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Phytoalexin Elicitor Activity of Carbohydrates from *Phytophthora megasperma* f.sp. *glycinea* and Other Sources

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ABSTRACT

Three unique classes of carbohydrates were isolated from the hyphal cell walls of *Phytophthora megasperma* f.sp. *glycinea* (Pmg) and compared with other substances for their activity as elicitors of the phytoalexin glyceollin in soybean tissues. Glucomannans extracted from cell walls with soybean β-1,3-endoglucanase were purified and proved to be the most active elicitors yet reported. They were approximately 10 times more active in soybean cotyledons than the heterogeneous β-glucan elicitor fraction extracted from Pmg walls. In addition, the glucomannan fraction gave race-specific elicitor activity in soybean hypocotyls. Pronase was found to be a suitable reagent for the mild extraction of glycopeptides from Pmg cell walls. All of the carbohydrates isolated from Pmg cell walls possessed significant elicitor activity, but other glucans, a glucomannan and mannan from other sources, were much less active. Chitin and chitosan, reported to function as elicitors in other plants, had low activity in soybean cotyledons. Arachidonic acid was inactive, despite its previously observed elicitor activity in potato tubers. The results indicated that, for Pmg, the carbohydrate elicitor most probably involved in the initiation of phytoalexin-mediated defense during fungus infection of soybean plants is the glucomannan fraction liberated by endoglucanase.

Elicitors are agents which initiate the production of phytoalexins by higher plant tissues (13, 26). Various chemicals possessing elicitor activity have been isolated from plant pathogenic microorganisms. These include peptides (26), enzymes such as polygalacturonases and pectate lyases (17), unknown plant enzymes (18), and unsaturated fatty acids such as arachidonic acid (7). With these few exceptions, however, the elicitors of microorganisms have generally been shown to be polysaccharides or glycoconjugates (13, 26).

Several carbohydrate elicitors have been isolated from the phytopathogenic fungus Pmg.[1] This pathogen has been of interest because its interaction with soybean plants exemplifies a gene-for-gene host-parasite relationship, and single gene resistance in the host is expressed by the postinfectional production of phytoalexins called 'glyceollin' (13, 29). It is suspected that the occurrence of the resistance reaction is initiated by a specific recognition of the incompatible pathogen by the host, and that one or more of the fungal carbohydrate elicitors are the ultimate biochemical products of pathogen avirulence alleles which interact with the dominant plant resistance gene products (1). It is not yet certain, however, which if any of the described fungal carbohydrates play this role.

Albersheim and co-workers (2, 4, 5, 24) isolated heterogeneous branched β-1,3-glucans from the culture medium and from isolated cell walls of Pmg that were efficient elicitors of glyceollin in soybean cotyledons. They concluded that the glucans accounted for the majority of the elicitor activity from the fungus, but noted no differences in the activity of the glucans from various races of the fungus on different soybean resistance genotypes. A related branched β-1,3-glucan with about 30 glucose residues, mycolaminaran, was also shown to be an elicitor in soybean cotyledons, but only at higher concentrations than reported for the glucans (14). In addition, we isolated cell surface glycoproteins from the same fungus which possessed elicitor activity and exhibited race-specificity (15). That is, preparations from races giving an incompatible or resistant reaction on a certain soybean resistance genotype were more efficient elicitors than those from compatible races. Finally, Yoshikawa *et al.* (28) observed that incubation of isolated cell walls from Pmg with a crude or purified soybean β-1,3-endoglucanase enzyme released water-soluble carbohydrates with potent elicitor activity. The objectives of the work reported here were to: (a) isolate and partially characterize the carbohydrate elicitor released by soybean endoglucanase; (b) compare the elicitor activity of the various polysaccharides thus far isolated from Pmg with structurally related carbohydrates; and (c) assess which elicitor(s) is of probable physiological significance in the plant-pathogen interaction.

MATERIALS AND METHODS

General Methods. *Phytophthora megasperma* f.sp. *glycinea* was grown and the cell walls isolated as previously described (15). Soybean plants of cultivars Harosoy (rps) or the near isogenic Harosoy 63 (Rps[a]) were grown from seed as previously reported. Fungus cell walls were tested for the binding of FITC Con A by the method previously reported (15).

Mycolaminaran was isolated from hyphal homogenates of Pmg or other *Phytophthora* spp. by the methods of Wang and Bartnicki-Garcia (25). The other elicitors were extracted from isolated cell walls of Pmg as indicated below or obtained as shown in Table I. Unless otherwise noted, race 1 of Pmg was used throughout.

