Plant Physiol. (1991) 96, 1167–1170
0032-0889/91/96/1167/04/$01.00/0

Received for publication February 6, 1991
Accepted April 13, 1991

Oxidized Oligogalacturonides Activate the Oxidation of Indoleacetic Acid by Peroxidase

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ABSTRACT

Partial hydrolysis of polygalacturonic acid with a purified α-1,4-endopolygalacturonase yielded oligogalacturonides and trace amounts of a series of modified oligogalacturonides. Three of the minor products were isolated and identified as oxidized oligogalacturonides possessing termini of galactaric acid. Oxidation of indole-3-acetic acid by peroxidases was activated by oxidized oligogalacturonides but not by normal analogs.

Oligosaccharides composed of 1,4-linked α-D-galacturonic acid residues (oligogalacturonides) have been included in a possible new class of regulatory molecules consisting of plant cell wall fragments that have been named oligosaccharins (1). They act as elicitors for the accumulation of proteinase inhibitors in tomato leaves (3) and of phytoalexins in several plants (6, 11). Evidence has been presented that oligogalacturonides of a specific Dp[1] regulate organ development in tobacco explants (14) and induce ethylene production in fruit tissues (5). There has not been an explanation of how these pectic fragments can induce such a variety of responses in plants. A clue to their mode of action may lie in a recent observation that certain anions are extraordinary activators of the oxidation of IAA by plant peroxidases (13). Mixtures of oligogalacturonides prepared by the controlled enzymic fragmentation of polygalacturonic acid also activate the oxidation of IAA, and this study deals with identification of the active components.

MATERIALS AND METHODS

Purification of Polygalacturonases

Endo- and exopolygalacturonases in Pectinase from *Aspergillus niger* (Sigma) were separated by chromatography on a column of Q-Sepharose (Pharmacia) and purified by fast protein liquid chromatography on a column of Mono Q (Pharmacia). Details of the purification and characterization of the enzymes will be published separately. A unit of exo- or endopolygalacturonase is defined as that amount liberating 1 μmol of reducing groups from polygalacturonic acid/min at 30°C.

[1] Abbreviations: Dp, degree of polymerization; HPAE-PAD, high performance anion-exchange chromotography with pulsed amperometric detection; KnA, 1000 nanoamp.

Preparation of Polygalacturonic Acid Hydrolyzate

Ten grams of polygalacturonic acid (P3889, Sigma) was dissolved in 1 L water, adjusted to pH 4.5, and warmed to 30°C. Endopolygalacturonase (280 units) was added to the solution and incubated at 30°C for 1 h. The enzyme was then inactivated by heating the solution at 100°C for 5 min and the small amount of precipitate was removed by centrifugation. The oligogalacturonides in the solution were precipitated by addition of 5 g $CaCl_2$ and a volume of 95% ethanol at pH 6.0. The precipitate was collected by centrifugation, washed with 50% ethanol, and dried under vacuum.

Analysis of Oligogalacturonides by High Performance Anion-Exchange Chromatography

Oligogalacturonides were analyzed by HPAE-PAD on a CarboPac PAI column in a Dionex system. Elution was conducted with 0.19 M sodium acetate in 65 mM NaOH for 5 min and then with a linear gradient of 0.19 to 0.96 M sodium acetate in 65 mM NaOH in 15 min at a flow rate of 1 mL/min. The pulsed amperometric detector was operated at a sensitivity of 1 KnA. Standard oligogalacturonides of Dp 2 to 8 were prepared and purified as described previously (12).

IAA Oxidase Assay

The reaction mixture consisted of 0.2 mL of 0.1 M sodium acetate, pH 4.0, 0.3 mL of 1 mM Mn^{2+}, 0.2 mL of 1 mM dichlorophenol, 0.3 mL of 1 mM IAA, and 0.1 mL of appropriately diluted tomato peroxidase (13). After 15 min at 30°C, 2 mL of Salkowski reagent (8) was added. The absorbance was read at 525 nm after 30 min storage in the dark, and the values were converted to μmol of residual IAA using a standard curve for IAA.

