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Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I

Patrice Lerouge [1], Malcolm A. O'Neill, Alan G. Darvill and Peter Albersheim *

Complex Carbohydrate Research Center and Department of Biochemistry, University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712 (USA)

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ABSTRACT

Rhamnogalacturonan I (RG-I) has been isolated from the walls of suspension-cultured sycamore cells (*Acer pseudoplatanus*), and additional structural features of the polysaccharide were elucidated. Treatment of RG-I with a purified endo-(1 → 5)-α-L-arabinanase released a series of arabinose-containing oligosaccharides with degrees of polymerization (dp's) between 2 and 20. These oligosaccharides were shown, by glycosyl-linkage composition analysis, to contain terminal, 5-, and (3 → 5)-linked Ara*f* residues. These results provide evidence that a branched arabinan is attached to the backbone of RG-I. RG-I was freed of 95% of its arabinosyl residues by treating the polysaccharide with a combination of endo-(1 → 5)-α-L-arabinanase and α-L-arabinosidase. No galacturonic acid was released by these enzymes, which is evidence that the arabinosyl-containing portions of the side chains do not contain galactosyluronic acid residues. The galactose-containing portions of the side chains of RG-I were not fragmented by an endo-(1 → 4)-β-D-galactanase. However, approximately 85% of the galactose and small amounts of galacturonic acid were released by digestion of arabinose-depleted RG-I with a combination of endo- and exo-β-D-galactanases. The galacturonic acid may have been released by small amounts of an exo-α-galactosyluronidase contaminating the galactanases. Treatment of RG-I with this mixture of endo- and exo-glycanases resulted in a relatively size-homogeneous, almost side chain-free backbone composed of the *O*-acetylated diglycosyl repeating unit → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*. A combination of ^{1}H NMR spectroscopy and periodate oxidation established that the backbone repeating unit contained a single *O*-acetyl substituent on C-2 or C-3 of each galactosyluronic acid residue.

INTRODUCTION

Rhamnogalacturonan I (RG-I) is a pectic polysaccharide[1] that is released from the walls of suspension-cultured sycamore (*Acer pseudoplatanus*) cells by treatment with endo-(1 → 4)-α-polygalacturonase (EPG)[2,3]. RG-I is composed of L-rhamnosyl, D-galactosyluronic acid, L-arabinosyl, D-galactosyl, and L-fucosyl residues, and

[1] Current address: LPTF-CNRS, 118 route de Narbonne, 31062 Toulouse, France.
* Corresponding author.

O-acetyl groups[3,4]. There are at least 20 differently linked glycosyl residues in RG-I[3]. RG-I has been shown, by chemical fragmentation analysis, to contain a backbone composed of the diglycosyl repeating unit →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*. Approximately 50% of the 2-linked rhamnosyl residues of the RG-I solubilized by endo-polygalacturonase are substituted through O-4 with monosaccharides and oligo- or poly-saccharides that are largely or entirely composed of neutral glycosyl residues[5]. Fragments of these side chains have been generated by treating RG-I with lithium in ethylenediamine[6]. Many of these fragments have been structurally characterized, and most of them contain 4-linked rhamnitol at the reducing terminus[6], leading to the conclusion that the side chains were freed by destruction of the galactosyluronic acid residues, followed by cleavage and then reduction of the 4-linked rhamnosidic linkages of the RG-I backbone. Some oligoglycosyl fragments that are also generated by lithium degradation of RG-I have arabinitol or galactitol at their reducing end[6]. Such fragments could be generated by lithium cleavage of the glycosidic linkages of arabinosyl or galactosyl residues, or by degradation of hexosyluronic acid residues if any are present in some of the side chains[7].

We now describe the use of purified endoglycanases for the (*i*) preparation of oligoglylcosyl side chain fragments from RG-I, (*ii*) generation of RG-I freed of most of its arabinosyl residues, and (*iii*) generation of the *O*-acetylated RG-I backbone freed of almost all its side chains.

RESULTS AND DISCUSSION

Preparation and isolation of homogalacturonan-free RG-I.—EPG-solubilized RG-I, which accounts for ~50% of the RG-I present in sycamore cell walls, was de-esterified and retreated with EPG to ensure the removal of homogalacturonan[8]. However, the molar ratios of galactosyluronic acid and rhamnosyl residues in several RG-I preparations obtained by this procedure were found to vary, which suggested that the removal of homogalacturonan residues was incomplete. Thus, RG-I that had been de-esterified and retreated with EPG was treated with a purified exo-α-D-polygalacturonase[9], and the products separated on a BioGel P-10 column. Galacturonic acid was the only monosaccharide detected, by GLC and HPAE–PAD analyses, in the column included volume. The exo-polygalacturonase-treated RG-I, which was recovered from the P-10 column void volume, contained galactosyluronic acid and rhamnosyl residues in an approximately equimolar ratio. This result is consistent with the previous finding that the RG-I backbone is composed of a diglycosyl repeating unit made up of galactosyluronic acid and rhamnosyl residues[5]. Homogalacturonan-free RG-I was used in all the experiments reported here.

