The Xylanase System of *Coniophora cerebella*

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1. The culture filtrate of the fungus *Coniophora cerebella* grown on poplar 4-*O*-methylglucuronoxylan as carbon source and enzyme inducer contained an enzyme system that degraded the polysaccharide to xylose, acidic and neutral oligosaccharides and an enzyme-resistant polymer. Free uronic acid was not produced. 2. Cold ethanol fractionation of the culture filtrate yielded two active fractions, one of which had only xylanase (EC 3.2.1.8) and the other both xylanase and xylosidase (EC 3.2.1.37) activities. Further fractionation on DEAE-cellulose resolved the xylanase and xylosidase activities. 3. The xylanase degraded poplar 4-*O*-methylglucuronoxylan in an essentially random manner, producing oligosaccharides, but some xylose residues in the vicinity of uronic acid side groups were protected from hydrolysis, preventing a truly random attack. The xylosidase attacked the polysaccharide very slowly, releasing xylose, but the oligosaccharides produced by the action of the xylanase were much more susceptible to hydrolysis by the xylosidase. 4. The products of xylanase action were separated into neutral and acidic fractions. The neutral oligosaccharides were separated by chromatography on charcoal-Celite, and the major products were characterized as xylobiose, xylotriose, xylotetraose and xylopentaose. Some of the acidic sugars were branched, having the uronic acid residue attached to a xylose residue other than the terminal non-reducing one. 5. Gel filtration of various xylanase fractions gave values for the molecular weight of the enzyme from 34 000 to 38 000.

Enzymes degrading the hemicellulose fraction of plant cell walls have not been as extensively studied as the cellulolytic or pectolytic enzymes. Probably the major reason for this is that, until comparatively recently, the chemistry of the components of the hemicellulose fraction was not in itself well understood. However, the main structural features of the 4-*O*-methylglucuronoxylan and of the glucomannan which make up the bulk of the hemicelluloses in woody tissues have now been determined with fair accuracy and methods of isolating these polysaccharides in good yield and in a pure state have been worked out (see Timell, 1964, 1965). It is now possible therefore to use fairly well-defined polysaccharides as substrates and from the products of enzymolysis to obtain information on the mode of action of the enzymes.

The 4-*O*-methylglucuronoxylan of poplar (*Populus tremuloides* Michx.) can be isolated in high yield by a simple alkaline extraction (Zinbo & Timell, 1965). Its structure has been characterized by Jones, Purves & Timell (1961) and Zinbo & Timell (1965). Unlike the xylans of cereal straw or of grasses it has a negligible arabinose content, which simplifies the

analysis of degradation products. For these reasons, this polysaccharide was chosen as the carbon source and enzyme inducer in the fungal cultures and as substrate in the enzyme studies described here. The enzyme source was the culture filtrate of the fungus *Coniophora cerebella*, which has been shown to produce extracellular hemicellulose-degrading enzymes (King, 1966).

MATERIALS AND METHODS

Materials. Amberlite IR-120 (analytical grade), Cyanogum 41, bovine serum albumin, peroxidase (horseradish) and trypsin were obtained from British Drug Houses Ltd., Poole, Dorset, De-Acidite FF-IP (3–5% cross-linking, 100–200 mesh), ovalbumin and β -lactoglobulin were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., haemoglobin (horse) and lysozyme (egg-white) were from Seravac Laboratories Ltd., Maidenhead, Berks., Blue Dextran 2000 was from Pharmacia (G.B.) Ltd., London, W. 13, and Bio-Gel P-100 was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; xylotriose and xylopentaose were gifts from Professor G. O. Aspinall, Trent University, Peterborough, Ont., Canada.