RESULTS

Composition of the Polygalacturonic Acid Hydrolyzate

Analysis of the polygalacturonic acid hydrolyzate using the HPAE-PAD system calibrated with standard oligogalacturonides revealed that it consisted primarily of galacturonic acid (I) and di- through pentagalacturonic acids (II–V) (Fig. 1). It contained much lower amounts of hexa-, hepta-, and octagalacturonic acids (VI, VII, and VIII). The polygalacturonic acid hydrolyzate also contained several minor components that did not correspond to normal oligogalacturonides. These have been designated as unknowns 1 through 5 (Fig. 1).

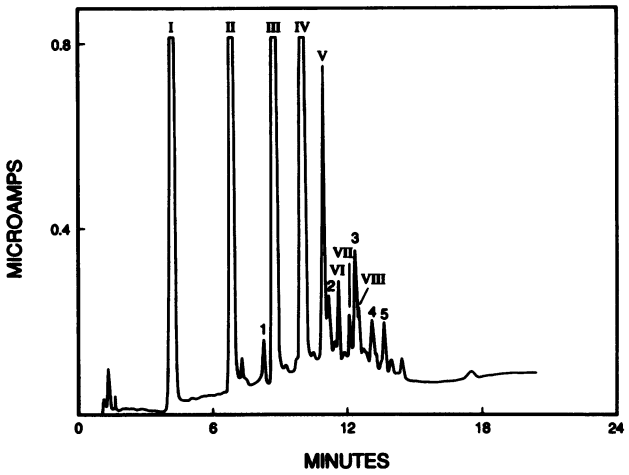


Figure 1. Anion-exchange chromatography of the polygalacturonic acid hydrolyzate. Eluant A was 65 mM NaOH and eluant B was 0.96 M sodium acetate in 65 mM NaOH. A CarboPac PAI column was equilibrated with 20% B. The polygalacturonic acid hydrolyzate (25 μL) was applied to the column and eluted for 5 min with 20% B, followed by a linear gradient of 20 to 100% B in 15 min. The flow rate was 1 mL/min. Oligogalacturonides were monitored with a pulsed amperometric detector at a sensitivity of 1 KnA. The column was calibrated with standard galacturonic acid and oligogalacturonides of Dp 2 to 8.

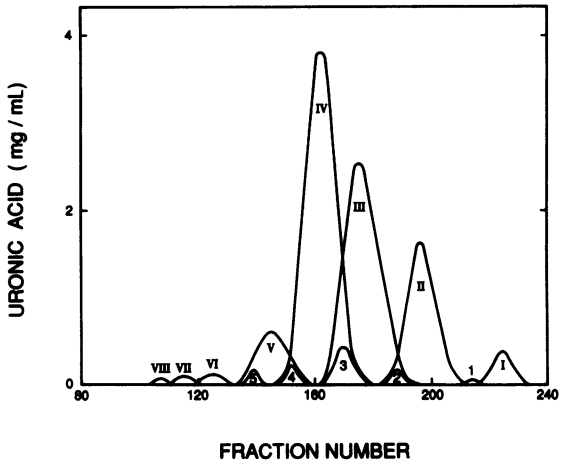


Figure 2. Gel filtration of polygalacturonic acid hydrolyzate. A 10 × 88 cm column of Bio-Gel P-4 was equilibrated with 0.15 M NaCl. A solution (50 mL) containing 5 g of polygalacturonic acid hydrolyzate, pH 6.0, was applied to the column and elution was conducted with 0.15 M NaCl. The flow rate was 520 mL/h and the fraction size was 25 mL. The fractions were analyzed for oligogalacturonides by HPAE-PAD.

Fractionation of the polygalacturonic acid hydrolyzate by gel filtration on a 2.4 × 90 cm column of Bio-Gel P-4 in 0.15 M NaCl showed that the strongest activator of IAA oxidation eluted between tri- and tetragalacturonic acids. There were several other peaks of activators but none of these corresponded to normal oligogalacturonides. When the same hydrolyzate of polygalacturonic acid was fractionated by ion-exchange chromatography on a column of Q-Sepharose calibrated with oligogalacturonide standards, the activators of IAA oxidation eluted in peaks corresponding to heptagalacturonic acid and the higher analogs. The discrepancy in apparent size of the activators as determined by gel filtration and ion-exchange chromatography confirmed that the normal oligogalacturonides were not responsible for the activation of IAA oxidation. It appeared that the minor components 1 through 5 (Fig. 1) were the actual activators.

