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STUDIES OF TWO LOW-MOLECULAR-WEIGHT ENDO-(1→4)-β-D-XYLANASES CONSTITUTIVELY SYNTHESISED BY THE CELLULOLYTIC FUNGUS *Trichoderma koningii*

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

Two endo-(1→4)-β-D-xylanases (xylanases 1 and 2), which were constitutively synthesised by the fungus *Trichoderma koningii*, were purified to homogeneity on gel-filtration media and by isoelectric focusing. They had molecular weights of 29,000 (xylanase 1) and 18,000 (xylanase 2), and isoelectric pHs of 7.24 (xylanase 1) and 7.3 (xylanase 2); neither enzyme was associated with carbohydrate. Xylanase 1 had an optimum at the remarkably high temperature of 60–65°. Each enzyme liberated a different range of oligosaccharides from oat-straw arabinoxylan, but only xylanase 1 released L-arabinose and D-xylose. Both xylanases were free from cellulase activity.

INTRODUCTION

Xylans are found in the hemicellulosic fraction of the cell walls of higher plants, and, in the main, they are heteroglycans consisting of D-xylopyranose, L-arabinofuranose, and D-glucopyranuronic acid (or its 4-methyl ether, or both) residues[1]. (1→4)-Linked β-D-xylopyranosyl residues make up the backbone, and (1→3)- and (1→2)-linked α-L-arabinofuranose and α-D-glucopyranuronic acid residues are present in the short side-chains. Some of the side chains may contain both β-D-xylopyranose and α-L-arabinofuranose residues. Many of the D-xylosyl residues in the native polysaccharide are acetylated, but these substituents are removed during extraction with alkali.

Enzymes which are capable of hydrolysing the xylans are synthesised by several micro-organisms[2,3]. Some xylanases from fungal sources are exo-(1→4)-β-D-xylanases, but most are endo-(1→4)-β-D-xylanases. We now report on the xylanase system which is elaborated, together with cellulase, by the fungus *Trichoderma koningii* when cellulose is the sole carbon source.

EXPERIMENTAL

Materials. — *T. koningii* IMI 73022 was obtained from the Commonwealth

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Mycological Institute (Kew, Surrey) and sodium carboxymethylcellulose (Cellofas B) was a gift from I.C.I. Stevenston (Ayrshire). Ampholine electrofocusing equipment, carrier ampholytes, and Ultrogels AcA44 and AcA202 were supplied by LKB Instruments. Sephadex and Concanavalin A–Sepharose were purchased from L.K.B. and Pharmacia (G.B.), respectively. Authentic samples of xylo-oligosaccharides were a gift from Professor O. Theander (University of Uppsala, Sweden).

Preparation of cellulase and xylanase. — Stationary cultures of *T. koningii* were prepared[4], and the extracellular enzymes were precipitated by the addition of solid $(NH_4)_2SO_4$ (80% saturation) and then redissolved in 0.1M acetate buffer (pH 5.0) which was 0.01% with respect to NaN_3, to yield a 50-fold concentrate. The enzyme was stored at 4°.

Extraction of oat-straw xylan. — Oat straw (Astor) was hammer-milled to go through a 2-mm sieve, then extracted continuously for 24 h with toluene–ethanol (2:1), and dried in air. A suspension of a sample (100 g) in distilled water (3 L) was stirred at 70° and delignified under nitrogen by adding glacial acetic acid (11 mL) and sodium chlorite (32 g) four times during 2 h. 1-Octanol was added to prevent frothing. The delignified straw was collected (porosity 0 sinter), washed with water (2 L) and ethanol (2 L), and dried in air.

Xylan was extracted from the delignified straw (100 g) by shaking with M KOH (1 L) under nitrogen. Residual solids were removed (porosity 0 sinter), the filtrate was cooled in an ice bath, and the pH was brought to 5.0 by the addition of glacial acetic acid. The precipitate was collected by centrifugation, suspended in water, dialysed against water at 1° until salt-free, and then freeze-dried. The xylan (21.5 g) contained rhamnose (0.4%), fucose (0.3%), arabinose (13.9%), xylose (80.2%), galactose (3.2%), glucose (2.3%), and uronic acid (3.1%).

