Glycopeptide Elicitors of Stress Responses in Tomato Cells

N-Linked Glycans Are Essential for Activity but Act as Suppressors of the Same Activity when Released from the Glycopeptides

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ABSTRACT

Induction of ethylene, an early symptom of the stress response in tomato (Lycopersicon esculentum) cells, was used as a bioassay to purify elicitor activity from yeast extract. The purified elicitor preparation consisted of small glycopeptides (mean relative molecular weight of approximately 2500) and induced ethylene biosynthesis and phenylalanine ammonia-lyase activity half-maximally at 15 nanograms per milliliter. Elicitor activity was partially abolished by pronase and almost completely by endo-β-N-acetylglucosaminidase, α-mannosidase, or periodate. The oligosaccharides released upon treatment with endo-β-N-acetylglucosaminidase competitively inhibited the elicitor activity of the glycopeptides. This suppressor activity was abolished by periodate oxidation and α-mannosidase treatment. The suppressors were chromatographically separated into four active fractions with sizes corresponding to 7 to 10 monosaccharides. They consisted predominately of mannose and contained also N-acetylglucosamine and glucose. The suppressors had no effect on the response of the tomato cells to a different elicitor, derived from cell walls of Phytophthora megasperma f. sp. glycinea. This strongly suggests that different recognition sites exist for different elicitors in tomato cells, and that the oligosaccharide suppressors act specifically on the perception of just one elicitor. The hypothesis is put forward that the suppressors bind to one of the elicitor recognition sites nonproductively, i.e. without producing a signal, thereby preventing induction of the stress responses by the corresponding elicitor.

Plants react to attack by pathogenic microorganisms and other irritations with a multiplicity of biochemical reactions, collectively known as defense or stress responses (7, 13). Elements of these defense or stress responses are the induction of enzymes of secondary metabolism like PAL (7, 13) and the enhanced biosynthesis of the plant hormone ethylene (1, 2). Studies with intact plants and plant cell cultures have shown that chemical stimuli derived from pathogens or from plant cell walls, so-called elicitors, can induce the same set of stress responses (1, 7, 13).

Elicitors of different structure have been isolated from culture filtrates, cell walls, and membranes of phytopathogenic fungi (1, 13). A landmark study identified a chemically pure heptasaccharide from cell walls of Pmg as an elicitor of defense reactions in soybean (23) and led to the concept that oligosaccharides are important signal molecules in plants. More recently, it was found that the component from Pmg cell walls eliciting defense reactions in parsley was not an oligosaccharide but a glycoprotein (21). Similarly, a glycoprotein derived from culture filtrates of Phytophthora parasitica acts as an elicitor of phytoalexin formation in tobacco (9). In the latter cases, the elicitor activity was found to reside in the protein part of the molecules (9, 21). Glycopeptide elicitors have also been isolated from Cladosporium fulvum (6) causing necrosis in tomato leaves and from Colletotrichum lagenarium (26) inducing ethylene biosynthesis in melon plants. Furthermore, elicitors unrelated to oligosaccharides and glycoproteins have been described. For example, arachidonic acid and eicosapentaenoic acid, isolated from the potato pathogen Phytophthora infestans, elicit phytoalexin formation in potato (3). In addition to microbial components recognized directly by the plant cells, microbial enzymes degrading plant cell walls can activate the defense response indirectly by releasing elicitor-active fragments, so-called endogenous elicitors, from plant cell walls (1, 7).

Although the molecular basis of elicitor perception remains unknown, it most likely occurs at the level of the plasma membrane. The plasma membrane of soybean cells contains binding sites for the glucan elicitor derived from Pmg (5, 22). Pathogen-derived elicitors are likely an important chemical cue for the plant to recognize and ward off potential pathogens. It has been hypothesized that virulent pathogens circumvent the plant defenses by secreting suppressors inhibiting elicitor recognition (4, 15). Fungal suppressors of the defense responses have been described, for example in the interactions between soybean and Pmg (29). The accumulation of the phytoalexin glyceollin, induced by a glucan elicitor derived from Pmg cell walls, was suppressed by invertase from a pathogenic race of Pmg. Suppressor activity appeared to be due to the carbohydrate moiety of invertase, because it was abolished by periodate oxidation and not by heat treatment (29). Other fungal suppressors include substances from P. infestans blocking the hypersensitive response of potato (8) or tomato (24), a protein fraction from culture filtrates of Ascochyta rabiei inhibiting phytoalexin accumulation in chick-
pea (17), and glycopeptides from germination fluids of *Mycosphaerella pinodes* (28) delaying induction of PAL and phytoalexin accumulation in pea. The mode of action of all of these suppressors remains to be elucidated. Although it has been proposed that suppressors bind to an elicitor receptor, thus preventing elicitor binding and induction of the defense response (4, 15), evidence for this intriguing mechanism is lacking.

