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Structural characterization of an arabinoxylan–rhamnogalacturonan complex from cell walls of *Zea* shoots*,†

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ABSTRACT

An arabinoxylan–rhamnogalacturonan complex, comprised of galacturonic acid, rhamnose, arabinose, xylose, and galactose in the ratios 75.9:4.6:5.2:3.5:5.4 and lesser amounts of other constituents, was dissociated from the water-insoluble matrix of cell walls of *Zea mays* by xylanase and glucuronoxylanase treatment. The solubilized complex retained its integrity when subjected to a series of separation procedures, and analysis of the sugar components throughout the elution profiles exhibited consistent ratios. The complex was subjected to controlled degradation by pectate lyase and pectin lyase, yielding two components comprised of rhamnose, fucose, arabinose, xylose, galactose, and galacturonic acid in the ratios 10.9:1.5:13.1:16.9:27.7:30.0 and 8.5:1.7:11.8:6.6:17.3:54.0, respectively, in addition to di-, tri-, and tetra-saccharides of galacturonic acid. The non-reducing terminals of the latter were characterized by the presence of 4,5-unsaturated hexuronic acid. The structural features of the two complex fractions were partially characterized.

INTRODUCTION

Enzymes have been employed to sequentially degrade maize cell-wall polysaccharides in order to probe for molecular associations that otherwise might be altered by alkali treatment. β-Glucanase [(1→3),(1→4)-β-D-glucan 4-glucanohydrolase] has proved effective in liberating most of the β-D-glucan, and endo-xylanase [endo-(1→4)-β-D-xylanase] releases at least a portion of the xylan[1]. However, most of the xylan is highly substituted and therefore resistant to xylanase hydrolysis. Glucuronoxylanase (glucuronoxylan xylanohydrolase) has therefore been employed to enhance the yield of soluble fragments[2]. Based on earlier studies, it was suggested that arabinoxylan and

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rhamnogalacturonan might be linked[2]. We now describe the partial characterization of two enzymically liberated components that are enriched in galacturonic acid, rhamnose, galactose xylose, and arabinose.

RESULTS AND DISCUSSION

The products of glucuronoxylanase treatment of cell walls were separated into seven fractions (I through VII) by DEAE Sephadex A-25 chromatography (Fig. 1). Each fraction was further resolved into components of larger (1) and smaller (2) mol. wt. Yields of all fractions and the sugar composition of the major fractions are summarized in Table I. Fraction IV-1, one that is enriched in acidic sugars and Rha, was purified by chromatography on a column of DEAE-Sepharose CL-6B. The following experiments were conducted using the purified fraction IV-1.

Fraction IV-1 in M ammonium acetate was chromatographed on a calibrated column of Sepharose CL-6B. The elution pattern of the phenol–H_2SO_4-positive material corresponds with that of the carbazole–H_2SO_4-positive material (uronic acid) (Fig. 2). The average mol. wt. of fraction IV-1 was estimated as 2.0×10^4 by comparison with standard dextrans.

Fraction IV-1 was hydrolyzed with CF_3CO_2H, and the hydrolyzate was resolved into seven fractions (AH-1 through AH-7) by chromatography on a Bio-Gel P-2 column (Fig. 3). Acidic and neutral sugar contents of all the fractions are summarized in Table II. On the basis of the total neutral and acidic sugar content of fraction IV-1 and

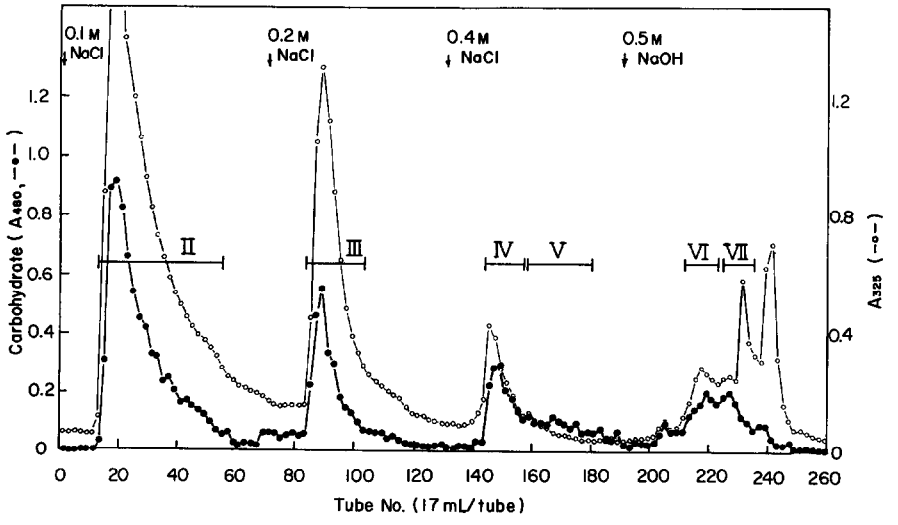


Fig. 1. Resolution of the products of enzyme degradation of the cell wall. The enzymic degradation products were applied to a column of DEAE-Sephadex A-25 pre-equilibrated with sodium acetate buffer. The column was washed with the same buffer, then stepwise elution was carried out; details are described in the text.

