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STRUCTURAL INVESTIGATION OF THE ARABINOXYLOGLUCAN FROM *Nicotiana tabacum*

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ABSTRACT

The structure of tobacco arabinoxyloglucan has been further studied by methylation analysis, by ^{1}H-, and ^{13}C-n.m.r., and by f.d. mass spectrometry, after complete digestion by cellulase. The results showed the polysaccharide molecule to be composed of two parts; a hexasaccharide component ($AraXyl_2Glc_3$, **1**) and an unsubstituted (1→4)-β-D-glucan region (4-*O*-linked glucosyl residues) in the molar ratio of ~1:2. Some heterogeneities of this structure in the arabinofuranosyl subgroup were also found.

INTRODUCTION

Xyloglucans are one of the major, matrix polysaccharides of the cell walls of dicotyledonous plants. The xyloglucans from various sources have been shown to have essentially the same heptasaccharide repeating-unit, consisting of cellotetraosyl residues to which are attached three mono-xylosyl side-chains, with or without galactosyl or fucosylgalactosyl substituents[1–4]. Recently, monocotyledonous plants have also been reported to contain a similar xyloglucan structure as one of their cell-wall components[5,6].

Our previous papers[7,8] have shown, however, that tobacco cell-wall xyloglucan seems to have a different structure: cellulase digestion of the arabinose-free xyloglucan prepared by mild acid hydrolysis gave essentially a pentasaccharide that contained one cellotriosyl and two substitutional xylosyl residues, and unsubstituted (1→4)-β-D-glucan fragments.

This paper reports complete, cellulase digestion of the intact tobacco arabinoxyloglucan to give a series of oligosaccharides. Chemical and spectrometric investigations of the resultant oligosaccharides and the intact polysaccharide demonstrated the structure of the latter.

RESULTS AND DISCUSSION

Acid hydrolysis of tobacco arabinoxyloglucan showed the presence of arabinose,

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xylose, and glucose in the molar ratios of 11.7:26.3:62.0, together with traces of galactose and mannose. The optical rotations of the sugars isolated from the hydrolyzate showed that the arabinose had the L configuration, whereas the xylose and glucose had the D. As described in our previous reports[7,8], the molar proportions of 2,3,6-tri-*O*-methyl-D-glucose and 2,3-di-*O*-methyl-D-glucose from methylation analysis of intact arabinoxyloglucan showed that ~40% of the (1→4)-β-D-glucan backbone was substituted at O-6.

Tobacco arabinoxyloglucan was completely digested by the cellulase of *Trichoderma viride*. The resultant mixture was fractionated on a column of Bio-Gel P-2. The elution profile (Fig. 1) showed the presence of 9 components, and each fraction (designated 1–8) was rechromatographed on the same column. Each fraction was then examined by t.l.c.

Fraction 6 showed two spots (A and B) in t.l.c. These two components, 6-A (major, having the higher R_{Glc} value) and 6-B, were collected by preparative p.c.

Fraction 7 showed three spots in t.l.c. The major component, having the highest R_{Glc} value, was also collected by preparative p.c. Fraction 1, corresponding to the region for monosaccharides, contained mainly glucose (96.6%) and a trace of galactose (3.4%). The yields (after the foregoing purification procedure), and R_{Glc} and M_{Glc} values of these oligosaccharides are summarized in Table I.

Characterization of the oligosaccharides. — The neutral-sugar compositions of the oligosaccharides, and the results of methylation analyses, before and after reduction by sodium borohydride, are summarized in Tables II and III, respectively.

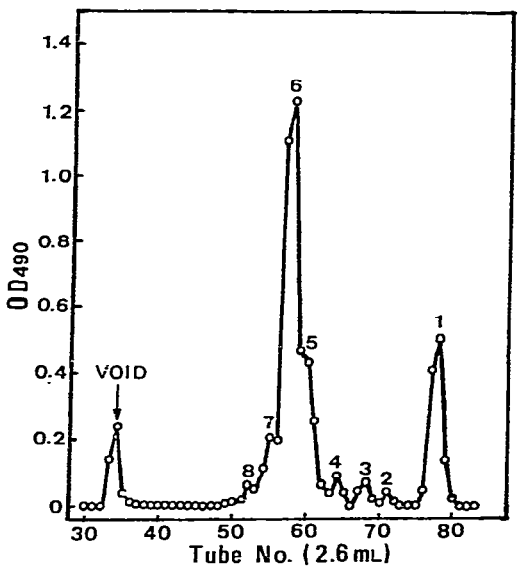


Fig. 1. Gel-filtration profile of the arabinoxyloglucan cellulase-digest on Bio-Gel P-2. Column, Bio-Gel P-2 (<400 mesh), 1.5 × 150 cm; water flow rate, 10 mL/h; column temperature, 50°; monitor, phenol–sulfuric acid method.

