

Analysis of pectate lyase-generated oligogalacturonic acids by high-performance anion-exchange chromatography with pulsed amperometric detection

ABSTRACT

Traditionally, UV absorption detection (A_{235}) of 4,5-unsaturated bonds has been used to evaluate the depolymerization of pectic polysaccharides by pectate lyase (PL). This approach ignores the generation of oligogalacturonic acids without a 4,5-unsaturated function that appear early in the time course of PL cleavage. We found that high-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC-PAD) could separate and detect oligogalacturonic acids both with and without 4,5-unsaturated functions at their nonreducing ends in PL digests of polygalacturonic acid (PGA) and tobacco cell walls. The "recombinant" PL used was free of endopolygalacturonase activity. Peaks were identified in PL digest chromatograms by retention time agreement with oligogalacturonic acid standards purified by preparative high-performance liquid chromatography and a PGA autoclave hydrolysate that contained oligogalacturonic acids up to degree of polymerization (dp) 40, which lacked the 4,5-unsaturated function. When comparing like dp oligogalacturonic acids, those without the 4,5-unsaturated function eluted earlier from the HPAEC CarboPac PA1 column than did their unsaturated counterparts. These techniques provide the first means for the dp assignment of oligogalacturonic acids generated by both hydrolytic and lytic cleavage of polygalacturonic acid and plant cell walls.

INTRODUCTION

Oligogalacturonic acids are 1,4-linked α -D-galactopyranuronosyl oligosaccharides that are released from plant pectin by enzymes secreted by pathogenic organisms. Two types of oligogalacturonic acids are produced depending on the type of pectin-degrading enzyme utilized. Endopolygalacturonase (EC 3.2.1.15)

cleaves the galacturonosyl glycosidic bonds by a hydrolytic mechanism, whereas pectate lyase (EC 4.2.2.2; PL) depolymerizes pectin by a transesterase reaction that produces oligogalacturonic acids containing a 4,5-unsaturated function at their nonreducing ends. PL is an endo-type enzyme that initially releases oligogalacturonic acids both with (unsaturated) and without ("normal") a 4,5-unsaturated function at their nonreducing ends. The ratio of "normal" to unsaturated oligogalacturonic acids should decrease during the time course of PL depolymerization. The amount of "normal" oligogalacturonic acids generated from pectin by PL will increase with decreasing substrate homogalacturonan dp or degree of branching.

The physiological role of oligogalacturonic acids in plants (reviewed in ref 2) includes elicitation of phytoalexins, proteinase inhibition, lignification, phenylalanine ammonia lyase activation³, and regulation of morphogenesis in tobacco thin-cell layer explant cultures⁴. Oligogalacturonic acids released by PL induce additional synthesis of PL in the soft-rot bacterium *Erwinia*, but when the unsaturated digalacturonic acid reaches a threshold concentration, it represses further PL synthesis^{5,6}. PL-generated oligogalacturonic acids have also been shown to inhibit the hypersensitive response to pathogens in tobacco⁷.

Oligogalacturonic acids have been chromatographically separated and detected by several different methods (reviewed in refs 8–10). High-performance anion-exchange chromatography and pulsed amperometric detection (HPAEC–PAD) utilizing the CarboPac PA1 column has been used to separate and detect oligogalacturonic acids up to dp 50 (ref 9). The high selectivity of the CarboPac PA1 column has been demonstrated for separations of various classes of monosaccharides and oligosaccharide positional isomers^{11,12}. Anion-exchange¹³ and reversed-phase ion-pairing^{14–16} high-performance liquid chromatography (HPLC) has been used to examine the kinetics of cell wall and polygalacturonic acid (PGA) depolymerization by PL. However, these reports used UV absorption at 235 or 245 nm (optimal for the detection of the 4,5-unsaturated function) to detect oligogalacturonic acids released by PL. We previously reported¹⁷ that HPAEC–PAD can separate and detect oligogalacturonic acids both with and without the 4,5-unsaturated function at their nonreducing ends in PL digests of cell walls and PGA, and we now present the supporting evidence.

