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Selective chemical depolymerization of rhamnogalacturonans

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Abstract—A method was developed to selectively methyl esterify and then cleave GalA residues in pectic polysaccharides. The method was optimized using a rhamnogalacturonan (RG) from *Arabidopsis* mucilage as a model compound. The carboxyl group of the GalA residues in the RG was selectively methyl esterified using tetrabutylammonium fluoride and iodomethane in Me_2SO containing 8% water. A 1D HMQC NMR method to determine the degree of methyl esterification was developed using ^{13}C-iodomethane as the methylating agent. The methyl-esterified pectins were fragmented by β-elimination in 0.2 M sodium borate, pH 7.3, at 125 °C. The resulting oligoglycosyl fragments, which contain a nonreducing 4-deoxy-β-L-*threo*-hex-4-enepyranosyluronic acid residue, were characterized using MALDI-TOF mass spectrometry, monosaccharide composition analysis, and 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. Application of this method to branched RG from potato generated low-molecular-weight fragments containing two residues from the RG backbone and a single side chain. In contrast, the fragments obtained when RG is treated with RG lyase contain a minimum of four backbone residues. The chemical method thus facilitates the release and structural characterization of the side-chain structures of RG obtained from various plant sources. The method also provides a convenient method for generating fully or partially methyl-esterified homogalacturonans.

Keywords: Rhamnogalacturonans; Pectic polysaccharide; Methyl esterification; β-Elimination; Selective depolymerization

1. Introduction

Rhamnogalacturonans (RGs) are a family of structurally related, highly branched, heteropolysaccharides present in the primary cell walls of gymnosperms and angiosperms.[1–4] The RG backbone is composed of a repeating disaccharide [-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)-].[5] Up to 80% of the backbone Rha*p* residues are substituted at O-4 with oligoglycosyl side chains. These side chains are composed predominantly of β-D-Gal*p* and α-L-Ara*f* residues linked together in various ways.[6,7] Some of the side chains are terminated by a Fuc*p*, Glc*p*A, or 4-*O*-methyl-Glc*p*A residue.[8,9] These differently linked glycosyl residues can combine to form a large number of side-chain structures that may be arranged in many different ways along the backbone.

The biological functions of RGs have not been established, although the results of several studies suggest that changes in the side-chain structures of these polysaccharides are correlated with their spatial and temporal locations in developing plant tissues.[2] Moreover, altering RG structure may have significant affects on plant growth and development.[10,11] Thus, increased knowledge of the structure of RG is required to understand its biological function, biosynthesis, and metabolism.

Determining the complete glycosyl sequence of a branched RG still remains a considerable challenge. Several enzymes that fragment the RG backbone have been characterized. For example, RG hydrolase catalyzes the hydrolysis of the α-Gal*p*A-(1→2)-α-Rha*p* linkage, generating fragments with a reducing GalA residue, whereas RG lyase catalyzes the β-elimination of the GalA residues and generates fragments terminated at the nonreducing end with a 4-deoxy-β-L-*threo*-hex-4-enepyranosyluronic acid residue.[12–16] However, these

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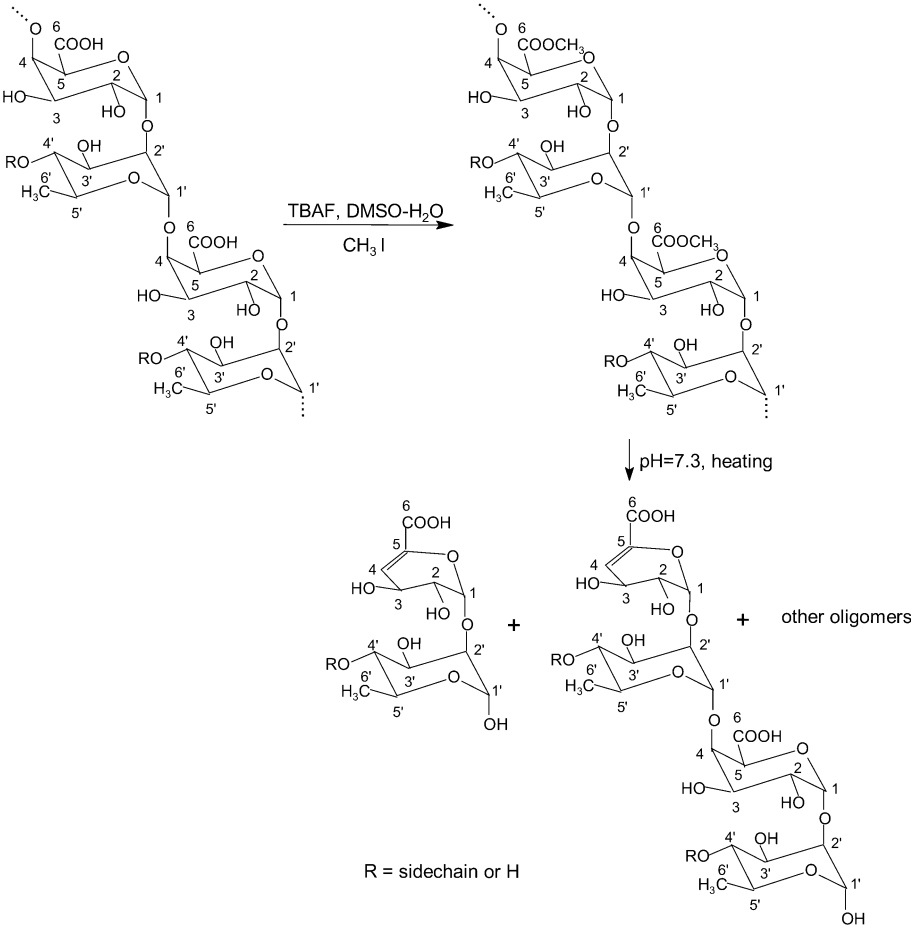
enzymes have limited use in the structural characterization of highly branched RG molecules, which are resistant to their action unless the side chains are partially removed by chemical or enzymatic treatments.[14,16,17]

Chemical fragmentation of RG using lithium metal in ethylenediamine has been used to generate oligoglycosyl-alditols that were sequenced using mass spectrometry and NMR spectroscopy.[8] This treatment cleaves the RG backbone by a free-radical mechanism,[18,19] but it also partially degrades some of the side chains. Moreover, the released oligoglycosyl-alditols are not readily labeled with a UV or fluorescent tag to facilitate their purification and structural characterization. Alternatively, pectic polysaccharides that contain methyl-esterified uronic acid residues can be fragmented by heating in a neutral aqueous solution, leading to the β-elimination of the substituent attached at O-4.[20,21] The results of these studies suggested to us that the availability of a method to efficiently methyl-esterify GalA residues in pectin, together with a facile method to generate fragments by β-elimination, would be of considerable value for sequencing RG. We now describe (Scheme 1) the use of tetrabutylammonium fluoride and iodomethane in Me_2SO containing 8% water to methyl-esterify GalA residues in rhamnogalacturonans and homogalacturonan. The methyl-esterified pectins are then fragmented by β-elimination at elevated temperature. Oligosaccharide fragments containing a single side chain are generated, providing a means to thoroughly characterize the structural features of complex RGs.