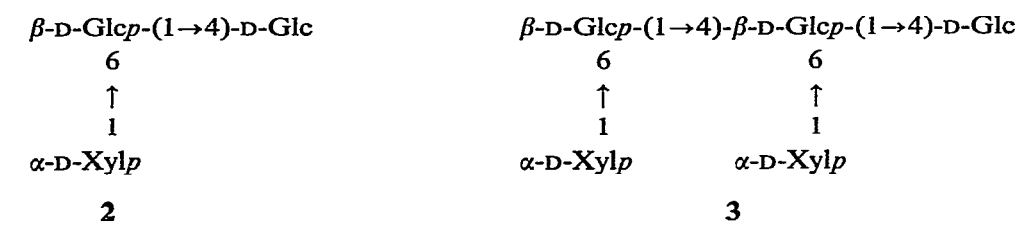
TABLE I

YIELDS AND MOBILITIES IN T.L.C. AND ZONE ELECTROPHORESIS OF THE OLIGOSACCHARIDES FROM ARABINOXYLOGLUCAN

Oligosaccharide	*Yields*		R_{Glc}-1[c]	R_{Glc}-2[d]	M_{Glc}[e]
	mg[a]	*weight %*[b]			
1	75.3	23.8	1.0	1.0	1.0
2	trace	trace	0.89[f], 0.75	0.74[f]	0.34[f]
3 (2)	4.7	1.5	0.71	0.68	
4	4.9	1.5	0.76, 0.58	0.81, 0.54, 0.45	
5 (3)	39.7	12.5	0.41	0.28	0.23
6-A (1)	163.3	51.6	0.49	0.42	0.22
6-B (4)	9.1	2.9	0.33	0.30	
7 (5)	15.5	4.9	0.56	0.55	
8	3.9	1.3	0.24	0.27, 0.15	
void	44.4				

[a]Dry weights of the Bio-Gel P-2 fractions. The data for 5, 6-A, 6-B, and 7 were the yields after purification by rechromatography and preparative p.c. [b]Calculated against the total weight of the cellulase-digested products, except void-volume material. [c,d]Mobilities of the components of each fraction relative to D-glucose in t.l.c. developed twice under conditions *A* and *B*, respectively. [e]Mobilities relative to 2,3,4,6-tetra-*O*-methyl-D-glucose in zone electrophoresis. [f]These data agreed with those for cellobiose.

Chemical and n.m.r. data for fractions 3 and 5 (12.5 wt.%) were in good agreement with those expected for the trisaccharide $XylGlc_2$ (**2**) and the pentasaccharide Xyl_2Glc_3 (**3**), respectively, as previously reported[8].



2 **3**

Fraction 6-A was the major component of the cellulase-digestion products (51.6 wt.%). Its homogeneity was confirmed by zone-electrophoresis and by t.l.c. under two different sets of conditions. This fraction contained arabinose, xylose, and glucose in the molar ratio of ~1:2:3 (Table II).

As shown in Table III, methylation analysis of 6-A showed the presence of one terminal arabinofuranosyl (2,3,5-tri-*O*-methyl-L-arabinose) and one 2-substituted xylopyranosyl (3,4-di-*O*-methyl-D-xylose) residue.

TABLE II

$[\alpha]_D^{23}$ VALUES AND NEUTRAL-SUGAR COMPOSITIONS OF ARABINOXYLOGLUCAN AND DERIVED OLIGOSACCHARIDES

Carbohydrates	$[\alpha]_D^{23}$	*Neutral-sugar compositions (mole %)*		
		Arabinose	*Xylose*	*Glucose*
Arabinoxyloglucan	+35.0° (*c* 0.5, water)	11.7	26.3	62.0
3 (2)			30.3	69.7
5 (3)	+82.2° (*c* 0.72, water)		36.2	63.8
6-A (1)	+50.5° (*c* 1.66, water)	16.8	30.9	52.3
6-B (4)			45.4	54.6
7 (5)		27.5	29.2	43.3

