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A HIGHLY SUBSTITUTED GLUCURONOARABINOXYLAN FROM DEVELOPING MAIZE COLEOPTILES

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ABSTRACT

A glucuronoarabinoxylan in which about six of every seven xylosyl residues were substituted with terminal arabinofuranosyl and glucosyluronic groups was extracted from developing maize coleoptiles with dilute alkali and purified by gel-permeation chromatography. Terminal arabinofuranosyl groups attached mostly to O-3 of the xylan backbone constituted most of the substitution. Indirect double-methylation analysis of mild acid-hydrolyzed xylan, direct methylation analyses of carbodiimide-activated, $NaBH_4$-reduced uronic acid groups, and isolation of the glucosyluronic-xylose aldobiouronic acids followed by 2-D COSY ^{1}H-n.m.r. spectroscopy indicated that most, if not all, of the D-glucosyluronic groups were α-(1→2)-linked to the xylan. Smith degradation indicated that at least a portion of this highly substituted xylan may have a repeating-unit structure.

INTRODUCTION

The principal hemicelluloses of primary cell-walls of developing maize coleoptiles are glucuronoarabinoxylans, mixed-linked glucans, and xyloglucans[1–3]. A fraction comprised mostly of a glucuronoarabinoxylan (GAX I) was discovered in dilute-alkali extracts of depectinated walls of rapidly growing coleoptile cells. Nearly six of every seven xylosyl residues of the xylan backbone were branch points for terminal arabinofuranosyl, terminal glucosyluronic , and possibly trace amounts of other disaccharide and trisaccharide side-groups[4]. This report examines the arabinosyl and glucosyluronic linkages of the purified, highly substituted GAX.

EXPERIMENTAL

Growth of seedlings and preparation of wall material. — Seedlings of maize (*Zea mays* L. cv. WF9 × Bear 38) were grown and wall material from excised

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coleoptiles was prepared as described previously[4].

Starch was extracted from the wall material with dimethyl sulfoxide[4–6], and pectic substances were removed by hot ammonium oxalate[4].

Hemicelluloses were extracted under N_2 with stepwise increases in concentrations from 0.01 to 4M KOH, supplemented with 3 mg/mL of $NaBH_4$ to prevent end-elimination[7]. Material extracted successively for 1 h each in 0.02, 0.03, and 0.045M KOH was pooled, made neutral with acetic acid, dialyzed for 18 h against running deionized water, and lyophilized. This material was dissolved in 1 mL of M KOH, and 200 μL of 0.1M Tris-Cl (pH 8.6) and 1 mL of M HCl were added. A small amount of undissolved material was removed by centrifugation, and the sample was loaded onto a 2.5-cm × 60-cm column of Sepharose 4B-200 (Pharmacia; repackaged by Sigma) equilibrated in 0.2M Tris-Cl (pH 8.6) supplemented with 0.2M KCl. Fractions (2.5 mL) were assayed for sugar by the phenol–H_2SO_4 method[8], and polymers comprising the major included fraction were pooled, dialyzed against running, deionized water for 18 h, and lyophilized. This fraction constituted the highly substituted glucuronoarabinoxylan (HS-GAX). Additional GAX was extracted by more-concentrated alkali; fractions extracted by 0.45, 0.6, and 0.8M KOH were pooled, made neutral with acetic acid, dialyzed as before, and lyophilized (GAX II).

Methylation analysis. — One to 3 mg of purified material was per-*O*-methylated according to Hakomori as described by Sandford and Conrad[9], except that the potassium dimethylsulfinyl anion was prepared, and anhydrous silylation-grade Me_2SO was from Pierce Chemical Co. Additional technical refinements were described previously[10]. After methylation, the Me_2SO mixture was diluted with water and applied to a C_{18} minicolumn (Sep-pak; Millipore) prewashed with methanol, and equilibrated with water. Me_2SO was washed from the column with excess water; *O*-permethylated polymers were eluted with methanol, and methanol was evaporated in a stream of N_2. Polymers were hydrolyzed in 2M CF_3CO_2H containing 1 μmol of *myo*-inositol (internal standard) for 90 min at 121°, and the acid was evaporated in a stream of N_2. Partially methylated sugars were reduced with $NaBD_4$ in Me_2SO and acetylated with acetic anhydride catalyzed by 1-methylimidazole as described by Blakeney *et al.*[11], except that the volumes of reagents were adjusted so that samples could be processed in 1-dram vials with caps lined with Teflon.