Substrate. 4-*O*-Methylglucuronoxylan was prepared from chlorite-delignified poplar sawdust (500 g.; 60–80 mesh) by the procedure of Zinbo & Timell (1965). The yield was 71 g., it had $[\alpha]_D^{25} - 72^\circ$ [c 1.0 in aq. 10% (w/v) NaOH], and it gave

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xylose, 4-*O*-methylglucuronic acid and traces of arabinose and mannose on hydrolysis. According to Zinbo & Timell (1965) this polysaccharide had $[\alpha]_D^{25} -76.5^\circ$ and contained xylose and uronic acid in the molar ratio 9:1.

Analytical methods. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) after precipitation with 5% (w/v) trichloroacetic acid. Total reducing sugar was determined by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952) with xylose as standard. The same reagent was used to determine the molecular weights of neutral oligosaccharides by reducing-end-group assay and also to determine the xylose content of the oligosaccharides after hydrolysis with 2*N*-H₂SO₄ for 1 hr. at 100°. Total hydrolysis of the 4-*O*-methylglucuronoxylan was carried out by the method of Saeman, Moore, Mitchell & Millet (1954). Hydrolysates were neutralized with BaCO₃, and Ba²⁺ was removed from the filtrates with Amberlite IR-120 (H⁺ form). Sugar-containing solutions were concentrated at 45° under reduced pressure in a thin-film rotary evaporator. Paper chromatography of mono-saccharides was carried out in ethyl acetate-pyridine-water (72:20:23, by vol.) and of neutral and acidic oligosaccharides in ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) (solvent A) and ethyl acetate-acetic acid-water (18:7:8, by vol.) (solvent B). Sugars were detected on paper chromatograms by an aniline phthalate reagent (Wilson, 1959). Neutral and acid sugar fractions in enzyme digests were separated by passing them first through a column (6 cm. × 0.8 cm.²) of Amberlite IR-120 (H⁺ form) and then through a column (12 cm. × 0.5 cm.²) of De-Acidite FF-IP (acetate form). The eluate and water washings were concentrated to small volume to give the neutral sugar fraction. The acidic sugars were eluted from the anion-exchange resin with 30% (v/v) acetic acid, the eluate was evaporated to dryness and the residue was taken up in a small volume of water.

Periodate oxidation. The procedure of Aspinall & Ferrier (1957) was used. When uptake of the oxidant had reached a constant value (usually about 48 hr.), ethylene glycol was added to reduce the excess of periodate, and the oxidized oligosaccharide was then reduced with excess of KBH₄. After hydrolysis by 1*N*-H₂SO₄ for 1 hr. at 100°, the solution was neutralized with BaCO₃, treated with Amberlite IR-120 (H⁺ form) and evaporated to dryness with methanol several times. The residue was examined for xylose by paper chromatography.

Electrophoresis. Polyacrylamide-gel electrophoresis was carried out according to Broome's (1963) modification of the original disc-electrophoresis technique (Davis, 1964) with 10% (w/v) Cyanogum 41 gels prepared according to the manufacturer's instructions. The buffer systems used were those described by Davis (1964). The applied current was maintained at 5 mA/tube, and the run was continued until the marker dye (bromophenol blue) had travelled to within 1 cm. of the bottom of the tube. The gels were fixed and stained for 1 hr. in 1% (w/v) Amido Black dissolved in 7% (v/v) acetic acid, and then de-stained electrophoretically.

Source of enzyme. *C. cerebella* F.P.R.L. culture no. 11E was grown in medium B for 18 days as previously described (King, 1966) with 1% 4-*O*-methylglucuronoxylan as carbon source and enzyme inducer. The crude enzyme solution was obtained by filtering the cultures through a no. 3 sintered-glass filter, the filtrate was immediately adjusted to pH 5.0 and NaN₃ (0.31 g./l.) was added as a preservative.

Fig. 1. Enzyme activity precipitated from the culture filtrate with 1-2 vol. of cold ethanol fractionated on a DEAE-cellulose column. The eluate was examined for enzyme activity (●) by the standard assay described in the text, and for E_{280} (▲). A, B, C and D, Fractions pooled for further study.

