Host-Pathogen Interactions
XXX. Characterization of Elicitors of Phytoalexin Accumulation in Soybean Released from Soybean Cell Walls by Endopolygalacturonic Acid Lyase*

Keith R. Davis**, Alan G. Darvill**, Peter Albersheim**

Department of Molecular, Cellular, and Developmental Biology and Department of Chemistry, University of Colorado, Campus Box 215, Boulder, CO 80309

and

Anne Dell

Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, England

Z. Naturforsch. **41c,** 39–48 (1986); received June 24, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Erwinia, Phytoalexins, Elicitors, Endopolygalacturonic Acid Lyase, Oligogalacturonides

Endopolygalacturonic acid lyase, purified from the phytopathogenic bacterium, *Erwinia carotovora,* induces phytoalexin accumulation in soybean (*Glycine max* L.) cotyledons. This pectin-degrading enzyme releases heat-stable elicitors of phytoalexin accumulation from soybean cell walls, citrus pectin, and citrus sodium polypectate. The most elicitor-active molecules obtained by treating soybean cell walls with endopolygalacturonic acid lyase have been purified and characterized. The cell-wall-derived elicitors are α-1,4-linked oligogalacturonides with degrees of polymerization of eight to twelve residues. The molecules with the highest specific elicitor activity were identified as α-1,4-linked deca- and undecagalacturonides that contained 4,5-unsaturated galactosyluronic acid at the nonreducing termini.

Introduction

Many plants, when confronted with potentially pathogenic microorganisms, synthesize and accumulate low-molecular-weight, antimicrobial compounds called phytoalexins [1, 2]. Phytoalexin accumulation can also be induced by treating plant tissues with molecules called elicitors [3, 4]. Several studies have demonstrated that oligosaccharides released from plant cell walls by either partial acid hydrolysis [5, 6] or treatment with pectin-degrading enzymes [7, 8] are elicitors of phytoalexin accumulation.

When soybean cotyledons are treated with oligosaccharides derived from plant cell walls [5, 6, 8], citrus pectin [6], and citrus sodium polypectate [8, 9], the cotyledons accumulate pterocarpan phytoalexins. The elicitors released from either soybean cell walls or citrus pectin by partial acid hydrolysis were oligogalacturonides that had degrees of polymerization between eight and thirteen residues; the dodecagalacturonide possessed the highest specific elicitor activity [6]. The elicitors released from citrus sodium polypectate by treatment with a purified bacterial PGA lyase were oligogalacturonides that contained the expected product of lyase degradation, 4-deoxy-β-L-5-threohexopyranos-4-enyluronic acid (4,5-unsaturated GalUA), at the nonreducing termini. Elicitor activity was associated with oligogalacturonides that had degrees of polymerization between eight and twelve residues; the decagalacturonide possessed the highest specific activity [9].

Previous experiments demonstrated that PGA lyase released heat-stable, uronic-acid-rich elicitors of phytoalexin accumulation in soybean cotyledons from isolated soybean cell walls [8]. Although it was likely that the elicitors solubilized from soybean cell walls by PGA lyase were α-1,4-linked oligogalacturonides, the molecules with the highest specific elicitor activity could have contained substituents other than α-1,4-linked GalUA residues that were essential for the elicitor activity of these molecules. For example, the elicitors could have contained a

* This research is supported by the United States Department of Energy (DE-AC02-76ERO-1426) and the paper is based in part on the Ph. D. thesis presented to the University of Colorado in 1985 by KRD.

** *Present address:* Complex Carbohydrate Research Center, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613, U.S.A.

Abbreviations: δ, chemical shift in ppm; FAB-MS, fast atom bombardment-mass spectrometry; GalUA, galactosyluronic acid; GLC, gas-liquid chromatography; Hz, hertz; *m/z,* mass-to-charge ratio; NMR, nuclear magnetic resonance; PGA lyase, α-1,4-D-endopolygalacturonic acid lyase (EC 4.2.2.2).

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0100–0039 $ 01.30/0

glycosyl residue other than GalUA, or modifications of the GalUA residues such as methyl esterification of the carboxyl groups or O-acetylation at any of the unsubstituted hydroxyl groups of the component glycosyl residues. It is possible that some of these types of modifications could have been removed during the partial acid hydrolysis procedures used in the previously published study [6] to obtain oligogalacturonide phytoalexin elicitors from soybean cell walls. Such modification might also have been absent in the commercial citrus sodium polypectate preparations used as the substrate to generate PGA lyase-released oligogalacturonide elicitors [9].

To characterize the elicitors of phytoalexin accumulation that are likely to be released from plant cell walls during microbial infection, purified PGA lyase from the phytopathogenic bacterium, *Erwinia carotovora*, was used to solubilize elicitors from soybean cell walls. This report describes the purification and characterization of the most active elicitors solubilized from soybean cell walls by PGA lyase.

Materials and Methods

Chemicals

Imidazole (Grade I), QAE Sephadex (A-25-120), Sephadex G-10, streptomycin sulfate, and Tris were from Sigma Chemical Co. Bio-Gel P-10 (200–400 mesh) was from Bio-Rad Laboratories. Tri-Sil silanizing reagent was from Pierce Chemical Co. Deuterium oxide (99.7–99.8 atom % ^2H) was from Aldrich Chemical Co. or Stohler Isotope Chemicals (Waltham, MA). Solvents used for carbohydrate structural analyses were of Spectroanalyzed or HPLC grade and were purchased from Fisher Scientific Co. All other chemicals and solvents were of reagent grade or better.

