Carbohydrate Research 344 (2009) 1852–1857

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

# Improved procedures for the selective chemical fragmentation of rhamnogalacturonans

Chenghua Deng [a], Malcolm A. O'Neill [a], Michael G. Hahn [a,b,c], William S. York [a,b,c,*]

[a] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA
[b] Department of Plant Biology, University of Georgia, Athens, GA 30602, USA
[c] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA

ARTICLE INFO

*Article history:*
Received 24 July 2008
Received in revised form 11 November 2008
Accepted 13 November 2008
Available online 3 December 2008

*Keywords:*
Polysaccharides
Pectin
Rhamnogalacturonan
Depolymerization
β-Elimination
*Lepidium sativum* (peppergrass) seed mucilage

ABSTRACT

The structural characterization of branched rhamnogalacturonans (RGs) requires the availability of methods that selectively cleave the Rha*p*-(1→4)-α-GalA*p* linkage and thereby generate oligosaccharide fragments that are suitable for mass spectrometric and NMR spectroscopic analyses. Enzymic cleavage of this linkage is often ineffective, especially in highly branched RGs. Therefore, we have developed an improved chemical fragmentation method based on β-elimination of esterified 4-linked Gal*p*A residues. At least 85% of the carboxyl groups of the GalA residues in *Arabidopsis thaliana* seed mucilage RG is esterified using methyl iodide or 3-iodopropanol in $Me_2SO$ containing 8% water and 1% tetrabutylammonium fluoride. However, β-elimination fragmentation at pH 7.3 and 120 °C is far more extensive with hydroxypropyl-esterified RG than with methyl-esterified RG. The non-reducing 4-deoxy-β-**L**-*threo*-hex-4-enepyranosyluronic acid residue formed by the β-elimination reaction is completely removed by treatment with aqueous *N*-bromosuccinimide, thereby simplifying the structural characterization of the chemically generated oligoglycosyl fragments. This newly developed procedure was used to selectively fragment the branched RG from peppergrass seed mucilage. The products were characterized using MALDI-TOF mass spectrometry, glycosyl residue composition analysis, and 1 and 2D NMR spectroscopy. Our data show that the most abundant low-molecular weight fragments contained a backbone rhamnose residue substituted at O-4 with a single sidechain, and suggest that peppergrass seed mucilage RG is composed mainly of the repeating unit 4-O-methyl-α-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→4)-[→4)-α-D-Gal*p*A-(1→2)-]-α-L-Rha*p*-(1→.



## 1. Introduction

Rhamnogalacturonans (RGs) are a family of structurally related, often highly branched, heteropolysaccharides present in the primary cell walls of seed-bearing plants,[1–4] and are components of many plant exudate gums.[5] The RG backbone is composed of the repeating disaccharide [-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-].[6] Up to 80% of the Rha*p* residues is substituted at O-4 with oligoglycosyl sidechains composed primarily of β-D-Gal*p* and α-L-Ara*f* residues. These differently linked glycosyl residues can combine to form a large number of sidechain structures that may be arranged in many different ways along the backbone. However, little is known about the biological functions of the sidechains or their biosynthesis and metabolism. Progress in these areas is limited by our incomplete knowledge of RG chemical structures and the structural modifications of RGs that occur during plant cell growth and differentiation.

Determining the complete glycosyl sequence of a branched RG still remains a considerable challenge. One approach is to selectively depolymerize the RG backbone using enzymatic[7–9] or chemical fragmentation,[10–12] and then structurally characterize the released oligosaccharides. Unfortunately, enzymic cleavage of the backbone of highly branched RG using RG-hydrolase or RG-lyase has had only limited success, as the presence of sidechains typically prevents these enzymes from cleaving adjacent regions of the backbone.[13,14]

We had previously described a chemical method to generate RG fragments that contain a single sidechain by selective depolymerization of the methyl-esterified RG backbone by β-elimination.[15] However, a single round of methyl esterification and β-elimination does not result in complete depolymerization of the RG backbone (see Fig. 1A). The procedure must be repeated to obtain single sidechain fragments in high yield.[15] The β-elimination reaction is incomplete because the reaction conditions used also lead to hydrolysis of the methyl ester. The methyl ester itself is required for the first step of the β-elimination reaction (i.e., abstraction of H-5 of the GalpA residue).[16,17]

* Corresponding author. Tel.: +1 706 542 4401; fax: +1 706 542 4412.
*E-mail address:* will@ccrc.uga.edu (W.S. York).

