHEN, *FEBS Lett.*, 58 (1975) 254–258.
23 O. OUCHTERLONY, in D. M. WEIR (Ed.), *Handbook of Experimental Immunology*, Blackwell, Oxford, 1967, pp. 656–706.
24 C. B. MARSH, G. V. MEROLA, AND M. E. SIMPSON, *Text. Res. J.*, 23 (1953) 831–841.
25 G. BELDMAN, M. F. SEARLE-VAN LEEUWEN, F. M. ROMBOULTS, AND F. G. J. VORGEN, *Eur. J. Biochem.*, 146 (1985) 301–308.
26 M. L. NIKU-PAAVOLA, A. LAPPALAINEN, T.-M. ENARI, AND M. NUMMI, *Biochem. J.*, 231 (1985) 75–81.
27 U. HAKANSSON, L. G. FAGERSTAM, L. G. PETTERSSON, AND L. ANDERSON, *Biochem. J.*, 179 (1979) 141–149.
28 J. C. SADANA, A. H. LACHKE, AND R. U. PATIL, *Carbohydr. Res.*, 133 (1984) 297–312.
29 A. P. MALONEY, S. I. MCCRAE, T. M. WOOD, AND M. P. COUGHLAN, *Biochem. J.*, 225 (1985) 365–374.
30 H. MERIVOURI, K. M. SIEGLER, J. A. SANDS, AND B. S. MONTENECOURT, *Biochem. Soc. Trans.*, 13 (1985) 411–414.
31 T. M. ENARI, M.-L. NIKU-PAAVOLA, L. HARJIS, A. LEPPALAINEN, AND M. NUMIN, *J. Appl. Biochem.*, 3 (1981) 157–163.
32 P. L. HURST, P. A. SULLIVAN, AND M. G. SHEPHERD, *Biochem. J.*, 169 (1978) 389–395.
33 S. P. SHOEMAKER AND R. D. BROWN, JR., *Biochim. Biophys. Acta*, 523 (1978) 133–146.
34 A. A. KLYOSOV, O. V. MITKEVICH, AND A. P. SINITSYN, *Biochemistry*, 25 (1986) 540–542.