Here we describe and characterize a model system that provides strong evidence for the existence of suppressors competitively inhibiting elicitor binding. We have previously used yeast extract as a convenient source of elicitor-active compounds, as suggested by Hahn and Albersheim (14), and found that it contained potent elicitors of stress responses in suspension cultured tomato cells (10–12). The work presented here shows that the elicitor-active fraction from yeast extract contains glycopeptides that need the carbohydrate and the peptide parts in combination for elicitor activity. The oligosaccharide part of the glycopeptides is inactive as elicitor but competitively and specifically inhibits elicitor activity of the glycopeptide from which it originates, demonstrating that the oligosaccharide acts as a suppressor of elicitation, most likely by competing for the elicitor binding site.

**MATERIALS AND METHODS**

**Plant Cells**

A cell suspension culture was prepared from a tomato (*Lycopersicon esculentum* [L.] Mill) callus, line Msk8 (19), kindly provided by Dr. M. Koornneef. It was grown at 27°C in a Murashige-Skoog-type liquid medium supplemented with 5 μM 1-naphthylacetic acid, 1 μM 6-benzyladenine, and vitamins as described (10), and subcultured in intervals of 3 weeks. Cells were used for experiments 10 to 16 d after transfer, at the beginning of the stationary phase (10).

**Pmg-Elicitor and Oligosaccharides**

An elicitor derived by partial acid hydrolysis from cell walls of Pmg, called Pmg-elicitor, was kindly supplied by Prof. J. Ebel (Biologisches Institut II, Universität Freiburg i. Br., Germany). The carbohydrate GlcNAC-Man₅ and the glyco-amino acid Asn(GlcNAC)₃Man₉ were obtained from Biocarb (Lucerne, Switzerland).

**Assays of Elicitor andSuppressor Activities**

Tomato cells (0.2 g fresh weight in 2 mL medium) were mixed with 20 μL of appropriate dilutions of the elicitors (or corresponding control fractions) and, where appropriate, with 20 μL of appropriate dilutions of suppressors (or appropriate control fractions), enclosed in reagent tubes capped with a rubber septum, placed horizontally on a shaker, and incubated at room temperature for 4 h. At the end of the incubation period, a gas sample (1 mL) was withdrawn from the reagent tubes and assayed for ethylene by gas chromatography (10). Where required, cells were collected immediately thereafter on a filter, frozen in liquid nitrogen, homogenized in 100 mM Tris (HCl), pH 8.8, containing 5 mM β-mercaptoethanol, and the extracts used for assays of PAL activity as described (12).

**Partial Purification of an Elicitor**

Yeast extract (800 g), an autolysate of *Saccharomyces cerevisiae* (Difco Co., Detroit, MI), was dissolved in 2000 mL water and subjected to differential ethanol precipitation at room temperature. The fraction soluble in 60% (v/v) ethanol but insoluble in 80% ethanol (14) was dialyzed extensively against water at 6°C, using Spectra/Por 3 dialysis membranes (Spectrum Medical Instruments, Los Angeles, CA) with a molecular mass cutoff of approximately 3.5 kD. The precipitate that formed inside the dialysis tubes was removed by centrifugation. The clear supernatant was adjusted to pH 8.0 and chromatographed on a column (5.5 × 20 cm) containing DEAE-trisacryl M (Industries Biologiques Françaises, Ville-neuve-La-Garenne, France) equilibrated with 3 mM Tris (HCl), pH 8.0. The nonbinding material was adjusted to 3 mM acetate (Na⁺), pH 5.0, and applied to a column (5.5 × 20 cm) of Sulfoethyl-Sephadex C-25 (Pharmacia, Uppsala, Sweden) equilibrated with 3 mM sodium acetate, pH 5.0. Elicitor-active material was eluted with 0.4 M NaCl in the same buffer, adjusted to 100 mM acetate (Na⁺) buffer, pH 5.2, containing 150 mM NaCl, 1 mM MgCl₂, 1 mM MnCl₂, and 1 mM CaCl₂, and applied to a Con A-agarose (Bio-Rad, Richmond, CA) column (2.5 × 20 cm) at 6°C. Elicitor-active material bound to the column was eluted with 200 mM α-methyl-d-mannopyranoside in the same buffer. After desalting the preparation on a column (2.5 × 50 cm) of Bio-Gel P-4, 400 mesh (Bio-Rad), at room temperature with distilled water as an eluent, the elicitor was freeze-dried, redissolved, and subjected to HPLC on a C18 ultraspHERE column (5 μm) column (4.6 × 250 mm) obtained from Beckman, eluting at a flow rate of 1 mL min⁻¹ with 10 mM phosphate (Na⁺) buffer (pH 2.5) for 10 min, followed by a linear gradient to 25% acetonitrile in 120 min. Fractions with the elicitor activity were collected, desalted on a Bio-Gel P-4 column, and freeze-dried. This preparation was designated YE-C18. YE-C18 was applied to a MicroPak AX-5 column (4 × 300 mm) obtained from Varian (Sunnyvale, CA). The column was equilibrated with acetonitrile:water (65:35, v/v), and elution was performed by increasing the water content at a rate of 0.5% min⁻¹ for 14 min and then by 0.27% min⁻¹ for 30 min at a flow rate of 1 mL min⁻¹. The fractions containing the highest elicitor activities were pooled and freeze dried. This elicitor preparation was designated YE-AX.