Elicitor Preparation.

Preparation of the Elicitor Released from Fungus Cell Walls by β-1,3-Endoglucanase. Purified hyphal cell walls (0.6 g) were incubated in 60 ml of 10 mM Na-acetate (pH 5.3) containing 20 or 50 $\mu g\ ml^{-1}$ of soybean endoglucanase obtained by electrofocusing and gel filtration or by CM Bio-gel fractionation and gel filtration (16). In some experiments, partially purified enzyme prepared through the ammonium sulfate step of Yoshikawa *et al.* (28) was employed in the cell wall incubations. The reaction mixtures were incubated at 35°C and monitored for liberation of soluble carbohydrates as previously described (16) until maximum release occurred after 50 to 80 min. They were then centrifuged, supernatant fluids collected, and the wall residue washed once with water. The pooled supernatants were passed through Millipore filters (0.22 μm), dialyzed against water, and brought to pH 4.4

[1] Abbreviations: Pmg, *Phytophthora megasperma* f.sp. *glycinea*; FITC Con A, fluorescein-labeled concanavalin A.

Table I. *Elicitor Activity of Various Fractions from Pmg Cell Walls and Other Metabolites in the Soybean Cotyledon Bioassay*

Fraction	Source	Concentration for Half-Maximal Activity[a]
		$\mu g\ ml^{-1}$
Glucomannan, fraction I	Pmg, race 1	0.3
Glucomannan, fraction II	Pmg, race 1	10.0
Glucomannan	*Pseudomonas syringae* pv. *glycinea*, race 1	>10,000[b]
Glycopeptides	Pmg, race 1	5.2
β-Glucan	Pmg, race 1	2.0
CM-cellulose	Sigma	>10,000[b]
CM-pachyman	B. Stone	>1,500[b]
Pustulan	B. Stone	>2,000[b]
Laminarin	Calbiochem	50
Mycolaminaran	Pmg, race 1	43
Cell walls	Pmg, race 1	9.0
Chitin	Eastman	4,000
Chitosan	Eastman	4,000
Yeast mannan	Sigma	>5,000[b]
Arachidonic acid	Sigma	>1,000[b]

[a] Saturation value determined using the glucomannan of Pmg as in Figure 4.

[b] These compounds exhibited activity equivalent to or less than 0.1 the maximum at the highest tested concentration.

with dilute HCl. To remove the glucanase enzyme, the preparations were passed through 0.7- × 5.0-cm columns of CM Bio-gel A equilibrated and washed with 5 mM K-acetate (pH 4.4). The effluent collected by washing with the acetate buffer was then concentrated to approximately 0.05 the original volume *in vacuo*. This was applied to a 1.4- × 35-cm column of Sephacryl S-200 equilibrated with 0.2 M NaCl. In some experiments, the fraction I carbohydrate peak from the column was collected and rechromatographed on the same column following reincubation with the enzyme as above. Pooled fractions were dialyzed against distilled H_2O and lyophilized, yielding white amorphous powders.

Release of Glycopeptide Elicitors from Cell Walls with Pronase. In earlier work, glycoproteins were extracted with 0.1 N NaOH at 0°C (15). It was observed here that pronase or papain also efficiently released glycoprotein elicitors from fungus cell walls. In the method routinely used, cell walls (5 or 10 mg ml^{-1}) were incubated at 35°C with crystalline pronase (Pronase CB, Calbiochem, 50 $\mu g\ ml^{-1}$ in 10 mM Tris-HCl, pH 7.2). Aliquots were withdrawn and passed through Millipore filters as before. Carbohydrate release from the walls was monitored by the anthrone method, and protein was monitored by measurement of A at 280 nm. When maximum release was reached after approximately 1 h, cell walls were removed from the bulk reaction mixture by centrifugation, the walls were washed once with water, and the pooled supernatant fluids passed through 0.22 μm Millipore filters. Following dialysis against water, the preparations were concentrated *in vacuo* at 45°C and chromatographed on Sephacryl S-200 columns as described above. The pooled carbohydrate-containing fractions were dialyzed, lyophilized, and adsorbed to 0.9- × 10-cm columns of DEAE Bio-gel equilibrated with 5 mM K-phosphate (pH 6.8). Glycopeptides were then eluted with the same buffer containing 0.2 N NaCl, dialyzed *versus* water, and lyophilized.

