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MODE OF ACTION OF A XYLANASE AND ITS SIGNIFICANCE FOR THE STRUCTURAL INVESTIGATION OF THE BRANCHED L-ARABINO-D-GLUCURONO-D-XYLAN FROM REDWOOD (*Sequoia sempervirens*)

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ABSTRACT

The substitution pattern of the water-soluble L-arabino-(4-*O*-methyl-D-glucurono)-D-xylan from redwood (*Sequoia sempervirens*) has been studied by enzymic degradation. Exhaustive hydrolysis by an endo-xylanase (EC 3.2.1.8) from a Basidiomycete *Sporotrichum dimorphosporum* left a residue accounting for 20% of the original D-xylan. In the dialyzable material, oligosaccharides having arabinose or 4-*O*-methylglucuronic acid residues attached to the non-reducing D-xylosyl end-group of xylobiose or xylotriose, respectively, were the smallest branched oligomers released. Action of the xylanase appears to involve a region of the polysaccharide backbone having three xylosyl residues. A mode of action is proposed that requires unsubstituted hydroxyl groups at C-2, C-3, and C-2′ of a xylobiosyl residue. The binding site seems to correspond to a shallow cavity. The composition and structure of the final residue of attack shows that the enzyme has no action when the xylosyl residues branched through O-2 are separated by only one, unsubstituted xylose residue. This pattern of action, the nature of the dialyzable products, and the production of a final residue in which the substituents are accumulated, suggest that the arabinosyl and glucosyluronic groups are irregularly distributed on the main chain of the xylan from redwood and that in some regions they are in close vicinity when not actually on adjacent xylosyl residues.

INTRODUCTION

Accurate determination of the proportions of the substituents in L-arabino-D-glucurono-D-xylans may be achieved by complete hydrolysis of the carboxyl-reduced polysaccharide. The linkage-sequence may be demonstrated by classical methylation analysis or by periodate oxidation. Nevertheless, none of these methods indicate the distribution pattern of substituents on the xylosyl backbone, because they provide only averaged data. More-precise features of the macromolecule would help in the understanding the molecular relations between the macromolecular constituents of the plant cell-wall. For xylans, details of structure have been established by chemical

techniques and by specific enzymic hydrolysis. The purification and mode of action of xylanases from various origins have been reviewed by Dekker and Richards[1].

Most of the substituents in the 4-*O*-methyl-D-glucurono-D-xylan from birch wood are irregularly distributed on the main chain, as demonstrated by Havlicek and Samuelson[2] by isolation of xylo-oligosaccharides of d.p. 2–18 after partial acidic hydrolysis. This result was confirmed by Rosell and Svensson[3], using a specific degradation technique based on β-elimination. A similar conclusion was reached earlier by Timell[4] who used enzymic hydrolysis. Conversely, analysis of the products of the action of a xylanase from *Trametes hirsuta* on the 4-*O*-methyl-D-glucurono-D-xylan from white willow has revealed a regular distribution of the uronic acid groups attached to the xylan backbone[5].

The L-arabino-D-glucurono-D-xylans from softwoods are known to contain about twice as many 4-*O*-methyl-D-glucuronic acid groups as do the xylans from deciduous woods. Shimizu *et al.*[6], using partial acidic hydrolysis of a xylan from larch wood, demonstrated that a large proportion of the uronic acid side-chains were located on two, contiguous D-xylose residues. In this study on enzymic hydrolysis of a xylan from another softwood, redwood (*Sequoia sempervirens*), with a xylanase from a Basidiomycete, our aim was first to obtain more information on the fine structure of the polysaccharide and, second, to gain some insight on the mode of action of the xylanase.

EXPERIMENTAL

General methods and instrumentation. — Paper chromatography was performed on Whatman paper No. 1 for analytical or No. 3 MM for preparative purposes. The solvent systems were: *A*, 10:4:3 ethyl acetate–pyridine–water and *B*, 6:3:4 ethyl acetate–acetic acid–water. The compounds were detected with aniline oxalate[7]. The mobilities (R_x) are expressed relative to that of xylose.

