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Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II ☆

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Abstract

An octasaccharide was released from sycamore cell wall rhamnogalacturonan-II (RG-II) by selective acid hydrolysis of the glycosidic linkages of apiosyl residues and purified to homogeneity by gel-permeation and high-performance anion-exchange chromatographies. The octasaccharide **1** contains a terminal nonreducing β-L-arabinofuranosyl residue linked to position 2 of the α-L-rhamnopyranosyl residue of the aceric acid-containing heptasaccharide **2** that had been previously isolated from RG-II [M.W. Spellman et al. *Carbohydr. Res.*, 122 (1983) 131–153]. Heptasaccharide **2** and octasaccharide **1** were found to be mono- or di-*O*-acetylated. The *O*-acetyl groups were located, by ESMSMS, on the terminal nonreducing 2-*O*-methyl-α-L-fucosyl residue and/or on the 2-linked β-L-aceryl acid residue. Octasaccharide **1** and heptasaccharide **2** have the following structures:

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† Dedicated to Andrew J. Whitcombe (1963–1995) who died after this paper was accepted for publication.

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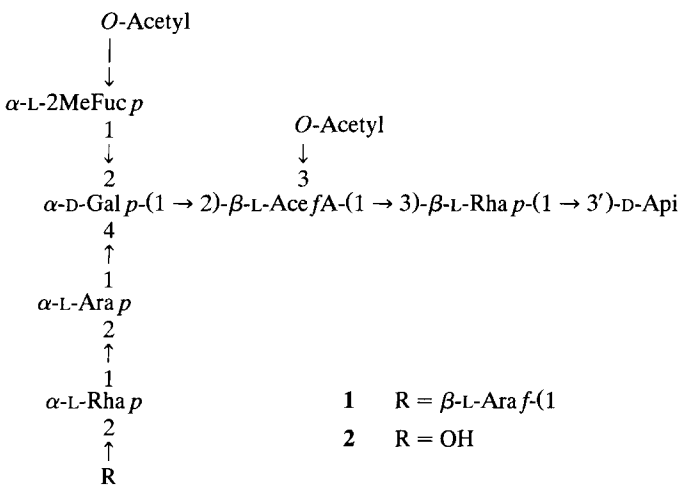

Six other oligosaccharides related to **1** and **2** were also generated by the acid hydrolysis of RG-II, and their partial structures were determined. The backbone of RG-II was shown, using a combination of *exo-* and *endo*-polygalacturonase digestions and partial acid hydrolysis, to be composed of up to eleven linear 1,4-linked α-D-galactosyluronic acid residues. These results provide additional information about the structure of RG-II.

Keywords: Pectic polysaccharide; Rhamnogalacturonan-II; Sycamore

1. Introduction

Rhamnogalacturonan-II (RG-II) is a small, structurally well-defined, complex pectic polysaccharide that is released from the walls of plant cells by treatment with *endo*-α-1,4-polygalacturonase [2]. RG-II has also been isolated from the cell walls of sycamore (*Acer pseudoplatanus*) [3], Douglas fir (*Pseudotsuga menziesii*) [4], rice (*Oryza sativa*) [5], onion (*Allium cepa*) [6], and kiwi fruit (*Actinidia deliciosa*) [7], and is present in the medium of cultured sycamore cells [8], in the commercial enzyme preparation Pectinol AC [9], and in red wine [10].

RG-II contains eleven different glycosyl residues [11], including the unusual monosaccharides apiose, 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid), 3-deoxy-D-*manno*-octulosonic acid (Kdo), and 3-deoxy-D-*lyxo*-heptulosaric acid (Dha). RG-II also contains the seldom observed methyl-etherified sugars 2-*O*-methyl xylose and 2-*O*-methyl fucose. Many of the glycosidic linkages and ring forms of the glycosyl residues of RG-II, including β-D-galactosyluronic acid, β-L-arabinofuranose, α-L-arabinopyranose, and a fully substituted rhamnosyl residue, are also unusual. Some of the glycosyl residues in RG-II are *O*-acetylated, although which residues are *O*-acetylated has not been determined [1]. The backbone of RG-II has been shown to be composed of at least seven 1,4-linked α-D-galactosyluronic acid residues [12].

Chemical fragmentation of RG-II led to the isolation and structural characterization of four oligoglycosyl side chains [11]. In particular, heptasaccharide **2** is generated by partial acid hydrolysis of RG-II [1]. We now report that partial acid hydrolysis of RG-II

also generates at least seven other oligoglycoses structurally related to heptasaccharide **2**. The complete structure of one octasaccharide **1** and the partial structures of six other oligosaccharides have been determined. In addition, we provide evidence for the sites of *O*-acetylation of heptasaccharide **2** and octasaccharide **1**, and that the backbone of RG-II is composed of up to eleven linear 1,4-linked α-D-galactosyluronic acid residues.

2. Results and discussion

Isolation of side chain oligosaccharides released by partial acid hydrolysis of RG-II.—Previous studies have shown that about 35% of the apiosidic linkages of RG-II are hydrolyzed by treatment with 0.1 M trifluoroacetic acid (TFA) for 16 h at 40° C[1]. Thus, purified RG-II (125 mg) was subjected to those conditions. Chromatography of the released oligosaccharides on a BioGel P-10 column yielded Fractions A–C (see Fig. 1).

Fraction A, which accounted for 60% by weight of the material applied to the column, eluted in the void volume and was shown by glycosyl-residue composition analysis to correspond to unfragmented RG-II as well as fragments representing large portions of RG-II (Table 1). Fraction B, which accounted for 21% by weight of the material applied to the column, was shown by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to contain two major and several minor acidic oligosaccharides. Fraction B has a glycosyl-residue composition similar to RG-II (Table 1). Fraction C, which accounted for approximately 19% of the material applied to the column, eluted in the region for an acidic oligosaccharide with a dp < 6 and was shown by HPAEC-PAD (data not shown) to contain a mixture of mono- and oligosaccharides with dp < 6.