Treatment of RG-I with an α-L-fucosidase.—Approximately 50% of the terminal α-L-fucopyranosyl residues (~2 mol% of RG-I) were hydrolyzed by treating RG-I with an α-L-fucosidase. The remaining fucosyl residues were not cleaved even after

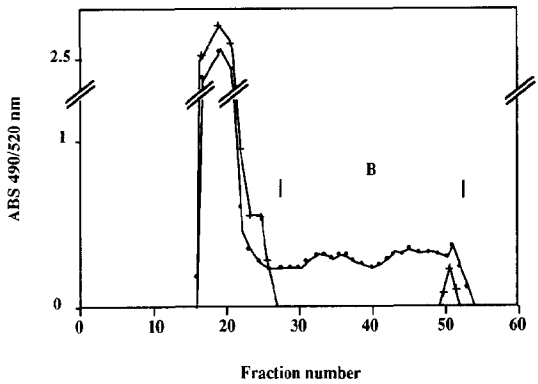

Fig. 1. Chromatography of endo-arabinanase-treated RG-I on a BioGel P-10 column (1.5×100 cm) in water. Column fractions (3 mL) were collected and assayed colorimetrically for neutral glycosyl[21] (•) and uronosyl[20] (+) residues. The fractions eluting between the included and void volumes of the column (Fraction B) were pooled and lyophilized.

repeated treatment with the enzyme. It is not known whether the remaining fucosyl residues are β-linked or sterically inaccessible to the enzyme.

Treatment of RG-I with a purified endoarabinanase.—A commercially available endo-(1 → 5)-α-L-arabinanase was found to contain trace amounts of α-L-arabinosidase, EPG, exo-galactanase, and exo-xylanase activities[10]. Therefore, the endo-arabinanase was further purified as described[10]. The purified endo-arabinanase had no detectable α-L-arabinosidase or EPG activities. However, traces of exo-galactanase and xylosidase activities were still detectable[10].

Arabinosyl-containing oligosaccharides were generated by treating RG-I with the "purified" endo-arabinanase. The oligoarabinosides were separated from the RG-I residue by chromatography on BioGel P-10 (Fraction B, see Fig. 1). Glycosyl-residue composition analysis showed that arabinose accounted for 94% of the material (Table I, column a) in Fraction B (see Fig. 1). No galacturonic acid was detected in Fraction B, which indicates that galactosyluronic acid residues are not present in the arabinose-containing side chains. The ^{1}H NMR spectrum of Fraction B contained signals (broad singlets) for anomeric protons at δ 5.05, 5.08, 5.12, and 5.15, which were assigned to α-L-arabinofuranosyl residues[11]. Two downfield signals (δ 5.23 and 5.28) were assigned to H-1α and H-1β of a reducing arabinosyl residue by comparison with the ^{1}H NMR spectrum of authentic arabinotriose and published data[11].

Purification of the arabinosyl-containing oligosaccharides.—The arabinosyl-containing oligosaccharides released from RG-I were converted to their corresponding oligoarabinosyl arabinitols by reduction with $NaBD_4$. The latter were desalted on a Sephadex G-15 column. The mixture of oligoarabinosyl arabinitols was analyzed by HPAE–PAD (Fig. 2a) using a CarboPac PA1 column. The column was calibrated with diarabinosyl arabinitol, heptaarabinosyl arabinitol, and the mixture of oligoarabinosyl alditols generated by partial enzymic fragmentation and $NaBD_4$

TABLE I

Glycosyl composition [a] of digested RG-I and material released by enzymic hydrolysis

Glycosyl residue	RG-I	a	b	c	d	e
	(mol%)					
Ara	32	94	1	93	1	0
Fuc	2	0	1	0	1	1
Xyl	1	3	1	2	0	1
Rha	16	0	26	0	45	1
GalA	17	0	30	0	44	3
Gal	31	3	40	5	9	92

[a] a, Endo-arabinanase-released material. b, Arabinose-free RG-I. c, Arabinosidase- and endo-arabinanase-released material. d, Arabinanase- and galactanase-treated RG-I. e, Arabinanase- and galactanase-released material.