Purification of Unknown 3

A suspension of 5 g of polygalacturonic acid hydrolyzate (Ca^{2+} salts) in 50 mL of water was dissolved by deionizing with Dowex-50 (H^+). The resin was removed by filtration, the solution was adjusted to pH 6.0 with NaOH, and applied to a 10 × 88 cm column of Bio-Gel P-4 (100–200 mesh) equilibrated with 0.15 M NaCl. Elution was conducted with 0.15 M NaCl. The eluate was analyzed for individual oligogalacturonides and unknowns using HPAE-PAD (Fig. 2). It should be noted that the unknowns eluted between the peaks of oligogalacturonides. The fractions (164–177) corresponding to the peak for unknown 3 were pooled and treated with 0.5 g $CaCl_2$ and one volume of 95% ethanol. This yielded 1.2 g of a white powder.

The sample of unknown 3 containing considerable amounts of tri- and tetragalacturonic acids was dissolved in 25 mL of water by deionizing with Dowex-50 (H^+). The solution was adjusted to pH 6.0 and applied to a 2.5 × 36 cm column of Q-Sepharose equilibrated with 0.02 M sodium acetate, pH 6.0. Elution was conducted with 1 L of a linear gradient of 0 to 0.4 M NaCl in 0.02 M sodium acetate, pH 6.0. The eluate was analyzed for oligogalacturonides and unknowns using HPAE-PAD (Fig. 3). The fractions (62–66) containing unknown 3 were pooled and 52 mg of the Ca^{2+} salt were isolated. The unknown was further purified by dissolving in 10 mL of water

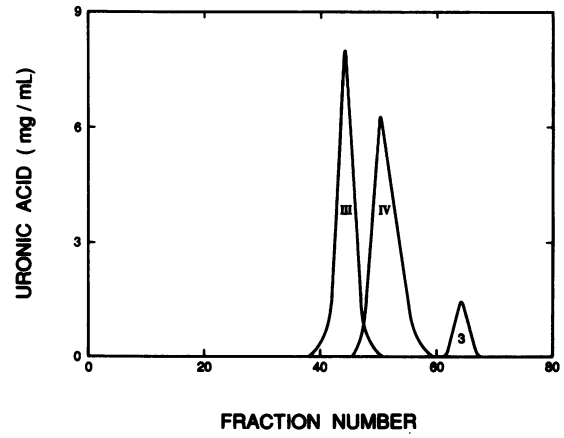
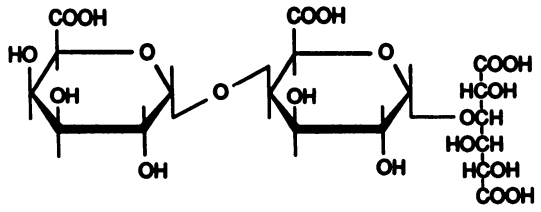


Figure 3. Purification of unknown 3 by ion-exchange chromatography. A 2.5 × 36 cm column of Q-Sepharose was equilibrated with 0.02 M sodium acetate, pH 6.0. Unknown 3 in 25 mL of water, pH 6.0, was applied to the column and elution was conducted with 1 L of a linear gradient of 0 to 0.4 M NaCl in 0.02 M sodium acetate, pH 6.0. The flow rate was 56 mL/h. The fractions were analyzed for oligogalacturonides by HPAE-PAD.



D - Gal A $\xrightarrow{\alpha}$ 4 - D - Gal A $\xrightarrow{\alpha}$ 4 - Galactaric Acid

Figure 4. Structure of unknown 3.

and chromatographing on a 2.5 × 95 cm column of Bio-Gel P-4. This step removed traces of unknown 4 and pentagalacturonic acid.