TABLE III

METHYLATION ANALYSES OF ARABINOXYLOGLUCAN AND DERIVED OLIGOSACCHARIDES, BEFORE AND AFTER REDUCTION BY SODIUM BOROHYDRIDE

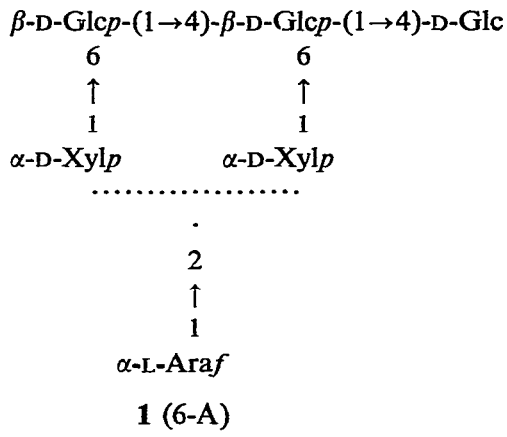
Methylated sugars[a]	T[b]	*Mole %*									
		AXG[c]	*3*	*5*[c]	*5-R*[c]	*6-A*	*6-A-R*	*6-B*	*6-B-R*	*7*	*7-R*
2,3,5-Ara	0.74	10.1				18.4	15.9			31.9	18.0
2,3,4-Xyl	0.80	14.9	34.6	47.2	44.7	17.8	23.3	32.8	31.5		
3,4-Xyl	0.95	14.6				14.4	14.1	17.0	15.0	26.3	35.6
2,3,6-Glc	1.18	36.9	28.3	12.7		12.9		15.0		10.6	
2,3,4-Glc	1.21		37.1	21.9	26.8	18.5	19.1	18.1	20.1	17.1	22.0
2,3-Glc	1.40	23.5		18.2	23.3	17.9	12.3	17.1	19.6	14.1	21.5
1,2,3,5,6-Glc-OH	0.79				9.7[d]		15.3		13.8[d]		2.9[d]

[a]2,3,5-Ara = 2,3,5-tri-*O*-methylarabinose and so on, 1,2,3,5,6-penta-*O*-methylglucitol. [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol on an OV-101 glass capillary column (40 m). Program; from 150°, 3° per min to 210°. [c]AXG = intact arabinoxyloglucan, 5 = the fraction 5,5-R = the sodium borohydride-reduced fraction 5. [d]Part of this volatile ether and derivatives were probably lost during isolation.

However, as our preceeding report[8] revealed that the Xyl_2Glc_3 structure was the basic unit in tobacco xyloglucan, it appeared probable that the arabinofuranosyl residue of 6-A was linked to O-2 of one of the two xylosyl residues of the Xyl_2Glc_3 structure.

As shown in Table IV, the ^{1}H-n.m.r. spectrum of 6-A indicated four signals for anomeric protons; these were observed at δ 5.16 ($J_{1,2}$ 1.5 Hz), 5.04 ($J_{1,2}$ 3.7 Hz), 4.91 ($J_{1,2}$ 2.7 Hz), and 4.52 (2 H, $J_{1,2}$ 7.1 Hz), and two signals were observed for anomeric protons of reducing groups, at δ 5.21 ($J_{1,2}$ 2.9 Hz) and 4.64 ($J_{1,2}$ 7.8 Hz). This result suggested that 6-A had the same configurations of the Xyl_2Glc_3 groups

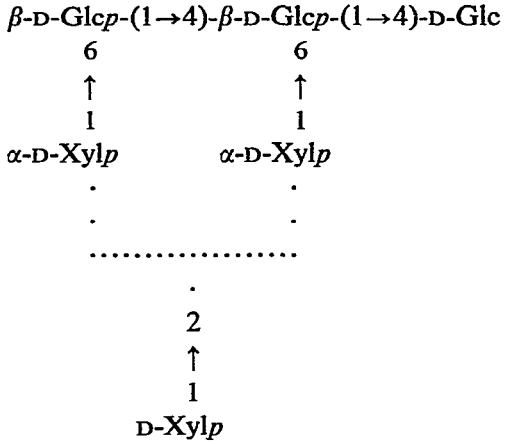
and that one of the two xylosyl residues was substituted with one arabinofuranosyl residue. The assignment of anomeric configuration of the furanosidic sugar residue from the chemical-shift and $J_{1,2}$ coupling-constant data[9,10] is difficult. However, as shown in Table V, in the ^{13}C-n.m.r. spectrum of 6-A, the C-1 arabinofuranosyl signal was well separated from the ^{13}C signals of the other residues. The chemical shift (100.3 p.p.m.) clearly indicated the α configuration of this residue[9,10]. From the foregoing results, although the methylation analysis and n.m.r. data of 6-A provided no proof for the absolute point of attachment of the arabinosyl residues, the structure of 6-A ($AraXyl_2Glc_3$, **1**) was established as follows:



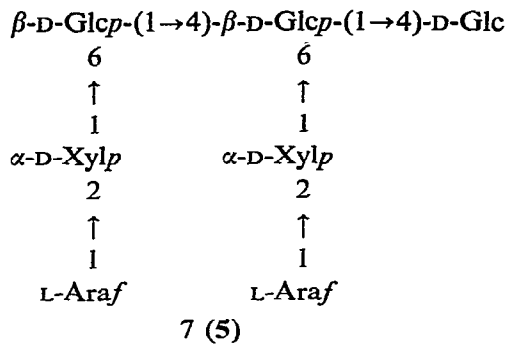
1 (6-A)

Dotted lines indicate that the arabinofuranosyl residue is attached to O-2 of either of the two xylosyl residues.

Similar considerations led to the tentative structures proposed for the minor components 6-B and 7 as follows:



6-B (**4**)



7 (5)

TABLE IV

^{1}H-N.M.R. DATA FOR ARABINOXYLOGLUCAN AND RELATED OLIGOSACCHARIDES

Carbohydrate	*Chemical shifts[a] (coupling constants in Hz and ratio of integrals in parentheses)*			
	Araf[b]	*Xylp*	*Glcp*	*Glcp (reducing terminal)*
Arabinoxyloglucan	5.09(1.5)	5.05(3.9) 4.92(3.0)	~4.5(7–8)	
AraXyl$_2$Glc$_3$ (6-A, **1**)	5.16(1.7)	5.04(3.7) 4.91 (2.7)	4.52(7.1, 2 H)	5.21(2.9) 4.64(7.8)
Xyl$_2$Glc$_3$ (5, **3**)		4.91(3.2, 2 H)	4.53(7.6, 2 H)	5.20(4.0) 4.64(7.8)
Cellotriose			4.52(7.8) 4.49(7.3)	5.21(3.7) 4.64(7.8)

[a]Chemical shifts were measured in p.p.m. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate as internal standard. [b]Methyl α-L-arabinofuranoside, 4.98(3.3); methyl β-L-arabinofuranoside, 4.88(< 1.0).

TABLE V

^{13}C-N.M.R. DATA OF ARABINOXYLOGLUCAN AND RELATED OLIGOSACCHARIDES

Carbohydrates	*Chemical shifts[a] (^{13}C–^{1}H coupling constants in Hz in parentheses)*									
	C-1				*C-6*		*C-5*		*C-4*	
	Araf[b]	*Xylp*	*Glcp*	*Glc-R[c]*	*Glcp*	*Glc-R*	*Xylp*	*Araf*	*Glcp*	*Glc-R*
Arabinoxyloglucan	111.2	100.8 100.6	~104.2		~68.5 ~62.3		X63.6 X63.3		~80.6	
AraXyl$_2$Glc$_3$ (6-A, **1**)	110.3 (174)	99.7 (170) 99.4 (170)	104.0 (164) 103.6 (164)	96.8 (159) 93.0 (168)	67.5 67.1	61.3	62.3	62.3	80.7 70.6	80.7
Xyl$_2$Glc$_3$ (5, **3**)		99.6 99.0	103.5 103.1	96.4 92.6	66.8	60.7	62.2 60.0		80.1 70.2	79.9
Cellotriose			103.4 103.2	96.6 92.7	61.8 61.1	61.1			79.3 72.1	79.3

[a]Chemical shifts measured in p.p.m. downfield from Me_4Si as external standard. [b]Methyl α-L-arabinofuranoside, 109.5 p.p.m.; methyl β-L-arabinofuranoside, 103.3 p.p.m. [c]Glucosyl residue located at reducing terminal.

The absolute point of attachment and the anomeric configuration of the D-xylopyranosyl residue in component 6-B, and the anomeric configurations of the L-arabinofuranosyl residue in component 7, are not known.