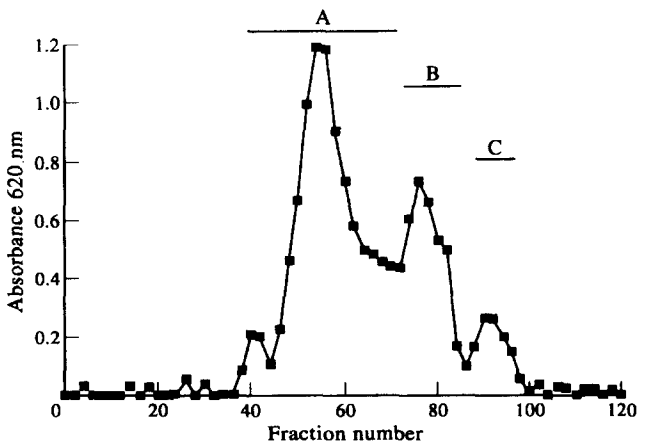

Fig. 1. Bio-Gel P-10 size-exclusion chromatography of the products generated by partial acid hydrolysis (0.1 M TFA, 24 h at 40° C) of RG-II. Fractions (1.5 mL) were collected and portions (50 μL) assayed colorimetrically (OD_{620}) for neutral sugars by the anthrone assay [17]. Fractions A–C were pooled as shown by the bars.

Table 1
Glycosyl-residue compositions of $NaBD_4$-reduced Fractions A and B generated by partial acid hydrolysis of RG-II and of the oligoglycosyl alditols B1 through B6 purified by semipreparative HPAEC-PAD of Fraction B

	A	B	B1	B2	B3	B4	B5	B6
	mol%	mol%						
Apiitol [a]	10	9	8	8	8	10	9	7
2-*O*-Me-Fuc	7	9	16	11	14	9	5	11
Rha	20	26	34	23	31	25	27	21
Ara	29	27	22	36	28	36	35	42
AceA	2	5	6	4	5	2	3	4
Gal	17	16	15	14	14	10	13	12
Fuc	6	5	0	2	1	2	5	1
2-*O*-Me-Xyl	9	5	0	2	tr [b]	5	3	2

[a] Apiitol was the only deuterium-reduced alditol detected by glycosyl-residue composition analysis.
[b] tr = < 1%.

Semipreparative HPAEC-PAD purification of the oligoglycosyl alditols generated by $NaBD_4$ reduction of Fraction B.—HPAEC-PAD is a powerful technique for separating complex mixtures of acidic oligosaccharides [13]. However, the alkaline eluant used to elute the carbohydrates from the CarboPac column results in base-catalyzed degradation of reducing glycoses [14]. Thus, the acidic oligosaccharides in Fraction B were reduced with $NaBD_4$ to generate their corresponding oligoglycosyl alditols. Apiitol was shown, by glycosyl-residue composition analysis, to account for 10 mol% of $NaBD_4$-reduced Fraction B and was the only deuterium-reduced alditol detected (Table 1). Thus, apiose is the reducing terminus of all the oligosaccharides in Fraction B.

The oligoglycosyl alditols generated by $NaBD_4$ reduction of Fraction B were purified using a semipreparative CarboPac PA1 column. Six fractions (B1–B6, see Fig. 2) were collected, and each was shown, by glycosyl-residue composition analysis, to contain, in addition to apiitol, L-rhamnosyl, L-arabinosyl, D-galactosyl, 2-*O*-methyl L-fucosyl, and

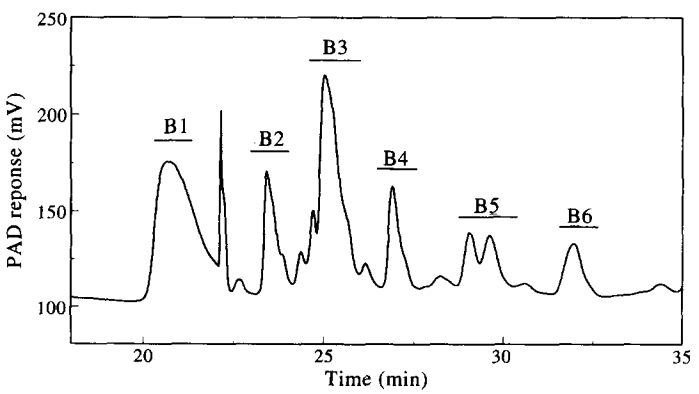

Fig. 2. Semipreparative HPAEC-PAD of the acidic oligoglycosyl alditols generated by $NaBD_4$ reduction of Fraction B (see Fig. 1 and text for experimental details). The peaks which eluted from the HPAE column were collected as shown, neutralized, dialyzed, and then freeze-dried.

Table 2
Glycosyl-linkage compositions of the oligoglycosyl alditols present in Fractions B1 through B6 isolated by semipreparative HPAEC-PAD

Linkage [a]	B1	B2	B3	B4	B5	B6
	mol %					
T-Rha	18	0	0	0	3	8
2-Rha	0	9	17	10	15	17
3-Rha	18	7	19	10	19	19
2,3-Rha	0	7	0	15	0	0
T-Fuc	0	0	0	0	11	0
T-(2Me)Fuc	18	14	14	15	0	14
4-(2Me)Fuc [b]	0	6	0	0	0	0
3,4-(2Me)Fuc [b]	0	12	0	15	0	9
T-Ara*f*	0	7	11	10	13	0
2-Ara*p*	17	14	15	0	16	10
2,3-Ara*p* [b]	4	12	0	5	3	0
2-AceA	6	tr [c]	5	0	tr	0
4-Gal	0	0	0	0	5	0
2,4-Gal	19	12	19	20	15	23

[a] The methylated 3′-linked apiitol derivative (3′-*O*-Ac-1,2,3,4-Me_4 apiitol), which is considerably more volatile than the other methylated alditol acetates, was not recovered. [b] These glycosyl residues are likely to have arisen from undermethylation. [c] tr = < 1%.

aceryl residues (Table 1). L-Fucosyl and D-xylosyl residues were also present in Fractions B2–B6, but together these residues accounted for < 10% of those fractions. The oligoglycosyl alditols present in Fractions B1–B6 and heptasaccharide **2** have similar glycosyl-residue compositions (Table 2). These results indicate that partial acid hydrolysis of RG-II generates a series of structurally related oligosaccharide side chains.