Extraction of β-Glucan Elicitors from Cell Walls by Autoclaving. Isolated Pmg cell walls or walls previously extracted with soybean glucanase and/or pronase were autoclaved in water according to Ayers *et al.* (5). Walls were centrifuged as above, and the supernatant fluids were passed through Millipore filters. The filtrates were concentrated *in vacuo*, dialyzed against water, and passed through 0.9- × 10-cm columns of DEAE Bio-gel equilibrated with 3 mM Tris-HCl (pH 7.2). The neutral fraction which passed through the column was collected, dialyzed against water, and lyophilized to yield the β-glucan elicitor fraction.

Chemical Techniques. Total hexose was determined by the anthrone reagent (6) with D-glucose as the standard. Glucomannans were methylated by the Hakomori method, following the procedure of Lindberg and co-workers (8). The methylsulfinyl sodium reagent was prepared according to the method of Sandford and Conrad (21). The polysaccharide (methylated or nonmethylated) was first soaked with 1.0 ml of 11.25 M H_2SO_4 at room temperature for 1 h, the acid was diluted to 425 mM, and the container was sealed and heated at 105°C for 8 to 10 h. The acid was neutralized with $BaCO_3$, and the hydrolysate was deionized through a double-bed column of Dowex-50 (H^+) and Dowex-1 (acetate) resins. The sugars in the hydrolysate were reduced with $NaBH_4$ for 2 h. The removal of borate and subsequent acetylation of alditols were done as described elsewhere (25). Monosaccharide components were separated and determined as alditol acetates by GLC (22) using a glass column (180 × 0.3 cm) packed with 3% (w/w) ECNSS-M on Gas Chrom Q (100–120 mesh). A Perkin-Elmer Gas Chromatograph, model 990, with a flame-ionization detector was employed. The identity of glucose and mannose was confirmed by cochromatography using alditol acetates of authentic glucose and mannose. Mol wt estimates were made by gel filtration on a Sephacryl S-200 column (1.4 × 35 cm) calibrated with *Leuconostoc mesenteroides* dextrans from Pharmacia (3).

Elicitor Bioassays.

Cotyledon Bioassay. Six cotyledons freshly harvested from 8- to 9-d-old Harosoy 63 plants were wounded on the undersurfaces as previously described (11, 28). Serial dilutions of the elicitor solutions were made with water. Rifampicin and penicillin were added to 10 and 300 $\mu g\ ml^{-1}$, respectively, for control of microbial growth and exhibited negligible elicitor activity in water (1% v/v of a 100× stock solution in 70% aqueous ethanol was added to all tubes just prior to assay). The solutions were placed onto the cut surfaces, and the cotyledons were incubated at room temperature under lights for 24 h. Then, five representative cotyledons were added to 20 ml of distilled H_2O which was thoroughly mixed, and glyceollin was immediately determined at 285 nm. The elicitor concentration giving half-maximal phytoalexin production was reported as a measure of elicitor potency (Fig. 4).

Hypocotyl Bioassay. The hypocotyl bioassay was modified from that reported by Yoshikawa *et al.* (29). Solutions of elicitors containing rifampicin and penicillin as above were introduced into 1-cm hypocotyl wounds on intact 6-d-old soybean plants grown in 10-cm pots. Wounds were made about 1 cm below the cotyledons with a No. 20 hypodermic needle and elicitor solutions were injected into the wounds from a syringe. Plants were covered with plastic bags and incubated for 18 to 24 h at 22°C in a lighted growth chamber. The wounded areas were then excised, weighed, and placed into 18-mm test tubes with 6 ml of 95% ethanol. Tubes were placed in a boiling water bath for 1 min, and the fluids were immediately decanted into a second tube which was taken to dryness *in vacuo* at 50°C. The entire contents of the dried tube were dissolved in 0.15 ml of $CHCl_3$ and applied as approximately 1-cm streaks to 0.375-mm-thick silica gel GF_{254} TLC plates which were developed with hexanes:ethyl acetate:methanol (60:40:1, v/v). Glyceollin zones were scraped from the plates and dissolved in 2 ml of 95% ethanol prior to centrifuging out the silica gel and quantitating the phytoalexin by UV spectrophotometry. Data were computed according to Yoshikawa *et al.* (29) and expressed as μg glyceollin g^{-1} fresh weight hypocotyl tissue.

RESULTS

Isolation and Partial Characterization of the Elicitor Released from Walls by Glucanase. Reaction products from Pmg cell walls

incubated with purified soybean endoglucanase were passed through CM Bio-gel and chromatographed on S-200 Sephacryl columns as two carbohydrate peaks (Fig. 1A). When these were collected, both fractions I and II gave elicitor activity (Table I), but fraction I was the most active. Upon rechromatography, fraction I eluted as a single near-symmetrical carbohydrate peak, at the same position as a dextran T-40 standard (Pharmacia) of 40,000 average mol wt. Reincubation of the fraction I carbohydrate with soybean endoglucanase did not alter its subsequent elution position from the S-200 Sephacryl column (Fig. 1B). This indicated that it is not further degraded by the endoglucanase and that fraction II is not a degradation product of fraction I.