Carbohydrates

Sodium polypectate (sodium salt of citrus polygalacturonic acid; Grade II) and D-galactose were from Sigma Chemical Co. D-GalUA was from Aldrich Chemical Co. and D-glucose was from Fisher Scientific Co. A high-molecular-weight mixture of phytoalexin elicitor-active β-D-glucans, partially purified from a partial acid hydrolysate of cell walls of the phytopathogenic fungus, *Phytophthora megasperma* f. sp. *glycinea* (void material from a low resolution Bio-Gel P-2 gel filtration column described in [10]), was provided by J. K. Sharp of this laboratory. A nona- and a decagalacturonide, purified by anion-exchange chromatography of a partial acid hydrolysate of citrus pectin [6], were provided by E. Nothnagel of this laboratory.

Enzymes

Homogeneous α-1,4-D-endopolygalacturonic acid lyase (EC 4.2.2.2) was prepared from cultures of *Erwinia carotovora* var. *carotovora* (ATCC No 495) as described [8]. PGA lyase activity was measured spectrophotometrically [8]. One unit of PGA lyase activity was the amount of enzyme required to release 1 μmol 4,5-unsaturated GalUA per minute at 30 °C.

Colorimetric assays

Uronic acid content was determined by the *m*-hydroxydiphenyl method [11], with GalUA as the standard. Hexose content was determined by the anthrone method [12], with glucose as the standard.

Assay for phytoalexin elicitor activity

The modification of the soybean-cotyledon assay previously described was used to determine elicitor activities [8, 9]. All samples assayed for elicitor activity were desalted by gel-permeation chromatography on Sephadex G-10 columns (1.5 × 18 cm) equilibrated with distilled H_2O. The data are expressed as the A_{286} of the test sample relative to the A_{286} of a partially purified high-molecular-weight glucan elicitor assayed at 0.18 μg/cotyledon, a concentration of glucan elicitor that induces maximum phytoalexin accumulation (A_{286}/A_{286max}). This procedure corrects for the day-to-day variability of the bioassay [5]. The A_{286} is directly proportional to the amount of pterocarpan phytoalexins produced by the cotyledons [5].

Analysis of glycosyl-residue compositions

Glycosyl-residue compositions were determined by preparing the trimethylsilyl methyl glycoside, methyl-ester derivatives as described [13]. Derivatives were prepared from 10 μg GalUA equivalents of desalted samples and were analyzed by GLC. GLC analyses of these derivatives were performed on a 0.25 mm × 15 m fused-silica capillary column (DB-1; J. and W. Scientific Co.) fitted in a Hewlett-Packard 5880A gas chromatograph with the following temperature program: 2 min at the injection temperature of 120 °C, 15 °C/min to 150 °C, 2 °C/min

to 190 °C, 30 °C/min to 240 °C, and then 10 min at 240 °C. Peak areas were quantitated and adjusted for differences in yield and detection with correction factors obtained by analyzing known amounts of the appropriate sugar standards. The relative amount of each glycosyl residue, expressed as weight percent (wt %), was calculated by dividing the peak areas of the derivatives of a particular glycosyl residue by the total area of all the derivatives detected and multiplying by 100.

^{1}H-NMR spectrometry

^{1}H-NMR spectra were recorded on a Bruker WM-250 Fourier transform spectrometer operating at 250 MHz. Prior to analysis, samples (200 μg GalUA equivalents) were lyophilized in the presence of imidazole-HCl buffer – three times from 99.7–99.8 atom % ^2H_2O and once from 99.96 atom % ^2H_2O. The samples were then dissolved in 0.5 ml of 99.96 atom % ^2H_2O and transferred to an NMR tube for analysis. The final concentration of imidazole-HCl was approximately 0.2 M (pH 7.0). A standard containing 0.2 M imidazole HCl (pH 7.0) and sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate was used to calibrate the spectrometer so that the chemical shift of sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate was equal to 0.00. The temperature of the sample was maintained at 75 °C while data was collected.

FAB-MS

The molecular weights of the underivatized oligogalacturonides were determined by FAB-MS as described [6, 9]. Prior to analysis, the samples were dissolved in 5–10 μl of 5% (v/v) aqueous acetic acid and one μl was added to a drop of a thioglycerol-glycerol mixture on the target. After partial evaporation of the solvent in the vacuum of the mass spectrometer source, one μl of 0.1 M HCl was added to the target and the analysis was performed in the negative-ion mode. Adding 0.1 M HCl to the sample greatly inhibited salts from forming and thereby promoted the detection of molecular ions ($[M-H]^-$).

Preparation of soybean cell walls

Cell walls were prepared from stems of eight- and nine-day old soybean seedlings as described [5]. The cotyledons and leaves were excised from the seedlings and the stems cut from the remaining plant approximately 1 cm above the soil. The stems were placed in liquid nitrogen for at least 15 min. A stem powder was prepared by wrapping the frozen stems in a fine nylon screen and pounding the stems into a fine powder with a wooden mallet. The stem powder was stored at −30 °C until used to prepare cell walls.