reduction of a linear (1 → 5)-α-L-arabinan (Fig. 2b). Monoarabinosyl arabinitol and triarabinosyl arabinitol were the most prevalent products detected in the $NaBD_4$-reduced endo-arabinanase digest of RG-I (Fig. 2a). Significant amounts of oligoarabinosyl arabinitol fragments with dp's between 6 and 20 were also detected (Fig. 2a). We have assumed that structural isomers of arabinosyl oligomers with the

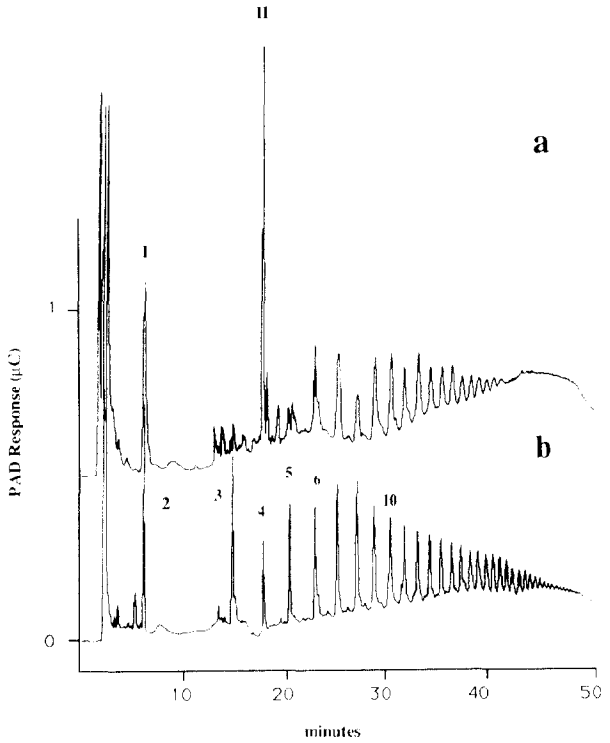

Fig. 2. HPAE–PAD chromatograms of reduced oligoarabinosyl alditols released from RG-I (a) and from a linear (1 → 5)-α-L-arabinan (b) by enzymic hydrolysis (see Experimental section). The numbers above the peaks in (b) correspond to the dp of the oligosaccharide in that peak.

same dp have similar retention times on the CarboPac column under the elution conditions used. In addition, it should be noted that the response of the PAD is proportional to the molar concentration of carbohydrate. Thus, the small peaks corresponding to oligoarabinosyl-arabinitols of dp > 6 are quantitatively significant.

Approximately 2 mg of the mixture of arabinosyl arabinitols generated by endo-arabinanase treatment of RG-I was fractionated by HPAE-PAD, and the two major peaks (peaks I and II, see Fig. 2a) were collected, desalted, and methylated. GLC–CIMS (NH_4 reagent gas) analysis of peak I gave an ion at m/z 400 that corresponds to $[M + 18]^+$ of a methylated monoarabinosyl arabinitol derivative. The GLC–EI mass spectrum and the results of glycosyl-linkage analysis of the methylated monoarabinosyl alditol derivative were consistent with the sequence Ara-(1 → 5)-arabinitol (data not shown). GLC–CIMS (NH_4 reagent gas) analysis of peak II gave an ion at m/z 720 that corresponds to $[M + 18]^+$ of a methylated triarabinosyl arabinitol. GLC–EIMS analysis and glycosyl-linkage analysis indicated that two triarabinosyl arabinitol derivatives were present, but unambiguous sequence data were not obtained. The larger oligoarabinosyl alditols also appeared to be mixtures, because they eluted as relatively broad peaks when analyzed by HPAE–PAD (compare Figs. 2a and 2b).

The point of substitution of the alditol moiety in the oligoarabinosyl arabinitols was determined by methylation analysis. The methylated oligoarabinosyl arabinitols were hydrolyzed, reduced, acetylated, and analyzed by GLC–EIMS and quantified by GLC (Table II). The deuterium-reduced derivative 1,2,3,4-tetra-*O*-methyl-5-*O*-acetylarabinitol was the only component detected that contained *O*-methyl groups at positions 1 and 4. Thus, only 5-linked arabinose was reduced prior to methylation, which confirms that the endo-arabinanase specifically cleaves

TABLE II

Arabinosyl-linkage composition of oligosaccharides isolated from RG-I and from plant arabinans

Arabinosyl linkage	RG-I [a]	Sycamore [b]	Apple[17]	White willow[19]	Mustard seed [16]	Rose[18]
	(mol%)					
Pre-reduced 5-linked	4					
t-Ara*f*	30	32	36	40	40	34
t-Ara*p*	0.7	0	0	3	0	1
5-Ara*f*	22	23	32	24	25	30
3-Ara*f*	4	0	4	4	0	7
3,5-Ara*f*	20	18	20	9	29	14
2,5-Ara*f*	8	8	0	6	0	6
2,3,5-Ara*f*	8	7	8	14	6	8

[a] Oligoarabinosides generated by endo-arabinanase treatment of RG-I. [b] Permethylated arabinan isolated from a mixture of methylated polysaccharides obtained from sycamore cell walls (McNeil, Darvill, and Albersheim, unpublished results).