For g.l.c., Packard Becker 417 or Hewlett–Packard 5710 A instruments, fitted with flame-ionization detectors and connected to a Hewlett–Packard 3380 A integrator, were used. Separations were performed on glass columns (180 × 0.15 cm) of: *A*, 3% of ECNSS-M on gas Chromosorb Q at 180° for alditol acetates; column *B*, 3% of SP 2340 on Chromosorb W AW at 215° for alditol acetates and programmed from 165 to 220° at 1°/min for the partially methylated alditol acetates, and column *C*, 3% of OV 225 for g.l.c.–m.s. of the methylated derivatives. Mass spectra were recorded with an A.E.I. model MS-30 mass spectrometer at an ionizing potential of 70 eV. The temperature in the ionizing chamber was 150 or 200°. Hydrolyses of the polysaccharide were performed with 72% sulfuric acid at room temperature for 30 min, followed by 0.5M sulfuric acid at 100° for 4 h (ref. 8). Methylations were performed according to Hakomori[9]. The methylated derivatives were hydrolyzed with 90% formic acid for 1 h at 100°, followed by 2M trifluoroacetic acid for 3 h at 100°, and the products analyzed by g.l.c. and g.l.c.–m.s. of their alditol acetate derivatives[10] (columns *B* and *C*).

The products released by the enzymic hydrolyses were further analyzed after hydrolysis for 4 h at 100° with 0.5M sulfuric acid.

Enzyme. — The xylanase was purified from a commercial enzyme preparation (cellulase La Rapidase, France) from the culture medium of a Basidiomycete *Sporotrichum dimorphosporum*. The fraction used[11] in this study, referred to as *xylanase II*, was desalted on a Diaflo UM 10 membrane (Amicon Ltd.) and freeze dried. It was obtained by elution from DEAE-Sephadex A 50 resin by stepwise elution with a 0.01M, pH 7 phosphate buffer 0.06M in sodium chloride, after washing with 0.02M sodium chloride in the same phosphate buffer. Proteins were made visible by electrofocusing (Multiphor apparatus, LKB) on a 5% thin layer of poly(acrylamide) gel containing 2% Ampholine carrier ampholytes (pH 4–6), after staining with 0.05% Coomassie Brilliant Blue R 250. Isoelectric focusing of xylanase II revealed a strong, double spot centered at pI 5.5 and the presence of three other spots of weaker intensities. Xylanase predominated, but *O*-(carboxymethyl)cellulase and amylase activities were also present[11].

Substrate: L-*Arabino*-(*4*-O-*methyl*-D-*glucurono*)-D-*xylan from redwood* (Sequoia sempervirens). — The polysaccharide was extracted with 24% potassium hydroxide from the holocellulose and further purified by treatment of the crude extract at room temperature with sodium chlorite. The accompanying galactoglucomannan was separated *via* its barium complex. The xylan was purified by repeated precipitation with ethanol as previously described[12]. The polysaccharide was thoroughly dialyzed and freeze dried before being subjected to enzymic treatment.

Uronic acid determinations. — (*a*) *Determination of equivalent weight.* Carboxylate groups were converted into the free acids by ion exchange on Amberlite IR-120 (H^+) resin. The polysaccharide (50 mg) was dissolved in water and titrated with 0.1M sodium hydroxide.

(*b*) *Decarboxylation.* This was achieved by treating the polysaccharide (50 mg) with hydroiodic acid and determining the carbon dioxide evolved by conductimetry[13].

(*c*) *Colorimetry.* The ratio of pentoses to uronic acids was determined, after standardization, by the harmine–cysteine–HCl method[14]. Best results were obtained when the mixture was left for 30 min in the dark after the addition of harmine, and 1 h in the dark after addition of cysteine hydrochloride. This method allowed rapid determination of the ratio of pentoses to uronic acids without contributions from the hexose contaminants.