0008-6215/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.carres.2008.11.011

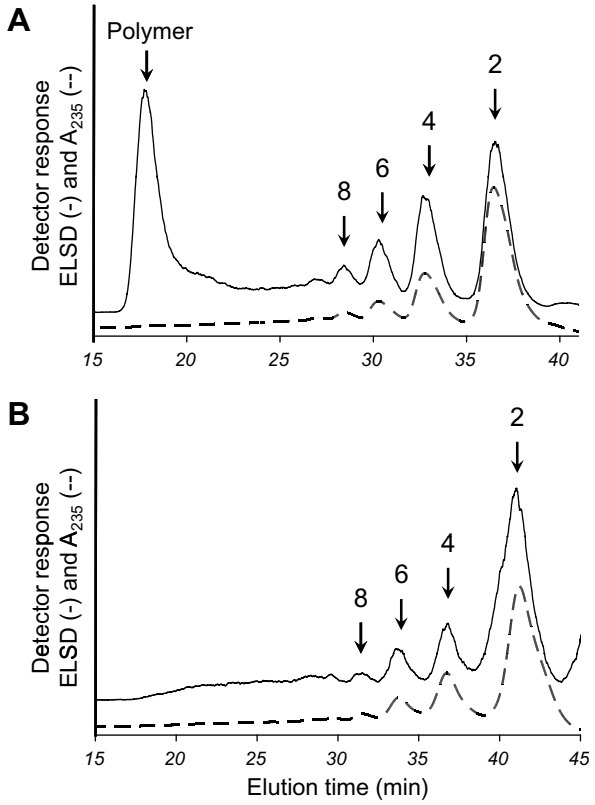



**Figure 1.** Size-exclusion chromatography (Superdex Peptide column) of the products obtained by β-elimination of the methyl and hydroxypropyl-esterified RG from *A. thaliana* seed mucilage. (**A**) Products obtained from β-elimination (pH 7.3, 3 h at 120 °C) of the methyl-esterified RG backbone. The intact polysaccharide and/or large fragments of the polysaccharide were eluted at the column void volume. $^1$H NMR spectroscopy indicated that all the methyl esters of D-Gal*p*A are hydrolyzed during the reaction, leading to incomplete fragmentation (see text for details). (**B**) Products obtained upon β-elimination (pH 7.3, 3 h at 120 °C) of the 3-hydroxypropyl-esterified RG backbone. Polysaccharide and oligosaccharide products were detected by evaporative light scattering detection (solid lines), and β-L-$\Delta^{4,5}$Hex*p*A residues generated by the β-elimination reaction were detected by their $A_{235}$ (dashed lines). The number of glycosyl residues in the oligosaccharide products, as determined by MALDI-TOF MS, is indicated above each peak. Minor differences in the retention times in the two profiles are the result of using two different Superdex Peptide columns.

We now describe procedures to improve the efficiency of β-elimination fragmentation of RGs by forming the bulky and polar 3-hydroxypropyl esters of the 4-linked Gal*p*A residues. The β-elimination-generated oligosaccharide fragments contain a non-reducing 4-deoxy-β-L-*threo*-hex-4-enepyranosyluronic acid (β-L-$\Delta^{4,5}$Hex*p*A) residue, and a reducing rhamnose residue bearing a single sidechain. The β-L-$\Delta^{4,5}$Hex*p*A residue is completely removed by treatment with aqueous *N*-bromosuccinimide, thereby simplifying structural characterization of the products.

## 2. Results and discussion

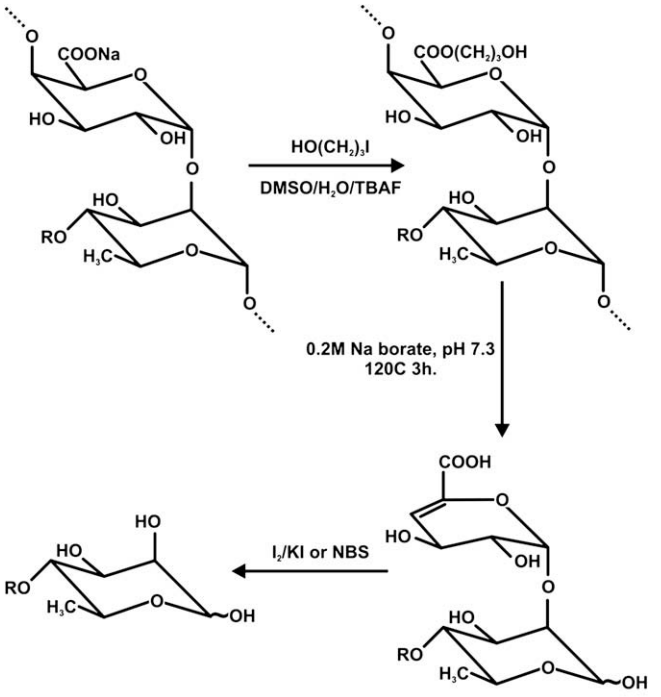
### 2.1. The efficiency of β-elimination fragmentation of RG is improved by forming the 3-hydroxypropyl-esterified polysaccharide