5-linked arabinofuranosyl residues. Terminal nonreducing, 5-, 3,5-, 2,5-, 2,3,5-, and 3-linked arabinosyl residues accounted for the remaining glycosyl linkages of the oligoarabinosides generated by endoarabinanase treatment of RG-I (Table II, column I). These results are consistent with the presence of a 5-linked arabinan often substituted through O-3 with a mono- or oligo-arabinosyl side chain.

Isolation of an arabinose-"free" RG-I.—RG-I was treated with a mixture of purified arabinanase and arabinosidase, and the products separated by BioGel P-10 gel-permeation chromatography. The RG-I residue was recovered in the column void volume. This material was again treated with the mixture of arabinanase and arabinosidase and re-isolated by BioGel P-10 gel-permeation chromatography. Approximately 95% of the arabinosyl residues of RG-I were removed by these treatments (Table I, column b). Glycosyl-residue composition analysis of the material released by the enzymes showed that arabinose accounted for 93% of the material (Table I, column c). No galactosyluronic acid residues were detected in the released material, which provides additional evidence that galactosyluronic acid residues are not present in the arabinosyl-composed side chains or those portions of the side chains composed of arabinosyl residues. However, the enzyme-released material was shown by HPAE–PAD to contain free galactose, which may have arisen by partial hydrolysis of the galactose-composed side chains by an exo-β-D-galactanase contaminating the arabinanases[10]. The demonstration that ~ 95% of the arabinosyl residues of RG-I could be removed without releasing significant amounts of galactosyl residues provides strong evidence for the presence of homoarabinosyl side chains or homoarabinosyl regions of side chains. Furthermore, most of the arabinosyl residues of the heteroglycosyl side chains must be located at the nonreducing ends of the chains.

Treatment of RG-I with endo- and exo-galactanases.—No galactose-containing oligosaccharides were released when either RG-I or arabinose-free RG-I was treated with a purified endo-(1 → 4)-β-D-galactanase. However, approximately 75% of the galactosyl residues were released by treating arabinose-"free" RG-I (Table I, columns d and e) with a combination of endo- and exo-galactanases. The resulting digest was shown, by HPAE–PAD, to contain free galactose and small amounts of free galacturonic acid. Thus, the galactose-hydrolyzing enzymes appear to contain small amounts of exo-galacturonase activity.

Structure of the RG-I backbone.—Approximately 95% of the side-chain glycosyl residues were released by treatment of RG-I with a combination of endo- and exo-arabinanases and galactanases (Table I, column e). The RG-I backbone eluted from a Superose 12 column as a single peak, corresponding to a molecular weight between 40 and 80 kDa. This is approximately one third of the estimated size (200 kDa) of EPG-solubilized RG-I[3]. The ^{1}H NMR spectrum (Fig. 3A) of the RG-I backbone contained signals for anomeric protons at δ 5.22 (broad singlet) and 4.96 ($J_{1,2}$ 3.5 Hz) that were assigned to α-L-rhamnopyranosyl and α-D-galactosyluronic acid residues, respectively[12,13]. Signals at δ 4.60 and 4.35 were assigned to the H-5 and H-4, respectively, of galactosyluronic acid residues.

