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Purification and characterization of biologically active 1,4-linked α-D-oligogalacturonides after partial digestion of polygalacturonic acid with endopolygalacturonase *,†

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ABSTRACT

A procedure is described for generating and purifying biologically active (1 → 4)-linked α-D-oligogalacturonides (oligogalacturonides). Oligogalacturonides are generated by treatment of polygalacturonic acid (PGA) with a homogeneous fungal α-(1 → 4)-endopolygalacturonase (EPG). Oligogalacturonides with a dp greater than seven were selectively precipitated in the presence of sodium acetate and ethanol. Oligogalacturonides with a dp less than 11 and modified oligogalacturonides remained soluble. Oligogalacturonides with a dp between 10 and 15 were purified from the resolubilized NaOAc–ethanol-precipitated material using Q-Sepharose fast-flow anion-exchange chromatography followed by semipreparative high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD). Approximately 70 mg of each homogeneous oligogalacturonide (dp between 10 and 15) was obtained from 10 g of EPG-treated PGA. The tridecagalacturonide fraction was selected for chemical and structural characterization and was shown to be homogeneous by glycosyl-residue composition analysis, HPAEC–PAD, FABMS, and ^{1}H NMR spectroscopy. The purified tridecagalacturonide elicited phytoalexin accumulation in soybeans and induced flower formation and inhibited root formation in tobacco thin-cell-layer explants.

INTRODUCTION

Treatment of plant cell walls or polygalacturonic acid (PGA) with acid or enzymes releases linear oligosaccharides composed of (1 → 4)-linked α-D-galactosyluronic acid residues (oligogalacturonides)[1–3]. Partially purified oligogalactur-

* In this paper, the term "oligogalacturonides" is used to describe oligosaccharides composed exclusively of (1 → 4)-linked α-D-galactosyluronic acid residues.

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onides have been shown to elicit defense responses and morphogenetic changes in plants[4,5]. Many of those biological effects are maximally induced by preparations enriched in oligogalacturonides of dp 10 to 15, which we refer to as bioactive oligogalacturonides[4]. However, none of those fractions were homogeneous. Thus, there is a need to develop a method for the preparation of pure oligogalacturonides of dp 10 to 15 to demonstrate that these homogeneous fragments are indeed bioactive.

PGA, a commercial product obtained by chemical deesterification of citrus pectin, has been the material of choice for generating bioactive oligogalacturonides[1–3]. However, each bioactive oligogalacturonide of dp between 10 and 15 accounts for only a small proportion of the compounds generated by either chemical or enzymic fragmentation of PGA[1–3]. Additional products include oligogalacturonides containing galactaric acid, the C-1 oxidized derivative of galacturonic acid, at their reducing end[3]. The modified and unmodified oligogalacturonides are not resolved by low-pressure anion-exchange and gel-permeation chromatographies[3]. Thus, it was apparent that the purification of milligram quantities of homogeneous oligogalacturonides requires the combination of high capacity and high resolution chromatographic procedures.

We now describe a procedure, based on selective NaOAc–ethanol precipitation, Q-Sepharose fast-flow anion-exchange chromatography (Q-Sepharose), and semipreparative high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), for the purification of milligram quantities of homogeneous, bioactive oligogalacturonides.

RESULTS AND DISCUSSION

Optimizing the enzymic generation of oligogalacturonides with dp 10 to 15.—The optimal conditions for generating bioactive oligogalacturonides (dp 10 to 15) were determined by HPAEC–PAD analysis of the products released at given time intervals during α-(1 → 4)-endopolygalacturonase (EPG) digestion of PGA. Portions (1 mL) of the digest were removed after 2, 4, 6, 8, 10, and 20 h and then immediately autoclaved (15 min at 121°C) to inactivate the EPG. The relative proportions of oligogalacturonides (dp 10 to 15) in each enzymic digest were determined by integration of the PAD response. The maximum yield of bioactive oligogalacturonides (dp 10 to 15) was obtained by treating PGA with EPG for 8 h (Fig. 1A). At longer times the majority of the oligogalacturonides were enzymically fragmented to biologically inactive fragments (dp < 10).