2. Results

2.1. Selective methyl esterification of the carboxyl group in the RG backbone

A water-soluble RG consisting predominantly of the repeating disaccharide unit [-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-] was isolated from *Arabidopsis thaliana* seed mucilage. This unbranched polysaccharide was used as a model compound to develop a fragmentation method that selectively cleaves the backbone by β-elimination to generate products similar to those produced by treatment with RG lyase. Chemically induced β-elimination of a 4-linked Gal*p*A residue does not occur efficiently unless the free carboxyl group is substituted, for



Scheme 1. Methyl esterification and β-elimination of RG.

example, by the formation of an alkyl ester. Therefore, a facile method was developed to selectively methyl esterify the carboxyl group of the GalA residues using tetrabutylammonium fluoride and iodomethane in Me_2SO. The method does not modify the hydroxyl groups of the polysaccharide, thus maintaining its water solubility, which is important for its efficient β-elimination in aqueous media.

The selective methyl-esterification procedure is based on a report[22] that treatment of ball-milled cell walls with iodomethane (MeI) and tetrabutyl ammonium fluoride (TBAF) in Me_2SO methylates phenolic hydroxyl groups (pK_a 6–7) of the lignin components, leaving the hydroxyl groups (pK_a >12) of the polysaccharides unmodified. This result suggested to us that comparable conditions would selectively methylate the carboxylate of GalA (pK_a 3–4), which has a relatively acidic proton. This is consistent with previous reports[23,24] of the selective methyl esterification of pectic polysaccharides by treatment of the tetrabutyl ammonium salts of these polysaccharides with iodomethane in Me_2SO. In our preliminary experiments, approximately 20% of the GalA residues of the mucilage RG were methyl esterified using Me_2SO containing MeI and TBAF. However, the addition of water (8% by volume) to the reaction mixture resulted in a degree of methyl esterification (DM) between 85% and 88% and a yield of greater than 85% of methyl-esterified polymer.

In the initial experiments, the DM was estimated by comparing the ^1H NMR signal areas of the methyl ester protons (δ 3.828) and the methyl protons of the rhamnosyl residue (δ 1.251). However, signal integration was difficult because the methyl ester resonance is not resolved from other resonances in the ^1H NMR spectrum. Therefore, an improved NMR method was developed to quantify the DM. This involved the use of ^{13}C-iodomethane as the methylating agent and analysis of the products by a modified 1D HMQC experiment (Fig. 1). One-dimensional HMQC experiments typically filter out the resonances of protons that are directly attached to ^{12}C atoms by subtraction of two spectra (Fig. 1A and B) recorded using opposite phases for the first ^{13}C pulse in the sequence. Alternatively, the two spectra can be added together to filter out the resonances of protons directly attached to ^{13}C atoms. This spectral editing technique makes it possible to obtain a spectrum (Fig. 1C) that includes only the resonances of the ^{13}C-labeled methyl group and a spectrum (Fig. 1D) that contains all of the other resonances of the polysaccharide. The proton resonances of the ^{13}C-labeled methyl group appear as a doublet due to the scalar interaction with ^{13}C ($^1J_{C,H}$ 148 Hz). The signal integrals from the two spectra can be directly compared because both spectra are derived from the same data set. The DM of the product is determined by comparing the total signal area of the ^{13}C-labeled methyl protons to that of another well-resolved

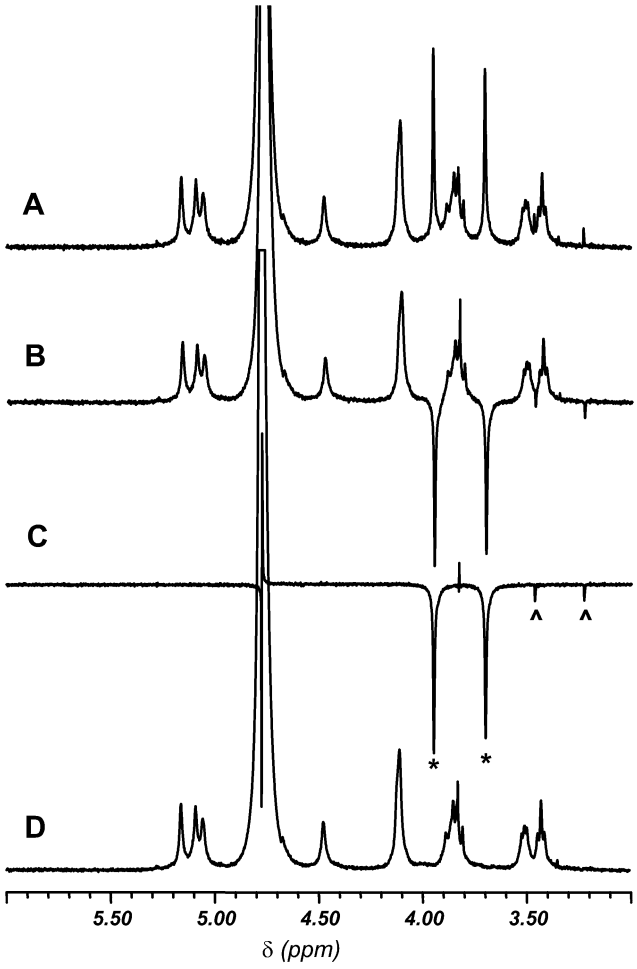


Figure 1. One-dimensional HMQC spectra of the RG backbone methyl esterified using ^{13}C-iodomethane. (A, B) Spectra recorded using opposite phase for the first ^{13}C pulse. (C) Subtraction of spectrum A from spectrum B produces a spectrum containing only resonances due to protons that are directly attached to ^{13}C atoms. (D) Addition of spectra A and B produces a spectrum containing only resonances of protons that are not directly attached to a ^{13}C atom. Signal integrals from spectra C and D can be directly compared for quantitative purposes. Methyl ester resonances are indicated by an asterisk (*) and methanol protons are indicated by a carat (^).

resonance, such as the methyl resonance of the rhamnosyl residues. The magnetic relaxation properties of the sample should be kept in mind when choosing the resonance to be used for comparison. A long relaxation delay was used when this method was applied to methyl-esterified polygalacturonic acid (PGA, see below) in order to completely recover longitudinal magnetization of the anomeric protons, whose measured spin–lattice relaxation time (T_1) is significantly longer (~12 s) than those of the ring protons (~2 s).