**Size Exclusion Chromatography**

Preparations of elicitors or suppressors were chromatographed on a Bio-Gel P4 column (1.0 × 150 cm) eluted with 0.1 M acetic acid. ³H-Labeled α-1,4-glucan oligomers, obtained by partial acid hydrolysis of dextran followed by reduction with [³H]NaBH₄ (20), were used as molecular mass standards.

**Enzymatic Treatments**

Deglycosylation of elicitor was performed either by incubation of YE-AX (1 mg) with 0.025 units Endo H from *Streptomyces pilatus* (Boehringer-Mannheim, Germany) in 50 mM citrate (Na⁺) buffer, pH 5.5, containing 0.02% SDS and 1 mM phenylmethanesulfonylfluoride at 37°C for 18 h.
under toluene, or by treatment of YE-AX (50 μg) with 1.7 units of N-glycanase from Flavobacterium meningosepticum (Genzyme Corp., Boston, MA) in 200 mm phosphate (Na+) buffer, pH 8.6, containing 0.17% SDS and 1.25% Nonidet P-40 (Sigma, St. Louis, MO) at 37°C for 16 h. Prior to deglycosylation, YE-AX was boiled in SDS-containing incubation buffer for 3 min. Proteinase treatment was performed by incubating YE-AX (100 μg) with 100 μg pronase from Strep.

tomyces griseus (Calbiochem, San Diego, CA) in 0.1 m Tris (HCl) buffer, pH 8.0, containing 5 mM CaCl₂, at 37°C under toluene for 24 h, followed by addition of another 100 μg of pronase and continuation of the digestion for 24 h. Treatments with α-mannosidase from jack bean (Sigma) were performed in 100 mM acetate (Na+) buffer, pH 4.5, containing 5 mM ZnSO₄ and 0.2 units of the enzyme/μg of the substrate at 37°C for 16 h under toluene. All incubations were terminated by boiling for 3 min, and samples were subsequently freeze dried.

Treatment with Periodate
YE-AX (100 μg mL⁻¹) or Pmg-elicitor (1 mg mL⁻¹) were incubated in 67 mM acetate (Na+) buffer, pH 5.5, containing 10 mM sodium periodate (Sigma) at room temperature in the dark for 30 min. The reaction was stopped by addition of sodium disulfite (Sigma) to a final concentration of 20 mM. Endo H-treated YE-AX (30 μg mL⁻¹) was incubated under the same conditions and the reaction stopped with sodium disulfite after 1 h. For mock treatments, sodium periodate was preincubated with sodium disulfite for 10 min before it was added to the elicitor preparations.

Determination of the Carbohydrate and Amino Acid Composition
Methanolysis of each sample (5–10 μg), spiked with an internal standard (1 μg of myo-inositol), was carried out in 400 μL methanol containing 1 m HCl at 85°C for 5 h. After cooling and addition of 50 μL of i-butanol, the reaction mixture was evaporated to dryness. Hexosamines were N-acetylated by adding 250 μL methanol, 25 μL pyridine, and 25 μL acetic anhydride and stirring for 15 min at room temperature. The samples were evaporated to dryness, dried over P₂O₅, and trimethylsilylated by adding 100 μL of pyridine:hexamethyldisilazane:trimethylchlorosilane (10:2:1) and allowing the reaction to proceed for 1 h at room temperature. GC-MS analysis was carried out on a fused silica column (20 m) with OV-1207 as the stationary phase and helium as carrier gas, using a temperature gradient from 116 to 280°C at 4°C min⁻¹ and a Finnigan TSQ-70 mass spectrometer for detection. The identity and the response factors for mannose, glucose, N-acetylglucosamine, and myo-inositol were determined by subjecting reference monosaccharides to the same methanolysis and derivatization protocol.