Isolation and partial characterization of the minor components present in the EPG digest of PGA.—The tridecagalacturonide-containing fraction, prepared by partial EPG digestion of PGA followed by QAE-Sephadex and Q-Sepharose chromatographies[6], was shown, by HPAEC–PAD, to contain significant amounts (30% w/w) of modified oligogalacturonides (Fig. 2). The components of the tridecagalacturonide-containing fraction were isolated by semipreparative HPAEC–PAD using a

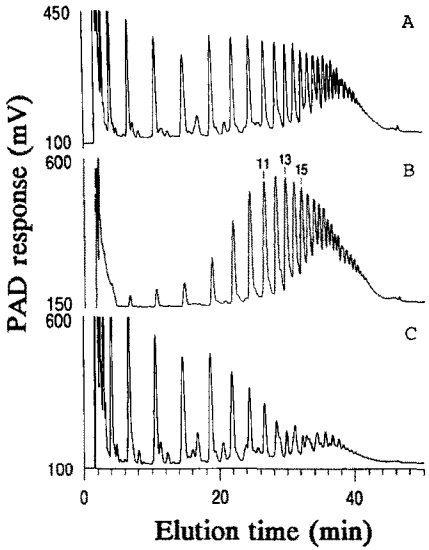

Fig. 1. HPAEC–PAD elution profiles of enzymically digested PGA and the products resulting from selective precipitation of the digest with NaOAc and ethanol: A, EPG-digested PGA; B, precipitate resulting from NaOAc–ethanol precipitation of the EPG digest; C, supernatant after NaOAc–ethanol precipitation of the EPG digest. Approximately 1 mg of each fraction was loaded onto the HPAEC column. The labels in (B) correspond to the dp of the oligogalacturonide in that peak. The peaks eluting between the homogalacturonides most likely are the modified oligogalacturonides.

NaOAc, pH 8, concentration gradient (550–700 mM). These compounds were structurally characterized by glycosyl-residue and glycosyl-linkage composition analyses, FABMS, and ^{1}H NMR spectroscopy.

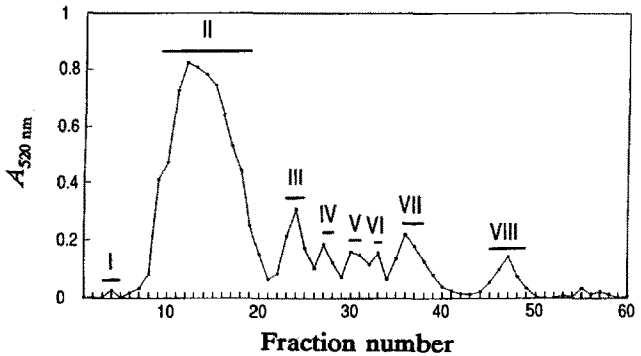

Fig. 2. Semipreparative HPAEC chromatography of the tridecagalacturonide fraction (~ 8 mg) isolated by QAE-Sephadex and Q-Sepharose chromatographies. The components were resolved at 5 mL/min with a linear gradient of 550–700 mM NaOAc, pH 8, beginning at 2 min and ending at 40 min. Fractions (2.5 mL) were collected after a 10-min delay, and portions (20 μL) analyzed colorimetrically for uronic acid[16]. Peak II contains the tridecagalacturonide. See text for the identification of other peaks.

Negative-ion FABMS of the major component in the tridecagalacturonide-containing fraction (peak II, see Fig. 2) gave a signal at m/z 2305 corresponding to $[M - H]^-$ from an oligosaccharide containing 12 galactosyluronic acid residues and galacturonic acid. The ^1H NMR spectrum of peak II contained signals for anomeric protons[7,8] at δ 5.23 (H-1 reducing α-Gal*p*A), δ 5.10 (H-(1 → 4)-linked α-Gal*p*A), and δ 4.64 (H-1 reducing β-Gal*p*A). Glycosyl-residue and glycosyl-linkage composition analyses established that the component in peak II was composed of terminal nonreducing Gal*p*A and 4-linked Gal*p*A in the ratio of 1.0: 12.4. Thus, the component in peak II is the tridecagalacturonide.