Carboxymethylxylan. — Oat-straw xylan (20 g) was dissolved in oxygen-free (flushed with nitrogen) aqueous 25% NaOH (500 mL) with stirring. 1-Octanol was added to prevent frothing, and nitrogen was continuously bubbled through the solution. Chloroacetic acid (100 g) was added, and the mixture was stirred at room temperature for 5 h, then dialysed against distilled water until chloride-free, and freeze-dried. The resulting carboxymethylxylan (24.6 g) had a d.s. of 0.57 determined by the method of Eyler *et al.*[5].

Xylo-oligosaccharides and arabinoxylo-oligosaccharides. — Xylo-oligosaccharides were prepared by partial acid hydrolysis (0.2M trifluoroacetic acid, 100°, 1 h) of oat-straw xylan and arabinoxylo-oligosaccharides by using the cellulase from the fungus *Myrothecium verrucaria*[6]. The oligosaccharides were fractionated[6] on a column of charcoal–Celite (1:1), using aqueous ethanol with stepwise increases in ethanol concentration. Final purification was performed on Whatman 17MM paper with ethyl acetate–acetic acid–formic acid–water (18:3:1:4).

Sugar analyses. — Hydrolysis products were analysed qualitatively by (*a*) p.c. with *1*, ethyl acetate–pyridine–water (10:4:3); or *2*, ethyl acetate–acetic acid–formic acid–water (18:3.1:4); and detection with silver nitrate[7]; (*b*) t.l.c. on

Kieselgel G with ethyl acetate–2-propanol–water (18:13:9) and detection with anisaldehyde–sulphuric acid[8].

G.l.c. of the alditol acetates[9] of neutral sugars (obtained by hydrolysis[10] with 2M trifluoroacetic acid for 1 h at 120° was performed with a glass column (1.5 m × 6.0 mm) of 5% of SP 2340 coated on Gas-Chrom Q in a Pye Unicam Model 104 gas chromatograph.

Isoelectric focusing. — Polyacrylamide gels and columns were used[11,12]. The ampholytes (pH 3.5–10) were prefocused in 7.5% gels for 30 min before the addition of the enzyme to the polyacrylamide gels. The enzymes were focused after 2 h at 2 mA/gel. After washing, each gel was sliced into 2-mm discs, added to acetate buffer (1 mL, pH 5.0) containing 0.02% of NaN_3, and left overnight at 4°. The contents of the tubes were mixed and centrifuged, and the supernatant solutions were assayed for xylanase activity.

Column electrofocusing using 1% ampholyte (pH 5–8) was performed[11] in an LKB column (110 mL) at 5° and power (15 W) was maintained throughout (24 h). The enzyme was precipitated with $(NH_4)_2SO_4$ (80% saturation), isolated by centrifugation, desalted on a column of Sephadex G-25, freeze-dried, and redissolved in 0.1M acetate buffer (pH 5.0).

Determination of molecular weight by SDS–polyacrylamide gel electrophoresis. — An LKB Multiphor was used, as recommended in LKB Application Note 306, with the standard reference proteins, cytochrome C (mol. wt. 12,400), ribonuclease (ox pancreas, 13,700), β-lactoglobulin A (18,300), trypsin inhibitor (soybean, 21,500), chymotrypsinogen (25,000), and ovalbumin (45,000). A plot of log mol. wt. against mobility gave a straight line.

Determination of protein and carbohydrate: cellulase and xylanase activities. — Protein was determined by the method of Lowry *et al.*[13] with bovine serum albumin as the standard. Cellulase activity was routinely measured by using carboxymethylcellulose as substrate[14], and the reducing sugar released was determined by the method of Nelson[15]. Total carbohydrate was determined by the phenol–H_2SO_4 method[16].

Xylanase activity was measured by using Sigma larchwood-xylan as substrate. To a solution of the substrate (1 g) in M NaOH (100 mL), the pH of which had been adjusted to 5.0 with M acetic acid, was added aqueous 2% NaN_3 (10 mL), and the volume was made up to 1 L. The slightly cloudy solution was centrifuged (76,000*g*, 45 min) and the supernatant was stored at 4°. Substrate solution (1.5 mL) was incubated at 37° for 1 h with enzyme in a total volume of 2 mL. The hydrolysis was stopped by the addition of Somogyi reagent[15] (2 mL), and the xylanase assay was completed by determining the reducing sugar liberated.

