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ISOLATION, PROPERTIES, AND POSTULATED ROLE OF SOME OF THE XYLANASES FROM THE BASIDIOMYCETE *Sporotrichum dimorphosporum*

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ABSTRACT

Xylanases excreted by the fungus *Sporotrichum dimorphosporum* have been separated by chromatography on DEAE-Sephadex at pH 7 into Fractions I and II. Gel filtration did not further resolve the enzymes, and preparative electrofocusing was necessary to isolate xylanases of pI 7.1, 7.2, 7.35, and 7.95 in fraction I and pI 3.9, 4, 4.4, 4.7, and 5.5 in fraction II. The latter fraction was studied in detail, and afforded two pure xylanases (pI 4.4 and 4.7); the others were contaminated by *O*-(carboxymethyl)cellulases. The xylanases of pI 3.9, 4, 4.4, and 4.7 had molecular weights of 32,000 and that of the xylanase of pI 5.5 was 26,000. The optimal reaction temperature was 65–70° for the former enzymes and 50° for the latter. The optimum pH of action was 4.5–5 for all of these xylanases. The xylanases of pI 4.7 and 5.5, the major constituents of the system, exhibit different properties and different values of their Michaelis constants. The end-products of the reaction affect the reactivity of the enzymes. Xylobiose and 4-thioxylobiose are competitive inhibitors of the xylanase of pI 5.5, but they are activators of the xylanase of pI 4.7 in the presence of xylan. This observation is suggestive of a new mechanism for regulation of hydrolysis.

INTRODUCTION

A challenge at the end of this century will certainly lie in the large-scale exploitation of biomass as a source of raw material for chemical industry and for energy production. It will, however, be necessary to develop new and economically competitive processes that will allow utilization of most of the constituents of the lignocellulosic materials with the best yield, and involve the least energy consumption. In this respect, enzymic degradation seems more promising than degradation by chemical hydrolysis or physical treatment. However, progress in the development of new schemes for enzymic hydrolysis is limited by the lack of a detailed knowledge of the structure of the plant cell-wall. Although microfibrils of cellulose are described as embedded in a matrix of amorphous hemicelluloses and lignin[1–3], the ultrastructural localization of these constituents and the nature of their interrelations remain to be elucidated[4].

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Considering the higher reactivity of hemicelluloses and their postulated intermediary position between cellulose and lignin, the study of hemicelluloses under different conditions becomes important in understanding the fine structure of the plant cell-wall. (1→4)-β-D-Xylan, the most common hemicellulose, was selected as the substrate, either isolated[5,6] or *in situ*[7], for study of the action of xylanases [(1→4)-β-D-xylan xylanohydrolase E.C. 3.2.1.8.] from a basidiomycete, *Sporotrichum dimorphosporum*. This paper reports an isolation scheme and the main physicochemical parameters of some of the enzymes of the xylanase system of this fungus.

EXPERIMENTAL

Materials and methods. — Column chromatography was performed on Biogel P-2 and P-60 resins, or Sephadex G-100 and DEAE-Sephadex A-50 resins. Electrofocusing experiments were conducted with an LKB Multiphor apparatus, using LKB Ampholine carrier ampholytes, equipped with a Huber Ministat thermostat. Desalting and concentrations were performed in an Amicon ultrafiltration stirred-cell fitted with Diaflo membranes (UM-10). Gas chromatography of neutral sugars as alditol acetate derivatives was effected with a Packard Becker instrument, using a 3% ECNSS-M column or 3% of SP-2340 on a column of Gas-Chrom Q. Paper chromatography employed Whatman no. 1 paper with either *A*: 10:4:3 ethyl acetate–pyridine–water, or *B*: 6:3:4 ethyl acetate–acetic acid–water. The sugars were detected with aniline spray-reagent.

Enzyme. — The crude enzyme constituted the culture filtrate of a basidiomycete, *Sporotrichum dimorphosporum**, kindly provided by "La Rapidase" 59, Seclin, France, under the trade name "Cellulase de basidiomycète".

Substrates. — For determining cellulase activities, an *O*-(carboxymethyl)cellulose (CMC), d.s. 0.7, "blanose cellulose gum", 7 H 3 SX F, from Hercules S.A. (France) was used. Xylanase activities were measured with a borohydride-reduced xylan from birch wood. The xylan was directly extracted according to Zinbo and Timell[10] with an alkaline solution (24% potassium hydroxide) containing 1% of sodium borohydride under nitrogen. The resulting polysaccharide was dialyzed against distilled water and freeze-dried; yield 18.5%. Hydrolysis with sulfuric acid[11] and g.l.c. of the alditol acetate derivatives on a column of ECNSS-M at 215° gave the relative molar proportions of the following sugars: rhamnose (0.9), arabinose (0.2), xylose (95.8), mannose (0.1), 4-*O*-methylglucose (2.0), galactose (0.6), and glucose (0.4). The 4-*O*-methylglucose arises from the *in situ* reduction of those uronic acid groups involved in ester linkages[12]. When extracted in the absence of reducing agent, the birch xylan shows a xylose:uronic acid ratio of ~10. Xylobiose was isolated by gel filtration of an enzymic hydrolyzate of the xylan on

*This strain has previously been related tentatively to a Basidiomycete of the genus *Poria* (see also refs. 6–9 and 13). However in a recent and more-accurate identification and comparison to the strain QM 806, it was established as *Sporotrichum dimorphosporum*.

a column of Biogel P-2. 4-Thioxylobiose, 4-*S*-β-D-xylopyranosyl-4-thio-D-xylopyranose, was synthesized in this Institute[13].

Enzyme assays. — Xylanase activity was routinely measured by incubating under shaking 0.1 mL of appropriately diluted enzyme solution with 1.9 mL of a suspension of xylan (5 mg) in 0.1M acetate buffer (pH 5, containing 5mM sodium azide as preservative) for 30 min at 40°. Concentrations of reducing sugars were determined colorimetrically (Somogyi–Nelson)[14]. The absorbance was read at 500 or 660 nm on the supernatant solution following centrifugation at 27,000*g*. A calibration curve was established with known concentrations of xylose. *O*-(Carboxymethyl)cellulase (CMCase) activity was determined by the same procedure, with D-glucose as reference standard. For better reproducibility, the suspension of xylan and the solution of *O*-(carboxymethyl)cellulose were stirred for at least 3 h before use.

Protein determination. — Protein concentration was determined by the method of Lowry *et al.*[15] and of Bradford[16], with bovine serum albumin as the standard.