Three components in the tridecagalacturonide-containing fraction were identified as oligosaccharides containing between 8 and 10 galacturonic acid residues with galactaric acid at their reducing ends (Fig. 2). Negative-ion FABMS of peak V gave a signal at m/z 1617 corresponding to $[M - H]^-$ from an oligosaccharide containing eight galactosyluronic acid residues and hexaric acid. No anomeric signals from a reducing galactosyluronic acid residue were present in the ^1H NMR spectrum of peak V. The hexaric acid was shown by glycosyl-residue and glycosyl-linkage composition analyses to be a 4-linked galactaric acid. Negative-ion FABMS of peak VIII gave signals at m/z 1793 and 1969 which correspond to $[M - H]^-$ from oligosaccharides containing nine galactosyluronic acid residues and galactaric acid, and ten galactosyluronic acid residues and galactaric acid, respectively. Galactaric acid, the C-1 oxidized derivative of galacturonic acid, may have been formed during the commercial preparation of polygalacturonic acid or may be a naturally occurring component of pectin, as a known plant oxidase converts the reducing end galacturonic acid residue of oligogalacturonides into galactaric acid[9]. Oligosaccharides composed of galactaric acid and between two and four 4-linked galactosyluronic acid residues have been generated by EPG treatment of polygalacturonic acid and shown to activate the oxidation of indole-3-acetic acid by tomato peroxidase[10]. In contrast, the results of preliminary studies (data not shown) have indicated that the modified oligogalacturonides isolated in this study do not possess biological activity, as they do not induce phytoalexin accumulation in soybean hypocotyls[1,2].

The tridecagalacturonide-containing fraction was also shown to contain an oligogalacturonide with tetraric acid at its reducing end (Fig. 2, peak VII). Negative-ion FABMS of peak VII gave an ion at m/z 1556 corresponding to $[M - H]^-$ from an oligosaccharide containing eight galactosyluronic acid residues and tetraric acid. Tetraric acid is a four-carbon analogue of galactaric acid.

Two additional components in the tridecagalacturonide-containing fraction were shown, by FABMS, to have molecular weights corresponding to oligosaccharides containing only glycosyluronic acid residues. However, these compounds did not cochromatograph with their corresponding oligogalacturonides (Fig. 2). Peak III contains a tridecauronide, and peak IV a dodeca- and a trideca-uronide. These oligohexuronides must contain at least one component that is not a (1 → 4)-linked α-D-galactosyluronic acid. Since the CarboPac PA-1 column is efficient in separat-

ing epimeric mixtures of oligosaccharides[11,12], these oligouronides may contain one or more epimerized galactosyluronic acid residues. The alkaline conditions used during the commercial de-esterification of citrus pectin may have catalyzed epimerization (C-2 epimer, talosyluronic acid; C-3 epimer, gulosyluronic acid; C-4 epimer, glucosyluronic acid; C-5 epimer, L-altrosyluronic acid). Another possibility is that the modified oligogalacturonides contain a differently linked galactosyluronic acid residue. The structural characterization of these modified oligogalacturonides was not further pursued.

Selective, size-specific precipitation of oligogalacturonides with NaOAc and ethanol.—The presence of significant quantities of modified oligogalacturonides in the oligogalacturonide-containing fractions isolated by low-pressure anion-exchange chromatography necessitated the development of a procedure for their removal on a large scale. Polysaccharides in aqueous solution can be precipitated by the addition of salt and ethanol[13]. Therefore, the ability of ethanol and NaOAc to precipitate oligogalacturonides based on size and structural differences was investigated.