Reduction of glycosyluronic linkages. — Glycosyluronic residues in depectinated wall material were reduced with $NaBH_4$ and $NaBD_4$ and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC) according to Taylor and Conrad[12], prior to alkali extractions. Material was dialyzed against running, deionized water, and lyophilized. Hemicelluloses were fractionated with increasing concentrations of alkali as before, and appropriate fractions were pooled, made neutral with acetic acid, dialyzed against deionized water, and lyophilized. Partially methylated alditol acetates were prepared as before, except that CD_3I was used to differentiate glucosyluronic from endogenously methylated glycosyluronic units.

Characterization of the glucosyluronic linkages. — The positions of glucosyluronic linkages were determined indirectly by double methylation[13]. The GAX was subjected to mild acid hydrolysis in 0.5M CF_3CO_2H for 2 h at 80° to remove the labile arabinofuranosyl groups. The xylan containing predominantly glucosyluronic groups was *O*-permethylated with CD_3I; the *O*-perdeuteriomethylated polymers were purified as before and redissolved in the dimethylsulfinyl anion solution to induce β-elimination of the partially methylated glycosyluronic residues. The newly generated hydroxyl groups were *O*-perethylated with CH_3CH_2I. Partially methylated, partially ethylated alditol acetates were prepared as described earlier.

Additional GAX (100 mg) was hydrolyzed in 12M CF_3CO_2H for 30 min at 80° to yield neutral sugars and acid-resistant aldobiouronic acids. The acid was evaporated off under diminished pressure, and the sugars were dissolved in water and applied to a column (0.9 × 10-cm) of Dowex 1-X8 (acetate form). Neutral sugars were eluted with at least 150 mL of water, and uronic acid-containing oligosaccharides were eluted with 6M acetic acid. The acid was evaporated off under diminished pressure, and the residue was dissolved in 80% ethanol (v/v) and spotted onto Whatman 3MM filter paper. Glycosyluronic derivatives were separated[14] by descending chromatography in 18:4:3:1 (v/v/v/v) ethyl acetate–water–acetic acid–formic acid for 42 h. Sugars were detected in a portion of the strip by alkaline $AgNO_3$: the major sugar-containing regions were excised, and sugar were eluted with water. The eluate was filtered through Whatman GF/F glass-fiber filter paper and the filtrate lyophilized.

^{1}H-N.m.r. spectroscopy of GlcA-Xyl. — About 1 mg of purified GlcA-Xyl was dissolved in 0.5 mL of D_2O. Spectra were obtained with a Varian Associates XL-200 n.m.r. spectrometer operating at 200 MHz. A 600-Hz sweep-width, 1-D spectrum was obtained in 617 transients of 2398 data points, weighted with a Lorentzian-to-Gaussian resolution-enhancement[15], and Fourier-transformed. The large HOD resonance was suppressed by presaturation with the decoupler. A 600 × 600 Hz 2-D COSY spectrum was obtained in a 512 × 512 point data-matrix with 546 transients for each t_1 increment. Lorentzian-to-symmetrical Gaussian weighting was used in both dimensions[16]. The 2-D experiment time was 10.5 h, and the 90° pulse-time was 13 μs.

Reduction and methylation of GlcA-Xyl oligosaccharides. — The glycosyluronic residues of several glycosyluronic-oligosaccharides resolved by paper chromatography were reduced with $NaBD_4$ and CMC as described for the native polymers[12]. After reduction, the solution was acidified with acetic acid to pH 4.5 and evaporated under diminished pressure. Borate was removed as trimethyl borate by repeated evaporation of methanol under diminished pressure. The residue was dissolved in water, the solution was deionized with Amberlite IRA-45, and neutral sugars were eluted with water. Neutral oligomers were separated by l.c. on a 4.5-mm × 30-cm HPX-42A l.c. column (Bio-Rad) at 85° at 0.5 mL/min with water as eluant. Fractions containing sugars were lyophilized, and partially deuteriomethylated alditol acetates prepared with CD_3I as described.