2.2. β-Elimination of methyl-esterified RG backbone

Heating the methyl-esterified mucilage RG that had been dissolved in aqueous buffers with pH values from

7 to 8 resulted in β-elimination of the uronic acids. The elimination reaction was more efficient in water than in Me_2SO (data not shown) presumably as a result of the increased solubility of pectin in water. The extent of fragmentation depends on the relative rates of the β-elimination and de-esterification reactions. At pH values greater than 8, the reaction rates for β-elimination and methyl ester hydrolysis are both likely to be elevated, whereas low pH (<7) may result in the cleavage of acid-labile Ara*f*-containing side chains. For example, Kravtchenko et al.[21] investigated the effects of pH, temperature, and buffer concentration on the β-elimination of lemon peel pectin (DM 72%) and concluded that maximum fragmentation (increase in A_{235}) occurred when the pectin was heated to 115 °C for 24 h in 0.1 M citrate, pH 5. In a subsequent paper, these authors report the use of 0.2 M ammonium carbonate, pH 6.8, for 8 h at 80 °C to fragment lemon and apple pectins by β-elimination.[25] This treatment generated a range of differently sized GalA-containing oligosaccharides, which were not characterized in detail. The small amount of RG present in the pectin was not fragmented, presumably because the GalA residues of this polysaccharide are not methyl esterified.

We found that significant elimination occurs when the methyl-esterified RG backbone is dissolved in borate buffer (pH 7.3) and then heated for 150 min at 125 °C. ^{1}H NMR spectroscopy indicated that all of the methyl esters of GalA are hydrolyzed by this treatment. This leads to incomplete fragmentation, as the de-esterified GalA residues do not undergo efficient β-elimination. This conclusion is supported by the time-dependent increase in the UV-absorption at 235 nm (Fig. 2), which did not change significantly after heating (125 °C) for 90 min, at which time no methyl esters could be detected.

RG lyase catalyzes the β-elimination of non-methyl-esterified α-GalA residues in the RG backbone, breaking the α-L-Rha*p*-(1→4)-α-D-Gal*p*A linkage in a manner comparable to the chemical β-elimination. The SEC profile of the products generated by treating RG from *A. thaliana* seed mucilage with RG lyase (Fig. 3C), together with the results of MALDI-TOF MS and

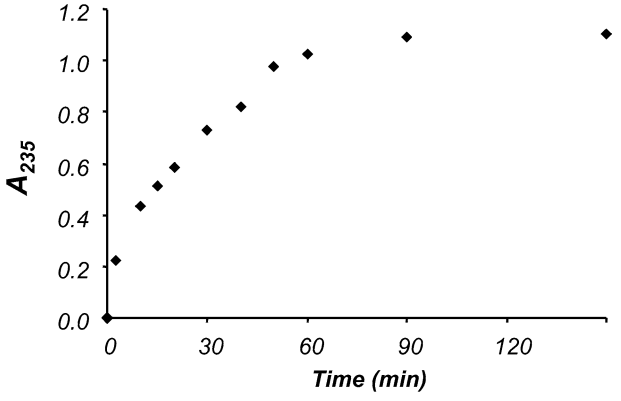


Figure 2. Time-dependent increase in $A_{235\,nm}$ during β-elimination of methyl-esterified RG backbone in 0.2 M sodium borate, pH 7.3, at 125 °C.