Solutions of EPG-digested PGA, pH 5 (Fig. 1A), were treated with different concentrations of NaOAc and ethanol. The resulting precipitates and supernatants were analyzed by HPAEC–PAD, using a CarboPac column that had been calibrated with standard oligogalacturonides, to determine the relative abundance of each oligogalacturonide. The addition of 11% ethanol and 50 mM NaOAc yielded a precipitate enriched in the bioactive oligogalacturonides. Under these conditions the precipitate was enriched in oligogalacturonides of dp 8 to 25 (Fig. 1B). The supernatant which was enriched in galacturonic acid and oligogalacturonides of dp 2 to 7 also contained small amounts of oligogalacturonides with dp 8 to 10 (Fig. 1C) as well as additional components that did not cochromatograph with the standard oligogalacturonides (compare Figs. 1B and 1C); these components were not characterized but are believed to be modified oligogalacturonides. Thus, the fractional precipitation of the EPG-digested products with ethanol and NaOAc is a rapid and efficient large-scale method for obtaining material enriched in bioactive oligogalacturonides.

Separation of the ethanol-precipitated oligogalacturonides on Q-Sepharose.—A portion of the NaOAc–ethanol-precipitated oligogalacturonides (450 mg galacturonic acid equivalents) in 300 mM ammonium formate, pH 6.5 (50 mL), was fractionated by Q-Sepharose chromatography (Fig. 3). This anion-exchange matrix, which has a high-loading capacity, does not fully resolve oligogalacturonides with a dp > 9. Thus, relatively large amounts (~ 15 mg of each oligomer) of size-enriched oligogalacturonides with dp 10 to 15 were obtained by a single chromatographic run on the Q-Sepharose. The compositions of the pooled peaks were determined by HPAEC–PAD using a CarboPac column calibrated with standard oligogalacturonides. Each pool analyzed contained a major component that accounted for ~ 80% of the material. In addition, two and sometimes three minor components were present.

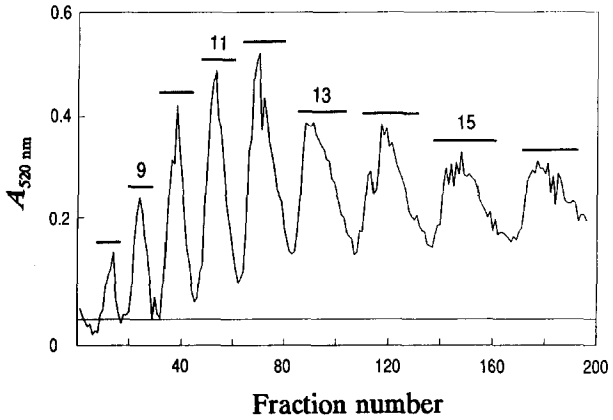

Fig. 3. Q-Sepharose fast-flow anion-exchange chromatography of 50 mM NaOAc–11% ethanol-precipitated, EPG-digested PGA. Fractions (10 mL) were collected, and portions (50 μL) analyzed colorimetrically for uronic acid[16]. Fractions were pooled as shown by the bars. The labels above the bars correspond to the dp of the predominant oligogalacturonide in that peak, as determined by HPAEC–PAD on a CarboPac PA-1 column calibrated with oligogalacturonides of known dp.

Purification of the Q-Sepharose-isolated oligogalacturonides by semipreparative HPAEC–PAD.—The semipreparative CarboPac PA-1 column is able to fully resolve mixtures containing homogalacturonides up to at least dp 16 and their corresponding modified oligogalacturonides. However, this column has a loading capacity of less than 6 mg for the oligogalacturonides in the pooled Q-Sepharose peaks. A portion of the Q-Sepharose tridecagalacturonide-enriched pool (5 mg) was fractionated by semipreparative HPAEC–PAD using a KOAc, pH 8, concentration gradient (Fig. 4). The cation used as the eluting buffer is important, as it greatly influences the solubility of the oligogalacturonides. We found that the solubility of oligogalacturonides in aqueous salt solutions decreases in the order $NH_4OAc > KOAc > NaOAc$. The recovery of oligogalacturonides was less than 50%, and the oligogalacturonides often precipitated in the sample collection tubes when the CarboPac column was eluted with NaOAc, pH 8.

