Hop cell wall material (CWM) extracted from hop leaves (*Humulus lupulus*) was purified and characterized. The total sugar content, uronic acid content and monosaccharide composition of the CWM were determined. Galacturonic acid is the major component in the CWM. A mixture of unsaturated oligogalacturonides were released from purified hop CWM by autoclaving. The biological activity of these oligomers was tested for their ability to elicit phytoalexins. The oligomer with hexagalacturonic acid possessed the greatest biological activity. Column chromatography and high-pH anion exchange chromatography were used for the sample separation and purification. Fast-atom-bombardment mass spectrometry (FAB-MS) was used for the structure elucidation. The FAB-MS spectrum showed that the unsaturated galacturonosyl residue was located at the nonreducing terminus of the oligomer.
Isolation, Purification, and Structure Elucidation
of Hop Plant Elicitor

by
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This thesis is dedicated to my husband Weixuan, my daughter Vivian and my parents.
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Introduction

Introduction to Phytoalexins

For the past few decades many aspects of the topic of plant disease resistance have been studied extensively. Plants are exposed to attack by many environmental stresses. One of these stresses is infection by pathogens. Plants are unable to escape from these threats by physically removing themselves from the hostile environment because of their sedentary nature. Fortunately, during the course of evolution plants have developed many ways for survival. Biochemical and chemical defense processes are carried out by the plants to protect themselves from attacks. Since plants do not possess an immune system as do humans and animals, the question is what functional methods do plants have to protect themselves and how do they work? Accumulation of phytoalexins is one of the mechanisms for disease resistance in plants. The accumulation only occurs after cell damage, by physical processes, chemical exposure, or after infection by microorganisms.

The studies of Muller and Borger on the phytophthora resistance in potatoes were among the first experiments that elucidated the biochemical defense mechanism. A purified defense substance was called "phytoalexin" (phyto = plant, alexin = to defend). Later, Paxton redefined the term. Today, phytoalexins are considered to be low molecular weight, antimicrobial compounds that are either synthesized by plants or accumulated in plants after exposure to microorganisms. The first phytoalexin to be
identified was pisatin, which was extracted from seed pods of peas (*Pisum sativum*) that were infected with *Monilinia fructicola.*

**Structures of Phytoalexins**

There are more than 200 phytoalexins discovered to date. The types of chemical structures of phytoalexins tend to be associated with particular plant families. Phytoalexins including isoflavonoids, sesquiterpenes, furanoterpenoids, polyacetylenes, dihydrophenanthrenes and other substances have been identified in various plants. Compounds 1-8 (Scheme 1) have been known for a long period of time, and recently compounds 9-12 (Scheme 2) have been identified as phytoalexins. The dianthalexine 1 from carnation and avenalumin 2 from oats are nitrogen-containing phytoalexins. Brassilexin 9 from *Cruciferae* is a sulfur containing indole derivative, and the oryzalexins 11 from rice is a biphenyl phytoalexin. Capsidiol 4 from the *Solanaceae* is a sesquiterpenoid phytoalexin. Pisatin 3 and glyceollin 7 are the legume isoflavonoid phytoalexins. Obviously, various plants may produce phytoalexins with different structures. Nevertheless, phytoalexin activity is structure related. Some investigations have shown that minor structural changes will cause decreasing antibiotic activity.

**Accumulation and Resistance Functions of Phytoalexins**

The accumulation of phytoalexins can be induced, or elicited, by a broad spectrum of substances and environmental conditions. Phytoalexins are produced by plants not only in response to interactions with fungi, bacteria, viruses, nematodes and other living organisms, but also by chemical and physical treatments and exposure to the products of microbial metabolism. By studying phytoalexin accumulation, it has been
Scheme 1. Some examples of structures of phytoalexins: dianthalexine (1), avenalumin I (2), pisatin (3), capsidiol (4), casbene (5), safynol (6), glyceollin (7), and viniferin (8).
observed that different plants produce different structures of phytoalexins, and different inducing agents elicit different compositions of the phytoalexin mixture in plants that produce several phytoalexins.

The theory about accumulation of phytoalexin stimulated research in this field. VanEtten and coworkers have described a pathway for the pea- interaction. Most of the key events that occur during the interaction of a plant with a pathogen are illustrated in Figure 1. In addition it was observed that the induction of phytoalexin accumulation and phytoalexin activity against microorganisms were of low specificity. So far the functional role of phytoalexins in plant disease resistance is only
beginning to be understood. It is also clear that phytoalexins do not account for all cases of disease resistance. Phytoalexin disease resistant mechanism studies has been discussed by Keen.5,6 There are two general mechanisms expected to be involved. One type, called general resistance, is effective against a wide range of microorganisms. The other type, called specific resistance, is controlled by single disease resistance genes. The observation of plant resistance and susceptibility to pathogens may be explained as follows with the help of Figure 2.10 An incompatible plant-pathogen interaction exists in a resistant plant. During this interaction the recognition of the infecting pathogen by the plant occurs, and presumably results in plant resistance. Whereas compatible interaction exists in a susceptible plant. In this event the recognition of the infecting
Figure 2. Schematic elucidation of plant cell resistance to incompatible parasites. 
a: The interaction, or recognition, between plant and the compatible pathogen does not occur due to lack of suitable surface structures. The plant resistance cannot be initiated. b: The interaction between plant and the incompatible pathogen can induce the accumulation of phytoalexins, which in turn resist the pathogen.\textsuperscript{10}
pathogen by the plant does not occur and plant resistance does not happen. The pathogen develops easily in the plant tissue, so the disease occurs.

**Classification and Characteristics of Elicitors**

The term "elicitors" was introduced by Keen and his colleagues. These substances can induce phytoalexin production. Unknown compositions were prepared in the earlier studies of elicitor function. In 1962 the first elicitors from Monilinia fructicola, a fruit parasite, were isolated and characterized by Cruickshank and Perrin. This elicitor, named monilicolin A, is a peptide with a molecular weight of approximately 8000 Daltons. To date many investigations have demonstrated conclusively that elicitors are widespread in nature and that a single elicitor is able to induce phytoalexins from different plants. Three types of elicitors have been described by Bailey and Brooks and coworkers. These three types and their characteristics are described below.

**Abiotic Elicitors**

Very diverse materials have been found to act as abiotic elicitors. Abiotic elicitors cannot be found in the natural elicitation process, but they have frequently been used for studies of mechanisms in the elicitation process. They can be separated into several kinds of materials as follows. Chemicals: i) salts of heavy metals, e.g. mercury and copper; ii) respiratory inhibitors, e.g. sodium iodoacetate, sodium fluoride, potassium cyanide and 2,4-dinitrophenol; iii) compounds with plant growth regulating properties, e.g. ethylene, indolyl-3-acetic acid, 2,4-dichlorophenoxyacetic acid. Physical treatments, include exposure to UV light and partial freezing, but not wounds from cutting, bruising or pricking.
Biotic Elicitors

Elicitors of biological origin are referred to as biotic elicitors. Biotic elicitors, including those from fungi, bacteria, and viruses, have been studied extensively and many of them have been isolated and characterized. They can be obtained by heating or partial hydrolysis of purified cell walls but still possess biological activity. In other words, culture filtrates, or killed fungal cells, are as effective as the living organism. Three types of compounds have been studied: peptides, glycopeptides and polysaccharides, which includes glucans, glucomannans, chitosan and oligomers of glucosamine. Two of the best studied polysaccharides are described below.

**β-Glucan** Many fungi produce elicitors with β-glucan structure which can be isolated following autoclaving of their mycelial cell wall. Signal function of the fungal cell wall components was first observed by Albersheim and his colleagues in the mid 1970s. One of the best-characterized elicitors is the β-glucan released from the cell walls of *Phytophthora megasperma*. Soybean tissues or cell suspension cultures were used for biological assays to detect elicitor activity. The chemical nature of the elicitor was later described by Albersheim. The elicitor-active molecules that were isolated from *Phytophthora megasperma* f. sp. *soyja* culture fluid and the one that was isolated from mycelial wall of *Phytophthora megasperma* f. sp. *soyja* had the same chemical and biological properties. The β-glucans were prepared from the culture fluid and mycelial cell wall by autoclaving for 3 h at 121 °C. Their elicitor activity was still detectable demonstrating that these elicitors were heat stable. These elicitors do not bind to anion or cation exchange columns, and they are stable at room temperature in the range pH 2 to 10. The most active elicitor is the one with a hepta-β-glucoside structure and the aldehyde-reduced form (compound 17) as shown in scheme 5. The elicitor can induce accumulation of large amounts of phytoalexins such as glyceollin from soybean tissues.
Scheme 3. Structure of chitin oligomer (13) and chitosan oligomer (14)

Chitin and Chitosan  Fungal cell wall polymers can also produce elicitors with chitin (compound 13) and chitosan (compound 14) components as shown in scheme 3. This subject is reviewed by Ryan and references are described therein.21 Chitin and chitosan fragments are quite active elicitors, but they are not as functionally effective β-glucan elicitors. These types of elicitors have not been investigated as thoroughly as the β-glucans. Chitin and chitosan are insoluble polymers, but the soluble fragments can still be obtained from fungal cell wall polymers by acid hydrolysis or by using enzymes such as chitin deacetylase, chitinase, and/or chitosanase. Chitosan was the most active elicitor of pisatin, one of the phytoalexins, obtained from cell walls of Fusarium solani.22