TABLE I

Yields and neutral sugar composition of subfractions obtained from a mixture of cell-wall fragments derived from glucuronoxylanase

Fraction	*Yield*[a] *(mg)*	*NS:AB*[b]	*Neutral sugar composition (mol %)*				
			Rha	*Ara*	*Xyl*	*Gal*	*Glc*
I-1	102.3	96.4:3.6		46.0	46.0	8.1	Trace
-2	381.1	ND[c]		37.6	47.7	14.7	Trace
II-1	246.6	94.6:5.4		42.7	51.5	3.8	Trace
-2	49.4	ND			ND		
III-1	75.0	89.1:10.1		44.6	52.6	2.8	Trace
-2	8.3	ND			ND		
IV-1	46.4	73.4:26.6	14.3	35.8	29.6	19.4	1.0
-2	1.1	ND			ND		
V-1	28.5	71.4:28.6	16.7	31.7	30.5	19.3	1.8
-2	5.9	ND			ND		
VI-1	28.6	95.2:4.8		44.2	53.8	2.0	Trace
-2	2.2	ND			ND		
VII-1	26.3	97.4:2.6		47.3	49.3	3.4	Trace
-2	0.7	ND			ND		

[a] Determined by the phenol–H_2SO_4 method and expressed as xylose equivalent. [b] Acid hydrolyzate was passed through a column of Dowex 1 (acetate form), yielding the neutral sugar fraction (NS). Acidic sugars (AS) were eluted from the column with 6M acetic acid. Contents of NS and AS were determined by the phenol–H_2SO_4 method. [c] ND, not determined.

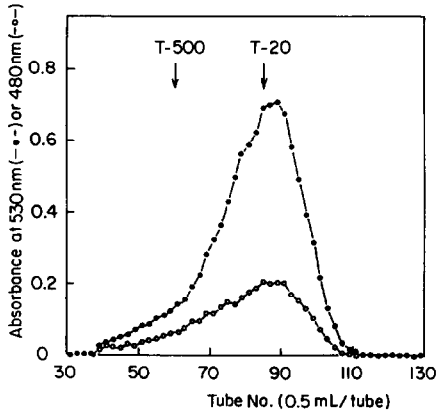


Fig. 2. Mol. wt. distribution of fraction IV-1. Fraction IV-1 was chromatographed on Sepharose CL-6B equilibrated with M ammonium acetate. Details are described in the text: -O-, total carbohydrate; -●-, uronic acid; T-500 and T-20 indicate the elution positions of Dextran T-500 and T-20 used for column calibration.

the overall percentages of neutral sugars, the ratios of GalA, Rha, Fuc, Ara, Xyl, Man, Glc, Gal, UI (unidentified sugar)-1, and UI-2 were 75.9:4.6:1.1:5.2:3.5:0.5:1.5:5.4:1.1:1.2 (Table II). Fractions AH-1 to AH-4 were analyzed by p.c. (method B) (Fig. 4); AH-4 is indistinguishable from authentic GalA. A substance having R_{GalA} 0.70 was detected on the chromatograms of AH-2 and AH-3. Upon hydrolysis of AH-2 and AH-3, GalA and Rha were disclosed, suggesting an oligosaccharide, GalA-(1→2)-Rha (R_{GalA} 0.75)[3].

Linkage analysis of the neutral sugars of fraction IV-1 showed that non-reducing terminal Ara (4.6%), non-reducing terminal Xyl (0.8%), 5-linked Ara and/or 3-linked Xyl (1.0%), 4- and/or 2-linked Xyl (5.3%), non-reducing terminal Gal (5.1%), 2,4-linked Rha (12.1%), 3,4- and/or 2,4-linked Xyl (10.2%), 4-linked Glc (0.8%), 3-linked Gal (1.8%), 6-linked Glc (3.8%), and 6-linked Gal (5.8%) were the prominent structural units.

These results, especially the presence of 2,4-linked Rha and isolation of GalA-(1→2)-Rha, indicate that all or part of the acidic polysaccharide in fraction IV-1 is a rhamnogalacturonan, to which poly- or oligo-saccharide composed of other neutral sugar residues is attached at O-4 of some of the Rha residues of the Rha–GalA backbone.

Partial acid hydrolysis of fraction IV-1. — Fraction IV-1 was partially hydrolyzed

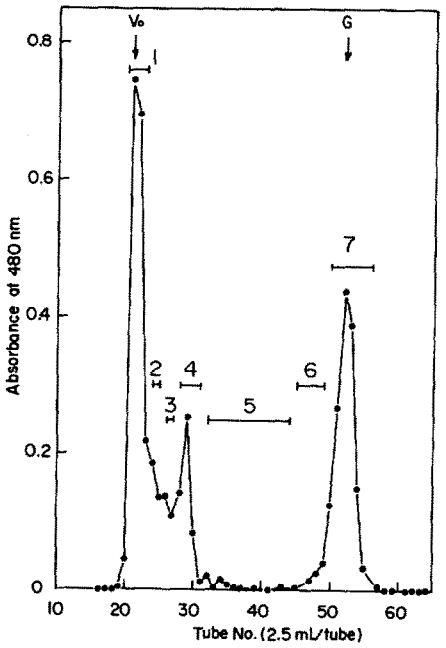


Fig. 3. Resolution of the acid hydrolyzate of fraction IV-1: the hydrolyzate was chromatographed on Bio-Gel P-2 and seven fractions, AH-1 (1) through AH-7 (7), were obtained. Details are described in the text; Vo and G indicate the elution positions of Blue Dextran and Glc used for column calibration.

with oxalic acid, and the hydrolyzate was resolved into five fractions (PAH-1 through PAH-5) by chromatography on Bio-Gel P-2 (Fig. 5).