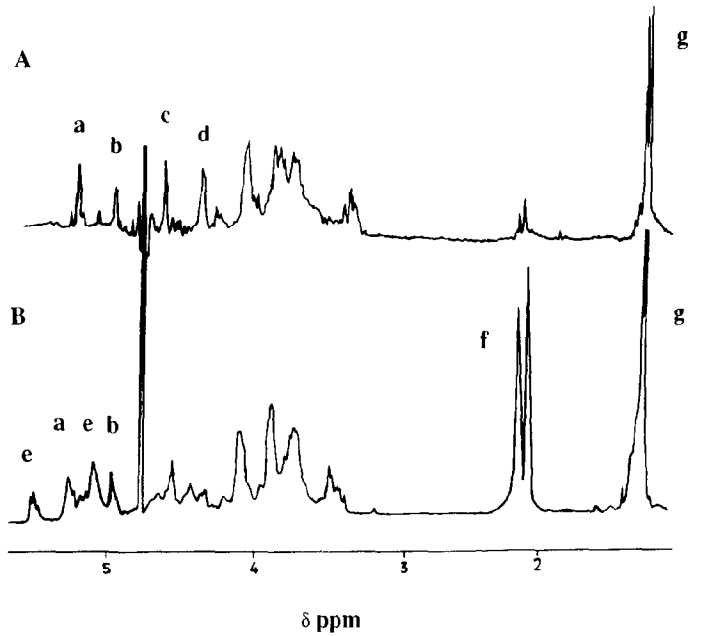

Fig. 3. ^1H NMR spectra (500 MHz) in D_2O of the saponified (A) and unsaponified (B) RG-I-backbone. The identified signals are labeled (a) H-1 of α-L-Rha*p*, (b) H-1 of α-D-Gal*p*A, (c) H-5 of α-D-Gal*p*A, (d) H-4 of α-D-Gal*p*A, (e) H linked to acetylated carbons, (f) CH_3 of acetyl groups, and (g) CH_3 of rhamnosyl residues.

The RG-I backbone was shown, by glycosyl-residue composition analysis, to contain GalA, Rha, and Gal in the ratios 1.0:1.0:0.2. The residual galactosyl residues could not be hydrolyzed despite repeated treatments with a combination of exo- and endo-galactanases. Glycosyl-linkage composition analysis of the methylated, carboxyl-reduced, and remethylated RG-I backbone showed terminal nonreducing and 4-linked Gal*p*A, 2- and 2,4-linked Rha*p*, and terminal and 4-linked Gal*p* in the molar ratios 1:18:17:2:1:1. This data confirms that the galactosyl residues are not fully removed by the exo- and endo-glycanase treatment of RG-I. A small amount of the terminal nonreducing galactosyluronic acid residues probably originates from the ends of the RG-I backbone, while others may be located at the nonreducing termini of galactosyl-containing side chains. Galactosyl-containing side chains terminated with a galactosyluronic acid residue would not be hydrolyzed by the exo- and endo-galactanase treatment unless a contaminating exo-galacturonidase removed the Gal*p*A residue. This is a possible explanation for the inability of the exo- and endo-galactanases to remove all of the galactosyl residues from the RG-I backbone.

Location of O-acetyl groups on the backbone of RG-I.—Previous studies have shown that RG-I isolated from cotton plant cell walls contains *O*-acetyl groups[4]. To determine whether sycamore cell wall RG-I also contains *O*-acetyl groups,

EPG-solubilized RG-I was isolated without alkaline saponification. The ^{1}H NMR spectrum of unsaponified RG-I contained two broad signals between δ 2.1–2.3 that are characteristic of the methyl protons of the *O*-acetyl groups. These signals (δ 2.1–2.3) were still present after removal of the side chain oligosaccharides with the combination of exo- and endo-glycanases (Fig. 3B). Thus, the *O*-acetyl groups are located on the RG-I backbone. The signals corresponding to the methyl protons of the *O*-acetyl groups and the H-6 signals (δ 1.1–1.3) of the rhamnosyl residues were shown by integration to be present in a 1 : 1 ratio which established that the backbone of RG-I contains, on average, one *O*-acetyl group per diglycosyl repeating unit.

The ^{1}H NMR spectrum of *O*-acetylated RG-I also contained signals at δ 5.10 and 5.40 (Fig. 3B). These signals were absent in saponified RG-I (Fig. 3A) and were therefore assigned to protons linked to *O*-acetylated carbons. Thus, a portion of at least two of the four free hydroxyl groups of the diglycosyl repeating unit is *O*-acetylated.

The locations of the *O*-acetyl groups were determined by a combination of periodate oxidation and glycosyl-residue composition analysis. The presence of an *O*-acetyl group on C-3 or C-4 of the rhamnosyl residues or on C-2 or C-3 of the galactosyluronic acid residues would protect those residues from periodate oxidation. Samples of *O*-acetylated and of saponified RG-I were treated with 50 mM Na periodate for 48 h. The rhamnosyl and galactosyluronic acid residues of saponified RG-I were both completely destroyed by periodate oxidation. In contrast, only the rhamnosyl residues were destroyed by oxidation of *O*-acetylated RG-I. Thus, the *O*-acetyl groups are located on the galactosyluronic acid residues. The results of ^{1}H NMR analysis and periodate oxidation of unsaponified RG-I provide strong evidence that every galactosyluronic acid residue is *O*-acetylated at either C-2 or C-3. *O*-Acetyl groups have previously been located at O-3 of the galactosyluronic acid residues in cotton RG-I (ref. 4). Examination of the ^{1}H NMR spectrum of the enzymically generated backbone of RG-I showed that the galactosyluronic acid residues were not measurably methyl esterified. This is in contrast to homogalacturonan, which has a linear α-(1 $\rightarrow$ 4)-galacturonan backbone in which many of the galactosyluronic acid residues are methyl esterified[1,8,17].

