Structure Elucidation of Hop Plant (*Humulus lupulus*) Phytoalexin Elicitors by Fast Atom Bombardment Mass Spectrometry

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Cell wall material (CWM) derived from hop leaves (*Humulus lupulus*) was extracted and characterized by fast atom bombardment mass spectrometry (FABMS). The material consists of monosaccharides, fucose, rhamnose, arabinose, galactose, glucose, and xylose, and galacturonic acid oligomers ranging from trimers to octamers, each with a double bond. The unsaturated galacturonic acids showed elicitor activity for phytoalexins in soybean cotyledons. By HPLC analysis it was observed that the pentagalacturonide is present in greatest amount, but the compound with the greatest biological activity is the unsaturated hexagalacturonide. The relative elicitor activity was found to be roughly 3:3:4:10:2 for the trimer, tetramer, pentamer, hexamer, and octamer, respectively. Structural analyses by FABMS of the peracetylated reductively aminated pentaglacturonide showed that the unsaturated galacturonosyl residue is located at the nonreducing end of the oligomer with unsaturation deduced to be at the 4,5-position.

INTRODUCTION

The accumulation of phytoalexins is the primary means of self-protection against a variety of insults in higher plants (Brooks and Watson, 1985; Keen, 1986; Grisebach and Ebel, 1978; Kuć and Rush, 1985). Phytoalexin production is induced by infection of plant tissues with microorganisms such as fungi, bacteria, and viruses. Infection by higher organisms such as nematodes or insects also has been shown to result in phytoalexin production (Paxton, 1981; Russell, 1986; Russell et al., 1978; Green and Ryan, 1972; Loper, 1968; Sutherland et al., 1980).

Molecules that are capable of inducing phytoalexin synthesis are called elicitors (West, 1981; Ryan, 1988; Keen, 1990). They are produced by the hydrolysis of cell wall polysaccharides from the infecting microorganisms (exogenous elicitors) as well as from the cell wall polysaccharides of the host plant (endogenous elicitors) (McNeil et al., 1984; Ryan, 1987; Albersheim et al., 1992).

Plant cell wall substances not only provide a structural framework for the plant but also contain information components (Hahn et al., 1981; Bishop and Ryan, 1987). Oligosaccharides with regulatory activities are called oligosaccharins (Darvill et al., 1985). These compounds are generated at infection or wound sites and may be early signals to activate genes whose products enhance the plants' defense against pathogens and herbivores (Ryan, 1987). Among the plant cell wall components that function as elicitors, the family of oligogalacturonic acids are of tremendous importance (Darvill et al., 1985; Davis et al., 1986). They can be isolated from pectic polysaccharides which are part of the plants' primary cell wall (Aspinall and Fanous, 1984; Selvendran and O'Neill, 1987). In this paper we report on the extraction, purification, and identification of the phytoalexin elicitor mixture, oligogalacturonic acids derived from hops (*Humulus lupulus*).

EXPERIMENTAL METHODS

Preparation of Cell Wall Material (CWM) from Hop Leaves. The procedure of Selvendran and O'Neill (1987) was followed. Frozen hop leaves (variety 64107, 50 g) were cut into small pieces and blended for 3 min with aqueous 1% sodium deoxycholate (SDC) (200 mL) containing 5 mM $Na_2S_2O_5$ using a Brinkmann homogenizer. A few drops of poly(ethylene glycol) were added to minimize frothing. After the material was cooled in an ice bath for 2 min, blending was repeated for an additional minute. The hop slurry was centrifuged in the cold and the supernatant discarded. The pellet was washed twice with cold 0.5% SDC (300 mL) containing 3 mM $Na_2S_2O_5$. The pellet then was repeatedly washed with acetone maintained at 4 °C until the material appeared colorless. The crude product was further blended for 3 min in 0.5% SDC (300 mL) containing 3 mM $Na_2S_2O_5$ and poly(ethylene glycol) (1.5 mL) using a bead-beater (Biosper Products) fitted with an ice jacket to obtain optimal cell disruption. The beads were separated from the suspension, and the suspension was centrifuged at 4 °C. The pellet was washed twice with cold water. For deproteination the residue was washed twice with phenol–acetic acid–water (PAW 2:1:1, 100 mL). To remove starch, the pellet was sonicated for 10 min in 90% DMSO (90 mL) and then stirred at 25 °C for 16 h. The remaining material was washed six times with water (100 mL each) until a starch–iodine test was negative. The residue was recovered by centrifugation. To remove traces of DMSO, the substance was suspended in water and dialyzed (Spectra/por1, MW cutoff 6000–8000) for 16 h against water. After lyophilization, 5.12 g of CWM was recovered.

Total Hydrolysis of CWM. Hop CWM (5.02 mg) was suspended in 2 N TFA (2 mL) and heated in a sealed reaction vial at 121 °C for 2 h (Selvendran et al., 1979). The suspension was filtered and the filtrate concentrated to dryness. The residue was redissolved in 0.5 mL of water.

Monosaccharide Analysis of Hop CWM. High-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) was performed using a Dionex BioLC Model 4500i instrument equipped with a 4 × 250 mm Dionex Carbopac PA-1 pellicular column and a 3 × 25 mm Carbopac PA guard column. Neutral carbohydrates were separated using water mixed with 100 mM NaOH (90:10) as eluent. For separation of acidic monosaccharides a solution with final concentrations of 150 mM NaOAc and 100 mM NaOH was used as eluent. The flow rate was 1 mL/min. Sodium hydroxide solution (300 mM) was added through a mixing tee at a flow rate of 1 mL/min by the Dionex postcolumn delivery system. The detector sensitivity was set to 1K. Monosaccharides were identified using standard solutions. The peak area was determined to obtain the monosaccharide composition (Table I).

Partial Hydrolysis of Hop CWM. The CWM was suspended in 10 mM Na_2HPO_4 (pH 7.4) and autoclaved for 20 min at 121

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°C. After filtration, the filtrate was adjusted to 0.06 M Tris-HCl buffer (pH 7.9) (Robertsen, 1986).

Isolation of Elicitor Fractions from Hop Leaves. Separation of the fractions obtained by partial hydrolysis was performed on a QAE-Sephadex A-25 anion-exchange chromatography column (2.5 × 25 cm), equilibrated with 0.06 M Tris-HCl buffer. The flow rate was 2 mL/min. After using 500 mL of 0.06 M Tris-HCl, a linear gradient from 0.06 to 0.6 M Tris-HCl buffer (2000 mL) was applied. All effluent fractions (12.5 mL each) were assayed for total sugar (Dubois et al., 1956) and uronic acid content (Blumenkranz and Asboe-Hansen, 1973).

Desalting of the Elicitor Fractions. Fractions 1–9 (Figure 2A) were desalted individually on a Bio-Gel P-2 column (2.5 × 50 cm). Approximately 6 mg of sample was applied for each run, and water was used for elution. Aliquots (ca. 1 μL) of all fractions (10 mL each) were spotted on a TLC plate and tested for carbohydrates by the orcinol method. Carbohydrate-containing fractions were pooled, the solvent was removed on a Buchi rotavapor, and the residues were lyophilized. Native carbohydrate samples were dissolved in 10 μL of 5% HOAc at a concentration of 10–20 mg/mL. These solutions were used for FABMS analyses.