PAH-4 and PAH-3 were subjected to preparative p.c. (method *A*). Oligosaccharides having R_{Xyl} values of 0.70 and 0.32 were obtained from PAH-4 and PAH-3, respectively. Paper chromatographic analysis of the partial and complete acid hydrolyzates of these oligosaccharides showed that the oligosaccharides having R_{Xyl} values 0.70 and 0.32 were β-Xyl-(1→4)-Xyl and β-Xyl-(1→4)-β-Xyl-(1→4)-Xyl, respectively.

When the major fraction PAH-1 was subjected to DEAE-Sephadex A-25 chromatography, it yielded a single peak eluting between 0.25 and 0.4M NaCl. PAH-1 was further hydrolyzed with CF_3CO_2H, and the hydrolyzate was resolved into three fractions (PAH-1-a, -b, and -c) by chromatography on Bio-Gel P-2 (Fig. 6A). Chromatographic analysis of PAH-1-a on Bio-Gel P-2 equilibrated with M ammonium acetate showed that the oligomer consisted of oligo-galacturonans having various d.p.'s (Fig. 6B). PAH-1-b was analyzed by p.c. (method *B*). The oligosaccharide with R_{GalA} 0.70, upon acid hydrolysis, gave Rha and GalA, consistent with the structure GalA-(1→2)-Rha. PAH-1-c (monosaccharide fraction) consisted of Rha, Fuc, Ara, Xyl, UI-2, Man, Glc, and Gal in the ratios 20.2:2.7:7.2:21.3:0.2:0.9:2.8:44.8.

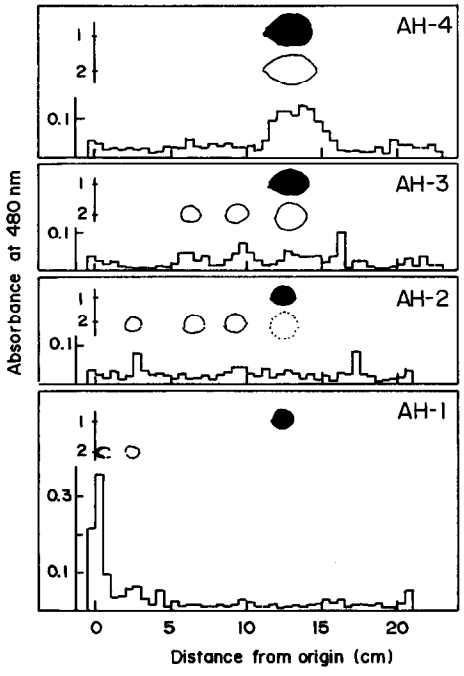


Fig. 4. Paper chromatographic analysis of AH-1 through AH-4 obtained in Fig. 3. AH-1 (650 μg as GalA equiv.), AH-2 (72 μg), AH-3 (62 μg), and AH-4 (110 μg) were individually subjected to p.c. (method *B*). Then the filter paper was cut into strips (1 × 0.5 cm), carbohydrates on the strips were extracted with 0.5 mL of distilled water, and the carbohydrate content in each solution was determined by the phenol–H_2SO_4 method. A guide strip was treated with alkaline silver nitrate: 1, authentic GalA; 2, samples.

TABLE II

Yields and sugar composition of fractions obtained from the acid hydrolyzate of fraction IV-1 after chromatography on Bio-Gel P-2 (Fig. 3)

Fraction	*Tube Nos. in Fig. 3.*	*Sugar content*		*Neutral sugar composition (wt%)*								
		As[a] *(μg)*	*NS*[b] *(μg)*	*Rha*	*Fuc*	*UI-1*[c]	*Ara*	*Xyl*	*UI-2*[c]	*Man*	*Glc*	*Gal*
AH-1	20–23	2600	233.0	25.5	10.3	5.8	14.8	4.1	8.7	4.9	8.7	17.3
AH-2	24–25	240	31.6	40.5	7.3	0	1.6	2.2	3.8	4.7	19.0	20.9
AH-3	26–27	210	33.7	40.9	7.1	0.6	2.7	2.7	2.1	4.7	21.1	18.1
AH-4	28–31	610	28.6	11.2	2.4	3.1	6.3	1.0	9.4	3.5	27.3	35.7
AH-5	32–44	22	26.8	1.9	1.5	4.5	10.1	4.5	11.6	7.1	28.4	30.6
AH-6	45–49	0	116.4	9.4	2.1	29.9	9.4	4.6	24.2	2.7	8.2	9.6
AH-7	50–56	0	698.0	17.6	3.0	0.3	28.8	21.7	0	0.7	2.4	25.5
Total		3682	1168.1	19.1	4.6	4.5	21.6	14.5	4.8	2.2	6.4	22.3

[a] Acidic sugar (AS) was determined by the carbazole–H_2SO_4 method. [b] Neutral sugar (NS), Rha + Fuc + UI-1 + Ara + Xyl + UI-2 + Man + Glc + Gal.
[c] Unidentified sugar 1 or 2.

