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Carbohydrate Research 343 (2008) 1041–1049
Carbohydrate RESEARCH

Isolation and structural characterization of a novel oligosaccharide from the rhamnogalacturonan of *Gossypium hirsutum* L.

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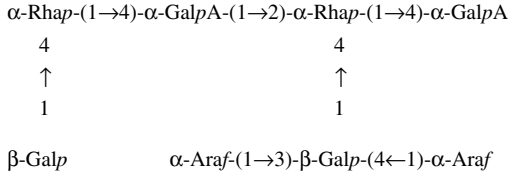
Received 14 November 2007; received in revised form 20 February 2008; accepted 26 February 2008
Available online 4 March 2008

Abstract—An alkali extract of cell walls of *Gossypium hirsutum* L. was sequentially digested by *endo*-polygalacturonase (EC 3.2.1.15), arabinofuranosidase AN1571.2 (EC 3.2.1.55), *endo*-arabinase (EC 3.2.1.99), and rhamnogalacturonan hydrolase AN9314.2 (EC 3.2.1.15). The rhamnogalacturonan hydrolase-generated oligosaccharides were separated by ultrafiltration, size-exclusion, and anion exchange chromatography. Fractions from the anion exchange chromatography were pooled, lyophilized, and screened by MALDI-TOFMS. A new oligosaccharide (RGS29), which contained a rhamnogalacturonan dimer backbone with two galactose and two arabinose residues in the side chains, was found. Its structure was identified by 1D and 2D NMR spectra as follows:



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Keywords: Rhamnogalacturonan; Enzymatic degradation; Oligosaccharide; Pectin; *Gossypium hirsutum*

1. Introduction

Rhamnogalacturonan I (RG-I), a complex pectic polysaccharide comprised of mainly neutral oligosaccharide side chains attached to a backbone of alternating rhamnosyl and galacturonosyl units, is an important component of the primary cell walls of dicotyledonous plants. The side chains typically contain α-arabinose and β-galactose residues.[1,2] Many researchers have focused on the structure of rhamnogalacturonan by chemical or enzymatic methods,[2–7] and models of pectin structures were proposed.[8,9] However, we still do not know exactly how the neutral sugar side chains, especially the arabinan, are linked to the rhamnogalacturonan backbone, due in part to their lability to traditional acid hydrolysis.[10] This problem has been partially overcome since a collection of cloned cell wall polysaccharide degrading enzymes has become available.[11] Because of the complexity and relatedness of the oligosaccharides from enzymatic degradation products from RG-I, it was very difficult to separate them. In this paper, we describe the purification and structural characterization of a new octasaccharide (RGS29) isolated from cell walls of *Gossypium hirsutum* L. It contains a rhamnogalacturonan dimer backbone with two galactose and two arabinose residues in two side chains. The glycosyl linkages between sugars were identified by TOCSY, COSY, HMQC, HMBC, and NOESY spectra.

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0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.02.021

2. Results and discussion

The scheme of production and purification of the new rhamnogalacturonan oligomer RGS29 is illustrated in Figure 1. In the alkali extraction, 0.1% sodium borohydride was used to prevent the peeling reaction.[12] The alkali extract was passed through a DEAE-650S column for separation into three fractions. The neutral polysaccharides were eluted in water and acidic polysaccharides in 0.5 and 2.0 M ammonium acetate buffer. According to GLC analysis, the sugar composition of the fraction eluted with water was Glc:Xyl:Gal:Fuc:Ara = 4.1:1.0:0.4:0.2:0.5 (Table 1). The most abundant component was identified as starch because there was a strong signal at δ 5.41 ppm in the ^{1}H NMR spectrum[13] and the glucose content was very high. From the sugar composition one can deduce that xyloglucan was also present. The fraction eluted with 0.5 M acetate contained Ara, Rha, Xyl, GalA, Gal, and Glc in a molar ratio of 5.2:1.0:1.5:2.2:6.2:16.0 (Table 1), and the fraction eluted with 2 M acetate was composed of Ara, Rha, Xyl, GalA, Gal, and Glc in a molar ratio of 2.9:1.0:0.5:4.5:1.8:0.5 (Table 1). CZE analysis indicated that all fractions released xyloglucan oligomers when digested with xyloglucan specific endoglucanase.[14] The fractions which eluted with 0.5 M and 2 M acetate might contain rhamnogalacturonan and xyloglucan covalently linked together.[15,16] In this project, we focused our research on the 2 M acetate-eluted fraction. The chemical shifts of signals from the C-6 methyl of rhamnopyranoses in the RG-I backbone appear at 1.22–1.25 ppm if the rhamnopyranose is unsubstituted, but at 1.28–1.32 ppm if the rhamnopyranose residue is substituted at O-4 by a β-D-Gal*p* residue.[17] The relative areas of these two chemical shift regions indicated that approximately 54% of the rhamnose was branched at O-4. There were four major types of arabinose evident in its HMQC spectrum, which contained four α-arabinofuranose anomeric signals: (5.24, 107.3), (5.18, 107.9), (5.15, 107.8), and (5.08, 108.3) ppm,[18,19] and a weak signal at 5.45 ppm in the ^{1}H NMR spectrum might also belong to arabinose. The areas of the five peaks in the ^{1}H NMR spectrum, in order of decreasing chemical shift, were 1.0:4.6:7.1:6.8:6.1.