An unbranched RG, from *Arabidopsis thaliana* seed mucilage,[15] was used as a model polysaccharide to investigate more efficient methods for the β-elimination of GalA residues in the RG backbone. The esters of bulky alcohols are often more resistant to hydrolysis than esters of small alcohols, and β-elimination of RG is much more efficient in water than in other solvents.[15] Therefore, we examined β-elimination of RG after formation of the bulky and polar 3-hydroxypropyl ester. Formation of this ester by treating RG with 3-iodopropanol and tetrabutyl ammonium fluoride (TBAF) in DMSO containing 8% water (Scheme 1) did not appear to have any effect on the water solubility of the RG. However, β-elimination of the product resulted in extensive fragmentation of the RG, producing oligosaccharides in high yield (Fig. 1B). Virtually no high-molecular weight products remained after this treatment. In comparison, ~30% of the RG remained as high-molecular weight material, when methyl-esterified RG was fragmented by β-elimination (Fig. 1A).

A second advantage of esterifying RG with 3-iodopropanol is that the degree of esterification (DE) can be directly determined by $^1$H NMR spectroscopy without the use of $^{13}$C-labeled alkylating reagents.[15] The H-2 resonance of the hydroxypropyl ester ($\delta$ 1.95) is well-resolved from the carbohydrate resonances (Fig. 2). Thus, the DE is obtained by comparing the signal integrals for H-2 of the hydroxypropyl ester and the H-6 of rhamnose ($\delta$ 1.26). The data shown in Figure 2 indicate that the RG has a DE of 85%.

### 2.2. Application of improved β-elimination methods to selectively fragment a highly branched RG from peppergrass seed mucilage

We tested the efficacy of the improved β-elimination protocol using a highly branched RG isolated from peppergrass (*Lepidium sativum*) seed mucilage. A major constituent of this mucilage is a complex acidic polysaccharide that contains 4-*O*-methyl glucuronic acid and galacturonic acid.[18] A disaccharide (β-L-$\Delta^{4,5}$Hex*p*A-(1-2)-L-Rha*p*), referred to as lepidimoide, has also been isolated from *L. sativum* seeds that had been incubated for two days after water inbibition.[19] The disaccharide was most likely formed by enzyme-catalyzed fragmentation of the seed mucilage. Taken together these results suggest that peppergrass seed mucilage is a branched RG.




**Scheme 1.** Esterification and β-elimination of RG.

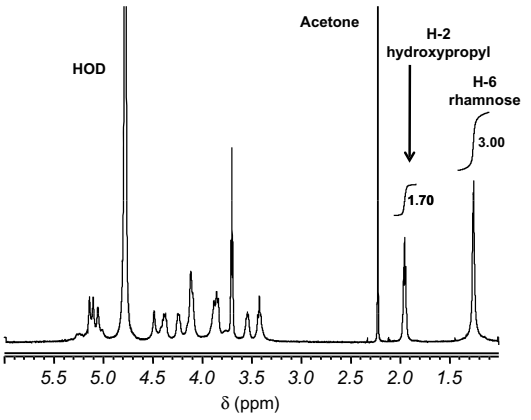



**Figure 2.** [1]H NMR spectrum of the 3-hydroxypropyl-esterified Arabidopsis seed mucilage RG. The upfield region of the spectrum includes the hydroxypropyl H-2 and rhamnosyl H-6 resonances. These well-resolved resonances can be used to determine the degree of esterification of the RG (see text for details).

In preliminary experiments, peppergrass RG was obtained by extracting *L. sativum* seeds with aqueous ammonium formate in the presence or absence of RG-lyase. Extraction with lyase yielded more than twice the amount of soluble RG than extraction with buffer alone, and solutions generated by lyase extraction had a much lower viscosity than those generated by buffer extraction. Size-exclusion chromatography revealed that the buffer-extracted RG was excluded from the column matrix due to its high-molecular mass (Fig. 3A). RG obtained by lyase extraction was dialyzed (MWCO 3500) to remove low-molecular weight components, and the retentate was analyzed by SEC (Fig. 3B), showing that it had been partially fragmented. The dialyzed material was used in all subsequent chemical fragmentation experiments. The high-molecular mass material that eluted at the same time (~18 min, see Fig. 3B) as the buffer-extracted RG only accounted for ~5% of the total material and was not further analyzed.