MATERIALS AND METHODS

Chromatography.—The chromatographic system included a Dionex (Sunnyvale, CA) Bio-LC apparatus consisting of a quaternary gradient pump, eluant degas (He) module, post-column delivery system (DQP-1 pump), and pulsed amperometric detector II (with gold working electrode). Chromatograms were recorded with a Hewlett–Packard (Palo Alto, CA) 3390A integrator. Oligogalacturonic acids were separated on a CarboPac PA1 (4 × 250 mm) pellicular anion-exchange column (Dionex) by nonlinear gradient (25–500 mM in 120 min) elution with an oxalate buffered (K⁺, pH 6) mobile phase as reported earlier⁹.

Preparation of oligogalacturonic acids.—PL digests of PGA (Sigma) and a tobacco leaf cell wall preparation were generously provided by C. Jacyn Baker (USDA–BARC)⁷. PL digest conditions included incubating 0.1% PGA in 1 L of 5 mM Tris, pH 8, containing 0.1 mM CaCl₂ and 0.0073 U · mL⁻¹ PL, for 10 min at 27°C. Cell walls (500 mg) were suspended in 15 mL of 0.5 mM Tris, pH 7.0, containing 0.1 mM CaCl₂ and 0.2 U · mL⁻¹ PL, for 24 h at 27°C. The PL used was isolated from *Escherichia coli* strain CSR2 (containing a cloned pectate lyase gene, *pelC*, from *Erwinia chrysanthemi*) osmotic shock fluids as previously described¹⁸.

Retention times of PL-generated oligogalacturonic acids were compared with those observed in chromatograms of untreated PGA, an autoclave hydrolysate of PGA, and highly purified oligogalacturonic acids isolated by preparative HPLC. Autoclave hydrolysis¹⁹ involved autoclaving 1% PGA in deionized water (adjusted to pH 4.4 with KOH) at 121°C for 40 min. Oligogalacturonic acid standards (“normal” and unsaturated) up to dp 7 were purified by preparative anion-exchange (aminopropyl silica gel) HPLC¹⁰ and AG MP-1 anion-exchange column chromatography with molecular weights confirmed by FABMS²⁰.

RESULTS AND DISCUSSION

Briefly (10 min) treating PGA with PL released a series of oligogalacturonic acids that were separated and detected by HPAEC–PAD (Fig. 1a). When the retention times were compared with those obtained from untreated PGA (Fig. 1b), an autoclave hydrolysate of PGA (Fig. 1c), and oligogalacturonic acid standards (dp 6 “normal” shown in Fig. 1d), the elution order of “normal” and unsaturated oligogalacturonic acids was determined. Oligogalacturonic acids lacking a 4,5-unsaturated function at the nonreducing end represented the major peaks in the PGA autoclave hydrolysate (Fig. 1c), and minor amounts of these oligosaccharides were also present in untreated PGA (Fig. 1b). However, in the PL-treated PGA chromatogram, “normal” oligogalacturonic acid peaks had less peak area than another series of peaks that represented unsaturated oligosaccharides based on retention time agreement with unsaturated oligogalacturonic acid standards. PL released oligogalacturonic acids from PGA up to dp 40–45 based on “normal” oligogalacturonic acid retention times. The “normal” oligogalacturonic acid peak area increased by an average of 3.15 (± 1.36 SD) times following the 10-min PL treatment (compare Figs. 1a and 1b). The dp 10 “normal” oligogalacturonic acid had the largest (5.45 \times), and the dp 3 oligogalacturonic acid had the smallest (1.35 \times), PL-generated increases in peak area.