The 2 M ammonium acetate-eluted fraction was digested by *endo*-polygalacturonase. The sugar composi-

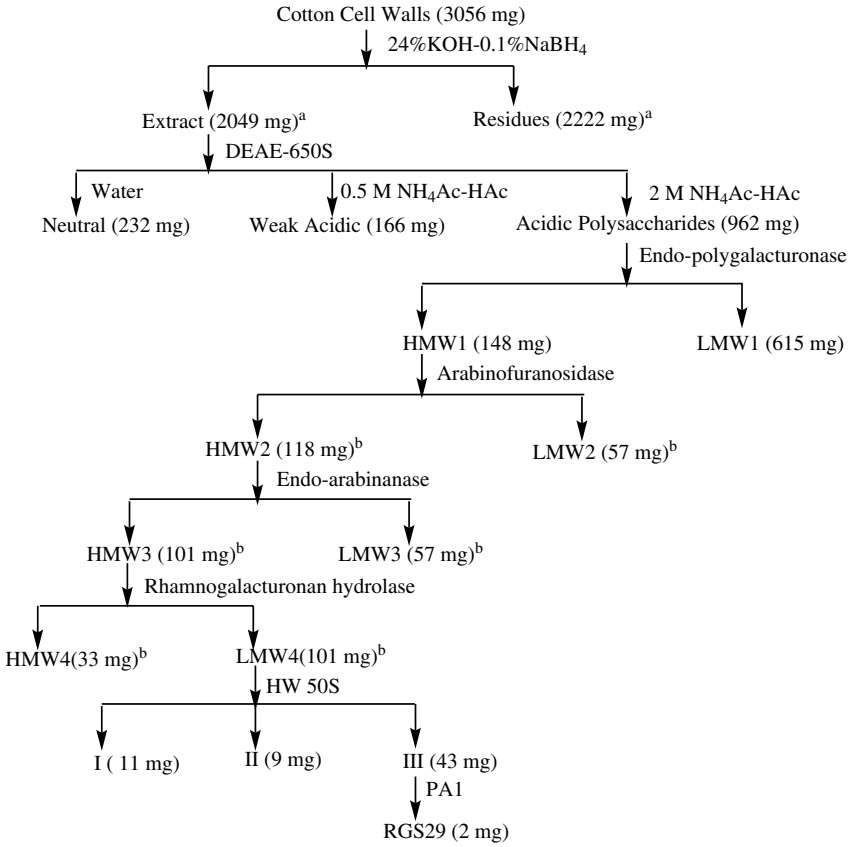

Figure 1. Flow chart of isolation and purification of RGS29 from cotton cell walls. [a]The alkali extracted residues still contained salts; [b]large volumes of enzymes relative to samples were added due to the low activity of enzymes and to commercial enzymes being preserved in 3.2 M sodium sulfate solution. Protein and salt in the preparations made the sum of the weights of high molecular weight (HMW) and low molecular weight (LMW) saccharide fractions more than those of the original saccharides. Denatured enzymes and salts could be easily removed by anion exchange chromatography. EPGase, *endo*-polygalacturonase; RGase, rhamnogalacturonan hydrolase.

Table 1. Carbohydrate compositions of saccharides in Figure 1

Saccharides	Monosaccharide composition (mol %)						
	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc
DEAE-650S fractions							
Neutral	7.2		3.3	16.1		6.7	66.7
Weak acidic	5.2	1.0		4.5	6.9	19.3	50.1
Acidic	25.9	8.9		4.6	40.1	16.1	4.4
HMW1	30.0	13.9		4.4	23.2	23.3	5.2
HMW2	17.5	19.2		4.4	27.6	26.7	4.6
HMW3	8.6	22.7		4.0	26.5	33.7	4.5
RGS29	16.7	23.8			33.3	26.2	

tion of the retentate (HMW1) after ultrafiltration indicated that it contained Ara, Rha, Xyl, GalA, Gal, and Glc in the molar ratio of 2.2:1.0:0.3:1.7:1.7:0.4 (Table 1), so about 60% of the galacturonic acid in the 2 M acetate-eluted fraction was digested into small pieces. After treatment of HMW1 with arabinofuranosidase, 60% of the arabinose was digested away and the sugar composition (HMW2) changed into Ara:Rha:Xyl:GalA:Gal:Glc = 0.9:1.0:0.2:1.4:1.4:0.2 (Table 1). The ^{1}H NMR spectrum also indicated that the signal at 5.08 ppm, which was assigned to H-1 of (1→5)-linked arabinose, became the dominant arabinose anomeric signal. The (1→5)-linked arabinose signal in the ^{1}H NMR spectrum decreased drastically after *endo*-arabinase treatment, and the peak at 5.45 ppm became a much higher proportion of the total signal. The decrease of arabinose was also indicated by the sugar composition of HMW3, which was Ara:Rha:Xyl:GalA:Gal:Glc = 0.4:1.0:0.2:1.2:1.5:0.2 (Table 1).