The structures of 6-B and 7 were also Xyl_2Glc_3, but their sub-groups were different from 6-A. For 6-B, xylopyranosyl residues were attached to O-2 of one of two xylopyranosyl residues of Xyl_2Glc_3. In component 7, both of the two xylosyl residues of Xyl_2Glc_3 were substituted at O-2 with arabinofuranosyl residues. These oligosaccharides may be considered to be microheterogeneity products of the sub-group $AraXyl_2Glc_3$.

Characterization of the intact arabinoxyloglucan. — The ^{1}H-n.m.r. spectrum of the polysaccharide was relatively poorly defined in the anomeric region, and did not seem useful for structural investigation. However, by comparing with the data of the corresponding oligomers ($AraXyl_2Glc_3$, Xyl_2Glc_3, and cellotriose), the signals of anomeric protons of the polysaccharide were assigned as shown in Table IV.

In the ^{13}C-n.m.r. spectra, the anomeric-carbon signals, C-6 of the hexose residues and C-5 of the pentose residues, were well separated from signals of the other carbon atoms, and could thus be readily assigned using literature data[11–16]. *O*-Glycosylated carbon atoms (C-4 and C-6 of glucosyl, and C-2 of xylosyl residues) could also be assigned by comparing their displacements with those of appropriate model compounds. Thus the *O*-glycosylated C-4 of glucosyl residues was readily assigned by comparing the data with those for cellobiose and cell-oligosaccharides[11,12]. As the *O*-glycosylated C-6 signals of the glucosyl residues were in good agreement with those of isomaltose [α-(1→6)-linked glucobiose], the signals were assignable[11]. The remaining *O*-glycosylated ring-carbon signal at 79.3 p.p.m. (see Fig. 2) may be assigned to C-2 of the xylosyl residue which was attached by an arabinosyl residue. The spectrum of $AraXyl_2Glc_3$ (**1**) and partial assignments of the signals are shown in Fig. 2. The ^{1}H- and ^{13}C-n.m.r. data for the oligosaccharide were compatible with their structures proposed from the results of chemical analyses.

Chemical investigations on the polysaccharide (see Tables I and II) suggested

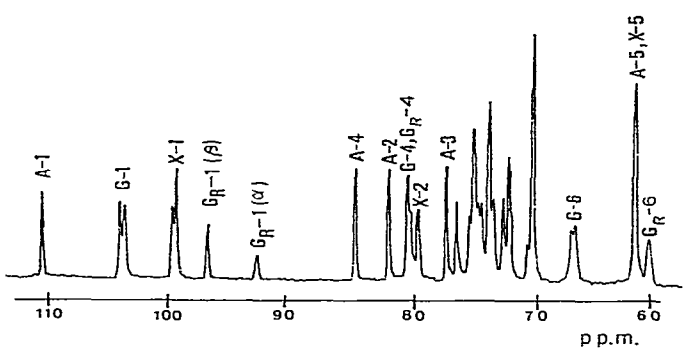


Fig. 2. ^{13}C-n.m.r. spectrum of 6-A ($AraXyl_2Glc_3$) in D_2O at 23°, and partial assignments (45° pulse, repetition time 1 sec, 5000 accumulations). Signals designated by G, G_R, X, and A refer to those of glucosyl, glucosyl (reducing terminal), xylosyl, and arabinosyl residues, respectively.