The 3-hydroxypropyl ester of peppergrass RG was prepared using 3-iodopropanol and TBAF. The esterified RG (DE 85%) was then treated for 3 h at 120 °C and pH 7 to β-eliminate the esterified Gal*p*A residues. The products that formed were separated on a Superdex Peptide SEC column (Fig. 4A). The small amount (~5% of the total area in the SEC profile) of material detected at the column void volume is likely to be arabinoxylan,[18] as arabinose and xylose were the major components detected by glycosyl residue composition analysis.

The most abundant products of the β-elimination reaction were oligosaccharides that form a regular series, whose members are separated by a mass difference of approximately 674 Da. Glycosyl

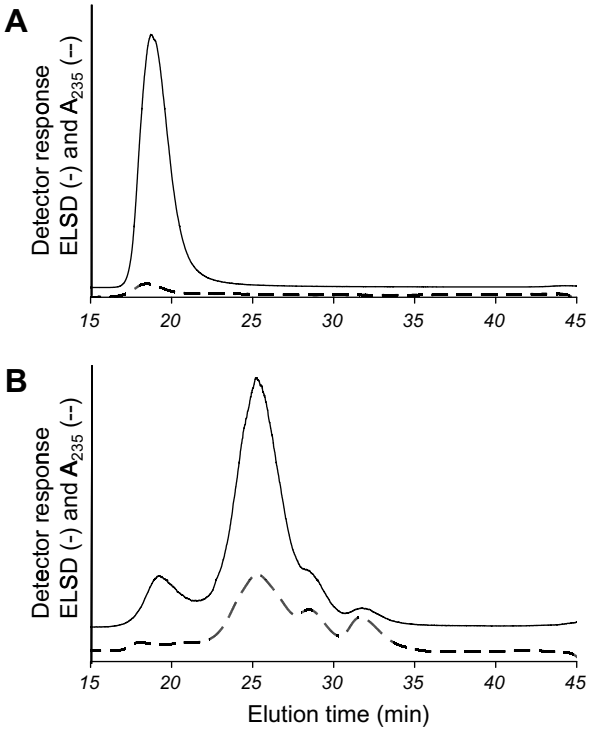



**Figure 3.** SEC chromatography of material solubilized from peppergrass seed mucilage in the presence or absence of RG-lyase. The soluble materials were dialyzed to remove low-molecular weight components and then chromatographed on a Superdex Peptide column. Products were detected by evaporative light scattering detection (solid lines). β-L-$\Delta^{4,5}$Hex*p*A residues generated by the lyase-catalyzed β-elimination reaction were detected by their $A_{235}$ (dashed lines). (A) Material solubilized in the absence of RG-lyase (B) Material solubilized in the presence of RG-lyase.