Endogenous Elicitors

The denomination of endogenous elicitors was based on the hypothesis that plant cell wall fragments containing oligosaccharide residues can be released by infection or physiological stress.23 These fragments elicit accumulation of phytoalexins. It was also expected that plant cell wall fragments were cleaved by enzymes which were either secreted by pathogens or activated by pathogen attacking. The first endogenous elicitor was discovered by Bailey, Hargreave and Selby24,25,26 from damaged pea or bean tissue. It is heat stable and dialyzable. Later fragments possessing elicitor activity were
purified from soybean cell walls and from citrus pectin. It was demonstrated that both fragments are composed of about 98% \(\alpha\)-1,4-linked galacturonide residues. The fragments were released from soybean cell wall by partial acid hydrolysis without loss of elicitor activity, but they could be destroyed by endo-1,4-galacturonase treatments. It was observed that the most active elicitors obtained from both soybean and citrus pectin were the components with 12 residues of galacturonic acid (compound 15 as shown Scheme 4). Similar fragments with elicitor activity were isolated from cell walls of tobacco, sycamore, castor bean, cowpea, tomato and cucumber. Lignin formation was induced in cucumber hypocotyls. Elicitor activity of unsaturated \(\alpha\)-1,4-D-oligogalacturonides with nonreducing termini, such as \(\Delta\)-4,5-galacturonic acid (Scheme 4, 16), have been discovered. It was observed that the elicitors with unsaturated form were as active as the same size oligogalacturonides in inducing soybean phytoalexins. Endogenous elicitors with unsaturated oligogalacturonides could be released from the plant cell wall by pectic lyases, which were secreted by a number of plant pathogens.

\[ \text{Scheme 4. Structure of galacturonide oligomers (15) and unsaturated galacturonide oligomers (16)} \]
Specificity Studies with Elicitors

Almost all investigations indicate that elicitors lack functional specificity. An example is provided by Albersheim. It shows that the activities of elicitors which were purified from the three different *Phytophthora megasperma* f. sp. *soyja* races, were similar using three separate bioassays. More research has been focused on the search for race specific elicitors, and some progress has been made.

General Role of Host-Parasite Interaction in Plants

Up to now the materials used to stimulate the phytoalexin responses in the studies were unnatural material such as abiotic elicitors or natural products such as oligosaccharides which are more or less affected by different kinds of treatments. During elicitation, conditions occurring in natural host-parasite interactions are lacking. The use of such unnatural elicitors, however, may still give an insight into the process occurring in nature. Several factors about the interaction between a host plant and a parasite, presented by Brooks and coworkers, are as follows. (1) The cell wall components from the parasite can interact with hosts such as a plants by binding to the host cell wall. This binding depends on the structure of the cell wall of the host. This process induces the accumulation of phytoalexins. (2) The cell wall components from the parasite can interact with genes of the host which may elicit the formation of phytoalexins. (3) Parasites can secret enzymes in the host cell walls by penetration, which releases elicitors from the host cell walls. (4) Elicitors such as endogenous elicitors may be released by indirect ways which combine one or more of the three previous factors. On the other hand, it was considered in these studies that components from the cell wall of the parasite and toxins produced by the parasite can suppress the accumulation of phytoalexins. Scientists now are seeking different ways to uncover the mechanism of recognition and
elicitation of plant resistance. One of the studies is focused on how oligosaccharides can alter gene expression patterns. Hopefully, these studies will be able to show that the regulation of the expression genes is accomplished by an oligosaccharide-based recognition-communication system which may exist in the plant. It then means expression genes play major roles in inducing defense responses.

**Elicitor Structures and Biological Activity**

\(\beta\)-Glucans are among the best-studied elicitors. Albersheim recently reviewed the relationship between \(\beta\)-glucan structures and their biological activity. The elicitor-active aldehyde-reduced form of hepta-\(\beta\)-glucoside 17 was obtained as described by Sharp and coworkers and its structure was confirmed by chemical synthesis. It was also found that reduced hepta-\(\beta\)-glucoside 17 obtained from fungal cell wall and the unreduced form obtained from the chemical synthesis have the same ability to induce phytoalexin accumulation in soybean cotyledons. Both components possess high elicitor activity, and their activity can be detected by bioassay at concentrations of \(~10\) nM. Several elicitor-inactive hexa-\(\beta\)-glucosyl glucitols (not shown) were purified at the same time, but had detectable activity only at high concentrations (> 400 mM). Oligoglucosides 17-20 in scheme 5 are the four most active elicitors. As shown in the scheme part of their structures are similar to that of elicitor-active hepta-\(\beta\)-glucoside 17. Among these four active elicitors the hexa-\(\beta\)-glucoside 19 possesses the least active elicitor. Although structures of oligoglucosides 21-25 look similar to oligoglucosides 17-20, these oligoglucosides are not elicitor active compounds. Significant reduction of elicitor activity was observed when extra groups are added at certain positions on the compounds, e.g. at the nonreducing terminal backbone glucosyl residue or the side chain glucosyl residue adjacent to the nonreducing terminus (21-24), and the different
Scheme 5. Structure of the hepta-β-glucoside elicitor (compound 17) and structurally related oligoglucosides. The scheme was organized by Albersheim.20
arrangement of the side chain as compound 9. It is also interesting to note that adding extra groups such as an aromatic group to the reducing terminus of the oligosaccharide (compound 26) does not affect elicitor activity. The structure-activity studies proved that elicitation of phytoalexin accumulation are strongly structure related.

For endogenous elicitors from plants many investigations indicated that elicitor-active components had linear structures and were composed of 1,4-linked α-D-galacturonic acid residues. Oligogalacturonic acids with twelve residues are the most active elicitors isolated from both soybean cell walls and from citrus pectin as mentioned under the section on Endogenous Elicitors. Jin and West\textsuperscript{31} found casbene synthetase-elicitor activity for oligogalacturonic acids with nine or more residues. The highest activity was found for the thirteen-residue galacturonides. The oligogalacturonides were prepared by partial digestion of polygalacturonic acid with \textit{R. stolonifer} endopolygalacturonase. Robertson\textsuperscript{32} reported that oligogalacturonic acid with ten and eleven residues possessed maximal elicitor-activity. The oligogalacturonides were prepared by limited degradation of polygalacturonic acid with the \textit{C. cucumerium} enzyme. The unsaturated α-1,4-D-oligogalacturonides showed elicitor-activity with ten and eleven residues.\textsuperscript{29} The unsaturated oligogalacturonides were prepared by limited degradation of polygalacturonic acid with pectic lyases or endopolygalacturonic acid lyase. Recently, Komae\textsuperscript{33} also found that unsaturated α-1,4-D-oligogalacturonic acid residues with five to seven possessed elicitor activity. These unsaturated oligogalacturonides were prepared by treating polygalacturonides of \textit{Ficus awkeotsang} with the purified endo-pectate lyase from \textit{Erwinia carotovora}. Little is known about how elicitors act in plant resistance, but it is quite clear that galacturonic acids not only have structural functions but also contain informational components that can activate synthesis of defensive chemicals.
Isolation, Purification and Analysis

To release elicitors from cell walls of different sources, e.g., fungi and plants, two methods have been used. These are enzyme degradation and physical or chemical treatment such as autoclaving or acid hydrolysis of cell wall material. All the fractions are randomly sorted, even though some polysaccharides are selectively degraded by certain enzymes. The solution obtained after depolymerization is a mixture of different size oligosaccharides or different kind of oligomers. To purify oligogalacturonic acids from the mixture, anion-exchange chromatography is the most powerful method since several carboxyl groups exist in these molecules. Recently, high performance liquid chromatography (HPLC) has become another effective technique, especially, high pH anion-exchange chromatography (HPAEC) with pulsed ampermetric detection (PAD) which has been shown to be very successful in the separation of carbohydrates. HPAEC is able to separate different hetero-oligosaccharides. It is also possible to separate homopolysaccharides with different degrees of polymerization. Separation and purification of β-glucan is accomplished by a combination of anion-exchange chromatography and affinity chromatography. To analyze the composition of carbohydrates, the m-hydroxybiphenyl assay for uronic acid concentration, the anthrone assay for neutral carbohydrate concentration, and the phenol-sulfuric acid assay for total carbohydrate concentration are used in most cases. If detailed information on the cell wall material composition is required, gas chromatography is a very useful technique after permethylation, peracetylation, or other derivatization of the saccharides. For structural elucidation of oligosaccharides, fast atom bombardment mass spectrometry (FAB-MS) and gas chromatography mass spectrometry have proven to be very useful. Nothnagel provided detailed procedures for applying FAB-MS analysis for oligogalacturonic acid samples. The mass spectrum of the most active elicitor
Figure 3. Negative fast atom bombardment mass spectrum of soybean cell wall elicitors. The molecular ion (M-H)$^-$ with m/z 1953 represents an elevenmer galacturonic acid. Glycerol was used as a matrix as peaks labeled G. The spectrum was adopted from Nothnagel.27
Figure 4. Negative fast atom bombardment mass spectra of unsaturated oligogalacturonic acids from seeds of *Ficus awkeotsang*. a: Pentamer. b: Hexamer. The spectrum was adopted from Kimae.33
with twelve galacturonic acid residues is shown in Figure 3. Another spectrum of Δ-4,5-unsaturated oligogalacturonides, which possess biological activity, were obtained by Komae (Figure 4). Gas chromatography mass spectrometry is also able to provide detailed information about oligosaccharide structure such as linkage of different carbohydrate residues, which has been well studied.