(*d*) *Carboxyl reduction.* The polysaccharide (50 mg) was reduced by the carbodiimide procedure of Taylor and Conrad[15], and the material was recovered after dialysis. The treatment was repeated twice in order to achieve full reduction, as checked by the absence of reaction with the carbazole reagent[16]. For the final residue (R2), the dialysis step was omitted, and the salts arising from the reduction process were removed through concentration and treatment with cation-exchange resin.

Enzymic hydrolyses. — (*a*) *First hydrolysis.* The xylan from *Sequoia sempervirens* (330 mg) was incubated with shaking for 48 h at 40° with the xylanase (130 μg of protein[17]) in 0.1M sodium acetate buffer (pH 5, 33 mL) containing sodium azide

(5mM) as a preservative. At the end of the incubation period, the enzyme was inactivated for 10 min at 100°, and the mixture was dialyzed in Visking tubing against frequently renewed distilled water for 3 days at 5°. The non-dialyzable residue (R1) afforded 132 mg of lyophilized material.

(*b*) *Second hydrolysis.* The residue R1 (100 mg) was solubilized and subjected to a second enzymic attack under the same conditions (substrate concentration 1%, xylanase 40 μg of protein) and a residue (R2) was recovered (46 mg).

(*c*) *Third hydrolysis.* Residue R2 was incubated as before with the enzyme, and a residue R3 was recovered with no significant loss of weight. This residue (R3) was not used further.

Gel chromatography of the dialyzable material. — After each hydrolytic step, the dialyzates were combined and concentrated, and then passed through a column of Amberlite IR-120 (H^+) resin, and concentrated to low volume. The mixture was fractionated on a column (210 × 2.5 cm) of Bio Gel P2 (200–400 mesh) kept at 65°. Elution was performed with degassed distilled water at a flow-rate of 25 mL/h, and the emergent sugars were monitored with a differential refractometer[18]. The components of each peak were collected and analyzed by paper chromatography, and the proportion of the constituents estimated, after detection, by densitometry. The sugars were further purified by preparative paper-chromatography.

Assays on methyl L-*arabinosides.* — Methyl α L-arabinofuranoside and methyl α- and β-L-arabinopyranosides were prepared according to Augestad and Berner[19]. The enzymic assays were conducted for 65 h at 40°, with a concentration of xylanase 10 times higher than for the foregoing enzymic hydrolysis of the xylan. The concentration of reducing sugar (arabinose) released[20] was not significant.

RESULTS

Analysis of the xylan from redwood. — The L-arabino-D-glucurono-D-xylan extracted from the wood of *Sequoia sempervirens* afforded a polysaccharide which

TABLE I

ACID HYDROLYSIS OF REDWOOD XYLAN: MOLAR PROPORTIONS OF NEUTRAL SUGARS

Compound	*Unreduced xylan (mol. %)*	*Carboxyl-reduced xylan (mol. %)*
Rhamnose	0.8	0.6
Arabinose	4.95	3.6
Xylose	87.0	74.9
Mannose	1.1	0.6
4-*O*-methylglucose		15.7
Galactose	3.7	1.9
Glucose		
(contaminant)	2.45	1.2
(from reduction)		1.5

dissolved in water after prolonged stirring. Total acid hydrolysis of this xylan, followed by sugar analysis as the alditol acetates (g.l.c., Table I) showed the presence of contaminating polysaccharides (~4% by weight) constituted of mannose, galactose, and glucose, partly arising from residual galactoglucomannan and glucomannan[12].

Carboxyl reduction[15], followed by hydrolysis, gave an additional peak on the g.l.c. chromatogram corresponding to 4-*O*-methylglucitol, which originated from 4-*O*-methyl-D-glucuronic acid. It may also be noted that the relative amount of glucose increased after the carboxyl reduction, showing that a non-methylated glucuronic acid residue was also present in the original xylan, as demethylation was improbable.