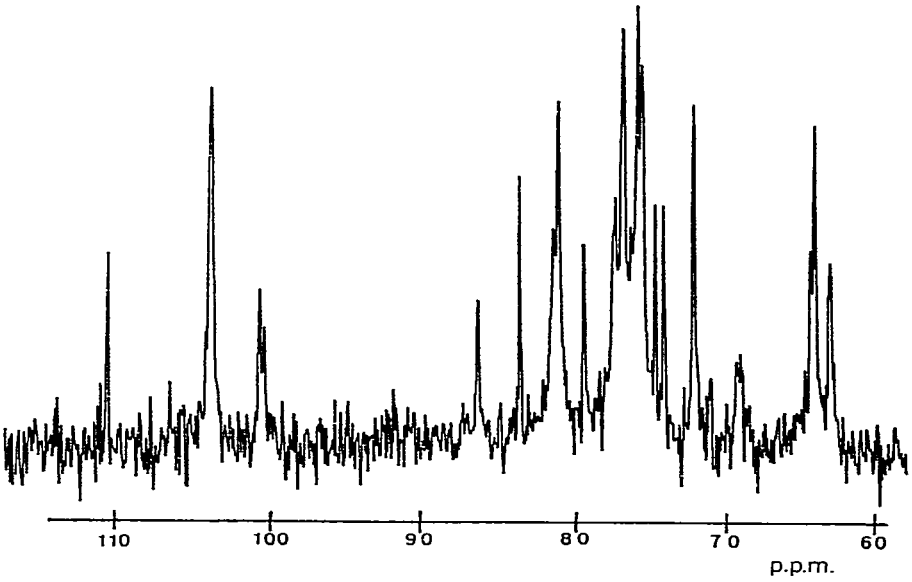


Fig. 3. ^{13}C-n.m.r. spectrum of intact arabinoxyloglucan in 0.2M NaOD–D_2O at 80°. (45° pulse, repetition time 1 sec, 57000 accumulations).

that the molecule was essentially composed of an $AraXyl_2Glc_3$ (**1**) (including Xyl_2Glc_3, **3**) part and unsubstituted glucan regions (4-*O*-linked glucosyl residues). The molar ratio of the oligosaccharide component was, on the average, ~1:2. The distribution of the oligosaccharide and the glucosyl residues in the polymer remained to be examined. Enzymic investigations confirmed the foregoing results; in the cellulase-digested products of the arabinoxyloglucan, the molar ratio of the monosaccharide component and oligomeric products was ~1:2. Consequently, the ^{13}C-n.m.r. spectrum of the polysaccharide could be considered as a combination of those of the oligosaccharide and of the unsubstituted glucan regions of the molecule.

In practice, as shown in Fig. 3, the ^{13}C-n.m.r. spectrum of the polysaccharide was in good agreement with the foregoing hypothesis and could be almost fully assigned by comparing the data with those of $AraXyl_2Glc_3$ (**1**) and cellotriose.

The signals for the glucosyl residues of the polysaccharide were broadened, but had relatively high peak-heights. The signals of C-1, C-4, and C-6 of the glucosyl residue appeared at ~104.2, ~80.6, and 68.6, 68.4 (*O*-glycosylated), and 62.3 (free) p.p.m., respectively.

As the glucosyl signals overlapped each other, the exact repeating-sequence in the polysaccharide molecule could not be determined.

Field-desorption mass spectroscopy (f.d.-m.s.) of Xyl_2Glc_3 (**3**) *and* $AraXyl_2Glc_3$ (**1**). — Determination of the molecular weights of **3** and **1** by f.d.-m.s. gave additional confirmation of the structural features of the oligosaccharides.

Most of the higher oligosaccharides (degree of polymerization 4) afforded the base peak as a cation cluster of molecular ions in f.d.-m.s.[17,18]. At emitter currents

of ~17 mA, the spectra of **3** and **1** showed $[M + {}^{23}Na]^+$ ions at *m/e* 791 and 923 as their base peaks, respectively. The corresponding $[M + {}^{39}K]^+$ ions (*m/e* 807 and 939) were also observed, with lower intensities. These data suggest that the molecular weights of **3** and **1** are 768 and 900, respectively. These results agree well with data obtained from chemical analyses.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. Optical rotations were measured at 23° with a Jasco DIP-181 polarimeter. Some of the oligosaccharides were purified by preparative p.c. on Whatman 3 MM paper with 5:3:3 butanol–pyridine–water as solvent. Purities of the oligosaccharides were checked by zone-electrophoresis and t.l.c. Zone electrophoresis was conducted on Whatman No. 1 paper (40 × 10 cm) at 2000 V for 100 min in 0.1M sodium tetraborate buffer (pH 9.2), and components were detected with aniline phosphate reagent[19]. Mobilities are expressed relative to 2,3,4,6-tetra-*O*-methylglucose. T.l.c. was performed (*A*) on cellulose plates (Avicel SF. Funakoshi Chemical) with 5:3:3 butanol–pyridine–water (developed twice), and the same detection reagent as before, or (*B*) on silica gel sheets (Eastman Chromatogram sheet, 13181 silica gel) with 3:12:4 (v/v) butanol–2-propanol–water developed twice)[20], and detection with 50% sulfuric acid.