Proposal

It should be noted, that most of the work on phytoalexins and phytoalexin elicitors has focused on fungi, bacteria and viruses; only a few studies concern the effects of phytoalexin on the development and behavior of insects and even less on their role in effecting resistance of plants to insect pests. Nevertheless, some investigations have been carried out in this field. For example, feeding deterrent phytoalexins have been found to affect aphid feeding, which may be responsible for the observed plant resistance. Besides research on nematodes as mentioned before, aphid infection seems to be another interesting area for studying plant resistance to higher organisms. Investigations with several hop varieties have shown that hop aphids feeding on resistant plants have the same chance of finding the phloem sap as when feeding on susceptible plants. In resistant plants, however, feeding stops earlier and so the animals grow slower and the overall population diminishes in comparison to aphid populations feeding on susceptible hop plants. It has been reported that in vascular plants, "inhibitors" accumulate in tissues far away from the "wounding site". This indicates that the phytoalexin was introduced into the vascular system of the plant. Based on these observations it is assumed that aphid feeding on hop plants triggers the plant defense mechanism. By penetrating the leaf tissue the aphid stylet destroys plant cells and so releases enzymes which produce endogenous elicitors. The elicitors are expected to be plant cell wall fragments like the oligogalacturonic acids described above. Further,
phytoalexins were found to be induced by the elicitors in hop plants and these work as feeding deterrents. Resistant plants will either provide higher phytoalexin concentrations or faster accumulation of these defense chemicals.

In the first step of our investigation, oligogalacturonic acids were isolated from hop cell walls, their elicitor activity was assayed by a bioassay method and their structures were elucidated by FAB-MS.
Experimental

Instrumentation and Materials

Fast atom bombardment mass spectrometry (FAB-MS), or liquid secondary ion mass spectrometry (LSIMS) was performed on a KRATOS MS50TC mass spectrometer fitted with a FAB source and an Ion-Tech atom gun. Xenon was used as the bombarding gas, and the atom gun was operated at 8 kV. High pH anion exchange chromatography (HPAEC) was performed on a Dionex BioLC Model 4500i system with a pulsed amperometric detector (Model PAD II) equipped with an electrochemical cell containing a gold working electrode. The Dionex Eluant Degas Module was used to sparge and pressurize the eluants with helium. Samples were separated on a Dionex Carbopac-PA1 pellicular anion exchange column (4 x 250 mm) with Carbopac-PA guard column (3 x 25 mm). The following pulse potentials and durations were used for detection of samples: $E_1 = 0.05 \text{ V} \ (t_1 = 300 \text{ ms}); E_2 = 0.65 \text{ V} \ (t_2 = 60 \text{ ms}); E_3 = -0.95 \text{ V} \ (t_3 = 180 \text{ ms}).$ The response time of the PAD was set to 3 s. Colorimetric measurements were performed on a Beckman Model 25 Spectrophotometer.

Trigalacturonic acid was purchased from CHI Carbohydrates International AB, Sweden. All other carbohydrate standards were purchased from Sigma or Aldrich. Thin layer chromatography plates with plastic backed sillicia gel 60F254, layer thickness 0.2 mm were used for the orcinol spot assay. Millipore purified water was used for all experiments.
Colorimetric Assays

Total Carbohydrate Assay

Total carbohydrate content was estimated by the phenol-sulfuric acid method, with β-cyclodextrin or cycloheptamylose (molecular weight 1134) as the standard. In the procedure 5% aqueous phenol solution (0.5 mL) and concentrated sulfuric acid (2.5 mL) were added in succession to 0.1 mL samples in test tubes. Samples were vortexed and cooled to room temperature. The absorbance of the characteristic yellow-orange color was measured at 490 nm on the UV-vis spectrophotometer. A mixture of 5% aqueous phenol solution and sulfuric acid served as background reference. The results of the assay were

![Graph showing calibration of total carbohydrate assay](image-url)

**Figure 5.** Calibration of total carbohydrate assay (monoGA = monogalacturonic acid, polyGA = polygalacturonic acid).
significantly affected by the composition of the sample. Monogalacturonic acid and polygalacturonic acid were tested. The response of the assay for the acidic sugars was much weaker than that for the neutral carbohydrate (cyclodextrin) as shown in Figure 5. For samples in which high acidic sugar concentrations were anticipated, the calibration curve for monogalacturonic acid was chosen.

Uronic Acid Assay

Uronic acid concentrations were determined by the \( m \)-hydroxylbiphenyl method\(^{35} \) using monogalacturonic acid as standard. In the procedure, 0.5\% (w/w) sodium tetraborate/concentrated sulfuric acid solution (3.5 mL) was added to a 0.2 mL sample in a test tube and cooled in an ice bath. The mixture was vortexed and heated in a 100 °C water bath for 5 min. After cooling in an ice bath 0.15\% (w/w) \( m \)-hydroxybiphenyl/1M sodium hydroxide reagent (0.4 mL) was added to the tube. The absorbance of the pinkish color was measured within 2 min on a UV-vis spectrophotometer at 520 nm. Also, a blank sample was measured without addition the \( m \)-hydroxybiphenyl reagent in which only 1 M sodium hydroxide solution (0.4 mL) was added instead.

Orcinol Spot Assay

Carbohydrate could be checked simply by the orcinol spot method. This assay is based on the principle of the normal orcinol assay procedure. Approximately 1 \( \mu L \) sample was spotted on a TLC plate. The plate was sprayed evenly with orcinol reagent (0.1\% orcinol in 4 N sulfuric acid) and baked on a hot plate (\(~115\) °C) for 15 min. A purple spot was observed for carbohydrate samples.
Preparation of Cell Wall Material (CWM) from Hop Leaves

A procedure adapted from Selvendran and O'Neill⁴⁶ was followed. Frozen hop leaves (variety: 64107, 50 g) obtained from the OSU green house were cut into small pieces and blended with aqueous 1% sodium deoxycholate (SDC, 200 mL) containing 5 mM sodium metabisulfite (Na₂S₂O₅) by a Brinkmann Homogenizer for 3 min. The blended material was cooled in an ice bath for 2 min and then blended one more minute; a few drops of polyethylene glycol were added to minimize frothing. The hop leaf slurry was centrifuged for 15 min at 13,800 g in the cold (4 °C). The supernatant solution was decanted and discarded. The pellet was washed twice with 0.5% sodium deoxycholate (300 mL) containing 3 mM sodium metabisulfite and collected by centrifugation as above. The pellet was then washed with cold acetone several times in a Buchner funnel with a Whatman GF/A glass microfibre filter until the filtrate was colorless. Both the funnel and the flask were kept on ice. The dried hop leaf material was mixed with 0.5% sodium deoxycholate (300 mL) containing 3 mM sodium metabisulfite, polyethylene glycol (1.5 mL) and the suspension was poured in two loads into the chamber of a Bead-beater (Biosper Products, 150 mL capacity) fitted with an ice water jacket. The material was further ground for 3 min to obtain optimal cell disruption. The derived suspension was decanted carefully and the beads were washed several times with water. The suspension was combined with the washing solution and centrifuged for 30 min at 23,000 g (4 °C). The pellet was washed twice with two bed volumes of water (2 x 150 mL) and recovered by centrifugation.

Phenol-acetic acid-water (PAW, 2:1:1, w/v/v, 100 mL) was used for deproteination. The pellet was suspended in the liquid with help of a Vortex mixer. The insoluble residue was washed twice with water (2 x 150 mL) and recovered by centrifugation.
To remove starch from the residue the deproteinized material was suspended in 90% aqueous dimethyl sulfoxide (v/v, 90 mL) and sonicated for 10 min, then stirred at room temperature overnight (16 h). A pellet was collected by centrifugation and was suspended in 90% dimethyl sulfoxide (60 mL), sonicated for 30 min (stopped twice and cooled to 20 °C), then stirred for 1 h. The final insoluble residue was washed six times with water (6 x 100 mL) until a starch-iodine test was negative and the residue was recovered by centrifugation. To obtain a material completely free of dimethyl sulfoxide the pellet was suspended in water and dialyzed (Spectra/por1, molecular weight cutoff 6,000-8,000) against water for 16 h. Finally, the dialysate was lyophilized yielding 5.12 g residue or CWM.