Purification of the enzyme. — The commercial mixture (30 g) was solubilized in 0.05M acetate buffer (pH 5, 1 L, with 5mM sodium azide) and stirred for 2 h at 4°. After centrifugation, the supernatant solution was desalted by ultrafiltration through a UM-10 Diaflo membrane and lyophilized to give the crude enzyme preparation.

Ion-exchange chromatography. A column (V_t = 400 mL) of DEAE-Sephadex A-50 was equilibrated at 4° in 0.01M phosphate buffer (pH 7 containing 0.05% chlorethone as preservative). The lyophilized, crude enzyme mixture, in the same buffer, was placed at the top of the column. Initial elution with the starting buffer gave the Xylanase I fraction. The column was washed with a solution of 0.02M sodium chloride in 0.01M phosphate buffer. A second elution with 0.06M sodium chloride in the same phosphate buffer then afforded the Xylanase II fraction. The elution conditions had been previously determined with a linear gradient of sodium chloride in the starting buffer[9]. Each Xylanase fraction was desalted and freeze-dried.

Gel-filtration chromatography. Two glass columns (100 cm × 2 cm, i.d.) were used with Biogel P-60 and Sephadex G-100 gels, respectively. The proteins were eluted at a flow-rate of 25 mL/h with 10mM acetate buffer (pH 5) at 4°, and 2-mL fractions were collected. Each fraction was tested for xylanase and CMCase activities. The molecular weights of the enzymes were estimated by reference to the elution volumes of Dextran Blue and of bovine serum albumin, ovalbumin, cytochrome C, and cyanocobalamin.

Preparative, isoelectric focusing. Preparative flat-bed electrofocusing was performed in Ultrodex granulated gel according to the LKB application note 198. The enzyme preparation was incorporated into the gel suspension before evaporation, and the running conditions were 8 watts constant power for 16–18 h at 10°. At the end of the experiment, the pH gradient was measured with the aid of a surface

glass-electrode on each of the 30 compartments of a fractionating grid applied on the gel as a template. The focused protein zones were made visible by a print technique with dry Whatman no. 1 paper, and the adsorbed proteins were stained with Coomassie Blue.

The gel sections corresponding to the compartments of the grid were transferred to 30 small columns (1 cm, i.d.) containing a bed of Biogel P-2 (1 cm). Distilled water (2 mL) was added and the gel allowed to settle in the column. Each column was then washed with distilled water, and the eluate (2 mL) kept frozen.

A narrower pH gradient, ranging between 5 and 6, was obtained by performing an electrofocusing experiment between pH 4 and 6 without any enzyme preparation, cutting the gel in its middle, and pouring the part corresponding to pH 5–6 onto another trough containing the enzymes.

Electrophoresis in flat poly(acrylamide) gels. The enzyme homogeneity was tested according to Laemmli[17] by electrophoresis on 15% poly(acrylamide) gel containing 0.1% sodium dodecylsulfate (SDS). The molecular weights were estimated after standardization with phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and alpha lactalbumin (Pharmacia calibration kit) proteins: mol. wts. 94,000; 67,000; 43,000; 30,000; 20,100; and 14,400, respectively.

Analysis of degradation products. Enzymic attack on the xylan (50 mg) was performed in 0.01M acetate buffer (pH 5, 10 mL) for 140 h, and the degraded product dialyzed against distilled water at 4°. The residue was freeze dried and weighted, and the dialyzable products were analyzed by paper chromatography in solvent systems *A* and *B*. Quantitative estimation of the products by l.c. was obtained after desalting by differential refractometry.

Kinetics and determination of the K_m value. — The kinetics were determined in test tubes each containing 0.1 mL of enzyme solution and various concentrations of xylan suspension in 0.1M acetate buffer (pH 5; 1.9 mL; 5mM sodium azide). They were incubated with shaking at 30° for periods of time ranging from 4–60 min, during which time the production of reducing sugar was linear. The results were expressed by a Lineweaver–Burk, reciprocal plot.

Role of disaccharides. — The action of xylobiose or of 4-thioxylobiose was studied at 30° on xylan as substrate in 0.1M acetate buffer (pH 5; 1.8 mL; 5mM sodium azide), with disaccharide (0.1 mL) and xylanase (0.1 mL). The final concentration of disaccharide was 0.25, 0.5, or 1.0mM. The variation in reducing power was determined against a blank by the Somogyi–Nelson method.

RESULTS

The crude, lyophilized enzyme-mixture shows several activities, including D-glucosidase, amyloglucosidase, amylases, xylanases, and cellulases. The strain is thus a very good source[9,18] of enzymes showing activity on soluble cellulose [*O*-(carboxymethyl)cellulose, CMC]. Manufacturers have also noted the presence of

TABLE I

SOME OF THE ENZYMIC ACTIVITIES FOUND IN THE CRUDE ENZYME PREPARATION FROM *Sporotrichum dimorphosporum* AND IN FRACTIONS OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY

Substrate	*Specific activity*[a]		
	Crude enzyme preparation	*Fraction*	
		Xylanase I	*Xylanase II*
Methyl α-D-glucopyranoside	0.009	[b]	[b]
Methyl β-D-glucopyranoside	0.24	[b]	[b]
Cellobiose	4.63	119.4	0
O-(Carboxymethyl)cellulose	57.9	231.5	76.9
Reduced cellulose powder	0.12	0.07	0
Methyl β-D-xylopyranoside	0	0	0
Reduced D-glucurono-D-xylan	8.1	17.6	137.8
Maltose	5.4	28 5	0
Amylopectin	23.6	87	23.1

[a]Expressed in terms of glycose equivalent (nmoles of glucose or xylose) produced per sec per mg of protein. [b]Not determined

proteases, galacturonanases, and (1→3)-β-D-glucanase in the culture filtrate. The preceding list of activities is certainly not exhaustive, as Eriksson *et al.* found enzymes involved in the degradation of lignin[19–21] in the culture filtrate of the related species *Sporotrichum pulverulentum*.

Fractionation on DEAE-Sephadex A-50 gave a fraction (Xylanase I) released by the starting buffer and a fraction (Xylanase II) that is eluted by 0.06M sodium chloride in the starting buffer[9]. Although this ion-exchange chromatography separated the xylanases in two groups, it was not able to achieve complete separation of the contaminant cellulase and amylase activities. However activities on maltose and cellobiose were found associated only with the Xylanase I fraction (Table I).

Gel filtration on Biogel P-60 gave elution diagrams exhibiting, for both fractions, three peaks having xylanase activity, one of which corresponding to the void volume. Gel filtration on Sephadex G-100 confirmed the existence of three xylanase constituents in each fraction.

The Xylanase I fraction contained xylanases having molecular weights of ~52,000, 25,000, and 20,000, whereas the Xylanase II fraction contained xylanases having molecular weights of ~75,000, 34,000, and 28,000. As the xylanases were eluted over the total elution-volume of the column this gel filtration step was omitted in the final scheme of purification.