Ammonium-containing eluants were found to interfere with the electrochemical detection of carbohydrates. Thus, ammonium acetate is not a suitable eluant for HPAEC–PAD.

Oligogalacturonides are also soluble in potassium oxalate, a salt compatible with PAD[14]. However, potassium oxalate is not easily removed from the oligogalacturonides, a factor that limits its use in isolating oligogalacturonides for biological studies.

KOAc was found to give satisfactory resolution of the oligogalacturonides. Furthermore, these components were recovered in yields greater than 80% from the CarboPac column. Therefore, a concentration gradient of KOAc, pH 8, was used for semipreparative HPAEC–PAD purification of oligogalacturonides. It should be noted that the resolution of oligogalacturonides is improved when

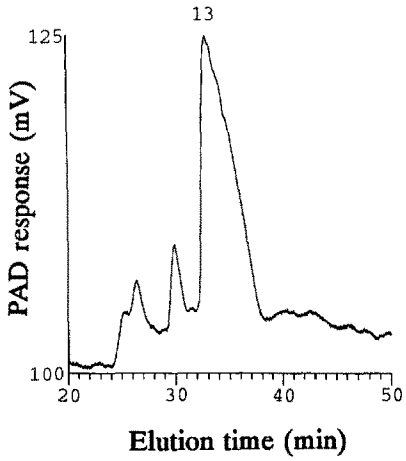

Fig. 4. Semipreparative HPAEC–PAD chromatography of a 5-mg portion of the tridecagalacturonide-containing peak prepared by Q-Sepharose chromatography (see Fig. 3). Components were resolved using a linear gradient from 550–675 mM KOAc (pH 8). The eluant was collected manually between 32–38 min. No peaks were detected before 20 min.

NaOAc rather than KOAc is used as the eluant. Thus, NaOAc is the preferred eluant for analytical HPAEC–PAD.

The oligogalacturonides eluting from the semipreparative CarboPac column were detected by PAD and collected manually. Since no postcolumn alkali was added, the pH of the eluant (pH 8) was below that required for an optimal PAD response but nevertheless allowed for the detection of the oligogalacturonides. The oligogalacturonide fractions purified by semipreparative HPAEC–PAD were desalted by dialysis [2000 molecular weight cut off (MW_{co})], concentrated to 2 mL, and stored frozen.

Characterization of the tridecagalacturonide purified by semipreparative HPAEC–PAD.—A portion of each semipreparative HPAEC–PAD-purified oligogalacturonide fraction was analyzed by HPAEC–PAD. In each case the fraction eluted as a single symmetrical peak with no other detectable components (for example, see Fig. 5). The tridecagalacturonide, which is in the middle of the size range of the bioactive oligogalacturonides, was selected for structural and chemical characterization.

The ^1H NMR spectrum of the tridecagalacturonide fraction contained signals (broad singlet) for anomeric protons at δ 5.08 that were assigned to (1 → 4)-linked α-D-galactosyluronic acid residues[7,8]. Signals for anomeric protons at δ 5.30 and 4.60 were assigned to H-1α and H-1β, respectively, of the reducing galactosyluronic acid residue[7,8]. Broad signals at δ 3.74, 4.00, 4.42, and 4.74 were assigned to H-2, H-3, H-4, and H-5, respectively, of 4-linked galactosyluronic acid residues[7,8].

Negative-ion FABMS analysis of the tridecagalacturonide fraction gave an ion at m/z 2305 corresponding to $[M - H]^-$ of an oligogalacturonide containing 12 hexosyluronic acid residues and a single hexuronic acid. No other ions were detected (data not shown).