**Monosaccharide Analysis of Hop Leaf Cell Wall Material**

**Complete Hydrolysis Study of Hop Leaf Cell Wall Material**

**Hydrolysis by 2 N Trifluoroacetic Acid**

Hop cell wall material (CWM, 5.02 mg) was suspended in 2 N trifluoroacetic acid (2 mL) and heated in a sealed reaction vial at 120 °C for 2 h as described by Selvendran and coworkers.48

**Hydrolysis by 2 N Sulfuric Acid**

Hop CWM (5.05 mg) was suspended in 2N sulfuric acid (2 mL) and heated in a sealed reaction vial at 100 °C for 2 h. This method was considered by Selvendran and coworkers48 as the optimal conditions for the stability of most carbohydrates.
Hydrolysis by the Saeman Method

The hydrolysis experiment followed a procedure described by Selvendran and coworkers.\textsuperscript{47} Hop leaf CWM (5.11 mg) was suspended in 72\% (w/w) sulfuric acid (0.25 mL) and stirred for 3 h at room temperature. The slurry was then diluted with water (2.7 mL) to produce a 2 N sulfuric acid solution and heated in a sealed reaction vial at 100 °C for 2 h. In addition a parallel experiment with 5.25 mg hop CWM was run omitting the heating step.

Hydrolysis by Concentrated Sulfuric Acid

A detailed description of the hydrolysis of uronic acid from fruit pulp was given by Ahmed and Labavitch.\textsuperscript{49} In a 20 mL beaker with a magnetic stir bar, hop leaf CWM (5 mg) was suspended in chilled concentrated sulfuric acid (2 mL). The beaker was placed in a water-ice bath that was situated on a stirring motor to swirl the suspension. Water (0.5 mL) was added dropwise to the beaker. After the suspension was stirred for 5 min, additional water (0.5 mL) was added dropwise. The suspension was stirred for 3 or 18 h (overnight).

Sample Preparation for Carbohydrate Content Determination

Carbohydrate samples from all the hydrolysis methods were prepared by filtering the hydrolysate through a Whatman GF/F glass microfibre filter paper. The filtrate from the concentrated sulfuric acid hydrolysate was then transferred to a 10 mL volumetric flask. The water that was used to wash the beaker was combined and used to fill the flask to its designated volume. Total carbohydrate and uronic acid content of all the filtrates were determined by the phenol and \textit{m}-hydroxybiphenol methods as described
before. The insoluble material was lyophilized and weighed. The results are shown in Table I.

High pH Anion Exchange Chromatography of Monosaccharides

Sample Preparation from the Hydrolysates for High pH Anion Exchange Chromatography

The trifluoroacetic acid hydrolysate (0.5 mL) prepared as described above was dried by blowing nitrogen gas in a 0.5 mL Eppendorf tube. Water (0.5 mL) was then added to the tube to dissolve the soluble material. The insoluble residue was either filtered off by a Millipore microfiltrate kit or was removed by centrifugation. For the concentrated sulfuric acid hydrolysate the prepared diluted filtrate (2 mL) was pipetted into a 100 mL beaker and 0.3 N barium hydroxide (48 mL) was then added to precipitate barium sulfate. The mixture was adjusted to pH 7 by adding either 0.3 N barium hydroxide or 2 N sulfuric acid. The beaker was placed in a warm water bath (37 °C) for about 18 h (overnight). The precipitate was removed by centrifugation and washed with water (10 mL). The supernatant layer was combined and concentrated to 0.5 mL on a Buchi rotary evaporator.

High pH Anion Exchange Chromatographic Analysis

For the separation of neutral carbohydrates a mixture of water and 100 mM sodium hydroxide (90:10) was run isocratically at a flow rate of 1 mL/min for 4 min. The eluant then was changed to 100% water (Figure 7a, b, and Figure 8). For the separation of acidic sugar from neutral sugars 150 mM sodium acetate/100 mM sodium hydroxide was used as eluant and run isocratically at a flow rate of 1 mL/min. In order to
minimize baseline distortion 300 mM sodium hydroxide was added to the postcolumn effluent via a mixing tee at a flow rate of 1 mL/min by a Dionex postcolumn delivery system. The detector sensitivity was set at 1 K. Standard solutions (approximately 0.300 mg/mL) of glucose, galactose, mannose, arabinose, rhamnose, fucose, galacturonic acid, xylose, myo-inositol and the mixture of all these sugars were prepared individually. They were used as internal standards and external standards to calibrate the HPAEC and to identify peaks in the HPAEC chromatogram (Figure 7a, b, and Figure 8).

Isolation of Elicitors from Hop Leaf Cell Wall Material

Partial Hydrolysis of Hop Cell Wall Material

The hop leaf CWM was hydrolyzed by the method of Robertsen.\textsuperscript{32} The CWM was suspended in 10 mM sodium phosphate buffer (500 mL) at pH 7.4. The suspension was autoclaved for 20 min at 121 °C and then filtered through a glass fibre filter. Tris-base and Tris-HCl were added to the filtrate to obtain a final concentration of 0.06 M (pH 7.9).

Separation of Partially Hydrolyzed Hop Cell Wall Material by Anion-exchange Chromatography

The filtered CWM hydrolysate was applied to a QAE-Sephadex A-25 anion-exchange column (2.5 x 25 cm) and equilibrated with Tris-HCl buffer (0.06 M). The column was first washed with 500 mL of buffer at a flow rate of 2 mL/min, and the eluted material was tested by both the total carbohydrate assay and the uronic acid assay methods. The column was then eluted with a linear gradient of Tris-HCl buffer (2000
28 mL) running from 0.06 to 0.6 M at pH 7.9 (Figure 12a). Finally, the column was eluted with 1.2 M Tris-HCl buffer. Effluent fractions (12.5 mL) were collected and assayed for total carbohydrate and uronic acid concentration.

**Desalting of the Elicitor Fractions**

Fractions 1-9 (Figure 12a) were pooled individually and desalted on a Bio-Gel P-2 column (2.5 x 50 cm). Approximately 6 mg sample was applied to the column each time. Millipore water was used as the eluant. Effluent fractions (10 mL) were collected, and carbohydrates were checked by the orcinol spot method as described. Fractions containing carbohydrates were pooled and concentrated on a Buchi rotary evaporator. Finally, the material was recovered by lyophilization and weighed. The sum of the material (from fractions 1-9) was approximately 80 mg.

**High pH Anion Exchange Chromatography of Hop Elicitors**

The analysis was performed on a Dionex system under the same conditions as mentioned above. Eluant A (100 mM sodium hydroxide) and eluant B (100 mM sodium hydroxide/1M sodium acetate) were used as solvents. Separation and analysis of elicitors was accomplished by a linear gradient increase of eluant B (50-75%) over a 30 min period followed by 75% B run isocratically for 5 min. No sodium hydroxide postcolumn solution was added this time. The reference cell of the electrochemical cell system was filled with 100 mM sodium hydroxide/1M sodium acetate. The detector was set at sensitivity 1K-3K, corresponding to the sample concentration or responsivity of the detector to the sample (Figure 10).
Depolymerization of Polygalacturonic Acid

Partial Hydrolysis of Polygalacturonic Acid

The hydrolysis method was similar to that described by Robertsen.\textsuperscript{32} Polygalacturonic acid (from oranges and purchased from Sigma, 2.5 g) was suspended in water (200 mL). The solution was adjusted to pH 4.2 with 1 N sodium hydroxide solution and autoclaved for 20 min at 121 °C. The solution was then adjusted to pH 2.0 by adding 1 N hydrochloric acid to precipitate high molecular weight polymers of galacturonic acid. The precipitate was removed by centrifugation.

Gel Filtration of Hydrolyzed Polygalacturonic Acids

Hydrolyzed polygalacturonic acid solution (approximately 30 mg in 1 mL) was adjusted to pH 3 by adding 2 N sodium hydroxide, centrifuged to remove insoluble material and applied to a Bio-Gel P-6 column (1.5 x 50 cm) and eluted with 0.5 N sodium chloride at flow rate of 18 mL/h. Fractions (2.5 mL) were collected and assayed for total carbohydrate. Dextrin, molecular weight 485,000, and galacturonic acid were used to determine the void volume (Vo) and the total volume (Vt), respectively. Fractions from number 18 to number 40 were pooled and concentrated by rotary evaporation (Figure 9).

Desalting of Hydrolyzed Polygalacturonic Acids

Polygalacturonic acid hydrolysate (~1.7 mg) had been purified by gel filtration as described above and desalted on a Bio-Gel P-2 column (1 x 25 cm). Water was used as
eluant at a flow rate of 0.2 mL/min. Fractions (1 mL) were collected and assayed by the orcinol spot method.

**High pH Anion Exchange Chromatography of Hydrolyzed Polygalacturonic Acids**

The analysis for polygalacturonic acids was run under the same conditions as described for the analysis of the hop CWM elicitors. Peaks 3 through 22 (Figure 10, bottom panel) were collected individually. They were further desalted procedures as described in "Sample Purification" and the molecular weight of the oligogalacturonic acids were measured by FAB-MS.