HPAEC-PAD Analysis of Oligosaccharides. Chromatographic analyses were carried out using the Dionex system as described above. The flow rate was set to 1 mL/min. Solvents used were as follows: solvent A, 100 mM NaOH solution; solvent B, 100 mM NaOH/1 M NaOAc solution. After a hold period of 5 min at 50% B, the concentration of B was changed to 75% over a period of 30 min. This eluting solvent composition was maintained for an additional 10 min. The detector sensitivity was set to 3K for fractions 3 and 4 and to 1K for the others (Figure 2 A).

Preparation of Oligogalacturonides from Commercial Poly(galacturonic acid). Poly(galacturonic acid) from oranges (2.5 g) was suspended in water (200 mL) and adjusted to pH 4.2 with 1 N NaOH (Robertsen, 1986). The suspension was autoclaved for 20 min at 121 °C. High molecular weight polymers were precipitated by adjusting the pH to 2 with 1 N HCl. The sample was centrifuged, and the polymers were removed.

Gel Filtration of Oligogalacturonides. The oligogalacturonides were subjected to size exclusion chromatography on a Bio-Gel P-6 column (1.5 × 50 cm). The material was eluted with 0.5 N NaCl at a flow rate of 18 mL/h. Effluent fractions (2.5 mL each) were collected and assayed for total carbohydrate (Dubois et al., 1956). Dextran (MW 485 000) and galacturonic acid (MW 194) were used to determine the void volume (v_0) and the total volume (v_t), respectively. Only the fractions between v_0 and v_t were collected, pooled, and concentrated by rotary evaporation. Desalting and HPAEC-PAD analysis (Figure 2A) were performed as described above.

Bioassay for Elicitor Activity. All fractions (Figure 2A) were assayed for biological activity using the soybean cotyledon assay (Hahn et al., 1981; Nothnagel et al., 1983; Smith and Goldering, 1989). Soybean cotyledons accumulate glyceollin in response to elicitors. The UV absorbance at 286 nm is linearly related to the phytoalexin concentration in the sample solution (Ayers, et al., 1976a,b). The assay was performed on cotyledons removed from 14-day-old soybean seedlings (*Glycine max* L. var. Evans) with the following modifications. Cotyledons were cut on the under surface and incubated in an Eppendorf tube that contained 100 μL of the elicitor solution (4 mg/mL). The elicitor solution also contained gentamycin sulfate (15 mg/mL). After 24 h in the dark, aliquots were pipetted from three vials, combined, and diluted 1:20 before the UV absorption was read. Each value was an average of three individual trials, and the values were corrected for background (gentamycin sulfate alone). The 100% (A_{max}) value was obtained using 10 mM $HgCl_2$ as abiotic elicitor.

Reductive Amination of Oligosaccharides. The procedure of Hogeland et al. (1992) was followed. In a 5-mL reaction vial unsaturated pentagalacturonic acid (ca. 200 μg) was dissolved in water (50 μL), and *n*-hexylamine (50 μL) was added. Ethanol (100%, 20 μL) was added to form a homogeneous solution. Glacial acetic acid (20 μL) was added, and the solution was kept at 25 °C for 3.5 h. After addition of $NaBH_3CN$ (1.5 M, 200 μL), ethanol (100%, 40 μL) was added and the solution was adjusted to pH 5 using glacial acetic acid. The reaction was terminated after 18 h at 25 °C by addition of water (1 mL). The solution was extracted with dichloromethane (500 μL) to remove excess *n*-hexylamine. The aqueous layer was washed twice with dichloromethane (300 μL each) and then concentrated to a final volume of 100 μL.

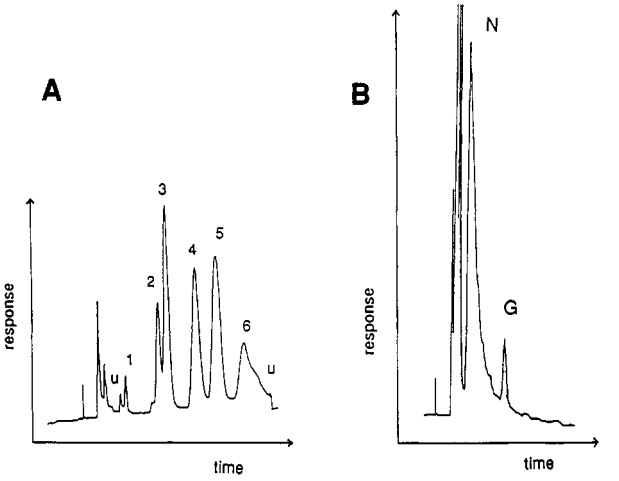

Figure 1. High-pH anion exchange chromatography pulsed amperometric detector (HPAEC-PAD) response for totally hydrolyzed cell wall material (CWM). (A) Neutral monosaccharides of the extracted hop CWM: 1, fucose; 2, rhamnose; 3, arabinose; 4, galactose; 5, glucose; 6, xylose; u, unknown. (B) Acidic monosaccharides: N, neutral monosaccharides; G, galacturonic acid.

Desalting of Chemically Derivatized Oligogalacturonic Acids. Desalting of derivatized oligogalacturonic acids was carried out on a Sephadex G-10 column (1.5 × 6 cm) The sample was eluted with water at a flow rate of 0.2 mL/min. All fractions (0.5 mL each) were tested for carbohydrate content using the orcinol method. Fractions containing carbohydrate were pooled and lyophilized.

Peracetylation of *n*-Hexylamine Derivatized Oligosaccharides. The procedure of Blakeney et al. (1983) was followed. 1-Methylimidazole (40 μL) and acetic anhydride (600 μL) were added to a solution of *n*-hexylamine-derivatized unsaturated pentagalacturonide dissolved in water (50 μL). The solution was kept at 25 °C for 15 min. The reaction was quenched by addition of water (1 mL). The solution was extracted three times with dichloromethane (300 μL each). The organic layer was washed with water (800 μL) and concentrated to a final volume of 10 μL. This solution was used directly for FABMS analysis.

Fast Atom Bombardment Mass Spectrometry (FABMS). Fast atom bombardment mass spectrometry (FABMS) experiments were carried out on a Kratos MS-50 TC double-focusing mass spectrometer in either the negative ion mode or the positive ion mode, operating at a resolution of 1000 for raw data acquisition. Xenon gas was used to generate the primary ionizing beam from an Ion-Tech gun operated at 7–8 kV. Ions were accelerated from the ion source at 8 kV. A modified Kratos postacceleration detector was operated at 25 keV. The scan rate was 30 s/decade. Cesium iodide in tetraethylene glycol was used as a calibration standard. Sample solution (2 μL) was mixed with glycerol matrix (2 μL) on the target.

