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Carbohydrate Research 264 (1994) 83–96

CARBOHYDRATE RESEARCH

Isolation and structural characterization of *endo*-rhamnogalacturonase-generated fragments of the backbone of rhamnogalacturonan I [1, 2]

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Received 21 February 1994; accepted 12 May 1994

Abstract

A combination of commercially available preparations of *Aspergillus niger* β-D-galactosidase, *endo*-α-L-arabinanase, α-L-arabinosidase, and *endo*-β-D-galactanase has been used to generate oligoglycosyl fragments of the backbone of rhamnogalacturonan I (RG-I) that had been isolated from the walls of suspension-cultured sycamore cells. The backbone-cleaving enzyme, which is present in the β-D-galactosidase preparation, only fragments the RG-I backbone when many of the neutral oligoglycosyl side chains have been removed by the other *exo*- and *endo*-glycanases. The oligosaccharides released from the backbone were separated from the partially fragmented RG-I and then purified, as their oligoglycosyl aldonic acids, by HPAEC–PAD. Those backbone fragments with degrees of polymerization (dp's) between 2 and 11 were characterized using one- and two-dimensional ^{1}H NMR spectroscopy, electrospray mass spectrometry, and glycosyl-residue and glycosyl-linkage composition analyses. Two series of oligoglycosyl fragments were identified. The quantitatively predominant series has the structure α-D-Gal*p*A-(1→2)-α-L-Rha*p*-[→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]$_n$-4-D-Gal*p*A, and the quantitatively minor series has the structure α-L-Rha*p*-[→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]$_n$-4-D-Gal*p*A (n = 1–5). Thus, the enzyme preparations contain an α-L-rhamnosidase in addition to the *endo*-rhamnogalacturonase. The products of the *endo*-rhamnogalacturonase provide additional evidence that the backbone of RG-I is composed of the diglycosyl repeating unit: →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. The *endo*-rhamnogalac-

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[1] This is number XXXVII of the series entitled, The Structure of Plant Cell Walls.

[2] Supported by U.S. Department of Energy grant DE-FG05-93ER20115 to AD and the DOE-funded (DE-FG09-93ER20097) Center for Plant and Microbial Complex Carbohydrates to PA and AD.

SSDI 0008-6215(94)00186-J

turonase from the *A. niger* β-D-galactosidase preparation and the *endo*-rhamnogalacturonase secreted by *Aspergillus aculeatus* [H.A. Schols et al. *Carbohydr. Res.*, 206 (1990) 117–129] have the same substrate specificities and generate similar oligoglycosyl fragments.

Keywords: Rhamnogalactouronan; *endo*-Rhamnogalactouronase; Characterization; Oligosaccharide; Polysaccharide

1. Introduction

The elucidation of the glycosyl sequence of rhamnogalacturonan I (RG-I), a pectic polysaccharide present in the cell walls of higher plants, is a research goal of this laboratory [1]. The backbone of RG-I, solubilized from suspension-cultured sycamore cell walls with an *endo*-α-(1→4)-polygalacturonase, has been shown [2] to consist of the diglycosyl repeating unit →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. Approximately 50% of the 2-linked rhamnosyl residues in the backbone of RG-I are substituted at C-4 with oligoglycosyl side chains composed primarily of arabinosyl and galactosyl residues [3,4]. No backbone fragments containing more than six glycosyl residues have been isolated from sycamore RG-I and characterized [3].

The availability of *endo*-glycanases that fragment the RG-I backbone would be of value in determining the distribution of arabinosyl- and galactosyl-containing oligoglycosyl side chains. Recently, an *endo*-rhamnogalacturonase was isolated from Ultra Sp., a commercial enzyme preparation from *Aspergillus aculeatus* [5]. The *A. aculeatus* enzyme fragmented the backbone of partially degraded apple juice rhamnogalacturonan but was not active on native, undegraded rhamnogalacturonan [5]. Some of the oligoglycosyl fragments generated by the *A. aculeatus endo*-rhamnogalacturonase (including a tetraglycosyl backbone fragment substituted with zero, one, or two galactosyl residues) were structurally characterized [6]. No backbone fragments larger than a tetrasaccharide were characterized.

We now report that an *endo*-rhamnogalacturonase is present in a commercially available preparation of an *A. niger* β-D-galactosidase. Treatment of sycamore RG-I with this enzyme preparation, together with preparations of *endo*-β-D-galactanase, α-L-arabinosidase, and *endo*-α-L-arabinanase, generates RG-I backbone fragments with degrees of polymerization (dp's) between 2 and 11. The purification to homogeneity and structural characterization of those backbone-derived oligosaccharides is described in this paper.

2. Results and discussion

An enzyme capable of fragmenting the backbone of RG-I is present in a commercial β-D-galactosidase-containing preparation.—Previous studies in our laboratory have shown that treatment of sycamore RG-I with a combination of *exo*- and *endo*-glycanases is a useful technique for generating oligoglycosyl fragments that can be structurally analyzed [4]. Such combinations of enzymes have also been used to generate an "arabinose-free" RG-I [4]. We have now found that acidic oligoglycoses, enriched in rhamnosyl and galactosyluronic acid residues, are also generated when sycamore RG-I is treated for 36 h with a

mixture of commercial preparations of β-D-galactosidase, β-D-galactanase, α-L-arabinanase, and α-L-arabinosidase. The dp's of those enriched oligoglycoses decreased when sycamore RG-I was treated for extended periods (> 48 h) with a mixture of *exo*- and *endo*-glycanases (data not shown). Those results suggest that one of the enzyme preparations contains an enzyme capable of fragmenting the RG-I backbone, since neither the rhamnosyl nor galactosyluronic acid residues are present in the side chains of RG-I. However, treatment of sycamore RG-I with each enzyme preparation by itself did not result in the generation of backbone fragments. This result is consistent with the findings of a previous study [5] showing that the *endo*-rhamnogalacturonase secreted by *A. aculeatus* fragments the rhamnogalacturonan backbone only when many of the neutral oligoglycosyl side chains are removed. Thus, to assay enzyme preparations for *endo*-rhamnogalacturonase activity, an RG-I is required that has a low degree of backbone substitution with oligoglycosyl side chains. Such an RG-I substrate has recently been isolated from the cell walls of pea seedlings [7]. Treatment of pea RG-I with individual *exo*- and *endo*-glycanase preparations showed that the *endo*-rhamnogalacturonase activity was present only in the β-D-galactosidase preparation.