Enzyme assay. The assay substrate was prepared by dissolving 4-*O*-methylglucuronoxylan (0.1 g.) in 4% (w/v) NaOH soln. (10 ml.), then acidifying to pH 5 with acetic acid and diluting to 100 ml. with water. Substrate (2 ml.) and enzyme solution (0.1 ml.) were mixed and incubated at 40° for 1 hr. Reducing sugar produced was estimated on the entire digest and the results were expressed in terms of xylose equivalent, i.e. the amount of xylose giving the same reducing-sugar value. Blanks contained enzyme solution (0.1 ml.) that had been inactivated by heating for 10 min. at 100°.

Enzyme fractionation with cold ethanol. Culture filtrate (935 ml.) was immersed in ice-salt freezing mixture and cooled to 0°. Ethanol precooled to -20° was slowly added with stirring to the culture filtrate, and the temperature of the mixture was allowed to fall to between -5° and -10°. The mixture was centrifuged at 3000*g* for 20 min. at -10° at various concentrations of ethanol, and the precipitated fractions were redissolved in water (100 ml.) and dialysed overnight at 4° against 10 mM-phosphate buffer, pH 5.8 (9.2 mM-KH₂PO₄ and 0.8 mM-Na₂HPO₄). The fractions were assayed for enzyme activity and examined by electrophoresis.

Digests of the fractions precipitated with 1-2 vol. and 2-3 vol. of cold ethanol were prepared, containing 4-*O*-methylglucuronoxylan (25 mg.), enzyme solution (1 ml.) and acetate buffer (0.1*N*-acetic acid adjusted to pH 5.0 with 4*N*-NaOH) to give a final volume of 25 ml. Samples (1 ml.) were removed after 1 and 17 hr., and the reducing-sugar content was determined. The remainders of the digests after 17 hr. were separated into neutral and acidic sugar fractions, and the neutral sugars were identified by paper chromatography with xylose, xylotriose and xylopentaose as standards.

Enzyme fractionation on DEAE-cellulose. The fraction obtained with 1-2 vol. of ethanol was passed through

a column (10cm. $\times$ 1.2cm.²) of DEAE-cellulose pre-equilibrated in 10mm-phosphate buffer, pH 5.8. After being washed with the equilibrating buffer (20ml.), the column was eluted by a linear gradient of phosphate buffer, pH 5.8 (10mm–0.5M), applied over a volume of 300ml. Fractions (5ml.) were collected and assayed for enzyme activity and extinction at 280m μ . Fractions exhibiting enzyme activity were further characterized by paper chromatography of the products; substrate (2mg.), enzyme (0.1ml.), water (1.0ml.) and acetic acid (0.1N; 0.05ml.) were incubated together for 20hr. at 40°. Fractions 8–11 (Fig. 1; 0.05ml.) were also incubated separately with substrate (2mg.), water (1.0ml.) and acetic acid (0.1N; 0.05ml.) for 20hr. at 40°, and the products were examined by paper chromatography. After dialysis against 10mm-phosphate buffer, pH 5.8, fractions with appreciable extinction at 280m μ were submitted to electrophoresis.

The fractions were pooled according to their enzyme activities and electrophoretic patterns to give fractions *A* (fractions 8–11), *B* (fractions 15–18), *C* (fractions 19 and 20) and *D* (fractions 22–26). A more elaborate examination of the sugars produced when 4-*O*-methylglucuronoxylan was incubated with the pooled fractions *B*, *C* and *D* was carried out. Substrate (25mg.) was dissolved in N-NaOH (5ml.) and acidified to pH 5.0 with acetic acid; enzyme solution (1ml.) was added and the mixture diluted to 25ml. with water. The digests were incubated at 40° with toluene added as preservative. Samples were removed from the digests at intervals and immediately heated for 10min. at 100° to inactivate the enzyme. The neutral and acidic sugars were fractionated, and the constituent sugars in each fraction were separated by paper chromatography.