Cell walls were prepared by suspending 35 g portions of the stem powder in 250 ml of 0.5 M sodium phosphate (pH 7.0). The stem-powder suspensions were homogenized in a ground glass homogenizer. The homogenates were centrifuged and the pellets recovered. The pellets were washed three times with 200 ml of 0.5 M sodium phosphate (pH 7.0), by suspending the pellets in buffer and repelleting the walls by centrifugation. The buffer washes were followed by three 200 ml washes with distilled H_2O. The pellets obtained after the distilled H_2O washes were combined and suspended in 800 ml boiling ethanol and stirred for 1 h to extract plant pigments. The insoluble walls were recovered by suction filtration on a sintered-glass funnel and washed once with 500 ml ethanol. The ethanol wash was followed by four 500 ml washes of chloroform/methanol (1:1; v/v) and four 500 ml washes of acetone. Air was drawn through the walls by suction for approximately 15 min. The walls were allowed to dry several days in a loosely covered evaporating dish. This procedure yielded 20.4 g white, fluffy cell walls from 335 g of frozen stem powder.

PGA-Lyase treatment of cell walls and isolation of cell-wall elicitors

Cell-wall elicitors were obtained by treating isolated soybean cell walls with purified PGA lyase. Cell walls (19.7 g) were suspended in a 2-l Fernbach flask containing 2 l of 5 mM Tris-HCl, 1 mM $CaCl_2$ (pH 8.5). The cell-wall suspension was preincubated in a shaking water bath at 30 °C for 30 min. After the preincubation period, 10 ml of purified PGA lyase I (35 units) was added to the cell-wall suspension and the reaction mixture was agitated by constant shaking. To monitor the reaction, 0.5 ml aliquots of the reaction mixture were removed at selected time intervals and the insoluble wall material removed by filtration through 1.2 μm Millipore filters (Type RA). The filtrate was collected in a test tube immersed in an ice bath to stop the enzyme reaction. The filtrates were diluted 1:10 with distilled H_2O and the A_{235} was determined.

After 2.5 h of incubation, the A_{235} showed no further increase, suggesting that the enzyme reaction

had approached completion. The reaction mixture was filtered with suction through two layers of glass-fiber discs (Whatman, GF/A) to remove the remaining insoluble cell-wall material. The pH of the filtrate was adjusted to 5.2 with 2 M HCl and the PGA lyase was inactivated by heating at 60–70 °C for 30 min. Results of colorimetric assays for uronic acid and hexose content indicated that approximately 920 mg total carbohydrate had been solubilized from the cell walls. This material constituted the PGA-lyase-solubilized cell-wall preparation used for the following studies.

Results

Anion-exchange chromatography of the PGA-lyase-solubilized cell-wall preparation

The material solubilized from soybean cell walls by PGA lyase was greater than 90% uronic acid, as was the material solubilized from sodium polypectate [9]. This suggested that anion-exchange chromatography would be a useful initial step towards isolating the most elicitor-active molecules.

The PGA-lyase-solubilized cell-wall preparation was adjusted to 5 mM imidazole-HCl by adding 10 ml 1 M imidazole-HCl (pH 6.0). The final pH of the cell-wall preparation was 5.8 and the salt concentration was approximately 10 mM. This material (890 mg total carbohydrate) was applied to a QAE-Sephadex column (3.1 × 20 cm) equilibrated with 10 mM imidazole-HCl (pH 6.0). The material that voided the column was collected as a single fraction. The column was then washed with 200 ml of 10 mM imidazole-HCl (pH 6.0). The eluant was also collected as a single fraction. The compounds remaining bound to the column were eluted stepwise with sequential washes of 200 ml each of 0.125 M, 0.25 M, 0.5 M, and 1.0 M imidazole-HCl (pH 6.0). The eluants of these imidazole-HCl washes were each collected as two 100-ml fractions. The uronic acid and hexose contents and the elicitor activities of the fractions are listed in Table I.

The carbohydrate applied to the column was recovered quantitatively. Approximately 1.6% of the carbohydrate was recovered in the void and 10 mM imidazole-HCl-wash fractions. These fractions were rich in neutral sugars. Approximately 85% of the carbohydrate applied to the column was recovered in the 0.5 M imidazole-HCl washes (QAE fractions 6 and 7). These fractions were rich in uronic acid.

Table I. Uronic acid and hexose content and phytoalexin elicitor activity of QAE-Sephadex column fractions.

QAE fraction	Imidazole [M]	Total uronic acid [mg]	Total hexose [mg]	Elicitor[a] activity	
Void	0.010	5.93	10.55	0.07	(0.8%)
1	0.010	0.05	0.15	nt[b]	–
2	0.125	3.29	1.67	0.02	(0.2%)
3	0.125	1.24	0.58	0.04	(0.1%)
4	0.250	32.55	6.43	0.03	(1.4%)
5	0.250	14.91	2.23	0.10	(1.0%)
6	0.500	629.70	41.14	0.15	(54.6%)
7	0.500	189.82	7.83	0.07	(9.6%)
8	1.000	10.15	2.35	0.01	(0.4%)
9	1.000	11.67	4.02	0.02	(0.5%)

[a] Samples were assayed at 10 µg/cotyledon total carbohydrate. The data shown is the average A_{286}/A_{286max} obtained from forty cotyledons. Percent of total elicitor activity applied to the column that was recovered in the fractions is in parentheses.