Detailed structural studies have not been performed on the enzyme-liberated carbohydrates. However, analysis of the sugars released by acid hydrolysis showed that fraction I elicitors from races 1, 4, and 7 contained glucose and mannose as the major neutral sugars. Preparations from two different isolates of race 1 gave a glucose/mannose ratio of 55% to 45% ± 1%. A single race 4 sample gave glucose/mannose at 52% to 48%, whereas a single race 7 sample yielded glucose/mannose at 98 to 1, with 1% arabinose tentatively identified. Preliminary methylation analyses of the fraction I glucomannan from race 1 suggest that mannose may constitute a backbone with both mannose and glucose occurring as terminal residues. This carbohydrate appears unique with respect to the relatively meager occurrence of both linear C-3 linked glucosyl residues and branched mannosyl residues.

Release of Glycopeptide Elicitors from Fungus Cell Walls. Pronase (Fig. 2) or papain released protein and carbohydrate from Pmg cell walls. The dialyzed and lyophilized product obtained with pronase was similar to preparations previously obtained by extraction of cell walls with weak base (12). However, the enzyme-extracted products were freely soluble in water and could be subsequently fractionated without loss on gel filtration or ion exchange columns in the absence of dissociating agents.

Chromatography of the products liberated by pronase on a Sephacryl S-200 column led to the recovery of heterogeneous peaks containing carbohydrate and protein (Fig. 3). The high-

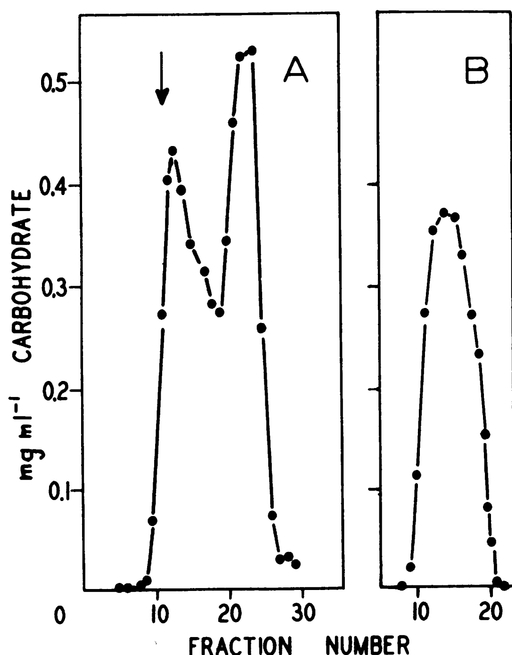

FIG. 1. A, Sephacryl S-200 gel filtration of reaction products from Pmg cell walls incubated with purified soybean β-1,3-endoglucanase. B, Fractions 11 to 16 from 'A' run on the same column after reincubation with endoglucanase. Arrow denotes the void volume; 2.5-ml fractions were collected.

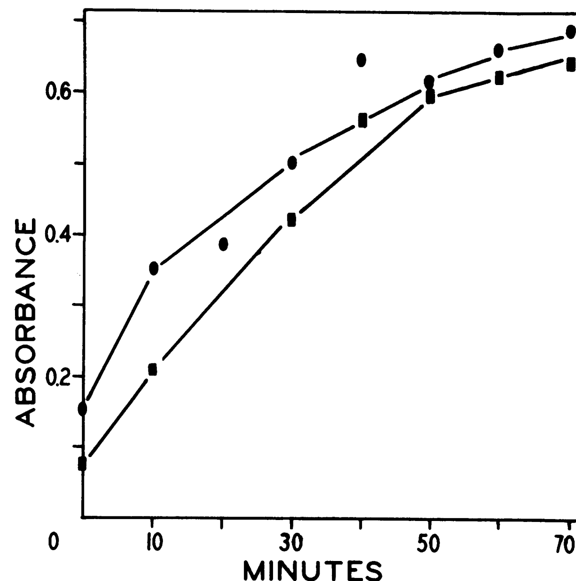

FIG. 2. Release of protein (as A at 280 nm, ■) and carbohydrate (as A at 625 nm, ●) from Pmg cell walls incubated with pronase. Race 1 walls (10 mg ml^{-1}) were previously extracted with soybean endoglucanase, washed, and incubated with CB-Pronase at 50 μg ml^{-1} at 35°C. Aliquots were withdrawn at the noted times and Millipore filtered to remove residual walls.