The oligoglycosyl alditols present in the quantitatively predominant Fractions B1 and B3 (see Fig. 2) were characterized by electrospray mass spectrometry (ESMS), ^{1}H NMR spectroscopy, and glycosyl-residue and glycosyl-linkage composition analyses. The

Table 3
Chemical shifts and coupling constants for the anomeric protons in the acidic oligoglycosyl alditols in Fractions B1 and B3 that were purified by semipreparative HPAEC-PAD

Residue	Linkage	Fraction B1		Fraction B3	
		δ(ppm) [a]	$J_{1,2}$(Hz)	δ(ppm)	$J_{1,2}$(Hz)
α-D-Gal*p*	2,4-	5.71	3.6	5.70	3.7
α-L-MeFuc*p*	T [b]	5.32	4.6	5.32	4.8
α-L-Rha*p*	T	5.13	< 1	np [c]	
α-L-Rha*p*	2-	np		5.14	< 1
β-L-Ara*f*	T	np		5.09	4.6
α-L-Ara*p* [d]	2-	4.72	6.7	4.72	6.2
β-L-Ace*f*A [d]	2-	4.70	7.9	4.70	3.7
β-L-Rha*p* [d]	3-	4.20	< 1	4.21	< 1

[a] Chemical shifts reported relative to internal acetone at δ 2.07 ppm. [b] T = terminal nonreducing. [c] np = not present. [d] These signals have not been unambiguously assigned.

oligoglycosyl alditols present in the quantitatively minor Fractions B2 and B4–B6 (see Fig. 2) were only available in amounts sufficient for ESMS and glycosyl-residue and glycosyl-linkage composition analyses.

Structural characterization of the oligoglycosyl alditols present in Fractions B1 and B3.—The electrospray (ES) mass spectrum of Fraction B1 contained ions at m/z 1060 and 1082, corresponding to the $[M + H]^+$ and $[M + Na]^+$ ions, respectively, of a hexaglycosyl alditol composed of two rhamnosyl residues, one arabinosyl residue, one 2-*O*-methyl fucosyl residue, one galactosyl residue, one aceryl acid residue, and apiitol (data not shown). The methylated 3′-linked apiitol derivative, which is extremely volatile, was not detected by glycosyl-linkage composition analysis. We have assumed that the apiitol residues are 3′-linked in all of the acidic oligoglycosyl alditols in Fractions B1–B6. The ^{1}H NMR spectrum of Fraction B1 contained signals for six anomeric protons (Table 3) and, in combination with the results of glycosyl-residue and glycosyl-linkage composition analyses (Tables 1 and 2), established that the hexaglycosyl alditol in Fraction B1 was generated by $NaBD_4$ reduction of previously characterized heptasaccharide **2**.

The ES mass spectrum of Fraction B3 contained ions at m/z 1192 and 1214, corresponding to the $[M + H]^+$ and $[M + Na]^+$ ions, respectively, of a heptaglycosyl alditol composed of two rhamnosyl residues, one arabinosyl residue, one 2-*O*-methyl fucosyl residue, one galactosyl residue, one aceryl acid residue, one pentosyl residue, and apiitol. The heptaglycosyl alditol in B3 contains one more pentosyl residue than the hexaglycosyl alditol in Fraction B1. The ^{1}H NMR spectrum of Fraction B3 contained signals for seven anomeric protons (Table 3). Six of those signals are also present in the ^{1}H NMR spectrum of Fraction B1 (see Table 3). Thus, the signal at δ 5.09 in the ^{1}H NMR spectrum of Fraction B3 (Table 3) must originate from the anomeric proton of the extra pentosyl residue of the heptaglycosyl alditol. This residue was shown, by glycosyl-linkage composition analysis, to be a terminal nonreducing arabinofuranosyl residue linked to position 2 of the α-L-rhamnosyl residue (Table 2). The arabinofuranosyl and arabinopyranosyl residues in the heptaglycosyl alditol (see Table 2) were both shown, by gas-liquid chromatography (GLC) analysis of the trimethylsilylated (*R*)-2-butyl glycosides, to have the L configuration (data not shown).

The anomeric configurations of furanosides are not easily determined by ^{1}H NMR spectroscopy [6], because the chemical shift of H-1 is not a good indentifier of the anomeric configuration. However, the $J_{1,2}$ values of the α- and β-L-arabinofuranosides differ significantly [6]. The $J_{1,2}$ coupling constant of the terminal arabinofuranosyl residue (4.6 Hz) in the heptaglycosyl alditol (Table 3) agrees with that of β-L-arabinofuranosides ($\approx$ 4.2 Hz) and not that of α-L-arabinofuranosides ($\approx$ 1.1 Hz). Thus, the heptaglycosyl alditol in Fraction B3 was generated by $NaBD_4$ reduction of octasaccharide **1**.

Octasaccharide **1** is equivalent to heptasaccharide **2** with the addition of a terminal nonreducing β-L-arabinofuranosyl residue attached to position 2 of the terminal nonreducing α-L-rhamnopyranosyl residue. Approximately equal amounts of **1** and **2** were recovered from sycamore RG-II following partial acid hydrolysis. Heptasaccharide **2** may be generated by hydrolysis of the terminal β-L-arabinofuranosyl residue in octasaccharide **1**.