Preparative electrofocusing was then applied to each fraction. This method offers the advantage of concentrating the proteins within narrow bands according to their isoelectric points (pI). Excellent resolution was obtained with a linear pH-gradient over the larger portion of the 24-cm length of the trough. Division of the gel into 30 different compartments with a cutting grid and elution led to isolation

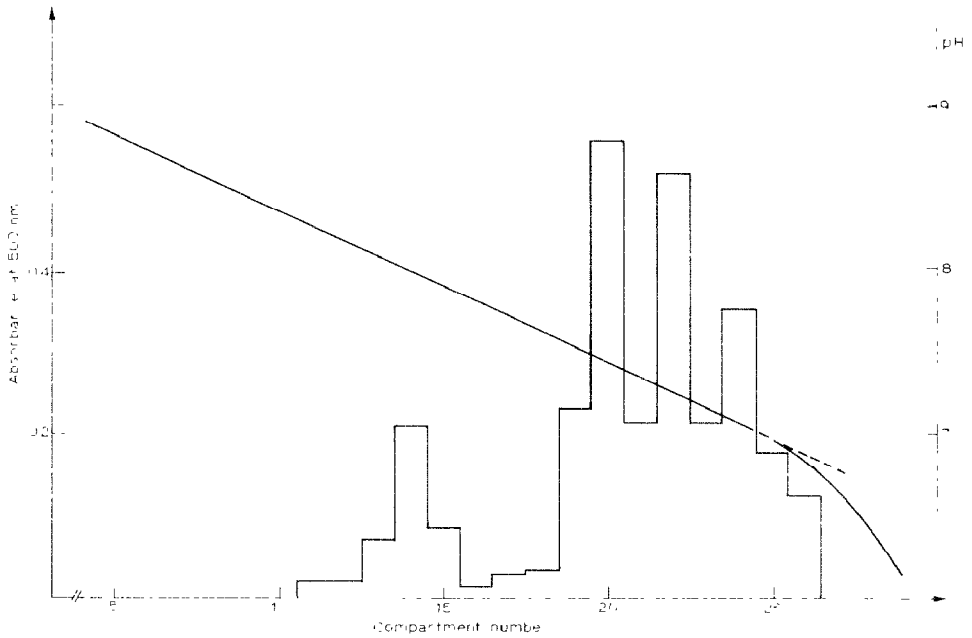

Fig 1 Preparative electrofocusing of Xylanase I fraction with LKB Ampholine 7–9 ampholyte Determination of xylanase activity in the pH range 7–9

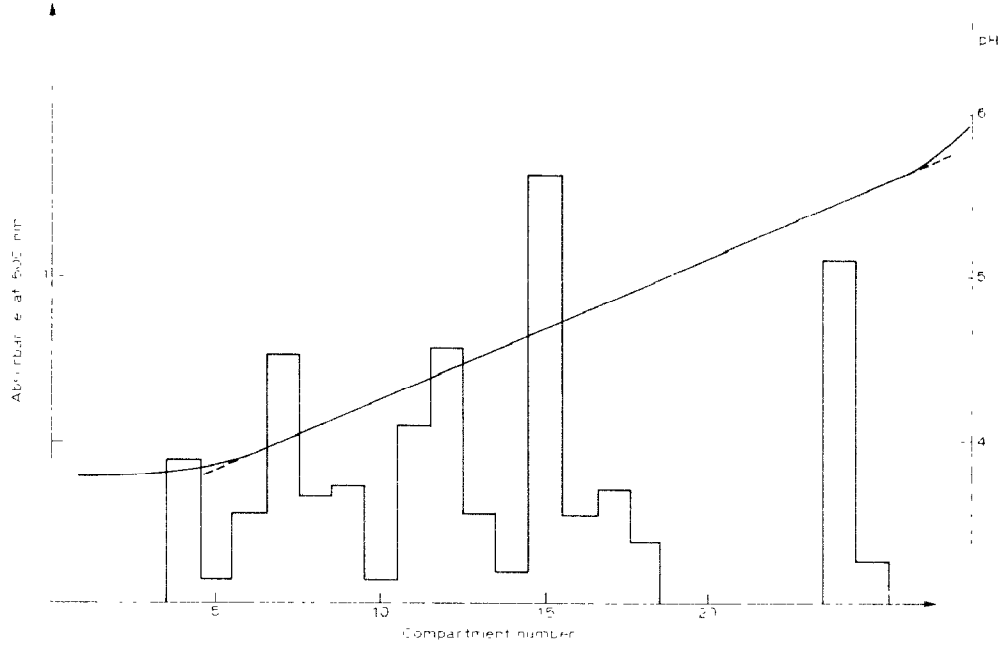

Fig 2 Preparative electrofocusing of Xylanase II fraction with LKB Ampholine 4–6 ampholyte Determination of xylanase activity in the pH range 4–6

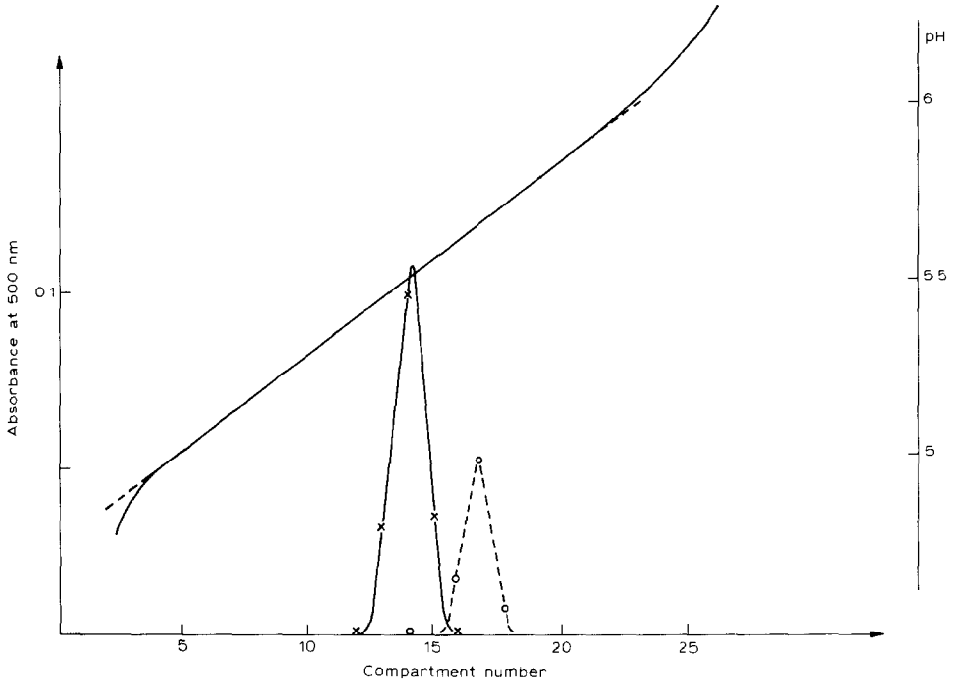

Fig. 3. Preparative electrofocusing over one pH unit of the proteins isolated at pI 5.5. ○, Xylanase activity; ×, CMCase activity.

