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Host-Pathogen Interactions[1]

XVII. HYDROLYSIS OF BIOLOGICALLY ACTIVE FUNGAL GLUCANS BY ENZYMES ISOLATED FROM SOYBEAN CELLS

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ABSTRACT

The ability of β-glucosylase I, a soybean cell wall β-glucosyl hydrolase, to degrade elicitors of phytoalexin accumulation was studied. Extensive β-glucosylase I treatment of the glucan elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae* results in hydrolysis of 77% of the glucosidic bonds of the elicitor and destruction of 94% of its activity. Soybean cell walls contain some additional factor, probably one or more additional enzymes, which can assist β-glucosylase I in hydrolyzing the glucan elicitor. This was demonstrated by the more rapid hydrolysis of the glucan elicitor by a mixture of soybean cell wall enzymes (containing β-glucosylase I). In a single treatment, the mixture of cell wall enzymes hydrolyzed 91% of the glucosidic bonds and destroyed 85% of the activity of the elicitor. The enzymes from soybean cell walls will also hydrolyze elicitor-active oligoglucosides prepared from the mycelial walls of *Phytophthora megasperma* var. *sojae*. The active oligoglucosides are more susceptible than the glucan elicitor to hydrolysis by these enzymes. The mixture of cell wall enzymes or β-glucosylase I, by itself, hydrolyzes more than 96% of the glucosidic bonds and destroys more than 99% of the activity of the oligoglucoside elicitor. Two possible advantages for the existence of these enzymes in the walls of soybean cells are discussed.

Pms,[4] a fungal pathogen of soybeans, produces a β-glucan which will stimulate soybeans and other higher plants to accumulate phytoalexins (2–4). Phytoalexin accumulation is thought to be a plant defense response to microbial attack (2).

We are interested in knowing if soybeans possess enzymes capable of hydrolyzing the glucan which elicits this response. Our results have shown that soybean cells have several wall-bound enzymes with the necessary β-1,3-glucanase and β-glucosidase activities (8). The dominant β-1,3-glucanase in soybean walls has been purified and characterized. This enzyme, which we call β-glucosylase I, is also a β-glucosidase (8).

The present study examines the hydrolysis of the glucan elicitor by β-glucosylase I and compares it to hydrolysis of the glucan elicitor by a crude mixture of soybean cell wall enzymes. Also reported here is the ability of these enzymes to hydrolyze elicitor-active oligoglucosides. This analysis was undertaken because Pms also produces in its culture fluid an elicitor with a mol wt of approximately 2,500 (15 glucosyl residues) (3). It may be that low molecular weight elicitors are important in stimulating phytoalexin accumulation *in situ*.

It is difficult to obtain the low molecular weight culture filtrate elicitor in quantities sufficient for enzyme hydrolysis experiments. Fortunately, for the studies reported here, substantial quantities of elicitor-active oligosaccharides have recently been prepared from Pms mycelial walls by partial acid hydrolysis (19). These acid-prepared oligosaccharides were used for the enzyme-hydrolysis experiments described in this paper.

MATERIALS AND METHODS

Chemicals. Bio-Gel P-2 was obtained from Bio-Rad. Sephadex G-100 was purchased from Sigma. Nitex nylon screen (37 μm pore opening) was obtained from Tetko Inc., N. Y. Seeds of the soybean cultivar Wayne were obtained from Wilkin Seed Grains Inc., Pontiac, Illinois.

Enzyme Preparations. β-Glucosylase I was purified by affinity-chromatography as described (8). A mixture of enzymes present in the walls of suspension-cultured soybean cells was obtained by extraction of intact cells (β-1,3-glucanase and β-glucosidase Extraction Method A) and was concentrated on CM-Sephadex as described (8). The preparation used here possesses a β-1,3-glucanase activity of 0.32 U/mg of protein and a β-glucosidase activity of 1.1 U/mg of protein.

Preparation of Pms Elicitors

Purification of Pms Mycelial Walls. Race 1 of Pms was cultured as described (3). The Pms mycelial walls were purified by a modification of the procedure of Ayers *et al.* (4) as follows: mycelia, resulting from approximately 14 days of growth, were collected by suction filtration through nylon screen (37 μm pore opening) which was supported on a Büchner funnel. The mycelial mat was washed on the funnel with distilled H_2O until the liquid passing through the filter became clear. The mycelial mat was then homogenized at full speed in a Waring Blendor with 5 volumes (ml/g mycelia) of 0.5 M NaCl. The mycelial walls were then collected by filtration on the nylon screen. Homogenization, followed by filtration, was repeated 3 times. The mycelial walls were then washed on a coarse sintered-glass funnel with distilled H_2O followed by organic solvents, and were air-dried as described (4). The air-dried walls were homogenized with 100 volumes (ml/g) of 0.5 M acetic acid and the resulting wall suspension dialyzed against three changes of 80 volumes (v/v) of 0.5 M acetic acid followed by five changes of 80 volumes (v/v) of distilled H_2O. Dialysis against acetic acid removes the ash which can account for up to 35% of the weight of undialyzed walls (4). Purification of mycelial walls by the above procedure results in a preparation which is approximately 93% carbohydrate and 7% protein.