The octasaccharide **1** side chain is probably not unique to sycamore RG-II, since 2-linked rhamnosyl residues are present in red wine RG-II [10], and an $[M + H]^+$ ion corresponding to octasaccharide **1** is present in the FAB mass spectrum of the products generated by partial acid hydrolysis of cotton cell wall RG-II [15].

Localization of O-acetyl groups on heptasaccharide 2 and octasaccharide 1 by electrospray-ionization in combination with tandem mass spectrometry (ESMSMS).— Previous studies have shown that some of the aceryl acid-containing heptasaccharides are mono-*O*-acetylated and some are di-*O*-acetylated, but the glycosyl residues substituted with acetyl groups were not identified [1]. We now confirm that both heptasaccharide **2** and octasaccharide **1** are partially *O*-acetylated. The partially *O*-acetylated hepta- and octasaccharides could not be purified by HPAEC-PAD since the pH of the eluants (pH $\approx$ 13) is sufficient to *O*-deacylate the oligosaccharides. The numbers and the locations of the *O*-acetyl groups were determined by ESMS and ESMSMS analyses, respectively, of the mixture of oligosaccharides present in Fraction B. The ES mass spectrum of Fraction B contained ions at m/z 1057, 1099, and 1141, corresponding to the $[M + H]^+$ ions of heptasaccharide **2** containing zero, one, or two *O*-acetyl groups, respectively, and ions at m/z 1189, 1231, and 1273 corresponding to the $[M + H]^+$ ions of octasaccharide **1** containing zero, one, or two *O*-acetyl groups, respectively (data not shown). The ESMSMS analyses were carried out by selecting, in the first quadrupole of the MS, the $[M + H]^+$ ions of the oligosaccharides containing zero, one, or two *O*-acetyl groups, and then, in turn, bombarding these with argon gas in the open-structured quadrupole collision cell. The oligosaccharide fragments generated by collision with the neutral gas were separated in the third quadrupole of the MS to obtain the daughter-ion spectra.

The daughter-ion spectra of di-*O*-acetylated (Fig. 3a) and mono-*O*-acetylated (Fig. 3b) heptasaccharide **2** both contained ions corresponding to the protonated molecular ion and major fragment ions corresponding to the elimination of a water molecule from the protonated parent ion. After elimination of water, less intense fragment ions, originating from both the reducing and nonreducing termini of the oligosaccharides, were generated by cleavage of glycosidic linkages. For example, elimination of water from the protonated molecular ion (m/z 1141) of di-*O*-acetylated heptasaccharide **2** gives a fragment at m/z 1123, which then undergoes the sequential loss of one apiosyl (m/z 132), two rhamnosyl (m/z 292), and one arabinosyl (m/z 132) residues to generate fragments at m/z 991, 845, 699, and 567, respectively (Fig. 3a). The protonated m/z 567 fragment ion contains a 2-*O*-Me-fucosyl residue (m/z 160), an aceryl acid residue (m/z 160), a galactosyl residue (m/z 162), and two *O*-acetyl groups (m/z 84). Fragmentation of the ion at m/z 567 results in the loss of either a mono-*O*-acetylated 2-*O*-Me-fucosyl residue or a mono-*O*-acetylated aceryl acid residue, which, in both cases results in an ion at m/z 365, as the 2-*O*-Me-fucosyl and aceryl acid residues have the same mass. The formation of an ion at m/z 203 from the ion at m/z 365 provides strong evidence that the galactosyl residue (m/z 162) is not *O*-acetylated.

The fragmentation pattern of the ESMSMS daughter-ion spectrum of mono-*O*-acetylated heptasaccharide **2** (Fig. 3b) is more complex than the corresponding daughter-ion spectrum of di-*O*-acetylated heptasaccharide **2** (Fig. 3a). This complexity was shown to be due, in part, to the presence in Fraction B of two mono-*O*-acetylated heptasaccha-