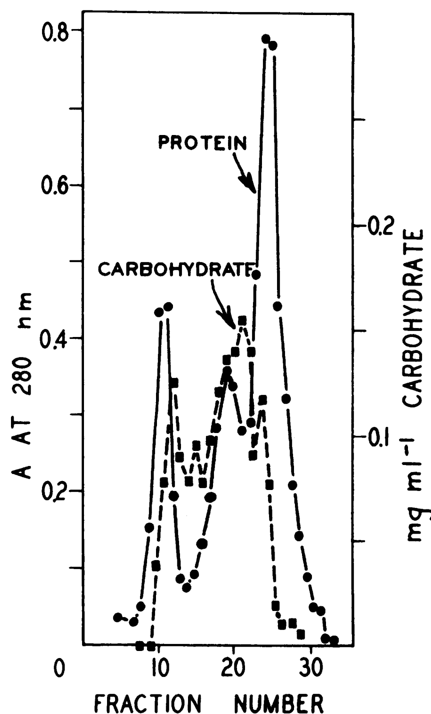

FIG. 3. Chromatography on S-200 Sephacryl of glycopeptides liberated from Pmg cell walls by pronase. The column was run as in Figure 1. Pronase activity was present in fractions 22 to 28.

weight fractions from the S-200 column were fractionated on DEAE Bio-gel as described in methods, yielding a major peak which was eluted by NaCl and contained both carbohydrate and protein.

Elicitor Activity of the Carbohydrates from Pmg. As noted by other workers (5, 10), the soybean cotyledon assay yielded saturation curves when relatively active elicitors were used (Fig. 4). On the other hand, some chemicals did not elicit the half-maximum glyceollin level at the highest tested concentration.

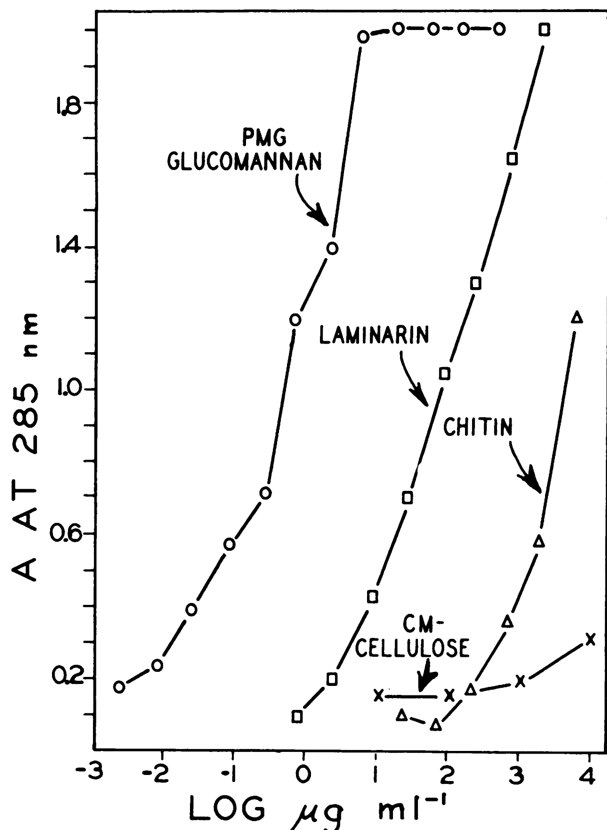

FIG. 4. The cotyledon elicitor bioassay, showing the production of glyceollin (as *A* at 285 nm) in response to different concentrations of carbohydrates with varying elicitor activity.

The purified fraction I glucomannan released from Pmg cell walls by endoglucanase was the most active elicitor of glyceollin in soybean cotyledons (Table I; Fig. 4). The glucan elicitor and glycopeptides released by pronase were both about 10-fold less active, while the intracellular Pmg oligosaccharide mycolaminaran was about 100-fold less active. Suspended cell walls of the fungus were also relatively active, giving half-maximal glyceollin production at about 10 $\mu g\ ml^{-1}$ (Table I). Among the other chemicals tested, laminarin gave activity comparable to mycolaminaran, while CM cellulose, CM pachyman, and pustulan were totally inactive. A glucomannan extracellular polysaccharide from the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* exhibited trace activity in the cotyledon bioassay, as did the preparations of chitin and chitosan (Table I). Suspensions of arachidonic acid lacked activity.