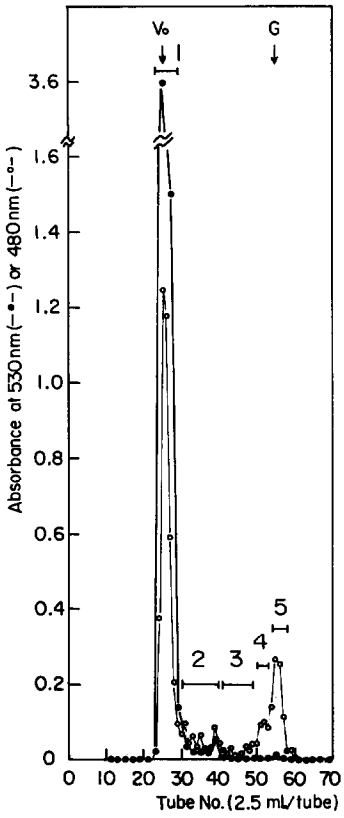


Fig. 5. Resolution of the partial acid hydrolyzate of fraction IV-1: the hydrolyzate was chromatographed on Bio-Gel P-2 and five fractions, PAH-1 (1) through PAH-5 (5), were obtained. Details are described in the text; -O-, total carbohydrate; -●-, uronic acid.

These results indicate that fraction IV-1 contains rhamnogalacturonan with other neutral sugar residues, and that Xyl residues detected in IV-1 are derived mostly from a β-(1→4)-xylan chain.

Enzymic hydrolysis of fraction IV-1. — When fraction IV-1 was treated with pectate lyase or with pectin lyase, an increase in absorbance at 235 nm was observed, indicating hydrolysis of the carbohydrate and the formation of oligomers with 4,5-unsaturated HexA at the non-reducing terminals. This absorbance was observed in products of both reactions. Therefore, fraction IV-1 was treated with pectate lyase and then with pectin lyase. The enzymic hydrolyzate was chromatographed on DEAE-Sephadex A-25 and two carbohydrate fractions, EH-1 and EH-2, were obtained (Fig. 7). EH-1 and EH-2 were separately subjected to Bio-Gel P-2 and P-6 chromatography: one major carbohydrate fraction. EH-A, was obtained from EH-1, and two major carbohydrate fractions, EH-B and EH-C, from EH-2 (Fig. 8). The ratios of EH-A, EH-B, and EH-C were 1:3.7:2, and the total amount of EH-A, -B and -C accounts for ~75% of the total carbohydrate in fraction IV-1.

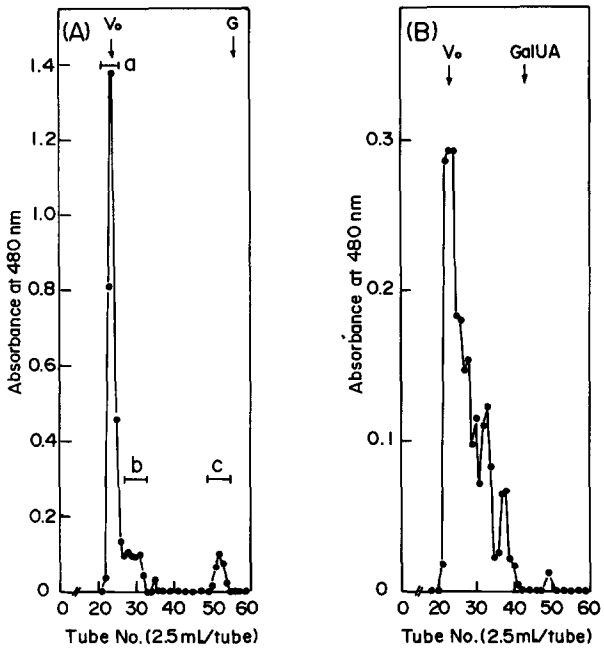


Fig. 6. A, Resolution of the acid hydrolyzate of fraction PAH-1: the hydrolyzate was chromatographed on Bio-Gel P-2 and three fractions, PAH-1-a, -b, and -c, were obtained. B, Chromatographic analysis of PAH-1-a was conducted on Bio-Gel P-2 equilibrated with M ammonium acetate. PAH-1-a obtained in A was chromatographed on Bio-Gel P-2 equilibrated with M ammonium acetate. Details are described in the text.

EH-C consisted of GalA and Rha in the ratio 98.5:1.5. When EH-C was analyzed by p.c. (method *B*), three compounds exhibiting R_{GalA} 0.52, 0.18, and 0.03 were detected. These products are thought to correspond to the oligosaccharides previously isolated from the pectate lyase hydrolyzate of a soluble *Zea* pectin, *i.e.*, di-, tri-, and tetrasaccharides of GalA, the non-reducing terminal residues of which are 4,5-unsaturated HexA[4].