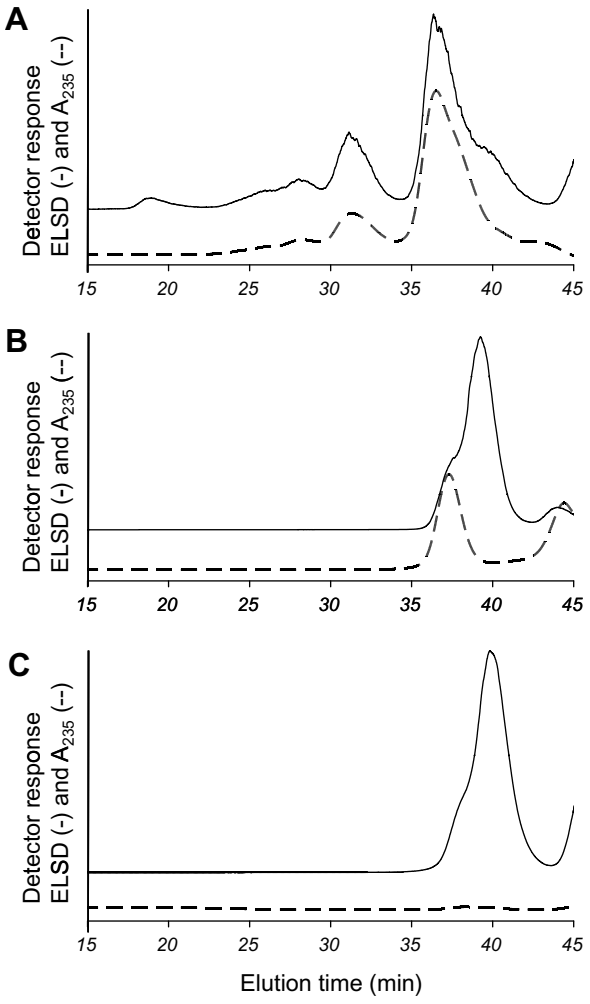



**Figure 4.** Size-exclusion chromatography of the products obtained by β-elimination of 3-hydroxypropyl-esterified peppergrass seed mucilage RG. Products were chromatographed on a Superdex Peptide column, and the eluent analyzed by evaporative light scattering detection (solid lines) and for the $A_{235}$ (dashed lines) of the β-L-$\Delta^{4,5}$Hex*p*A residues generated by the β-elimination reaction. (**A**) Products generated by the β-elimination reaction. (**B**) Products formed by treating the tetrasaccharide fraction in Panel A with $I_2$/KI. (**C**) Products formed by treating the tetrasaccharide fraction in Panel A with N-bromosuccinimide (NBS). The vertical scales are identical in Panels B and C, showing that NBS treatment results in the near complete removal of $\Delta^{4,5}$Hex*p*A residues from the tetrasaccharide.

residue composition and MALDI-TOF MS analyses indicated that the oligosaccharide with the lowest nominal mass ($[M+Na]^+$ at *m/z* 697) is a tetrasaccharide containing 4-*O*-methyl-glucuronic acid (4-*O*-Me-GlcA), galactose (Gal), rhamnose (Rha), and 4-deoxy-β-L-*threo*-hex-4-enepyranosyluronic acid (β-L-$\Delta^{4,5}$Hex*p*A). The presence of β-L-$\Delta^{4,5}$Hex*p*A in the tetrasaccharide was confirmed by the strong $A_{235\ nm}$ and NMR spectroscopic analyses.[15] Together, these data suggest that β-elimination of peppergrass RG generated oligosaccharides consisting of tandemly repeated tetrasaccharide subunits (each containing one Rha and one GalA in the backbone and a sidechain with one MeGlcA and one Gal). The non-reducing terminus of each oligosaccharide is a β-L-$\Delta^{4,5}$Hex*p*A residue generated by β-elimination of a 4-linked Gal*p*A residue in the original polysaccharide.

### 2.3. Partial removal of β-L-$\Delta^{4,5}$Hex*p*A residue by treatment with $I_2$/KI and structural characterization of the products

β-Elimination of GalA-containing polysaccharides generates oligosaccharides terminated at the non-reducing end by a β-L-$\Delta^{4,5}$Hex*p*A residue, which may complicate their structural analysis.

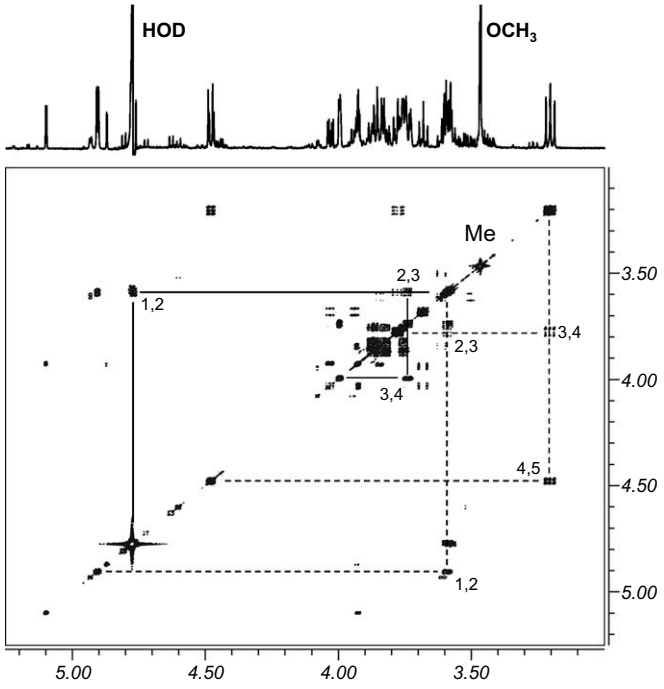



**Figure 5.** 1D and gCOSY NMR spectra of the trisaccharide generated by β-elimination and $I_2$/KI treatment of peppergrass RG-I. The spin system corresponding to the 4-*O*-methyl-α-D-Glc*p*A (dashed lines) and β-d-Gal*p* (solid lines) is shown. Numbers indicate specific scalar-coupled proton pairs giving rise to each crosspeak.