TABLE II

NUMBER OF PROTEINS AND MOLECULAR-WEIGHT DETERMINATION OF THE ENZYMES IN COMPARTMENTS SHOWING STRONG XYLANASE ACTIVITY AFTER ELECTROFOCUSING EXPERIMENTS

Fraction	*pI*	*Number of proteins*[a]	*Mol.wt (Sephadex G-100)*	*Activity*
Xylanase I fraction	7.1	[b]	26,000	xylanase
	7.2	[b]	20,000	xylanase
	7.35	[b]	19,000	xylanase
	7.95	[b]	[b]	xylanase
Xylanase II fraction	3.9	2	26,000	CMCase
			32,000	xylanase
	4	2	26,000	CMCase
			32,000	xylanase
	4.4	1	32,000	xylanase
	4.7	1	32,000	xylanase
	5.5	3	26,000	xylanase
			25,000[c]	activity[b]
			24,000[c]	CMCase

[a]Observed in SDS–poly(acrylamide) gel electrophoresis. [b]Not determined. [c]Estimated by comparison with standards in electrophoresis.

TABLE III

RECOVERY AND SPECIFIC ACTIVITIES DURING PURIFICATION

Purification step	*Desalted, lyophilized material (mg)*	*Protein as serum albumin (mg)*	*Specific activity (nanokatal mg^{-1})*	*Purification*	*Cumulative purification*
Commercial mixture	30,000				
Crude enzyme preparation	15,100	13542	8.1	1	1
Xylanase I fraction	3,690	3324	17.6	2.2	2.2
Xylanase II fraction	370	302	137.8	7.8	17
Electrofocusing of xylanase II fraction (pH range 4–6)					
Compartments containing					
xylanase pI 3.9		25.9	56.4	0.4	7
xylanase pI 4		24	84	0.6	10.3
xylanase pI 4.4		28	145.7	1.1	18
xylanase pI 4.7		34.3	282.5	2	34.9
xylanase pI 5.5		56.3	290.1	2.1	35.8

of solutions of pure enzyme and of solutions containing a few proteins possessing nearly identical pI values. Identification of xylanases in each compartment was carried out on properly diluted enzyme solution. Results are given in Figs. 1 and 2. The Xylanase II fraction (Fig. 2) was studied in more detail. The purity of the eluted bands having xylanase activity was checked by electrophoresis on SDS–poly(acrylamide) gel. The compartment at pI 5.5 gave rise to three proteins that could be later separated by using a one pH-unit gradient over the total length of the trough. The corresponding proteins were collected, a xylanase of pI 5.5 and a CMCase of pI 5.6 (Fig. 3) were identified by activity measurements. For all compartments having xylanase activities, molecular weights were determined by gel filtration on Sephadex G-100. The molecular-weight values were similar to those obtained by SDS–poly(acrylamide) gel electrophoresis (Table II). Enrichment during purification and specific activities are given in Table III.

Several authors have reported on the multiplicity of xylanases produced by fungi[22], but never has such a great number of different enzymes been demonstrated as in the present example of xylanases from a Basidiomycete. The results were confirmed when carrier ampholytes from another origin (Pharmacia) were used under different experimental conditions. The same multiplicity of xylanases having the same pI values was again observed in culture filtrate of the fungus that had been grown on a different carbon source under conditions that did not optimize the production of xylanases. The only difference seemed to be in the relative proportions of the proteins. These results suggest that the numerous xylanases detected by electrofocusing might arise from genuine heterogeneity in their structures, as was concluded by Fagerstam and Pettersson[23] for the cellulases from *Trichoderma reesei*.

PROPERTIES OF THE XYLANASES

To confirm the preceding conclusion, the individual characteristics and kinetics for each xylanase were studied.

Influence of pH. — Each xylanase was tested at 30° between pH 2.2 and 7.4 in the same buffer at constant ionic strength. The activity as a function of pH gave similar curves for all of the xylanases (Fig. 4). For each of the enzymes from fraction II, the maximum was at pH 4.5–5, when the net charge of the protein was nearly zero. As a general rule, the activity was zero for pH values <2.5 and >7.5. The results are in agreement with those reported in the literature for other xylanases isolated from fungi. Xylanases from fraction I had the same optimal pH values, but had a positive net charge on the protein molecule (pI >7). A similar result has been reported for xylanase HCI (pI 9.17) from *Ceratocystis paradoxa*[24] and that from *Trichoderma pseudokoningii*[25] (pI 9.7).

Influence of temperature at optimum pH. — The optimum temperature of action is rather high, 65–70° (Fig. 5), for all of the enzymes from fraction II except for the xylanase of pI 5.5, whose maximum is at 50°. The latter also differs in its lower molecular-weight (Table II). Optimal temperatures were generally 45–55°, although Dekker and Richards[26] observed a temperature optimum at 80° for xylanase HC II, pI 4.5, isolated from *Ceratocystis paradoxa.*

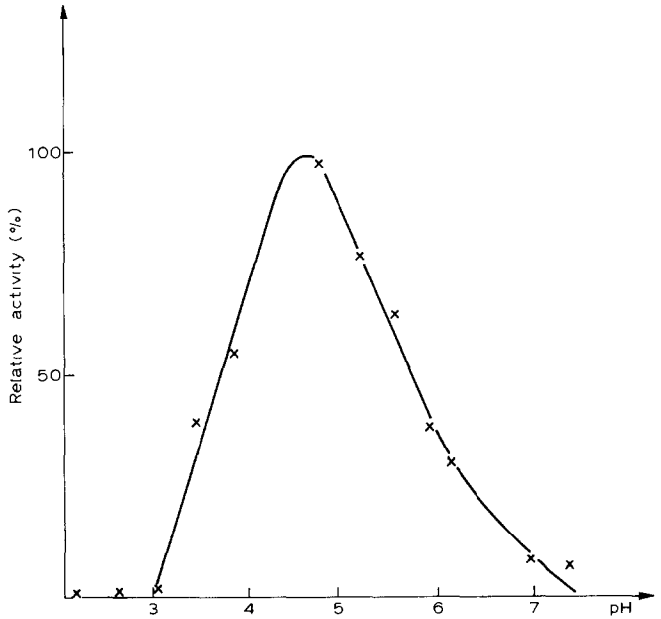

Fig. 4. Effect of pH on the enzymic activity against xylan at 30°. The test tubes contained an aqueous suspension of xylan (5 mg, 0.9 mL) and McIlvaine buffer (1 mL) at different pH values ranging from 2.2 to 7.4, and enzyme solution in water (0.1 mL). The ionic strength was maintained at 0.25M with solid potassium chloride. After incubation for 30 min at 30°, the increase in reducing power was measured at 660 nm. The curve is given for xylanase of pI 4.7.