EH-A and EH-B consisted of Rha, Fuc, Ara, Xyl, Gal, and GalA in the ratios 10.9:1.5:13.1:16.9:27.7:30.0 and 8.5:1.7:11.8:6.6:17.3:54.0, respectively. EH-A and EH-B were separately subjected to glycosidic linkage analysis of the neutral sugar moieties, and the results are summarized in Table III.

The results clearly show that fraction IV-1, liberated from wall fragments of *Zea* shoot cell-walls, contains a rhamnogalacturonan with associated neutral sugars. When fraction IV-1 was treated with pectate lyase and with pectin lyase, an increase in absorbance at 235 nm indicated hydrolysis of the carbohydrate into oligomers with 4,5-unsaturated HexA at the non-reducing terminals. However, the extent of hydrolysis of fraction IV-1 was lower than that of the *Zea* pectin[4] which was used here as a reference compound. This result suggests that the rhamnogalacturonan in fraction IV-1 has more branches than the soluble *Zea* pectin, and also that the mol. wt. of the associated pectin is smaller than that of the soluble pectin.

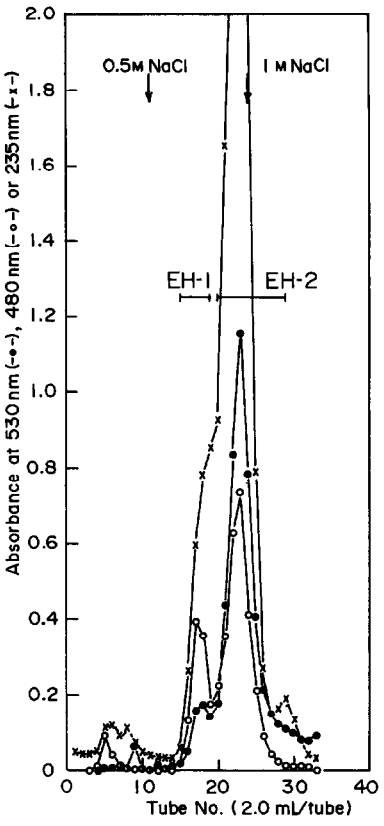


Fig. 7. Resolution of the enzymic hydrolyzate of fraction IV-1. After treatment with pectate lyase and pectin lyase, fraction IV-1 was chromatographed on DEAE-Sephadex A-25 and two fractions, EH-1 and EH-2, were obtained. Details are described in the text: -O-, total carbohydrate; -●-, uronic acid; -X-, 4,5-unsaturated GalA.

Upon hydrolysis with pectate lyase and pectin lyase, fraction IV-1 yielded mainly three carbohydrate fractions, EH-A through -C. EH-C consisted of di-, tri-, and tetra-saccharides of GalA, the non-reducing terminal residues of which are 4,5-unsaturated HexA. It is uncertain whether these di-, tri-, and tetra-saccharides are derived from polygalacturonic acid molecules or are covalently linked to rhamnogalacturonan chains present in EH-A and EH-B. From the results of the glycosidic linkage analysis of EH-A and EH-B (Table III) and the results of acid hydrolysis and partial acid hydrolysis of fraction IV-1, we can conclude that EH-B and EH-A contain at least the following structural units, although it is unclear whether these units are in one polymer.

(*a*) Detection of 2- and 2,4-linked Rha indicates the presence of rhamnogalacturonan in both EH-A and EH-B: Rha residues are inserted in galacturonan chains and a portion of the Rha residues serve as points of attachment for other neutral glycosyl residues.

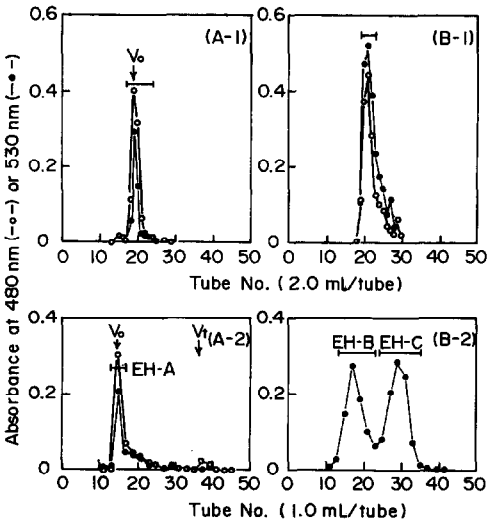


Fig. 8. Resolution of EH-1 and EH-2 obtained in Fig. 7. EH-1 was chromatographed on Bio-Gel P-2, and tubes 17–24 were combined and concentrated (A-1). The concentrate was then chromatographed on Bio-Gel P-6, and tubes 13–17 were combined to give EH-A (A-2). EH-B and EH-C were prepared from EH-2 after chromatography on Bio-Gel P-2 and P-6 (B-1 and B-2). Details are described in the text.