Pea RG-I was not available in quantities sufficient to generate backbone fragments in the amounts required for the structural studies described in this paper. Thus, sycamore RG-I was treated with a mixture of *exo*- and *endo*-glycanses. The resulting side chain-depleted acidic fragments were isolated and purified by semipreparative HPAEC–PAD, and then structurally characterized.

Generation of backbone fragments by treating sycamore RG-I with a mixture of exo- *and* endo-*glycanases*.—To obtain backbone fragments in quantities sufficient for structural analysis, sycamore RG-I (50 mg) was treated with a combination of commercial preparations of β-D-galactosidase, *endo*-β-D-galactanase, *endo*-α-L-arabinanase, and α-L-arabinosidase. The products were subjected to Bio-Gel P-10 gel-permeation chromatography yielding Fractions A–D (Fig. 1). Fraction A, which accounted for 23% of the material recovered from the gel-permeation column, is composed largely of galactosyl, rhamnosyl, and galactosyluronic acid residues (Table 1). Such a glycosyl-residue composition would

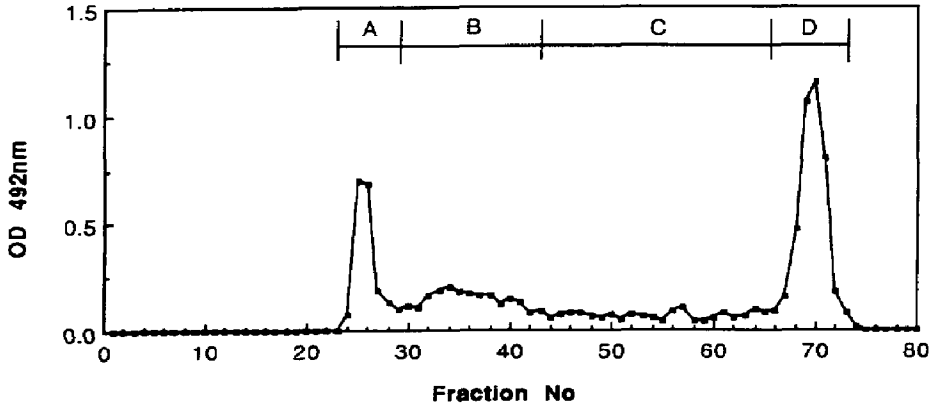

Fig. 1. Bio-Gel P-10 separation of the products generated by *exo*- and *endo*-glycanase treatment of sycamore RG-I. Fractions (3 mL) were collected, and portions (30 μL) analyzed colorimetrically ($OD_{492\,nm}$) for carbohydrate with the phenol–H_2SO_4 reagent [16]. The four fractions (A–D) were pooled as shown by the bars.

Table 1
Glycosyl-residue compositions of sycamore RG-I and the oligoglycose fractions isolated by Bio-Gel P-10 chromatography of the products generated by digestion of sycamore RG-I with a mixture of *exo*- and *endo*-glycanase preparations

Glycosyl residue	RG-I	Bio-Gel P-10 Fraction [a]			
		A	B	C	D
Ara	29	6	8	3	64
Gal	28	44	12	5	22
Rha	17	18	38	44	5
Fuc	1	2	1	nd [b]	nd
Xyl	3	1	nd	nd	nd
GalA	20	22	39	44	9
GlcA	1	3	2	2	nd
4-*O*-MeGlcA	1	4	nd	2	nd
Yield of all products recovered from Bio-Gel column (%)		23	13	13	51

[a] See Fig. 1 and text for details.
[b] Not detected.

be expected of enzymatically modified RG-I, as a previous study [4] has shown that 95% of the arabinosyl residues and up to 80% of the galactosyl residues are removed by treating sycamore RG-I with a mixture of *exo*- and *endo*-glycanses.

Rhamnosyl and galactosyluronic acid residues together account for 78 mol% of Fraction B (Table 1). This fraction, which accounts for 13% of the material recovered from the column, elutes in the region for oligoglycoses with a dp ~15 and is thus likely to contain RG-I backbone-enriched fragments. Since arabinosyl and galactosyl residues together accounted for 20 mol% of Fraction B (Table 1), some of the fragments must contain oligoglycosyl side chains.

Rhamnosyl and galactosyluronic acid residues account for 88 mol% of Fraction C (Table 1). This fraction, which also accounts for 13% of the material recovered from the column, elutes in the region for oligoglycoses with dp's between 2 and ~14. Thus, Fraction C is likely to contain enzymatically generated RG-I backbone fragments. Arabinosyl and galactosyl residues account for 86 mol% of Fraction D. This fraction, which accounts for 51% of the material recovered from the column, elutes in the included volume of the Bio-Gel P-10 column and was shown, by HPAEC–PAD (data not shown), to contain mono- and disaccharides, thereby confirming that a mixture of *exo*- and *endo*-glycanase does hydrolyze the neutral oligoglycosyl side chains of RG-I. Since Fraction D also contains rhamnosyl and galactosyluronic acid residues, the mixture of *exo*- and *endo*-glycanases used to fragment the RG-I backbone must contain *exo*-galacturonidase and *exo*-rhamnosidase activities.