Large-scale digestion of the 4-O-methylglucuronoxylan. Substrate (20g.) was dissolved in N-NaOH (250ml.), and the solution was acidified to pH 5 with acetic acid. The substrate was not precipitated but remained as a colloidal suspension. To this suspension contained in dialysis tubing was added enzyme solution (40ml.; a mixture of fractions *B*, *C* and *D*), and the digest was incubated at 40° for 4 days with toluene as preservative. The digest was continuously dialysed against 2l. of 10mm-acetate buffer (10mN-acetic acid adjusted to pH 5 with 4N-NaOH), the buffer being changed daily. The combined diffusates were concentrated to about 100ml., and then neutral and acidic sugar fractions were separated by using first a column (20cm. $\times$ 27cm.²) of Amberlite IR-120 (H⁺ form) and then a column (20cm. $\times$ 12cm.²) of De-Acidite FF-IP (acetate form).

The dialysis residue was further dialysed against water and recovered by freeze-drying.

The neutral sugar fraction was concentrated to about 50ml., and the constituent sugars were separated by

chromatography on a charcoal–Celite (1:1, w/w) column (70cm. $\times$ 50cm.²), with a linear gradient of aq. 0–50% (v/v) ethanol applied over a volume of 8l. Fractions (20ml.) were collected and examined by paper chromatography, and similar fractions were pooled, evaporated to dryness and weighed. Identification of these sugars was made by chromatographic comparison in solvent systems A and B with xylose, xylotriose and xylopentaose, and by their optical rotations, molecular weights and xylose contents.

An attempt to separate the acidic sugars in a similar manner with a linear gradient of aq. 5–60% (v/v) ethanol was less successful, all the fractions containing more than one component.

Gel filtration of enzyme fractions. Samples (0.5ml.) of fractions *B*, *C* and *D* and the precipitate obtained on treating the culture filtrate with 2–3 vol. of ethanol were submitted to gel filtration on a column (75cm. $\times$ 2cm.²) of Bio-Gel P-100. The column was pre-equilibrated with 0.1M-phosphate buffer, pH 5.8 (92mm-KH₂PO₄ and 8mm-Na₂HPO₄), and it had been calibrated for molecular-weight estimations by measuring the elution volumes of Blue Dextran 2000, bovine serum albumin, peroxidase, trypsin, ovalbumin, β -lactoglobulin, haemoglobin, lysozyme and glucose. Fractions (2ml.) were collected and the enzyme activity was detected by incubating 0.5ml. of each fraction with standard assay substrate (2.0ml.) and toluene (1drop) for 18hr. at 40° and estimating the reducing sugar produced.

RESULTS

The cold ethanol fractionation gave two fractions with appreciable enzyme activity, with 1–2 vol. of ethanol and 2–3 vol. of ethanol respectively. The xylose equivalent and apparent percentage hydrolysis of the substrate to xylose brought about by these two fractions after incubation for 1 and 17hr. are given in Table 1. The fraction precipitated with 1–2 vol. of ethanol appeared to degrade the substrate faster and probably to a greater extent than the fraction precipitated with 2–3 vol. of ethanol. However, an examination of the neutral sugars produced on hydrolysis of the substrate by the two fractions indicated that fractionation of the enzyme activity had occurred. The enzyme fraction precipitated with 1–2 vol. of ethanol gave xylose and xylotriose as the major products with a smaller amount of xylotriose and traces of higher oligosaccharides. The fraction precipitated with 2–3 vol. of ethanol yielded xylotriose, xylotriose