The fraction I glucomannan elicitor purified from the S-200 Sephacryl column and the crude glucomannan released from Pmg cell walls by soybean endoglucanase also gave elicitor activity in soybean hypocotyls (Table II). Preparations from race 1 of the fungus gave higher levels of glyceollin in incompatible Harosoy 63 hypocotyls than in the near-isogenic compatible cultivar Harosoy (Table II). Purified fraction I glucomannan preparations from race 4, which is compatible on both soybean cultivars, produced levels of glyceollin which were not significantly different. Unfractionated reaction mixtures prepared from partially purified soybean endoglucanase and Pmg cell walls also yielded a degree of race specificity (Table II); preparations from incompatible races 1 and 2 gave significantly higher levels of glyceollin in the incompatible cultivar Harosoy 63 than Harosoy, while races 3, 4, and 5, which are compatible on both soybean cultivars, gave levels of glyceollin in the two soybean cultivars that were not significantly different.

Con A Precipitin Tests. As noted previously in Con A precipitin tests with the glycoproteins extracted by base (15), pronase-liberated glycopeptides from races 1, 4, and 7 were strongly agglutinated by Con A. However, the carbohydrates released from cell walls of races 1, 4, and 7 with endoglucanase gave weak, but observable Con A precipitates, which did not form in the presence of 0.2 M α-methyl mannoside.

Sequential Extraction of Pmg Cell Walls and Their FITC Con A Staining. Native cell walls of Pmg were stained intensely by FITC Con A, but those from which glycoproteins were extracted with base did not (15). To determine whether the glucomannan elicitors released from fungus cell walls with endoglucanase were the carbohydrates of the glycoproteins or unique, sequential wall extractions were performed. The order of extraction with pronase or endoglucanase did not influence recovery of the respective elicitors (Table III). Further, incubation of isolated walls with the glucanase did not reduce appreciably their subsequent staining with FITC Con A, but extraction with pronase reduced the staining to essentially zero. Extraction of isolated cell walls with either enzyme decreased only slightly the subsequent yield of the crude β-glucan elicitor (Table III). These results were interpreted as indicating that the various elicitor-active carbohydrates represent unique components of the cell wall of the fungus, and that the glucomannans released by endoglucanase are different from the carbohydrates of the cell surface glycoproteins and the β-glucan fraction.

DISCUSSION

Considerable differences were observed in the elicitor activity of several carbohydrate polymers in soybean cotyledons. The most active elicitors were branched β-1,3-glucans and the glucomannan and glycopeptide fractions from Pmg cell walls (Table I; Fig. 4). The activity of these compounds was relatively specific. For instance, the β-1,6 branched, β-1-3-glucans (β-glucan, mycolaminaran, and laminarin) were at least two orders of magnitude more active than glucose homopolymers composed only of β-1,4 linkages (CM cellulose), β-1,3 linkages (CM pachyman), or β-1,6 linkages (pustulan). Likewise, the Pmg polymers that contain both glucose and mannose (glycopeptides and the glucomannan fractions) were 3 to 4 orders of magnitude more active as elicitors in soybean than the glycoprotein from yeast (yeast mannan) or the glucomannan extracellular polysaccharide from *Pseudomonas syringae* pv. *glycinea* (Table I). The latter glucomannan is of interest because it gives a pronounced Con A precipitin test, and our preliminary data indicate that the glucosyl residues are present in predominantly α-1,3 linkages. The elicitor activity of carbohydrate polymers in soybean therefore requires the presence of specific linkages, a conclusion consistent with the hypothesis that elicitor activity is mediated by specific receptors in soybean cells (13).

Arachidonic acid, recently reported to be a phytoalexin elicitor in potato (7), was devoid of similar activity in soybean cotyledons (Table I). Chitin and chitosan are elicitors in other plants (12, 19, 20) and exhibited activity in soybean cotyledons, but only at relatively high concentrations. Nevertheless, it would appear that soybean cells can recognize these wide-spread components of fungal cell walls and invoke phytoalexin production.