[b] nt = not tested.

Analyses of the elicitor activities of the QAE fractions indicated that 70% of the elicitor activity applied to the column was recovered after QAE-Sephadex chromatography. Fractions 5–7 had the highest specific elicitor activity. These fractions contained approximately 95% of the elicitor activity recovered from the column. The apparent loss of 30% of the elicitor activity applied to the QAE-Sephadex column was probably due to underestimation of the elicitor activity of fractions that contained larger oligogalacturonides. As previously discussed [9], larger, partially purified oligogalacturonides are insoluble under the bioassay conditions; their insolubility is likely to decrease their apparent elicitor activity.

Gel-permeation chromatography of QAE fraction 6

Since QAE fraction 6 exhibited the highest specific phytoalexin elicitor activity and contained 69% of the carbohydrate applied to the QAE-Sephadex column, the elicitor-active molecules in this fraction were purified further by gel-permeation chromatography.

Approximately 230 mg GalUA equivalents of QAE fraction 6 were concentrated to a final volume of 5 ml by rotoevaporation under reduced pressure at 40 °C. The concentrated material was applied to a Bio-Gel P-10 gel-permeation column. The elution profile obtained is shown in Fig. 1A. The GalUA

equivalents applied to the column were recovered quantitatively. The eluted materials were pooled as indicated by the bars. Approximately 37%, 40%, 12%, 8%, and 2% of the GalUA equivalents were recovered in P-10 fractions V_o, A, B, C, and D, respectively. Analyses of the phytoalexin elicitor activities of desalted samples from the P-10 fractions demonstrated that fractions C and D were the most elicitor-active; fraction C exhibited the highest specific elicitor activity (Table II). The elicitor activity of fraction C was abolished by further treatment with active PGA lyase, but was unaffected by treatment with heat-inactivated PGA lyase (data not shown). This indicated that the elicitor-active components in fraction C contained α-1,4-linked D-GalUA residues that were required for elicitor activity.

P-10 fractions C and D were individually concentrated to approximately 5 ml by rotoevaporation under reduced pressure after the addition of 5 ml 1 M imidazole-HCl (pH 6.0). The imidazole buffer was added to prevent the precipitation of larger oligogalacturonides that sometimes occurs when these molecules are concentrated in H_2O or in the presence of mono- or divalent cations. The concentrated P-10 fractions were separately rechromatographed on the same P-10 column. The elution profiles obtained for P-10 fractions C and D are shown

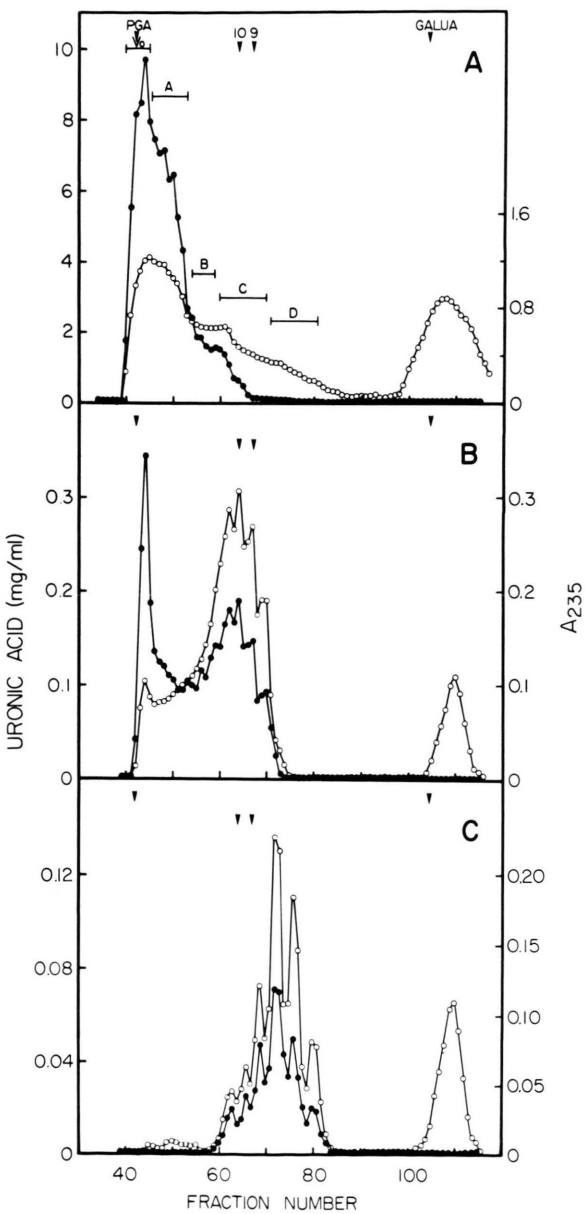

Table II. Phytoalexin elicitor activity of fractions obtained by P-10 gel-permeation chromatography of QAE fraction 6.