Characterization of Unknown 3

The absence of neutral sugars in unknown 3 was established by hydrolysis, reduction, acetylation, and analysis by GC (2). The unknown did not contain reducing groups, based on a negative reaction with Somogyi copper reagent (10) at a concentration of 100 μg/mL. A positive reaction with hydroxydiphenyl reagent (4) indicated the presence of uronic acid, but the absorbance was 0.64 of that for an equal weight of galacturonic acid. Elution of the unknown before trigalacturonic acid on Bio-Gel P-4 indicated that it was larger than the normal trimer.

Unknown 3 was hydrolyzed very slowly by tomato and *Aspergillus* endopolygalacturonases. However, it was readily degraded by *Aspergillus* exopolygalacturonase. The initial products were galacturonic acid and unknown 2 (see Fig. 1). Continued hydrolysis with the exoenzyme resulted in the disappearance of unknown 3. Unknown 2 then decreased with the formation of more galacturonic acid and a new product that corresponded to unknown 1. The latter was isolated by hydrolyzing 20 mg of unknown 3 with exopolygalacturonase for 16 h followed by chromatography on Q-Sepharose. Unknown 1 yielded negative reactions with reagents for reducing groups and uronic acids but was detected by HPAE-PAD. It was determined to be galactaric acid by coelution with a standard of galactaric acid on Carbo-Pac and Bio-Gel P-4 columns. A solution of the product was evaporated, yielding rhombic crystals as described for galactaric acid (9).

The identities of the unknowns as oxidized oligogalacturonides were confirmed by comparison with enzymatically oxidized analogs. An enzyme has been isolated from squash fruit that oxidizes galacturonic acid and oligogalacturonides in the presence of O_2 (my unpublished results). Galacturonic acid and di-, tri-, tetra-, and pentagalacturonic acids oxidized with the oxidase matched unknowns 1, 2, 3, 4, and 5, respectively, when chromatographed on the CarboPac column. Thus, the evidence is consistent with a structure for unknown 3 as the trisaccharide galacturonosyl-1,4-galacturonosyl-1,4-galactaric acid, or oxidized trigalacturonic acid (Fig. 4). Unknowns 4 and 5 have been isolated and identified as oxidized tetragalacturonic acid and oxidized pentagalacturonic acid, respectively.

Activation of IAA Oxidation

Galactaric acid activated the oxidation of IAA by tomato peroxidase in the same manner as numerous other polycarboxylic acids (13). A threshold concentration of about 0.3 mM galactaric acid was required, but higher concentrations of the acid increased the rate of oxidation sharply (Fig. 5). The reaction occurred optimally at pH 3.7. The oxidized oligogalacturonides were more effective than galactaric acid, with progressively lower threshold concentrations as the chain length of the oligomer increased. The pH optimum for IAA oxidation by peroxidase changed from pH 3.7 in the presence of galactaric acid to pH 4.25 in the presence of the oxidized pentamer. Normal oligogalacturonides were ineffective on the oxidation, even at much higher concentrations.

DISCUSSION

Commercial polygalacturonic acid contains some molecules with termini of galactaric acid instead of the usual galacturonic acid (7). Partial enzymatic hydrolysis of the polygalacturonic acid yields the expected series of oligogalacturonides as well as a series of oxidized analogs. Oxidized trigalacturonic acid accumulates during hydrolysis by *Aspergillus* endopolygalacturonase because this fragment is cleaved slowly by the enzyme, whereas larger oxidized fragments can be obtained by controlled enzymatic hydrolysis. The oxidized oligomers can be distinguished from the reducing oligogalacturonides by chromatography on a CarboPac PAI column and isolated by a combination of gel filtration and ion-exchange chromatography. Oxidized oligogalacturonides can be prepared from normal oligogalacturonides by oxidation with squash uronic acid oxidase, and this was helpful in the identification of the oxidized components in polygalacturonic

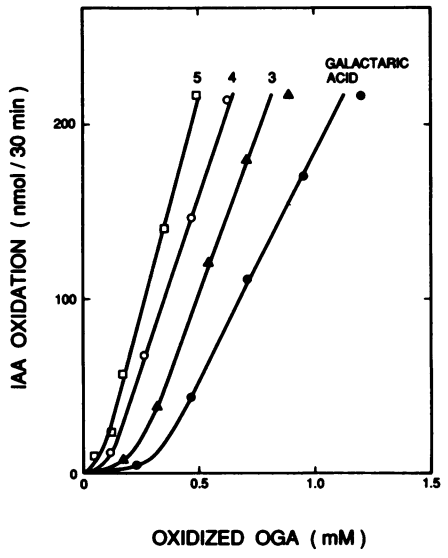


Figure 5. Effects of oxidized oligogalacturonides on the oxidation of IAA by tomato peroxidase.

acid hydrolyzates. The presence of the oxidase in squash fruit and other plant tissues (my unpublished results) indicates that plants have the potential to oxidize cell wall fragments released by endogenous or pathogenic pectic enzymes.