We investigated the use of $I_2$/KI[20] to selectively remove these residues. Thus, the tetrasaccharide, generated by β-elimination of peppergrass RG and purified by SEC (Fig. 4A) and ion-exchange chromatography (see Section 3), was treated for 48 h with aqueous $I_2$/KI in the absence of light. Analysis of the resulting products by SEC (Fig. 4B) and MALDI-TOF MS indicated that the removal of the unsaturated residue was incomplete, as both the original tetrasaccharide ($[M+Na]^+$ at *m/z* 697) and a trisaccharide ($[M+Na]^+$ at *m/z* 539) that lacks the unsaturated residue were detected. $^1$H NMR spectroscopic analysis of the purified trisaccharide confirmed that the unsaturated residue was no longer present, as the vinyl proton (H-4) resonance ($\delta$ 5.82) and other resonances[15] originating from the β-L-$\Delta^{4,5}$Hex*p*A residue were virtually absent.

The complete structure of the $I_2$/KI-generated trisaccharide was determined by 1 and 2D NMR spectroscopy. Four isolated spin systems were identified in the gCOSY and HSQC spectra of the trisaccharide and were assigned to a terminal 4-*O*-methyl Glc*p*A, an internal Gal*p*, and the α- and β-anomers of a reducing Rha*p* (Fig. 5 and Table 1). Glycosyl linkages were determined by identifying characteristic downfield glycosylation shifts for C-4 of the Gal*p* residue ($\delta$ 76.3) and C-4 of the α- and β-Rha*p* ($\delta$ 81.6 and $\delta$ 81.2, respectively). The linkage between the 4-*O*-Me-Glc*p*A and the internal Gal*p* residues and the location of the methyl group on the Glc*p*A were confirmed by 2D ROESY analysis of the trisaccharide, which revealed dipolar coupling between H-1 of 4-*O*-Me-Glc*p*A and H-4 of the Gal*p*, and between H-4 of the Glc*p*A and the *O*-methyl protons. The combined $^1$H NMR, gCOSY, ROESY, and HSQC analyses (Fig. 5 and Table 1) thus indicate that the trisaccharide is 4-*O*-methyl-α-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→4)-L-Rha*p*. The NMR spectra of the trisaccharide-containing fraction (Fig. 5) also indicated the presence of quantitatively minor amounts of a trisaccharide terminated with quinovose (6-deoxy glucose) that most likely arose by C-2 epimerization of rhamnose during the β-elimination reaction.[15] NMR spectroscopic analysis of the tetrasaccharide before $I_2$/KI treatment was consistent with the structure 4-*O*-methyl-α-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→4)-[β-L-$\Delta^{4,5}$Hex*p*A-(1→2)]-L-Rha*p*. The RG isolated from yellow mustard seed mucilage has been reported to have similar sidechains, in which 4-*O*-methyl-α-D-Glc*p*A is linked to O-6 of the β-D-Gal*p* residue.[21]

### 2.4. *N*-Bromosuccinimide removes the β-L-$\Delta^{4,5}$Hex*p*A residue more effectively than $I_2$/KI

It is likely that nucleophilic addition (iodination) to the double bond by $I^+$ is the initial step in the mechanism leading to cleavage of the unsaturated residue by $I_2$/KI. Thus, we examined the ability of various reagents that produce halogen cations in aqueous solution to completely remove the β-L-$\Delta^{4,5}$Hex*p*A residue. We found that aqueous *N*-bromosuccinimide (NBS, see Fig. 4C) was far more effective than $I_2$/KI (Fig. 4B) in removing this unsaturated residue. A significant $A_{235}$ still remained after treatment with $I_2$/KI, whereas the $A_{235}$ is virtually absent after treatment with NBS. Both treat-