Table 1. Cold ethanol fractionation of culture filtrates

Cold ethanol added to precipitate the fraction (vol.)	Vol. of fraction after dialysis (ml.)	Protein content (μ g./ml.)	Time of digestion (hr.)	Xylose equivalent (μ g.)	Apparent hydrolysis to xylose (%)
1.0–2.0	100	460	$\left\{ \begin{array}{l} 1 \\ 17 \end{array} \right.$	$\left\{ \begin{array}{l} 10.5 \\ 136.0 \end{array} \right.$	$\left\{ \begin{array}{l} 0.5 \\ 6.0 \end{array} \right.$
2.0–3.0	120	108	$\left\{ \begin{array}{l} 1 \\ 17 \end{array} \right.$	$\left\{ \begin{array}{l} 8.0 \\ 52.0 \end{array} \right.$	$\left\{ \begin{array}{l} 0.4 \\ 2.3 \end{array} \right.$

and xylo-tetraose as major products and other higher oligosaccharides in appreciable amounts; xylose was not present in digests containing this fraction. The two enzyme fractions were therefore not directly comparable by the assay method used to obtain the results shown in Table 1. The crude culture filtrate appeared to contain two types of enzyme acting on the 4-*O*-methylglucuronoxylan, one producing oligosaccharides by hydrolysis at various points along the chains of xylose residues and the other releasing single xylose units from the substrate, or, more probably, from the oligosaccharides produced by the action of the first enzyme.

Separation of the two types of enzyme activity was achieved on DEAE-cellulose. Fig. 1 shows the results for the standard assay and the extinction at 280m μ of the fractions. On paper chromatography of the products of hydrolysis of the substrate with these fractions, fractions 8-11 yielded very small amounts of xylose and little or no oligosaccharides, fractions 15-26 produced oligosaccharides and little xylose, and fractions 12-14 produced both xylose and oligosaccharides. The high value for xylose equivalent shown by fraction 14 in Fig. 1 is in agreement with the presence of both types of enzyme in this fraction.

When fractions 8-11 were incubated separately with substrate and with fraction 18 the amounts of reducing sugar produced were much larger than those with any of fractions 8-11 alone or of fraction 18 alone. Paper chromatography of the products of these digests showed that xylose was the major product with appreciable amounts of oligosaccharides. Thus it appeared that fractions 8-11 contained an enzyme with little activity on the 4-*O*-methylglucuronoxylan but which is capable of degrading xylo-oligosaccharides to xylose, whereas fractions 16-26 contained an enzyme able to

release xylo-oligosaccharides from the 4-*O*-methylglucuronoxylan but unable to degrade these further to xylose.

When the pooled fractions *B*, *C* and *D* (see Fig. 1) were incubated with the substrate, all three fractions produced very similar patterns of neutral and acidic sugars. In the early stages the major neutral sugars had R_{xylose} values corresponding to xylobiose and xylo-triose but, later, sugars corresponding to xylose and xylo-tetraose were also observed. The acidic sugars presented a more complex picture. Slow-moving sugars with presumably a fairly large degree of polymerization were produced initially. These were apparently broken down as the hydrolysis proceeded until, after 48hr., the major acidic sugars had R_{xylose} values of 0.26 and 0.12 respectively in solvent B.

The electrophoretic patterns of the various enzyme fractions were complex. The culture filtrate had at least 11 bands and the fractions obtained with 1-2 vol. and 2-3 vol. of cold ethanol at least seven and six bands respectively. The DEAE-cellulose fractions *A*, *B*, *C* and *D* had at least five, four, three and five bands respectively. Although it was not possible to identify specific bands with enzyme activity, the enzyme producing xylose from the substrate appeared to be associated with the slower-moving bands and the oligosaccharide-producing activity was associated with the faster-moving bands.

The results of the large-scale digestion of 4-*O*-methylglucuronoxylan with the purified oligosaccharide-producing activity are summarized in Tables 2 and 3. The recovery of material was 70%. The dialysis residue (9.7 g.) represented 69% of the recovered material and the neutral and acidic sugars represented 13% and 18% respectively.

As all the acidic sugar fractions contained more

Table 2. *Neutral sugars fractionated on a charcoal-Celite column*

The sugars were produced in a large-scale hydrolysis of 4-*O*-methylglucuronoxylan by oligosaccharide-producing enzyme activity and separated from the acidic sugars by treatment with ion-exchange resins. For details see the text. n.d., Not determined.