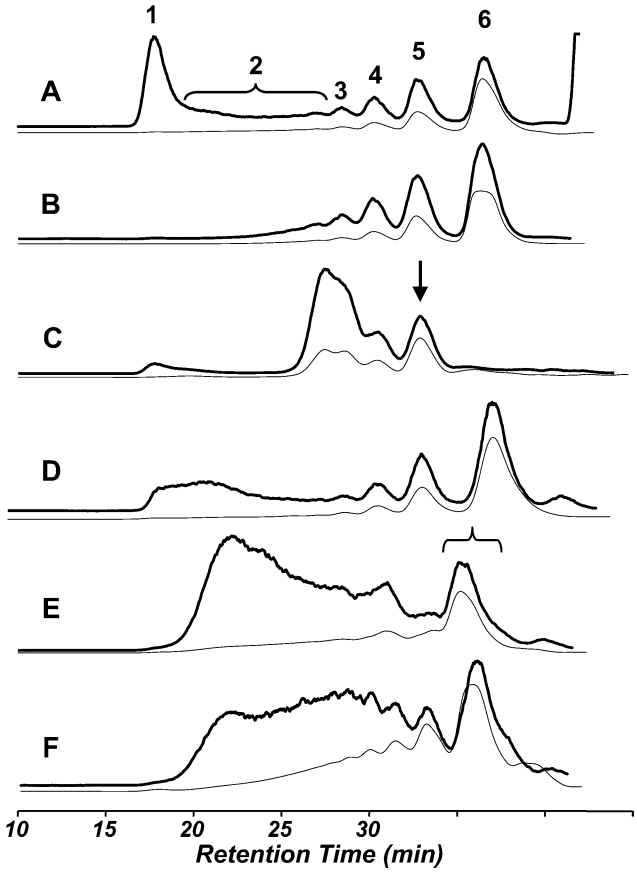


Figure 3. Size-exclusion chromatography profile of the products obtained upon β-elimination of methyl-esterified pectic polysaccharides. The responses of the ELSD detector (bold line) and UV detector at 235 nm (thin line) are shown. (A) Products of chemical elimination (0.2 M sodium borate, pH 7.3, 125 °C, 150 min) of the methyl-esterified RG backbone from *A. thaliana* seed mucilage (DM 88%). Fractions 5 and 6 contained the tetrasaccharide β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→2)-L-Rha and disaccharide β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-L-Rha, respectively. (B) Products obtained by re-methyl esterification and β-elimination (0.2 M sodium borate, pH 7.3, 125 °C, 150 min) of Fraction 1 (Panel A). The same oligosaccharides observed in Panel A were detected here. (C) Products obtained by RG lyase treatment of RG backbone from *A. thaliana* seed mucilage. The smallest of these (indicated by the arrow) is the same tetrasaccharide that is present in Fraction 5 (see Panel A). (D) Products obtained by chemical elimination of the methyl-esterified RG backbone from *A. thaliana* seed mucilage (DM 88%) after chemical elimination at higher temperature (0.2 M sodium borate, pH 7.3, 155 °C, 150 min). This led to increased fragmentation, but additional side products were also observed. (E) Products obtained by chemical β-elimination (0.2 M sodium borate, pH 7.3, 125 °C, 150 min) of methyl-esterified potato RG-I. Fractions pooled for subsequent purification and analysis are indicated by the bracket. (F) Products obtained by chemical β-elimination (0.2 M sodium borate, pH 7.3, 125 °C, 150 min) of methyl-esterified polygalacturonic acid. These consist of a series of oligouronides with the structure β-L-$\Delta_{4,5}$Hex*p*A-(1→4)-[α-D-Gal*p*A-(1→4)]$_n$-D-GalA, where *n* can be 0 or more.

NMR analysis (Table 3), indicated that oligosaccharides composed of 8–10 sugar residues (t_R 27–29 min) are the most abundant fragments generated by this enzyme under the conditions used. The smallest fragment detected corresponds to a tetrasaccharide (t_R 33 min, Fig. 3C). The ^{1}H NMR spectrum of the tetrasaccharide β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→2)-L-Rha is shown in Figure 4A and assigned in Table 3.

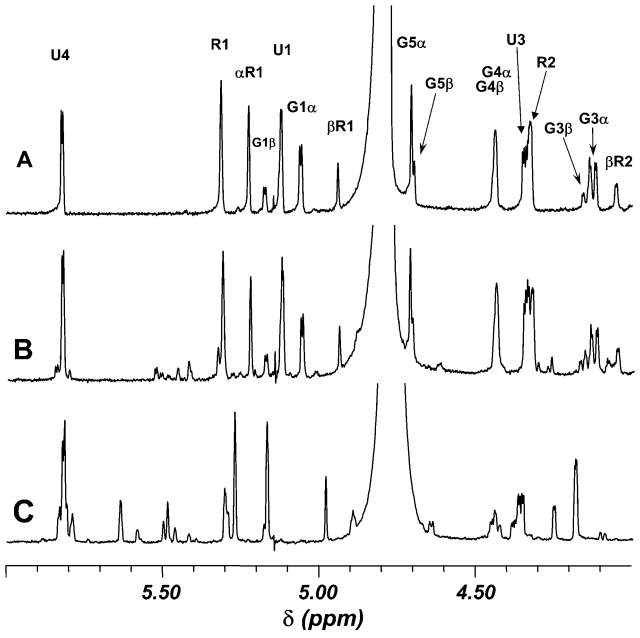


Figure 4. ^{1}H NMR spectra of the oligosaccharide fractions obtained by β-elimination of RG backbone. (A) Tetrasaccharide fraction obtained by RG-catalyzed elimination (see Fig. 3C, arrow). (B) Tetrasaccharide fraction obtained by chemical elimination (Fig. 3A, Fraction 5). (C) Disaccharide obtained by chemical elimination (see Fig. 3A, Fraction 6). Assignment abbreviations include an uppercase letter to indicate the residue, a Greek letter to indicate the anomeric configuration of the reducing Rha residue of the oligosaccharide, and an Arabic number to indicate the proton position. That is R1 and R2 represent H-1 and H-2, respectively, of internal rhamnosyl residues; U1, U3, and U4 represent H-1, H-2, and H-4, respectively, of unsaturated uronic acid residues; αR1 represents H-1 of reducing α-rhamnose residues; βR1 and βR2 represent H-1 and H-2, respectively, of reducing β-rhamnose residues; G1α, G5α, G4α, and G3α represent H-1, H-5, H-4, and H-3, respectively, of internal GalA residues linked to O-2 of reducing α-rhamnose residues; G1β, G5β, G4β, and G3β represent H-1, H-5, H-4, and H-3, respectively, of internal GalA residues linked to O-2 of reducing β-rhamnose residues.

Table 1. Relative peak areas of fractions obtained by SEC on a Sephadex-peptide column following β-elimination of the methyl-esterified RG backbone

Fraction	Range of retention time (t_R/min)	Area/%[a]
1	16.5–20.0	30.2
2	20.0–27.7	17.4
3	27.7–29.3	5.1
4	29.3–31.8	8.8
5	31.8–34.5	15.3
6	35.5–38.5	23.2

[a] Peak areas were obtained by integration of the evaporative light-scattering detector signal (see Fig. 2).

The products obtained by incubating the methyl-esterified RG backbone (DM 88%) at 125 °C for 150 min in 0.2 M sodium borate, pH 7.3, were fractionated by size-exclusion chromatography (SEC) using a Superdex-Peptide column (Fig. 3A). At least 60% of the polysaccharide was fragmented into low-molecular-weight oligosaccharides (Fractions 3–6). These fractions were shown, by MALDI-TOF MS, to contain oligosaccharides with glycosyl residues between 2 and 10 (Table 2). The structures for the disaccharide and tetrasaccharide products (Fractions 6 and 5, respectively, in Fig. 3A) were established using NMR analysis (Fig. 4A and B, Table 3). The intact polysaccharide and/or large fragments of the polysaccharide, which elute at the void volume of the SEC column (Fraction 1, Fig. 3A), accounted for ~30% of the total (Table 1). We assume that fragmentation of this material was minimal, as it had almost no absorption at 235 nm. Incomplete fragmentation is probably due to hydrolysis of the methyl esters. Indeed re-methyl-esterification of Fraction 1, followed by incubation at 125 °C for 150 min in 0.2 M borate, pH 7.3, resulted in its almost complete conversion into small oligosaccharides (Fig. 3B), with the disaccharide being the most abundant product.

