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THE STRUCTURES OF AN α-L-ARABINOSYL-β-D-XYLOTRIOSE AND -TETRAOSE OBTAINED FROM HEMICELLULOSE BY ENZYMIC HYDROLYSIS BY AN EXTRACELLULAR HEMICELLULASE PRODUCED BY *Cephalosporium sacchari*

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ABSTRACT

An extracellular endohemicellulase III from *Cephalosporium sacchari* degraded spear-grass (*Heteropogon contortus*) hemicellulose B to a series of mixed oligosaccharides. Two neutral oligosaccharides, $AraXyl_3$ and $AraXyl_4$, were isolated by preparative paper-chromatography, and characterised by enzymic and permethylation techniques.

INTRODUCTION

Arabinoxylose and D-xylose oligosaccharides having various degrees of polymerisation (d.p.) have been separated from hydrolysates of hemicellulose obtained by the action of hemicellulases[1–4]. Bishop and Whitaker[5] hydrolysed wheat-straw hemicellulose with a cellulase produced by the fungus *Myrothecium varrucaria* to give a series of oligosaccharides of d.p. 3–7. One of the oligosaccharides, $AraXyl_2$, was characterised to be 2^2-α-L-arabinofuranosyl-D-xylobiose[6]. Goldschmid and Perlin[7] degraded wheat hemicellulose with an enzyme produced by *Streptomyces QMB 814* and isolated arabinoxylose tetrasaccharide, $AraXyl_3$, which was characterised as 3^2-α-L-arabinofuranosyl-D-xylotriose. Takenishi and Tsujisaka[4] hydrolysed a rice-straw arabinoxylan with hemicellulase I and isolated $AraXyl_2$ and $AraXyl_3$. The arabinoxylose oligosaccharides were characterised as 2^1-α-L-arabinofuranosyl-D-xylobiose and 3^1-α-L-arabinofuranosyl-D-xylotriose, respectively. Dekker and Richards[8] degraded hemicellulose B with hemicellulase II (Hc II) produced by *Ceratocystic paradoxa* and isolated $AraXyl_2$ and $AraXyl_3$. These were shown to be 2^2-α-L-arabinofuranosyl- and 3^3-α-L-arabinofuranosyl-D-xylotriose, respectively[8].

This paper reports the characterisation of two L-arabinoxylo tetrasaccharides isolated from the hydrolysates of hemicellulose B, which had been degraded by hemicellulase III, obtained from *Cephalosporium sacchari*[9].

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EXPERIMENTAL

Materials. — Authentic samples of L-arabino-D-xylose and D-xylose oligosaccharides of d.p. 3 and 2–5, respectively, were provided by Dr. R. F. H. Dekker, and authentic arabinosylxylose oligosaccharides of d.p. 4–5 by Dr. R. J. Beveridge. Hc II and permethylated hemicellulose B were provided by Dr. R. F. H. Dekker and Professor G. N. Richards. Hemicellulase III and IV (Hc III and Hc IV) were purified as previously described[9]. Commercial silica gel, thin-layer-chromatographic plates (0.25 mm thickness) were obtained from E. Merck (Darmstadt, West Germany). All chemicals used in this work were either analytical reagent (AR) grade, or purified before use. Dimethylsulphinyl anion was prepared by the method of Garegg *et al.*[10].

General methods. — Oligosaccharide solutions were concentrated at 40° (2.7 KPa) in a rotory evaporator. Concentration of arabinosylxylose and D-xylose oligosaccharides was determined by the phenol–sulphuric acid method[11] using L-arabinose and D-xylose as standards; and that of reducing sugars by the Nelson–Somogyi method[12].