Gas chromatography–mass spectrometry. — Derivatives were separated in a 0.2-mm × 30-m SP-2330 vitreous silica WCOT capillary column (Supelco) temperature-programmed from 170 to 240° at 2°/min. The split ratio was 50:1, with He carrier flow at ~1.5 mL/min. Electron-impact mass spectra were obtained at 70 eV and a source temperature of 160° with a Finnigan/MAT 9610 g.l.c. instrument coupled to a Finnigan/MAT 4021 quadrupole mass spectrometer interfaced to a Finnigan/MAT 2100C INCOS data-system. Derivatives were identified by relative retention-time and fragmentation analysis after e.i.–m.s.; amounts of each derivative were quantified according to the effective carbon-response calculated by Sweet *et al.*[17].

Smith degradation. — Samples (~10 mg) of HS-GAX and GAX II were suspended in 2.0 mL of 0.1M sodium acetate, pH 3.9, at 4° and 2.0 mL 50mM sodium metaperiodate was added. The mixture was stirred in darkness for 8 days at 4° until periodate consumption as measured by absorption at 223 nm was virtually complete. The excess of periodate was decomposed by adding 0.5 mL of ethylene glycol, and the mixture was stirred for an additional 30 min, dialyzed against deionized H_2O, reduced with $NaBH_4$, and redialyzed. The material was then hydrolyzed with 0.5M CF_3CO_2H for 30 min at 80°, and the acid was evaporated off under diminished pressure. The residue was dissolved in H_2O, filtered through Whatman GF/F glass-fiber filter paper, and oligomers were separated by l.c. on a Bio-Rad HPX-42A column (4.5-mm × 30-cm) at 85° at 0.5 mL/min with water as eluant. The effluent was monitored by refractive index, and 0.5-mL fractions were assayed for sugars by the phenol–H_2SO_4 method[8]. Fractions containing sugars were lyophilized, and partially methylated alditol acetates prepared as described.

RESULTS AND DISCUSSION

Dilute alkali extracted a small amount of mostly GAX from walls of rapidly growing coleoptiles[4]; this material varied in abundance relative to other hemicellulosic material depending on the developmental state, but was maximal between 2 and 3 days of incubation when cell-elongation rates were highest[10]. This fraction comprised ~150 μg of the 750 μg of hemicellulosic material per coleoptile.

The GAX was contaminated by only ~4% glucose residues and comprised mostly arabinose, xylose, glucuronic acid, and small amounts of galactose. The material was fractionated further on Sepharose 4B-200 and resolved into polymers constituting the excluded fraction and a homogenous, included fraction (HS-GAX) corresponding to dextran standards of 40,000–100,000 mol.wt. Most of the glucosyl residues were in polymers comprising the excluded volume, and the distribution of neutral sugars in samples taken through the HS-GAX fractions was consistently ~50% xylose, 45% arabinose, 3% galactose, and 2% glucose residues.

Terminal-arabinofuranosyl (t-Ara), 4-xylosyl, and 2,4- and 3,4-xylosyl branch-points comprised >90% of the sugar residues recovered (Table I). Over six of every seven xylosyl residues were branch points, mostly for the t-Ara groups.

TABLE I

COMPOSITION OF PARTIALLY METHYLATED ALDITOL ACETATES OF THE HS-GAX AND DERIVATIVES[a]

Fraction	*Arabinose*					*Xylose*				*Galactose*			*Glucose*		
	t-	*2-*	*3-*	*5-*	*3,5*	*t-*	*4-*	*2,4-*[b]	*3,4*	*t-*	*3-*	*4-*	*t-*	*3-*	*4-*
	mol%														
HS-GAX	43	tr[c]	tr	2	tr	tr	7	3	41	tr	tr	2	tr	tr	1
Reduced HS-GAX	34	1	tr	4	tr	1	9	5	36	1	tr	1	6	tr	1
Mild-acid HS-GAX	3	n.d.[d]	n.d.	tr	n.d.	4	72	5	6	1	n.d.	n.d.	7	n.d.	1