Assuming that the ratio of mannose to glucose from contaminating glycans did not vary between the original and the carboxyl-reduced hemicellulose, and taking the percentage of mannose as a reference, it was possible to estimate the ratio of glucuronic acid to its 4-methyl ether as 1 : 11.

Similarly with arabinose as the reference compound and knowing the ratio of xylose to uronic acid (Table I), it was also possible to estimate that ~70% of the aldobiouronic acid (GlcA-Xyl) did not undergo acid hydrolysis. In this way, by using the ratio of pentoses to uronic acid (as determined by colorimetry[14]), a systematic correction for better estimation of xylose was made throughout the experiments, in order to establish more-accurate molar ratios between the different sugars in g.l.c. without carboxyl reduction of the samples.

Methylation analysis of the carboxyl-reduced xylan (Table II) demonstrated a high degree of substitution of the xylan chain by uronic acid and arabinose residues. Part of the arabinose is present in the (less common) pyranoid form. It may also be noted from the presence of unmethylated xylose that a few xylose residues carry two side-chains, unless this xylose arises from slight undermethylation. The average degree of polymerisation ($\overline{d.p.}$) is ~50, as previously established[12].

In the following study of the action of the enzyme on the xylan, it was important to rely on accurate determination of the uronic acid content. Table III gives the results obtained by different methods. As a result, the overall composition of the

TABLE II

SUGAR ANALYSIS[a] OF PERMETHYLATED, CARBOXYL-REDUCED XYLAN

Compound	*Mol. %*
2,3,4-Tri-*O*-methylrhamnose	0.7
2,3,5-Tri-*O*-methylarabinose	2.5
2,3,4-Tri-*O*-methylarabinose	1.2
2,3,4-Tri-*O*-methylxylose	1.7
2,3,4,6-Tetra-*O*-methylglucose	16.8
2,3-di-*O*-methylxylose	56.3
Mono-*O*-methylxylose	19.6
Xylose	1.2

[a]Only the methylated derivatives originating from the xylan are listed in this table.

TABLE III

MOLAR RATIOS OF THE CONSTITUENT SUGARS OF THE XYLAN FROM *Sequoia sempervirens*

Method	*Pentoses/acids*	*Xylose/acid*	*Xylose/arabinose*	*Xylose/rhamnose*
Equivalent-weight determination	4.0			
Decarboxylation	4.1			
Methoxyl determination[a]	4.1			
Harmine–cysteine · HCl	4.2			
Total hydrolysis			20.9[b]	129[b]
Carboxyl reduction		4.35	20.8	125
Carboxyl reduction and methylation		4.7	21.3	113

[a]Uronic acid content calculated as 4-*O*-methylglucuronic acid[12]. [b]Corrected for the aldobiouronic acid (see text).

L-arabino-D-glucurono-D-xylan from redwood is deduced to be as follows: 1 uronic acid per 4.3 xylose residues, 1 arabinose per 21 xylose residues, and one rhamnose per 120 xylose residues. To our knowledge, rhamnose has never been shown to be present in glucomannans, and it is deduced to be linked to the xylan[21]. The high level of substitution makes this xylan very well suited for study by enzymic hydrolysis of the localization of these substituents.

Hydrolysis of redwood xylan with a xylanase. — The first attack of the xylan from *Sequoia* with xylanase II for 48 h converted 60% of the substrate into dialyzable products, which were removed by dialysis. The yield in the hydrolysis was estimated from the weight of non-dialyzable residue (R1). A second attack, under the same conditions, on the residual material (R1) raised the hydrolysis yield to 82%. This yield was not significantly increased when a third attack was performed.

The contaminating glycans, which are only slightly degraded by a glucanase accompanying the xylanase, accumulated in the residues from the attack. Analysis of the neutral sugars released by hydrolysis with sulfuric acid of the products from

TABLE IV

SUGAR COMPOSITION OF THE PRODUCTS RELEASED BY ENZYMIC HYDROLYSES

Sugar	*First hydrolysis*		*Second hydrolysis*	
	Dialyzate	*Residue* R1	*Dialyzate*	*Residue* R2[a]
Rhamnose	—[b]	4.05	—[b]	3.9
Arabinose	4.25	7.4	6.15	6.0
Xylose	89.6	69.05	77.15	52.2
Mannose	1.9	2.1	2.1	2.3
Galactose	0.85	12.6	2.5	8.6
Glucose	3.4	4.8	12.1	7.5
4-*O*-Methylglucose				19.5

[a]After carboxyl reduction[15]. [b]Not detected.