Semipreparative HPAEC–PAD purification of the oligoglycosyl aldonic acids generated by $NaBH_4$ reduction of Fraction C.—HPAEC–PAD is a powerful technique for separating complex mixtures of acidic oligoglycoses [8,9]. However, the alkaline eluants used to elute the carbohydrates from the CarboPac column cause base-catalyzed degradation of reducing oligoglycoses [9]. Thus, the acidic oligoglycoses in Fraction C were partially purified using low-pressure DEAE-Sephadex anion-exchange chromatography and then reduced with

$NaBD_4$ to generate their corresponding oligoglycosyl aldonic acids prior to HPAEC–PAD. Those oligoglycosyl aldonic acids were purified to homogeneity by semipreparative HPAEC–PAD and then structurally characterized.

Galactonic acid (GalA'ol), which is the $NaBD_4$ reduction product of galacturonic acid, accounts for 9 mol% of $NaBD_4$-reduced Fraction C. Only trace amounts of rhamnitol were detected. Thus, galacturonic acid is the reducing-end residue of the acidic oligoglycoses in Fraction C. These results establish that most, if not all, of the enzyme-generated RG-I backbone fragments result from the cleavage of the glycosyl linkages between the 4-linked galactosyluronic acid residues and the 2-linked rhamnosyl residues.

The oligoglycosyl aldonic acids generated by $NaBD_4$ reduction of Fraction C were separated into 12 fractions (R1–R6 and G1–G6) on a semipreparative CarboPac PA-1 column (Fig. 2). The fractions were neutralized, desalted, and then freeze-dried. The components in Fractions R1–R5 and G1–G5 were characterized by electrospray mass spectrometry (ESMS), glycosyl-residue and glycosyl-linkage composition analyses, and ^1H NMR spectroscopy. Fractions R6 and G6 were only available in amounts sufficient for glycosyl-residue and glycosyl-linkage composition analyses.

ESMS analysis of the oligoglycosyl aldonic acids purified by HPAEC–PAD of $NaBD_4$-reduced Fraction C.—The positive-ion ESMS of Fractions R1–R5 and G1–G5 each contained two major ions corresponding to $[M+Na]^+$ and $[M+Na-18]^+$ (see Table 2) and a less intense ion corresponding to $[M+H-18]^+$. For example, the ESMS of R2 contained ions at m/z 688, 670, and 648 corresponding to $[M+Na]^+$, $[M+Na-18]^+$, and $[M+H-18]^+$, respectively, for a triglycosyl aldonic acid composed of two rhamnosyl residues, one galactosyluronic acid residue, and galactonic acid. The $[M+Na-18]^+$ and the $[M+H-18]^+$ ions originate from the lactone form of the aldonic acid located at the reducing end of each oligosaccharide. Aldonic acids are known [10] to form lactones at low pH; the solvent used for ESMS contained 1% HCl and had a pH of ~2.

The results of the ESMS analyses provide strong evidence that acidic oligoglycoses with dp's between 2 and 11 were generated by enzymatic fragmentation of the RG-I backbone. No ESMSs were obtained for Fractions R6 and G6. However, on the basis of their glycosyl-

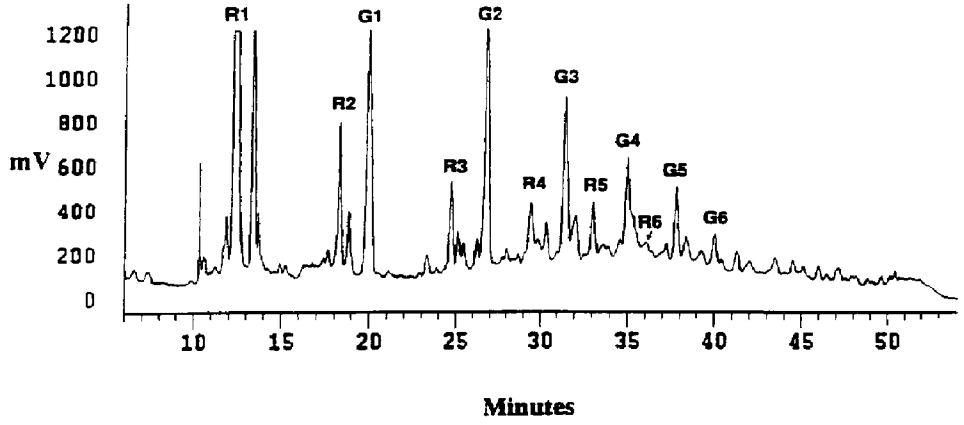

Fig. 2. Semipreparative HPAEC–PAD separation of the oligoglycosyl aldonic acids generated by $NaBD_4$ reduction of Bio-Gel P-10 Fraction C. The peaks which eluted from the HPAE column were collected as shown, desalted, and then freeze-dried.

Table 2
ESMS pseudomolecular ions of the oligoglycosyl aldonic acids separated by semipreparative HPAEC–PAD of Bio-Gel P-10 Fraction C

HPAE fraction [a]	Pseudomolecular ion (*m/z*)		Deduced number of glycosyl residues		
	$[M+Na]^+$	$[M+Na-18]^+$	GalA	Rha	GalA'ol [b]
R1	366 [c]	348 [d]	0	1	1
R2	688	670	1	2	1
R3	1010	992	2	3	1
R4	1332	1314	3	4	1
R5	1654	1636	4	5	1
G1	542	524	1	1	1
G2	864	846	2	2	1
G3	1186	1168	3	3	1
G4	1508	1490	4	4	1
G5	1830	1812	5	5	1

[a] See Fig. 1 and text for details.
[b] GalA'ol = galactonic acid.
[c] The pseudomolecular ion of the oligoglycosyl aldonic acid in which the galactonic acid at the reducing end was not converted into the lactone form by the acidic solvent (pH ~2) used for the ESMS.
[d] The pseudomolecular ion of the oligoglycosyl aldonic acid in which the galactonic acid at the reducing end was converted into the lactone form by the acidic solvent (pH ~2) used for the ESMS.

residue and glycosyl-linkage compositions (Tables 2 and 3) and their HPAEC–PAD retention times (see Fig. 2), we hypothesize that Fractions R6 and G6 contain acidic dodecaglycose and tridecaglycose backbone fragments, respectively.