[a]HS-GAX polymers were solubilized by dilute alkali from depectinated walls purified from developing maize coleoptiles. "Reduced HS-GAX" was from depectinated walls reduced with $NaBH_4$ after carbodiimide activation of uronic acid groups, as described by Taylor and Conrad[12] before alkali extraction. "Mild-acid HS-GAX" was from HS-GAX subjected to hydrolysis in 0.5M CF_3CO_2H for 2 h at 80° before carbodiimide-activated reduction of the uronic acid groups with $NaBD_4$. Partially methylated alditol acetates were separated in a vitreous silica WCOT capillary column (0.2 × 30-m SP-2330, Supelco), temperature-programmed from 170 to 240° at 2°/min. The split ratio was 50:1, with a He carrier flow of 1.5 mL/min. Derivatives were identified by relative retention-time and e.i.–m.s. and quantified from total ion-counts corrected to mol% by the effective carbon response-factors calculated by Sweet *et al.*[17]. [b]2,4- and 3,4-xylosyl derivatives were unresolved by GC but were determined by selective ion-monitoring. [c]Tr = trace amounts (<0.5%). [d]N.d. = not detected.

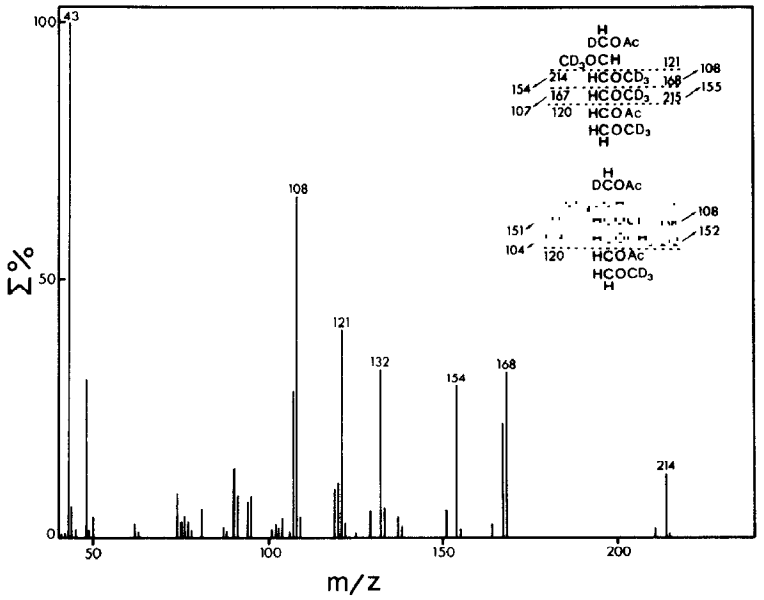

Fig. 1. Fragmentation analysis of terminal glucosyl derivative from carbodiimide-activated, $NaBH_4$ reduction of terminal glucosyluronic and 4-*O*-methylglucosyluronic groups. After reduction, the GAX was deuteriomethylated with CD_3I to differentiate between endogenously methylated and unmethylated glycosyluronic derivatives.

Only small amounts of 5-arabinosyl, 4-galactosyl, and 4-glucosyl, and trace amounts of other linkages were identified. Glycosyluronic acid comprised ~15–20% of the GAX, based on phenol–H_2SO_4 (ref. 8) and carbazole[18] assays. Because the aldobiouronic acid residues are resistant to acid hydrolysis, only a portion of the neutral sugar attachment-sites were recovered in the methylation analysis. Several investigators have reported GlcA-(1→2)-xylosyl aldobiouronic acids in xylans from several graminaceous species, including maize[14,19–23]. These linkages were examined in the HS-GAX to determine if they were different from those of the other maize xylans. Furthermore, various proportions of endogenous 4-*O*-methylated glucosyluronic residues have been identified in many xylans, but have not usually been quantified[14,19,20,23].