RESULTS AND DISCUSSION

Cell wall material (CWM) from hop leaves was prepared using a procedure that achieves maximum solubilization of starch and cytoplasmic components with minimum loss of the nonstarchy cell wall polysaccharides. Total hydrolysis of the CWM in either trifluoroacetic acid (TFA) or sulfuric acid confirmed that the material obtained consisted only of carbohydrates (Figure 1 and Table I). Using both high-pH anion-exchange chromatography with a pulsed amperometric detection (HPAEC-PAD) system and colorimetric assays (Dubois et al., 1956; Blumenkrantz and Asboe-Hansen, 1973), the monosaccharide composition of the CWM was established.

The high content of galacturonic acid confirms that the isolated material was indeed pectin from cell walls.

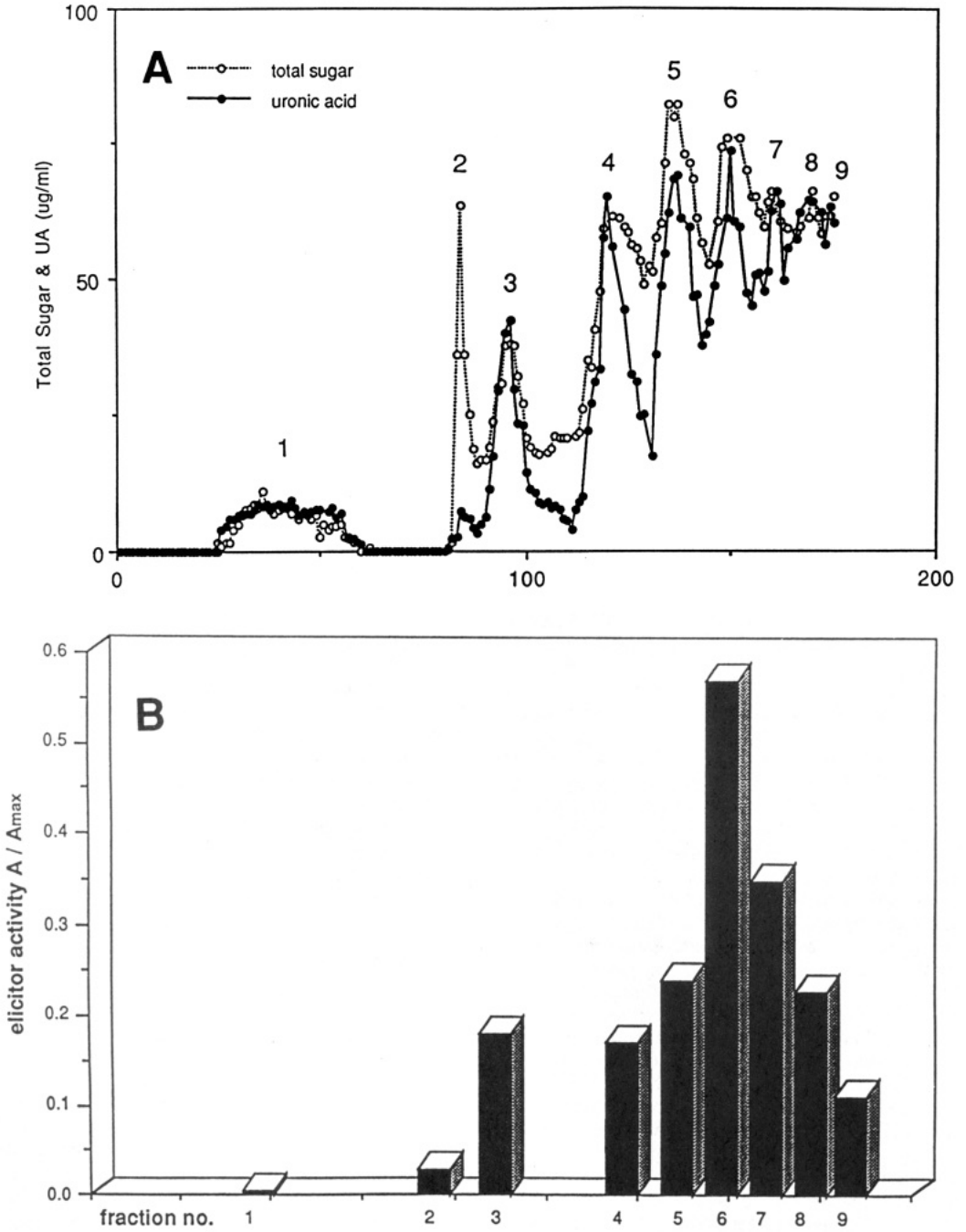

Figure 2. (A) Chromatogram of partially hydrolyzed cell wall material (CWM). After partial hydrolysis, the mixture was applied to a QAE-Sephadex A-25 ion-exchange column and eluted with the gradient described under Experimental Methods. Data point assays were for total sugar (○) and uronic acid (●). (B) Bioassay for elicitor activity in pooled fractions using a soybean cotyledon assay method (see Experimental Methods).

Table I. Monosaccharide Composition of Hop Cell Wall Material (Hop Variety 64107)

carbohydrate	% of total sugar	carbohydrate	% of total sugar
1, fucose	0.1	5, glucose	16.6
2, rhamnose	6.8	6, xylose	8.2
3, arabinose	14.8	G, galacturonic acid	39.5
4, galactose	12.9		

Homogalacturonans and rhamnogalacturonans are responsible for the high abundance of the monosaccharides galacturonic acid (39.5%) and rhamnose (6.8%) in the sample. Other pectin components such as arabinogalactans are reflected by the monosaccharides arabinose (14.8%) and galactose (12.9%). Also, xyloglucans were present in appreciable amounts as suggested by the relatively high content of the monosaccharides xylose (8.2%) and glucose (16.6%). Cellulose could also contribute to the high glucose content in the sample. The only acidic sugar present was shown to be galacturonic acid (Figure 1B).

Partial hydrolysis of the CWM led to a mixture of oligomers. Separation on a QAE-Sephadex A-25 anion-exchange column (Figure 2A) led to nine peaks as determined by colorimetric assays for galacturonic acid and total sugar (Dubois et al., 1956; Blumenkrantz and Asboe-Hansen, 1973) on the individually collected fractions. Determination of total sugar and uronic acid content in each of the fractions showed that all but fraction 2 contained acidic sugars. Since the peak maxima for total sugar and uronic acid are almost equivalent in all other fractions, it is assumed that these fractions contain mainly galacturonic acid. The later eluting fractions contain