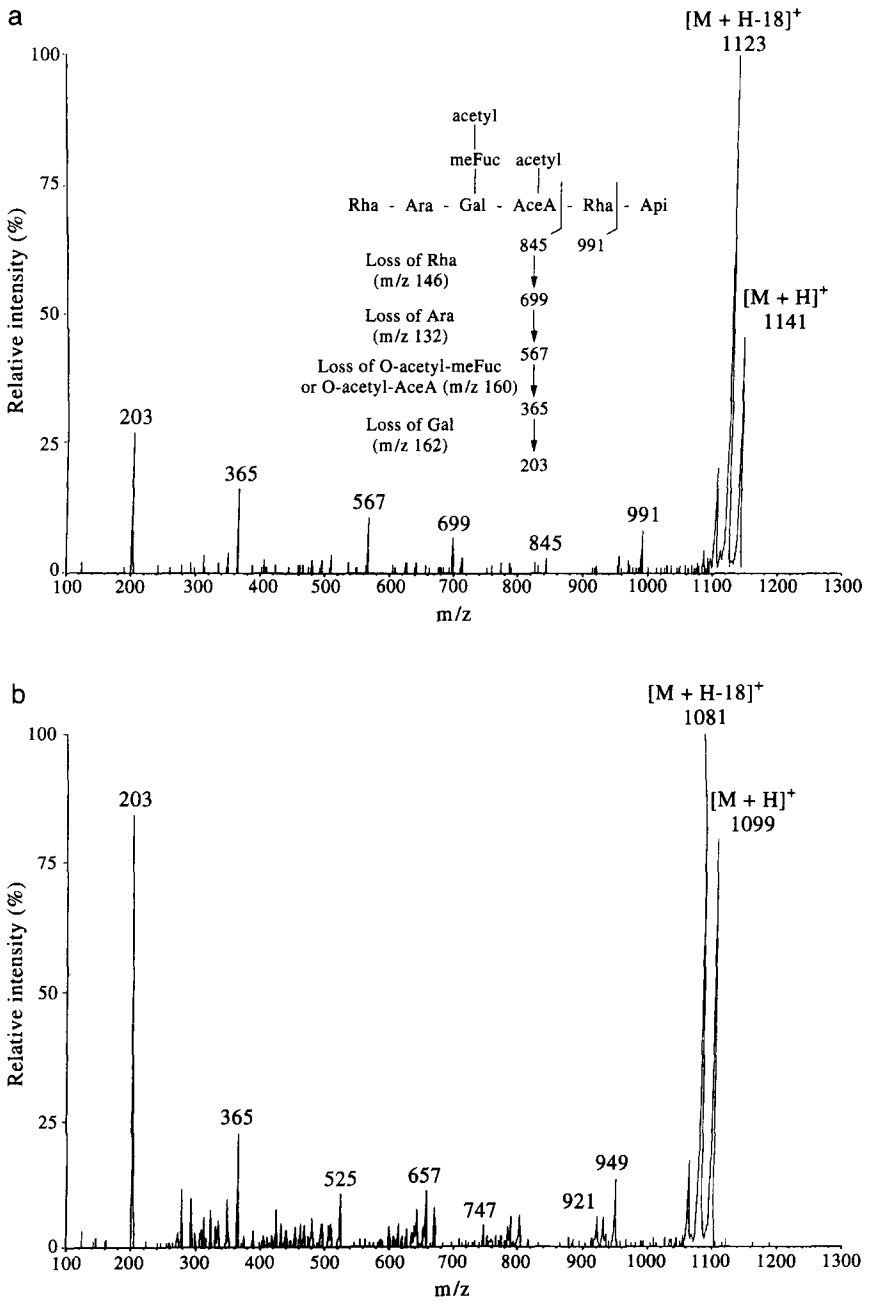

Fig. 3. Electrospray MSMS daughter-ion spectrum of partially *O*-acetylated heptasaccharide **2** present in Fraction B. A, the daughter-ion spectrum of di-*O*-acetylated heptasaccharide **2** (*m/z* 1141). The insert shows the glycosyl sequence of di-*O*-acetylated heptasaccharide **2** and the proposed origins of the fragment ions. B, the daughter-ion spectrum of mono-*O*-acetylated heptasaccharide **2** (*m/z* 1099).

rides that differ in the location of their *O*-acetyl groups. Those mono-*O*-acetylated heptasaccharide molecules were shown by collision-induced fragmentation of the protonated parent ion (*m/z* 1099), to contain either a mono-*O*-acetylated 2-*O*-Me-fucosyl residue or a mono-*O*-acetylated aceryl acid residue. Elimination of water from the ion at *m/z* 1099 generated a fragment ion at *m/z* 1081 (Fig. 3b) that then lost either an apiosyl (*m/z* 132) or a 2-*O*-Me fucosyl residue (*m/z* 160) to give fragment ions at *m/z* 949 and 921, respectively (Fig. 3b). The fragment ion at *m/z* 949 generated an ion at *m/z* 747 by the loss of 202 amu corresponding to a mono-*O*-acetylated 2-*O*-Me fucosyl residue. Thus, some of the 2-*O*-Me fucosyl residues are mono-*O*-acetylated, and some are not *O*-acetylated.

The protonated fragment ion at *m/z* 525 (Fig. 3b) contains a 2-*O*-Me fucosyl residue (*m/z* 160), an aceryl acid residue (*m/z* 160), a galactosyl residue (*m/z* 162), and one *O*-acetyl group (*m/z* 42). Further fragmentation of the ion at *m/z* 525 results in the loss of either the 2-*O*-Me-fucosyl or the aceryl acid residue (*m/z* 160) and then a galactosyl residue (*m/z* 162) to form ions at *m/z* 365 and 203, respectively. The fragment ion at *m/z* 203 corresponds to a mono-*O*-acetylated 2-*O*-Me-fucosyl or mono-*O*-acetylated aceryl acid residue. Both these residues have the same mass and therefore cannot be distinguished by ESMSMS. However, the loss of 162 amu from the ion at *m/z* 365 (Fig. 3b) provides additional evidence that the galactosyl residue (*m/z* 162) is not *O*-acetylated.

The corresponding ESMSMS daughter-ion mass spectra of the mono- and di-*O*-acetylated octasaccharide **1** (data not shown) similarly contained fragment ions that were consistent with the presence of *O*-acetyl groups on both the 2-*O*-Me fucosyl and/or aceryl acid residues. The results of the ESMSMS analyses provide evidence that mono-*O*-acetylated derivatives of both heptasaccharide **2** and octasaccharide **1** are partially mono-*O*-acetylated on their 2-*O*-Me fucosyl and aceryl acid residues.

Table 4
ESMS protonated molecular ions of the oligoglycosyl alditols present in Fractions B2, B4, B5, and B6 isolated by semipreparative HPAEC-PAD of Fraction B

HPAEC-PAD fraction	ESMS $[M+H]^+$	Deduced number of glycosyl residues [a]					
		DH	PT	HX	mDH	AceA	Api'ol
B2	913	1	1	1	1	1	1
	1337	3	2	1	1	1	1
B4	899	2	1	1	0	1	1
B5	899	2	1	1	0	1	1
	1031	2	2	1	0	1	1
	1177	3	2	1	0	1	1
B6	910	1	1	1	1	1	1 [b]
	1031	2	2	1	0	1	1

[a] DH = 6-deoxyhexose, PT = pentose, HX = hexose, mDH = mono-*O*-methyl 6-deoxyhexose, AceA = aceric acid, and Api'ol = apiitol. [b] The mass of the protonated molecular ion ($[M + H]^+$) corresponds to the mass of an oligosaccharide fragment containing apiose rather than apiitol at the reducing terminus. Such a fragment would result from the incomplete $NaBD_4$ reduction of apiose.