**Table 1**
Assignments of the proton and carbon chemical shifts for the trisaccharide 4-*O*-Me-α-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→4)-L-Rha generated by β-elimination/$I_2$-KI treatment of hydroxypropyl-esterified peppergrass seed RG

| Glycosyl residue | H-1 (C-1) | H-2 (C-2) | H-3 (C-3) | H-4 (C-4) | H-5 (C-5) | H-6 (C-6) | -$OCH_3$ |
|---|---|---|---|---|---|---|---|
| 4-*O*-Me-α-D-Glc*p*A-(1→4) | 4.906 | 3.592 | 3.777 | 3.205 | 4.481 | | 3.468 |
| | (99.342) | (71.526) | (71.761) | (82.153) | (71.917) | | (59.494) |
| (1→4)-β-D-Gal*p*-(1→4) | 4.772 | 3.589 | 3.736 | 3.998 | 3.753 | 3.825/3.866 | |
| | (103.873) | (71.214) | (71.761) | (76.293) | (75.121) | (59.494) | |
| (1→4)-α-L-Rha- | 5.099 | 3.924 | 4.033 | 3.679 | 3.940 | 1.333 | |
| | (93.404) | (70.511) | (69.886) | (81.606) | (66.526) | (16.834) | |
| (1→4)-β-L-Rha | 4.871 | 3.930 | 3.845 | 3.612 | 3.504 | 1.347 | |
| | (93.091) | (70.980) | (59.572) | (81.215) | (59.490) | (16.756) | |

ments resulted in the formation of 4-*O*-methyl-α-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→4)-L-Rha*p* as the major product. NBS treatment was also shown to quantitatively cleave the β-L-$\Delta^{4,5}$Hex*p*A residues from oligosaccharide fragments generated by RG-lyase treatment of Arabidopsis seed mucilage RG (data not shown).

We conclude that oligosaccharide fragments containing a single sidechain can be generated from branched RGs by the following sequence of reactions: (1) treatment with RG-lyase to partially cleave the sparsely branched regions of the RG-I backbone; (2) esterification with a bulky, hydrophilic alkyl halide such as 3-iodopropanol; (3) β-elimination at 120 °C in borate buffer, pH 7.3; (4) removal of β-L-$\Delta^{4,5}$Hex*p*A residues by treatment with aqueous NBS.

## 3. Experimental

### 3.1. General methods

Total carbohydrate content and uronic acid content were determined colorimetrically using the phenol-sulfuric acid[22] and 3-hydroxybiphenyl[23] methods, respectively. Size-exclusion chromatography was performed using an Agilent 1100 series liquid chromatograph and data system and a Superdex Peptide HR10/30 column eluted at 0.4 mL/min with 50 mM ammonium formate, pH 5. The column outlet was connected, in series, to an Agilent 1100 UV detector and a Sedex evaporative light scattering detector (ELSD).

### 3.2. Extraction and purification of RG from Arabidopsis and peppergrass seed mucilage

RG was isolated from *A. thaliana* seed mucilage as previously described.[15] RG from fine curled peppergrass (*L. sativum*) seeds (Strokes Seeds Inc, Buffalo, NY) was isolated by suspending the seeds (1 g) in 100 mM ammonium formate (pH 6, 15 mL), containing rhamnogalacturonan α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronide lyase (1 mg) from *Aspergillus aculeatus* (a gift from Novozymes A/S, Denmark) and chlorobutanol (25 mg). The mixture was gently stirred for 72 h at 25 °C. Seeds and insoluble material were removed by centrifugation, and the supernatant was then dialyzed against deionized water (Spectra/Por, molecular weight cut-off 3500). The retentate was lyophilized to yield 34 mg of polysaccharide. An extraction performed with buffer in the absence of the RG-lyase gave only 15 mg of highly viscous RG. The lyase-solubilized peppergrass RG was dialyzed, and the retentate was analyzed by HPLC on a Superdex peptide column eluted with 50 mM ammonium formate, pH 5, at a flow rate of 0.4 mL/min (Fig. 3B). Subsequent β-elimination experiments were performed using the dialysis retentate from the lyase-extracted seeds.

### 3.3. Esterification of Arabidopsis and peppergrass seed mucilage RGs

Arabidopsis RG was methyl-esterified by treatment with methyl iodide and tetrabutyl ammonium fluoride in 8% DMSO.[15] Briefly, a suspension of the sodium form of the polysaccharide (20 mg) in water (0.32 mL) and $Me_2SO$ (4 mL) containing tetrabutylammonium fluoride (40 mg) and MeI (20 μL) in a 5-mL capped-tube was stirred at room temperature for 18 h. The reaction mixture was poured into ice-cold water (12 mL), and the mixture was centrifuged to remove iodine. The supernatant was dialyzed (Spectra/Por, MWCO 12,000–14,000) against deionized water for 48 h and then lyophilized. The 3-hydroxypropyl esters of the Arabidopsis and peppergrass RGs were prepared by similar methods, except that 3-iodopropanol was used as the alkylating reagent, and the MWCO for the dialysis of peppergrass RG was 3500. The products were lyophilized and analyzed by $^1$H NMR.