The NMR spectrum of the disaccharide fraction generated by chemical β-elimination (Fig. 4C) is more complex than anticipated, indicating that side products are generated during the reaction. Signals originating from side products are also present, albeit in lower amounts, in the spectrum of the chemically generated tetrasaccharide (Fig. 4B). These may form as a result of degradation of the reducing Rha residue (due to its reactive

Table 2. MALDI-TOF MS data and proposed structures of the major components of SEC fractions of methyl-esterified, β-eliminated RG backbone (Fig. 2)

Fraction	*m*/*z*[a]	Deduced structure
3	1313.8 (1336.5)	$\Delta_{4,5}$Hex*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-Rha
4	991.3 (1014.1)	$\Delta_{4,5}$Hex*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-Rha
5	669.1 (691.2)	$\Delta_{4,5}$Hex*p*A-(1→2)-α-Rha*p*-(1→4)-α-Gal*p*A-(1→2)-Rha
6	346.3	$\Delta_{4,5}$Hex*p*A-(1→2)-Rha

[a] Data are *m*/*z* for the $[M+Na]^+$ ion and *m*/*z* for $[M+2Na-H]^+$ ion (in parentheses).

Table 3. Chemical shift assignments for oligosaccharides prepared by chemical and enzyme-catalyzed β-elimination reactions

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-L-Rha[a]						
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-[α][b]	5.167	3.813	4.355	5.816		
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-[β][b]	5.636	3.938	4.442	5.831		
Reducing (1→2)-α-L-Rha	5.269	4.180	3.862	3.414	3.886	1.277
Reducing (1→2)-β-L-Rha	4.978	4.250	3.641	3.315	3.426	1.295
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→2)-L-Rha[c]						
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)	5.123	3.800	4.342	5.826		
	(5.123)	(3.797)	(4.343)	(5.823)		
(1→2)-α-L-Rha-(1→4)	5.315	4.324	3.865	3.347	3.781	1.248
	(5.314)	(4.328)	(3.864)	(3.350)	(3.783)	(1.248)
(1→4)-α-D-GalA-(1→2)-[α][b]	5.062	3.942	4.129	4.439	4.714	
	(5.062)	(3.943)	(4.126)	(4.436)	(4.706)	
(1→4)-α-D-GalA-(1→2)-[β][b]	5.175	3.981	4.150	4.447	4.712	
	(5.174)	(3.982)	(4.150)	(4.445)	(4.697)	
Reducing (1→2)-α-L-Rha	5.225	3.969	3.891	3.472	3.879	1.285
	(5.228)	(3.969)	(3.895)	(3.471)	(3.880)	(1.285)
Reducing (1→2)-β-L-Rha	4.942	4.052	3.666	3.343	3.425	1.303
	(4.938)	(4.050)	(3.663)	(3.344)	(3.427)	(1.301)
β-L-$\Delta_{4,5}$Hex*p*A-(1→4)-D-GalA[d]						
β-L-$\Delta_{4,5}$Hex*p*A-(1→4)	5.125	3.743	4.294	5.795		
Reducing α-D-GalA	5.304	3.798	3.971	4.591	4.459	
Reducing β-D-GalA	4.603	3.469	3.738	4.536	4.101	
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-[β-D-Gal*p*-(1→4)-]-L-Rha[e]						
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-[α][b]	5.192	3.825	4.369	5.829		
	97.49[f]	69.99[f]	65.62[f]	106.87[f]		
β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-[β][b]	5.295	3.801	4.430	5.800		
β-D-Gal*p*-(1→4)	4.647	3.504	3.661	3.911	3.667	3.746
	102.50[f]	71.24[f]	74.99[f]	68.74[f]	72.49[f]	60.62[f]
Reducing α-L-Rha	5.267	4.171	4.089	3.682	3.962	1.341
	91.24[f]	77.49[f]	69.37[f]	79.99[f]	67.49[f]	17.17[f]
Reducing β-L-Rha	4.985	4.241	3.863	3.586	3.513	1.354

[a] Disaccharide produced by chemical elimination of the unbranched RG from *A. thaliana*.
[b] Residues attached to O-2 of the α and β reducing Rha residues are listed separately to account for the significant anomerization effect that they exhibit. The anomeric configuration of the reducing residue to which the listed residue is attached is indicated by a bracketed Greek letter ([α] or [β]).
[c] Tetrasaccharide produced by RG lyase treatment of the unbranched RG from *A. thaliana*. Assignments for the same tetrasaccharide generated by methyl esterification and chemical elimination of this polysaccharide are in parentheses.
[d] Disaccharide produced by chemical elimination of PGA.
[e] Trisaccharide produced by chemical elimination of potato RG-I.
[f] ^{13}C chemical shifts. ^{13}C-Resonances for the oligosaccharide with a reducing β-Rha*p* residue were too weak to assign.

aldehyde) or of the β-L-$\Delta_{4,5}$Hex*p*A residue (due to its reactive double bond).

The ^{1}H NMR spectrum of the disaccharide fraction (Fig. 4C) contained five assignable spin systems corresponding to $\Delta_{4,5}$Hex*p*A residues. Two abundant spin systems corresponding to reducing α- and β-Rha*p* residues were also observed in the spectrum, and these were assigned, by comparing signal integrals, as the residues to which the two most abundant $\Delta_{4,5}$Hex*p*A spin systems are linked. Thus, β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-α-L-Rha*p* and β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-β-L-Rha*p* were the two most abundant products in the disaccharide fraction. Five other, lower abundance spin systems were also present in the spectrum. All of these contained methyl resonances (δ 1.1–1.3), indicating they were derived from the reducing Rha residue generated by the elimination reaction. Two of these spin systems were consistent with the presence of α-L-quinovopyranose and β-L-quinovopyranose, respectively. (Quinovopyranose is 6-deoxy-glucopyranose, the 2 epimer of rhamnopyranose, which is 6-deoxy-mannopyranose.) The remaining three spin systems appear to originate from compounds that contain vinyl and/or keto groups that probably formed as a result of more extensive degradation of the reducing Rha. For example, the third spin system contained no resonances corresponding to H-1 or H-2, and its H-3 (δ 5.461) and H-4 (δ 4.095) resonances exhibited significant downfield shifts compared to H-3 (δ 3.865 and 3.644) and H-4 (δ 3.414 and 3.315) of reducing α- or β-Rha*p*, respectively. The fourth and fifth of these spin systems contained no H-1 resonance, but each did contain a significantly deshielded H-4 resonance (δ 4.645

and 4.893, respectively). Furthermore, these last two spin systems each included a moderately deshielded resonance (δ 5.498 and 5.487, respectively) similar to H-3 in the third spin system (described above) and a strongly deshielded resonance (δ 5.790 and 5.819, respectively) consistent with the presence of a vinyl proton.