P-10 Fraction	Elicitor activity[a] 5 μg/cot	10 μg/cot
Void	0.01	0.00
A	0.02	0.00
B	0.14	0.14
C	0.33	0.41
D	0.17	0.25

[a] Samples were assayed at 5 μg/cotyledon and 10 μg/cotyledon GalUA equivalents. The data shown is the average A_{286}/A_{286max} obtained from forty and twenty cotyledons (cots), respectively.

Fig. 1. (A) Gel-permeation chromatography of QAE fraction 6. Concentrated QAE fraction 6 (Table I) was chromatographed on a Bio-Gel P-10 (200–400 mesh) column (2.6 × 107 cm) that was equilibrated and eluted with 50 mM potassium phosphate (pH 6.0). Fractions of 4.8 ml were collected at a flow rate of 38 ml/h. The eluted compounds were detected by measuring the A_{235} (○) of each fraction and by determining the uronic acid content (●) of aliquots taken from selected fractions. The void (V_o) and included volumes (V_i) were determined in a separate experiment with polygalacturonic acid and GalUA, respectively. The elution volumes of deca- and nonagalacturonides, also determined in a separate experiment, are indicated by the arrows labelled 10 and 9, respectively. (B) Rechromatography of P-10 fraction C shown in Fig. 1A. (C) Rechromatography of P-10 fraction D shown in Fig. 1A. In all three elution profiles, the A_{235}-absorbing material that eluted after the included volume was due to the presence of imidazole in those fractions.

in Fig. 1B and 1C, respectively. In both cases, the GalUA equivalents applied to the column were recovered quantitatively.

The elution profile of P-10 fraction C demonstrated that it was a mixture of variously sized oligogalacturonides. Fractions 60–75 (Fig. 1B) contained a series of partially resolved oligosaccharides rich in uronic acid. The elution volumes of the partially resolved oligosaccharides were consistent with those expected of oligogalacturonides with degrees of polymerization of seven to eleven residues; undeca- and decagalacturonides were the major components. P-10 fraction D was also separated into a series of partially resolved oligosaccharides rich in uronic acid. The elution volumes of these oligosaccharides were consistent with the presence of oligogalacturonides with degrees of polymerization of six to eleven residues; the heptagalacturonides were the major component. FAB-MS and glycosyl-residue composition analyses confirmed that pooled fractions 72 and 73 from the P-10 chromatography of fraction D contained predominantly heptagalacturonides.

Aliquots of selected fractions from the P-10 chromatography of fractions C and D were desalted by gel-permeation chromatography on Sephadex G-10 columns and assayed for phytoalexin elicitor activity (Tables III and IV, respectively). Fractions 62 and 64 from the P-10 chromatography of fraction C (Fig. 1B) possessed the highest specific elicitor activity of the material in fraction C. Pooled fractions 62–63 and 65–66 from the P-10 chromatography of fraction D (Fig.1C) possessed the highest specific elicitor activity of the material in fraction D.

Table III. Phytoalexin elicitor activity of selected fractions obtained by P-10 gel-permeation chromatography of P–10 fraction C.

P-10 fraction no.	Elicitor activity[a]
43–45	0.19
46–50	0.12
51–55	0.11
56–59	0.31
62	0.41
64	0.47
67	0.27
70	0.13

[a] Samples were assayed at 5 μg/cotyledon GalUA equivalents. The data shown is the average A_{286}/A_{286max} obtained from forty cotyledons.

Table IV. Phytoalexin elicitor activity of selected fractions obtained by P-10 gel-permeation chromatography of P–10 fraction D.

P-10 fraction no.	Elicitor activity[a]
62–63	0.32
65–66	0.33
68–69	0.21
72–73	0.17
76–77	0.16
80–81	0.13

[a] Samples were assayed at 5 μg/cotyledon GalUA equivalents. The data shown is the average A_{286}/A_{286max} obtained from twenty cotyledons.

The elicitor activities of various components of the original cell-wall preparation are compared in Fig. 2. Fractions 62 and 64 exhibited very similar phytoalexin elicitor activities over the concentration range tested. The specific elicitor activity of these fractions was 5- to 10-fold greater than that observed for QAE fraction 6 and the unpurified cell-wall preparation. The maximum amount of phytoalexins induced by P-10 fractions 62 and 64 was 3- to 5-fold higher than

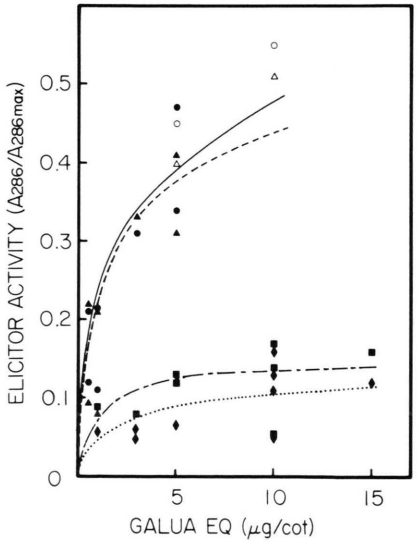

Fig. 2. Response of soybean cotyledons to various concentrations of elicitors in the PGA-lyase-treated cell-wall preparation (◆), QAE fraction 6 (■), and fractions 62 (▲, △) and 64 (●, ○) from the P-10 chromatography of P-10 fraction C. Each point represents the average A_{286}/A_{286max} obtained from twenty cotyledons (closed symbols) or from ten cotyledons (open symbols). The curves represent the best fit of the data to a linear regression analysis of the data shown (plotted as log GalUA equivalents [x] *vs* A_{286}/A_{286max} [y]).

that induced by the unpurified cell-wall preparation or by QAE fraction 6. The differences in the maximum amounts of phytoalexins induced by these fractions may be due to differences in their phytoalexin elicitor activities. It is also possible that the lower level of phytoalexin accumulation induced by QAE fraction 6 and the unpurifed cell-wall preparation was due to the insolubility of the larger oligogalacturonides in these fractions. Another possibility is that the smaller and larger oligogalacturonides in QAE fraction 6 and the unpurified cell-wall preparation inhibited the activity of the most elicitor-active oligogalacturonides in these preparations [9].