**Sample Preparation for Fast Atom Bombardment Mass Spectrometry**

**Sample Purification**

**Silanization of Glassware**

Some glassware used for analysis was silanized to minimize sample loss through adsorption. The procedure was identical to that of Waeghe and coworkers. The glassware was soaked in a solution of 2% dichlorodimethylsilane in toluene for at least 15 min. It then was soaked in methanol three times. Finally, the glassware was successively rinsed with hot tap water, distilled water, and dried in an oven.
Desalting of Oligogalacturonic Acids and Elicitor Fractions by Column Chromatography

The column (1.5 x 4 cm or 1.5 x 6 cm) used for desalting was packed with Bio-Gel P-2 gel (molecular weight cutoff 1800) or Sephadex G-10 gel (molecular weight cutoff 700) corresponding to the amount or molecular size of the sample molecule. For small samples (less than 50 µg) the smaller column was used. Samples obtained from the effluent of HPAEC (Figure 10, bottom panel) or from derivatization of carbohydrate were lyophilized and dissolved in water (0.5-1.0 mL). The solution was then applied to the column and eluted with water at a flow rate of 0.2 mL/min. Effluent fractions (0.5 mL) were collected and assayed by the orcinol spot method. Carbohydrate-containing fractions were pooled and lyophilized. The dried samples were dissolved in a suitable solvent for FAB-MS analysis directly or were analyzed after derivatization.

Desalting of Oligogalacturonic Acids and Elicitor Fractions by Tip Column Chromatography

For desalting very small amounts of sample (below 40 µg) a tip column, which is a packed pipet tip column, was employed as described by Chen and coworkers. A 1 mL pipet tip was packed with Bio-Gel P-2 gel or Sephadex G-10 gel. The tip was then placed in a disposable tube (10 x 75 mm) to elute the solvent and run on a centrifuge which was set at 1600 g. The sample was allowed to spin for 4 min. To insure that the tip column was packed properly the tip was loaded with water (60 µL) and run at the same centrifuge settings as used above. A sample (maximum volume 60 µL) was loaded on to the column, centrifuged, and collected in a small Eppendorf tube (400 µL).
sample was then dried by lyophilization, evaporated by a nitrogen gas and analyzed, or used for FAB-MS analysis directly.

Desalting of Oligogalacturonic Acids and Elicitor Fractions by Barium Precipitation

HPAEC fractions and elicitor fractions were purified to minimize the sodium adducts which prevented FAB-MS analysis. Solid barium chloride (40 mg) was dissolved in the desalted sample (~200 μg in 2 mL) solution according to the method described by Nagel and Wilson.51 Absolute ethanol (3 mL) was added to the sample to give a final concentration of 60% (v/v), and the solution was kept at 4 °C overnight. Precipitates were centrifuged, washed with ethanol (96%, 1 mL), and dried under vacuum. The precipitates then were suspended in water (0.5 mL) and a suspension of Bio-Rad 50W x 8 cation exchange resin (H+ form, 0.5 mL) was added. The mixture was stirred overnight to dissolve all the precipitates and then filtered on a sintered glass filter to remove the resin. The sample then was recovered by lyophilization.

Sample Derivatization

\( o-(2, 3, 4, 5, 6\text{-Pentafluorobenzyl}) \) Hydroxylamine (PFB) Derivatization

The PFB derivatization method was adopted from Nothnagel and coworkers.27 The oligogalacturonic acids sample (40 μg, 0.019 μmol-0.073 μmol) prepared previously was mixed with \( o-(2, 3, 4, 5, 6\text{-Pentafluorobenzyl}) \)-hydroxylamine hydrochloride (500 μg, 2.00 μmol) in pyridine (400 μL). The solution was heated in a 2 mL sealed reaction vial at 100 °C for 2 h and then dried by nitrogen gas.
Reductive Amination

The derivatization followed the method described by Hogeland and coworkers.\textsuperscript{52} In a 5 mL reaction vial, oligogalacturonic acids (200 µg, 0.0939 µmol-0.366 µmol) was dissolved in water (50 µL) and \(n\)-hexylamine (50 µL, 0.50 µmol) was added. Absolute ethanol (20 µL) was added to form a homogenized solution. To avoid precipitation of the oligogalacturonic acid in ethanol solution, a minimum amount of ethanol was added. Glacial acetic acid (20 µL) was added and the solution was kept at room temperature for 3.5 h before aqueous sodium cyanoborohydride (1.5 M, 200 µL) solution was added. Absolute ethanol (40 µL) was added to regain the homogeneity of the solution, and more glacial acetic acid was added to adjust to pH 5. This solution was kept at room temperature overnight (~18 h). Alternatively, the sodium cyanoborohydride solution was added right after the \(n\)-hexylamine, and the solution was heated at 75 °C for 2 h. The excess \(n\)-hexylamine was removed by extraction with dichloromethane (500 µL), using added water (1 mL) to increase the phase separation. The aqueous layer was washed twice with dichloromethane (2 x 300 µL) and then concentrated by blowing over it nitrogen gas. The concentrate (~100 µL) was desalted on a Sephadex G-10 column as described above.

Peracetylation of \(n\)-Hexylamine Derivatized Oligogalacturonic Acid

The procedure followed that of Blakeney and coworkers.\textsuperscript{53} The \(n\)-hexylamine derivatized oligogalacturonic acid was dissolved in water (50 µL, 2.8 µmol). 1-Methylimidazole (40 µL) followed by acetic anhydride (600 µL, 6.35 µmol) was added to the solution. The solution was mixed well at room temperature for 15 min and water (1 mL) was added to decompose the excess acetic anhydride. After cooling the solution was extracted with dichloromethane (3 x 300 µL). The organic layer was combined and
washed with water (800 μL), and then concentrated to ca.10 μL under a stream of nitrogen.

**Permethylolation of n-Hexylamine Derivatized Oligogalacturonic Acid**

The procedure described by Hogeland and coworkers\textsuperscript{52} was followed. n-Hexylamine derivatized trigalacturonic acid (100 μg, 0.158 μmol) was dried overnight in a vacuum desiccator over phosphorus pentoxide at room temperature. The sample was dissolved in dry dimethyl sulfoxide (300 μL) and stirred for 10 min under nitrogen. Finely ground sodium hydroxide (∼50 μg) was added and the suspension was stirred under nitrogen for 5 min at room temperature. Iodomethane (150 μL, 2.41 μmol) was then added and the mixture stirred for 10 min. After adding another portion of iodomethane (50 μL) and stirring for 30 min, the reaction mixture was quenched with water (1 mL). The solution was extracted with chloroform (3 x 400 μL). The combined organic layers were washed with water (3 x 400 μL) and concentrated to ca. 10 μL under a stream of nitrogen.

**Fast Atom Bombardment Mass Spectrometric Analysis**

Fast atom bombardment mass spectrometry was performed under positive or negative ion mode based on sample requirements. Trigalacturonic acid and oligogalacturonic acid prepared from hydrolysis of polygalacturonic acid were used as model compounds to test the protocol by FAB-MS. Oligogalacturonic acids of differing molecular weight were tested individually or as a mixture. Hop elicitors were then analyzed in the same way the model sample had been. To increase sensitivity and get structural information from the molecular fragments, derivatization of hop elicitors was attempted.
Results and Discussion

Hop Leaf Cell Wall Material (CWM) Preparation

Hop leaves (50 g)

- Blend in 1% SDC + 5 mM Na₂S₂O₅
- Wash with acetone

Crude product

- Bead beat in 0.5% SDC + 3 mM Na₂S₂O₅
- Extract with PAW (2:1:1)
- Extract with 90% DMSO
- Dialyze

Cell wall material (CWM, 7.09 g)

Figure 6. Extraction of cell wall material (CWM) from hop leaves

The method used for preparing CWM from hop leaf was developed by Selvendran and O'Neill⁴⁷ to achieve maximum dissolution of starch and cytoplasmic material with minimum loss of the non-starchy cell wall polysaccharides. The coprecipitation of a variety of alcohol insoluble materials from the leaves was avoided by using aqueous salt solutions [1% sodium deoxycholate (SDC)] instead of aqueous alcohol. Sodium metabisulfite (Na₂S₂O₅, 5 mM) was incorporated into the extraction medium to minimize the formation of polyphenol oxidation products. To achieve complete removal of proteins and starch it was necessary to ensure thorough disruption of tissue structures, and to use solvents that have a high affinity for these compounds. The first objective was accomplished by triturating the tissue with a bead-beater in an aqueous solvent (0.5% sodium deoxycholate containing 3 mM sodium metabisulfite). Proteins were removed by sequential treatment with 1% aqueous sodium deoxycholate
and phenol-acetic acid-water (PAW), and starch was removed with 90% dimethyl sulfoxide (DMSO). The extraction was done at low temperature (under 4 °C) to minimize enzyme activity before protein removal. Chlorophyll in the plant tissue also needed to be removed from the CWM, and this was accomplished by an acetone wash. Although the method was somewhat labor intensive, the final preparation is believed to have been free of intercellular compounds. Yields of 7.09 g (~14%) from 50 g leaves provided plenty of material for further research. The result is reproducible.