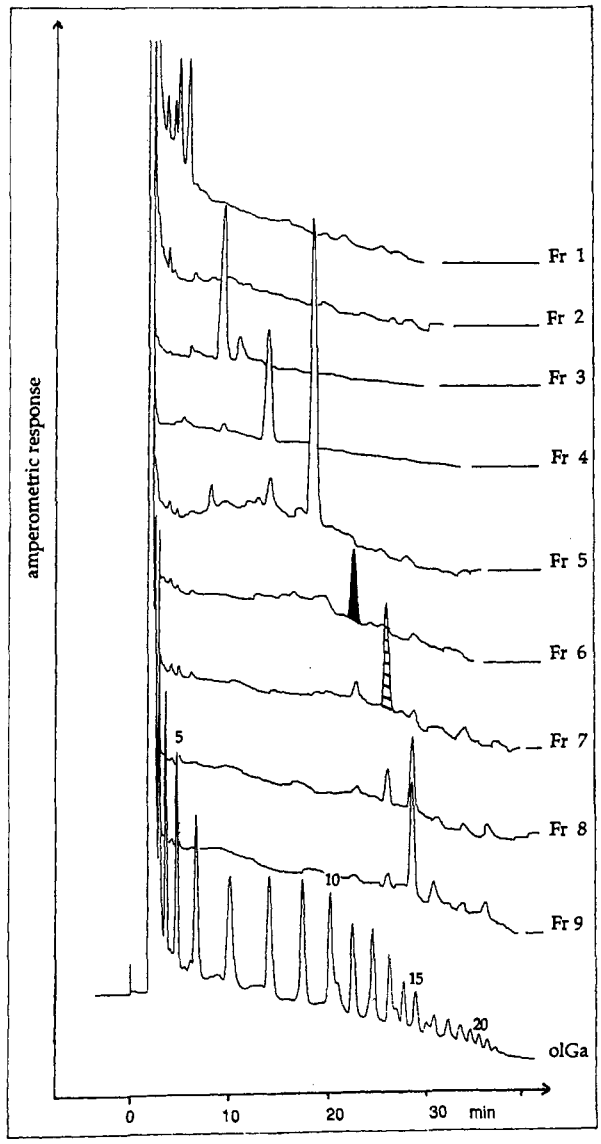

Figure 3. High-pH anion exchange chromatography pulsed amperometric detector (HPAEC-PAD) response to partially hydrolyzed cell wall material (CWM). Fr 1–9 represent the corresponding fractions from the QAE Sephadex column. olGa represents the oligogalacturonic acid mixture derived from partial hydrolysis of commercial poly(galacturonic acid). The number of monomeric units in oligomers is represented by numbers on top of peaks.

higher oligomers of acidic sugars and, therefore, are retained longer on the resin than the early eluting smaller oligomers.

Analysis of the nine fractions by HPAEC-PAD further substantiated that all but fraction 2 consisted of galacturonic acid oligomers (Figure 3). High-pressure liquid chromatograms (HPLC) with UV detection showed that the pooled fractions also were relatively free of extraneous absorbing components. In fraction 7 only a little of fraction 6 material was present. Fraction 8 shows a mixture of fractions 7 and 9. Each fraction showed peaks with increasing retention times, indicating that the fractions with higher numbers contained longer oligomers of the isolated galacturonides. The retention times of the hop fraction components were compared to a mixture of oligogalacturonic acids (olGa) prepared from commercially available poly(galacturonic acid) (bottom chromatogram, Figure 3). This comparison indicated that fraction 6 from the hop CWM, for example, eluted like an 11- or 12-mer.

It was observed by FABMS that the individual components represented by the chromatographic peaks from the mixture of partially hydrolyzed commercial poly(galacturonic acid) (olGa) contained a galacturonic acid oligomer of varying number of monomer units. Negative ion FABMS analysis of the galacturonic acid mixture confirmed the presence of galacturonic acid oligomers elongated by monomer units (Figure 4). Each of the mass peaks observed (m/z 545, 721, 897, 1073, 1249, and 1425) are separated by 176 D from one another, which is just the difference of one monomer unit.

The degree of oligomerization of the isolated elicitor fractions was unambiguously established by FABMS for the native carbohydrates. In addition the possibility of methyl esterification in the sample could be addressed. It is known that pectic polygalacturonides are methylated to a variable degree (Aspinall and Fanous, 1984; Selvendran and O'Neill, 1987). In the present studies FABMS analysis showed that the partially hydrolyzed hop fractions contained only unesterified galacturonic acid oligomers (Figures 5 and 6). Negative ion FABMS analysis of the separated hop fractions showed that fraction 3 contained a trimer, fraction 4 a tetramer, fraction 5 a pentamer (Figure 5), fraction 6 a hexamer, fraction 7 a heptamer, and fraction 9 an octamer of oligogalacturonic acid (Figure 6). These masses can only be accounted for by the absence of esterification, and nowhere in the spectra was there any hint for the loss of methyl groups. However, all recorded oligomers showed mass peaks 18 D lower than the standard mixture derived from commercial poly(galacturonic acid). For example, the trimer of the hop sample showed a molecular ion peak with m/z 527, the tetramer a molecular ion with m/z 703, and so on. This mass difference of 18 D can be explained by the existence of an unsaturated galacturonic acid oligomer.

Partial hydrolysis of the hop CWM cannot account for the formation of the unsaturated galacturonosyl residue, since the commercial poly(galacturonic acid) was subjected to a very similar procedure to test this possibility. This mixture, which served as a reference standard, showed no evidence of unsaturation. And since no methyl esterification was observed in the CWM, a β-elimination reaction (Albersheim et al., 1960) involving a methyl ester group at C-5 also can be ruled out. It is assumed that the unsaturated material in the hop samples originated directly in the plant source. The possibility of an endopectate lyase causing enzymatic cleavage during extraction, however, cannot be ruled out.

The presence of unsaturated galacturonosyl residues in the hop samples also explains their retention times by HPLC. It is known that unsaturated acidic sugars cause a dramatic shift toward longer retention times (Paskach et al., 1992) because of conjugation in which the electrons of the double bond can be delocalized into the carboxy group of the molecule. The double negatively charged carboxy function is assumed to interact more strongly with the ion-exchange resin, resulting in later retention times. Thus, the commercial poly(galacturonic acid) was not an appropriate standard with which to determine HPLC retention times. Comparison of the retention times with the saturated compounds initially led to misinterpretation of the actual degree of oligomerization in the hop oligomers. Only additional FABMS experiments were able to resolve this issue by allowing a determination of the exact molecular weights of the compounds and their degrees of oligomerization.