The *O*-acetyl group on the 2-*O*-Me fucosyl residue could be located either at O-3 or O-4. The exact location could not be determined by ESMSMS, since the carbon–carbon bonds in the sugar ring are not fragmented by the soft ionization technique used to generate the daughter-ions. In contrast, the *O*-acetyl group on the aceryl acid residue was located without fragmenting the carbon–carbon bonds in the sugar ring. This residue is 2-linked, and its only free hydroxyl group is O-3. Therefore, when the aceryl acid residue is *O*-acetylated, the O-acetyl group is on O-3.

Partial structural characterization of the semipreparative HPAEC-PAD purified oligoglycosyl alditols present in Fractions B2, B4, B5 and B6.—Fractions B2, B4, B5, and B6 were each shown by glycosyl-linkage composition analysis (Table 2) and ESMS (Table 4) to contain mixtures of several oligoglycosyl alditols structurally related to heptasaccharide **2** and octasaccharide **1**. Oligoglycosyl alditols with the same mass were present in more than one fraction (Table 4). For example, one or more pentaglycosyl alditols (m/z 899) present in Fractions B1, B4, and B5 may originate from heptasaccharide **2** by removal of the 2-*O*-Me fucosyl residue. These fractions were not available in sufficient quantities to determine the complete structure of the pentaglycosyl alditols. Thus, it is not known if the pentaglycosyl alditols in Fractions B1, B4, and B5 are structurally identical or have closely related structures.

Fraction B2 contained a pentaglycosyl alditol ($[M + H]^+ = m/z$ 913) that may have been formed from reduced heptasaccharide **2** by the removal of the terminal nonreducing rhamnosyl residue. Fraction B2 also contains an octaglycosyl alditol ($[M + H]^+ = m/z$ 1337) that is equivalent to reduced octasaccharide **1** with the addition of a 6-deoxyhexosyl residue. Fractions B5 and B6 contain one or more hexaglycosyl alditols ($[M + H]^+ = m/z$ 1031) that correspond to reduced octasaccharide **1** minus the 2-*O*-Me fucosyl residue (Table 4). Fraction B5 also contains a heptaglycosyl alditol ($[M + H]^+ = m/z$ 1177) that corresponds to reduced octasaccharide **1** in which a fucosyl or rhamnosyl residue has replaced the 2-*O*-Me fucosyl residue (see Tables 2 and 4). Fraction B6 contains a hexasaccharide ($[M + H]^+ = m/z$ 910) that has an apiose residue rather than an apiitol at its reducing terminus. Such a fragment would result from the incomplete reduction of apiose with $NaBD_4$.

Some of the oligosaccharides in Fractions B2, B4, B5, and B6 probably arose because the glycosidic linkage of the 2-*O*-Me fucosyl residue is acid labile and may have been cleaved when the oligosaccharides were generated by treating RG-II with 0.1 M TFA. However, those oligosaccharides with additional or new glycosyl residues could not have resulted from hydrolysis of heptasaccharide **2** or octasaccharide **1** and are probably present in minor amounts in cell wall RG-II.

Isolation and characterization of backbone fragments of RG-II.—Evidence was presented in a previous study that the backbone of RG-II is composed of at least seven, linear 1,4-linked α-D-galactosyluronic acid residues [12]. We now provide the results of further characterization of the backbone. Backbone fragments were generated by treating RG-II with 0.1 M TFA for 16 h at 80° C. A portion of the hydrolysate was analyzed by HPAEC-PAD and shown to contain a series of components (Fig. 4) that cochromatograph with standard 1,4-linked α-D-oligogalacturonides. To obtain the fragments in quantities sufficient for structural analysis, the total hydrolysate was chromatographed on a column of QAE-Sephadex by stepwise elution with increasing concentrations of

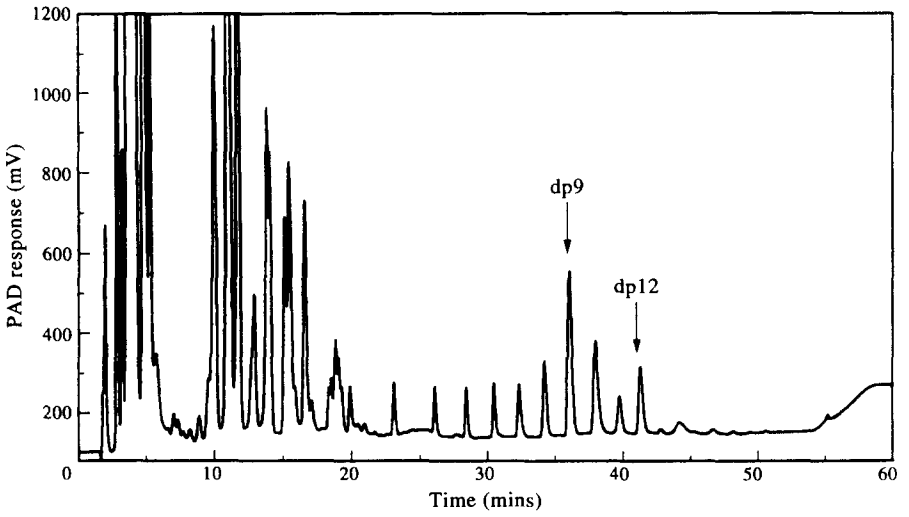

Fig. 4. HPAEC-PAD of the backbone fragments generated by partial acid hydrolysis (0.1 M TFA, 16 h at 80°C) of RG-II. The peaks labeled dp 9 and dp 12 correspond to the elution positions of the authentic nonagalacturonide and the dodecagalacturonide, respectively. The peaks eluting between 10 and 20 min correspond to oligosaccharide side chain fragments known to be generated by partial acid hydrolysis of RG-II.

ammonium bicarbonate. Three fractions, which eluted with 0.25, 0.5, and 1.0 M ammonium bicarbonate, respectively, were shown by glycosyl-residue composition analysis to be enriched (> 80 mol%) in galactosyluronic acid residues. The ^{1}H NMR spectrum of each fraction contained broad signals for anomeric protons at δ 5.29, 5.11, 5.02, and 4.52 that were assigned to the α-anomer of reducing galacturonic acid, terminal nonreducing α-D-galactosyluronic acid, 4-linked α-D-galactosyluronic acid, and to the β anomer of reducing D-galacturonic acid, respectively. Broad signals at δ 4.72, 4.37, 3.95, and 3.70 were assigned to H-5, H-4, H-3, and H-2, respectively, of the 4-linked α-D-galactosyluronic acid residues[13].