Hydrolysis Studies and Composition Analysis of Hop Leaf Cell Wall Material

To obtain the composition information on the polysaccharides from plant CWM, many hydrolysis methods were tested, but the results were not conclusive. According to Dutton, when different types of glycosidic linkages are present in the same molecule, and each monosaccharide has different stability to acid, no single method of hydrolysis will cleave every linkage and give each component in quantitative yield. Thus, conditions of hydrolysis have to be chosen which compromise some information. In our studies, several methods and conditions were tested as described in the Experimental section in order to find a method that can determine content of total carbohydrate and uronic acid of CWM (Table I). Hydrolysates can also be applied on HPAEC for detailed analysis as shown by the results in Table II and in Figures 7a, 7b, 8. The data in Table I give the following conclusions. First, in Saeman hydrolysis and concentrated sulfuric acid hydrolysis, 72% and concentrated sulfuric acid was used, respectively. The results indicate that extended reaction time and heat increased the solubility of CWM, but extensive decomposition of carbohydrates also occurred during hydrolysis. Thus, a low percentage of total carbohydrate and uronic acid in soluble material was observed as compared to the data from two different hydrolysis conditions for each of the other
methods. A second conclusion can be drawn from the diluted acid hydrolysis method. A higher percentage of carbohydrates, including uronic acid is solubilized by hydrolyses

Table I. Results of hydrolysis method studies

<table>
<thead>
<tr>
<th></th>
<th>T. Sugar(^a) (%)</th>
<th>UA(^b) (%)</th>
<th>Insol. Mc(^c) (%)</th>
<th>Sol. Md(^d) (%)</th>
<th>T. Sugar(^*) (%)</th>
<th>UA(^*) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2N TFA</td>
<td>9</td>
<td>4</td>
<td>41</td>
<td>59</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>2N H(_2)SO(_4)</td>
<td>9</td>
<td>6</td>
<td>62</td>
<td>38</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>Saemen (w/o heat)</td>
<td>17</td>
<td>9</td>
<td>58</td>
<td>42</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>Saemen (w/heat)</td>
<td>20</td>
<td>8</td>
<td>39</td>
<td>64</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>H(_2)SO(_4) (conc. 3 h)</td>
<td>17</td>
<td>11</td>
<td>63</td>
<td>37</td>
<td>46</td>
<td>30</td>
</tr>
<tr>
<td>H(_2)SO(_4) (conc. 18 h)</td>
<td>22</td>
<td>10</td>
<td>43</td>
<td>57</td>
<td>38</td>
<td>18</td>
</tr>
</tbody>
</table>

a. Total sugar contents were measured by the phenol-sulfuric acid assay.
b. Uronic acid contents were measured by the \(m\)-hydroxybiphenyl assay.
c. Insoluble material content.
d. Soluble material contents were calculated by subtracted insoluble material content from total sample.

* The data were calculated as percentile of soluble material contents.

with high concentration of sulfuric acid, even though it has been shown\(^{49}\) that part of the carbohydrate may become sulfated, leading to interpretations of results that might be erroneous. The hydrolysis method with concentrated sulfuric acid carried out for three hours provided the highest percentage of measurable carbohydrate, especially uronic
acid. The method was recommended by Ahmed as a simplified method for accurate determination of cell wall uronic acid which is consistent with our findings. Finally, the 2N trifluoroacetic acid method proved to be a very useful method. The time course for 2N trifluoroacetic acid hydrolysis of bean hypocotyl cell wall has already been reported by Albersheim and coworkers. For comparison, the carbohydrates resulting from 2N trifluoroacetic acid hydrolysis (2 hours) are shown in Table I and II. Although the method did not display its hydrolysis efficiency in this case, the advantage of easier work-up due to the high volatility of the acid, and the good reproducibility of results were obvious.

Table II. Carbohydrate composition of hop cell wall material

<table>
<thead>
<tr>
<th>Carbohydratea</th>
<th>% of total sugar</th>
<th>2N TFA hydrolysis</th>
<th>Conc. H₂SO₄ hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 = Fucose</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>2 = Rhamnose</td>
<td>6.8</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>3 = Arabinose</td>
<td>14.8</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>4 = Galactose</td>
<td>12.9</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>5 = Glucose</td>
<td>16.6</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>6 = Xylose</td>
<td>8.2</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>7 = Galacturonic acid</td>
<td>39.5</td>
<td>66.2</td>
<td></td>
</tr>
</tbody>
</table>

a. Neutral carbohydrates were calculated from the chromatogram of HPAEC (Figure 7a and 8). The galacturonic acid was measured by the m-hydroxylbiphenyl assay.

Concentrated sulfuric acid and 2N trifluoroacetic acid hydrolysis methods (3 hours) were used in studies for the determination of carbohydrates in the high pH anion
Figure 7. High pH anion exchange chromatography from 2N trifluoroacetic acid hydrolyzed cell wall material (CWM) with a pulsed amperometric detector. a: The neutral monosaccharides of the extracted hop CWM are shown as indicated. b: A chromatogram resolving acidic monosaccharides shows two peaks: neutral monosaccharides (N); galacturonic acid (G). For conditions see page 26.
Figure 8. High-pH anion exchange chromatography from concentrated sulfuric acid hydrolyzed cell wall material. The neutral monosaccharides of the extracted hop CWM are shown as indicated.

exchange chromatography (HPAEC) after colorimetric assays to analysis the composition of hop CWM (Figure 7a, 7b, and 8). The relative percentage of carbohydrates is provided in Table II. The high content of galacturonic acid confirmed that the isolated material was indeed pectin from cell walls. Based on the results from 2N trifluoroacetic acid hydrolysis, homo-galacturonans and rhamno-galacturonans 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 arabino-galactans 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 amounts of the
also contribute to the high glucose content in the sample. The only acidic sugar found was shown to be galacturonic acid (Figure 7b). The amount of galacturonic acid determined from high-pH anion-exchange chromatography was quite close to that measured by the colorimetric assay. A comparison between the measurements of galacturonic acids by HPAEC and by colorimetric assay method was made. However, the chromatogram from the concentrated sulfuric acid hydrolysis method (Figure 8) was one large peak cannot be identified and the results were not reproducible. The uncertainty of the results may result from an impurity from the barium precipitation procedure.

The results obtained in these studies may still poorly represent the whole hop CWM because the unusual stability of the glycosyl uronic acid linkage hinders quantitative depolymerization under normal acid hydrolysis conditions, which results in incomplete solubilization and hydrolysis of uronic acid-containing polymers. Nevertheless, by analogy to previous results obtained by composition analysis, our experimental data certainly provides valuable information about the material prepared from hop leaves.

**Fractionation of Polygalacturonic Acid**

The primary purpose in testing for the presence of polygalacturonic acid in hop CWM is to obtain standard samples for hop leaf elicitor analysis. In the partial hydrolysis procedure a sodium hydroxide solution was added to dissolve the polygalacturonic acid before autoclaving. In order to protect the HPAEC column, the soluble, high molecular weight polymers were removed using gel filtration chromatography on a Bio-Gel P-6 column (Figure 9) as described in the Experimental section.
Figure 9. Gel filtration chromatogram of partially hydrolyzed polygalacturonic acid. A Bio-Gel P-6 column was used and the sample was eluted with 0.5 N sodium chloride. Each fraction was assayed for total sugar content.

Using HPAEC to separate the different sizes of oligogalacturonic acid polymers was quite successful as shown in Figure 10. It is observed that polymers of up to 22 residues can be separated and detected with one residue resolution. Meanwhile, it is also obvious from Figure 10 that the height of the peaks decrease dramatically after oligomers with 13 residues. It may be that oligomers with a higher degree of polymerization may have lower response to the detector. Alternatively, the height change may be due to different sizes of polysaccharides existing in hop leaves or to an altered set of oligomers caused by the hydrolysis conditions used.
Figure 10. High pH anion exchange chromatography from partially hydrolyzed cell wall material (CWM) with a pulsed amperometric detector. Fraction (Fr.) 1 through 9 represent the corresponding fractions from the QAE Sephadex column. An oligogalacturonic acid mixture (olGalA) derived from partial hydrolysis of commercially available polygalacturonic acid serves as a standard (bottom panel). The degree of oligomerization is shown by the numerals on top of the peaks.
**Fractionation and Biological Activity of Hop Elicitors**

Cell wall material (CWM, 1 g)
- Suspend in 500 mL 10 mM Na₂HPO₄ (pH 7.4)
- Autoclave for 20 min at 121 °C
- Filter through glass fibre

Crude extract
- Make the solution of 0.06 M Tris-HCl (pH 7.9)
- Apply to QAE-Sephadex A-25 anion exchange chromatography
- Elute fractions with a linear gradient ranging from 0.06 M to 0.6 M Tris-HCl
- Assay for total carbohydrate and uronic acid content

Elicitor fractions (~80 mg)

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**Figure 11.** Isolation of elicitors from hop leaf cell wall

Hop leaf elicitors released from hop CWM by autoclaving were fractionated by anion-exchange column chromatography procedures as shown in **Figure 11**. In this procedure the first eluant from 500 mL 0.06 M Tris-HCl buffer was assayed for uronic acid and total carbohydrate. The assay results indicated that only neutral sugars were washed out at this time. After eluting with a linear gradient of Tris-HCl buffer (0.06-0.6 M) at pH 7.9 (**Figure 12a**), oligosaccharide peaks, different in size and/or composition, were obtained. It was observed from the elution profile that peaks become more closely spaced with increasing buffer concentration. Determination of the total sugar and uronic acids content in each of the fractions obtained showed that all but fraction 2 contained acidic sugars. Since peak maxima for total sugar and uronic acid are almost equivalent in all the other fractions, it is assumed that these fractions contain mainly galacturonic acid. The later eluting fractions contain higher oligomers of acidic sugars and therefore were retained more strongly on the resin than the smaller oligomers.
Figure 12. a: Chromatogram of partially hydrolyzed cell wall material. QAE Sephadex A-25 ion exchange column was used for separation and the hydrolyzed sample was eluted with the gradient described in the Experimental. Nine fractions (1-9) were pooled and assayed for elicitor activity. b: Results of the bioassay have been described. The soybean cotyledon assay was used for each fraction.
Nine fractions were pooled and assayed for biological activity, using the soybean cotyledon assay.46 Soybean tissues accumulate glyceollin in response to elicitors. Fraction 6 was identified as the sample with the greatest activity in phytoalexin elicitation. A lower elicitor activity was detected for fraction 7.