The ESMSs of those oligoglycosyl aldonic acids with dp's > 6 (R3–R5 and G3–G5, see Table 2) each contain, in addition to the $[M+Na]^+$, $[M+Na-18]^+$, and $[M+H-18]^+$ pseudomolecular ions, ions corresponding to fragmentation of the glycosidic linkage between the rhamnosyl and galactosyluronic acid residues and the linkage between the

Table 3
Glycosyl-residue compositions of the oligoglycosyl aldonic acids purified by semipreparative HPAEC–PAD of Bio-Gel P-10 Fraction C

Glycosyl residue	HPAEC fraction [a]											
	R1	R2	R3	R4	R5	R6	G1	G2	G3	G4	G5	G6
	Mol%											
Rha	49	40	43	47	46	48	35	40	42	43	44	44
GalA	nd [b]	30	35	37	39	37	34	40	43	43	44	46
GalA'ol [c]	51	30	22	14	12	11	31	20	15	12	9	6
Gal	nd	nd	nd	2	2	3	nd	nd	nd	2	2	3
Ara	nd	nd	nd	tr [d]	1	1	nd	nd	nd	tr	1	1

[a] See Fig. 2 and text for details.
[b] Not detected.
[c] Galactonic acid.
[d] Trace, present in less than 1 mol%.

rhamnosyl residue and galactonic acid. No fragment ions were detected from those oligoglycosyl aldonic acids with dp's < 6.

Fragment ions are not usually observed when oligosaccharides are analyzed by ESMS [11]. However, fragment ions have been observed in the ESMSs of peptides [12]. Those ions are believed to be generated by intermolecular collisions in the region between the capillary inlet of the electrospray source and the quadrupole mass analyzer [12]. The oligoglycosyl aldonic acids (analyzed by ESMS in aq 30% MeOH containing 1% HCl) were shown, by HPAEC–PAD, to each contain only a single component. Thus, the observed oligoglycosyl aldonic acid fragment ions are generated in the ES source and do not originate from fragments generated prior to ESMS by hydrolysis of the glycosidic linkages of the oligoglycosyl aldonic acids.

The fragment ions in the ESMSs of the oligoglycosyl aldonic acids originate from both the nonreducing and the galactonic acid-terminated portions (Z fragments) of each of the ($[M+Na]^+$, $[M+Na-18]^+$, and $[M+H-18]^+$) pseudomolecular ions. For example, fragment ions originating from the aldonic acid terminus of the $[M+Na-18]^+$ ion (m/z 1314) of heptaglycosyl aldonic acid R4 (see Fig. 3) correspond to the sequential loss of galactonic acid (m/z 1135), a rhamnosyl residue (m/z 989), a galactosyluronic acid residue (m/z 813), and then another rhamnosyl residue (m/z 667). The corresponding fragment ions originating from the aldonic acid terminus of the $[M+H-18]^+$ and $[M+Na]^+$ pseudomolecular ions were observed at m/z 1113, 967, 791, and 645 and at m/z 1007, 831, and 685, respectively (see Fig. 3). The fragment ions originating from the nonreducing terminus of the $[M+Na]^+$ ion (m/z 1332) of heptaglycosyl alditol R4 (see Fig. 3) correspond to the sequential loss of a rhamnosyl (m/z 1186), a galactosyluronic acid (m/z 1010), another rhamnosyl (m/z 864), and another galactosyluronic acid residue (m/z 688). The corresponding fragment ions originating from the nonreducing terminus of the $[M+H-18]^+$ and $[M+Na-18]^+$ pseudomolecular ions were observed at m/z 1146, 970, and 824 and at m/z 1168, 992, and 846, respectively (see Fig. 3). No fragment ions were observed corresponding to the loss of more than four residues from the aldonic acid-terminated or the nonreducing terminus of the oligoglycosyl aldonic acids (see Fig. 3). The fragmentation in the ES source of oligoglycosyl aldonic acids with dp's > 6 provides compelling evidence that those fragments are indeed composed of a diglycosyl repeating unit. However, our finding that oligosaccharide fragment ions are generated in the ES source demonstrates that the ESMSs of acidic oligosaccharides with unknown structures need to be interpreted with caution.

Glycosyl-residue and glycosyl-linkage composition analyses of the oligoglycosyl aldonic acids purified by HPAEC–PAD of Fraction C.—The ESMS analyses provide strong evidence that the oligoglycosyl aldonic acids are composed of a repeating diglycosyl unit. This conclusion is confirmed and extended by the results of glycosyl-residue and glycosyl-linkage composition analyses (Tables 3 and 4). The predominant components of Fractions R1–R6 and G1–G6 were each shown to be composed only of rhamnosyl and galactosyluronic acid residues and galactonic acid (Table 3). Galactosyl and arabinosyl residues were not detected in Fractions R1–R3 and G1–G3, but together those glycosyl residues account for between 2 and 4 mol% of Fractions R4–R6 and G4–G6. Thus, some of the oligoglycosyl aldonic acids with dp's > 8 contain small amounts of side chain glycosyl residues. However, the