HMW3 was finally treated with rhamnogalacturonan hydrolase. The resulting low molecular weight fraction (LMW4) was separated on a size exclusion column (HW-50) using aqueous trifluoroacetic acid (pH 2.5) as an eluent, which could protonate the galacturonic acid and be removed by lyophilization easily. Three major peaks appeared with retention times of 126, 135, and 145 min and were collected as fraction I, II, and III, respectively, as shown in Figure 2.

The last peak off the HW-50 elution (fraction III) was pooled, lyophilized, and separated further on an anion exchange column (PA1). The ^{1}H NMR spectra of the oligomers obtained after PA1 anion exchange column chromatography of fraction III were much simpler than those of oligomers separated by anion exchange chromatography without having included the HW 50S gel filtration chromatography first. The elution profile is shown in Figure 3. Fractions were collected every minute and freeze-dried separately in 20-mL scintillation vials and analyzed by MALDI-TOFMS. The masses of the components in the major peaks were consistent with them being Rha_3GalA_3 (peak k), Rha_2GalA_2 (peak f), and $Rha_2GalA_2Gal_2$ (peak d). These oligomers have been commonly found in previous studies on rhamnogalacturonan and their structures well characterized.[17,20,21]

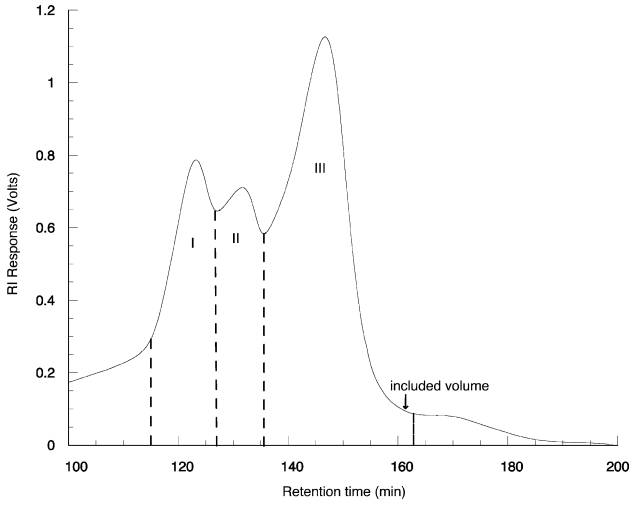

Figure 2. HW-50 size-exclusion chromatography profile of rhamnogalacturonan hydrolase-generated oligosaccharides. Void volume and included volume were at 75 and 162 min, respectively, when calibrated with pullulan (M_w = 788 kDa) and glucose. Only the portion of the profile showing significant material is shown.

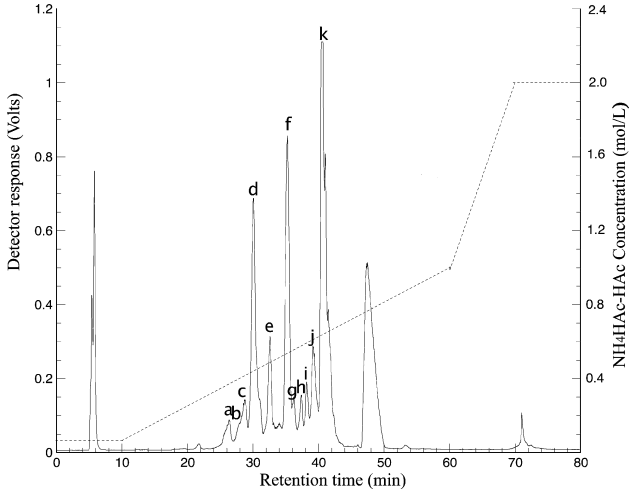

Figure 3. Evaporative light scattering profile of fraction III on anion exchange chromatography. The major components in each peak were identified as follows by MALDI-TOFMS spectra: (a) $Rha_2GalA_2Gal_4$; (b) $Rha_2GalA_2Gal_3$, $Rha_2GalA_2Gal_2Ara_2$, $Rha_2GalA_2Gal_3Ara_3$, $Rha_2GalA_2Gal_2Ara_4$; (c) $Rha_2GalA_2Gal_2Ara_2$, $Rha_2GalA_2Gal_3$; (d) $Rha_2GalA_2Gal_2$, $Rha_2GalA_2Gal_2Ara$; (e) Rha_2GalA_2Gal; (f) Rha_2GalA_2; (g) $Rha_3GalA_3Gal_3$; (h) Rha_2GalA_2, $Rha_2GalA_3Gal_4$, $Rha_3GalA_3Gal_2$; (i) $Rha_3GalA_3Gal_2$, $Rha_3GalA_3GalAra$, $Rha_2GalA_3Gal_3Ara_2$; (j) $Rha_2GalA_3Gal_3$, $Rha_3GalA_3GalAra_2$, Rha_3GalA_3Gal, $Rha_2GalA_3Gal_3Ara_2$, $Rha_2GalA_3Gal_3Ara_3$; (k) Rha_3GalA_3, $Rha_2GalA_3Gal_2$, $Rha_3GalA_4Gal_2$, $Rha_3GalA_4Gal_3$, $Rha_3GalA_4Gal_4$.