Neutral sugar and methylation analyses. — Methods for neutral sugar determination, and methylation analyses by combined g.l.c. and g.l.c.–m.s., have been described previously[7,8]. Methylation analyses of samples containing *O*-substituted xylosyl residues were verified by g.l.c.–m.s. of the alditol acetates prepared by using sodium borodeuteride. The results clearly demonstrated that all the xylosyl residues were 3,4-di-*O*-methylated.

Materials. — The arabinoxyloglucan was isolated from the dry midrib of flue-cured leaves of *Nicotiana tabacum* cv. BY, as previously described[8]; $[\alpha]_D^{23}$ +35.0° (*c* 0.5, water).

Cellotriose was isolated from the saponification product of an acetolyzate[21] of cellulose powder that have been successively fractionated on columns of charcoal, Bio-Gel P-2, and by preparative p.c.; $[\alpha]_D^{23}$ +21.9° (*c* 0.5, water); lit.[22] $[\alpha]_D$ 23.2°.

^1H- and ^{13}C-n.m.r. spectra. — ^1H- and ^{13}C-n.m.r. spectra (99.6 MHz) were recorded with a Jeol FX-100 spectrometer and 5-mm tubes. Before each measurement, the sample (~50 mg) was dissolved in deuterium oxide (D_2O) and freeze-dried, and this procedure was repeated three times, and the material was finally dissolved in 0.3 mL of D_2O or 0.2M NaOD/D_2O. Chemical shifts (δ values) were measured downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard. Normal ^{13}C-n.m.r. spectra (25.1 MHz) were recorded in 10-mm tubes with complete proton-decoupling. The spectrometer was equipped with Fourier-transform apparatus [spectral width, 6000 Hz; digitalization, 8192 data points; pulse width, 7 μsec (45°); and repetition time, 1 sec]. ^{13}C-^1H coupling constants

were determined by the gated, ^1H decoupler sequence to retain nuclear Overhauser enhancements (interval between pulses, 3.0 sec; decoupling time, 2.3 sec). The spectra of oligosaccharides were measured at 23° and that of the polysaccharide at 80°. To prevent degradation of polysaccharide during the measurement at elevated temperature in alkaline solution, the samples were treated with sodium borohydride overnight, followed by dialysis against water and freeze-drying before use.

Field desorption mass spectroscopy (f.d.-m.s.). — F.d.-m.s. were obtained by using a compact, double-focusing, g.l.c.–mass spectrometer, Jeol D-300, equipped with a combined f.d. ion source (MS-FD 03). Samples (0.5–1 μg) were applied in aqueous solution. The source temperature was 100°; the resolution was 800 and the accelerating voltage was 3 kV, with −6 kV on the cathode. A Jeol JMA-2000 data-processing system was used.

Cellulase digestion. — Cellulase from *Trichoderma viride* (Meicellase, kindly donated by Meiji Seika Ltd.) was used after purification on a gauze column according to Ogawa and Sotoyama[23]. The absence of interfering activities in the enzyme was checked on appropriate model substrates, namely, *p*-nitrophenyl glycosides of α-L-arabinofuranose, α-D-xylopyranose, and α- and β-D-glucopyranose. The results showed that the contaminating activity in it was negligible, but a trace of β-D-glucopyranosidase activity was present [0.3% of the (1→4)-β-D-glucanase activity].

Tobacco arabinoxyloglucan (500 mg) was suspended in 350 mL of 0.1M sodium acetate buffer (pH 4.5) and incubated together with the enzyme (245 mg, 350 units) for 4 days at 37° under a few drops of toluene. For such a long incubation, all operations were performed under sterile conditions on a clean bench, and toluene was added each day. From our previous results[7], incubation for two days was not sufficient to give a complex digest-mixture. After the digestion period, the reaction was stopped by heating for 30 min on a boiling-water bath. Under these conditions, L-arabinofuranosyl residues in the oligosaccharides were not hydrolyzed. The insoluble material was removed by centrifugation, and the supernatant solution was deionized by passage through a column of Dowex 50W (H^+), and freeze-dried.

The material (400 mg) was divided into 5 portions of 80 mg, each of which was dissolved in 2 mL of distilled water and applied to a column (1.5 × 150 cm) of Bio-Gel P-2 (<400 mesh) which was eluted with water at 50°. The elution profile is shown in Fig. 1. Each fraction was further purified by rechromatography on the same column until a single, symmetrical elution-peak was obtained.

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