TABLE V

MOLAR RATIOS OF THE CONSTITUENT SUGARS OF THE XYLAN FROM *Sequoia sempervirens* AT EACH STEP OF THE ENZYMIC ATTACK

Product	*Xylose/uronic acid*[a]	*Xylose/arabinose*[b]	*Xylose/rhamnose*
Original xylan	4.3	21.0	~120
Dialyzate from the first attack	5.0	24.5	V.h.[c]
Residue R1	2.4	13.1	~24[b]
Dialyzate from the second attack	3.1	16.2	V.h.[c]
Residue R2	1.8	8.7	~13

[a]Xylose and uronic acids estimated by colorimetry[14]. [b]Corrected for the aldobiouronic acid (see text). [c]V.h. = very high because only traces of rhamnose could be detected.

the enzymic digestions are given in Table IV. Molar ratios of the constituent sugars, including uronic acids, calculated as before, are listed in Table V.

Arabinose and uronic acid showed an approximately two-fold increase in the residue R1 and a five-fold enrichment in rhamnose. These increases were enhanced after the second attack. The residue R2 originating from a substrate of low $\overline{d.p.}$, and obtained after exhaustive enzymic digestion should be comprised of acidic compounds of short chain-lengths. Using a modified larchwood arabinoxylan* whose uronic acid content had been decreased to ~2% and further purified[22], we checked that the hydrolysis products of d.p. 13 or 14 were dialyzable. The electrostatic exclusion process[23] may have prevented highly acidic residues from diffusing across the dialysis membrane. Such acidic-substituted oligomers have previously been isolated by Shimizu *et al.*[6] following partial acid hydrolysis of a xylan from larchwood.

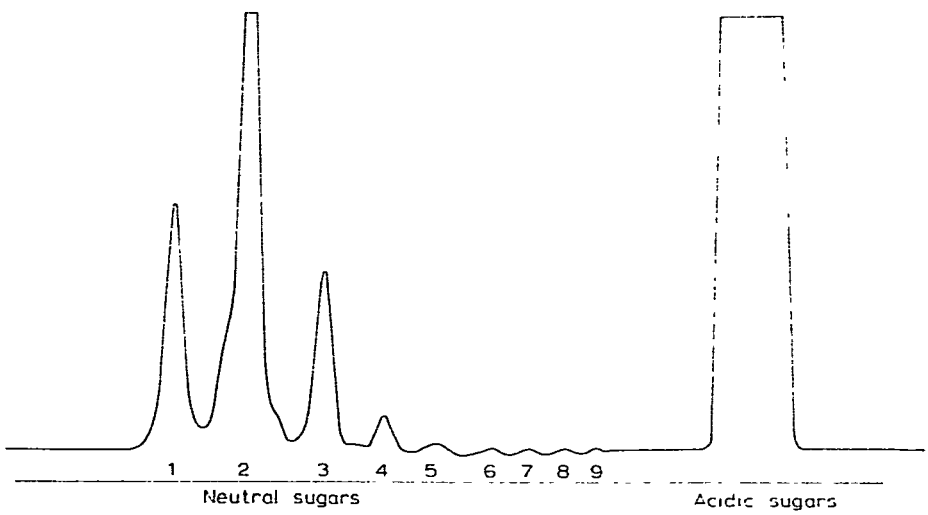

Fig. 1. Elution profile from a column of Bio-Gel P2 of the dialyzable products from the first enzymic hydrolysis. A large sample was applied in order to determine the relative proportions of neutral sugars.