Hydrolysis of peptides and analysis of the amino acids were performed according to the procedure of Knecht and Chang (18).

RESULTS AND DISCUSSION

Purification of Elicitor-Active Glycopeptides from Yeast Extract
Elicitor-active compounds were purified from yeast extract according to their ability to stimulate ethylene biosynthesis in tomato cells (Table I). Most of the elicitor activity did not bind to DEAE-trisacryl but bound to SP-Sephadex, indicating cationic properties. All elicitor activity bound to Con A-agarose and was eluted with α-methyl-D-mannopyranoside, indicating that it contained carbohydrates. During HPLC on a C18 reversed-phase column, elicitor activity was eluted at low acetonitrile concentrations (4–6%); the bulk of material absorbing at 214 nm eluted at higher concentrations (data not shown). After each purification step, the fractions containing high elicitor activity were pooled, desalted, lyophilized, and weighed. Dose-response curves for elicitor-active fractions at different stages of purification are shown in Figure 1. The most purified fraction, obtained from the C18 column and designated YE-C18, yielded half-maximal stimulation of ethylene biosynthesis at a concentration of approximately 15 ng mL⁻¹. Assuming a mean Mᵣ of the elicitor molecules of larger than 2000 (see below), this corresponds to less than 8 nm. The progress of purification was accompanied by a reduction of the stimulation of ethylene formation at saturating concentrations of elicitors (Fig. 1), possibly due to the separation of factors acting synergistically in stimulating ethylene production. The maximal level of ethylene production attained by saturating concentrations of YE-C18, 95 ± 8.5 pmol g⁻¹ h⁻¹ in the experiment shown in Figure 1, varied considerably depending on the age of the cells, as described previously (10). However, the amount eliciting 50% of the maximum remained approximately constant in each experiment. Therefore, we defined as one unit the amount of elicitor per liter of cell culture yielding a stimulation of ethylene production equivalent to 50% of the maximum attained by saturating concentrations of YE-C18. In these terms, one unit corresponded to 15 μg of YE-C18 or 8 mg of the crude dialyzed preparation of yeast extract (Fig. 1). Using this definition, we established a balance sheet for the purification procedure (Table I). The elicitor activity was purified approximately 500-fold with a yield of 15%.

<table>
<thead>
<tr>
<th>Table I. Balance Sheet of Elicitor Purification</th>
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<tr>
<td>Preparation</td>
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<tr>
<td>Crude (yeast extract after ethanol precipitation and dialysis)</td>
</tr>
<tr>
<td>Flow-through of DEAE-triacyl</td>
</tr>
<tr>
<td>Bound to SP-Sephadex C-25</td>
</tr>
<tr>
<td>Bound to Con A-agarose</td>
</tr>
<tr>
<td>Peak fractions after HPLC on C18</td>
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</table>

* Dry weight measured after lyophilization of an appropriate sample. † One unit is defined as the amount of elicitor stimulating 1 L of cell suspension to 50% of the maximal ethylene biosynthesis attained by saturating levels of purified elicitor (YE-C18).
Characterization of Purified YE-AX and Comparison with Pmg-Elicitor

To characterize YE-AX, we compared its sensitivity to various enzymatic treatments to that of an elicitor derived by partial acid hydrolysis from Pmg cell walls (Pmg-elicitor). We have previously shown that tomato cells react similarly to elicitors derived from yeast extract and Pmg (12). Enzyme treatments were stopped by boiling to inactivate the enzymes; both elicitor activities were stable to boiling. Extensive pro-nase digestion strongly reduced the activity of both elicitors, implying that the activity of both depends on the peptide part of the preparations. Treatment with α-mannosidase almost completely eliminated elicitor activity of YE-AX but had no effect on Pmg-elicitor.

The preparation of YE-C18 was further chromatographed on a MicroPak AX-5 column. On this column, elicitor activity eluted in three broad, overlapping peaks between 27 and 40 min, demonstrating that the elicitor-active molecules were heterogeneous (Fig. 2A). Fractions containing the bulk of elicitor activity were combined, lyophilized, and weighed. This preparation, designated YE-AX, was subjected to quantitative amino acid analysis after peptide hydrolysis and found to contain approximately 10% (w/w) amino acids, indicating that the elicitor-active molecules are glycopeptides. Threonine, serine, lysine, and aspartate/asparagine were the main amino acids. High contents of serine, threonine, and asparagine are typical for preparations of yeast cell wall phosphomannoproteins (25). Other amino acids were present in lower, uneven molar ratios in relation to aspartate/asparagine, indicating that the different glycopeptides present in YE-AX had different amino acid compositions.