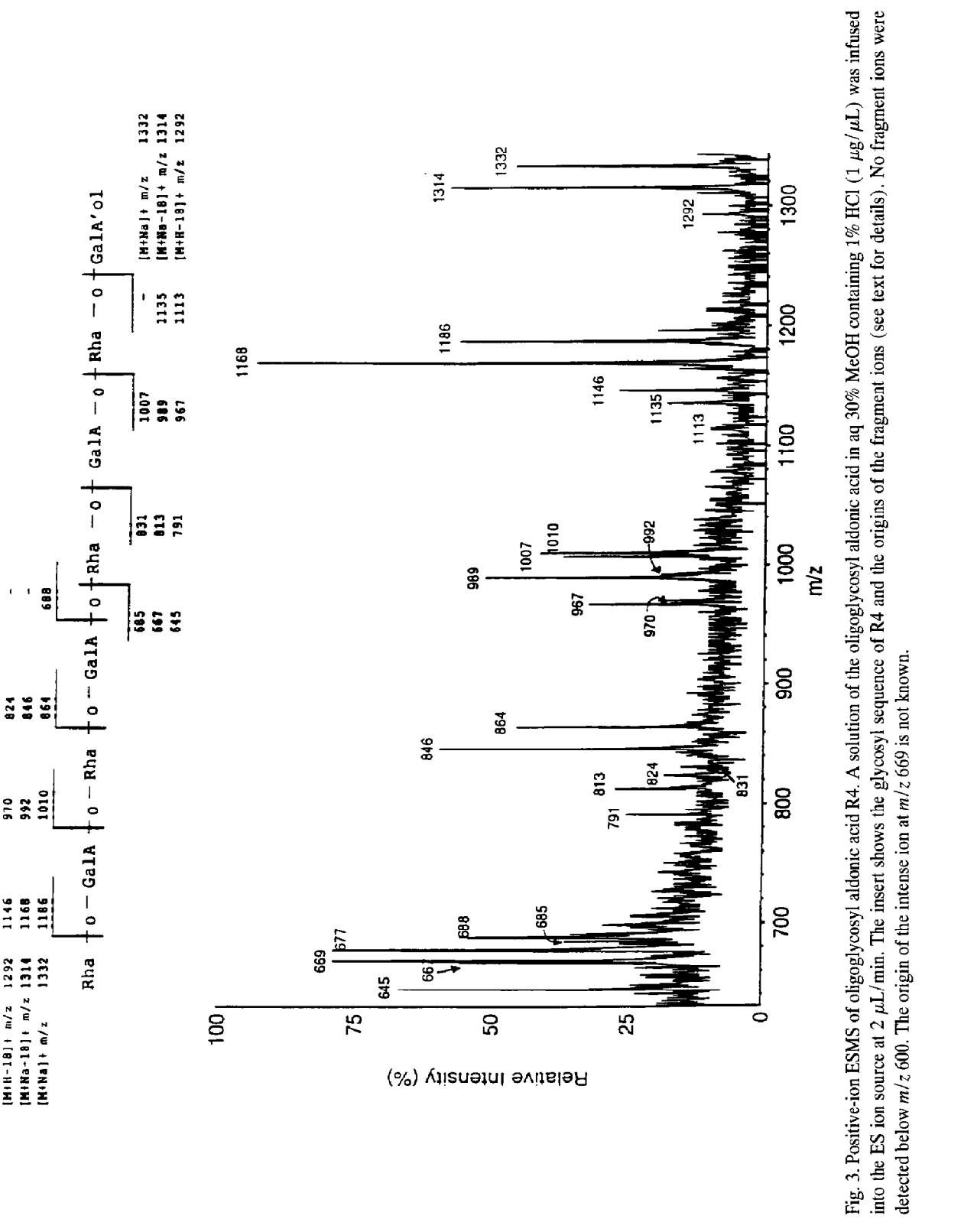

Fig. 3. Positive-ion ESMS of oligoglycosyl aldonic acid R4. A solution of the oligoglycosyl aldonic acid in aq 30% MeOH containing 1% HCl (1 $\mu g/\mu L$) was infused into the ES ion source at 2 μL/min. The insert shows the glycosyl sequence of R4 and the origins of the fragment ions (see text for details). No fragment ions were detected below m/z 600. The origin of the intense ion at m/z 669 is not known.

Table 4
Glycosyl-linkage compositions of the oligoglycosyl aldonic acids purified by semipreparative HPAEC–PAD of Bio-Gel P-10 Fraction C

Glycosyl residue	HPAEC fraction [a]											
	R1	R2	R3	R4	R5	R6	G1	G2	G3	G4	G5	G6
	Mol%											
T-Rha [b]	51	25	18	13	11	8	nd [c]	nd	nd	nd	tr [d]	tr
2-Rha	nd	25	34	39	42	43	34	40	42	43	44	45
T-GalA	nd	nd	nd	1	2	2	33	20	17	15	12	10
4-GalA	nd	22	22	34	36	36	nd	20	30	32	35	37
4-GalA'ol [e]	49	28	26	12	9	8	33	20	11	10	10	7

[a] See Fig. 2 and text for details.
[b] Terminal nonreducing rhamnosyl residue.
[c] Not detected.
[d] Less than 1 mol% detected.
[e] 4-Linked galactonic acid.

amounts of the oligoglycosyl aldonic acids substituted with neutral glycosyl residues were insufficient for structural analyses.

The quantitatively predominant series of backbone fragments (G1–G6, see Fig. 2) contain terminal nonreducing Gal*p*A, 2-linked Rha*p*, 4-linked Gal*p*A, and 4-linked galactonic acid (Table 4). The quantitatively minor series of fragments (R1–R6, see Fig. 2) contain terminal nonreducing Rha*p*, 2-linked Rha*p*, 4-linked Galp*A*, and 4-linked galactonic acid (Table 4). The latter series (R1–R6) was generated by the *endo*-rhamnogalacturonase present in the β-D-galactosidase preparation, which hydrolyzes the glycosyl linkages between the 4-linked Gal*p*A residues and the 2-linked Rha*p* residues. The more prevalent G1–G6 series is formed from the R1–R6 series by the action of an α-L-rhamnosidase that removes the terminal nonreducing Rha*p* residues and thereby generates terminal nonreducing Gal*p*A residues.