To further examine the structures of its components, the disaccharide-containing fraction, generated by chemical β-elimination, was treated with $NaBH_4$ to convert the reducing disaccharides into the corresponding monoglycosyl-alditols. This considerably simplified the ^1H NMR spectrum of the disaccharide-containing fraction. The ^1H NMR spectrum contained three spin systems consistent with L-$\Delta_{4,5}$Hex*p*A residues and three spin systems consistent with a 6-deoxy hexitol. The 6-deoxy hexitols were assigned as L-rhamnitol with a β-L-$\Delta_{4,5}$Hex*p*A substituent at O-2 (signal area 1.0), L-quinovitol with a β-L-$\Delta_{4,5}$Hex*p*A substituent at O-2 (signal area 0.25), and free L-rhamnitol (signal area 0.18). The free rhamnitol, identified by comparison to an authentic standard, was probably generated by acid-catalyzed hydrolysis of β-L-$\Delta_{4,5}$Hex*p*A-(1,2)-rhamnitol during removal of borate with acidified methanol. The spectrum also contained a deoxy-sugar spin system (signal area 0.38) with no observable H-1 or H-2 resonances, and downfield shifted H-3 (δ 5.226). This spin system is similar, but not identical, to spin system 3 found in the disaccharide prior to reduction with $NaBH_4$.

The $NaBH_4$-reduced disaccharide was hydrolyzed and the products were per-O-acetylated and analyzed by GC–MS. The expected product, rhamnitol penta-acetate (t_R 8.86 min), was detected along with another 6-deoxy-hexitol penta-acetate identified as quinovitol penta-acetate by comparing its retention time (9.54 min) and electron-impact mass spectrum to an authentic standard. The combined results of ^1H NMR spectroscopic and glycosyl residue composition analyses indicate that ~12% of the β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-L-Rha, was converted to $\Delta_{4,5}$Hex*p*A-(1→2)-L-Qui under the conditions used to chemically fragment the methyl-esterified RG backbone by β-elimination. Other side products generated from the reducing Rha residue (~23% of the total) give rise to resonances with chemical shifts consistent with the presence of vinyl and/or keto groups.

More extensive β-elimination fragmentation is promoted by temperatures higher than 125 °C (see Fig. 3A and D). Treatment at 155 °C for 150 min. (Fig. 3D) produces a larger amount of low-molecular-weight oligosaccharides and leaves a smaller fraction of material with a molecular weight large enough to void the SEC column than treatment at 125 °C. However, the ^1H NMR spectrum of the disaccharide generated by elimination at 155 °C (data not shown) is more complex than that of the disaccharide generated at 125 °C, indicating that additional side reactions occur at the higher temperature.

2.3. Methyl esterification and β-elimination of polygalacturonic acid

The method developed for the site-specific fragmentation of the RG backbone was used to selectively methyl esterify and depolymerize polygalacturonic acid (PGA). Treating PGA with ^{13}C-iodomethane and TBAF in Me_2SO containing 8% H_2O gave a product with a DM of ~100% as determined by 1D HMQC. Most of the methyl-esterified PGA was converted to oligosaccharides by heating at 125 °C for 150 min in 0.2 M sodium borate, pH 7.3 (Fig. 4). The fraction (retention time 36 min) that contained the most abundant product accounted for 22% of the total (light-scattering) peak area. The MALDI-TOF mass spectrum of this fraction contained an abundant ion at *m*/*z* 375, which corresponds to $[M+Na]^+$ of a disaccharide composed of a β-L-$\Delta_{4,5}$Hex*p*A residue and a GalA residue. Its structure, β-L-$\Delta_{4,5}$Hex*p*A(1,4)-Gal*p*A, was unambiguously determined by ^1H NMR analysis (Table 3).

2.4. Methyl esterification and elimination of branched RG-I from potato cell walls

Commercially available, highly branched RG-I from potato was shown by SEC on a Superdex-Peptide column to consist of a size-heterogeneous mixture of polysaccharides (data not shown). Glycosyl residue composition analysis of the fraction that was eluted in the void volume of the SEC column gave a GalA:Rha ratio of 2.5:1, indicating the presence of RG-I, together with some homogalacturonan. This high-molecular-weight fraction was selectively methyl esterified using ^{13}C-iodomethane, and the DM of the GalA residues was estimated to be 93% by 1D HMQC analysis. This value was calculated by comparing the signal integrals for the ^{13}C-methyl (ester) protons and the methyl protons of the rhamnosyl residues.

Most of the methyl-esterified potato RG-I was depolymerized to oligomers by heating at 125 °C for 150 min in 0.2 M sodium borate, pH 7.3 (Fig. 3E). The SEC fraction with the most intense absorption of 235 nm light (Fig. 3E, retention time 34.5–37 min), which accounted for ~17% of the total light-scattering peak area, was collected and further purified by anion exchange on a HiTrap Q HP column. The major ion-exchange fraction was eluted at 15.5–23.5 min and accounted for 97% of the total light-scattering peak area. An abundant $[M+Na]^+$ ion was observed at *m*/*z* 507 in the MALDI-TOF mass spectrum of this fraction, suggesting that its major component contained one $\Delta_{4,5}$Hex*p*A residue, one Rha residue, and one hexose residue. NMR analysis (Table 3), including 2D gCOSY and gHSQC experiments, are consistent with the structure β-D-Gal*p*-(1→4)-[β-L-$\Delta_{4,5}$Hex*p*A-(1→2)]-L-Rha. These results indicate that the methyl-esterification and β-elimination

reactions can be used to selectively break branched RG-I molecules into small fragments that contain a single side chain.

The minor ion-exchange fraction (t_R 58.5–63.5 min) accounted for <3% of the total light-scattering peak area. This fraction and the disaccharide fraction obtained by chemical fragmentation of methyl-esterified PGA had comparable 1D ^{1}H NMR spectra (Table 3), except for a difference in signal-to-noise ratio. The MALDI-TOF mass spectrum of this fraction contained an abundant ion at *m*/*z* 375. Thus, the dominant component of this fraction is β-L-$\Delta_{4,5}$Hex*p*A-(1→4)-D-GalA. This result confirmed that the potato RG-I fraction contained some homogalacturonan, which gives rise to this product after selective methyl esterification and elimination. No β-L-$\Delta_{4,5}$Hex*p*A-(1→2)-L-Rha was detected in the column eluant, suggesting that nearly all of the Rha residues in the potato RG-I backbone bear a side chain.