The units of cellulase and xylanase activity are defined as the amounts of enzyme required to liberate 1 μmol of glucose (cellulase) and 1 nmol of xylose (xylanase) equivalent/min, respectively, under the assay conditions.

Hydrolysis of xylans by xylanases. — Xylan (220 mg) was dissolved in M KOH (10 mL), the pH was adjusted to 5.0 with M acetic acid, and the solution was diluted

to 100 mL with distilled water. Substrate solution (4.5 mL) was incubated at 37° with either xylanase 1 or 2 (50 μg of protein) in a mixture (5 mL) containing 0.05M NaN_3 (0.1 mL). Samples were withdrawn periodically and assayed for reducing sugar.

RESULTS

Fractionation and purification. — Concentrated [80% saturated $(NH_4)_2SO_4$ fraction], cell-free culture fluid was fractionated on Ultrogel AcA44 (see Fig. 1). The elution profile showed two peaks of xylanase activity: *A*, associated with the high-molecular-weight endo-(1→4)-β-D-glucanase previously described[17]; and *B*, with a low-molecular-weight endo-(1→4)-β-D-glucanase[17]. Of the xylanase activity recovered (87%) from the column, 93% was found in the low-molecular-weight xylanase fraction.

The material in Fraction *B* was isolated and a solution in 0.01M ammonium acetate buffer (pH 5.0) was applied in 6-mL aliquots to a column (61.0 × 2.5 cm) of Sephadex G-25 equilibrated with 0.01M ammonium acetate buffer (pH 5.0). The low-molecular-weight xylanase was eluted near the void volume (not shown), and was partially separated from the low-molecular-weight endo-(1→4)-β-D-glucanase.

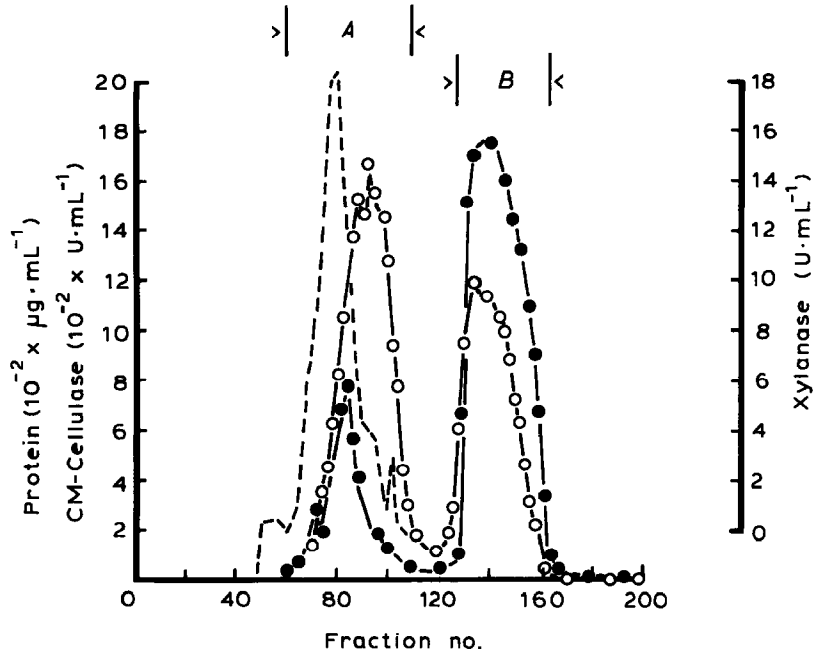

Fig. 1. Fractionation of the xylanase of *T. koningii* on Ultrogel AcA44. A sample (40 mL) of concentrated (see text) enzyme was applied to a column (5.0 × 87.5 cm) of Ultrogel AcA44 equilibrated with 0.01M ammonium acetate buffer (pH 5.0) and eluted at 51 mL/h. Fractions (12 mL) were assayed for xylanase (●), CM-cellulase (○), and protein (----). Fractions were combined as shown.