One- and two-dimensional ^1H NMR spectroscopy of the oligoglycosyl aldonic acids purified by HPAEC–PAD of Fraction C.—To confirm and extend the results of ESMS and glycosyl-residue and glycosyl-linkage composition analyses, the HPAEC-purified oligoglycosyl aldonic acids were analyzed by one- and two-dimensional ^1H NMR spectroscopy. The signals for anomeric protons in the ^1H NMR spectrum of each oligoglycosyl aldonic acid (R1–R5 and G1–G5) were assigned on the basis of their glycosyl-residue and glycosyl-linkage compositions and from published data [4,13,14]. The signals for ring protons were assigned using two-dimensional (COSY) ^1H NMR spectroscopy.

The partial 2D ^1H NMR spectrum of tetraglycosyl aldonic acid (G2) is shown, together with its corresponding 1D ^1H NMR spectrum in Fig. 4. The connectivities of the individual proton resonances are illustrated in this figure. The cross-peaks for H-5 and H-6 are not shown. Such analyses enabled all the proton resonances to be assigned for each of the glycosyl residues in the RG-I backbone oligosaccharides investigated (Table 5).

The chemical shifts of the anomeric and ring protons of the rhamnosyl and galactosyluronic acid residues were altered by the closeness of those glycosyl residues to the galactonic acid located at the reducing end of each oligoglycosyl aldonic acid. For example, the

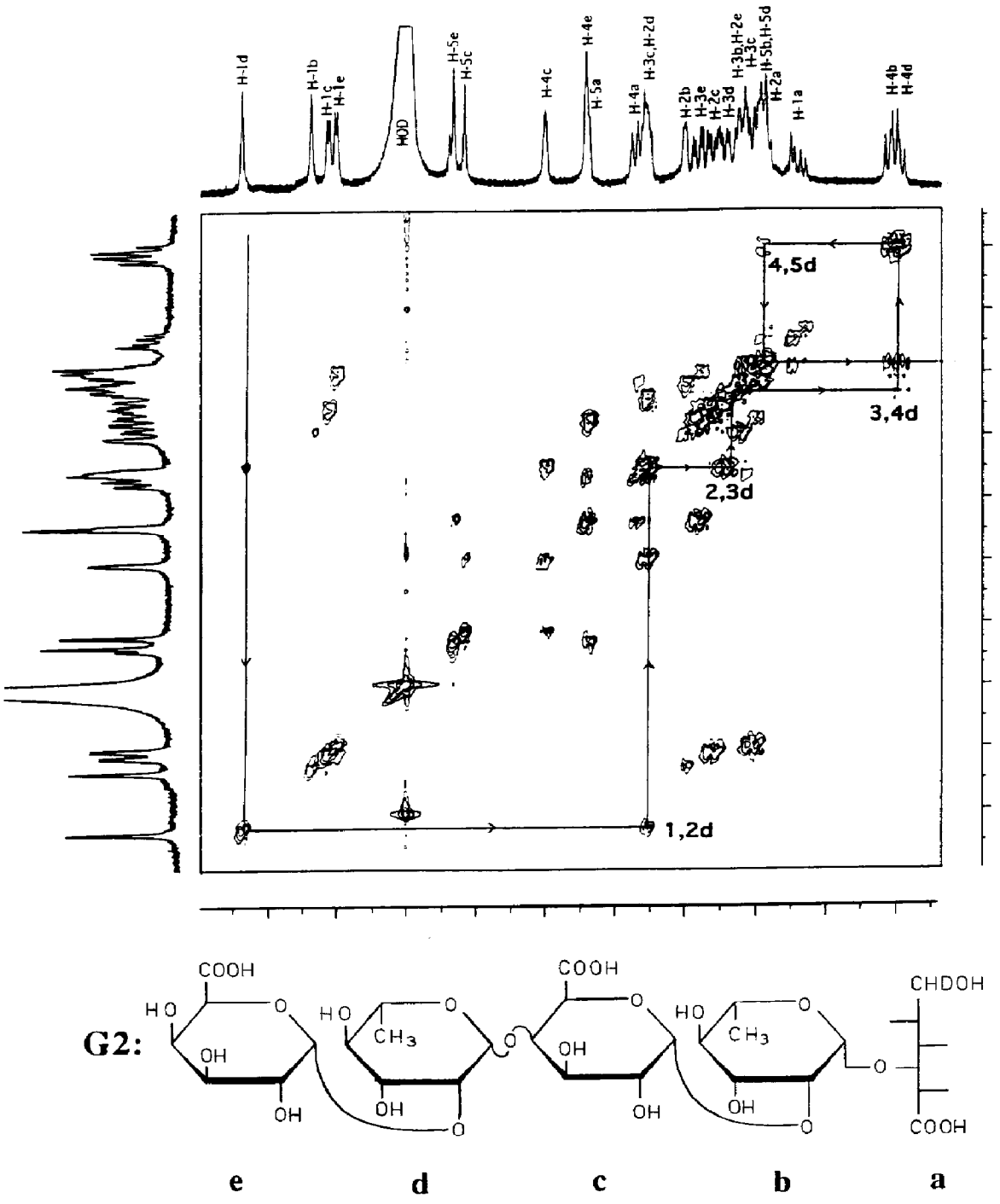

Fig. 4. COSY contour plot for a portion of the anomeric and ring proton resonances (δ 3.3–5.4) of tetraglycosyl aldonic acid (G2). The corresponding portion of the 1D ^{1}H NMR spectrum is also shown. The connectivity pathway for protons in the nonreducing rhamnosyl residue (residue **d**) and proton assignments are indicated. The structure of tetraglycosyl aldonic acid (G2) is shown below with the notation used to denote the individual glycosyl residues.