The rhamnogalacturonan oligomer RGS29 was eluted in the 29-min fraction (peak c). Its MALDI-TOFMS spectrum had two major ions at *m/z* 1249.5 and 1271.5, which correspond to $[Rha_2GalA_2Gal_2Ara_2-H]^-$ and $[Rha_2GalA_2Gal_2Ara_2-2H+Na]^-$. There were also two smaller peaks at *m/z* 1147.4 and 1169.4, which correspond to $[Rha_2GalA_2Gal_3-H]^-$ and

$[Rha_2GalA_2Gal_3-2H+Na]^-$. The peak abundance at *m/z* 1147.4 was 22% of the major peak at *m/z* 1249.5. Composition analysis indicated that the RGS29 fraction contained Ara, Rha, GalA, and Gal in a molar ratio of 0.7:1.0:1.4:1.1 rather than 1.0:1.0:1.0:1.0. This was probably due to the contamination with $Rha_2GalA_2Gal_3$ and some galacturonic acid monomer, which was identified by NMR spectroscopy.[22] In the TOCSY spectrum (Fig. 4), two sets of correlations to both the α and β reducing end galacturonic acid anomeric protons can be seen. One set of the correlation, for both the α and β anomers have chemical shifts identical to those of free galacturonic acid, whereas, the other sets, from the downfield shift of H-4, show that these galacturonic acid residues are linked through O-4 to another sugar. Free galacturonic acid does elute from the PA1 column at the same ionic strength as RGS29, but it is unclear how it came to be in the sample. No further purification was done before NMR analysis because of the low yield of RGS29 and the similarity of the molecular size and charge of the contaminating oligomer.

All 2D NMR spectra were obtained at 15 °C to avoid suppression of the anomeric proton signal of galacturonic acid (5.03 ppm) or the branched galactose (4.76 ppm) during water presaturation at 4.93 ppm. Nine anomeric signals were found in the HMQC spectrum (Fig. 5) of RGS29 and they were assigned to α and β anomers of the reducing end galacturonic acid, internal α-galacturonic acid, non-reducing end α-rhamnose, internal α-rhamnose, terminal β-galactose, internal β-galactose, and two α-arabinoses (Table 2). In the HMBC spectrum (Fig. 6), the correlations of internal galacturonic acid C-1 and internal rhamnose H-2, of reducing end galacturonic acid C-4 and internal rhamnose H-1, and of internal galacturonic acid C-4 and non-reducing end rhamnose H-1 indicated that the oligomer had a Rha_2GalA_2 backbone with alternating (1→2)-linked rhamnose and (1→4)-linked galacturonic acid as described before.[2] In the ^1H NMR spectrum, the signal at 1.22–1.25 ppm was almost absent and only 1.28–1.32 ppm remained.[17] This indicated that almost all rhamnoses were branched. That both rhamnoses of the major component RGS29 were branched was also supported by the HMBC spectrum in which we found signals corresponding to the correlation of internal rhamnose C-4 and terminal galactose H-1 and of non-reducing end rhamnose C-4 and branched galactose H-1. That is, the two galactoses were linked to the