Xylanase-rich fractions from several such separations were combined, freeze-dried, and fractionated on Ultrogel AcA202 (Fig. 2). A major endo-(1→4)-β-D-glucanase (*C*), and two major (*D* and *E*) and two minor xylanase components were obtained. Fractions containing the major xylanase (*D*, xylanase 1; *E*, xylanase 2) were combined, freeze-dried, and re-fractionated on Ultrogel AcA202. The recoveries of xylanase and protein were 75 and 76%, respectively.

Xylanase 1 was isoelectric at pH 7.24 when the density gradient was supported in polyacrylamide gels, was homogeneous with respect to xylanase activity, and contained only one major protein band when stained with Coomassie Blue (Fig. 3*a*). A small amount of contaminating protein was apparent only when large amounts of the xylanase 1 were applied to the gel (Fig. 3*a*).

Xylanase 2 was isoelectric at pH 7.3 (Fig. 3*c*). The separation profile showed that a small amount of another xylanase (fractions 35–37), which was isoelectric at pH 7.1, was present. Further purification was effected on an LKB isoelectric focusing column, using a pH gradient covering the pH range 5–8 (see Experimental).

Properties. — The physicochemical properties of the xylanases are compared in Table I. The most marked differences were in the pH optima and in the effect of heat. Xylanase 1 had a much higher temperature optimum (60°) than xylanase 2 (50°) and was much more thermostable. Xylanase 1 retained 76% of its activity

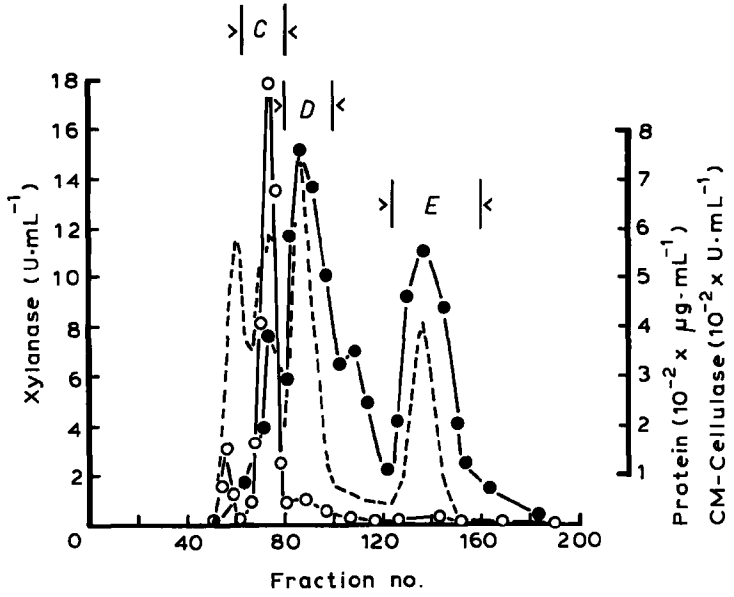

Fig. 2. Purification of low-molecular-weight xylanase on Ultrogel AcA202. Fraction *B* (Fig. 1), which had been purified on Sephadex G-25 (see text), was applied to a column (5.0 × 41.5 cm) of Ultrogel AcA202 equilibrated with 0.01M ammonium acetate buffer (pH 5.0) and was eluted at 14 mL/h. Fractions (5 mL) were assayed for xylanase (●), CM-cellulase (○), and protein (----), and combined to give xylanase 1 (D) and xylanase 2 (E).