GENERAL DISCUSSION

Treatment of sycamore cell wall RG-I with purified exo- and endo-glycanases has provided additional information about the structure of this complex pectic polysaccharide. Based on both the data reported here and the results of previous studies[5,6], we suggest that a branched arabinan is linked to the RG-I backbone, although unequivocal evidence was not obtained for the covalent linkage between the arabinan and a rhamnosyl residue. However, arabinosyl-containing oligosaccharides (dp $<$ 5) terminated at the reducing end with rhamnitol are released by lithium treatment of RG-I (ref. 6), which demonstrates that at least short arabi-

nosyl side chains are linked to the RG-I backbone[6]. Since we found that lithium treatment of a 5-linked arabinan results in a significant cleavage of the glycosidic linkages and generates a mixture of oligoarabionsyl arabinitols (dp's 2–25), some or all of the arabinosyl-containing oligosaccharide alditols released by lithium treatment of RG-I (ref. 6) may have originated from the covalent attachment of an arabinan to a rhamnosyl residue of the backbone.

Arabinans have been isolated from various plant sources[14–19], but, due to the harsh extraction conditions used, it is not known if the arabinans were originally covalently linked to a pectic polysaccharide, such as RG-I, or were present in situ as free arabinans. Our studies provide evidence that some, if not all, of the arabinan in sycamore cell walls is covalently linked to RG-I. These results, in combination with the structures of the fragments characterized after lithium fragmentation of RG-I, indicate that the arabinan is linked to the rhamnosyl residues of the RG-I backbone both directly and via galactosyl residues. The arabinosyl residues are always located at the non-reducing end of those side-chain fragments that have been characterized which contain both arabinosyl and galactosyl residues[6]. Thus, arabinosyl-containing side chains are located either entirely or almost always on the exterior of the RG-I molecule.

Plant arabinans appear to have conserved structures. Glycosyl-linkage composition analysis (see Table II) has shown that the arabinosyl-containing oligomers released by endo-arabinanase treatment of RG-I and arabinans isolated from the cell walls of other plants[14–19] all contain a 5-linked Ara*f* backbone. This backbone is branched at positions 2 and 3 with terminal nonreducing Ara*f* residues or with oligosaccharides composed of Ara*f*.

No oligogalactosyl fragments were released by treating arabinose-free RG-I with a commercial endo-(1 → 4)-β-D-galactanase, although the polysaccharide is known to contain (1 → 4)-linked β-D-galactosyl residues[6]. In contrast, a small amount of free galacturonic acid and approximately 85% of the galactosyl residues were released by treating arabinose-free RG-I with a combination of exo- and endo-β-galactanase. The presence of free galacturonic acid in the enzymic digest indicates that one of the enzymes is contaminated with small amounts of an exo-galacturonidase. These results provide evidence that the galactose-containing side chains possess galactosyluronic acid residues, which is in contrast with our results (see above) that show that the arabinose-containing side chains do not contain galactosyluronic acid residues.

The partial digestion of RG-I with endo- and exo-glycanases provided a selective method for the almost complete removal of the RG-I side chain oligosaccharides, generating a nearly linear RG-I backbone. The resulting RG-I backbone, which was primarily composed of the diglycosyl repeating unit → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 → , was approximately one third of the size of intact RG-I. Thus, the rhamnosyl and most of the galactosyluronic acid residues, which together account for ~30% of intact RG-I, are located in a single relatively size-homogeneous backbone.

The enzymic method of generating the *O*-acetylated RG-I backbone made it possible to locate the positions of *O*-acetyl substituents. A combination of ^{1}H NMR spectroscopy and periodate oxidation established that each repeating unit of the RG-I backbone is mono-*O*-acetylated on O-2 or O-3 of the galactosyluronic acid residues. The distribution of *O*-acetyl groups between O-2 and O-3 probably results from acetyl migration either during sample work-up or while RG-I is in the cell wall, although it is possible RG-I may be naturally *O*-acetylated at a mixture of these positions. *O*-Acetyl groups have previously been found at O-3 of galactosyluronic acid residues in other plant rhamnogalacturonans[4].