The three galactosyluronic acid-enriched fractions that eluted with ammonium bicarbonate were each shown by HPAECPAD to contain oligogalacturonides of varying dps that co-eluted with standard 1,4-linked α-D-oligogalacturonides. ESMS analysis established that oligogalacturonides with dps 3–6, 4–10, and 7–12 were the predominant components of those fractions that eluted with 0.25, 0.5, and 1.0 M ammonium bicarbonate, respectively. These backbone fragments are composed of linear 1,4-linked α-D-oligogalacturonides, since they are hydrolyzed by treatment with a homogeneous *endo*polygalacturonase (data not shown). These results suggest that the RG-II backbone contains up to twelve 1,4-linked α-D-galactosyluronic acid residues.

The nonreducing terminus of the RG-II backbone was shown to contain 1,4-linked α-D-galactosyluronic acid residues susceptible to *exo*-polygalacturonase by demonstrating a significant reduction in the relative proportion of the backbone fragment with dp 12 when RG-II was treated with *exo*-polygalacturonase prior to acid hydrolysis. Thus,

the backbone of RG-II generated by treatment with *endo*- and *exo*-polygalacturonase contains up to eleven 1,4-linked α-D-galactosyluronic acid residues with RG-II containing nine galactosyluronic acid residues being the most abundant.

3. Conclusions

The data presented here establish that sycamore RG-II contains an octasaccharide side chain **1** that is equivalent to previously characterized heptasaccharide **2** with an additional glycosyl residue [1]. Octasaccharide **1** contains a terminal nonreducing β-L-arabinofuranosyl residue linked to position two of the terminal nonreducing α-L-rhamnopyranosyl residue of heptasaccharide **2**. This is the second report showing an oligosaccharide side chain of RG-II containing a terminal nonreducing β-L-arabinofuranosyl residue; this residue is also a component of the diglycosyl side chain of RG-II that is composed of a β-L-arabinofuranosyl-(1 → 5)-3-deoxy-D-*lyxo*-2-heptulosaric acid [6].

Octasaccharide **1** and heptasaccharide **2** were each isolated containing zero, one, or two *o*-acetyl groups. The 2-*O*-Me-fucosyl and aceryl acid residues are each mono-*O*-acetylated in the di-*O*-acetylated molecules. Mono-*O*-acetylated derivatives of **1** and **2** were isolated as mixtures in which some of the molecules contained a mono-*O*-acetyl 2-*O*-Me-fucosyl residue and some a mono-*O*-acetyl aceryl acid residue. It is not known if mixtures of the mono-*O*-acetylated oligosaccharide side chains are present in vivo or if they are generated from the di-*O*-acetylated oligosaccharides during partial acid hydrolysis of RG-II.

We have obtained evidence that other structurally related oligosaccharide side chains, including a nonasaccharide, are obtained by mild acid hydrolysis of RG-II. Oligosaccharides with a dp < 7 could be generated by partial acid hydrolysis of heptasaccharide **2** or octasaccharide **1**. However, the nonasaccharide present in Fraction B2 and the octasaccharide present in Fraction B5 are likely to be genuine side chains of RG-II, although the quantity of these side chains in intact RG-II remains to be determined. Our results also show that RG-II has a backbone that contains between eight and eleven 1,4-linked α-D-galactosyluronic acid residues with nine residues being the most abundant. Determining the complete structure of this polysaccharide, which is a goal of this laboratory, remains a challenging task.

4. Experimental

Isolation of RG-II.—RG-II was isolated from the walls of suspension-cultured sycamore cells and purified as described [2,16].

Partial acid hydrolysis of RG-II.—A solution of RG-II (125 mg) in 0.1 M TFA (10 mL) was heated at 40° C for 24 h. The acid was evaporated under a stream of dry air at 30° C, and a solution of the residue in 50 mM NaOAc, pH 5.2 (2 mL) was fractionated on a BioGel P-10 column (100 × 2 cm) equilibrated in the same buffer. Fractions (1 mL) were collected, and portions (50 mL) were analyzed colorimetrically for neutral

sugars [17] and uronic acids [18]. Fractions containing carbohydrate were pooled, desalted, and then freeze-dried.

Generation and isolation of RG-II backbone fragments.—A solution of RG-II (20 mg) in 0.1 M TFA (10 mL) was heated at 80° C for 16 h. The acid was evaporated under a stream of dry air at 30° C, and a solution of the residue in water was freeze-dried. A portion (200 μg) of the hydrolyzate was analyzed by HPAEC-PAD. The remaining material (~ 18 mg) was fractionated by stepwise elution from a QAE-Sephadex column (15 × 2 cm) using 0.25, 0.5, and 1.0 M ammonium bicarbonate. Fractions (1 mL) were collected and portions (50 mL) analyzed colorimetrically for uronic acid[18]. Fractions containing uronic acid were pooled and freeze-dried.

High performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD).—HPAEC-PAD was performed with a Dionex BioLc interfaced to an AutoIon Series 400 data station. Carbohydrates were separated on a CarboPac PA1 column (Dionex Corp., Sunnyvale, California) and detected electrochemically (PAD II, Dionex). The electrochemical detector, with a gold working electrode, was operated in the pulsed amperometric mode ($E1$ 0.05 V, $E2$ +0.6 V, and $E3$ −0.3 V; $T1$ 480 ms, $T2$ 180 ms, and $T3$ 360 ms). Eluants were prepared using ultrapure water, carbonate-free 50% NaOH (J.T. Baker, Phillipsburg, New Jersey), and $NaOAc{\cdot}3H_2O$ (Sigma, St. Louis, Missouri). Eluants were filtered (0.2 μm Nylon 66 membranes, Rainin, Woburn, Massachusetts) and degassed with helium using an eluant degas module (Dionex).