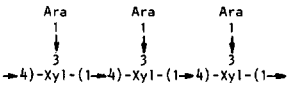
TABLE III

Sugar linkage composition [a] of EH-B and EH-A

G.l.c. peak No.	*Sugar linkage*	*Percentage of total area*	
		EH-B	*EH-A*
1	T-Ara[b]	10.5	7.5
2	T-Rha	10.6	2.3
3	T-Xyl	9.8	6.6
4	T-Fuc	4.3	3.4
5	2-Rha	5.1	3.7
6	3-Ara	0.9	3.2
7	3-Rha	8.4	4.3
8	3-Xyl	2.6	7.5
9	5-Ara	6.8	6.0
10	T-Gal and/or 4(2)-Xyl	10.0	14.8
11	Unidentified	7.2	1.2
12	Unidentified	3.4	6.7
13	3,4-Rha	3.7	0.9
14	2,4-Rha	3.6	5.0
15	3,4-Xyl and/or 2,4-Xyl	5.6	17.9
16	2,4-Gal	7.4	8.8

[a] The partially methylated alditol acetates obtained from the acid hydrolyzates of the methylated EH-B and EH-A were analyzed by g.l.c. on a glass capillary column (0.25 mm × 15 m) of DB-225. The column oven temperature was raised linearly from 140 to 200° at 2°/min. [b] T connotes a terminal residue.

(*b*) Although terminal Gal and 2- and 4-linked Xyl were not separated by the column used for analysis of partially methylated alditol acetates, peak 10 of EH-B (Table III) was shown to be free from 2- and 4-linked Xyl by g.l.c.–m.s. This finding and the detection of 3-linked Xyl and terminal Ara indicate the presence of oligoarabinoxylan as shown in structure **1**. The length of the xylan chain was calculated from the ratio of 3- and 3,4-linked Xyl. However, peak 10 of EH-A indicates 2- and/or 4-linked Xyl together with terminal Gal. This combination suggests that the xylan chain of the oligoarabinoxylan in EH-A is longer than EH-B and that unsubstituted xylosyl regions do persist in EH-A (see structure **2**).



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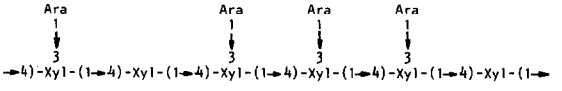

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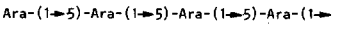

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(*c*) Detection of 5-linked Ara indicates the presence of an arabinan chain (structure **3**), the length of which may be as many as four sugars in EH-B. The length was calculated from the equation [(total terminal Ara) − (terminal Ara in oligoarabinoxylan)]/ 5-linked Ara.

Arabinan and galactan side chains are known[5,6] to be linked to Rha residues of rhamnogalacturonan main-chains through O-4.

We detected oligoarabinoxylans in EH-B and EH-A. These structures are considered to be remnants of the glucuronoarabinoxylan, which persist after sequential hydrolysis with purified endo-xylanase[1] and glucuronoxylanase[2,7]. Recently, there was a report of a relationship between rhamnogalacturonan and arabinoxylan, suggesting an association[7]. A definitive relationship between oligoarabinoxylan and the rhamnogalacturonan remains to be established, but the affinity of the components in fractions EH-A and EH-B supports this hypothesis.

Because the components of EH-B and EH-A, especially 3- and 3,4-linked Rha, are similar to the "rhamnogalacturonan II" found in suspension-cultured sycamore cell walls[8], the possibility that rhamnogalacturonan II is present cannot be excluded. The results of these studies point to the possibility that glucuronoarabinoxylan is covalently associated to rhamnogalacturonan in *Zea* shoot cell-walls.

EXPERIMENTAL

General methods. — Concentration of carbohydrate solutions was performed under reduced pressure at 35–40°. P.c. was performed on Toyo No. 50 filter paper by the multiple ascending method using 1-butanol–pyridine–water (6:4:3) (method *A*), or by the descending method using ethyl acetate–water–acetic acid–formic acid (18:4:3:1) (method *B*). Sugars on the chromatogram were detected with alkaline silver nitrate[9]. Total carbohydrate and uronic acid were determined by the phenol–H_2SO_4 method[10] and the carbazole–H_2SO_4 method[11], respectively. G.l.c. was conducted with a Yanagimoto Model G-80 gas chromatograph equipped with a flame-ionization detector and operated at a nitrogen flow rate of 15 mL per min.

Analysis of neutral sugars in poly- and oligo-saccharides. — Polysaccharides (10–50 μg) were hydrolyzed with 2M CF_3CO_2H for 5–6 h at 100°. Oligosaccharides (5–10 μg) were hydrolyzed with M CF_3CO_2H for 4–5 h at 100°. In each case, the hydrolyzate was evaporated to dryness. Alditol trifluoroacetates derived from sugars were analyzed by g.l.c.[12] on a column (0.4 × 200 cm) packed with 1.5% QF-1 on Chromosorb W at 140°.

Glycosidic linkage analysis of poly- and oligo-saccharides. — Each sample was methylated by the method of Hakomori[13], and the neutral-sugar linkage composition was determined by g.l.c. analysis of the derived alditol acetates[1].