*Xylan obtained from J. T. Baker, Deventer, Holland.

TABLE VI

DIALYZABLE PRODUCTS FROM THE ENZYMIC HYDROLYSES

Peak number	*Compound*	*First attack (%)*[a]	*Second attack (%)*[a]
1	Xylose	21.7	19.5
	Arabinose	0.5	0.3
2	Glucose	1.0	33.4
	Xylobiose	55.0	34.0
3	Xylotriose	5.1	N.d.[b]
	Ara-Xyl_2[c]	10.9	12.8
4	Xylotetraose	1.0	N.d.
	Ara-Xyl_3[c]	2.4	N.d.
5,6,7,8.9	Higher terms	2.4	N.d.

[a]In weight percent as determined by differential refractometry. [b]N.d., not detected. [c]Ara-Xyl_2 and Ara-Xyl_3 denote arabinosylxylobiose and arabinosylxylotriose respectively.

The dialyzable products from the first attack were separated on a column of Bio-Gel P2 (Fig. 1). Acidic sugars (55% by weight of the mixture) were obtained in the void volume[23], as confirmed by colorimetric monitoring[14]. Neutral sugars (45%) were fractionated according to their molecular weights, and the oligomers of d.p. 1–5 could be located[18] by plotting log $K_{av} = f$(d.p.). Each peak was then analyzed by paper chromatography, and the proportions of the different constituents are given in Table VI.

Xylose and arabinose present in the mixture were determined by g.l.c. following reduction and subsequent acetylation, without pre-hydrolysis of the products. Only 7% of the arabinose present in the dialyzable mixture existed as the monomer, and appeared in peak **1** (Fig. 1). Xylobiose was eluted in peak **2** with free glucose, and was identified by paper chromatography (R_x = 0.60 and 0.75 in solvent systems *A* and *B*, respectively). Glucose was identified in the mixture as described for arabinose and xylose. Xylotriose was eluted under peak **3** and had R_x = 0.31 and 0.53 in solvent systems *A* and *B*, respectively. Xylobiose and xylotriose were further characterized by the fragmentation in mass spectrometry of their permethylated derivatives. Peak **3** also contained a product (R_x = 0.78 and 0.84 in solvent systems *A* and *B*, respectively), which was isolated by preparative paper-chromatography. This compound behaved as a neutral trisaccharide on the column of Bio-Gel P2, and was comprised of arabinose and xylose. Methylation analysis revealed that all of the arabinose was present as terminal Ara*f* groups linked to O-3 of a xylobiose residue (Ara-Xyl_2). This structure was confirmed by the mass spectrum of the permethylated saccharide: *m/z* 335 (0.54), 303 (0.56), 235 (12.53), 175 (29), 143 (29.15), 115 (22.8), 101 (90.9), 88 (100), 45 (29.8%) corresponding to *O*-α-L-Ara*f*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl (Ara-Xyl_2), which has already been found in the enzymic hydrolysis products from arabinoxylans[24,25].

A tetrasaccharide (R_x = 0.42 and 0.63 in solvent systems *A* and *B*, respectively) eluted in the same manner with peak **4**, was characterized as *O*-α-L-Ara*f*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl (Ara-Xyl_3).

In peaks 4, 5, and 6, oligomers of higher d.p. were present in small amount and were tentatively identified as xylotetraose (Xyl_4) and arabinosylxylotetraose (Ara-Xyl_4) by their R_x values in paper chromatography, using a plot[4,25] of log $R_x/1 - R_x$ (R_x = 0.14 and 0.33 for the former and R_x = 0.15 and 0.35 for the latter in solvent systems *A* and *B*, respectively).