The analysis of each of the fractions by FABMS resulted in fragment ion peaks in addition to the molecular ion signal. Fraction 9 was chosen to demonstrate the observed fragmentation by negative ion FABMS (Figure 7). The molecular ion with m/z 1407 represents the native unsaturated octagalacturonide. Fragmentation from the

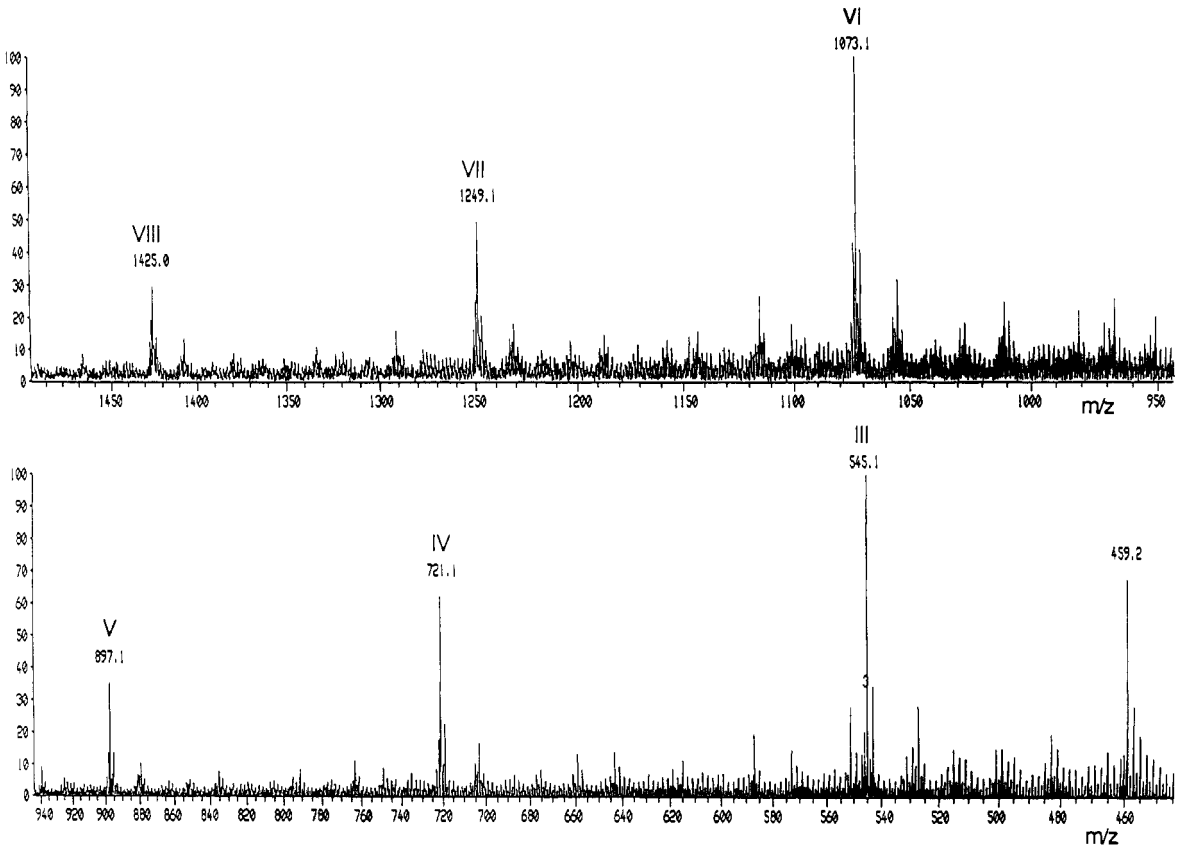

Figure 4. Negative ion fast atom bombardment mass spectrum (FABMS) of oligogalacturonic acid mixture derived from partially hydrolyzed poly(galacturonic acid) showing molecular ion peaks ($[M - H]^-$) for the trimer (m/z 545), tetramer (m/z 721), pentamer (m/z 897), hexamer (m/z 1073), heptamer (m/z 1249), and octamer (m/z 1425).

reducing end of the molecule produces peaks 176 D lower for the loss of each saturated monomer. This is apparent from the sequential fragment ion peaks with m/z 1231 and 1055. Fragmentation from the nonreducing end produces a peak 158 D lower (peak with m/z 1249), thereby indicating loss of the terminal unsaturated galacturonosyl residue. Further loss of saturated galacturonosyl residues is observed (e.g., peak with m/z 1073). The observed fragmentation that occurred during recording of the spectra proved that the site of unsaturation was a terminal galacturonosyl residue (Figure 7). The parent molecule and the fragments that still bear the unsaturated ring in the chain show a secondary fragmentation producing peaks 44 D lower (peaks with m/z 1363, 1187, and 1011). This can be rationalized by the loss of carbon dioxide at the unsaturated ring. With this information the structure of the unsaturated octagalacturonide was deduced (Figure 7).

Previous studies by NMR on endopolygalacturonic acid lyase-released elicitors from soybean cell walls showed unsaturation at the 4,5-position (Davis et al., 1986b). It is assumed that the double bond of the hop galacturonic acids also is at the 4,5-position in the terminal nonreducing end ring.

Negative ion FABMS experiments of the native oligogalacturonides showed that fragmentation occurs from both ends of the molecule. To further examine whether the unsaturated ring is located at the nonreducing end or at the reducing end, derivatization reactions to label the reducing end were carried out. Fraction 5 gave the highest yield of carbohydrate oligomers, and this fraction, accordingly, was used for the derivatization reactions. The unsaturated pentagalacturonide was reductively aminated with n-hexylamine (Hogeland et al., 1992), desalted, and then peracetylated. When the reductively aminated oligosaccharide was peracetylated, the derivative could be extracted into an organic layer to give a salt-free sample.

The native oligogalacturonic acids gave good quality negative ion FABMS spectra. In the positive ion mode carbohydrates do not fragment as easily, but it was possible to record good quality spectra of the derivatized oligogalacturonides in the positive ion mode. Derivatization by reductive amination followed by peracetylation not only adds a tag at the reducing end of the molecule but also alters the fragmentation characteristics of the compounds to provide useful structural information.

The FABMS spectrum of reductively aminated and peracetylated unsaturated pentagalacturonic acid shows (Figure 8) that the expected molecular ion at m/z 1470 was not present. However, characteristic losses of acetic acid units (m/z 60) from this ion were recorded ($MH^+ - 60$ with m/z 1410, $MH^+ - 120$ with m/z 1350, $MH^+ - 180$ with m/z 1290) (Domon et al., 1989; Hogeland et al., 1992). A second set of peaks separated by 60 D was observed at m/z 1151 and 1091, respectively. The fragment ions represented by these peaks resulted from the loss of the terminal unsaturated peracetylated galacturonosyl residue (259 D increment) located at the nonreducing end of the molecule. These results support the conclusion that the unsaturated galacturonosyl residue is at the nonreducing end of the oligomer.

A peak at m/z 1453 appears to be due to the presence of a trans anhydride. A carboxy function of the oligomer could have reacted with excess acetic anhydride to give a mixed anhydride, thereby adding 42 D to the mass. Loss of acetic acid from this species could produce the peak at m/z 1393.