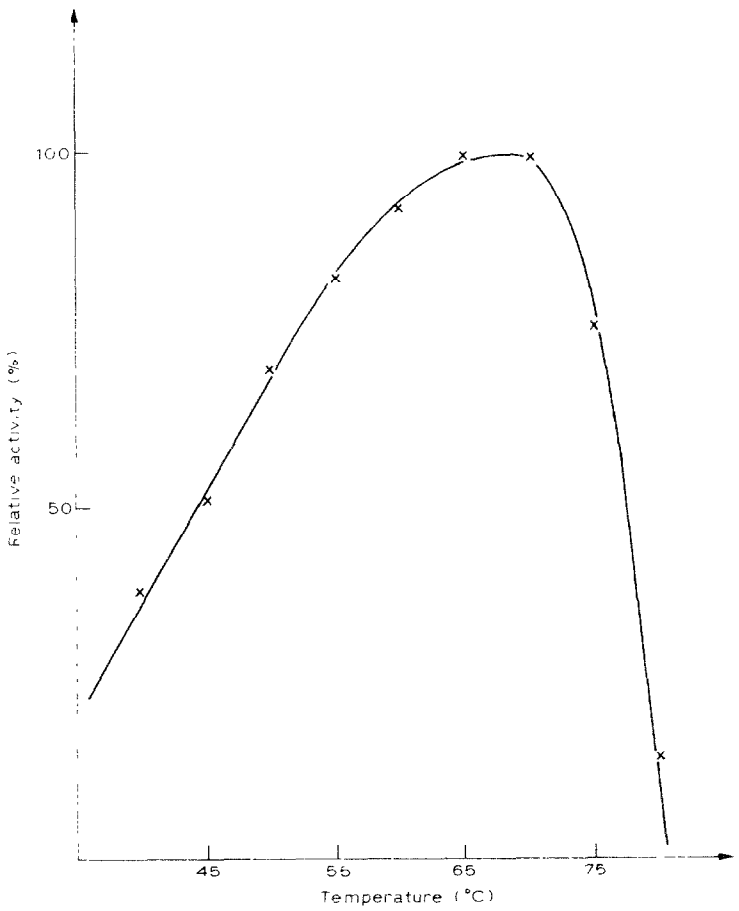

Fig 5 Effect of temperature on the enzymic activity against xylan in 0 1M acetate buffer at the optimum pH of action The test tubes containing both buffer and substrate were equilibrated for 10 min at the temperature investigated Incubation with the xylanase was then performed as previously described for the assays The curve is given for xylanase of pI 4.7

The activation energy was calculated from the rate of hydrolysis of xylan at pH 5. A linear Arrhenius plot was obtained between 30 and 50° for the xylanase of pI 5.5, giving an activation energy of 52.3 kJ.mol^{-1}. Activation energies for the other xylanases of pI 4, 4.4, and 4.7 were 42.2, 42.2, and 56.8 kJ.mol^{-1}, respectively. The enzymes of pI 4 and 4.4 gave linear plots between 30 and 60°, whereas the xylanase of pI 4.7 showed a deviation above 48°. This deviation could arise from partial inactivation of the enzyme during the incubation period, which could be related to the slight loss of activity of this enzyme between 50 and 60° because of the effect of temperature (Fig. 6). However, the xylanase of pI 4.7, which has the highest activation-energy, seems to be the principal xylanase of the Xylanase II fraction.

The same value observed for the activation energies of the xylanases pI 4 and

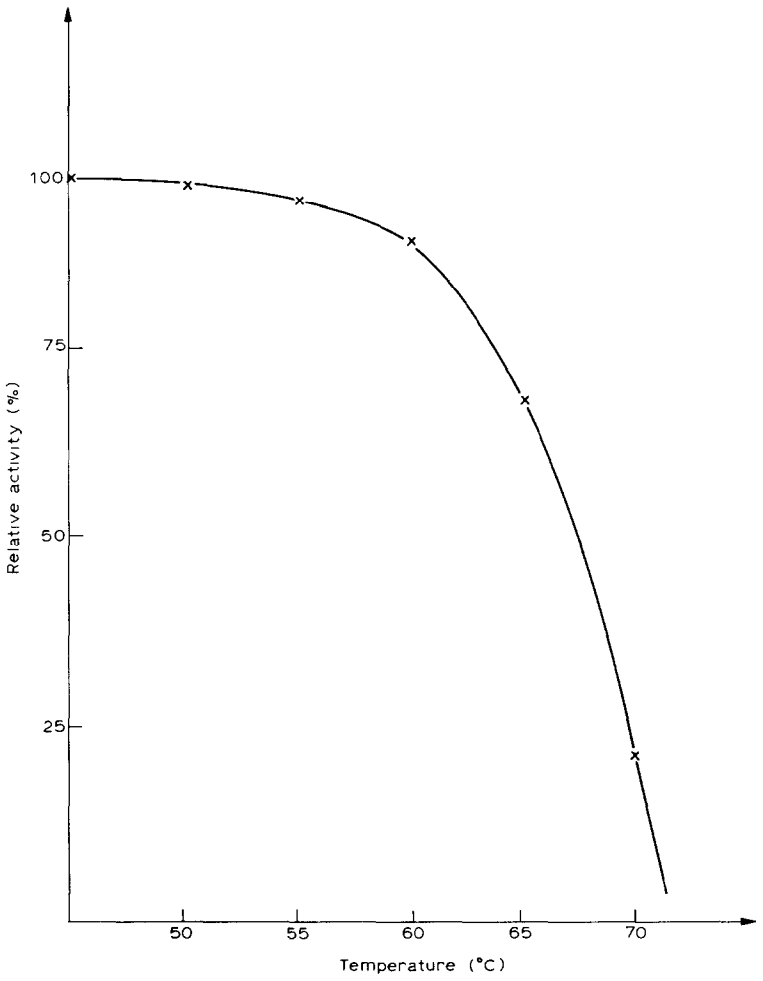

Fig. 6. heat stability of the xylanase of pI 4.7 in 0.1M acetate buffer (pH 5) during 30 min.