Suspensions of Pmg cell walls were relatively potent elicitors of glyceollin (Table I), which may be accounted for by one or more of the three carbohydrate fractions present in them. Two of these are the branched β-1,3-glucans described by Albersheim *et al.* (2, 4, 5, 24) and the glycoproteins previously extracted by us (15). Finally, we observed that the fraction I glucomannan released by soybean endoglucanase is the most active elicitor (Table I). It should be noted that the glucomannans constitute about 5.5% of the Pmg cell wall (Table III) and are approximately 30 times more active on a weight basis as elicitors than the walls themselves. Accordingly, the glucomannans may account for the majority of the elicitor activity of Pmg cell walls. It is also significant that the

Table II. *Elicitor Activity in Soybean Hypocotyls of Purified Glucomannans and Crude Glucomannans Released from Cell Walls of Pmg by Soybean Endoglucanase*

Elicitor	Fungus Race	Harosoy		Harosoy 63	
		Disease reaction	Glyceollin	Disease reaction	Glyceollin
			μg g^{-1} fresh wt		*μg g^{-1} fresh wt*
Purified glucomannan, fraction I[a]	1	C[c]	380 ± 160	I[c]	895 ± 105
Purified glucomannan, fraction I[a]	4	C	285 ± 75	C	260 ± 50
Crude soluble fraction[b]	1	C	131 ± 28	I	234 ± 33
Crude soluble fraction[b]	2	C	150 ± 19	I	277 ± 82
Crude soluble fraction[b]	3	C	118 ± 22	C	146 ± 30
Crude soluble fraction[b]	4	C	133 ± 7	C	170 ± 31
Crude soluble fraction[b]	5	C	168 ± 28	C	180 ± 37

[a] Bioassayed in hypocotyls at 100 μg ml^{-1} in water; data are means ± SE of three replicate bioassays; experiments shown are representative of four identical experiments.

[b] Soybean endoglucanase partially purified by ammonium sulfate precipitation (28) was incubated with cell walls of the noted races, and following Millipore filtration, the reaction mixtures were directly bioassayed in soybean hypocotyls.

[c] C, compatible or susceptible host reaction; I, incompatible or resistant host reaction when plants are inoculated with the living fungi.

Table III. *Material Extracted from Pmg Cell Walls by Enzymes or Autoclaving and the FITC Con A Staining of Walls after Extraction*

Prior Treatment of Walls	Cell Wall Dry Wt Extracted		
	Pronase	Endoglucanase	Autoclaving
		%	
Not extracted	2.5 (−)[a]		
Not extracted		5.6 (+)	
Not extracted			4.0 (−)
Pronase		4.5 (−)	3.5 (−)
Endoglucanase	2.7 (−)		3.0 (−)
Pronase + endoglucanase			2.0 (−)

[a] FITC Con A staining after extraction was recorded as (+) or (−) following observation of walls under the fluorescence microscope as outlined in Ref. 15.

glycoprotein and glucomannan elicitors taken collectively account for almost all of the mannose content of the race 1 Pmg cell wall, reported to be about 6% (5). This and their liberation by purified enzymes are consistent with our view that the mannose-containing polysaccharides are located at the cell-surface.

The fraction I glucomannan released by endoglucanase was highly active in the elicitor bioassay, chromatographed as a single symmetrical peak on the S-200 Sephacryl column, and did not contain detectable amounts of protein or lipid. It is therefore unlikely that the elicitor activity can be accounted for by impurities or that the glucomannan is a mixture of glucan and mannan. Since the glucomannans were released from Pmg cell walls by highly purified β-1,3-endoglucanase, they are presumed to be covalently linked to the β-1,3-linked backbone of the native Pmg cell wall. Based on the gel filtration patterns (Fig. 1), the glucomannans occur as relatively homogeneous polymers of about 40,000 average mol wt (fraction I) and a lower mol wt fraction II, also containing glucose and mannose, but with lesser biological activity.

Only preliminary studies have been done on the structures of the enzymically released glucomannan elicitors. However, reincubation of the fraction I elicitor with soybean endoglucanase decreased neither the apparent mol wt nor the elicitor activity. This indicates that the glucomannan does not contain long unsubstituted and repeating sequences of β-1,3-linked glucose residues, as is the case with mycolaminaran and the β-glucan elicitor fraction. This, coupled with its approximately 10-fold greater activity and the fact that mannose is a constituent of the elicitor released by endoglucanase indicates that it is distinct from the β-glucan elicitor. Preparations of the glucomannan elicitor from races 1 and 4 of Pmg contained similar quantities of glucose and mannose, but the preliminary data obtained with those from race 7 showed a much reduced mannose content. The significance of these differences is not yet clear, but they suggest that the released carbohydrates from various races may differ in structure. It is noteworthy that the carbohydrate structures of certain glycoproteins from the fungus have also been shown to differ among races (27, 31).