Table 5
Chemical shifts (in δ-units) for the proton resonances of the glycosyl residues in the oligoglycosyl aldonic acids (R1–R6 and G1–G6) purified by HPAEC–PAD of Bio-Gel P-10 Fraction C

Residue	Linkage	Chemical shift [a]					
		H-1	H-2	H-3	H-4	H-5	H-6
α-Rha*p*	terminal	5.23	4.05	3.78	3.34	3.81	1.25
	terminal [b]	5.02	3.97	3.80	3.43	3.81	1.25
	2-linked	5.27	4.12	3.89	3.41	3.80	1.25
	2-linked [c]	5.07	4.01	3.84	3.41	3.80	1.26
α-Gal*p*A	terminal	5.00	3.81	4.00	4.29	4.67	
		5.02 [d]				4.65 [d]	
	4-linked	5.00	3.90	4.11	4.40	4.65	
	4-linked [e]	5.02	3.92	4.10	4.40	4.64	
						4.62 [f]	
GalA′ol [g]	4-linked	3.68	3.80	3.84	4.14	4.30	
	4-linked [b]	3.63	3.70	3.83	3.97	4.33	

[a] Measured to an accuracy of ±0.01 ppm.
[b] In Rha-GalA′ol.
[c] Linked directly to GalA′ol.
[d] In GalA-Rha-GalA′ol.
[e] Linked directly to the 2-linked Rha residue which is linked to GalA′ol.
[f] In Rha-GalA-Rha-GalA′ol.
[g] The H-1 signal of GalA′ol was a complex multiplet.

chemical shift of H-1 (δ 5.02) of the terminal Rha*p* residue of R1 (Rha-GalA′ol) is upfield from H-1 (δ 5.23) of the terminal Rha*p* residue in fragments R2–R6 (see Table 5). Small but discernible upfield shifts in the H-2, H-3, and H-4 resonances of the terminal nonreducing Rha*p* residue attached to galactonic acid (in R1) were also observed (Table 5). The H-1, H-2, H-3, and H-6 resonances of the 2-linked Rha*p* residue (residue **b** in Fig. 4) directly attached to galactonic acid differed from the corresponding resonances of the other 2-linked Rha*p* residues in the larger backbone fragments (residue **d** in Fig. 4 and see Table 5). The chemical shifts for the protons in the 2-linked Rha*p* residue directly attached to galactonic acid are similar to those of the terminal Rha*p* residue in Rha*p*-(1→2)-Gal′ol (R1, see Table 5). Thus, the galactonic acid alters the chemical shifts of the glycosyl residue directly attached to it.

The effect of galactonic acid is also seen on the Gal*p*A residue (residue **c** in Fig. 4), which is linked to the 2-position of the Rha*p* residue attached to galactonic acid. The resonances for H-1 (δ 5.02) and for H-2 (δ 3.92) in this 4-linked Gal*p*A residue are shifted slightly downfield compared to the corresponding resonances in the 4-linked Gal*p*A residues that are distant from the galactonic acid (Table 5). The terminal Gal*p*A in G1 and the 4-linked Gal*p*A penultimate to galactonic acid have H-1 resonances with identical chemical shifts (δ 5.02), in contrast to the chemical shift of the H-1 resonance (δ 5.00) of the terminal Gal*p*A residue in the oligoglycosyl aldonic acids G2–G6 (see Table 5). Although the H-1 signals of terminal Gal*p*A and the 4-linked Gal*p*A residues that are not affected by galactonic acid both have the same chemical shift (δ 5.00), they can be distinguished because the resonances of their corresponding ring protons differ (Table 5).

The results of ^{1}H NMR spectroscopy are consistent with the results of a previous study [6] and provide confirmatory evidence that both the rhamnosyl and galactosyluronic acid residues are α-linked and that the RG-I backbone is composed of the specified diglycosyl repeating unit. Furthermore, the assignment of all the proton resonances in the enzymatically generated backbone fragments provides a basis for the structural characterization of larger backbone fragments, some of which contain oligoglycosyl side chains attached to C-4 of one or more of the 2-linked rhamnosyl residues.

3. Conclusions

The results of this study have shown that a mixture of *A. niger exo-* and *endo*-glycanases can be used to generate backbone fragments from sycamore RG-I. The *endo*-rhamnogalacturonase, which is present in a commercial β-D-galactosidase preparation, did not cleave the sycamore RG-I backbone unless many of the neutral oligoglycosyl side chains were removed by *exo-* and *endo*-glycanses present in the enzyme mixture. The *endo*-rhamnogalacturonase isolated from the culture medium of *A. aculeatus* is also unable to fragment RG-I unless many of the neutral oligoglycosyl side chains are removed. However, those naturally occurring RG-I's that have a low degree of backbone substitution with oligoglycosyl side chains, such as those present in the cell walls of pea seedlings [7], are fragmented without alteration by the *A. niger endo*-rhamnogalacturonase.

The inability of the *endo*-rhamnogalacturonases from *A. niger* and *A. aculeatus* to fragment RG-I backbones that are highly substituted with oligoglycosyl side chains limits the direct use of these enzymes in structural studies. However, *endo*-rhamnogalacturonases will be useful for the structural analysis of RG-I's, such as those isolated from pea cell walls [7], that do not have highly substituted backbones. *endo*-Rhamnogalacturonases also have considerable potential for generating useful fragments from RG-I when used in combination with purified *exo-* and *endo*-glycanases or in combination with selective chemical modifications.

4. Experimental

Materials.—*endo*-1,5-α-L-Arabinanase-, *exo*-α-L-arabinosidase-, and *endo*-β-D-galactanase-containing preparations from *A. niger* were obtained from Megazyme (North Rocks, Australia). The β-D-galactosidase-containing preparation from *A. niger* was purchased from Sigma. *endo*-α-(1 → 4)-Polygalacturonase purified from *A. niger* [15] was a gift of C. Bergmann (this laboratory).