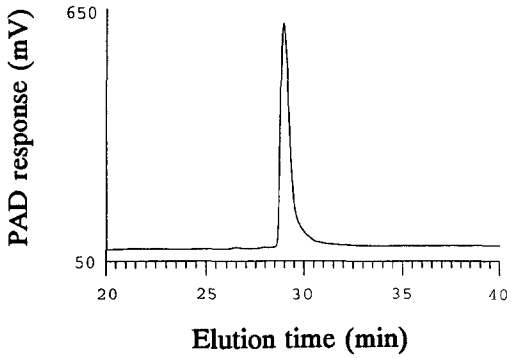

Fig. 5. Analytical HPAEC–PAD chromatography of the tridecagalacturonide purified by semipreparative HPAEC–PAD (see Fig. 4). No peaks were detected before 20 min.

The glycosyl-residue compositions of the tridecagalacturonide fraction, the NaOAc–ethanol-precipitated EPG digest of PGA, and PGA were determined by GLC and GLC–MS (EI and CI modes) analyses of their constituent trimethylsilyl–methyl ester–methyl glycoside derivatives (Table I). Galacturonic acid was the only sugar detected in the purified tridecagalacturonide. In contrast, the NaOAc–ethanol-precipitated material contained galacturonic acid and galactaric acid in the ratio of 1:0.003. PGA contained galacturonic acid, rhamnose, galactaric acid, arabinose, and xylose in the ratios of 1:0.03:0.02:0.005:0.006.

The combined evidence of spectroscopic data and chemical analyses indicates that the semipreparative HPAEC–PAD purified fraction contains only a homogeneous tridecagalacturonide.

Biological activity of the apparently homogeneous oligogalacturonides.—The HPAEC–PAD-purified oligogalacturonides (dp between 10 and 15) each elicited phytoalexin synthesis in soybean cotyledons[1,2]. The dodeca-, trideca-, and tetradeca-galacturonides were the most active of the homogeneous oligogalacturonides (manuscript in preparation). This result is consistent with previous studies[1,2] which established that a partially purified dodecagalacturonide was the most active elicitor of phytoalexin production in soybeans.

TABLE I

Glycosyl-residue compositions of PGA, the NaOAc–ethanol precipitated EPG-digested PGA, and the HPAEC–PAD purified tridecagalacturonide

Fraction	Glycosyl-composition (Mol%)				
	GalA	Ara	Galactaric acid	Rha	Xyl
PGA	94	0.5	1.7	3.2	0.6
NaOAC–ethanol precipitate	99.7	0	0.3	0	0
Purified tridecagalacturonide	100	0	0	0	0

The semipreparative HPAEC–PAD purified dodeca- and trideca-galacturonides were both shown to promote flower formation and inhibit root formation (manuscript in preparation) in a tobacco thin-cell-layer morphogenesis assay[15]. Both oligogalacturonide fractions demonstrated approximately equal activities, which is consistent with results obtained using partially purified dodeca- and trideca-galacturonides[15].

GENERAL DISCUSSION

We have herein described a method for the preparation of milligram quantities of homogeneous bioactive oligogalacturonides. Approximately 14 mg of each homogeneous oligogalacturonide (dp between 10 and 15) can be prepared in three weeks from 2 g of PGA. The amount of PGA digested with EPG can be increased to 20 g, and large quantities ($\sim$ 6 g) of material enriched in bioactive oligogalacturonides can be prepared by selective NaOAc–ethanol precipitation. In our experience, it is possible to scale up the Q-Sepharose chromatography step to fractionate 900 mg of NaOAc–ethanol precipitate on a 400-mL bed volume column without affecting the resolution. The low loading capacity ($\sim$ 6 mg) of the semipreparative CarboPac PA-1 column limits the amounts of homogeneous oligogalacturonides that can be prepared, although each chromatographic run is complete in 1 h.

The main emphasis of this study was the development of a method for the purification of homogeneous bioactive oligogalacturonides. However, smaller fragments (dp < 10) are fully resolved using Q-Sepharose chromatography. Thus, relatively pure ($\sim$ 95% purity) oligogalacturonides (dp < 9) can be obtained in large quantities (50–100 mg) without using the semi-preparative CarboPac column.