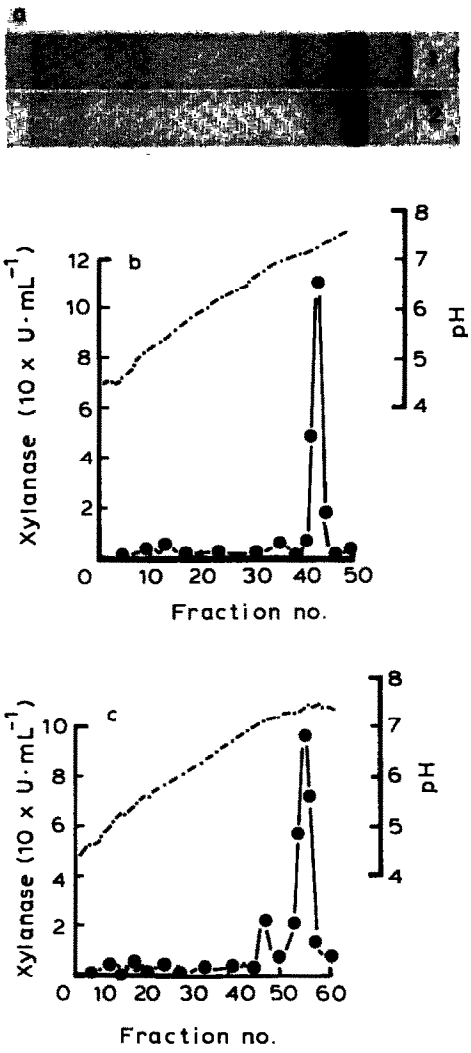

Fig. 3. Isoelectric focusing of xylanases 1 and 2 on polyacrylamide gels (see Experimental): (*a*) polyacrylamide gels stained with Coomassie Blue: gel 1, xylanase 1; gel 2, xylanase 2; (*b*) xylanase 1; and (*c*) xylanase 2.

after 1 h at 60° in the absence of substrate, whereas xylanase 2 had lost 75% of its activity at this temperature after 10 min. Both enzymes were stable at −18°.

The Lineweaver–Burk plots were obtained for the enzymic reactions with carboxymethylxylan and oat-straw xylan as substrates (Table I): K_m values for carboxymethylxylan were 0.63 and 2.3 mg/mL for xylanases 1 and 2, respectively. K_m values for oat-straw xylan were 1.4 and 4.2 mg/mL for xylanase 1 and 2, respectively.

Neither enzyme was adsorbed on Concanavalin A–Sepharose and each was

TABLE I

PHYSICOCHEMICAL PROPERTIES OF THE LOW-MOLECULAR-WEIGHT XYLANASES PRODUCED BY *T. koningii*

Property	*Xylanase 1*	*Xylanase 2*
pH optimum	4.9–5.8[a] 4.7–5.5[b]	4.9–5.5[a] 4.7[b]
Temperature optimum (degrees)	60[a]	50[a]
Isoelectric point	7.24	7.3
Molecular weight (SDS)	29,000	17,700
K_m (oat-straw xylan)	1.4 mg/mL	4.2 mg/mL
K_m (CM-xylan)	0.63 mg/mL	2.3 mg/mL
Effect of heat (residual activity after 20 min at 60°)	100%	10%

[a]Oat-straw xylan. [b]CM-xylan.

devoid of carbohydrate (phenol–H_2SO_4 method).

Breakdown of the xylans from oat straw by the purified xylanases. — Oat-straw xylan was extensively hydrolysed by both xylanase 1 and 2. The rate of production of reducing sugar increased rapidly during the first 5 h (Fig. 4) and then decreased. Xylanase 1 was more active than xylanase 2 when evaluated in this way, but there was no synergistic effect. Fresh enzyme added at intervals did not increase the reducing power of the solutions significantly (not shown in Fig. 4). After incubation for 30 min with either enzyme, 20–22% of the xylan substrate still had a chain length that was sufficiently long to be precipitated by the addition of 4 vol. of ethanol, but this fell to 10–12% after 24 h. Hydrolysis of the residues by trifluoroacetic acid followed by p.c. (solvent *1*) showed that, for each enzyme, the arabinose–xylose ratio was increased significantly but was particularly marked for xylanase 2.

The two xylanases hydrolysed oat-straw xylan by a different mode of action, as shown by t.l.c. and gel filtration on Biogel P-2 of the incubates after 4, 24, and

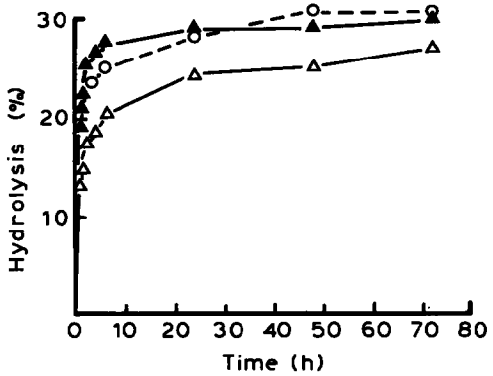

Fig. 4. Hydrolysis of oat-straw xylan by xylanases 1 and 2 (see Experimental): ▲, xylanase 1; △, xylanase 2; ----, xylanase 1 + 2.