4.4, which also have the same molecular weight and show identical Michaelis constants on the xylan substrate (Table IV), is in favor of their close relationship.

Because of the high thermal stabilities of the xylanases as illustrated in Fig. 6, heating for 10 min at 100° was necessary to ensure complete arrest of the enzymic reaction in the assays of exhaustive degradation of the polysaccharide.

Influence of metal ions on stability. — The xylanase of pI 4.7 was totally inhibited by mercuric ions at concentrations >1.25mM and by zinc ions at concentrations >90mM. Calcium ions at 100mM concentrations caused only partial inhibition (35%). All xylanases were inhibited by mercuric ions at concentrations >1.5mM.

Mode of action of the xylanases. — All of the xylanases from fraction II are endoenzymes, as shown by the rate of hydrolysis and by the nature of the products released during hydrolysis of xylan. Xylose and homologous oligomers up to d.p.

TABLE IV

PHYSICOCHEMICAL PROPERTIES AND K_m VALUES OF THE XYLANASES FROM XYLANASE II FRACTION

Isoelectric point	3.9	4	4.4	4.7	5.5
pH optimum	4.5–5	4.5–5	4.5–5	4.5–5	4.5–5
Temperature optimum	65–70°	65–70°	65–70°	65–70°	50°
Michaelis constant					
K_m *xylan g/L*	5	5	5	2.6	7.8

4 were tentatively identified by paper chromatography[5] (R_{Xyl} values of 1, 0.60, 0.30, and 0.14 in solvent *A*) and similarly an aldotetraouronic acid (R_{Xyl} 0.59 in solvent *B*).

Degradation with xylanase of pI 5.5. — Prolonged hydrolysis of birch xylan for 140 h was limited to 57% degradation as estimated by the weight of non dialyzable residue. L.c. fractionation of the dialyzable sugars gave a chromatogram where acidic products were first eluted, followed successively by xylose, xylobiose, xylotriose, and traces of xylotetraose (Fig. 7). The presence of oligosaccharides and the low extent of hydrolysis show that there was inhibition of the reaction. An estima-

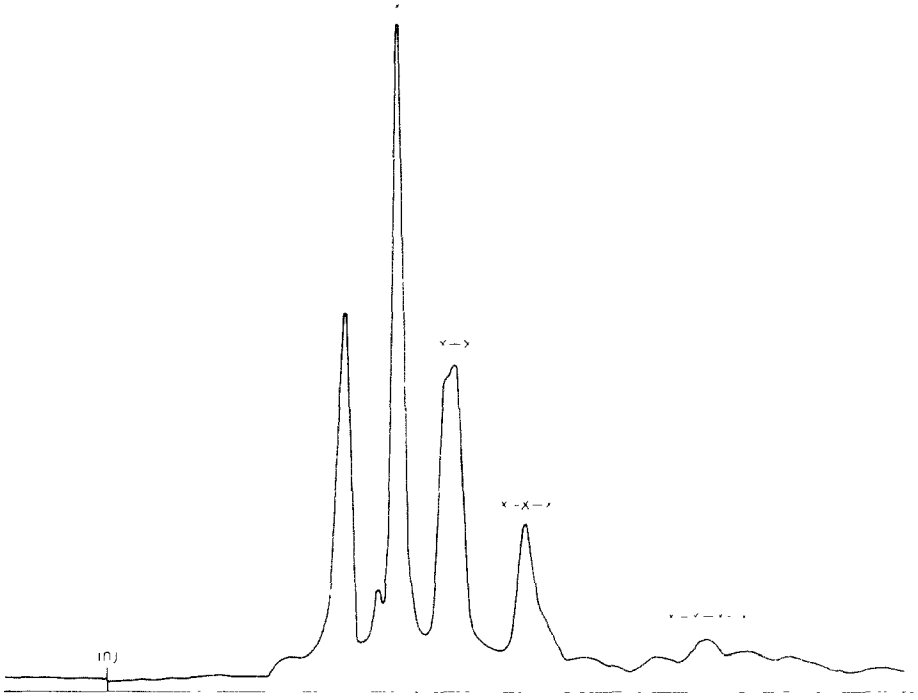

Fig. 7. L.c. analysis of the dialyzable products from the hydrolysis of xylan by pI 5.5 xylanase. The experiment was performed with a Waters liquid chromatograph using a C_{18} μ-Bondapak column kept at 20° and eluted with distilled water at the flow rate of 0.5 mL min^{-1}. The dialyzate was desalted on Amberlite IR-120 resin prior to injection. The xylo-oligosaccharide markers were isolated by filtration on Bio-Gel P-2 of an enzymic hydrolyzate of xylan.

tion based on the l.c. fractionation gave a concentration of xylobiose of ~4mM. This value agrees well with the xylobiose concentration (5mM) that limits to 54% the enzymic hydrolysis of the arabinoglucuronoxylan from sequoia[5].

Kinetics. — From the properties determined for xylanases from fraction II (Table IV), two groups may be distinguished. The first constitutes the enzymes of pI 3.9; 4; 4.4; and 4.7 and the second the enzyme of pI 5.5. Kinetics for the hydrolysis of birch xylan confirms this distinction into two groups, and the demonstration of different K_m values for the isolated xylanases again establishes that the multiplicity of xylanases is genuine. The determination of a Michaelis constant expressed in $g.L^{-1}$ when an insoluble substrate is used may be unusual, as the value of K_m for a given enzyme may be affected by the physical state of the substrate. This factor points out the problem of the lack of a standard soluble substrate for the xylanases. The values reported here are thus proper to the present experimental conditions, but the comparison established for the different xylanases from *Sporotrichum dimorphosporum* is still correct, as the reproducibility in the results was good, provided care was taken in the dispersion of the lyophilyzed xylan according to the conditions described here.