The carbohydrates liberated by β-1,3-endoglucanase are also clearly distinct from the previously described surface glycoproteins. Walls extracted with endoglucanase subsequently gave the same yield of glycopeptides with pronase as unextracted walls (Table III). Walls that were extracted first with pronase likewise gave the expected yield of glucomannan when reincubated with soybean endoglucanase. Further, FITC Con A intensely stained both native Pmg cell walls (15) and those previously extracted with the endoglucanase. When glycoproteins were removed by extraction with weak NaOH (15) or pronase, however, the binding of FITC Con A was entirely abolished (Table III). Finally, the extracted glycopeptides, but not the carbohydrates released with endoglucanase, gave strong Con A precipitin reactions. All of these observations demonstrate that the glycoproteins and glucomannans released by endoglucanase are distinct cell surface molecules. However, since they both appear to give race-specific elicitation of glyceollin in soybean hypocotyls (Ref. 15; Table II), they may share common structural features.

It is difficult to state which, if any, of the elicitors from Pmg is physiologically important in the interaction of infecting fungus hyphae with soybean cells. The relatively weak activity of the mycolaminarans, as well as their intracellular nature (25), argue against their involvement. However, mycolaminaran (approximately 30 glucose residues) gives similar elicitor activity to algal laminarin (Table I).

Albersheim *et al.* (2, 4, 5, 24) concluded that the branched β-1,3-glucans extracted from cell walls and present in the extracellular fluids of old, autolysing Pmg cultures are important physiological elicitors during the invasion of soybean plants by the

fungus. One reason was the high reported elicitor activity (5). We have confirmed that the β-glucan fraction is indeed relatively active, giving half-maximal activity at about 2 $\mu g\ ml^{-1}$ (Table I). This is comparable to the observations of Ayers *et al.* (5), but somewhat lower than the value of approximately 10 $\mu g\ ml^{-1}$ for half-maximal activity noted by Ebel (10). Despite the relatively high activity of the β-glucan fraction in soybean cotyledons, several considerations lead us to question their physiological role: (a) the glucans have not been shown to exhibit any race or cultivar specificity as elicitors; (b) the glucan elicitors are not as active as the glucomannans described in this paper and have not been shown to be liberated from the fungus cell wall during pathogenesis; (c) the glucans have indeed only been extracted from cell walls by harsh and artefact-inducing treatments such as autoclaving for long periods or partial acid hydrolysis; it is in fact likely that they are largely masked by the surface glycoproteins and glucomannans of the native cell wall and accordingly may not be detected by plant cells; (d) the β-glucans are degraded by soybean glucanases (9, 16) to adducts with little elicitor activity—if the glucans were released in infected plant tissue, they would be expected to be attacked by such enzymes. These factors all argue against the probable importance of the glucan elicitor in the natural interaction of Pmg and soybean plants.

The mannose-containing carbohydrates of Pmg appear to give race-specific elicitation of glyceollin in soybean hypocotyls (Ref. 15; Table II). Ziegler and Pontzen (31) recently reported that invertase of Pmg, an extracellular glycoprotein with a carbohydrate portion composed mainly of mannose, conferred race-specificity in phytoalexin elicitor bioassays in combination with the nonspecific β-glucan elicitor. Although any physiological role for invertase in the natural host-parasite interaction is uncertain, the results confirm the association of mannose-containing carbohydrates of Pmg with the conferral of race specificity.

The cell surface glycoproteins and glucomannans seem to be the major candidates for the fungus elicitors that are physiologically important in the initiation of phytoalexin production in infected soybean plants. The fact that they are surface molecules on the Pmg cell wall would facilitate their possible detection by plant cells. A major theoretical problem, however, has been the mechanism by which cell surface elicitors of a pathogen are presented to plant receptors, since the latter are thought to occur on the plasma membrane (1, 13). We have not detected release of the cell wall glycoproteins of Pmg by enzymes present in soybean cotyledons and hypocotyls. This may mean that the glycoproteins do not function in the physiological interaction of infecting Pmg hyphae and soybean cells. On the other hand, the glucomannans have been shown to be readily released from fungus cell walls and from living fungus hyphae by soybean β-1,3-endoglucanase. The fraction I glucomannan elicitor studied here is about 40,000 average mol wt, a size that would be expected to permeate higher plant cell walls, based on recent studies (23). In addition, the glucomannans are the most active elicitors yet detected from the fungus, are released by the endoglucanases within minutes (28), and appear to give race-specific elicitation of glyceollin in soybean hypocotyls (Table II). These factors all suggest that they may be the physiologically important elicitors in the relatively rapid interactions which are known to occur between soybean cells and infecting fungus hyphae (13).

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