Carbodiimide-activated glycosyluronic units were reduced[12] with $NaBH_4$ before alkaline extractions. Dilute alkali extracted a similar HS-GAX from the wall material, except for an increase in t-Glc, as well as in 5-arabinose and 2,4-xylose (Table I), and loss of detectable uronic acid (data not shown). Fragmentation analyses of the terminal glucosyl groups of polymers deuteriomethylated with CD_3I revealed *m/z* 164 (4.0%), 167 (22.1%), and 168 (32.3%) of *m/z* 43 (base peak), and indicate that ~15% of the terminal glucosyluronic groups were 4-*O*-methylated (Fig. 1).

The nature of the linkage of the terminal glucosyluronic groups to the xylan was examined by both direct and indirect methods. To simplify analyses, the labile

arabinofuranosyl groups were removed from the xylan by mild acid hydrolysis. The hydrolysis left a relatively unbranched xylan substituted primarily by the acid-resistant uronic acid residues. Carbodiimide-activated reduction of the latter with $NaBD_4$, followed by *O*-permethylation with CH_3I resulted again in the appearance of t-Glc groups, comprising ~7 mol% of the polymer (Table I). The terminal glucosyl was unequivocally the result of reduction of terminal glucosyluronic groups on the xylan chain, as demonstrated by detection of *m/z* 207 instead of *m/z* 205 and *m/z* 163 in addition to *m/z* 162 upon fragmentation during e.i.–m.s. Likewise, about one-half of the 4-glucosyl also detected was also the result of reduction of glucuronic acid, because of the detection of about equal amounts of *m/z* 235 and 233. The novel fragments arise from double deuteration of the C-6 carboxylic group upon reduction with $NaBD_4$.

Other samples of unreduced, mild-acid hydrolyzed HS-GAX were subjected to a "double methylation" as described by Darvill *et al.*[13] in which the second introduction of the potassium dimsyl anion induced β-elimination of the partially methylated glucosyluronic groups from the xylan and liberated new hydroxyl groups that were subsequently ethylated to indirectly reveal former attachment-sites of the glycosyluronic units. The methylation analyses demonstrated the presence of primarily 4-Xyl and only trace amounts of t-Ara, t-Xyl, 2,4-Xyl, and 3,4-Xyl units remaining after hydrolysis (Fig. 2). Two additional peaks resulted from a doubly ethylated xylose and two chromatographically unresolved, single

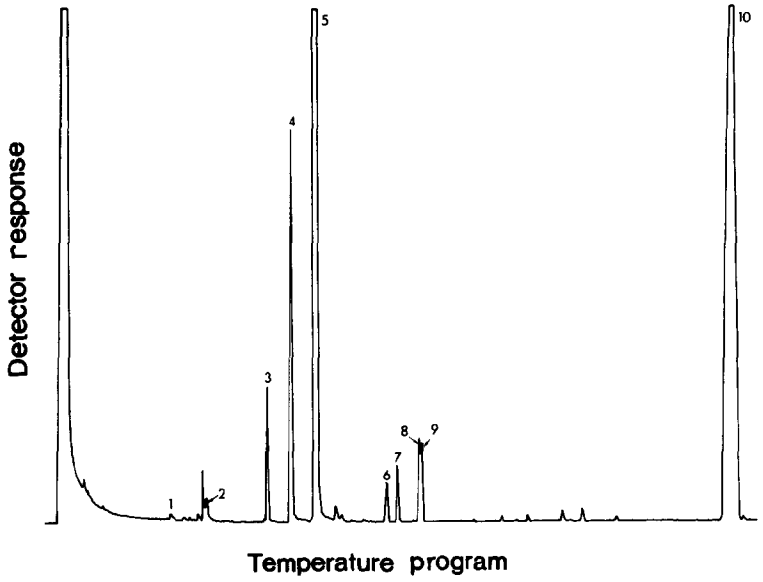

Fig. 2. Gas-chromatographic separation of partially methylated, partially ethylated alditol acetates prepared from HS-GAX subjected to mild acid hydrolysis. Peaks identified by retention time and fragmentation analysis were: 1, t-Ara; 2, t-Xyl; 3, doubly-ethylated 4-Xyl; 4, singly ethylated 4-Xyl; 5, 4-Xyl; 6, 4-Gal; 7, 4-Glc; 8, 2,4-Xyl; 9, 3,4-Xyl; and 10, *myo*-inositol (internal standard).