Materials. — The water-insoluble fraction (8.76 g dry wt) of *Zea mays L.* (B73 × Mo17, Brayton Seed C., Ames, IA) shoot cell-walls was treated with purified β-glucanase and purified endo-xylanase as described previously[1,14]. The resulting water-insoluble product was suspended in 500 mL of 20mM sodium acetate buffer (pH 5.5) and incubated for 24 h at 35° with a glucuronoxylanase preparation[15] in the presence of a few drops of toluene. After incubation, the suspension was filtered (Whatman No. 1 paper), and the residue was washed with 100 mL of water. The filtrate and wash solution were combined and heated in a boiling water bath for 20 min. The residue, after washing with water, was incubated 5 additional times in sequence with the enzyme preparation under the same conditions. A total of ~3.1 L of combined filtrate and wash solution was obtained.

Resolution of the products of glucuronoxylanase treatment of cell walls. — The products (1245.4 mg as Xyl equiv./ 3 L of 50mM sodium acetate buffer, pH 5.0) was introduced to a DEAE-Sephadex A-25 column (5.5 × 18 cm) pre-equilibrated with 50mM sodium acetate buffer (pH 5.0). The column was washed with the same buffer (3 L). Absorbed materials were eluted stepwise with 0.1M NaCl (850 mL), 0.2M NaCl (1,020 mL), and 0.4M NaCl (1,020 mL) in sodium acetate buffer, and 0.5M NaOH (1,190 mL): 17-mL fractions were collected and assayed for carbohydrate and ferulic acid (Fig. 1). Unabsorbed materials (~6 L, fraction I) were concentrated to 337 mL, treated with Dowex 1 (H^+) resin, and concentrated to 50 mL. Methanol (250 mL) was added to the concentrate, and the solution was maintained at −20° overnight, then centrifuged. The precipitate was washed successively with methanol and acetone, and dried to yield fraction I-1. The supernatant solution was concentrated and dried, to yield

fraction I-2. Fractions II (tubes 14–55), III (83–103), IV (144–157), V (158–180), VI (211–223), and VII (224–235) were obtained upon elution of the DEAE-Sephadex column with NaCl or NaOH, and each was dialyzed against three changes of 500 mL of distilled water (12 h per cycle) in order to separate the fractions of smaller and larger mol. wt. Larger polymers (non-dialyzable fraction) and smaller oligomers (dialyzable fraction) were designated fractions 1 and 2, yielding the series II-1 and II-2 through VII-1 and VII-2.

Purification of fraction IV-1. — Fraction IV-1 (122 mg dry wt) was dissolved in 20 mL of 20mM sodium acetate buffer (pH 5.0) and centrifuged. The supernatant solution was applied to a DEAE-Sepharose CL-6B column (1.8 × 16 cm) equilibrated with the same buffer. The column was first eluted with 20mM sodium acetate buffer (pH 5.0) and then with a linear salt gradient (0–0.5M NaCl), followed by elution with 0.5M NaCl; the same buffer was used in the presence of salt. Fractions (3 mL) were collected and assayed for uronic acid, total carbohydrate, and ferulic acid. Tubes 63–70 (the elution pattern of total carbohydrate corresponded with that of uronic acid and with that of the ferulic acid) were combined and concentrated to a small volume. The concentrate was applied to a Bio-Gel P-4 column (2.7 × 16 cm) followed by elution with water. Fractions (3 mL) were collected and assayed for total carbohydrate. The phenol–H_2SO_4-positive fractions (the void fraction of the column) were combined and designated as fraction IV-1.

Mol. wt. distribution of fraction IV-1. — Fraction IV-1 was applied to a column (1.5 × 72 cm) of Sepharose CL-6B equilibrated with M ammonium acetate followed by elution with M ammonium acetate. Fractions (0.5 mL) were collected and assayed for uronic acid and total carbohydrate (Fig. 2). Dextrans T-500 (average mol. wt. 50×10^4) and T-20 (average mol. wt. 2×10^4) were used to calibrate the column.

Acid hydrolysis of fraction IV-1. — Fraction IV-1 (1300 μg as Xyl equiv. and 4.450 μg as GalA equiv.) was hydrolyzed with 2 mL of M CF_3CO_2H at 100° for 4.5 h. The resulting solution was evaporated to dryness in the presence of methanol. The hydrolyzate dissolved in 1 mL of water was applied to a Bio-Gel P-2 column (1.5 × 100 cm) and eluted with water at 45°. Fractions (2.5 mL) were collected and assayed for total carbohydrate. Tubes 20–23, 24–25, 26–27, 28–31, 32–44, 45–49, and 50–56 were separately combined to give fractions AH-1 through AH-7 (Fig. 3).

Partial acid hydrolysis of fraction IV-1. — Fraction IV-1 (5 460 μg as Xyl equiv. and 18 690 μg as GalA equiv.) was hydrolyzed with 4 mL of 0.1M oxalic acid at 100° for 3 h. The resulting solution was neutralized with NH_4OH and concentrated to a small volume. Resolution of the hydrolyzate was performed using a Bio-Gel P-2 column under the conditions described above. Tubes 23–29, 30–40, 41–49, 50–53, and 54–58 were separately combined to give fractions PAH-1 (yield: 21 000 μg as GalA equiv.) PAH-2 (780 μg as Xyl equiv. and 572 μg as GalA equiv.), PAH-3 (288 μg as Xyl equiv.), PAH-4 (430 μg as Xyl equiv.), and PAH-5 (1 050 μg as Xyl equiv.) (Fig. 5).