All fractions were isolated and assayed for biological activity. Since the phytoalexins from hops are not known yet, the potential elicitor activity of the isolated hop CWM

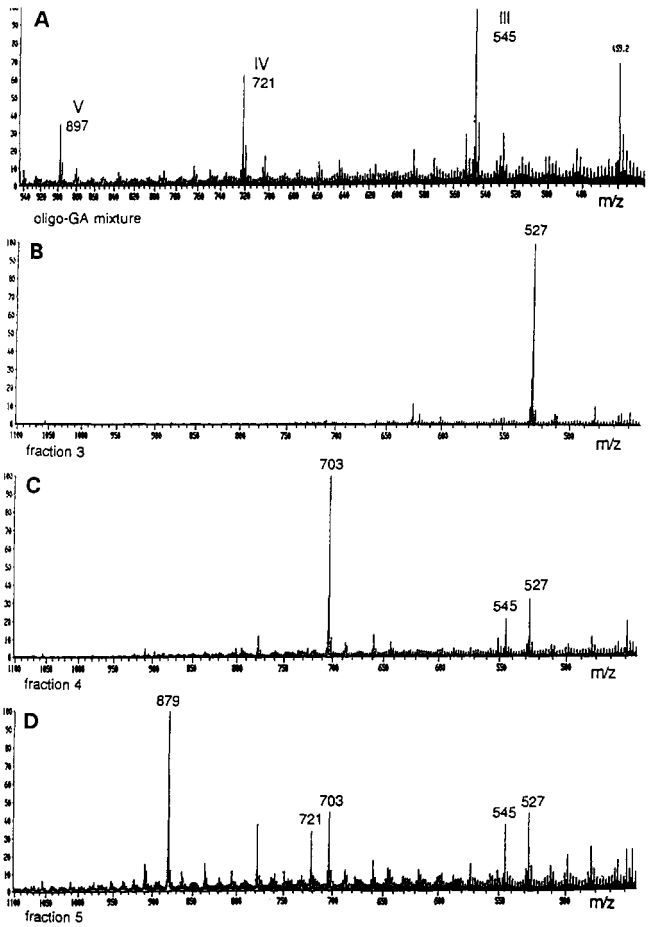

Figure 5. Negative ion fast atom bombardment mass spectrum (FABMS) of native hop elicitor fractions. (A) Oligogalacturonic acid mixture derived from poly(galacturonic acid) with molecular ion $(M - H)^-$ peaks for the trimer (m/z 545), tetramer (m/z 721) and pentamer (m/z 897). (B) Elicitor fraction 3 showing an unsaturated trigalacturonide molecular ion peak $(M - H)^-$ with m/z 527. (C) Fraction 4 showing an unsaturated tetragalacturonide molecular ion peak $(M - H)^-$ with m/z 703 and fragment ions. (D) Fraction 5 showing the unsaturated pentagalacturonide molecular ion peak $(M - H)^-$ with m/z 879 and fragment ions.

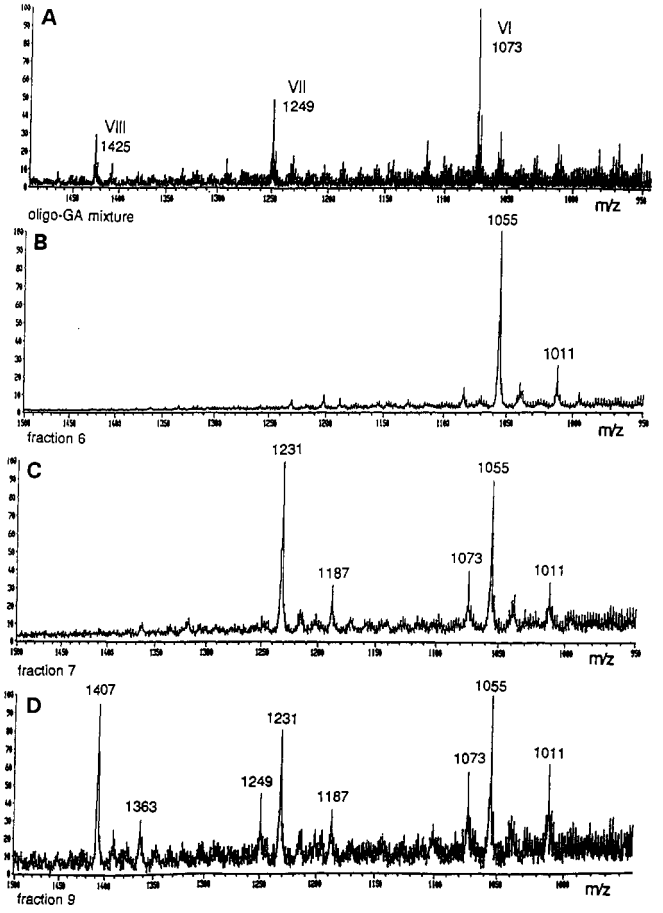

Figure 6. Negative ion fast atom bombardment mass spectrum (FABMS) of native hop elicitor fractions. Molecular and fragment ion peaks appear for (A) oligogalacturonic acid mixture derived from poly(galacturonic acid) including the hexamer (m/z 1073), heptamer (m/z 1249), and octamer (m/z 1425). Molecular ion and fragment ion peaks for unsaturated galacturonides are shown in (B) fraction 6, hexagalacturonide (m/z 1055), (C) fraction 7, heptagalacturonide (m/z 1231), and (D) fraction 9 octagalacturonide (m/z 1407).

fractions was tested using the soybean cotyledon assay (Figure 2B). It is well established that the phytoalexin, glyceollin is accumulated in soybean tissues in response to elicitors (Albersheim and Valent, 1978). This model system is well described, and the increase in phytoalexin concentration is easy to monitor (Hahn et al., 1987; Nothnagel et al., 1983; Smith and Goldring, 1989). The UV absorbance at 286 nm is linearly related to the phytoalexin concentration in the sample solution (Ayers et al., 1976a,b). Using this assay, fraction 6 was identified as the sample with the greatest activity in phytoalexin elicitation. This same fraction was used to show the dependency of the elicitor activity in the soybean cotyledon on elicitor concentration (Table II). These results clearly demonstrate the elicitor activity, and though the phytoalexin was not isolated, it it is assumed to be glyceollin (Albersheim and Valent, 1978).

An accurate comparison of the elicitor activities among the different oligomers is difficult because the fractions isolated (Figure 2A) are not pure (Figure 3). However, a rough comparison can be made. If fractions 7 and 8 are excluded, because they clearly contain significant amounts of the hexamer and heptamer, respectively, the relative elicitor activities are approximately 3:3:4:10:2 for the trimer, tetramer, pentamer, hexamer, and octamer. In a recent study Komae et al. (1990) have shown that unsaturated galacturonic acids with $n = 3$–12 isolated from

Table II. Effect of Amount of Oligomer in Fraction 6 on Elicitor Activity in Soybean Cotyledons

elicitor (μg)/ cotyledon[a]	elicitor activity[b]	elicitor (μg)/ cotyledon[a]	elicitor activity[b]
0	0	100	0.22
10	0.009	200	0.40
20	0.01	400	0.57
50	0.12		

[a] Cotyledons from 14-day-old soybean seedlings. [b] Elicitor activity = $A/A_{max(10mM\ mercuric\ chloride)}A_{286nm}$.

the seeds of *Ficus awkeotsang* Makino function as phytoalexin elicitors in soybeans. Activity for the different oligomers varies widely, but the unsaturated hexa- to undecagalacturonides represent activity maxima in groups penta- to heptagalacuturonides and nona- to dodecagalacturonides. The oligosaccharide with the highest elicitor activity solubilized from soybean cell walls by endopolygalacturonic acid lyase was the unsaturated undecagalacturonide (Davis et al.,1986b).