48 h. After 4 h, xylanase 1 gave xylose and arabinose in the ratio 4:1, but the principal products were xylobiose and xylotriose. Xylotetraose and xylopentaose were present, in small amounts only, after incubation for 4 h but had virtually disappeared after 48 h when xylobiose, xylotriose, xylose, and arabinose had accumulated. During the course of the hydrolysis only small amounts of arabinoxylotetraose and arabinoxylotriose were formed, suggesting that most of the arabinose was liberated directly from the xylan backbone. The purified enzyme had no action on *p*-nitrophenyl α-L-arabinofuranoside or on arabinoxylotetraose or arabinoxylotriose generated by the *M. verrucaria* cellulase.

Xylanase 2 incubation mixtures contained only a trace of xylose and no arabinose. After 4 h, xylobiose was the principal product, but large amounts of xylotriose, xylotetraose, and arabinoxylotetraose were present. As hydrolysis proceeded, xylobiose, xylotriose, and arabinoxylotetraose accumulated.

Neither xylobiose nor xylotriose was attacked by xylanase 2. Xylanase 1 had no action on xylobiose, and xylotriose was attacked only very slowly.

DISCUSSION

Xylanase 1 and 2 were separated from each other and from a low-molecular-weight cellulase [endo-(1→4)-β-D-glucanase] on Sephadex G-25 and Ultrogels AcA44 and AcA202. With Ultrogel AcA202 and Sephadex G-25, it was clear that adsorption of the xylanases and the endo-(1→4)-β-D-glucanase was an important factor in the separation. Indeed, it appeared that adsorption and not gel permeation was the governing factor. Xylanases 1 and 2, for example, were readily separated from each other on Ultrogel AcA202, which has an operating range for globular proteins of 1000–5000. Yet xylanases 1 and 2, according to SDS–polyacrylamide gel electrophoresis, had mol. wts. of 29,000 and 18,000, respectively, and would have been expected to have been eluted at the void volume of the Ultrogel AcA202 column.

On Sephadex G-25 (operating range 1,000–5,000 for globular protein), it was the low-molecular-weight endo-(1→4)-β-D-glucanase (mol. wt. 12,000[18]) that behaved in an anomalous fashion, and was adsorbed sufficiently to permit some separation from the xylanase components. Xylanases from *Schizophyllum commune*[19] and *Cephalosporium sacchari*[20] have some affinity for Sephadex gels.

The mol. wts. of 29,000 and 18,000 for xylanases 1 and 2 are in the range previously reported for microbial xylanases. Thus, xylanases from *Aspergillus niger* have been reported to have mol. wts. of 22,000[21,22] and 24,000–33,000[19]; a mol. wt. of 22,000–24,000 has been reported for a xylanase from *Trametes*[23], and xylanases[24] of *Trichoderma pseudokoningii* induced with larchwood xylan had mol. wts. of 15,000–22,000.

Xylanase 2 had a remarkably high temperature optimum of 60–65° when assayed over 1 h; the temperature optima of fungal xylanases reported so far are normally 5–10° lower than this value. A non-fungal xylanase from a *Streptomyces* species[25] had a temperature optimum of 60°.

Neither of the xylanases 1 and 2 was a glycoprotein. In this respect they were similar to the xylanases from *S. commune,* but different from most others[3]. The *Schizophyllum* enzyme complexed with carbohydrate in a non-covalent manner. Thus, carbohydrate is not essential for enzyme activity in these two sources.

Both xylanases 1 and 2 were endo-(1→4)-β-D-xylanases since the xylo-oligosaccharides produced during the early stages of hydrolysis of oat-straw xylan were of medium chain-length. However, the mode of action of the two xylanases was quite different. Only xylanase 1 could cleave L-arabinofuranosyl linkages in the polysaccharide. Debranching enzymes of this type have been purified from cultures of *Aspergillus niger*[26,27] and *Cephalosporium*[20], but knowledge of this type of enzyme is not extensive[3].