Chromatography. — Samples to be chromatographed were de-ionised with mixed Amberlite resin IR-120 (H^+) and IR-45 (OH^-) for 30 min, and then concentrated. Qualitative paper chromatography (p.c.) was carried out on Whatman No. 1 paper, and preparative p.c. on Whatman No. 17 paper. The solvents (all v/v) used were: (*A*) 10:4:3 ethyl acetate–pyridine–water; (*B*) 3:1:1 1-butanol–ethanol–water; (*C*) 6:2:5 1-butanol–pyridine–water for sugar and oligosaccharide separation; (*D*) 4:1:5 (upper layer) 1-butanol–ethanol–water; and (*E*) 23:2 2-butanone–water for methylated sugar separation.

For t.l.c., the plates were activated for 30 min at 100°, and allowed to cool before samples were applied. The solvent systems employed were (*E*) as in p.c. for methylated sugars, and (*F*) 8:2:1 ethyl acetate–pyridine–water for oligosaccharide separation.

The spray reagents were: (*a*) Alkaline silver nitrate[13], (*b*) *p*-anisidine hydrochloride[14], and (*c*) permanganate–sodium periodate[15], for detection of sugars on duplicate p.c.; and reagents (*a*) and (*c*) for detection of alditols. The sugars separated by t.l.c. were detected by spraying with 50% sulphuric acid or saturated ammonium sulphate, followed by heating for 20 min at 100 and 140°, respectively. Methylated sugars were detected in t.l.c. with spray (*b*). Me_4Glc refers to 2,3,4,6-tetra-*O*-methyl-D-glucose.

Enzymic degradation of hemicellulose B and oligosaccharides. A mixture containing 0.5% hemicellulose B (400 mL) in 0.05M acetate buffer (pH 6.0), Hc III (8.0 mL), and toluene (0.04 mL) was incubated for 72 h at 37°, and then heated for 10 min at 100° to deactivate Hc III. Unhydrolysed hemicellulose B was precipitated by addition of ethanol (3 vol.), followed by centrifugation at 16 700*g* for 1 h. The pH of the solution was adjusted with 0.05M sodium hydroxide to 7·2, and the solution concentrated and made up to 2.0 mL.

Enzymic hydrolysis of 2 mmol of reduced or unreduced $AraXyl_4$ in 0.05M

TABLE I

PRODUCTS[a] OF HYDROLYSIS OF REDUCED AND UNREDUCED $AraXyl_4$ BY Hc II, Hc III, AND Hc IV

Enzyme	*Sub-strate*[b]	*Time (h)*	*Spray reagent*[c]	*Sugar component*							
				Ara	*Xylitol*	*Xyl*	*$AraXyl_2$*	*Xyl_2*	*$AraXyl_3$*	*Xyl_3*	*$AraXyl_4$*
Hc II	R	8	I		4		2	[d]	10		[d]
Hc II	R	8	II				1		10		
Hc III	R	2	I	1	3				10		10
Hc III	R	2	II	1					10		7
Hc III	U	2	I	3		10		3	8		10
Hc III	U	2	II	3		10		3	8		10
Hc IV	R	10	I				2	[d]			10
Hc IV	R	10	II				1				10
Hc IV	U	10	I				2	2			10
Hc IV	U	10	II				2	2			10

[a]Identified by p.c. in Solvent A; visual observation based on a scale of 10. [b]R, reduced; U, unreduced. [c]I, alkaline silver nitrate; II, *p*-anisidine hydrochloride. [d]Trace.

TABLE II

SPECIFIC ROTATION OF PURIFIED OLIGOSACCHARIDES PRODUCED BY THE ACTION OF Hc III ON HEMICELLULOSE B

Oligosaccharide	*Conc. ($g \cdot cm^{-3}$)*	*$[\alpha]_D^{25}$ obs. (degrees)*	*$[\alpha]_D^{25}$ reported[a] (degrees)*
$AraXyl_2$[b]			−17, −15
Xyl_2	0.01	−25.4	−25
$AraXyl_3$	0.01	−69.0	−74
Xyl_3	0.03	−59.2	−60
$AraXyl_4$	0.03	−72.7	−75
Xyl_4	0.03	−61.2	−61

[a]Refs. 16, 17, and 18. [b]The yield of $AraXyl_2$ was too small (8.0 mg) for the specific rotation to be determined.

sodium acetate buffer (pH 6.0) was carried out by incubating a solution (0.20–0.40 mL) of the oligosaccharide with Hc II or Hc III (0.10 mL), or Hc IV (0.40 mL), for 8, 2, and 10 h, respectively, and then heating to 100° for 3 min to deactivate the enzyme. The hydrolysate was de-ionised, and then developed by p.c. in solvent *A*; spray reagents *a* and *b* were used to detect the products. Table I shows products of hydrolysis of $AraXyl_4$ by Hc II, Hc III, and Hc IV.