Analysis of the nine fractions by HPAEC further substantiated that all but fraction 2 consisted of galaturonic acid oligomers (Figure 10). The chromatograms also showed that the pooled fractions were relatively clean. In fraction 7 only a small amount of fraction 6 material was present. Fraction 8 showed a mixture of fraction 7 and fraction 9. Each fraction showed peaks with increasing retention times confirming that the higher numbered fractions contained longer oligomers of the isolated galacturonide. The retention times of the hop fractions were compared to the mixture of oligogalacturonic acids prepared from commercially available polygalacturonic acid (bottom chromatogram of Figure 10). This comparison indicated that fraction 6 from the hop CWM, for example, eluted like an 11-mer or 12-mer. The results from FAB-MS analysis proved that this interpretation was misleading (see next section).

**Fast Atom Bombardment Mass Spectrometry Analysis Studies of Oligogalacturonic Acids and Hop Elicitors**

**Degree of Oligomerization of Hydrolyzed Polygalacturonic Acids**

Different sizes of oligomers (bottom chromatogram of Figure 10) were collected individually. Purified samples from fractions 4, 5, and 9, were analyzed by FAB-MS (Figure 13a, b, c). The peak with m/z 721 (Figure 13a) and m/z 1602 (Figure 13c) correspond to the molecular weight of the galacturonic acid tetramer and nonamer. Mass ratio m/z 941 (Figure 13b) corresponds to the molecular weight of the pentamer
Figure 13. Negative fast atom bombardment mass spectra of oligogalacturonic acids. a: a spectrum of tetragalacturonic acid. The molecular ion peak (M-H)\(^{-}\) with m/z 721 represents the tetramer. b: a spectrum of pentagalacturonic acid. The (M-H)\(^{-}\) with m/z 941 represents the pentamer with two sodium adducts. c: a spectrum of nonagalacturonic acid. The (M-H)\(^{-}\) with m/z 1602 represents the nonamer. For detail see Results and Discussion.
Figure 14. Negative fast atom bombardment mass spectrum of an oligogalacturonic acid mixture. The degree of oligomerization is as indicated by roman numerals.
with two sodium adducts. All spectra show oligomer peaks as sodium adducts as observed in the figures. The results indicate that the chromatograms in the bottom of Figure 10 presented a sequence of oligogalacturonic acids with each peak representing an oligomer elongated by one residue. Negative FAB-MS analysis of the galacturonic acid mixture also confirmed the results obtained above (Figure 14). Each of the mass peaks observed (m/z 545, m/z 721, m/z 897, m/z 1073, m/z 1249, m/z 1425, m/z 1601, m/z 1777, m/z 1953, and m/z 2130) differs from each other by 176 Daltons. The degree of oligomerization is indicated with roman numerals.

**Degree of Oligomerization and Structural Analysis of Hop Elicitors**

The degree of oligomerization of the isolated elictor fractions was unambiguously established by FAB-MS of the native carbohydrates. Only non-esterified galacturonic acid oligomers were observed in FAB-MS spectra (Figure 15 and 16). Negative FAB-MS analysis of the separated hop CWM fractions showed that fraction 3 contained a trimer, fraction 4 a tetramer, fraction 5 a pentamer (Figure 15), fraction 6 a hexamer, fraction 7 a heptamer and fraction 9 an octamer of oligogalacturonic acid (Figure 16). However, all recorded oligomers showed mass peaks 18 Daltons lower than the standard mixture derived from commercial polygalacturonic acid. For example, the trimer from the hop sample shows a molecular ion peak with m/z 527, the tetramer a molecular ion with m/z 703, etc. This difference of 18 mass units can be explained by the existence of an unsaturated carbohydrate unit. Loss of water by an elimination reaction or by an enzymatic cleavage reaction would produce unsaturated galacturonic acid oligomers each containing a double bond.

The presence of unsaturated galacturonosyl residues in the hop samples also explains their behaviour on the HPAEC. A dramatic shift towards longer retention times
Figure 15. Negative fast atom bombardment mass spectrum of the native elicitor fractions. a: Spectrum for the oligogalacturonic acid mixture derived from polygalacturonic acid. The degree of oligomerization of the oligomers are as indicated. b: Elicitor fraction 3 shows an unsaturated trigalacturonic acid with m/z 527. c: Elicitor fraction 4 shows an unsaturated tetragalacturonic acid with m/z 703. d: Elicitor fraction 5 shows an unsaturated pentagalacturonic acid with m/z 879.
Figure 16. Negative fast atom bombardment mass spectrum of the native elicitor fractions. a: Spectrum for the oligogalacturonic acid mixture derived from polygalacturonic acid. The degree of oligomerization is indicated. b: Elicitor fraction 6 shows an unsaturated hexagalacturonic acid with m/z 1055. c: Elicitor fraction 7 shows an unsaturated heptagalacturonic acid with m/z 1231. d: Elicitor fraction 9 shows an unsaturated octagalacturonic acid with m/z 1407.
due to unsaturated acidic sugars has been discussed. Conjugation allows the electrons of the double bond to delocalize into the carboxyl group of the molecule. The double negatively charged carboxy function is assumed to interact more strongly with the ion exchange resin, resulting in longer retention times. Comparison of the retention times between fully saturated and unsaturated compounds leads to misinterpretation of the actual degree of oligomerization in the hop oligogalacturonic acids. Thus, by HPAEC experiments the commercial polygalacturonic acid was not an appropriate standard. Only FAB-MS experiments were able to determine the actual molecular weight of the compounds and their exact degree of oligomerization.

Partial hydrolisis of the hop CWM cannot account for the formation of the unsaturated galacturonosyl residues, since the commercial polygalacturonic acid was subjected to a very similar procedure in order to produce the oligogalacturonic acid mixture. This mixture showed no evidence unsaturation. It is assumed that the unsaturated material in the hop samples has its origin in the plant. Hop CWM may contain more unsaturated galacturonosyl residues than the CWM from other plants. Alternatively, during the extraction of the hop CWM, enzymatic cleavage may have occurred.

In a recent publication Komae and coworkers have shown that unsaturated penta- through hepta-galacturonic acids isolated from *Ficus awkeotsang* function as phytoalexin elicitors in soybeans. They also indicated that the elicitor with the most biological activity is an unsaturated hexagalacturonic acid as mentioned in the Introduction section (Figure 4).

Each of the hop fractions showed fragmentation peaks in addition to the molecular ion signal. Fraction IX was chosen to demonstrate the observed fragmentation in negative ion FAB-MS (Figure 17). The molecular ion with m/z 1407 represents the native unsaturated octagalacturonide. Fragmentation from the reducing end of the molecule produces peaks 176 mass units lower for the loss of saturated monomers.
Figure 17. Negative fast atom bombardment mass spectrum of the fragmentation pattern for unsaturated galacturonides from hop fraction 9 (Figure 10). The molecular ion peak (M-H)^- with m/z 1407 shows the unsaturated octagalacturonide. For details see Results and Discussion.
Peaks with m/z 1231 and m/z 1055 are observed in the spectra. Fragmentation from the nonreducing end produces a peak 158 mass units lower (peak with m/z 1249) indicating the loss of an unsaturated galacturonosyl residues. The observed fragmentation showed that the site of unsaturation was a terminal galacturonosyl residue (Figure 17). The parent molecule and the fragments that still bear the unsaturated ring in the chain show a secondary fragmentation producing peaks 44 mass units lower (peaks with m/z 1363, m/z 1187, and m/z 1011). This can be rationalized by the loss of carbon dioxide from the unsaturated ring. A tentative structure is proposed which is the unsaturated octagalacuronide as shown at the bottom of Figure 17.