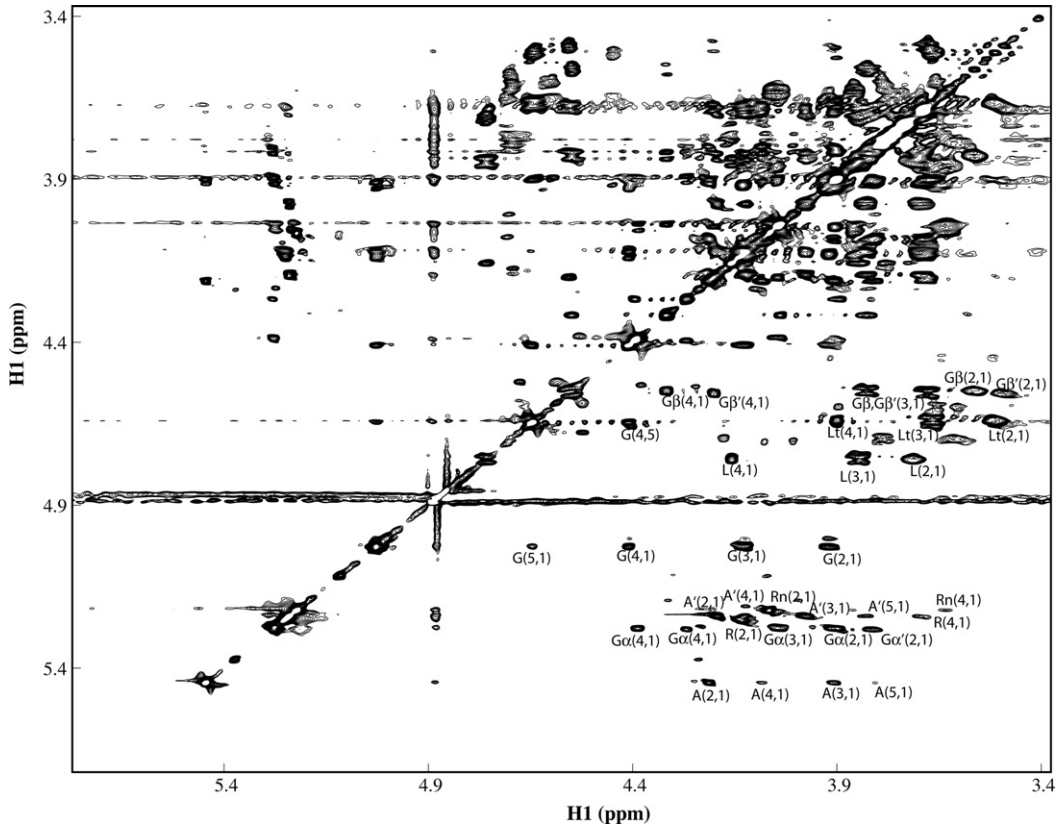

Figure 4. TOCSY spectrum of RGS29. Gα, reducing end α-galacturonic acid; Gβ, reducing end β-galacturonic acid; G, internal galacturonic acid; Rn, non-reducing end rhamnose; R, internal rhamnose; Lt, terminal galactose; L, (1→3,4)-linked galactose; A, terminal arabinose linked to O-4 position of (1→3,4)-linked galactose; A′, terminal arabinose linked to O-3 position of (1→3,4)-linked galactose; Gα′, α-galacturonic acid monomer; Gβ′, β-galacturonic acid monomer.

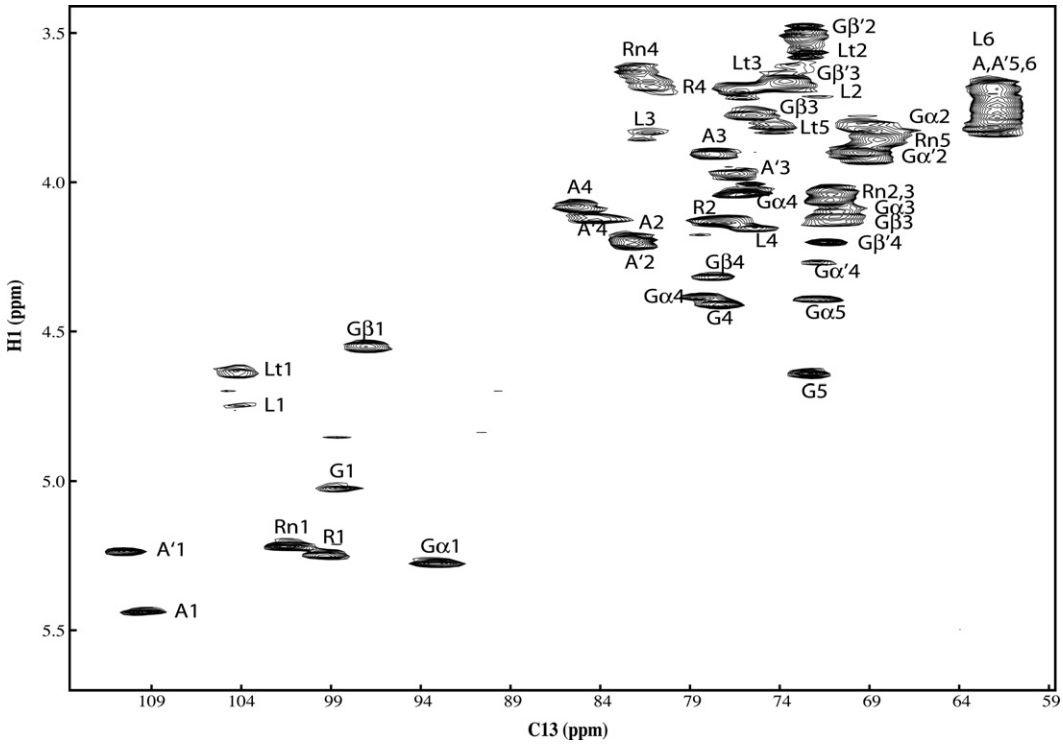

Figure 5. HMQC spectrum of RGS29. Symbols in the spectrum represent the different glycosyl residues as described in Figure 4.

Table 2. Assignments of chemical shifts of RGS29

	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5	C-6/H-6
→4)-α-Gal*p*A	93.2/5.28	68.5/3.90	70.9/4.04	78.1/4.38	71.5/4.40	176.6
→4)-β-Gal*p*A	97.0/4.56	72.3/3.56	74.2/3.82	77.6/4.31	75.9/4.03	175.9
→4)-α-Gal*p*A-(1→	98.3/5.03	69.4/3.91	70.8/4.13	77.1/4.40	72.3/4.61	176.1
→4)-α-Rhap	101.1/5.23	70.9/4.06	70.7/4.04	81.8/3.63	68.4/3.84	17.7/1.30
→2,4)-α-Rha*p*-(1→	99.3/5.25	77.4/4.14	70.8/4.12	81.2/3.69	68.4/3.85	17.7/1.31
β-Gal*p*-(1→	104.2/4.65	72.4/3.51	73.5/3.67	69.5/3.90	74.4/3.65	62.1/3.82, 3.70[c]
→3,4)-β-Gal*p*-(1→	104.1/4.75	71.7/3.71	81.6/3.85	75.6/4.16	75.1/3.78	61.8/3.69, 3.77[c]
α-Ara*f*-(1→[a]	110.5/5.24	82.0/4.19	76.4/3.98	84.1/4.12	62.0/3.69, 3.83[c]	
α-Ara*f*-(1→[b]	109.3/5.45	82.1/4.21	77.6/3.90	85.2/4.08	62.1/3.69, 3.81[c]	

[a] Terminal arabinose linked to the O-3 position of (1→3,4)-linked galactose.
[b] Terminal arabinose linked to O-4 position of (1→3,4)-linked galactose.
[c] The assignments of galactose H-6 and arabinose H-5 were interchangeable.