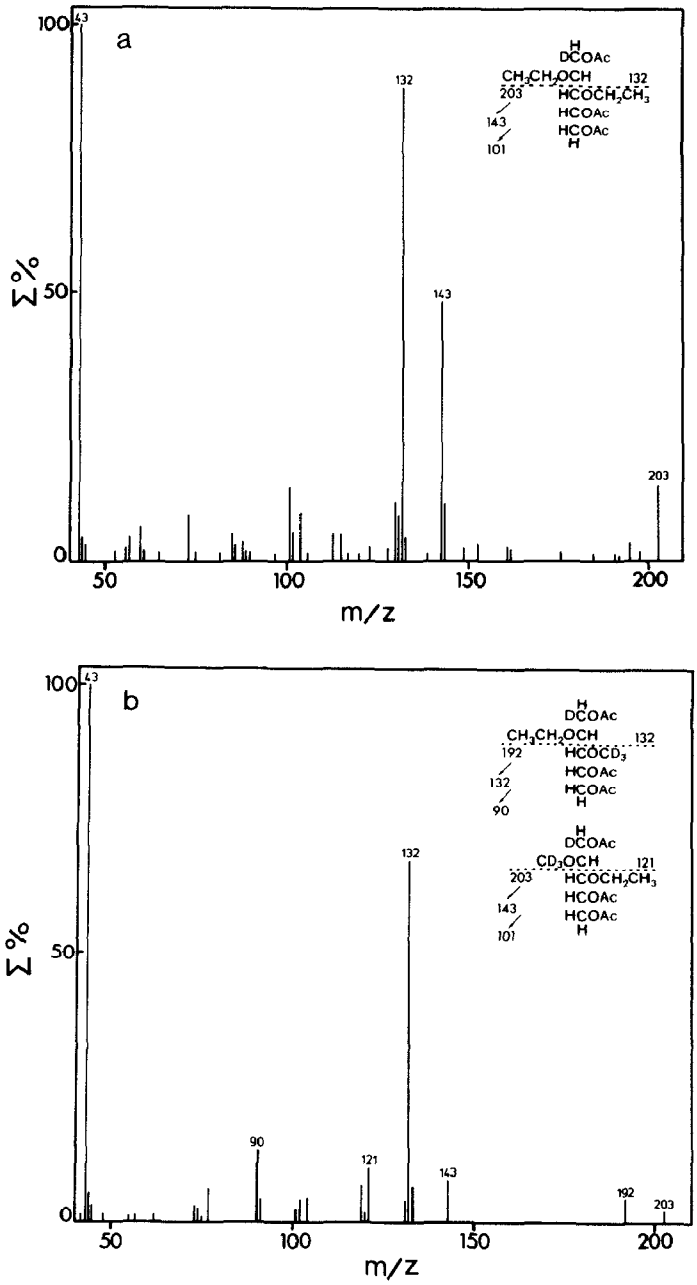

Fig. 3. Fragmentation analysis of partially ethylated, partially methylated alditol acetate derivatives (*a*) Spectrum and expected fragmentation of 1,4,5-tri-*O*-acetyl-2,3-di-*O*-ethyl-xylitol; (*b*) Spectrum and expected fragmentations of 1,4,5-tri-*O*-acetyl 2-*O*-deuteriomethyl-3-*O*-ethyl xylitol and 1,4,5-tri-*O*-acetyl-3-*O*-deuteriomethyl-2-*O*-ethyl xylitol.

ethylated xylose derivatives (Fig. 2); from mass spectrometry, the preponderance of *m/z* 132 in the singly-ethylated xylose derivative indicated that GlcA was linked primarily at O-2 of the xylan chain (Fig. 3). The small amounts of doubly ethylated xylose and O-3 ethylated xylose may indicate alternative linkages or could have resulted artifactually from initial minor undermethylation of the xylan. Hence, more-direct determinations of the linkages were made.