Oligogalacturonides prepared by hydrolysis of polygalacturonic acid have been evaluated as elicitors of various responses in plants (1, 3, 5, 6, 11, 14), and it has been assumed that the responses are due to normal oligogalacturonides. The present study shows that mixtures of oligogalacturonides contain oxidized analogs, and that individual oligogalacturonides purified by ion-exchange chromatography are contaminated by shorter oxidized analogs. It will be necessary to reexamine earlier studies to determine if normal or oxidized oligogalacturonides are responsible for the responses in plants. Davis *et al.* (7) found that active peaks separated from polygalacturonic acid partially degraded with a lyase were enriched for galactaric acid, although a purified heptagalacturonide with an unsaturated galacturonic acid residue at the nonreducing terminus and a galactaric acid at the reducing terminus had low elicitor activity. If oxidized oligomers are found to function as elicitors, their activation of IAA oxidation may provide an explanation for their action. Increased IAA destruction by introduction of oxidized oligogalacturonides into plant tissues could lead to the observed physiological changes.

LITERATURE CITED

1. **Albersheim P, Darvill AG** (1985) Oligosaccharins. Sci Am **253:** 58–64
2. **Albersheim P, Nivens DJ, English PD, Karr A** (1967) A method for the analysis of sugars in plant cell-wall polysaccharides by gas-liquid chromatography. Carbohyd Res **5:** 340–345
3. **Bishop PD, Pearce G, Bryant J, Ryan CA** (1984) Isolation and characterization of the proteinase inhibitor-inducing factor from tomato leaves. J Biol Chem **259:** 13172–13177
4. **Blumenkrantz N, Asboe-Hansen G** (1973) A new method for quantitative determination of uronic acids. Anal Biochem **54:** 484–489
5. **Brecht JK, Huber DJ** (1988) Products released from enzymatically active cell wall stimulate ethylene production and ripening in preclimacteric tomato (*Lycopersicon esculentum* Mill.) fruit. Plant Physiol **88:** 1037–1041
6. **Bruce RJ, West CA** (1982) Elicitation of casbene synthetase activity in castor bean. Plant Physiol **69:** 1181–1188
7. **Davis KR, Davill AG, Albersheim P, Dell A** (1986) Host-pathogen interactions. XXIX. Oligogalacturonides released from sodium polypectate by endopolygalacturonic acid lyase are elicitors of phytoalexins in soybean. Plant Physiol **80:** 568–577
8. **Hinman RL, Lang J** (1965) Peroxidase catalyzed oxidation of indole-3-acetic acid. Biochemistry **4:** 144–148
9. **McCready RM** (1963) α-D-Galacturonic acid monohydrate from polygalacturonic acid. *In* RL Whistler, ML Wolfrom, eds, Methods in Carbohydrate Chemistry, Vol II. Academic Press, New York, pp 27–29
10. **Nelson N** (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem **153:** 375–380
11. **Nothnagel EA, McNeil M, Albersheim P, Bell A** (1983) Host-pathogen interactions. XIII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol **71:** 916–926
12. **Pressey R, Avants JK** (1975) Modes of action of carrot and peach exopolygalacturonases. Phytochemistry **14:** 957–961
13. **Pressey R** (1990) Anions activate the oxidation of indoleacetic acid by peroxidases from tomato and other sources. Plant Physiol **93:** 798–804
14. **Tran Thanh Van K, Toubart P, Cousson A, Darvill AG, Gollin DJ, Chelf P, Albersheim P** (1985) Manipulation of the morphogenetic pathways of tobacco explants by oligosaccharins. Nature **314:** 615–617