Chromatography of YE-AX on a Bio-Gel P-4 column (Fig. 2B) also showed the heterogeneity of the elicitor-active compounds: they spread over a range where dextran standards of a degree of polymerization of 8 to approximately 20 (Mr = 1300–3200) would migrate. The mean Mr of the elicitor-active compounds was estimated to be 2500, although this probably represents an underestimation taking into account their nature as glycopeptides (see below): glycopeptides generally have higher Mr values than estimated from the Mr values of glucan standards (A. Sturm, personal communication).

Figure 1. Dose-response curves for induction of ethylene biosynthesis in tomato cells by elicitor preparations at different stages of purification: ●, Crude elicitor (yeast extract after ethanol precipitation and dialysis); ▼, fraction not absorbed on the DEAE-trisacyl column; ▽, fraction eluted with α-methyl manno-pyranoside from the Con A-agarose column; ○, preparation YE-C18, purified by reversed-phase HPLC on a C18 column. Dotted lines, levels of ethylene biosynthesis corresponding to water controls and one unit of elicitor activity, respectively (see Table I for definition of one unit).

Figure 2. Chromatography of partially purified elicitor preparations. A, Chromatography of elicitor preparation YE-C18 (1 mg) on MicroPak AX-5. Aliquots (0.75 μL) of fractions (0.5 mL) were tested for induction of ethylene biosynthesis in tomato cells (●). Thin line, continuous registration of the optical density at 195 nm. Fractions indicated by the double arrow were pooled to yield the elicitor preparation YE-AX. B, Chromatography of preparation YE-AX (50 μg) on Bio-Gel P-4. Aliquots (0.25 mL) of fractions (0.5 mL) were evaporated to dryness and tested for induction of ethylene biosynthesis (●). Positions of [3H]dextran size markers are marked at the top of the figure by vertical lines labeled with the respective degree of polymerization. V0 indicates the void volume of the column.
Two endoglucanases were used to test if the elicitor activity is due to N-linked glycopeptides: Endo H hydrolyzes N-linked glycans between the two GlcNAc residues that are linked to the peptide, releasing oligosaccharides with one GlcNAc residue at its reducing end (27). N-Glycanase hydrolyzes various classes of N-linked oligosaccharides between asparagine and GlcNAc (16) and produces oligosaccharide fragments with two GlcNAc residues at the reducing end. Both Endo H and N-glycanase almost completely destroyed elicitor activity of YE-AX (Table II), indicating that the union of the carbohydrate and the peptide part is important for elicitor activity in the yeast extract-derived elicitor. In contrast, the activity of the Pmg-elicitor was resistant to treatment with Endo H and N-glycanase (Table II).

Periodate is known to degrade glycosyl residues with adjacent unsubstituted hydroxyl groups and, therefore, is expected to attack terminal or 1,6- or 1,4-linked glycosyl residues. Periodate oxidation of YE-AX resulted in a complete abolition of elicitor activity, indicating that the structure of the glycosyl side chain is important for elicitor activity (Fig. 3A). The same treatment did not affect Pmg-elicitor (Fig. 3B). The activity of YE-AX was resistant to boiling in 20 mM β-mercaptoethanol (Fig. 3A), a treatment known to reduce S-S bridges in peptides, as expected from the absence of cysteine found by the amino acid analysis. In contrast, Pmg-elicitor was completely inactivated by the same treatment (Fig. 3B), indicating that S-S bridges are involved in the activity of Pmg-elicitor.

In summary, these data show that purified YE-AX consists of glycopeptides requiring the union of the carbohydrate and peptide part for activity. We are not aware of a precedent for such an elicitor, although glycopeptides have been described as elicitors in other instances, usually with the untested contention that the carbohydrate part is essential for activity (reviews: 1, 7). The elicitor-active component of crude Pmg-elicitor differs in various respects and probably consists of polypeptides without essential carbohydrate parts. This is reminiscent of the situation in parsley, in which the carbohydrate part of a glycoprotein elicitor from a Pmg cell wall preparation was not needed for activity (21).