Isolation and characterisation of L-*arabinosyl*-D-*xyloses and* D-*xylose. — Oligosaccharides from enzymic hydrolysis of hemicellulose B.* The oligosaccharides produced by enzymic degradation of hemicellulose B were separated by preparative paper

TABLE III

PAPER CHROMATOGRAPHY DATA[a] OF THE PURIFIED OLIGOSACCHARIDES

Oligosaccharides	*Solvents*[b]		
	A	B	C
$AraXyl_2$	0.75(0.78)	0.53	0.84(0.83)
Xyl_2	0.63(0.60)	0.49(0.53)	0.68(0.68)
$AraXyl_3$	0.43(0.39)	0.25(0.29)	0.56(0.50)
Xyl_3	0.32(0.31)	0.22(0.28)	0.40(0.41)
$AraXyl_4$	0.26(0.22)	0.11(0.13)	0.31(0.32)
Xyl_4	0.15(0.15)	0.06	0.18(0.21)

[a]R_{Xyl}. [b]Literature[16,17] values in parentheses.

chromatography in solvent *A*. The chromatographic paper was loaded with 300 mg and developed for 9 h by descending paper-chromatography. The various components were located, strips of paper cut, and eluted separately with 3:1 (v/v) ethanol–water. Each solution was concentrated, and the residue further resolved by descending paper-chromatography in solvent *B*. Xylose, arabinose, galactose, glucose, xylobiose, and L-arabinosyl-D-xylose trisaccharides were purified by running the chromatograms for 16 h. The L-arabinosyl-D-xylose tetrasaccharide was purified by running the chromatogram for 24 h, whereas the D-xylose trisaccharide and higher oligosaccharides were purified by running chromatograms for 60 h.

The specific optical rotation of the isolated hydrolysates was measured at 25 ±2° with a Perkin-Elmer Model 141 Polarimeter (Table II). Partial acid hydrolysis of the hydrolysates was carried out with 5mM sulphuric acid for 30 min at 100°, and total acid hydrolysis with 0.5M sulphuric acid for 4 h at 100°. The hydrolysates were analysed by p.c. in solvents *A*, *B*, and *C*, or t.l.c. in solvent *F*; t.l.c. plates were scanned with a Kipp and Zonen densitometer DD_2. The results are shown in Table III.

Reduction of L-*arabinosyl*-D-*xylose and* D-*xylose oligosaccharides with sodium borohydride*. — A sample of oligosaccharide (3.0 mg) was dissolved in distilled water (2.0 mL); sodium borohydride (25 mg) was added, and the solution was kept for 3 h at 25°. The temperature of the solution was lowered to 10°, and excess sodium borohydride destroyed by dropwise addition of 0.5M acetic acid. The solution was de-ionised and filtered through sintered glass, and the borate ions were removed as volatile methyl borate by codistillation with methanol (4 × 50 mL). The residue was dried in the presence of phosphorus pentaoxide, in a vacuum oven, for 20 h at 60°.

Permethylation. — Permethylation was performed by the method of Hakomori[19] as modified by Garegg *et al.*[20]. The oligosaccharides (reduced $AraXyl_3$ and $AraXyl_4$), Xyl_2, and cellobiose (1.0–5.0 mg) were dried at 60° for 20 h in a vacuum oven in the presence of phosphorus pentaoxide before methylation. Hemicellulose B (4.0–7.0 mg) was passed through a 210-μm-mesh sieve, and similarly dried before methylation.