These results suggested that the most elicitor-active molecules solubilized from soybean cell walls by PGA lyase were oligogalacturonides with degrees of polymerization from eight to twelve residues. Further studies were conducted to more precisely characterize the phytoalexin elicitors with the highest specific elicitor activity.

Glycosyl-residue composition analysis of P–10 purified elicitors

Glycosyl-residue composition analyses of fractions 62, 64, 67, and 70 of the P–10 chromatography of fraction C (Fig. 1B) established that each of these fractions contained greater than 99% GalUA residues. Trace amounts of rhamnosyl and xylosyl residues (<0.2%) were also detected in these fractions. Analysis of pooled fractions 62–63, 65–66, 68–69, 72–73, 76–77, and 80–81 from the P-10 chromatography of fraction D (Fig. 1C) established that each of these fractions also contained greater than 99% GalUA residues and trace amounts of rhamnosyl and xylosyl residues (<0.5%). These results clearly established that oligogalacturonides were the predominant molecules in the elicitor-active material solubilized from soybean cell walls.

FAB-MS analysis of the oligogalacturonide elicitors

FAB-MS analyses in the negative-ion mode were conducted on fractions 62, 64, 67, and 70 from the P-10 chromatography of fraction C (Fig. 1B). These fractions gave abundant molecular ions ($[M–H]^-$) and the mono- and dipotassium salts of the molecular ion ($[M\text{-}2H + K^+]^-$ and $[M\text{-}3H + 2K^+]^-$, respectively).

The FAB mass spectrum of fraction 62 had a molecular ion at *m/z* 1935 and the monopotassium salt of the molecular ion at *m/z* 1973. These signals are consistent with the presence of an undecagalacturonide with an unsaturated GalUA residue at the nonreducing terminus. A minor signal, which has not been assigned, was detected at *m/z* 1891. The spectrum of fraction 64 had a molecular ion at *m/z* 1759 and the monopotassium salt of the molecular ion at *m/z* 1797. These are the expected signals for a decagalacturonide containing an unsaturated GalUA residue at the nonreducing terminus. The spectrum of fraction 67 had a molecular ion at *m/z* 1583 and the mono- and dipotassium salts of the molecular ion at *m/z* 1621 and 1659, respectively. These are the expected signals for a nonagalacturonide containing an unsaturated GalUA residue at the nonreducing terminus. The mass spectrum of fraction 70 had a molecular ion at *m/z* 1407 and the mono- and dipotassium salts of the molecular ion at *m/z* 1445 and 1483, respectively. These are the expected signals for an octagalacturonide containing an unsaturated GalUA residue at the nonreducing terminus.

^{1}H-NMR analysis of the elicitor-active oligogalacturonides

Fractions 62 and 64 from the P-10 chromatography of fraction C (the fractions with the highest specific elicitor activity) were analyzed by ^{1}H-NMR. The ^{1}H-NMR spectra obtained for fractions 62 and 64 (Fig. 3A and 3B, respectively) are compared in Figure 3 to the spectrum obtained for the elicitor-active decagalacturonide isolated from PGA-lyase-treated sodium polypectate (Fig. 3C). The chemical shifts (δ) and proton assignments of the spectra obtained for P-10 fractions 62 and 64 are presented in Table V. The assignment of protons to specific signals was based on the relative peak areas of the signals and on comparison of the observed chemical shifts with those obtained for α-1,4-linked tri-D-GalUA and α-1,4-linked penta-D-GalUA, each of which contained 4,5-unsaturated GalUA at their nonreducing termini (W. York and K. Davis, manuscript in preparation). The spectra obtained for fractions 62 and 64 were indistinguishable from those obtained for the undeca- and decagalacturonides (isolated from sodium polypectate) containing terminal 4,5-unsaturated GalUA residues (Fig. 3). The very close