Although considerable progress has been made in elucidating the structure of RG-I, determining the complete glycosyl sequence of this molecule remains a tremendous challenge. Some of the important structural features of RG-I that remain to be established include elucidating the structures of the galactosyl-containing oligosaccharides; confirming that galactosyluronic acid residues are located within the galactosyl-containing side chains, and, if they are, exactly where they are located; whether side chains exist that contain only galactosyl residues; and determining the distribution of the various side chains along the RG-I backbone.

EXPERIMENTAL

Materials.—The endo-α-(1 → 5)-arabinanase, α-L-arabinosidase, and endo-(1 → 4)-β-D-galactanase were obtained from Megazyme (Australia) and purified as described[10]. The exo-β-D-galactanase from *Aspergillus niger* and the α-L-fucosidase from bovine epididymis were from Sigma Chemical Co. The endo-(1 → 4)-α-D-polygalacturonase (EPG) was purified from *A. niger* and was a gift of Dr. C. Bergmann (this laboratory). The exo-α-D-polygalacturonase (exoPG) was purified from *A. niger* and was a gift of Dr. R. Pressey (Richard B. Russell Agricultural Research Center, U.S. Department of Agriculture, Athens, GA 30613, USA). Linear (1 → 5)-α-L-arabinan, (1 → 5)-α-L-arabinotriose, and (1 → 5)-α-L-arabinooctaose were obtained from Megazyme.

Isolation of RG-I.—RG-I was isolated from the purified walls of suspension-cultured sycamore cells by treatment of the walls with a purified EPG from *A. niger*. The solubilized polysaccharides were fractionated on a BioGel P-30 column by elution with 50 mM NaOAc, pH 5.2. The material eluting in the column void volume was dialyzed, concentrated, and incubated at pH 12 for 4 h at 4°C to remove methyl and acetyl esters of the galactosyluronic acid residues. The solution was then adjusted to pH 5.2 and fractionated on a BioGel P-30 column. The material eluting in the column void volume was concentrated and again treated with EPG. The EPG-treated material was fractionated on an Agarose A-5M column by elution with 50 mM NaOAc, pH 5.2. RG-I eluted as a single partially included peak that was further purified by anion-exchange chromatography on a DEAE-Sephadex column. RG-I eluted as a single peak. *O*-Acetylated RG-I was isolated using the same conditions, except that the material was not saponified.

Exo-α-D-polygalacturonase treatment of RG-I.—A solution (5 mL) of RG-I (20 mg) that had been saponified and treated twice with EPG in 50 mM NaOAc, pH 5.0, was treated for 16 h at 25°C with 50 μg of exoPG. The mixture was neutralized and lyophilized. A solution (2 mL) of the products in 50 mM NaOAc, pH 5.0, was fractionated on a BioGel P-10 column. Fractions (1 mL) were collected and portions (25 μL) were assayed colorimetrically for uronic acid[20].

α-L-Fucosidase treatment of RG-I.—A solution (1 mL) of RG-I (100 μg) in 50 mM NaOAc, pH 6.5, was treated for 18 h at 40°C with α-L-fucosidase (0.5 Units). The reaction mixture was dialyzed against water, and the amount of free fucose in the dialysate was determined by HPAE–PAD.

Endo-arabinanase treatment of RG-I.—A solution (5 mL) of exoPG-treated RG-I (50 mg) in 50 mM NH_4OAc, pH 4.0, was treated for 4 h at 40°C with purified endo-arabinanase (100 μg). The mixture was neutralized and lyophilized. A solution (2 mL) of the products in water was fractionated on a BioGel P-10 column (1.5 × 100 cm) by elution with water. Fractions (3 mL) were collected, and portions (20 μL) were assayed colorimetrically for neutral sugars[21] and uronic acid[20]. Approximately 4 mg of a mixture of arabinose-containing oligosaccharides was obtained.

Preparation of arabinose-free RG-I.—A solution (1 mL) of RG-I (5 mg) in NH_4OAc, pH 4.0, was treated for 4 h at 40°C with a mixture of endo-arabinanase and α-L-arabinosidase (50 μg each). The mixture was neutralized and lyophilized. A solution (2 mL) of the products in water was fractionated on a BioGel P-10 column by elution with water. Fractions (2 mL) were collected, and portions were assayed colorimetrically for neutral sugars[21] and uronic acid[20]. The partially digested RG-I was recovered in the column void volume, retreated with the mixture of endo-arabinanases and arabinosidase, and then isolated by BioGel P-10 chromatography. Approximately 4.5 mg of arabinose-"free" RG-I was recovered.