### Table II. Effect of Enzymes on the Activities of YE-AX and Pmg-Elicitor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Elicitor Activity Remaining&lt;sup&gt;a&lt;/sup&gt;</th>
<th>YE-AX</th>
<th>Pmg-elicitor</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Pronase</td>
<td></td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Pronase control&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>126</td>
<td>61</td>
</tr>
<tr>
<td>α-Mannosidase</td>
<td></td>
<td>3</td>
<td>89</td>
</tr>
<tr>
<td>α-Mannosidase control&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>120</td>
<td>90</td>
</tr>
<tr>
<td>Endo H</td>
<td></td>
<td>120</td>
<td>94</td>
</tr>
<tr>
<td>Endo H control&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>111</td>
<td>106</td>
</tr>
<tr>
<td>N-Glycanase</td>
<td></td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>N-Glycanase control&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td></td>
<td>257</td>
<td>49</td>
</tr>
</tbody>
</table>

<sup>a</sup> A dilution series was prepared of a known amount of elicitor after incubation with various enzymes, followed by boiling.<br><sup>b</sup> Controls were obtained by incubating elicitor in the appropriate buffer in the absence of enzymes, adding a boiled enzyme preparation at the end of the experiment. <br><sup>c</sup> The detergent, NP-40, increased ethylene accumulation in the presence of YE-AX.

Suppressor Activity of Oligosaccharides Released from Glycopeptide Elicitors from Yeast Extract

When different amounts of the reaction mixture of Endo H with YE-AX were added to tomato cells together with a constant amount of the untreated elicitor YE-AX, induction of ethylene biosynthesis was inhibited in a concentration-dependent manner (Fig. 4A). Almost complete inhibition was obtained by addition of 20 μL of the reaction mixture, yielding a final concentration of 1000 ng mL<sup>-1</sup> Endo H-digested YE-AX. Control experiments showed that Endo H-digested YE-AX did not affect ethylene biosynthesis in the absence of elicitor, and that reaction mixtures of Endo H without YE-AX had no inhibitory effect (Fig. 4A).

The reaction mixture of Endo H with YE-AX was chromatographed on a Con A-agarose column. The fraction not bound, containing peptides, displayed neither suppressor nor elicitor activity. All suppressor activity was bound to the Con A-agarose column, indicating that the suppressor activity resided in the oligosaccharides released upon Endo H digestion (data not shown). In agreement with this, periodate oxidation completely destroyed the suppressor activity (Fig. 4B). Control experiments showed that sodium disulfite-inactivated periodate itself did not affect suppressor activity, had no elicitor activity, and did not affect the elicitor activity of YE-AX (Fig. 4B).

Carbohydrates released from YE-AX by N-glycanase and purified by HPLC on a MicroPak AX-5 column similarly
further of YE-AX the amounts different for induced ethylene were added to presence experiment B, cells tomato 4.

Figure 4. Dose-response curves for the suppression of elicitor-induced ethylene biosynthesis by (A) Endo H-treated elicitor or (B) Endo H-treated elicitor subjected to periodate oxidation. A reaction mixture containing Endo H-treated elicitor was obtained by incubation of YE-AX with Endo H, followed by boiling. In experiment A, different amounts of this reaction mixture were added to tomato cells in the presence (O) or absence (V) of 33 ng mL⁻¹ YE-AX. For controls, different amounts of a similar reaction mixture, obtained by incubation of the same quantity of Endo H without YE-AX followed by boiling, were added to cells in the presence of 33 ng mL⁻¹ YE-AX (V). In experiment B, different amounts of reaction mixtures obtained by further incubation of the Endo H-treated elicitor with 10 mM NaIO₄ for 1 h, followed by 20 mM sodium disulfite (O), or with 10 mM NaIO₄ previously reduced with 20 mM sodium disulfite (O), were added to tomato cells and assayed for their effect on ethylene biosynthesis induced by 33 ng mL⁻¹ YE-AX. For controls, different amounts of similar reaction mixtures, obtained by incubation of 10 mM NaIO₄ without substrate and then inactivated by 20 mM sodium disulfite, were added to cells and assayed for their effect on ethylene biosynthesis in the presence (V) or absence (V) of 33 ng mL⁻¹ YE-AX. Dotted lines, ethylene production rates induced by YE-AX at the concentrations stated above the lines. Top x-scale, amount of reaction mixture added; bottom x-scale, concentration of Endo H-treated YE-AX, where present.

reduced elicitor-induced ethylene formation in a concentration-dependent manner (data not shown). Taken together, these results demonstrate that hydrolysis of the elicitor-active glycopeptides into the glyco- and -peptide parts by endoglycanases yields oligosaccharides that act as suppressors of the elicitor activity.