Fraction (ml. of eluent)	Wt. (g.)	R_{xylose} in solvent system B	$[\alpha]_D^{25}$	Mol.wt. from reducing end-group assay	Xylose produced on hydrolysis (moles/mole of oligosaccharide)	Identification
Washing water and 0-1500	0.10	1.00	+18°	148	n.d.	Xylose
1950-3000	0.23	0.60	-27	282	1.99	Xylobiose
3000-3340	0.01	0.59 and 0.27	n.d.	n.d.	n.d.	Mixture of xylobiose and xylo-triose
3340-4400	0.63	0.25	-48	407	2.98	Xylo-triose
4400-5340	0.39	0.11	-64	558	4.13	Xylo-tetraose
5340-6320	0.23	0.05	-68	698	5.14	Xylo-pentaose
6320-8865	0.25	0-0.06	n.d.	n.d.	n.d.	Xylo-pentaose and higher oligosaccharides
Total	1.84					

Table 3. *Acidic sugars fractionated on a charcoal-Celite column*

The acidic sugars were produced in a large-scale hydrolysis of 4-*O*-methylglucuronoxylan by oligosaccharide-producing enzyme activity, and separated from the neutral sugars by treatment with ion-exchange resins. For details see the text. n.d., Not determined.

Fraction no.	Vol. of fraction (ml.)	Wt. (g.)	R_{xylose} of main component in solvent system B	Xylose after periodate oxidation and hydrolysis
A_1	0-3075	0.88	0.25	+
A_2	3075-3975	0.11	0.14	n.d.
A_3	3975-4200	0.09	0.06	n.d.
A_4	4200-4725	0.05	0.03	+
A_5	4725-4875	0.04	Mixture	n.d.
A_6	4875-5175	0.13	0.29	+
A_7	5175-5250	0.02	Mixture	n.d.
A_8	5250-5625	0.16	0.13	n.d.
A_9	5625-6025	0.24	0.07	+
A_{10}	6025-6375	0.22	0.02	+
A_{11}	6375-8075	0.52	0-0.02	n.d.
	Total	2.46		

than one component, rigorous characterization of these sugars was not possible. Timell (1962) obtained a similar series of acidic oligosaccharides from digests of birch 4-*O*-methylglucuronoxylan with a commercial enzyme. However, most of the acidic sugars obtained in the present study differed in R_{xylose} values in solvent B from those given by Timell (1962), and a test was performed on some of the purer fractions to see if they had the same general structure:

found by Timell (1962) for his series of oligosaccharides. Oligosaccharides with this structure should not yield xylose on hydrolysis after periodate oxidation. All the acid sugar fractions examined in this way gave xylose (Table 3).

The molecular weights of the various oligosaccharide-producing enzyme activities were estimated by gel filtration to be 37 000, 34 000 and 37 000 respectively for fractions *B*, *C* and *D*, and 38 000 for the enzyme precipitated from the culture filtrates with 2-3 vol. of ethanol.

DISCUSSION

C. cerebella grew well in a medium containing the 4-*O*-methylglucuronoxylan as carbon source and produced an extracellular enzyme system capable of degrading the polysaccharide. From the enzyme-fractionation experiments and the analyses of the products of enzymolysis, two types of enzyme activity were identified in the culture filtrates. One type, a xylanase (β -1,4-xylan xylanohydrolase, EC