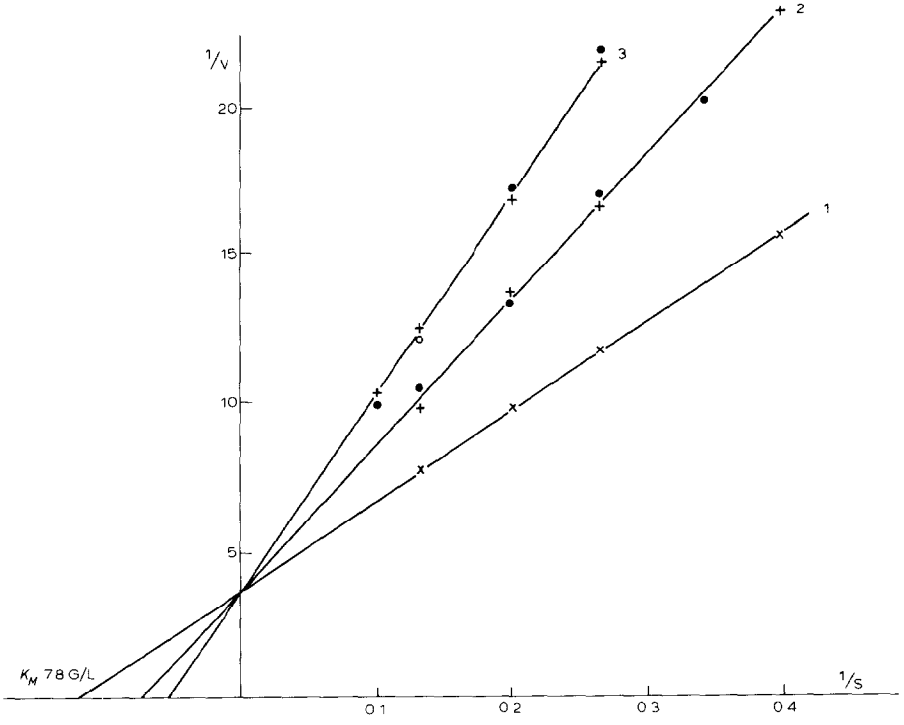

Fig. 8. Kinetics of the hydrolysis of xylan by pI 5.5 xylanase as expressed in a Lineweaver–Burk reciprocal plot. The concentration of substrate is expressed as $g.L^{-1}$ and the velocity as $nanokatal.mL^{-1}$. Hydrolysis of xylan alone: (*1*); hydrolysis of xylan in the presence of xylobiose + (or 4-thioxylobiose ●) at the final concentration of 0.5mM: (*2*) or of 1mM: (*3*).

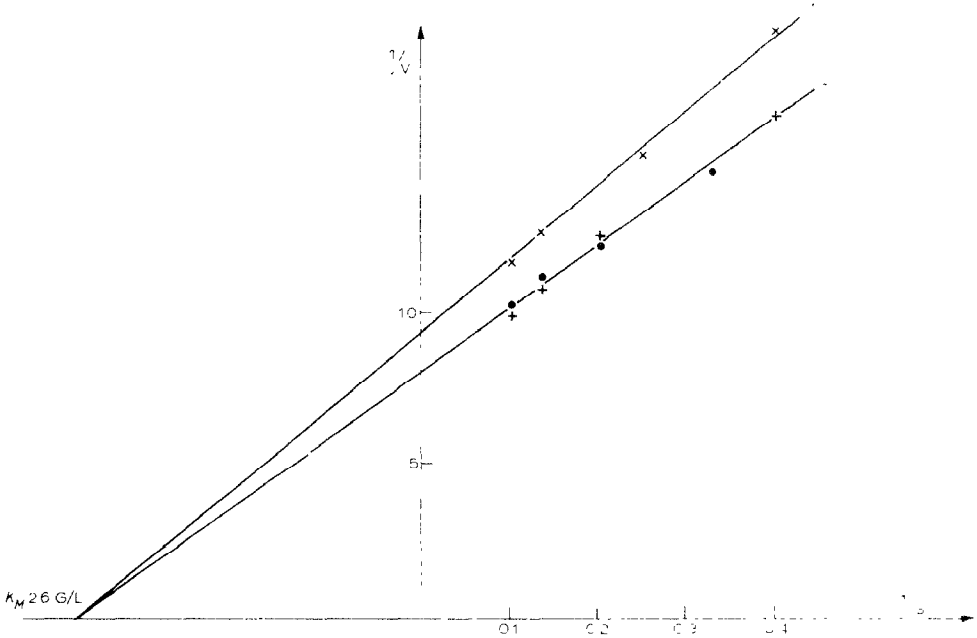

Fig. 9. Kinetics of the hydrolysis of xylan by pI 4.7 xylanase as expressed in a Lineweaver–Burk reciprocal plot. The concentration of substrate is expressed as g L^{-1} and the velocity as nanokatal mL^{-1}. Hydrolysis of xylan alone (*1*), hydrolysis of xylan in the presence of xylobiose + (or 4-thioxylobiose ●) at the final concentration of 0.25mM (*2*).

Influence of the hydrolysis products. — Product inhibition is a common feature of many enzymes. The influence of xylobiose and 4-thioxylobiose on the activity of xylanase was therefore tested. Surprising results were obtained, as both disaccharides showed the expected effect on the xylanase of pI 5.5 (Fig. 8) but not on the xylanase of pI 4.7. Fig. 8 shows that xylobiose and 4-thioxylobiose act identically as competitive inhibitors for the xylanase of pI 5.5 (K_I 0.725mM), whereas they are activators for the xylanase of pI 4.7 as K_m is unmodified and the rate of hydrolysis of the xylan is increased (Fig. 9). In neither case is xylobiose or 4-thioxylobiose degraded.

Analysis of the carbohydrate moiety of the isolated xylanases. — The pure xylanases of pI 4.4 and 4.7 were hydrolyzed in 0.5M sulfuric acid for 4 h at 100° and the neutral sugars analyzed in g.l.c. as their alditol acetate derivatives. The relative molar proportions of the major sugars xylose, mannose, and glucose were 1:1.10 and 1:3:7 for the xylanase of pI 4.4 and 4.7, respectively. The exact amount of carbohydrate in the xylanases was not determined because insufficient sample was hydrolyzed.

DISCUSSION

The basidiomycete fungus *Sporotrichum dimorphosporum* excreted at least 9 distinct xylanases from its hyphae. The isolated enzymes may be divided into two