Rha_2GalA_2 mainchain through the C-4 positions of the two rhamnoses. In the TOCSY spectrum, we found correlations from H-1 of the branched galactose at 4.75 ppm to H-4 at 4.16 ppm, H-3 at 3.85 ppm, and H-2 at 3.71 ppm. H-3, 3.85 ppm, had a downfield shift compared to the H-3 of β-(1→4)-linked galactan[23] and coincided well with the chemical shift of H-3 of the (1→3,4)-linked galactose reported previously.[18] The signal at (75.6, 5.45) ppm in HMBC was assigned unambiguously to a correlation of branched galactose C-4 and arabinose H-1. We also found a cross peak at (81.5, 5.24) ppm which should be the correlation of the branched galactose C-3 and the other arabinose H-1. Thus, both arabinose residues are linked to the branched galactose, one at O-3 position and one at O-4 position. The full chemical shifts assignments are listed in Table 2. The TOCSY, HMQC, and HMBC and the assignments of the signals in the spectra are shown in Figures 4–6.

Based on all of the results described above, the structure of RGS29 was

α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A
4 4
↑ ↑
1 1
β-Gal*p* α-Ara*f*-(1→3)-β-Gal*p*-(4←1)-α-Ara*f*

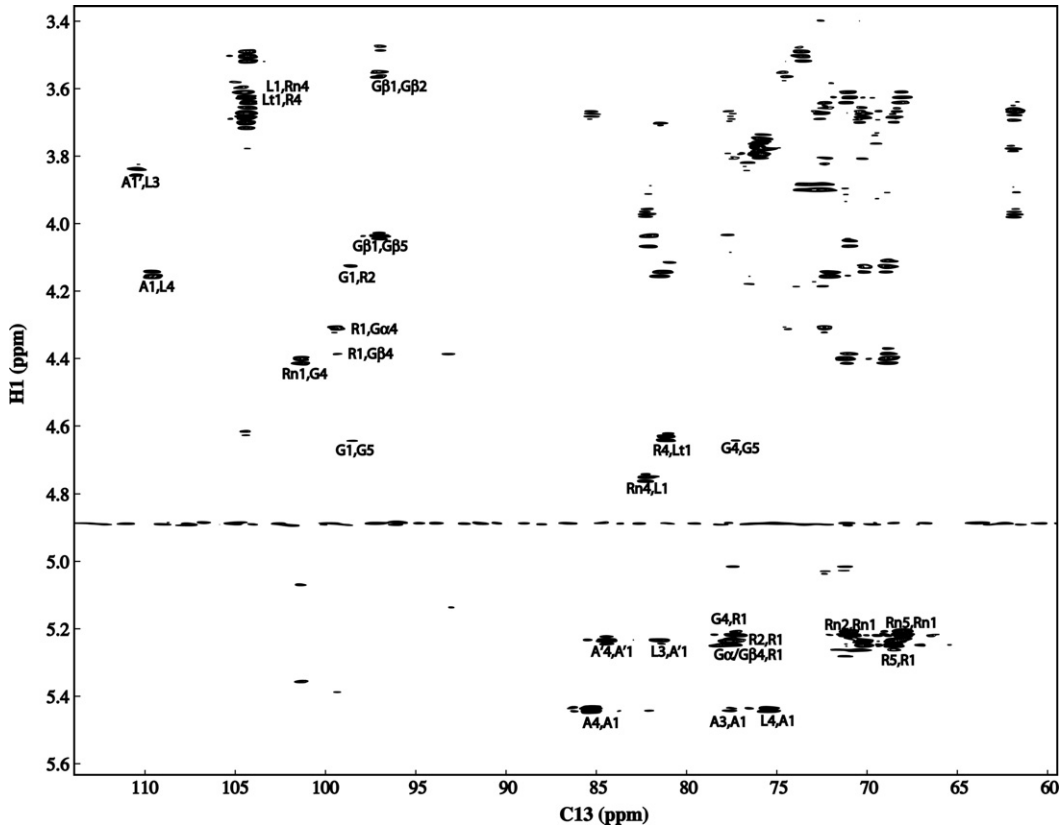

Figure 6. HMBC spectrum of RGS29. Symbols in the spectrum represent the different glycosyl residues as described in Figure 4.