In the void volume of the Bio-Gel P2 column, a mixture of acidic oligosaccharides contained the aldotetraouronic (GlcA–Xyl_3) and aldopentaouronic (GlcA–Xyl_4) acids (R_x = 0.59 and 0.42, respectively, solvent system *B*). They were identified as previously described[26], by the mass spectra of their permethylated derivatives. In both acids, the 4-*O*-methylglucuronic acid residue was linked to O-2 of the non-reducing, terminal D-xylosyl group of xylotriose or xylotetraose, respectively. Two other acidic compounds, constituting ~5% were separated by paper chromatography (R_x 0.86 and 0.71 solvent *B*), and contained xylose and arabinose in addition to uronic acid. Considering the mode of cleavage of the enzyme, their structures should be complex pentasaccharides at least, but the small amounts available precluded characterization.

The second attack, performed on residue R1 under conditions the same as the first, led to 54% of hydrolysis, or a total of 82% for the two successive enzymic treatments. The mixture of dialyzable saccharides was resolved on column of Bio-Gel P2 (Fig. 2). Acidic sugars amounted to 80% by weight of the mixture. No neutral oligomers of d.p. >3 were present. Neutral sugars totalled 20% (Table VI) of the dialyzable product, as expected under the conditions of incubation for exhaustive

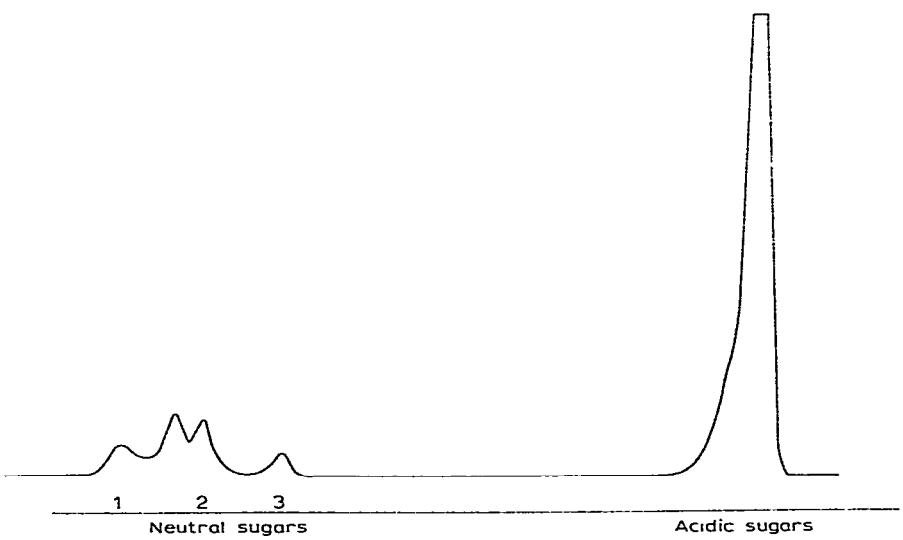

Fig. 2. Elution profile from a column of Bio-Gel P2 of the dialyzable products from the second enzymic hydrolysis.

hydrolysis. The final concentration of xylobiose was 1.3mM, whereas in the first hydrolysis it was 5mM, and higher oligomers (Table VI) were also present. This result suggests that xylobiose, when present at such a concentration in the medium, inhibits the action of the xylanase. Xylobiose was shown independently to act as a competitive inhibitor for the xylanase[27].

The arabinose in the dialysis mixture was present as the free sugar (1.5%), 20% as the trisaccharide Ara-Xyl_2, and ~80% in the acidic compounds. Paper chromatography (solvent *B*) afforded the aldotetraouronic acid (R_x 0.59), aldopentaouronic acid (R_x 0.42), and an acidic, complex saccharide containing arabinose (R_x = 0.86), in the approximate proportions 4:2:1.