groups having pI values above 7 or below 6. In each group, xylanases having very close pI values and properties, may be distinguished from other xylanases differing in pI value and molecular weight. The multiplicity of enzymes has been demonstrated, but not their exact number, as xylanase activities corresponding to mol.wts. of ~52,000 for fraction I and ~75,000 for fraction II have been identified by gel filtration on Sephadex G-100. These might have corresponded to enzymes of low activity that escaped detection in the compartments of electrofocusing, or to dimer complexes in solution that were decomposed in the electrofocusing experiment; this point remains to be demonstrated. In any event the aggregate results related to both the enzymic and physical properties suffices to establish the existence of several different xylanases, and leads to the fundamental question as to why does a fungus secrete so many enzymes having the same type of activity in order to hydrolyze a (1→4)-β-D-xylan. The same question likewise applies for cellulases. The conditions of action of these hydrolases outside the microbial cells do not correspond to the formation of complex multienzymic systems that could explain this multiplicity. A synergistic effect between the xylanases would be a more plausible interpretation, and we consider that the multiplicity is an illustration of the response of the microorganism for the need to act in a heterogeneous, solid phase. Isolated (1→4)-β-D-xylans, unsubstituted and of low d.p., crystallize in a hexagonal unit-cell structure with the chain in a threefold helical conformation[27]; oval single-crystals have recently been obtained[28], but the chain conformation still remains to be established. Moreover, left-handed, double helices are not excluded[29]. In the biological state, we do not know whether these conformations exist or whether the distribution of uronic acid and arabinose substituents on the xylan backbone[5] induces the adoption of particular conformations, as do the L-arabinose side-chains in arabinoxylans[30]. All xylosidic linkages are evidently not equivalent and equally accessible to a single a xylanase that would find specific arrays of hydroxyl groups and hydrogen atoms on the xylosyl residues near the bond to be cleaved, and thus a range of xylanases is needed to achieve complete hydrolysis. Similar stereochemical considerations have been proposed by Wood[31] to account for the multiplicity of endoglucanases and cellobiohydrolases in fungi.

According to Nakayama *et al.*[32], limited proteolysis of enzyme components may be responsible, at least in part, for the multiplicity of cellulases. Proteases are indeed present in our enzyme complex, and Eriksson[33] has found, in the culture solutions of the strain *Sporotrichum pulverulentum*, two proteases that enhanced the activity of the endo-(1→4)-β-D-glucanases. Whether or not these proteases are responsible for the multiplicity of the endoglucanases was not established[33]. Proteolysis would lead to numerous fragments lacking enzymic activity, unless the specific sites of attack were protected.

The kinetic studies with xylan in the presence of xylobiose (or 4-thioxylobiose) show the effect of products on the xylanase activities. The xylanase of pI 5.5 is competitively inhibited, whereas that of pI 4.7 is activated under the same circumstances.

The combined operation of competitive inhibition and activation seems to act as a regulation factor on the hydrolysis of substrate beyond the domain of the fungal cell.

CONCLUSION

This study of the xylanase system of a basidiomycete fungus *Sporotrichum dimorphosporum*, showing great activity on plant cell-walls, brings new knowledge on the wall-degrading enzymes. As with cellulases, xylanases exist in more than one form, and this multiplicity of enzymes having similar properties leads to the conclusion that each enzyme of the xylanase system may accommodate a particular, rigidly constrained, conformation of the substrate. This interpretation implies the need for synergistic action in order to achieve significant hydrolytic conversion.

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REFERENCES

1 E. B. Cowling and T. K. Kirk, *Biotechnol. Bioeng. Symp.*, *No. 6*, Wiley, 1976, pp. 95–123.
2 A. J. Kerr and D. A. I. Goring, *Cellulose Chem. Technol.*, 9 (1975) 563–573.
3 K. Ruel, F. Barnoud and D. A. I. Goring, *Wood Sci. Technol.*, 12 (1978) 287–291.
4 K. Ruel and F. Barnoud, *Int. Symp. Wood Pulping Chem.*, The Eckman Days, 1981, Stockholm, 1, 11–16.
5 J. Comtat and J.-P. Joseleau, *Carbohydr. Res.*, 95 (1981) 101–112.
6 H. Chanzy, J. Comtat, M. Dube and R. H. Marchessault, *Biopolymers*, 18 (1979) 2459–2464.
7 K. Ruel, J.-P. Joseleau, J. Comtat and F. Barnoud, *J. Appl. Polym. Sci.* 28 (1976) 971–981.
8 J. Comtat and F. Barnoud, *C. R. Acad. Sci. Paris, Ser. C*, (1973) 61–64.
9 J. Comtat, K. Ruel, J.-P. Joseleau and F. Barnoud, *Symposium on Enzymatic Hydrolysis of Cellulose*, S.I.T.R.A., Helsinki, Finland, (1975) 351–373.
10 M. Zinbo and T. E. Timell, *Sven. Papperstidn.*, 68 (1965) 647–662.
11 J. F. Saeman, W. E. Moore, R. L. Mitchell, and M. A. Millett, *Tappi*, 37 (1954) 336–343.
12 J. Comtat and J.-P. Joseleau, *Cellulose Chem. Technol.*, 7 (1973) 653–657.
13 M. Blanc Muesser, J. Defaye, H. Driguez, and E. Ohleyer in W. Palz, P. Chartier and D. O. Hall (Eds.), *Energy from Biomass*, Applied Science Publishers, 1980, pp. 312–318.
14 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
15 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
16 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248–254.
17 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
18 D. Sternberg, *Biotechnol. Bioeng. Symp. No. 6*, Wiley, 1976, pp. 35–53.
19 U. Westermark and K.-E. Eriksson, *Acta Chem. Scand., Ser. B*, 28 (1974) 209–214.
20 P. Ander and K.-E. Eriksson, *Sven. Papperstidn.*, 18 (1975) 643–652.
21 K.-E. Eriksson, *Biotechnol. Bioeng.*, 20 (1978) 317–332.
22 R. F. H. Dekker and G. N. Richards, *Adv. Carbohydr. Chem. Biochem.*, 32 (1976) 277–352.
23 L. G. Fagerstam and L. G. Pettersson, *FEBS Lett.* 98 (1979) 363–367.

24 R. F H. DEKKER AND G. N. RICHARDS, *Carbohydr. Res.*, 39 (1975) 97–114.
25 C. J. BAKER AND C. H. WHALEN, *Phytopathology*, 67 (1977) 1250–1258.
26 R. F. H. DEKKER AND G. N. RICHARDS, *Carbohydr. Res.*, 42 (1975) 107–123.
27 I. A. NIEDUSZYNSKI AND R. H. MARCHESSAULT, *Biopolymers*, 11 (1972) 1335–1344.
28 H. CHANZY, M. DUBE, AND R. H. MARCHESSAULT, *Polymer*, 20 (1979) 1037–1040.
29 T. L. BLUHM AND A. SARKO, *Carbohydr. Res.*, 54 (1977) 125–138.
30 I. C. M. DEA, D. A. REES, R. J. BEVERIDGE, AND G. N. RICHARDS, *Carbohydr. Res.*, 29 (1973) 363–372.
31 T. M. WOOD, *Int. Symp. Wood Pulping Chem.*, The Eckman Days, 1981, Stockholm, III, 31–38.
32 M. NAKAYAMA, Y. TOMITA, H. SUSUKI, AND K. NISIZAWA, *J. Biochem. (Tokyo)*, 79 (1976) 955–966.
33 K.-E. ERIKSSON, in ref. 31, pp. 60–65.