3. Discussion

A method was developed to specifically methyl esterify and then degrade GalA residues in pectic polysaccharides to generate low-molecular-weight fragments of RG backbone from *Arabidopsis* mucilage, the backbone of a branched RG-I from potato, and homogalacturonan. The chemical reaction leads to the β-elimination of 4-substituted Gal*p*A residues and is comparable to the action of a pectate lyase and/or rhamnogalacturonan lyase. However, the chemical procedure has an important advantage over the use of these enzymes. Chemical β-elimination of branched RG-I from potato generates fragments containing two backbone residues and a single side chain. In contrast, fragments containing a minimum of four backbone residues are obtained when RG is treated with RG hydrolase or RG lyase. Moreover, highly branched RG molecules are typically resistant to these enzymes unless some of the side chains are removed.[14,17] Extensive depolymerization of the RG backbone should be attainable by performing multiple, sequential methyl-esterification and elimination reactions, whereas repeated enzyme treatments does not lead to significant additional depolymerization.

The ease with which pectic polysaccharides are methyl esterified by treatment with TBAF and iodomethane in Me_2SO containing 8% H_2O makes this method an attractive alternative to treatment with diazomethane (an environmentally hazardous reagent) in diethyl ether, treatment with acidified methanol, or reaction with activated esters generated by treatment with a carbodiimide.[26] Pectins are only sparingly soluble in the organic solvents used in the first two reactions, and complete methyl-esterification is often difficult to achieve. Furthermore, treatment with acidified methanol may lead to solvolysis of acid-labile residues. Unwanted side reactions often occur during carbodiimide-based esterification reactions[26] making it difficult to isolate the desired product.

The methyl-esterification reaction is carried out in a mixed solvent system (8% H_2O in Me_2SO) in which pectic polysaccharides are readily soluble. The use of anhydrous Me_2SO gave inferior results, possibly due to incomplete solubilization or to the formation of strong intramolecular hydrogen bond networks that reduce the flexibility of the pectic polysaccharide, leading to steric or conformational barriers to the methyl-esterification reaction.[27] It has been previously shown that conversion of pectic polysaccharides to their tetrabutyl ammonium salts increases their solubility in Me_2SO and facilitates their esterification with alkyl halides.[23,24]

The methylation reaction is specific in that it forms methyl esters of the carboxyl groups of uronic acids but does not generate methyl ethers by reaction with the hydroxyl groups of these residues. This specificity is clearly seen in the 1D HMQC spectra of products generated by methyl esterification with ^{13}C-iodomethane. The only resonances observed in the ^{13}C-filtered spectra (Fig. 1C) are due to methyl esters (δ 3.801 for methyl-esterified polygalacturonic acid and δ 3.826 for methyl-esterification RG backbone), free methanol (δ 3.346) and residual HDO (due to incomplete subtraction, δ 4.75). The methanol resonance appeared and increased in intensity when the sample was stored in aqueous solution at room temperature for several weeks, indicating that it arose due to hydrolysis of the methyl esters. Methylation of hydroxyl groups would give rise to a collection of discrete resonances in the ^{13}C-filtered spectra. However, such resonances were not observed. Other reactions, such as transesterification or the formation of methyl glycosides were not detected.

The elimination reaction is carried out in an aqueous buffer capable of completely dissolving pectic polysaccharides. It is likely that the use of an aqueous buffer, which competes with intramolecular hydrogen bonds, leads to increased flexibility of the pectic polysaccharide.[28,29] This increased flexibility probably facilitates the elimination reaction by making the transition state geometry more energetically accessible.

β-Elimination of methyl-esterified pectin at 125 °C with 0.2 M borate generates small amounts of side products that are likely to be produced by rearrangement of the newly exposed, reactive, reducing rhamnose residue to form quinovose (6-deoxyglucose) and other more extensively modified products. Formation of quinovose is not surprising, as abstraction of the acidic H-2 of the reducing rhamnose residue, followed by reprotonation, would lead to C-2-epimerization of the residue. The side products are most abundant in the disaccharide fraction obtained by β-elimination of GalA residues linked to a rhamnose residue that does not have a

substituent at O-4. The products containing a reducing 2,4-linked rhamnose (derived from branch points in the RG backbone) appear to be less susceptible to side reactions. The increased stability of the 2,4-linked rhamnose is important, because this branch point contains the structural information that is required for sequencing RGs.

The NMR spectra of the oligosaccharides generated by the chemical β-elimination are somewhat complex, but can be significantly simplified by converting the oligosaccharides to the corresponding oligoglycosyl-alditols. Nevertheless, the presence of a reducing glycose is of value as it facilitates the preparation of a fluorescent derivative via reductive amination.[30] Such derivatives can be detected with high sensitivity, which is essential for microanalysis of the oligosaccharides.

The selective methylation procedure described here provides a facile method for methyl esterifying pectins and has considerable potential as an analytical tool for the structural characterization of pectic polysaccharides. In addition, this method will facilitate the preparation of fully methyl-esterified oligogalacturonides that can then be selectively de-esterified and used in biological and physiochemical studies. Similarly, the combination of the methyl-esterification and β-elimination procedures to selectively fragment RG isolated from the walls of different tissues at different developmental stages, and from the walls of plants carrying mutations that affect pectin structure, will provide key information regarding the relationships between the specific structural features of rhamnogalacturonans and their role(s) in plant cell development.

4. Experimental procedures

4.1. General methods

Total carbohydrate content and uronic acid content were determined colorimetrically using the phenol–sulfuric acid[31] and 3-hydroxybiphenyl[32] 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 0.45 mL/min with 50 mM ammonium formate, pH 5. The column outlet was connected in series to an Agilent 1100 UV detector ($A_{235\,nm}$) and a SEDEX evaporative light-scattering detector.