**Derivatization studies**

In the negative FAB-MS experiments on the native oligogalacturonides, fragmentation from both ends of the molecule was observed. In order to examine whether the unsaturated ring is located at the nonreducing end or at the reducing end, a derivatization reaction to label the reducing end was carried out. o-(2, 3, 4, 5, 6-Pentafluorobenzyl)hydroxylamine (PFB) derivatization was first tested as described in the Experimental section. The method studied by Dell et al.\(^3\) has shown that incorporation of functional groups such as PFB, which stabilizes a negative charge, will enhance the sensitivity of negative ion FAB-MS. One successful example is shown in Figure 3. The PFB tail at the reducing end of the molecule could provide some information about the fragmentation process. Unfortunately, the results from FAB-MS analysis (spectra not shown) were not as good as expected. Two general problems limit the method. First, the very poor solubility of oligogalacturonides in pyridine solution seriously affected the reaction. The PFB derivatives of higher molecular weight oligogalacturonides were barely visible in the spectra. Second, the fragmentation obtained was obviously occurs from both ends of the molecule. Possible explanations
for this result include random fragmentation which can occur even with the reducing end labeled, and the other that the hydrolysis of the oligomer happened before the reaction. The same results (not shown) are observed for PFB derivatives of hop elicitors.

Reductive amination has been successfully used on neutral oligosaccharides.\textsuperscript{52} In our case the reaction worked out quite well. FAB-MS spectra obtained in the positive mode for reductively aminated samples are shown in Figures 18 and 19. Derivatized trigalacturonic acid provides a clean spectrum (Figure 18). Beside the major mass ratio with m/z 0f 632, a di-hexylamine derivative is present at m/z 715. Fragments m/z 280 and m/z 456 indicate that the fragmentation begins at the nonreducing end of the galacturonides. Peaks responsible for the loss of water are also present 18 Daltons below each sequential ion. Figure 19 gives the spectrum of a reductively aminated oligogalacturonide mixture. Among them, the highest mass ratio m/z 1864 and m/z 1846 correspond to derivatives of the decamer with and without loss of water. A difference of 176 D between each pair of sequential peaks was observed, and each mass ratio peak was accompanied by the one with loss of water. The utility of the method is sometimes limited, however, because of the formation of sodium adducts, which result from using sodium cyanoborohydrate as the reducing agent. This problem may become even more severe for small amounts of sample. This was exactly what happened to the reductively aminated elicitor samples. In the FAB-MS spectra (not shown), no mass ratio corresponding to derivatized elicitors was observed; only a series of sodium adduct ion peaks were present.

To diminish the salt interference reductively aminated oligogalacturonides were peracetylated. The resulting derivative could then be extracted into an organic solvent and thus give a sodium free sample. The method was tested on reductively aminated trigalacturonic acids (Figure 20) and unsaturated pentagalacturonic acids (Figure 21). In Figure 20, the molecular ion for the fully derivatized, reductively aminated
Figure 18. Positive fast atom bombardment mass spectrum of reductively \( n \)-hexyl aminated trigalacturonic acid. The molecular ion peak \((\text{MH})^+\) with \(m/z\) 632 represents the derivatized trigalacturonic acid. For details see Results and Discussion.
Figure 19. Positive fast atom bombardment mass spectrum of reductively $n$-hexyl aminated mixed oligogalacturonic acids. The molecular ion peak $(\text{MH})^+$ from m/z 808 to m/z 1864 represents the derivatized tetramer to decamer.
Figure 20. Positive fast atom bombardment mass spectrum of peracetylation of reductively aminated trigalacturonic acid. The molecular ion peak (MH)$^+$ with m/z 1032 represents the derivatized trimer with one sodium adduct. The molecular ion peak (MH)$^+$ with m/z 972 and 912 show the sequence losses of mass 60 D (HOAc). For details see scheme 6.
Figure 21. Positive fast atom bombardment mass spectrum of peracetylation of reductively aminated pentagalacturonic acid from hop fraction 5. The molecular ion peak (MH)$^+$ with m/z 1410 shows the derivatized pentamer with loss of mass 60 D (HOAc). For details see Results and Discussion.
trigalacturonic acid with m/z 1010 is absent, instead a peak with m/z 1032 corresponding to the sodium ion adduct was observed. Consecutive losses of acetic acid (m/z 60) from this ion were also observed as [(M+Na)H-60]^+ with m/z 972 and [(M+Na)H-120]^+ with m/z 912. Fragmentation of the derivatized molecule is also observed, and similar losses

Scheme 6. Fragmentation of peracetylated reductively aminated trigalacturonic acid [mass unit of acetic acid (HOAc) = 60 Daltons]
of acetic acids were also detected. A detailed illustration of the fragmentation is provided in Scheme 6. Losses of acetic acid only occurred at the residue with the amine tail. Spectra for the peracetylation of reductively aminated unsaturated pentagalacturonic acid are more difficult to interpret. Partial spectra and fragmentations are shown in Figure 21. The expected molecular ion for the fully derivatized, reductively aminated substrate with m/z 1470 is not present. However, characteristic losses of acetic acid (m/z 60) from this ion were recorded. It was shown as (MH-60)+ with m/z 1410, (MH-120)+ with 1350, and (MH-180)+ with 1290. A second set of peaks separated by 60 mass units is observed with m/z 1151 and m/z 1091, respectively. These fragment ions result from the loss of the terminal unsaturated peracetylated galacturonosyl residue (m/z increment 259) located at the nonreducing end of the molecule. The peak with m/z 1453 is due to mixed-anhydride formation. One of the carboxyl groups of the unsaturated pentagalacturonides reacted with excess acetic anhydride to give a mixed anhydride thereby adding 43 mass units. Loss of acetic acid from this species produces a peak with m/z 1393.

In contrast to the native oligogalacturonic acids and the pentafluorobenzyl hydroxylamine derivatizatives which gave FAB-MS spectra in the negative ion mode, the other derivatized oligogalacturonides were recorded in the positive ion mode. Reductive amination not only added a tag to the reducing end of the molecule, it also altered the ionization characteristics of the compounds. In the positive ion mode carbohydrates do not fragment as easily as in the negative ion mode, and only a few fragment ions were detected, especially, for the larger oligmers.

Desalting Studies

Interferences due to the presence of salts was a problem in each step of the studies. Several methods were used to try to solve this problem. Gel filtration column
chromatography is the most popular method used for this purpose. In our case, a different size of Bio-Gel P-2 column and a Sephadex G-10 column was used as described in the Experimental section. For small samples, such as 40 μg or less, desalting with a normal column causes significant sample losses. Accordingly, a tip gel filtration column was used. But neither a normal column nor a tip column can remove salt completely. Only excess salt, e.g. sodium acetate, sodium hydroxide, can be removed. It is not possible to completely remove the sodium ion associated with the carboxyl groups of the galacturonides. Barium precipitation is a useful method for desalting particularly for acidic sugars.\textsuperscript{31} It was found that if a sample contains a huge amount of salt, such as the fractions collected from HPAEC, primary desalting had to be done before the barium precipitation could be carried out. However, the results obtained from the ion-exchange step with barium precipitation method were not reproducible, presumably, because of the very small amount of sample (micrograms) and the contamination from a variety of sources, e.g. from the ion-exchange resin, or from coprecipitation of materials such as impurities in the sample or in the reagent.

The formation of sodium adducts can sometimes be inhibited by using different matrixes when the sample is applied to the probe tip for FAB-MS analysis. Adding HCl to the target during the sample application, as described by Davis and coworkers, was attempted.\textsuperscript{28} The results with this method are shown in Figures 22a,b. The molecular ions of tetragalacturonic acid, m/z 721, which was barely observed in the bottom spectrum (Figure 22b), was increased dramatically after 0.4 N HCl was added (Figure 22a). The highest peak shifted from m/z 787 [(M+3Na)H\textsuperscript{+}, Figure 22b] to m/z 743 [(M+Na)H\textsuperscript{+}, Figure 22a]. A similar result (not shown) was obtained by adding p-toluene sulfonic acid, but it was not as effective as adding HCl. Strong acids added as a partial matrix kept salt from forming and thereby promoted the detection of the molecular ion. For the larger oligomers (pentamer and greater) with several sodium adducts on the carboxyl groups, adding acid was not enough to increase the detection
Figure 22. Negative fast atom bombardment mass spectrum of desalting studies by using different matrixes. a: Glycerol and 0.4 N HCl were used as a matrix. The molecular ion peak (M-H)⁻ with m/z 721 represents a tetragalacturonic acid. b: Glycerol only was used as a matrix. The molecular ion peak (M-H)⁻ with m/z 765 represents tetragalacturonic acid with two sodium adducts.
sensitivity of the molecular ion. Also, additional loss of water molecules was observed after addition of strong acids.

Conclusions

It was observed from this investigation that the hop elicitors released from hop leaf extracts by autoclaving were unsaturated galacturonide oligomers. The structure of oligomers are likely the same as those obtained by treating apple pectin with the endopectate lyase. The oligomer possessing the highest biological activity is the unsaturated hexagalacturonic acid, and the double bond is located at the nonreducing terminus of the oligomer. These elicitors can strongly bind to an anion-exchange resin. They are also quite heat stable. This has been the first reported attempt to isolate and to characterize elicitors from hop plants. This research will contribute to an understanding of plant resistance towards microbial and insect pests. Further studies will focus on the isolation and characterization of the hop phytoalexins and to the biological activity in the protection of the plant.
Bibliography


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