### 3.4. β-Elimination of the esterified RGs

β-Elimination of the methyl or 3-hydroxypropyl esters of RG was performed as described.[15] Briefly, a solution of the esterified RG (20 mg) in 0.2 M sodium borate (pH 7.3, 4 mL) was treated for 3 h at 120 °C in a sealed tube. The solution was then acidified (pH 6) with carbon dioxide gas. The products were fractionated by SEC on a Superdex Peptide column (Fig. 4A) eluted with 50 mM ammonium formate, pH 5, at a flow rate of 0.4 mL/min. Fractions were collected manually, and volatile buffer salts were removed by repeated lyophilization. The fractions were collected, lyophilized, and analyzed by NMR spectroscopy and MALDI-TOF mass spectroscopy. The smallest fragment (a tetrasaccharide) from peppergrass seed mucilage was also the most abundant. This fragment was further purified by ion-exchange chromatography using a HighTrap Q HP cartridge. The cartridge was eluted with a gradient (50 mM to 1 M) of aqueous ammonium formate. Fractions containing anionic material as detected by ELSD were combined, lyophilized, and then desalted on a Sephadex G-25 column.

### 3.5. Removal of the non-reducing end β-L-$\Delta^{4,5}$Hex*p*A residue from the β-elimination products using $I_2$/KI

Solutions of the tetrasaccharide (1 mg) obtained by β-elimination of peppergrass RG in 25 mM ammonium formate, pH 4.5 (500 μL), containing 0.036 M $I_2$ and 2% KI were maintained for 48 h at room temperature in the absence of light.[18] The mixture was concentrated to dryness under a stream of dry air. Deionized water (3 × 500 μl) was added and evaporated to remove most of the iodine. The residue was then dissolved in water (500 μl), and was purified by SEC on a Superdex Peptide column eluted with 50 mM ammonium formate, pH 5, at a flow rate of 0.4 mL/min (Fig. 4B). The eluant was monitored by ELSD and spectrophotometrically for its $A_{235}$. Fractions were collected manually, and volatile buffer salts were removed by repeated lyophilization.

### 3.6. Removal of the non-reducing end β-L-$\Delta^{4,5}$Hex*p*A residue from the β-elimination products using *N*-bromosuccinimide

A solution of the oligosaccharide (2 mg) obtained by β-elimination fragmentation of peppergrass RG in 0.2 M ammonium formate, pH 5.3 (1 mL), was cooled in an ice-water bath. *N*-Bromosuccinimide (total 4 mg) was added to the solution in 1 mg portions at intervals of 60 min. The mixture was kept in the ice-water bath for an additional 2 h and, then maintained at room temperature for 4 h. The mixture was purified by SEC on a Superdex Peptide column with ELSD and $A_{235}$ detection (see Fig. 4C).

### 3.7. Matrix-assisted laser-desorption ionization-time of flight mass spectrometry

Aqueous solutions (2 μL) of the oligosaccharides (1 μg/μL) were mixed with an equal volume of 2,5-dihydroxybenzoic acid (100 mM in 90% aq MeOH). A portion of the mixture (1 μL) was then applied to the MALDI target plate and air dried. MALDI-TOF spectra were recorded with a Voyager DE-STR mass spectrometer (Applied Biosystems, Boston, MA, USA) with an $N_2$ laser (337 nm), operating at a source pressure of 1.0e−007 Torr, an accelerating voltage of 20 kV, a grid voltage of 90% of accelerating voltage, an extraction delay time of 500 ns, and a mass range of 320–5000. Typical mass spectra were acquired by averaging 100 laser shots.

### 3.8. Nuclear magnetic resonance spectroscopy

Oligosaccharides were dissolved in $D_2O$ (99.96% D), and spectra were recorded at 298 K using a Varian INOVA 600 spectrometer (operating at 599.739 MHz for $^1H$ and 150.816 MHz for $^{13}C$). Acetone was used as an internal standard ($^1H$ $\delta$ 2.225; $^{13}C$ $\delta$ 29.92). Standard Varian pulse sequences were used to record gCOSY, HSQC, and ROESY spectra.

## Acknowledgments

This work was supported by the US Department of Agriculture (Grant # 2003-35318-13644), the US Department of Energy Center for Plant and Microbial Complex Carbohydrates (Grant # DE-FG02-93ER20097), and the National Science Foundation (Grant # DBI-0421683). We thank Novozymes A/S (Bagvaerd, Denmark) for supplying the RG-lyase.

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