Rhamnogalacturonan oligomers containing a single galactose residue linked to O-4 of one or more of the rhamnose residues have been isolated and characterized several times,[17,24,25] and recently oligomers with up to galactosyl trisaccharides linked to the rhamnose have been produced from flax pectin by a combination of galactanase and rhamnogalacturonan hydrolase from *Aspergillus aculeatus*.[20] However, rhamnogalacturonan oligomers with oligomeric side chains containing both galactose and arabinose have not previously been reported. The rhamnogalacturonan hydrolase we used was cloned from *Aspergillus nidulans* and expressed in *Pichia pastoris*.[11] Unlike the enzyme from *A. aculeatus*, which appears to be highly progressive, the enzyme we used attacks RG-I randomly, producing initially a wide range of lengths of oligomers which get shorter as the digestion progresses until only dimers and trimers of the RG-I repeat unit remain. It may be that differences in the specificity of the rhamnogalacturonan hydrolase which allowed us to produce the arabinose-containing oligomers, or it could be the combination of the starting pectin's structure and the mix of enzymes we used. Lau et al.[2] isolated rhamnitol-attached oligosaccharides after the degradation of rhamnogalacturonan from the walls of suspension-cultured sycamore cells (*Acer pseudoplatanus*) with lithium in ethylenediamine. Three of them contained Ara*f* linked to O-3 of galactose on O-4 of the rhamnitol. No arabinose on O-4 of galactose was found. They characterized 12 different rhamnitol-linked oligomers to varying extents. It is surprising that there have been no other reports on such complex short side chains in rhamnogalacturonans of other plant species.

Fractionation of the lowest molecular weight oligomers recovered from the HW-50 column on a PA1 ion exchange column followed by MALDI-TOFMS analysis showed them to consist of three abundant ones: Rha_2GalA_2, Rha_3GalA_3, and $Rha_2GalA_2Gal_2$; one intermediately abundant one: Rha_2GalA_2Gal; and an array of less abundant ones. Most of the side branches on the rhamnogalacturonan were single galactose residues. Among the less abundant oligosaccharides, all of them appeared by MS to contain galactose. Some also contained enough arabinose which could be the start of an arabinan chain in them. Since the oligomers were produced after extensive digestion of arabinans, we cannot tell if the RGS29 oligomer side chains had been modified from arabinan chains or if they were unchanged from how they were in the original pectin. It is likely that all the side chains start with a single galactose linked to O-4 of rhamnose in the backbone of the rhamnogalacturonan, but during biosynthesis some are elongated by the addition of galactose to generate the well-known β-(1→4)-linked galactan whereas others are arabinosylated and then extended

into the well-known α-(1→5)-linked arabinan. It will be interesting to learn what influences the type of polymer that is initiated. Lau et al.[2] suggested that the oligomers they derived from sycamore RG-I fell into certain groups, depending on how the elongation from the galactose attached to the rhamnose proceeded during the biosynthesis. In the case of sycamore, arabinose was linked to O-3 of the initial galactose, and galactose was linked through O-4 or O-6 of the initial galactose.

3. Experimental

3.1. Preparation of cell walls

Cotton (*G. hirsutum* L. OK1.2) suspension cells were cultured as described by Ruyack et al.[26] Cells were collected on a nylon mesh (35 μm, Small Parts, IL), then washed with 5 vol of 100 mM and 4 vol of 500 mM potassium phosphate buffer (pH 7.0) to remove extracellular debris. The washed cells were suspended in 1 vol of 500 mM potassium phosphate buffer (pH 7.0) and then broken by a Polytron homogenizer (Brinkmann Instruments, NY) for 12 min at 4 °C with stopping every 3 min for cooling. The broken cells were washed with 5 vol of 500 mM potassium phosphate buffer (pH 7.0), 10 vol of water, 5 vol of 1:1 $CHCl_3$–MeOH, and 10 vol of acetone sequentially. The cell walls were then put into a vacuum oven and dried at room temperature.

3.2. Alkali extraction

The crude cotton cell walls were suspended in 24% KOH–0.1% $NaBH_4$, to a concentration of approximately 10 mg/mL, and gently stirred overnight at room temperature. The suspension was centrifuged at 20,000*g* for 20 min, washed with distilled water twice and centrifuged again. The supernatants were combined and acidified with glacial acetic acid to pH 5.5 and then dialyzed (COMW = 12–14 kDa, Spectra/Por, CA) against distilled water for two days. After dialysis, the supernatant was concentrated by ultrafiltration in an Amicon 8400 ultrafiltration cell (Millipore, MA) with a 10 kDa COMW ultrafiltration membrane (Millipore, MA) and then lyophilized.

3.3. Liquid chromatography

The dialyzed alkali extract was applied to a TSK-GEL Toyopearl DEAE-650S column (22 × 250 mm). The column was eluted with water, 0.5 M, and 2 M ammonium acetate buffer (pH 5.2). Eluents were changed only after no more sugar, as determined by the phenol–sulfuric acid method,[27] was eluting from the column. The eluates were pooled, concentrated by ultrafiltration, and then freeze-dried.

The oligosaccharides from rhamnogalacturonan hydrolase digestion were separated on Toyopearl TSK HW-50S (Supelco, Bellefonte, PA) packed in a stainless steel column (50 × 2.4 cm, Alltech Associates, KY). The column was equilibrated with TFA/water (pH 2.5) with a flow rate of 1.0 mL/min and calibrated with pullulan standards (M_w = 180, 738, 5900, 11,800, 22,800, 47,300, and 78,800, Xpertek, P. J. Cobert Associates, MO). The sugar content was monitored by a refractive index detector (Shodex RI-71, Japan). Fractions were collected every 2 min on a Gilson fraction collector. The elution profile is shown in Figure 2; the fractions corresponding to the three peaks were pooled and lyophilized separately.