Chromatographic analysis on DEAE-Sephadex A-25 of PAH-1. — PAH-1 (7 778 μg as GalA equiv. in 6 mL of water) was applied to a column (1.7 × 7.5 cm) of DEAE-Sephadex A-25 equilibrated with 20mM sodium acetate buffer (pH 5.0). The

column was first eluted with this buffer, and then with a linear salt gradient (0–0.5M NaCl) followed by 0.5M NaCl in the same buffer. Fractions (3 mL) were collected and assayed for total carbohydrate and uronic acid.

Acid hydrolysis of PAH-1. — PAH-1 (10 500 μg as GalA equiv.) was hydrolyzed with 2 mL of M CF_3CO_2H at 100° for 3 h. The hydrolyzate was concentrated to dryness, and the concentrate was dissolved in 2 mL of water and centrifuged. The supernatant solution was chromatographed on Bio-Gel P-2 under the conditions described above. Tubes 21–26, 27–33, 49–55 were separately combined to give fractions PAH-1-a, -b, and -c (Fig. 6A).

Chromatography of PAH-1-a on Bio-Gel P-2. — A solution of PAH-1-a in 1 mL of M ammonium acetate was applied to a column (1.5 × 83.5 cm) of Bio-Gel P-2 equilibrated with M ammonium acetate and operated at 45°, followed by elution with M ammonium acetate. Fractions (2.5 mL) were collected and assayed for total carbohydrate (Fig. 6B). Apple pectin and GalA were used to calibrate the column.

Enzymic hydrolysis of fraction IV-1. — *Erwinia carotovora* pectate lyase (PAL) and *Pseudomonas marginalis* pectin lyase (PPL)[16], purified to single bands upon electrophoresis, were used. PAL (0.1 mg) and PPL (0.1 mg) were separately dissolved in 0.1 mL of 50mM Tris-HCl buffer (pH 8.2). A portion of purified fraction IV-1 (1 525 μg as GalA equiv.) dissolved in 3 mL of 50mM Tris-HCl buffer was incubated with PAL (10 μL) at room temperature. *Zea* pectin (3 180 μg as GalA equiv.), a reference compound[4], was incubated with 20 μL of PAL under the same conditions. Another portion of purified fraction IV-1 (1 525 μg as GalA equiv.) dissolved in 3 mL of Tris-HCl buffer (pH 8.2) was incubated with PPL (10 μL) at room temperature. At intervals, the incubation mixture was monitored for 4,5-unsaturated HexA by measuring the absorbance at 235 nm.

Resolution of PAL- and PPL-treated fraction IV. — Fraction IV-1 (7 125 μg as GalA equiv.) dissolved in 3 mL of 50mM Tris-HCl buffer (pH 8.2) was incubated with PAL (30 μL) at room temperature for 5 h, then with PPL (20 μL) for 5 h. The incubation mixture was applied to a Bio-Gel P-2 column (1.8 × 44 cm), followed by elution with water. Fractions (2 mL) were collected and assayed for total carbohydrate. The phenol–H_2SO_4-positive fractions (the void fraction of the column) were combined and concentrated to dryness. The dried material was dissolved in 1 mL of 50mM sodium acetate buffer (pH 5.0) and applied to a column (1.8 × 5.0 cm) of DEAE-Sephadex A-25 equilibrated with 50mM sodium acetate (pH 5.0). The column was washed with the same buffer (20 mL), and then eluted stepwise with 0.5M NaCl in the same buffer (26 mL) and M NaCl in the same buffer (24 mL). Fractions (2 mL) were collected, and assayed for total carbohydrate, uronic acid, and 4,5-unsaturated HexA. Tubes 15–19 and 20–29 were separately combined and concentrated to give EH-1 and EH-2 (Fig. 7).

Resolution of EH-1 and EH-2. — EH-1 (300 μg as GalA equiv. and 373 μg as Xyl equiv.) was dissolved in 1 mL of water and applied to a column (1.8 × 44 cm) of Bio-Gel P-2, followed by elution with water: 2-mL fractions were collected. Tubes 17–24 were combined and concentrated to dryness. The dried sample was dissolved in 100 μL of water and applied to a column (1.0 × 46.5 cm) of Bio-Gel P-6 equilibrated with M

ammonium acetate, followed by elution with M ammonium acetate: 1-mL fractions were collected. Tubes 13–17 were combined, concentrated, and desalted with Bio-Gel P-2, to give material designated EH-A (Fig. 8, A-1 and A-2).

EH-2 (2 400 μg as GalA equiv. and 1 000 μg as Xyl equiv.) was dissolved in 1 mL of water and applied to the same P-2 column, followed by elution with water. Tubes 19–23 were combined and concentrated to dryness. The dried sample was dissolved in 100 μL of water and applied to the same P-6 column, followed by elution with M ammonium acetate. Tubes 13–23 and 24–35 were separately combined, concentrated, and desalted with Bio-Gel P-2 to give EH-B and EH-C (Fig. 8, B-1 and B-2).

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