The methylated products were hydrolysed with M sulphuric acid, and the solution made neutral with barium carbonate, and then de-ionised. The products were analysed by p.c. in solvent *D* and *E*, or by t.l.c. in solvent *E*, and the spots scanned with a Kipp and Zonen densitometer DD_2.

The hydrolysate of methylated Xyl_2 was divided into two parts: (a) One half was de-ionised, and (b) the other half was extracted with hexane, the aqueous layer de-ionised, and both phases were combined. This was done to observe the effect of resins on fully methylated sugars. T.l.c. was carried out separately on (a) and (b) in solvent *E*, and the spots were scanned as just described.

The neutral components obtained from the hydrolysate of methylated hemicellulose B were used, as reference standards, for identifying the components formed by the hydrolysis of the methylated oligosaccharides, since the structure of speargrass hemicellulose B is known[20].

RESULTS AND DISCUSSION

As assayed by the Nelson method after 72 h, enzymic hydrolysis of hemicellulose B gave 25% of the reducing sugars. Oligosaccharides of d.p. >5 were not separated.

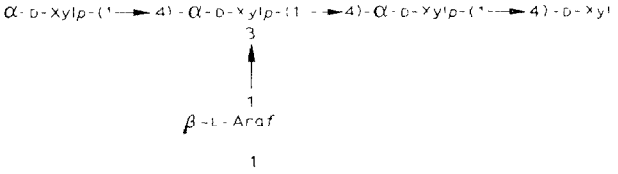
The isolated oligosaccharides $AraXyl_3$ and $AraXyl_4$ co-chromatographed, in solvents *A*, *B*, and *C*, with corresponding authentic samples. Partial acid hydrolysis of $AraXyl_2$ gave arabinose and a product that co-chromatographed, in solvents *A*, *B*, and *C* (p.c.), and *F* (t.l.c.) with authentic Xyl_2. Total acid hydrolysis gave arabinose and xylose only, in the ratio 1.0:1.9. $AraXyl_3$ gave arabinose, and a product that co-chromatographed in solvents *A*, *B*, and *C* (p.c.), and solvent *F* (t.l.c.) with authentic D-xylose. The ratio of L-arabinose to D-xylose was 1.0:2.9. Partial acid hydrolysis of $AraXyl_4$ gave arabinose, and a product that co-chromatographed with authentic Xyl_4 in the aforementioned four solvents. The ratio of L-arabinose to D-xylose was 1.0:3.7. Total acid hydrolysis of xylose oligosaccharides gave only xylose as the product. Degradation products of hemicellulose B by Hc II, Hc III, and Hc IV have been reported earlier[8,9]. The results obtained from preparative paper chromatography revealed that Xyl_2 (267.0 mg) was the major component, followed by $AraXyl_3$ (96.8 mg). The yield of Xyl_3, Xyl_4, and $AraXyl_4$ were 60.0, 66.0, and 67.6 mg, respectively. Hc II hydrolysed reduced $AraXyl_4$ into xylitol, $AraXyl_2$, and $AraXyl_3$. Both $AraXyl_2$ and $AraXyl_3$ were detected by alkaline silver nitrate and *p*-anisidine hydrochloride (Table I), showing that the α-L-arabinofuranosyl group was linked either to the third or fourth D-xylose unit of $AraXyl_4$. Hc III hydrolysed reduced $AraXyl_4$ into L-arabinose, xylitol, and $AraXyl_3$ (Table I). L-Arabinose and $AraXyl_3$ were detected by both alkaline silver nitrate and *p*-anisidine hydrochloride. This again eliminates the possibility of a 4^1-α-L-arabinofuranosyl-D-xylotetraose structure for $AraXyl_4$. Hc IV hydrolysed reduced $AraXyl_4$ into $AraXyl_2$, which was detected by both alkaline silver nitrate and *p*-anisidine hydrochloride, and reduced xylitol, which was detected by alkaline silver nitrate but not *p*-anisidine

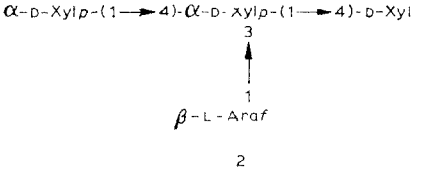
hydrochloride (Table I). These results show that the structure of $AraXyl_4$ is either that of a 4^3- or 4^4-α-L-arabinofuranosyl-β-D-xylotetraose.