The purification of larger oligogalacturonides (dp > 16) presents some major difficulties, since the solubility of oligogalacturonides decreases as their dp increases. The larger oligogalacturonides tend to precipitate on, and irreversibly bind to, anion-exchange media in the presence of high concentrations of salt. We have found that the top 5 cm of the Q-Sepharose gel must be replaced after four or five runs to maintain the efficiency of the column. Further studies would be required to optimize the chromatographic conditions for the purification of oligogalacturonides with a dp > 16.

MATERIAL AND METHODS

Polygalacturonic acid (Na^+ salt) was purchased from Sigma (St. Louis, MO) and used without further purification. SpectraPor-7 1000 and 2000 MW_{co} tubing was purchased from Fisher Scientific. Dialysis was performed at 4°C. All buffers were prepared using ultra-pure water (Continental Ultrafiltration Water System, San Antonio, TX). All other materials were obtained from Sigma unless otherwise stated.

Treatment of polygalacturonic acid with a purified fungal α-(1 → 4)-endopolygalacturonase (EPG).—A solution of polygalacturonic acid (2 g) in 20 mM NaOAc, pH 5.0 (100 mL), containing bovine serum albumin (1 mg) as a carrier protein, was treated for 8 h at 23°C with α-(1 → 4)-endopolygalacturonase (15 units; 1 unit releases 1 mM reducing sugar per minute at 24°C). The EPG had been purified to homogeneity from the culture medium of *Fusarium moniliforme*[6] (a gift of C. Bergmann of this laboratory). The enzymic reaction was terminated by autoclaving the solution for 15 min at 121°C. An aliquot (50 μL) of the enzymic digest was analyzed by HPAEC–PAD to determine the size distribution of oligogalacturonides.

Selective precipitation of oligogalacturonides with a dp > 6 by treatment of EPG-digested PGA *with EtOH and NaOAc.*—The partial EPG digest of PGA was adjusted to contain 0.5% galacturonic acid residues (w/v), 50 mM NaOAc, and 11% EtOH (v/v) by the sequential addition of water, solid NaOAc, and abs EtOH while stirring. The mixture was kept for 16 h at 4°C. The precipitate that formed was collected by centrifugation (30 000*g* for 30 min at 4°C). The supernatant was decanted, dialyzed (1000 MW_{co}), concentrated to dryness, and the residue stored at −20°C. The pellet was washed with cold aq 50% EtOH (2 × 20 mL) and stored at −20°C. Aliquots of the EtOH-soluble and EtOH-precipitated material (100 μg each) in water (100 μL) were analyzed by HPAEC–PAD to determine the size distribution of the oligogalacturonides.

Q-Sepharose chromatography of the oligogalacturonides precipitated by EtOH and NaOAc.—A solution of the NaOAc–EtOH-precipitated oligogalacturonides (450 mg galacturonic acid equivalents) in 50 mM ammonium formate (50 mL) was adjusted to pH 6.5 with 10 mM ammonium hydroxide. Ammonium formate (1 M) was added until the conductivity of the solution was equal to that of 300 mM ammonium formate. The mixture was loaded onto a Q-Sepharose column (2.2 × 50 cm) that had been equilibrated with 300 mM ammonium formate, pH 6.5. The oligogalacturonides were eluted by a two-stage concentration gradient of ammonium formate, pH 6.5, at a flow rate of 5 mL/min. During the first stage of the gradient, the ammonium formate concentration was increased from 300 to 420 mM over 120 min, and the eluant was discarded. The ammonium formate concentration was then raised to 470 mM over the next 400 min, and fractions (10 mL) collected. Aliquots (50 μL) of every second fraction were assayed colorimetrically[16] for uronic acid. Fractions corresponding to uronic acid-containing peaks were pooled, dialyzed (2000 MW_{co}), and freeze dried. Aliquots (100 μg) of each of the pooled peaks were analyzed by HPAEC–PAD to determine the dp of the major component and the degree of purity of the sample.