Comparing oligogalacturonic acids of like size, unsaturated oligogalacturonic acids eluted later than “normal” oligogalacturonic acids (Fig. 2). Similar observations were also made by Lieker et al.²¹ using a high-pH mobile phase. Two mechanisms that have been proposed to explain carbohydrate retention on the CarboPac PA1 column operated at high pH may also be involved in the elution of “normal” and unsaturated oligogalacturonic acids at slightly acidic pH. An inverse

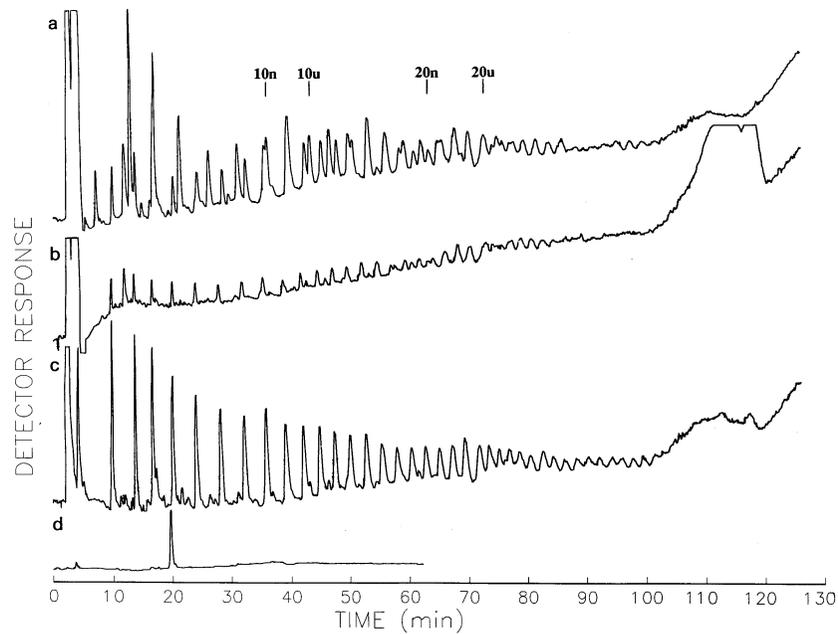


Fig. 1. HPAEC-PAD separation of oligogalacturonic acids. (a) A 10-min PL digest of PGA. (b) The untreated PGA. (c) An autoclave hydrolysate of PGA. (d) Dp 6 “normal” oligogalacturonic acid standard. Conditions included a CarboPac PA1 column, nonlinear 25–500 mM pH 6 potassium oxalate gradient mobile phase (0.8 mL/min) and post-column addition of 500 mM potassium hydroxide. Oligogalacturonic acid dp values are listed over the peaks (n = “normal”, u = unsaturated).

relationship has been observed between monosaccharide retention and their pK_a values¹². The pK_a values of unsaturated oligogalacturonic acids were proposed to be lower than “normal” oligogalacturonic acid pK_a values due to conjugation

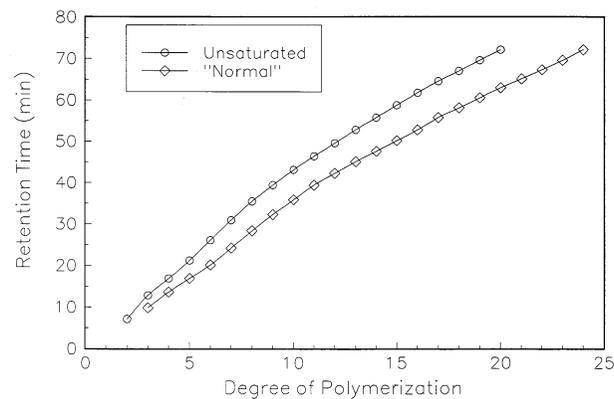


Fig. 2. The elution order of “normal” and unsaturated oligogalacturonic acids separated by HPAEC-PAD.