The aldobiouronic acids and acidic oligosaccharides of the xylan were prepared by vigorous acid hydrolysis in CF_3CO_2H and purified by anion exchange and paper chromatography. In addition to GlcA and a small amount of 4-*O*-methylglucosyluronic-xylose, there were four $AgNO_3$-positive spots corresponding to the GlcA-Xyl and GlcA-Xyl[Xyl]$_n$ series; from methylation analyses after carbodiimide-activated deuteriomethylation of the glycosyluronic groups, only terminal-glucosyl, 2-xylosyl, and 2-xylitol derivatives were identified, in addition to 4-xylosyl residues of oligosaccharides and some t-Xyl (data not shown). No derivatives were detected that would indicate uronic acid groups attached to O-3 or both O-2 and O-3 of the xylose residues. Our results were consistent with other observations that xylans of graminaceous species vary markedly with respect to both the degree of substitution of uronic acid side-groups and the proportion of endogenous 4-*O*-methylation of the uronic acid residues. However, the uronic acid groups are usually linked to O-2 of the xylan backbone[14,19–23].

From ^{1}H-n.m.r. 2-D COSY spectra of the purified GlcA-Xyl derivative, the α linkage was deduced unequivocally from the $J_{1,2}$ value of only 3.6–3.8 Hz for the uronic acid residues, whereas both α and β orientations of the free anomeric carbon atom of xylose were observed (Table II).

Other investigators have sought to determine if the arabinosyl and uronic acid groups were attached to the xylan backbone randomly or in a repeating sequence much like that observed for xyloglucans of dicotyledonous species[1]. Endoxylanases require several contiguous, unbranched xylosyl residues for their action, in part because of the steric hindrance of O-2 or O-3 linked side-group[24–26]. Because of this problem, it is unresolved whether or not oligosaccharide fragments obtained from GAX from grasses or other species display any repeating-unit structure[25,26]. From pulse-chase studies[10], radioactivity in the HS-GAX was possibly accumulated into GAX with less branching, and it was proposed that the HS-GAX was the precursor for other xylans via *in situ* loss of uronic acid and arabinosyl side-groups. If so, then random hydrolysis of the side-groups may obscure any repeated structure originally present in the HS-GAX. Because of the potential steric hindrance problem for hydrolysis by endo-xylanase, Smith degradation was employed to elucidate the number and proportion of contiguously branched xylose residues.

About 30% of the Smith-degraded material was recovered, based on phenol–H_2SO_4 sugar determination of fractions collected upon l.c. Degradation of HS-GAX yielded a large proportion of xylan oligomers ~5–6 residues long, whereas the degradation products of GAX II were mostly shorter than 5 residues (Fig. 4).

TABLE II

PROTON CHEMICAL SHIFTS AND COUPLING CONSTANTS FOR GLUCURONIC ACID AND XYLOSE IN THE ALDOBIOURONIC ACID[a]

Sugar residue	*Parameter*	*Proton*					
		H-1	*H-2*	*H-3*	*H-4*	*H-5*	
α-GlcA-1	δ	5.29	3.48	3.66	3.39	4.22	
	J	3.83	9.85	9.05	10.10	—	
α-GlcA-2	δ	4.99	3.49	3.70	3.40	4.11	
	J	3.61	9.88	9.06	10.09	—	
		H-1	*H-2*	*H-3*	*H-4*	*H-5e*	*H-5a*
α-Xyl	δ	5.30	3.52	3.4–3.6			
	J	2.8	10	N	N	N	N
β-Xyl	δ	4.60	3.29	3.37	3.51	3.83	3.22
	J	8	9	10	5.2	11.2	10.2

[a]Assignments were made primarily from the 2-D COSY spectrum. Chemical shifts are in p.p.m. from Me_4Si, and coupling constants are in Hz. The coupling constants in column H-1 are $J_{1,2}$ in column H-2 are $J_{2,3}$, and so forth; the β-xylose $J_{4,5a}$ values are given in column H-5a. Because the chemical shifts of the α-Xyl protons H-4, H-5e, and H-5a were poorly resolved, coupling constants were not determined (N). The ratio of α-Xyl and β-Xyl in the disaccharide was nearly 1:1, and hence, it was not determined which set of GlcA resonances was associated with the α and β anomers.