Hydrolysis of permethylated $AraXyl_4$ produced 2-*O*-methyl- and 2,3-di-*O*-methyl-D-xylose, and tri-*O*-methylpentoses that co-chromatographed in solvents *D* and *E* with authentic samples supplied by Dekker and Richards[8]. Thus, on the basis of the permethylation results, the structure of $AraXyl_4$ is either 4^2- or 4^3-α-L-arabinofuranosyl-β-D-xylotetraose (**1**), but the results obtained by Hc II and Hc IV degradation, however, rule out the former structure. The ratios of 2-*O*-methyl-D-xylose to 2,3-di-*O*-methyl-D-xylose to tri-*O*-methylpentoses was found to be 1.00:0.97:0.37. The results obtained by hydrolysis of permethylated Xyl_2 (with and without deionisation) showed that 2,3,4-tri-*O*-methyl-D-xylose and 2,3,5-tri-*O*-methyl-L-arabinose were selectively absorbed by the resin(s), which may be explained by glycosylamine formation[21].

Hydrolysis of reduced, permethylated $AraXyl_3$ gave 2-*O*-methyl-D-xylose and a tri-*O*-methylpentose. The absence of 2,3-di-*O*-methyl-D-xylose confirmed that $AraXyl_3$ is 3^2-α-L-arabinofuranosyl-β-D-xylotriose (**2**), thus the same compound as that characterised by Goldschmid and Perlin[7], but different from 3^3-α-L-arabinofuranosyl-[8] and 3^1-α-L-arabinofuranosyl-β-D-xylotriose[4].

The absence of $AraXyl_2$ and Xyl_3 from the hydrolysis products of $AraXyl_4$ by Hc III (Table I), and the equivalent proportions of L-arabinose and Xyl_2, together with the hydrolysis products of reduced $AraXyl_4$ by Hc III degradation, strongly





suggests that Hc III attacks $AraXyl_4$ at the reducing end to produce **2**. Then Hc III degrades $AraXyl_3$ to give L-arabinose, but does not release Xyl_3 until it is hydrolysed into Xyl_2 and D-xylose, as shown in reaction *1*. The mechanism of hydrolysis of

$$\begin{array}{l} \overset{\text{slow}}{} \quad \overset{\text{fast}}{} \\ E + AraXyl_4 \rightarrow EAraXyl_4 \rightarrow E + Xyl + AraXyl_3 \\ \overset{\text{slow}}{} \quad \overset{\text{slow}}{} \quad \overset{\text{fast}}{} \\ \rightarrow EAraXyl_3 \rightarrow Ara + EXyl_3 \rightarrow Xyl_2 + Xyl + E \end{array} \qquad (1)$$

oligosaccharides by Hc II has earlier been explained[8]. The hydrolysis of $AraXyl_4$ by Hc IV can be expressed by reaction *2*.

$$E + AraXyl_4 \xrightarrow{\text{slow}} EAraXyl_4 \xrightarrow{\text{fast}} E + AraXyl_2 + Xyl_2 \qquad (2)$$

The proposed mechanism (*1*) of Hc III hydrolysis of hemicellulose B explains the low production of $AraXyl_2$ and the high production of $AraXyl_3$, reaction *3*

$$E + AraXyl_4 \xrightarrow{\text{slow}} EAraXyl_4 \xrightarrow{\text{slow}} AraXyl_2 + Xyl_2 + E \qquad (3)$$

taking place but to a negligible extent. The large proportion of Xyl_2 detained is most probably due to preferential cleavage in branched regions.

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