It was expected that an L-arabinose debranching enzyme would act synergistically with the non-debranching enzyme. However, no synergism was observed using oat-straw xylan.

Significant was the observation that most of the xylanase secreted by the fungus, using cellulose as the sole carbon source, could be separated from the cellulase enzymes. The possibility that xylanase and cellulase activities are found in the same enzyme protein continues to be a topic of discussion. It is clearly possible that a cellulase enzyme that can accommodate the CH_2OH group of D-glucopyranose will also be able to form a complex with xylans since the only substituent at position 5 is hydrogen. Toda *et al.*[28] and Kanda *et al.*[29] have interpreted data obtained with the endo-(1→4)-β-D-glucanases of *T. viride* and *Polyporus tulipiferae,* respectively, to indicate that xylanase and cellulase originate in the same active site.

A cellulase component free from xylanase and xylanases free from cellulase are potentially useful tools for the study of the structure of plant cell walls.

REFERENCES

1 K. C. B. WILKIE, *Adv. Carbohydr. Chem. Biochem.*, 36 (1979) 215–264.
2 R. F. DEKKER AND G. N. RICHARDS, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
3 P. J. REILLY, in A. HOLLAENDER AND R. RUBSON (Eds.), *Trends in the Biology of Fermentations for Fuels & Chemicals,* Plenum, New York, 1981, pp. 111–129.
4 T. M. WOOD, *Biochem. J.*, 109 (1969) 217–227.
5 R. W. EYLER, E. D. KLUG, AND F. DIEPHUIS, *Anal. Chem.*, 19 (1947) 24–27.
6 C. T. BISHOP AND D. R. WHITAKER, *Chem. Ind. (London),* (1955) 119.
7 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London),* 166 (1950) 444–445.
8 E. STAHL AND U. KALTENBACH, *J. Chromatogr.*, 5 (1961) 351–352.
9 J. H. SLONEKER, *Anal. Biochem.*, 43 (1971) 539–546.
10 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
11 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 128 (1972) 1183–1192.
12 T. M. WOOD, S. I. MCCRAE, AND C. M. MCFARLANE, *Biochem. J.*, 189 (1980) 51–65.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. C. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
14 T. M. WOOD AND S. I. MCCRAE, *Carbohydr. Res.*, 57 (1977) 117–133.
15 N. NELSON, *J. Biol. Chem.*, 153 (1952) 376–380.
16 M. DUBOIS, K. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
17 T. M. WOOD AND S. I. MCCRAE, *Biochem. J.*, 171 (1978) 61–72.
18 T. M. WOOD, *Biochem. J.*, 109 (1968) 217–227.

19 M. G. PAICE, L. JURASEK, M. R. CARPENTER, AND L. B. SMILLIE, *Appl. Environ. Microbiol.*, 36 (1978) 802–811.
20 G. N. RICHARDS AND T. SHAMBE, *Carbohydr. Res.*, 49 (1976) 371–381.
21 M. SINNER AND H. H. DIETRICHS, *Holzforschung*, 21 (1925) 207–225.
22 M. M. FREDERICK, J. R. FREDERICK, A. R. FRATZKE, AND P. J. REILLY, *Carbohydr. Res.*, 97 (1981) 87–103.
23 M. KUBACKOVA, S. KARACSONYI, L. BILISKS, AND R. TOMAN, *Carbohydr. Res.*, 76 (1979) 177–183.
24 C. J. BAKER, C. H. WHALEN, AND D. F. BATEMAN, *Phytopathology*, 67 (1977) 1250–1258.
25 H. IIZUKA AND T. KAWAMINAMI, *Agric. Biol. Chem.*, 29 (1960) 520–525.
26 M. JOHN, B. SCHMIDT, AND J. SCHMIDT, *Can. J. Biochem.*, 57 (1979) 125–128.
27 N. A. RODIONOVA, I. V. GORBACHEVA, AND V. A. BUIVID, *Biokhimiya*, 42 (1977) 659–664.
28 S. TODA, H. SUZUKI, AND K. NISIZAWA, *Hakko Kogaku Zasshi*, 49 (1971) 499–521.
29 T. KANDA, K. WAKABAYASHI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 79 (1976) 989–995.