An aliquot (~ 200 μg) of the oligosaccharides generated by partial acid hydrolysis of RG-II were analyzed by HPAEC-PAD on a CarboPac PA1 column (4 × 250 mm) eluted at 1mL/min and detected by PAD operated at 1 μamp sensitivity. The gradients were formed from 0.1 M NaOH and from 1.0 M NaOAc containing 0.1 M NaOH. The column was eluted with 0.1 M NaOH (0–2 min), followed by a linear gradient (2–30 min) of NaOAc (0–0.2 M) in 0.1 M NaOH. The column was then eluted with a linear gradient (30–54 min) of NaOAc (0.2–1.0 M) in 0.1 M NaOH. The column was washed with 1.0 M NaOAc in 0.1 M NaOH for 5 min and then re-equilibrated with 0.1 M NaOH for 20 min.

The oligoglycosyl alditols (~ 2.5 mg) formed by $NaBD_4$ reduction of the side chain oligosaccharides, which were themselves generated by partial acid hydrolysis of RG-II, were purified by HPAEC-PAD at 5 mL/min using a semipreparative CarboPac PA1 column (7.6 × 250 mm) with PAD operated at 30 μamp sensitivity. The gradients were formed from 0.1 M NaOH and from 1.0 M NaOAc containing 0.1 M NaOH. The column was eluted with 0.1 M NaOH (0–2 min), followed by a linear gradient (2–20 min) of NaOAc (0–0.05 M) in 0.1 M NaOH. The column was then eluted with a linear gradient (20–35 min) of NaOAc (0.05–0.08 M) in 0.1 M NaOH. The column was washed with 1.0 M NaOAC in 0.1 M NaOH for 5 min and then reequilibrated in 0.1 M NaOH for 20 min. The samples eluting in the peaks were collected manually (see Fig. 2), neutralized, dialyzed (1000 mwco), and then freeze-dried.

RG-II backbone fragments generated by partial acid hydrolysis were analyzed by HPAEC-PAD using a CarboPac PA-1 column (4 × 250 mm). The column was eluted with 0.1 M NaOAc in 0.1 M NaOH (0–5 min), followed by a linear gradient (5–25 min) of NaOAc (0.1–0.5 M) in 0.1 M NaOH. The column was then eluted with a linear gradient (25–50 min) of NaOAc (0.5–0.7 M) in 0.1 M NaOH. The column was washed

with 1.0 M NaOAc in 0.1 M NaOH for 10 min and then reequilibrated in 0.1 M NaOAc and 0.1 M NaOH for 20 min.

Glycosyl–esidue and glycosyl–linkage composition analyses.—The oligosaccharides (100 μg) released by partial acid hydrolysis of RG-II were analyzed for neutral sugars by GLC of their alditol acetates [2] and for neutral and acidic sugars by GLC of their per-*O*-trimethylsilylated methyl glycoside methyl esters [2]. The glycosyl-linkage compositions of the oligoglycosyl alditols (200 μg) were determined by methylation analysis and GLC-MS as described [2], except that trideuteriomethyl iodide was used as the alkylating reagent.

Determination of the absolute configuration of the glycosyl residues.—The absolute configurations of sugars were determined by a modification of the procedure of Gerwig et al. [19]. Solutions of the oligosaccharides or oligoglycosyl alditols (~ 100 μg) in (*R*)-2-butanol containing 1.0 M HCl (200 μL) were heated at 80° C for 16 h. The solutions were cooled and the solvents evaporated under a flow of air. The residual acid was removed by codistillation with MeOH (3 × 500 μL). The residue was then treated with Tri-Sil (Pierce, Rockford, Illinois, 200 μL) at 80° C for 20 min. The resulting trimethylsilylated (R)-2-butyl glycosides were extracted into hexanes (1 mL), concentrated (100 μL), and analyzed by GLC using a 30 m DB-1 column (J & W Scientific, Folsom, California).

Electrospray mass spectrometry.—ESMS was performed with a PE-Sciex API III biomolecular analyzer operated in the positive ion mode. Solutions of oligosaccharides or oligoglycosyl alditols (100 μg) in aq 30% MeOH containing 0.75% HCl (100 μL) were infused into the electrospray source at 4 μL/min using a Harvard 22 syringe pump. The ionspray was operated at 5000 V with an orifice potential of 35 V. Ten scans (100–1500 amu) were collected and averaged.

ESMSMS was performed by selecting the appropriate protonated parent ion ($[M + H^+]$) in the first quadrupole and then bombarding it with argon gas (collision gas thickness $200–300 \times 10^{12}$ molecules/cm^2) in the open-structured quadrupole collision cell. The fragment ions generated by collision with the neutral gas molecules were separated in the third quadrupole to obtain the daughter-ion mass spectra. Between 50 and 100 scans (100–1300 amu) were collected and averaged.

^{1}H NMR spectrsocopy.—^{1}H NMR spectroscopy was performed with a Bruker AM 500 spectrometer operated at 500 MHz. Carbohydrates were repeatedly exchanged with D_2O prior to analyses. Chemical shifts (δ) are reported in ppm relative to internal acetone (δ 2.07).

Enzyme treatments of RG-II and RG-II fragments.—Enzyme treatments were performed with solutions of RG-II (250 μg) in 100 mM NaOAC, pH 5.2, containing 0.02% thimerosal at 30° C for 16 h. Purified *exo*-polygalacturonase (tomato) was a gift of R. Pressey (USDA, Richard Russell Research Center, Athens, Georgia), and *endo*-polygalacturonase (*Aspergillus niger*) was a gift of C. Bergmann (CCRC).

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