A quantitative comparison cannot be made with data in the Komae paper, because the oligomer mixture was used. The reported elicitor activity–oligomer concentration response is somewhat lower than the response observed for the hexaglacturonide (Table II). This reduced elicitor response to the mixture of oligomers in comparison to the pure hexaglacturonide is consistent with dilution by less active elicitors. The agreement of the data here with the reported elicitor activities is remarkable, especially

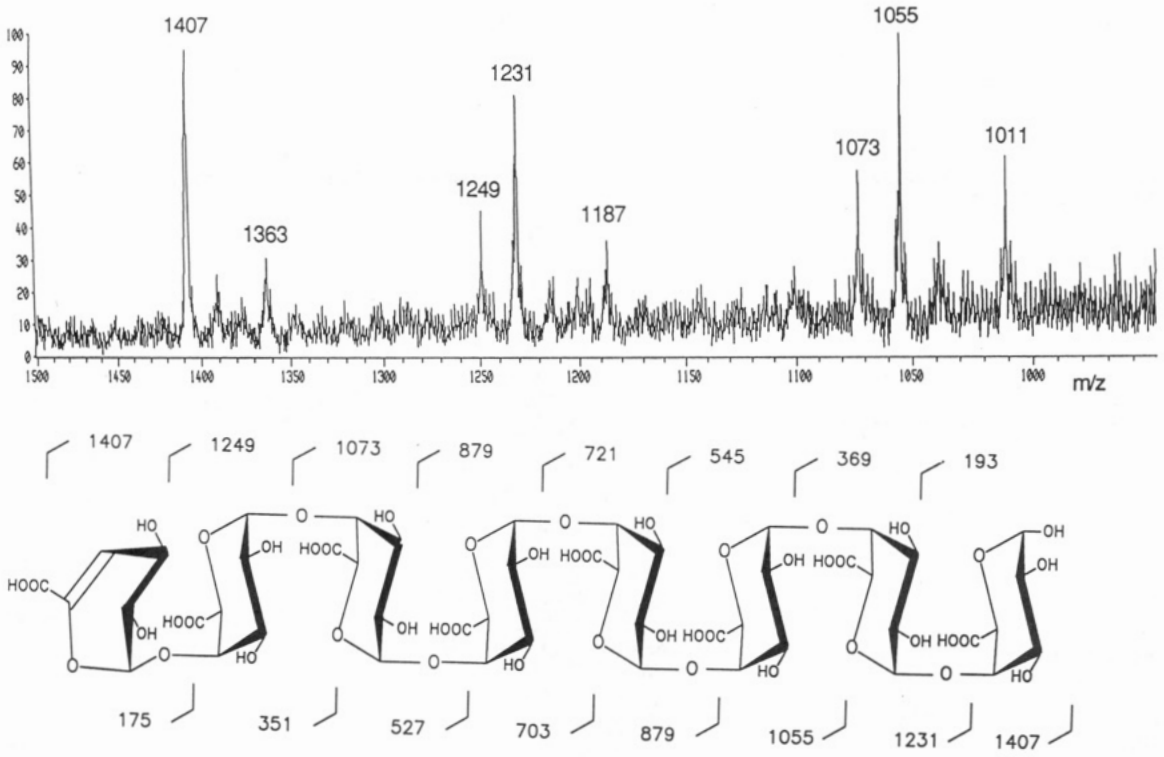

Figure 7. Partial negative ion fast atom bombardment mass spectrum and fragmentation pattern for the unsaturated galacturonide in hop fraction 9 (octagalacturonide).

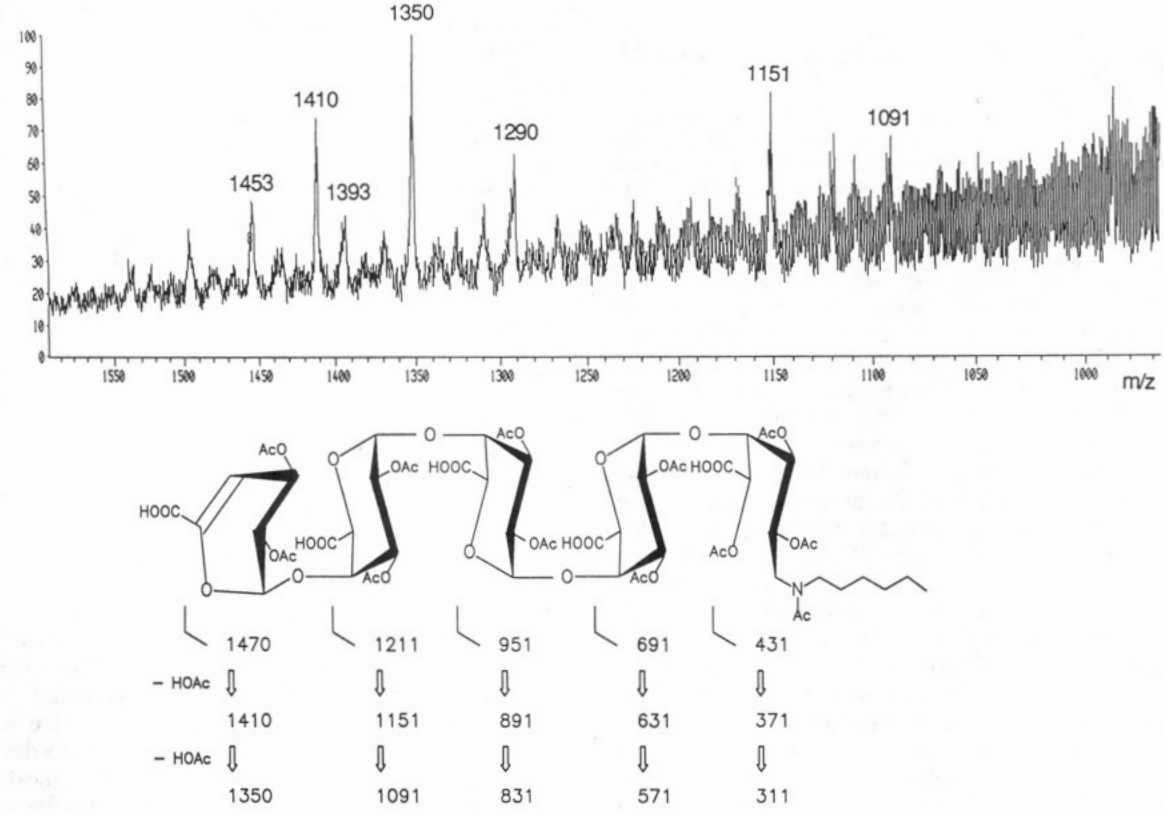

Figure 8. Positive ion fast atom bombardment mass spectrum (FABMS) of derivatized unsaturated pentagalacturonic acid. Reductively *n*-hexylaminated and peracetylated hop fraction 5 shows peaks for $MH^+ - 60$ (m/z 1410), $MH^+ - 120$ (m/z 1350), and $MH^+ - 180$ (m/z 1290).

considering that A_{max} values depend highly on the reference compound used, which in the present case was 10 mM mercuric chloride.