Both degradations resulted in unidentified, phenol–H_2SO_4-positive degradation products that coeluted with the monosaccharide fraction (Fig. 4), but little xylose was detected in these fractions. Hence, HS-GAX apparently has at least a portion of repeating blocks of consecutively branched xylose residues along the xylan chain. Based on the ratio of 4-Xyl to t-Xyl determined from methylation analysis, the average length of the HS-GAX major xylan oligomers (Fig. 4a, Fractions 12–15) was 5.8, whereas that of the GAX II oligomers (Fig. 4b, Fractions 15–18) was only 2.2; these data confirm the apparent size of xylan oligomers as judged by l.c.

The results of our experiments indicate that the HS-GAX is related structurally to other GAX in the maize primary-wall. Both t-Ara and t-GlcA groups are attached to the xylan chain through similar linkages, and differ only with respect to the proportion of side-group substitution. The function of the HS-GAX is unknown, but the apparent repeating nature of consecutively branched xylose residues lends support to the hypothesis that HS-GAX is the precursor to other GAX via *in muro* hydrolysis of arabinosyl and glucosyluronic side-groups, and hence, may represent a synthesized and secreted GAX of maize primary cell-walls[10]. The inference of arabinosidase activity in maize walls[27], the capacity of maize cells to metabolize arabinose possibly hydrolyzed from GAX[28], and the observation of turnover of the HS-GAX in pulse-chase studies[10] each support the hypothesis.

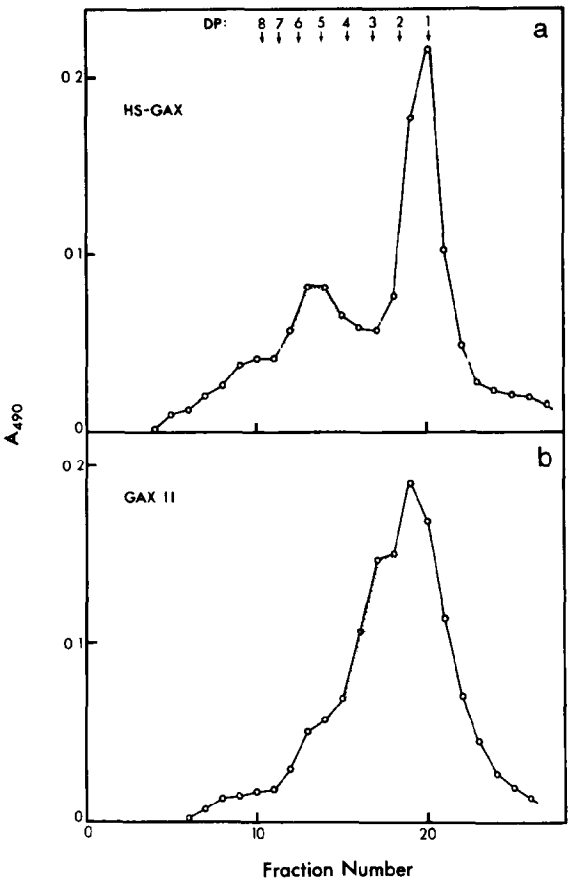

Fig. 4. Separation of xylan oligomers generated by Smith degradation of HS-GAX and GAX II. After periodate oxidation, polymers were reduced with $NaBH_4$ and hydrolyzed with 0.2M CF_3CO_2H for 30 min at 70°. Oligomers were separated on an column of HPX-42A (Bio-Rad) at 85° at 0.5 mL/min, with water as eluant. The 0.5-mL fractions were assayed for sugars by the phenol–H_2SO_4 method[8]. Standard xylan oligomers were prepared by strong acid hydrolysis of purified unbranched xylan. Shaded fractions were pooled for methylation analyses.

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