4.2. Matrix-assisted laser-desorption-ionization time-of-flight spectrometry (MALDI-TOF MS)

Aqueous solutions (2 μL) of oligosaccharides (1 μg/μL) were mixed with an equal volume of 100 mM 2,4-dihydroxybenzoic acid in 90% aq MeOH. One microliter of this mixture was applied to the MALDI 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 with the following parameters: source pressure 1.0×10^{-7} Torr, accelerating voltage 20 kV, grid voltage 90% of accelerating voltage, extraction delay time of 500 ns, and mass range of m/z 320–2500. Mass spectra were acquired by averaging 100 laser shots.

4.3. NMR spectroscopy

Samples were dissolved in D_2O (99.96% D) and spectra were recorded at 298 K using a Varian INOVA 600 spectrometer (operating frequency 599.739 MHz for ^1H and 150.816 MHz for ^{13}C). Acetone was used as the internal standard (^1H δ 2.225; ^{13}C δ 29.92). The DM was determined by using the standard Varian pulse program for a 1D HMQC experiment except that the two free-induction decays that are generated by inverting the first ^{13}C pulse in the sequence were saved separately. Addition of these FIDs provides an edited spectrum that includes only ^1H resonances of those protons that are directly attached to ^{13}C atoms (see Fig. 1C); subtraction provides an edited spectrum that contains the remaining ^1H resonances (see Fig. 1D). Complete subtraction of unwanted signals in these FIDs required precise calibration of the pulse widths and placing the decoupler frequency exactly at the center of the methyl proton doublet generated by scalar coupling to the ^{13}C nucleus ($^1J_{CH}$ 148 Hz). Furthermore, slow longitudinal (T_1) relaxation of the anomeric resonances of polygalacturonic acid (~12 s) made it necessary to include long relaxation delays (>80 s) between transients when this anomeric resonance was used for quantitation.

4.4. Materials

A water-soluble polysaccharide composed almost exclusively of the RG backbone was isolated from the mucilage of *A. thaliana* seeds. Seeds (50 g) were suspended in 800 mL of 0.5% ammonium oxalate (pH 6.5) and gently stirred for 4 h at 55 °C. Insoluble material was removed by centrifugation, the supernatant was concentrated to 200 mL, extensively dialyzed against deionized water (Spectra/Por, molecular weight cutoff [MWCO] 12,000–14,000 Da) and lyophilized to yield 870 mg of polysaccharide. Polygalacturonic acid (PGA) was obtained from Sigma Chemical Co. Commercial rhamnogalacturonan from potato was obtained from Megazyme and partially purified by SEC using a Superdex-Peptide column eluted with 50 mM ammonium formate, pH 5, at a flow rate 0.45 mL/min. High-molecular-weight material that was eluted at column void volume was collected manually, and buffer salts were removed by repeated lyophilization.

4.5. Methyl esterification of RG backbone

A suspension 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. Preliminary experiments had established that the addition of water to 8% by volume resulted in the highest levels of methyl esterification. The cloudy mixture became a clear solution after stirring for 6–8 h. After incubation for an additional 10 h, a light-yellow product, which was identified as iodine (arising from the oxidation of iodide), appeared in the solution. 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 Da) against deionized water for 48 h and then lyophilized. The polymer was desalted on a Superdex G-15 column (3 cm × 65 cm) to remove traces of TBAF, which remained even after dialysis for 48 h. The methyl-esterified polysaccharide was lyophilized and analyzed by ^{1}H NMR. In some cases, ^{13}C-labeled MeI was used, and the resulting ^{13}C-methyl-esterified polysaccharide was analyzed by an HMQC experiment to determine the DM.

4.6. β-Elimination of methyl-esterified RG backbone

A solution of the methyl-esterified RG backbone (10 mg) in 0.2 M sodium borate (pH 7.3, 5 mL) was heated (125 or 155 °C) in a sealed tube for 150 min. The actual pH of the reaction mixture at elevated temperature was not known, as changes in the pH of the solution that occurred upon heating were not measured. The reaction was monitored by recording the increase in $A_{235\,nm}$ as a function of time (Fig. 2). Sodium borate was found to be a better buffer for the elimination reaction than sodium phosphate, sodium citrate, or Tris–HCl, at the same pH. The reaction was stopped after 150 min by immersing the tubes in cold water. The products formed were then fractionated by SEC on a Superdex-Peptide column (Fig. 3). Six fractions were collected, lyophilized, and analyzed by NMR spectroscopy and by MALDI-TOF MS. The disaccharide-containing Fraction 6 was reduced with $NaBH_4$. The reaction was quenched by the addition of glacial HOAc and the products were desalted by SEC on Sephadex G-10, lyophilized, and analyzed by ^{1}H NMR. The $NaBH_4$-reduced product was then hydrolyzed by heating (120 °C, 1.5 h) in 2 M TFA. The alditols that were released were per-O-acetylated and analyzed by GC–MS.[33]

The high-molecular-weight material (Fraction 1 in 3A), which was not fragmented by the first β-elimination reaction, was re-methyl esterified and then β-eliminated. The products were analyzed by SEC (Fig. 3B).

4.7. Methyl esterification and β-elimination of RG-I from potato and PGA

The methyl esterification and elimination of these two polymers was performed using the conditions optimized for the RG backbone. Samples (methyl-esterified RG-I from potato or methyl-esterified PGA, 10 mg) were dissolved in 5 mL of 0.2 M sodium borate buffer, pH 7.3, and incubated at 125 °C for 150 min in a sealed tube. The depolymerized products were fractionated by SEC on a Superdex-Peptide column. The potato RG-I oligosaccharide fraction having the greatest absorbance at 235 nm (retention time 34.5–37.0 min on the Superdex-Peptide column) was further purified by anion exchange on a 5-mL HiTrap Q HP column (prepacked with Q Sepharose, High Performance, Amersham Biosciences) eluted isocratically with 50 mM ammonium formate, pH 6.3, and a flow rate of 0.45 mL/min. Fractions collected were lyophilized and analyzed by NMR and MALDI-TOF MS.

4.8. Degradation of RG backbone with RG lyase

Enzyme-catalyzed depolymerization of RG backbone was performed using rhamnogalacturonan α-L-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronide lyase (rhamnogalacturonase B) from *Aspergillus aculeatus* (a gift from Novozymes A/S, Denmark). A solution of RG backbone (6 mg) in 0.1 M ammonium formate (1.5 mL), pH 6.0, was treated for 48 h at 30 °C with 30 μg of RG lyase. The products were separated by SEC on a Superdex-Peptide column, as described above. Fractions were collected, lyophilized, and analyzed by NMR and MALDI-TOF MS.

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