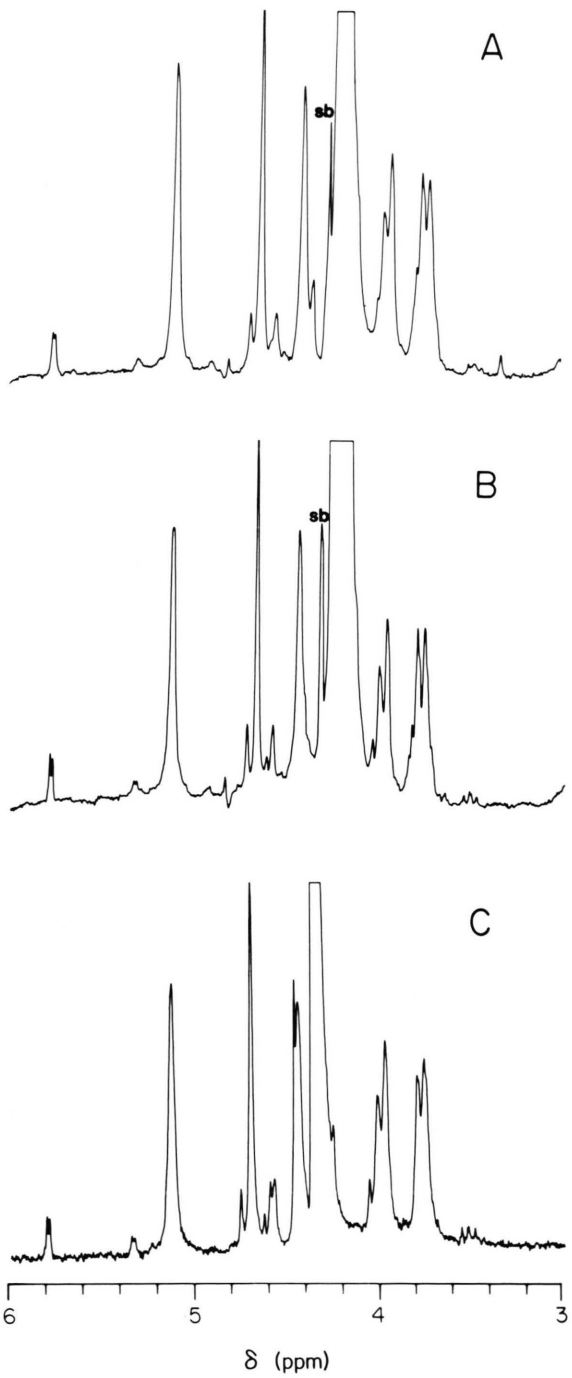

Fig. 3. (A) ^{1}H-NMR spectra of fraction 62, (B) fraction 64, (C) the decagalacturonide-rich fraction obtained from PGA-lyase treated sodium polypectate (fraction A_{10}, [9]). The spectrometer was calibrated so that the chemical shift (δ) of sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate was equal to 0.00. The signals marked sb are spinning side bands caused by the presence of ^{2}HHO.

Table V. Chemical shifts (δ) and proton assignments of ^{1}H-NMR spectra of fractions 62 and 64 from P-10 chromatography of P-10 fraction C.

Chemical Shift (δ)[a]		
Fraction 62	Fraction 64	Proton assignment[b]
5.78	5.78	H4 un GalUA
5.32	5.32	H1 red GalUA – α
5.13	5.14	H1 un GalUA H1 int GalUA
4.74	4.74	H5 int GalUA – system 2
4.68	4.67	H5 int GalUA – system 1
4.60	4.60	H1 red GalUA – β H4 int GalUA system 2
4.43	4.43	H4 int GalUA system 1
4.00	4.00	H3 int GalUA system 1
3.96	3.96	H3 int GalUA system 2
3.73	3.73	H2 int GalUA system 1 H2 int GalUA system 2
3.50	3.50	H2 red GalUA – β

[a] Chemical shift relative to 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (δ = 0.00).

[b] Glycosyl residues are designated as follows: un GalUA, 4,5-unsaturated GalUA; red GalUA, reducing GalUA in either the α or β conformation; int GalUA, internal 1,4-linked GalUA. System 1 int GalUA residues include all internal GalUA except the GalUA residues to which the terminal 4,5-unsaturated GalUA residues are glycosidically linked, which is designated as int GalUA-system 2.

similarities of these spectra were convincing evidence that the major components in P-10 fractions 62 and 64 were oligosaccharides composed only of α-1,4-linked D-GalUA that contained 4,5-unsaturated GalUA at their nonreducing termini.

Discussion

The results presented demonstrate that the molecules with the highest elicitor activity solubilized from soybean cell walls by PGA lyase were oligogalacturonides with degrees of polymerization from eight to twelve residues. Chemical characterization of those purified oligogalacturonides that possessed the highest specific elicitor activity (P-10 fractions 62 and 64, Fig. 1B) demonstrated that these fractions contained predominantly decasaccharides and undecasaccharides of α-1,4-linked D-GalUA that contained 4,5-unsaturated GalUA residues at their nonreducing termini.

The phytoalexin elicitor activities of cell-wall-derived undeca- and decagalacturonide-rich fractions were very similar over the concentration range tested

(Fig. 2). Both of these elicitors exhibited maximum elicitor activity at 5–10 μg GalUA equivalents/cotyledon (~ 30–40 μM). This is very similar to the elicitor activity of the undeca- and decagalacturonide-rich fractions obtained by PGA lyase treatment of sodium polypectate [9]. The slight differences in the response of the cotyledons to the elicitors derived from cell walls and those derived from sodium polypectate are within the variability of the elicitor bioassay.