Semipreparative HPAEC–PAD purification of the oligogalacturonides isolated by NaOAc–EtOH precipitation and Q-Sepharose chromatography.—Semipreparative HPAEC–PAD was performed with a Dionex metal-free BioLC interfaced to an AutoIon series 400 data station. Portions (5 mg) of the pooled peaks of Q-Sepharose-resolved oligogalacturonides between dp 10 and 15 in water (500 μL) were

separated on a semipreparative CarboPac PA-1 column (9 × 250 mm; Dionex, Sunnyvale, CA) and detected using a pulsed amperometric detector equipped with a gold working electrode (Dionex, Sunnyvale, CA). The electrochemical detector was operated in the pulsed amperometric mode (E1, 150 mV; E2, 700 mV; and E3, −300 mV; T1, 480 ms; T2, 120 ms; and T3, 360 ms) at 3 μA sensitivity. Eluants were filtered (0.2-μm Nylon 66 membranes; Rainin, Woburn, MA) and degassed with He using an eluant degas module (Dionex, Sunnyvale, CA). The column was eluted at 5 mL/min with a linear concentration gradient (550–675 mM) of KOAc, pH 8. No postcolumn NaOH was added in order to minimize base-catalyzed modification of the sample. The oligogalacturonides were collected manually while monitoring the PAD output. The resulting fractions were desalted by dialysis (2000 MW_{co}), concentrated to 2 mL, and stored at −20°C.

Analytical HPAEC–PAD of oligogalacturonides.—Solutions of oligogalacturonides (100–500 μg) in water (250 μL) were separated on a CarboPac PA-1 column (4.6 × 250 mm) using a linear gradient from 400–800 mM NaOAc, pH 8, at 1 mL/min over 40 min. The column was re-equilibrated with 400 mM NaOAc for 15 min before loading the next sample. To facilitate the detection of carbohydrates and to minimize baseline drift, NaOH (400 mM) was added postcolumn at a flow rate of 0.5 mL/min using a pressurized reagent delivery system (Dionex, Sunnyvale, CA). The electrochemical detector was operated at 1000 nA sensitivity in the pulsed amperometric mode as described for semipreparative HPAEC–PAD. This procedure separates oligogalacturonides between dp 3 to 25.

Glycosyl-residue composition analysis.—PGA and the oligogalacturonide-containing samples (100 μg) were separately treated with M HCl in MeOH (250 μL, 16 h at 80°C). The resulting methyl ester—methyl glycosides were then trimethylsilylated and analyzed on an HP 5880A GC using a DB-1 30 m column with split injection[17]. The peaks were identified by comparison of their retention times to those of standard monosaccharide derivatives and by GLC–MS (EI and CI modes; ammonium as the reagent gas) using an HP 5985 GLC–MS system.

Fast-atom-bombardment mass spectrometry (FABMS).—FAB-mass spectra were recorded with a VG ZAB-SE mass spectrometer (VG Analytical, Altringham, UK) operating in the negative-ion mode with an accelerating voltage of 8 kV. The oligogalacturonides were converted to their ammonium salt forms using Chelex 100 ion-exchange resin (ammonium form; Bio-Rad, Richmond, CA). A portion (1 μL) of the ammonium-oligogalacturonate in water (10 mg/mL) was applied to a mixture of M HCl (0.5 μL) and thioglycerol (1 μL) on the probe tip of the mass spectrometer.

^1H NMR spectroscopy.—^1H NMR spectroscopy was performed with a Bruker AM 250 spectrometer. The oligogalacturonide samples were exchanged three times with ^2H_2O (Aldrich, 99.96%) prior to ^1H NMR spectroscopy. Chemical shifts are reported in δ-units (ppm) downfield from Me_4Si. HOD (δ 4.8) served as the internal reference.

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