DISCUSSION

The foregoing results show that exhaustive enzymic degradation of the arabinoglucuronoxylan from redwood converts 80% of the starting material into dialyzable degradation-products. Because both arabinosyl and uronic acid substituents are present in xylans from coniferous trees, these products are mixtures of different series of homo- and hetero-oligosaccharides of limited chain-length. All of the heterooligosaccharides substituted by arabinosyl or glucosyluronic residues carry the substituent on their nonreducing end. Among these, two hetero-oligosaccharides are characteristic of the mechanism of action of the xylanase. One is the trisaccharide *O*-α-L-Ara*f*-(1→3)-*O*-β-D-Xyl*p*-(1→4)-D-Xyl (Ara-Xyl_2) and the other is the tetrasaccharide aldotetraouronic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylotriose (GlcA-Xyl_3). Not found were 4-*O*-methyl-D-glucuronic acid, the aldobiouronic acid: 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose, the aldotriouronic acid: 2-*O*-(4-*O*-methyl-D-glucopyranosyluronic acid)-D-xylobiose, nor the neutral disaccharide α-L-Ara*f*-(1→3)-D-Xyl.

This fact agrees with the observation made by several authors[4,5,28,29] that the glycosidic linkage to the "left" of the branch-point is "more easily hydrolysed by the enzyme". However, this apparent preferential cleavage could actually be the normal consequence of the mode of action of the xylanase.

As xylobiose accumulates as an end product of hydrolysis and was shown to be a competitive inhibitor of the enzyme, and as Ara-Xyl_2 and GlcA-Xyl_3 are the smallest heterosaccharides resulting from exhaustive digestion, it is possible to conceive that a xylotriosyl group is required for hydrolysis to take place. In order to justify the presence of all oligomers, this trisaccharide component must have its xylosyl residue (I) *on the left* (normal formula conventions) and unsubstituted at O-2, the central residue (II) unsubstituted at O-2 and O-3, and residue III branched or unbranched. Cleavage takes place between residues II and III. It seems, therefore, that the binding site of the xylanase accommodates a xylobiose residue without taking into account the presence of a substituent at O-3 of the xylosyl residue (I). This hypothesis would agree with a shallow binding-cavity, as is also suggested by the attack observed on the compact structure of single crystals of xylan[30]. When the

substituent is at O-2 of xylosyl residue I, the two xylosidic linkages *on the right* of this branched residue are not accessible to the enzyme. Only the position of the substituent on O-2 is determinative, as we have already demonstrated[26] by reduction of the carboxyl group that the nature of the substituent is not involved in the hydrolysis.

A series of methyl arabinosides were tested with the xylanase in order to explain the presence of some arabinose in the hydrolysis products from the xylan. As yields of hydrolysis products were insignificant, it may be concluded that the arabinose was not released by the action of contaminating arabinosidases. This sugar could therefore arise by partial acid hydrolysis after treatment of the buffered solution of the dialyzable material with cation-exchange resin before gel filtration. According to Dekker's classification of xylanases[1], our enzyme falls then in the group of the "non-arabinose-liberating-endo-xylanases".

The recovery at the end of three successive hydrolyses by the enzyme of a residue accounting for 20% of the starting material, and where the degree of substitution with arabinosyl and glucosyluronic residues is considerably increased, indicates that the enzyme attacks those regions of the xylan backbone that are "more open" to access by the xylanase. This observation, in addition to the preceding conclusions on the mode of action of the enzyme, is of interest when considering the fine structure of the substrate.

The isolation of significant amounts of dialyzable, acidic oligosaccharides on the one hand and of a residue of attack containing an average of one acid group per 1.8 xylosyl residues on the other, constitutes evidence for the irregularity of the distribution of the uronic acid substituents on the xylosyl backbone. There must be hydrolyzable regions having uronic acid groups more than three xylosyl residues apart, and non-hydrolyzable blocks having a higher density of substituents. The same arguments apply to the arabinose residues, which must also be irregularly distributed. These results are in agreement with those of Shimizu[6], and show that the distribution mode of uronic acid side-chains is different for xylans from hardwoods[2,5] and softwoods.

The xylan from redwood is thus comprised of a xylan backbone on which some regions are highly substituted by arabinosyl and glucosyluronic groups, in either close vicinity of or contiguous with xylosyl residues. As it is established[19,30] that the carboxyl groups of xylans are often esterified, this disposition of the substituents must play a role in the macromolecular interactions existing in the plant cell-wall.

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