The fraction III was separated further on an anion exchange PA1 column (22 × 250 mm, Carbo Pac, Dionex, CA). The column was eluted with an ammonium acetate gradient with a flow rate of 5 mL/min. An evaporative light scattering detector was used to monitor the sugar content in the eluate split 1:25 between the detector and the fraction collector. The fractions were collected every minute on a Gilson fraction collector. The elution pattern was shown as in Figure 3. Every fraction was collected separately in a 20-mL vial and freeze-dried four times to remove ammonium acetate completely before MALDI-TOFMS and NMR analyses.

3.4. Enzymatic degradations

endo-Polygalacturonase and *endo*-arabinase were bought from Megazyme, Ireland. Arabinofuranosidase, rhamnogalacturonan hydrolase, and xyloglucan-specific endoglucanase AN0452.2 (EC 3.2.1.151) were cloned from *A. nidulans* into *Escherichia coli* and expressed in *P. pastoris*.[11,28]

Enzyme activities were assayed by the modified spectrophotometric method described previously.[29] Four microliter substrate, 1 μL enzyme, and 4 μL buffer were mixed together and reacted for 30 min. 100 μL 0.1 M borate buffer, 50 μL 2-cyanoacetamide, and 40 μL water were added and heated in a boiling water bath for 10 min. After the reaction mixture cooled down, the absorbance at 276 nm was measured on a NanoDrop ND-1000 spectrometer (NanoDrop Technology, DE).

Samples were suspended in 50 mM ammonium acetate buffer (pH 4.0 for *endo*-polygalacturonase and rhamnogalacturonan hydrolase, pH 5.0 for other enzymes) to a concentration of approximately 10 mg/mL. An estimated fourfold excess of enzyme activity was added to ensure complete digestion.

For testing whether the samples could be digested by xyloglucan-specific endoglucanase, 1 mg samples (10 mg/mL) were used and excess enzyme was added. After 1-day digestion, 5-μL aliquots were taken out and analyzed by CZE.

The 2 M acetate-eluted fraction from HW-50 chromatography was first digested by 2.3 μL *endo*-polygalacturonanase (652 U/mL on Aldrich pectic acid, 30 min, 37 °C, pH 4.0) for two days. The enzyme was denatured at 90 °C for 20 min before the sample was subjected to ultrafiltration on a 10 kDa COMW membrane. The liquid remaining above the membrane contained high molecular weight (HMW1) polysaccharide, which was used for further enzymatic digestion, and the liquid that passed through the membrane was designated as low molecular weight (LMW1) oligosaccharides. Arabinofuranosidase, 22 μL (13.1 U/mL on Megazyme sugar beet arabinan, 30 min, 37 °C, pH 5.0), 103 μL *endo*-arabinase (1.40 U/mL on Megazyme debranched sugar beet arabinan, 37 °C, pH 5.0), and 0.49 mL rhamnogalacturonan hydrolase (0.2 U/mL on *endo*-polygalacturonase-digested citrus pectin rhamnogalacturonan high molecular weight fraction,[30] 37 °C, pH 4.0) were used sequentially following the same procedure as for *endo*-polygalacturonase digestion.

3.5. Gas liquid chromatography (GLC) sugar composition analysis and capillary zone electrophoresis (CZE)

Sugar compositions were determined by GLC analysis of trimethylsilyl methyl glycosides. Methanolysis and derivatization were performed using the protocol of Chaplin modified by Komalavilas and Mort.[31] Enzymatic degradation-generated oligosaccharides were reductively aminated with 8-aminopyrene-1,3,6-trisulfonate (APTS) or 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). Capillary zone electrophoresis was performed as described previously.[32]

3.6. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOFMS)

Samples, 0.25 μL (~10 mg/mL), were spotted on top of 0.25 μL matrix (100 mg/mL 2,4,6-trihydroxy acetophenone in MeOH) on 96 × 2 sample plates and dried in air. Spectra were obtained on a Perseptive Biosystems Voyager DE PRO matrix-assisted laser desorption time-of-flight mass spectrometer in the negative ion mode.

3.7. Nuclear magnetic resonance (NMR) spectroscopy

^{1}H NMR and TOCSY, COSY, HMBC, HMQC, and NOESY spectra were recorded at 15 °C on a Varian Unity Inova 600 NMR spectrometer using Varian VNMRJ software. Samples were dissolved in 99.9% D_2O (Sigma–Aldrich, MO). Proton decoupled ^{13}C NMR spectra (HMQC and HMBC) were recorded using a Nalorac 5 mm probe at 150.82 MHz. Nuclear Overhauser enhancement spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) experiments were performed with a mixing time of 500 ms and 100 ms, respectively. All spectra used presaturation mode to suppress the water peak and saturation power at −10.

Acknowledgments

This work was supported DOE Grant DE-FG02-96ER20215 and has been approved for publication by the Director of the Oklahoma Agricultural Experiment Station. We thank Margaret Pierce for helpful comments on the manuscript.

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