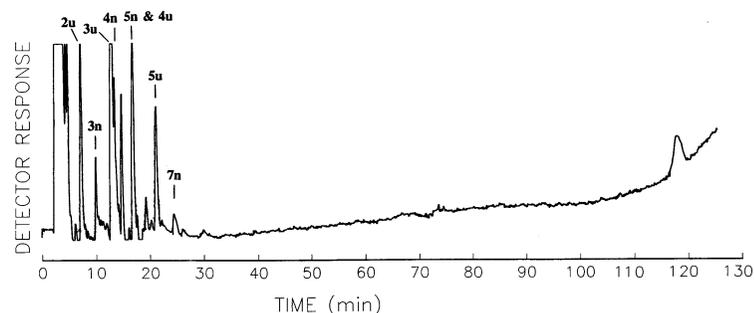


Fig. 3. HPAEC-PAD separation of oligosaccharides released from tobacco leaf cell walls following a 24-h digestion with PL. Conditions as in Fig. 1.

between the 4,5-double bond and the carboxyl group²⁰. The interaction between the oligosaccharide three-dimensional conformation and the stationary phase anion-exchange sites also has been proposed to be significant in their retention on the CarboPac PA1 column^{22,23}.

An extensive treatment (24 h) of tobacco cell walls with PL released a relatively small number of oligosaccharides, the majority of which had retention times that corresponded to either “normal” or unsaturated oligogalacturonic acids (Fig. 3). The highest molecular weight oligosaccharides observed in the cell wall digest corresponded to dp 7 “normal” and dp 5 unsaturated oligogalacturonic acids. The identity of some peaks (14.61 and 19.17 min) in this chromatogram could not be accounted for with oligogalacturonic acid retention times. The presence of rhamnose residues or other pectin-constituent neutral sugars in these oligosaccharides may explain the different retention times. The exact identity of all the cell wall oligosaccharides will await technical developments which allow for the collection of enough of these oligosaccharides for further compositional analysis.

The HPAEC-PAD method is most appropriate for the analysis of PL depolymerization of substrate patterns. A significant amount of the PL depolymerization end products would be missed if detection was based solely on UV absorption at 235 nm. For example, recombinant PLc, derived from osmotic shock fluids of *Escherichia coli* that contained a cloned *Erwinia chrysanthemi* EC16 *pelC* gene, has been reported to produce primarily dp 3 (with lesser amounts of dp 2 and 4) unsaturated oligogalacturonic acids from PGA substrate in limit digests using UV absorption detection¹⁶. Dp 3 unsaturated oligogalacturonic acid is also the major peak in our 24 h cell wall digest (Fig. 3), which is approaching the limit of depolymerization by PLc. However, significant amounts of dp 3–5 “normal” and dp 5 unsaturated oligogalacturonic acids were also detected in this chromatogram. The oligosaccharides present in the 24 h digest may be too small to be further depolymerized by PLc. If the dp value of limit products is to be used to infer the minimum substrate domain size that an enzyme can recognize¹⁶, then it seems appropriate to use a method that will provide the most complete limit product

oligosaccharide profile possible. We feel that the above example illustrates that the HPAEC-PAD method provides the most complete limit product profile possible.

Several soft-rot disease producing bacteria produce both PL and endopolygalacturonase²⁴. In order to determine which oligogalacturonic acids were produced by PL alone, it was essential to use a source of PL free of endopolygalacturonase. Otherwise we could not rule out the possibility that oligogalacturonic acids lacking a 4,5-unsaturated function at the nonreducing end in the PL digests were produced by minor amounts of endopolygalacturonase activity. By comparing the pattern of oligogalacturonic acids released by various pectinases with the HPAEC-PAD method, it will then be possible to determine the relative contributions of each enzyme during mixed enzyme depolymerization of pectin.

Both the PGA and cell wall digests were observed to inhibit the hypersensitive response to *Pseudomonas syringae* in tobacco leaf panels and to elevate the extracellular pH in tobacco suspension cultures, with the PGA digest having highest specific activity⁷. It appears that the oligogalacturonic acid with highest activity in these physiological responses is larger than dp 7. We are currently isolating pure oligogalacturonic acids larger than dp 7 to try to determine which dp value has the highest activity.

ACKNOWLEDGMENTS

We thank Elizabeth Orlandi, Norton Mock, and C. Jacyn Baker for providing the PGA and cell wall PL digests and Rebecca Haines for technical assistance.

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