3.2.1.8), hydrolysed chains of β -(1 $\rightarrow$ 4)-linked xylopyranose residues in an essentially random manner, producing large amounts of oligosaccharides but little xylose. The other type of activity, a β -xylosidase (β -D-xyloside xylohydrolase, EC 3.2.1.37), released xylose from xyloligosaccharides but degraded the 4-*O*-methylglucuronoxylan only very slowly, yielding small amounts of xylose. Similar xylan-degrading systems have been reported in *Chaetomium globosum* (Sorensen, 1952), *Aspergillus foetidus* (Whistler & Masak, 1955), *Aspergillus batatae* (Fukui & Sato, 1957) and *Diplodia viticola* (Strobel, 1963). Free 4-*O*-methylglucuronic acid was not produced by the action of the culture filtrate or by any of the enzyme preparations on the polysaccharide or derived oligosaccharides, and it was evident that the fungus did not produce an extracellular enzyme catalysing the hydrolysis of the uronic acid residues from the chains of xylopyranose units.

The fractionation of the xylanase activity into at least three components by chromatography on DEAE-cellulose may not have represented a separation of three different enzymes with xylanase activity. Thus it was not possible to distinguish any differences in activity towards the substrate, 4-*O*-methylglucuronoxylan, and the molecular weights of the xylanase fractions, as determined by gel filtration, were very similar. Although it is possible that the xylanase activity represents an isoenzyme system, it is known (see Jermyn, 1962) that some polysaccharide-degrading enzymes are difficult to separate from associated carbohydrates, and different degrees of complex-formation with carbohydrate could be responsible for the apparent fractionation on DEAE-cellulose. Such protein-

carbohydrate associations may also cause the complex band patterns on polyacrylamide-gel electrophoresis of the fractionated material.

The action of the xylanase on poplar 4-*O*-methylglucuronoxylan was essentially a random hydrolysis, producing neutral and acidic oligosaccharides and a resistant polymer. The neutral products were mainly xylobiose, xylotriose, xylotetraose and xylopentaose, but the series of acidic sugars had probably somewhat higher degrees of polymerization, as shown by their relatively low mobilities on paper chromatograms. The fastest-moving acidic oligosaccharide had a considerably smaller R_{xylose} value than the acidic tetrasaccharide isolated by Timell (1962) from digests of birch 4-*O*-methylglucuronoxylan with a commercial fungal 'pectinase'. It seems therefore that the uronic acid groups attached to the main chains of xylose residues in the polysaccharide modified the random action of the enzyme by protecting from hydrolysis some of the bonds between xylose units close to the uronic acid groups. This modifying influence of the acidic residues may account for the relatively small yield of neutral oligosaccharides with degrees of polymerization greater than 4, since, from the uronic acid content (Zinbo & Timell, 1965), even if the acidic residues were uniformly distributed along the xylan chains, there should be runs of at least eight or nine unsubstituted xylose units capable of yielding appreciable amounts of neutral oligosaccharides with degrees of polymerization greater than 4, if a truly random hydrolysis occurred. It is possible that any large neutral oligosaccharides that were produced were particularly susceptible to further enzymolysis, but the dialysis system used in the large-scale experiment should have helped to protect such oligosaccharides. Samples of hydrolysates taken in the early stages did not show the presence of large neutral oligosaccharides that were further degraded as the hydrolysis proceeded.

Although the acidic oligosaccharides produced in the present study were not rigorously characterized, the periodate-oxidation results showed that some did not have the uronic acid group attached to the terminal xylose unit at the non-reducing end of the chain, i.e. they were branched oligosaccharides.

Timell (1962) did not obtain major amounts of such products, but his enzyme preparation probably contained much more β -xylosidase than that used in the present study, since xylose represented over 30% by weight of the products isolated. If the xylose residues on the non-reducing side of the branch-point in branched oligosaccharides were particularly susceptible to the action of the β -xylosidase, a series of acidic oligosaccharides with the general structure proposed by Timell (1962) could result.

The enzyme-resistant polymer was not investigated, but its resistance may have been due, at least in part, to retrogradation, as the substrate did not remain dispersed throughout the experiment. Inaoka (1961) showed that an enzyme-resistant retrograded xylan was rendered susceptible to attack by fresh enzyme after autoclaving to redisperse the xylan.

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