**Purification and Characterization of Oligosaccharides with Suppressor Activity**

The reaction products of Endo H with YE-AX were separated by gel filtration on Bio-Gel P-4. Suppressor activity, detected by the reduction of ethylene production induced by a standard amount of untreated YE-AX, eluted as a broad peak in the range of dextran size markers with a degree of polymerization of 7 to 12 hexose units (Fig. 5). Taking into account the findings that the molecules have one GlcNAc (see below) and that free GlcNAc behaves as a disaccharide in the Bio-Gel P-4 column (A. Sturm, personal communication), the actual size of the suppressors corresponded to 6 to 11 monosaccharides. Fractions with an estimated size of 10, 9, 8, and 7 monosaccharides (dextran standards of 11, 10, 9, and 8 glucose units, respectively) were combined into four pools, termed preparations I through IV (Fig. 5). The slight enhancement of elicitor-induced ethylene biosynthesis by fractions 151 to 153 (Fig. 5) was not reproducible and therefore was not further investigated.

Each of the pooled preparations I through IV was further purified individually by chromatography on a MicroPak AX-5 column (Fig. 6, A–D). The elution profile showed that the suppressor activity (arrows in Fig. 6, A–D) coincided with a single peak of absorption at 195 nm in each chromatogram. These fractions were designated I1, I2, I3, and I4. The increased retention times reflect increased molecular sizes from I4 to I1.

Aliquots of the components I1, I2, I3, and I4 were analyzed by GC-MS. All four samples contained 80% (w/w) mannose, 10 to 20% (w/w) glucose, and small amounts of GlcNac, which could not be quantitatively recovered in the procedure. Thus, the suppressors are most probably derived from N-linked glycans and contain one GlcNac, five to eight Man, and one Glc. The small overall size of the elicitor molecules (see Fig. 2B) indicates that they consist of one short peptide and one N-linked glycan. The high amount of mannose is typical of high-mannose glycan side chains. We have tested a few high-mannose glycans obtained from other sources than from yeast extract as potential suppressors. The following preparations showed no activity as elicitors at concentrations up to 10 µg mL⁻¹ and did not reduce elicitor-induced ethylene formation at 100- to 300-fold excess over elicitor: (a) high mannose glycopeptides released from ovalbumin by trypsin; (b) the purified hexasaccharide Man α 1–3 (Man α 1–3 (Man α 1–6) Man α 1–6) Man β 1–4 GlcNAc; (c) the glyco-amino acid Asn(GlcNAc)₂Man₂ from soybean lectin; (d) the high mannose oligosaccharide GlcNAcMan₄ released from this glyco-amino acid by Endo H.

Figure 5. Separation of Endo H digestion products of YE-AX (1 mg) on Bio-Gel P-4. Aliquots (50 µL) of each fraction (0.5 mL) were evaporated to dryness and tested for their ability to suppress induction of ethylene formation by YE-AX (33 ng mL⁻¹). Fractions pooled for further analysis are indicated (I–IV). Dotted lines, ethylene production rates induced by YE-AX at the concentrations stated above the lines.
Determining the Inhibition Constant of Suppressor I2 and Inhibition of PAL Induction

To test the nature of inhibition, we assayed a series of concentrations of suppressor I2 in combination with three different elicitor concentrations. Analysis of the data in a Dixon plot (Fig. 8) indicated that inhibition by the suppressor was competitive with a $K_i$ of approximately 60 ng mL$^{-1}$ or 40 nM, assuming that I2 is composed of one GlcNAc and eight mannose/glucose residues ($M_r = 1517$).

Suppressor I2 competitively inhibited not only induction of ethylene biosynthesis, but also induction of PAL by the glycopeptide elicitor (data not shown). Although it has been hypothesized that elicitor-induced ethylene production is a signal for the induction of PAL (review: 2), this is not the case in our model system. Rather, elicitors normally induce PAL activity in tomato cells even when induction of ethylene biosynthesis is completely blocked by cobalt ions (12). Hence, induction of ethylene biosynthesis and PAL are separate, independent elicitor responses. Our results show that the two responses are not only induced in parallel by the purified elicitor preparations, but that they are also suppressed in parallel by purified suppressors, indicating that elicitors and suppressors compete for a single elicitor recognition site.