These results indicate that the elicitors solubilized from soybean cell walls by PGA lyase are indistinguishable from those obtained by PGA lyase treatment of sodium polypectate [9]. There was no evidence for the presence of glycosyl residues other than α-1,4-linked D-GalUA. However, it remains possible that the elicitor-active molecules are minor components of these fractions. If this is the case, the elicitors would be modified oligogalacturonides. The selectivity of the purification procedures used and the sensitivity of the chemical analyses conducted probably would not have revealed the presence of 10% or less of an oligogalacturonide that contained a single structurally modified residue. The isolation, from soybean cell walls [6], citrus pectin [6], and citrus polygalacturonic acid [9, 14], of very similar α-1,4-linked oligogalacturonides that possess very similar specific elicitor activities strongly supports the conclusion that the elicitor-active molecules are, in fact, the predominant unmodified oligogalacturonides in these fractions. It is unlikely that partial acid hydrolysis [6] and treatment with pectin-degrading enzymes [9, 14] would have produced, from these different starting materials, similar yields of elicitor-active modified oligogalacturonides. The fact that the specific elicitor activity of oligogalacturonides obtained by enzymic degradation of soybean cell walls was the same as that of oligogalacturonides obtained by partial acid hydrolysis of soybean cell walls demonstrates that neither acid-labile substituents such as methyl esters nor acid-labile glycosyl residues are important for elicitor activity. Furthermore, the elicitor activity of these oligogalacturonides is destroyed by treatment with homogeneous preparations of pectin-degrading enzymes that specifically degrade α-1,4-linked oligogalacturonides [5, 6, 8]. This demonstrates an absolute requirement for consecutive α-1,4-linked D-GalUA residues in elicitor-active oligogalacturonides. However, only the chemical synthesis of pure α-1,4-linked oligogalacturonides will conclusively demonstrate whether they are indeed the elicitor-active molecules.

There is now considerable evidence that oligogalacturonides are important regulatory molecules that play a role in inducing defense responses in plants. Recent studies demonstrating that pectin-degrading enzymes from both fungal [15, 16] and bacterial [8] sources are able to elicit phytoalexins in plants support the proposed physiological role of oligogalacturonides in the induction of phytoalexins during microbial attack [3, 5, 7]. These pectin-degrading enzymes released very similar elicitor-active oligogalacturonides from plant cell walls and polygalacturonic acid [9, 14]. Further evidence that oligogalacturonides contribute to phytoalexin accumulation is provided by the observations that most, if not all, plants contain homogalacturonans within their cell walls [17] and that GalUA-rich elicitors can be obtained from cell walls of various plant species [5]. Since all phytopathogenic microorganisms studied secrete pectin-degrading enzymes [18], it is likely that elicitor-active oligogalacturonides are released from the cell wall during attempted infection.

Acknowledgements

The authors gratefully acknowledge William York for assistance with the ^{1}H-NMR analyses. The authors thank Jill Skarstad for typing the manuscript and Leigh Kirkland for editorial assistance. A. Dell is grateful for financial support from MRC and SERC grants awarded to Professor Howard Morris of Imperial College.

[1] J. A. Bailey, in: J. A. Bailey and J. W. Mansfield, eds. Phytoalexins, pp. 289–318, John Wiley and Sons/New York 1982.
[2] R. A. Dixon, P. M. Dey, and C. J. Lamb, in: A. Meister, ed. Advances in Enzymology. pp. 1–136, John Wiley and Sons/New York 1983.
[3] A. G. Darvill and P. Albersheim, Ann. Rev. Plant Pathol. **35,** 243–275 (1984).
[4] C. A. West, Naturwissenschaften **68,** 447–457 (1981).
[5] M. G. Hahn, A. G. Darvill, and P. Albersheim, Plant Physiol. **68,** 1161–1169 (1981).
[6] E. A. Nothnagel, M. McNeil, A. Dell, and P. Albersheim, Plant Physiol. **71,** 916–926 (1983).
[7] R. J. Bruce and C. A. West, Plant Physiol. **69,** 1181–1188 (1982).
[8] K. R. Davis, G. D. Lyon, A. G. Darvill, and P. Albersheim, Plant Physiol. **74,** 52–60 (1984).
[9] K. R. Davis, A. G. Darvill, P. Albersheim, and A. Dell, Plant Physiol., in press.
[10] J. K. Sharp, B. Valent, and P. Albersheim, J. Biol. Chem. **259,** 11312–11320 (1984).
[11] N. Blumenkrantz and G. Asboe-Hansen, Anal. Biochem. **54,** 484–489 (1973).
[12] Z. Dische, in: R. L. Whistler and M. L. Wolfrom, eds. Methods in Carbohydrate Chemistry, Vol. I, pp. 478–481, Academic Press, New York 1962.
[13] M. F. Chaplin, Anal. Biochem. **123,** 336–341 (1982).
[14] D. F. Jin and C. A. West, Plant Physiol. **74,** 989–992 (1984).
[15] S.-C. Lee and C. A. West, Plant Physiol. **67,** 633–639 (1981).
[16] S.-C. Lee and C. A. West, Plant Physiol. **67,** 640–645 (1981).
[17] M. McNeil, A. G. Darvill, S. C. Fry, and P. Albersheim, Ann. Rev. Biochem. **53,** 625–663 (1984).
[18] D. F. Bateman and D. F. Basham, in: R. Heitefuss iology. Physiological Plant Pathology, New Series Vol. IV, pp. 316–355, Springer Verlag, New York 1976.