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LITERATURE CITED

Albersheim, P.; Valent, B. S. Host-Pathogen Interactions in Plants. *J. Cell Biol.* **1978**, *78*, 627–643.

Albersheim, P.; Neukom, H.; Deuel, H. Splitting of pectin chain molecules in neutral solutions. *Arch. Biochem. Biophys.* **1960**, *90*, 46–51.

Albersheim, P.; Darvill, A.; Augur, C.; Cheong, J.-J.; Eberhard, S.; Hahn, M. G.; Marfa, V.; Mohnen, D.; O'Neill, M. A.; Spiro, M. D.; York, W. S. Oligosaccharins: Oligosaccharide regulatory molecules. *Acc. Chem. Res.* **1992**, *25*, 77–83.

Aspinall, G. O.; Fanous, H. K. Structural investigations on the non-starchy polysaccharides of apples. *Carbohydr. Polym.* **1984**, *4* (3), 193–214.

Ayers, A. R.; Ebel, J.; Valent, B.; Albersheim, P. Host-pathogen interactions. *Plant Physiol.* **1976a**, *57*, 760–765.

Ayers, A. R.; Valent, B.; Ebel, J.; Albersheim, P. Host-pathogen interactions. *Plant Physiol.* **1976b**, *57*, 766–774.

Bishop, P. D.; Ryan, C. A. Plant cell wall polysaccharides that activate natural plant defenses. *Methods Enzymol.* **1987**, *138*, 715–724.

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291–299.

Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484–489.

Brooks, C. J. W.; Watson, D. G. Phytoalexins. *Nat. Prod. Rep.* **1985**, *2*, 427–459.

Darvill, A. G.; Albersheim, P.; McNeil, M.; Lau, J. M.; York, W. S.; Stevenson, T. T.; Thomas, J., Doares, S.; Gollin, D. J.; Chelf, P.; Davis, K. Structure and function of plant cell wall polysaccharides. *J. Cell Sci. Suppl.* **1985**, *2*, 203–217.

Davis, K. R.; Darvill, A. G.; Albersheim, P.; Dell, A. Host-Pathogen interactions. *Plant Physiol.* **1986a**, *80*, 568–577.

Davis, K. R.; Darvill, A. G.; Albersheim, P.; Dell, A. Host-Pathogen Interactions. *Z. Naturforsch.* **1986b**, *41C*, 39–48.

Domon, B.; Müller, D. R.; Richter, W. J. Identification of interglycosidic linkages and sugar constituents in disaccharide subunits of larger glycosides by tandem mass spectrometry. *J. Org. Mass Spectrom.* **1989**, *24*, 357–359.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars of related substances. *Anal. Chem.* **1956**, *28*, 350–356.

Green, T. R.; Ryan, C. A. Wound-induced proteinase inhibitor in plant leaves: A possible defense mechanism against insects. *Science* **1972**, *175*, 776–777.

Grisebach, H.; Ebel, J. Phytoalexins, chemical defense substances of higher plants? *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 635–647.

Hahn, M. G.; Darvill, A. G.; Albersheim, P. Host-pathogen interactions. *Plant Physiol.* **1981**, *68*, 1161–1169.

Hogeland, K. E., Jr.; Arbogast, B.; Deinzer, M. L. Liquid secondary ion mass spectrometry analysis of permethylated, n-hexylamine derivatized oligosaccharides. Application to baculovirus expressed mouse interleukin-3. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 345–352.

Keen, N. T. Phytoalexins and their involvement in plant disease resistance. *Iowa State J. Res.* **1986**, *60*, 477–499.

Keen, N. T. Phytoalexins and their elicitors. *ACS Symp. Ser.* **1990**, *No. 439*, 114–129.

Komae, K.; Komae, A.; Misaki, A. A 4,5-unsaturated low molecular oligogalacturonide as a potent phytoalexin-elicitor isolated from polygalacturonide of *Ficus awkeotsang*. *Agric. Biol. Chem.* **1990**, *54*, 1477–1484.

Kuć, J.; Rush, J. S. Phytoalexins. *Arch. Biochem. Biophys.* **1985**, *236*, 455–472.

Loper, G. M. Effect of aphid infestation on the coumestrol content of alfalfa varieties differing in aphid resistance. *Crop Sci.* **1968**, *8*, 104–106.

McNeil, M.; Darvill, A. G.; Fry, S. C.; Albersheim, P. Structure and function of the primary cell walls of plants. *Annu. Rev. Biochem.* **1984**, *53*, 625–663.

Nothnagel, E. A.; McNeil, M.; Albersheim P.; Dell, A. Host-pathogen interactions. *Plant Physiol.* **1983**, *71*, 916–926.

Paskach, T. J.; Lieker, H.-P.; Reilly, P. J.; Thielecke, K. High-performance anion-exchange chromatography of sugars and sugar alcohols on quaternary ammonium resins under alkaline conditions. *Abstracts of Papers*, 203rd National Meeting of the American Chemical Society, San Francisco, April 5–10, 1992; American Chemical Society, Washington, DC, 1992; No. 86.

Paxton, J. D. Phytoalexins-A working definition. *Phytopath. Z.* **1981**, *101*, 106–109.

Robertsen, B. Elicitors of the production of lignin-like compounds in cucumber hypocotyls. *Physiol. Mol. Plant Pathol.* **1986**, *28*, 137.

Russell, G. B. Phytochemical resources for crop protection. *N. Z. J. Technol.* **1986**, *2*, 127–134.

Russell, G. B.; Sutherland, O. R. W.; Hutchins, R. F. N.; Christmas, P. E. Vestitol: A phytoalexin with insect feeding-deterrent activity. *J. Chem. Ecol.* **1978**, *4*, 571–579.

Ryan, C. A. Oligosaccharide signalling in plants. *Annu. Rev. Cell Biol.* **1987**, *3*, 295–317.

Ryan, C. A. Oligosaccharides as recognition signals for the expression of defense genes in plants. *Biochemistry* **1988**, *27*, 8879–8883.

Selvendran, R. R.; O'Neill, M. A. Isolation and analysis of cell walls from plant material. *Methods Biochem. Anal.* **1987**, *32*, 25–153.

Selvendran, R. R.; March, J. F.; Ring, S. G. Determination of aldoses and uronic acid content of vegetable fiber. *Anal. Biochem.* **1979**, *96*, 282–292.

Smith, C. J.; Goldring, P. Endogenous elicitor of phytoalexin synthesis from glycine max L. *Biochem. Soc. Trans.* **1989**, *17*, 200–201.

Sutherland, O. R. W.; Russell, G. B.; Biggs, D. R.; Lane, G. A. Insect feeding deterrent activity of phytoalexin isoflavonoids. *Biochem. Systems Ecol.* **1980**, *8*, 73–75.

West, C. A. Fungal elicitors of the phytoalexin response in higher plants. *Naturwissenschaften* **1981**, *68*, 447–457.

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