Specificity of Suppressor Activity

Suppressor I3 inhibited the induction of ethylene biosynthesis by YE-AX in a dose-dependent manner (Fig. 9). How-

\[ \text{Reaction mixture added, } \mu\text{L} \]

\[ \begin{array}{c|c}
    \text{Inhibitor, ng/mL} & \text{Ethylene, umol/h} \\
    \hline
    300 & 33 ng/mL \\
    100 & 3.3 ng/mL \\
    0 & \text{Water control} \\
\end{array} \]

**Figure 6.** HPLC of the suppressor pools I, II, III, and IV on a MicroPak AX-5 column equilibrated with acetonitrile:water (65:35) and eluted with the same mixture, increasing the water content by 0.5% min$^{-1}$ for 60 min at a flow rate of 1 mL min$^{-1}$. A, Pool I; B, pool II; C, pool III; D, pool IV (see Fig. 5). Continuous trace, optical density at 195 nm. Fractions comprising peaks of $A_{vis}$ were assayed for their effect on induction of ethylene formation by 33 ng mL$^{-1}$ YE-AX (○). Arrows indicate positions of peaks with suppressor activity. Numbers on x-axis, elution time (min).

**Figure 7.** Inactivation of the suppressors I3 and I4 with $\alpha$-mannosidase. Different amounts of reaction mixtures, obtained by incubation of I3 (𝑉, ⬇) and I4 (○, ◆) with (𝑉, ○) or without (𝑉, ◆) $\alpha$-mannosidase followed by boiling, were added to tomato cells and tested for their effect on ethylene biosynthesis in the presence of 33 ng mL$^{-1}$ YE-AX elicitor. As a control, different amounts of a reaction mixture containing $\alpha$-mannosidase incubated under the same conditions in the absence of suppressor were similarly tested (○). Dotted lines, ethylene production rates induced by YE-AX at the concentrations stated above the lines. The carbohydrate contents of I3 and I4 were calculated from the result of the GC-MS analysis by comparison with the internal standard.

$\alpha$-Mannosidase Treatment of Suppressors

Suppressors I3 and I4 were treated with $\alpha$-mannosidase. Control preparations of suppressors I3 and I4, incubated in buffer without enzyme, reduced the effect of 33 ng mL$^{-1}$ elicitor by about 66% at concentrations of 78 and 30 ng mL$^{-1}$, respectively (Fig. 7). $\alpha$-Mannosidase destroyed the inhibitor activity of I4 almost completely and that of I3 by more than 90%. This indicates that mannose residues are as important for suppressor activity as they are for elicitor activity of the glycopeptides (see Table II).
ever, when Pmg-elicitor was employed to induce ethylene biosynthesis, suppressor I3 did not suppress induction up to the highest concentration assayed but rather had a slight additional stimulating effect (Fig. 9). As discussed above (see Table II), the active compounds in Pmg-elicitor are proteinaceous in nature and differ from the yeast extract-derived elicitor. The results show that suppressor activity is highly specific, and that tomato cells contain at least two distinct elicitor recognition sites with different specificities. We are currently studying the structural requirements for elicitor and suppressor activities at the different elicitor recognition sites.

CONCLUSION

The results presented demonstrate that, in tomato cells, stress responses can be elicited by glycopeptides and that their action can be suppressed by oligosaccharides derived from the elicitor-active glycopeptides. They provide a model for the longstanding hypothesis that recognition of elicitors may be blocked by specific suppressors (4, 15). Although the biological significance of these observations is not clear because we have employed a model system without relation to a natural plant-pathogen interaction, the biochemical implications are interesting. They strongly suggest that the glycopeptide elicitors and the oligosaccharide suppressors compete for a single elicitor recognition site functioning as a receptor, and that interaction of this receptor with suppressors is non-productive, i.e. does not produce a signal, whereas its interaction with elicitors is productive and initiates the signal transduction chain leading to the stress responses. Thus, experiments with suppressors may not only help in the identification of elicitor receptors, but may also shed light on the elements in signal transduction immediately following elicitor-receptor interactions.

Figure 8. Inhibition of the YE-AX elicitor-dependent induction of ethylene biosynthesis by suppressor I2, presented in a Dixon plot. Different concentrations of suppressor I2 were tested for their capacity to suppress ethylene formation induced by YE-AX at concentrations of 33 ng mL\(^{-1}\) (●), 100 ng mL\(^{-1}\) (○), and 333 ng mL\(^{-1}\) (△). The carbohydrate content of I2 was calculated from the result of the GC-MS analysis by comparison with the internal standard.

Figure 9. Specificity of the effect of suppressor I3. Different concentrations of I3 were tested in a single experiment for their capacity to suppress ethylene formation induced (A) by YE-AX (33 ng mL\(^{-1}\)) or (B) by Pmg-elicitor (1 μg mL\(^{-1}\)). The carbohydrate content of I3 was calculated from the internal standard used in the GC-MS analysis. Dotted lines, ethylene production rates induced by YE-AX (A) or by Pmg-elicitor (B) at the concentrations stated above the lines.

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