Isolation of RG-I.–RG-I was purified as described from the material solubilized by *endo*-α-(1 → 4)-polygalacturonase treatment of the purified walls of suspension-cultured sycamore cells [16].

endo- *and* exo-*Glycanase treatment of RG-I.*—Separate portions of the *endo*-α-L-arabinanase (50 μg), α-L-arabinosidase (50 μg), *endo*-β-D-galactanase (50 μg), and β-D-galactosidase (50 μg) preparations were dialyzed (SpectraPor 6; 6 000–8 000 MW cutoff dialysis tubing) against 25 mM NaOAc, pH 4.5, at 4°C for 24 h to remove ammonium

sulfate. A solution of RG-I (50 mg) in 50 mM NaOAc, pH 4.5 (10 mL), was then treated at 30°C for 36 h under toluene with the combined enzyme preparations. The solution containing the enzymatically treated RG-I was adjusted to pH 7, desalted by elution through a column (1 × 10 cm) containing Dowex 50 (H^+) resin, and then freeze-dried.

Fractionation of the products released by enzymatic treatment of RG-I.—A solution of the enzymatically treated RG-I (50 mg) in 25 mM NaOAc, pH 5.2 (2 mL), was fractionated on a Bio-Gel P-10 column (1.5 × 100 cm) by elution with 25 mM NaOAc, pH 5.2, at a flow rate of 0.3 mL/min. Fractions (3 mL) were collected and portions (30 μL) were assayed colorimetrically for carbohydrate content with the phenol–H_2SO_4 reagent [17]. Four fractions containing carbohydrates (Fractions A–D, see Fig. 1) were individually pooled, separately desalted by passage through columns (1 × 10 cm) containing Dowex 50 (H^+) resin, and then freeze-dried. Separate solutions of each oligoglycosyl-containing fraction in water (1 mL) were applied to columns (1.0 × 5.0 cm) containing DEAE-Sephadex A-25 ($HCOO^-$ form). The neutral monosaccharides and neutral oligoglycoses were eluted with water. The acidic monosaccharides and acidic oligoglycoses were then eluted with aq 10% formic acid. The acidic portions of Fractions A–D were concentrated to dryness, dissolved in water, and freeze-dried.

Purification, using semipreparative HPAEC–PAD, of the backbone oligosaccharides generated by enzymatic treatment of RG-I.—A solution of the acidic oligoglycoses in Fraction C (~3 mg) in M NH_4OH (500 μL) was reduced with $NaBD_4$ as described [18] to yield the corresponding oligoglycosyl aldonic acids. The $NaBD_4$-reduced backbone oligosaccharides were then purified by HPAEC–PAD using a Dionex BioLc interfaced to an AutoIon series 400 data station and a semipreparative (9 mm × 25 cm) CarboPac PA1 column. Carbohydrates were located with a pulsed electrochemical detector equipped with a gold working electrode [8]. The column was eluted at 5 mL/min with a gradient of NaOAc (0–700 mM) in 100 mM NaOH as follows: 100 mM NaOH (0–2 min), 0–500 mM NaOAc (2–45 min), and 500–700 mM NaOAc (46–50 min). The column was then reequilibrated in 100 mM NaOH for 15 min prior to the next injection. Fractions were collected manually, neutralized with AcOH, desalted by elution through columns (0.5 × 5 cm) containing Dowex 50W × 12 (H^+) resin, and then freeze-dried.

Glycosyl-residue composition analysis.—Glycosyl-residue compositions were determined by formation of the trimethylsilyl methyl glycosides and analysis of the derivatives by GLC [16]. The derivatives were separated on a DB-1 column (0.25 mm × 30 m) using a HP 5880 gas chromatograph.

Glycosyl-linkage composition analysis.—Solutions of the oligoglycosyl aldonic acids (50 μg) in Me_2SO (300 μL) were methylated with butyllithium and MeI as previously described [19]. The methyl-esterified and per-*O*-methylated oligoglycosyl aldonic acids were then purified using Sep Pak C-18 cartridges [18]. The methyl-esterified carboxyl groups of the galactosyluronic acid residues and galactonic acid were carboxyl-reduced with lithium triethylborodeuteride [16]. The glycosyl-linkage compositions of the oligoglycosyl alditols were then determined by GLC–MS analyses of the derived partially methylated alditol acetates [16].

Electrospray mass spectrometry.—Electrospray mass spectrometry (ESMS) was performed with an API III Biomolecular Mass Analyzer (PE-Sciex, Thornhill, ON, Canada) interfaced to a Macintosh IIFx data station. The mass spectrometer was operated in the

positive-ion mode with an ion spray voltage of 5 000 volts and an orifice potential of 35 volts. Solutions of the carbohydrate (1 μg/μL) in aq 30% MeOH containing 1% HCl were introduced into the ES source at 2 μL/min using a Harvard 22 syringe infusion pump. The mass range was scanned from 200–2 000 amu. Ten scans were collected and averaged.

^{1}H NMR spectroscopy.—^{1}H NMR spectroscopy was performed with a Bruker AM 500 spectrometer. Spectra were obtained (in D_2O) at 500 MHz and 295 K. Chemical shifts are reported in ppm downfield (δ-units) relative to the signal for free acetate (δ 1.908).

Two-dimensional double quantum filtered homonuclear chemical shift correlation spectra (DQF-COSY) were obtained at 295 K with the assistance of an Aspect 3000 computer using standard Bruker software. In a typical experiment, spectra were collected using a 512×1024 data matrix, and 80 transients were acquired for each of 512 t_1 values. Spectral widths of 2645.5 Hz were used in both dimensions.

Acknowledgements

We thank Andrew Whitcombe and Jing Huang of this center for providing the sycamore RG-I used in this study and Rosemary Nuri and Lori Hadaway for assistance in preparing the manuscript.

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