Isolation of the RG-I backbone.—A solution (2 mL) of arabinose-"free" RG-I (4.5 mg) in 50 mM NH_4OAc, pH 4.0, was treated for 16 h at 30°C with a mixture of endo-β-(1 → 4)-galactanase (30 μg) and exo-β-D-galactanase (50 μg). The mixture was neutralized and lyophilized. A solution (1 mL) of the products in water was fractionated on a BioGel P-10 column by elution with water. Fractions (2 mL) were collected, and portions (20 μL) assayed colorimetrically for neutral sugar[21] and uronic acid[20]. The RG-I backbone eluted in the column void volume.

Estimation of the molecular weights of RG-I and the isolated RG-I backbone.—The molecular weights of RG-I and of the RG-I backbone were estimated by FPLC in a Superose 12 column (1 × 30 cm) by elution with M NH_4OAc at a flow rate of 0.2 mL/min. Fractions (500 μL) were collected and assayed colorimetrically for uronic acid[20]. The Superose 12 column was calibrated with dextrans of known molecular weight.

Sodium periodate treatment of the RG-I backbone.—Separate solutions (1 mL) of saponified RG-I (250 μg) and unsaponified RG-I (250 μg) in 50 mM Na periodate were reacted for 48 h at 4°C in the dark. The excess periodate was then destroyed

by the addition of ethylene glycol (100 μL), and the mixture was dialyzed and lyophilized. The glycosyl-residue compositions of the two samples were determined by GLC analysis of their methyl per-*O*-trimethylsilyl-glycoside derivatives.

Glycosyl-residue composition analysis.—The glycosyl-residue compositions of RG-I and its oligoglycosyl fragments were determined by formation of the methyl per-*O*-trimethylsilyl-glycosides and analysis of the derivatives by GLC[8]. The derivatives were separated on a DB-1 column (0.25 mm × 30 m) using an HP-5880 gas chromatograph.

Glycosyl-linkage composition analysis.—The glycosyl-linkage compositions of oligoglycosyl alditols were determined by GLC–MS of the partially methylated alditol acetates as described[8], except that potassium methanesulfinylmethide (dimsyl) anion was used. The RG-I backbone was methylated, carboxyl-reduced with lithium triethylborodeuteride (Superdeuteride™, Aldrich), and remethylated before glycosyl-linkage composition analysis.

GLC–MS analysis of the methylated oligoarabinosyl arabinitols.—Monoarabinosyl arabinitol and triarabinosyl arabinitols isolated by HPAE–PAD were methylated as described[6] and purified on SepPak C-18 cartridges[6]. The derivatives were separated on a DB-1 column (0.25 mm × 15 m) with on-column injection and analyzed by GLC–MS using an HP-5985 GC–MS in both the CI (ammonia as reagent gas) and EI modes.

Preparation of a standard mixture of oligoarabinosyl arabitols.—A solution (1 mg) of arabinan in 50 mM NH_4OAc, pH 4.0 (1 mL) was treated at 40°C with the purified endo-arabinanase (5 μg). After 2 min, the reaction was stopped by neutralization. The mixture of arabinose-containing oligosaccharides was converted to the corresponding oligoarabinosyl arabinitols by reduction with $NaBH_4$. The mixture of oligoarabinosyl arabinitols was desalted on a Sephadex G-15 column by elution with water and lyophilized.

HPAE–PAD analysis of the products released by enzymic treatment of RG-I.—HPAE–PAD was performed with a Dionex metal-free BioLC column interfaced to an AutoIon Series 400 data system. Carbohydrates were separated in a CarboPac PA1 column (4.6 mm × 25 cm) and detected with a pulsed electrochemical detector equipped with a gold working electrode. To facilitate the detection of carbohydrates, NaOH (400 mM) was added post-column at a flow rate of 0.6 mL/min with a pressurized reagent delivery system. Carbohydrates were eluted at 1 mL/min with 100 mM NaOH for 5 min followed by a linear 40 min gradient of NaOAc (0–600 mM) in 100 mM NaOH. Oligoarabinosyl arabinitols were eluted over 40 min at 1 mL/min using a gradient of NaOAc (0–500 mM) in 20 mM NaOH. The fractions corresponding to the mono-, tri-, and tetra-arabinosyl arabinitols were manually collected and desalted by elution through OnGuard H cartridges (Dionex). The eluates were neutralized with M NH_4OH and lyophilized.

^1H NMR spectroscopy.—^1H NMR spectroscopy was performed with a Bruker AM 500 spectrometer. Spectra were obtained (in D_2O) at 500 mHz and 298 K. Chemical shifts are reported in ppm relative to the HOD signal (δ 4.76).

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