Extraction and Purification of the Glucan Elicitor from Mycelial Walls. A glucan elicitor was solubilized from the acetic acid-purified Pms walls by hot water extraction and was purified by the procedure described for the Fraction I elicitor (4). The glucan elicitor so obtained represents approximately 7% of the total

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[4] Abbreviations: Pms, *Phytophthora megasperma* var. *sojae*.

carbohydrate present in the walls and has a carbohydrate to protein ratio of 70. The carbohydrate portion of the glucan is entirely glucosyl residues. Therefore, the glucan elicitor purified from acetic acid-dialyzed walls is compositionally different from the Fraction I elicitor described by Ayers *et al.* (4) which contains, in addition to glucosyl residues, approximately 6% mannosyl residues and which has a carbohydrate to protein ratio of about 4. However, the glucan elicitor from acetic acid-dialyzed walls is similar to the Fraction I elicitor both in molecular size distribution on Agarose A 0.5 M and in elicitor activity in the cotyledon assay (4).

A high molecular weight form of the glucan elicitor was also prepared for the studies reported here. This was achieved by Sephadex G-100 gel filtration chromatography of the purified glucan elicitor. The high molecular weight form was obtained by pooling carbohydrate-containing fractions of the void volume of the column. This material is comprised of glucans with mol wt in excess of 100,000 and represents approximately 50% of the carbohydrate of the unfractionated glucan elicitor.

Preparation of Oligosaccharide Elicitors from Pms Mycelial Walls. Oligosaccharide elicitors were derived from Pms mycelial walls by partial acid hydrolysis using a modification of the procedure of Valent (19). Acetic acid-dialyzed walls were suspended in 100 volumes (ml/g) of 0.5 M trifluoroacetic acid, and the suspension was heated at 85 C for 5 h. The wall residue remaining after this partial hydrolysis was removed by centrifugation at 20,000*g* for 20 min. The oligosaccharide-containing supernatant solution was evaporated to dryness under reduced pressure at room temperature. This material consists of a heterogeneous mixture of oligo- and polysaccharides (19). A relatively size-homogeneous oligosaccharide elicitor was prepared by Bio-Gel P-2 gel filtration chromatography of the above acid-released material. A preparation with an average apparent mol wt of 2,200 was obtained by pooling the carbohydrate which elutes from the P-2 column with volumes ranging from $1.1 \times V_0$ to $1.25 \times V_0$.

Assay for Elicitor Activity. The cotyledon assay for elicitor activity (3) was used with the following modifications. Cotyledons were obtained from 7-day-old soybean seedlings (cultivar Wayne) which were grown as described by Valent (19). Samples to be assayed for elicitor activity were dissolved in 20 mM Na-acetate (pH 5.2) (without antibiotics). Ninety μl of a solution to be assayed was applied to the cut surface of each cotyledon. Ten cotyledons were used to assay each sample. The cotyledons were incubated in the dark at 25 C and 100% RH for 19 h. The cotyledon assay is terminated by transferring the 10 cotyledons of each assay plate to 20 ml of distilled H_2O (3) and measuring the A_{285} of the resulting aqueous solution. The A_{285} was shown to be proportional to the amount of the phytoalexin, glyceollin, in the inoculation droplet (3). In the cotyledon assays conducted for the studies reported here, the inoculation droplets on approximately 10% of the cotyledons dried up during the incubation period. When this occurred, the dried cotyledons were discarded, and the remaining cotyledons were transferred to an amount of water equal to 2 ml per remaining cotyledon. The A_{285} of the resulting aqueous solution was measured.

Treatment of the cotyledons with increasing amounts of elicitor results in a nonlinear increase in A_{285} until saturation is reached (3). At saturation, increases in the amount of applied elicitor do not result in an increase in A_{285}, and the response is said to be maximum.

Elicitor activity of unknown samples was determined using standard curves which relate the fraction of maximum response, *i.e.* $A_{285} \div A_{285}$ max, to the amount of elicitor applied. The standard curves were generated with either the glucan elicitor or with the oligosaccharide elicitor.

Standard curves were not generated each day samples were assayed. Therefore, elicitor values obtained from the standard curve were adjusted by multiplying them by a factor. This factor corrects for daily fluctuations in the sensitivity of cotyledons to elicitor. The correction factor was obtained by assaying a known amount of standard elicitor in parallel with the samples to be tested. From the A_{285} resulting from the standard elicitor, an apparent amount of eliator was obtained from the standard curve. The correction factor for that day is the actual amount of standard elicitor divided by the apparent amount obtained from the standard curve.

All elicitor values reported here were conducted in assays in which the A_{285} resulting from both the unknown and the standard elicitor were between 40 and 80% of saturation. We found that when the A_{285} values are between 40 and 80% of saturation, this method of correction results in errors for single point assays of less than 30% of the value which would have been obtained had a standard curve been obtained at the same time as the single point assay. This was assessed by assays of varying quantities of standard elicitor on 3 separate days.

Saturation A_{285} values for each day's assay were obtained from at least two assay plates, using 1 μg standard elicitor per cotyledon. The correction factor for each day's assay is an average value obtained from 4 to 5 assay plates of standard elicitor. Glucan elicitor was used as the standard for assays of samples derived from the glucan elicitor. Oligosaccharide elicitor was used as the standard for assays of samples derived from the oligosaccharide elicitor. The values reported for elicitor activity of various samples were determined from a total of 9 to 10 assay plates per sample resulting from 2 days of assay. High and low values were eliminated, and the average and standard deviations were computed from the remaining seven to nine values.

One unit of elicitor activity is arbitrarily defined as the amount of elicitor per cotyledon which yields an A_{285} which is 50% of saturation under the corrected conditions.

Other Assays. Carbohydrate (hexose) was measured by the anthrone method (11) or, when sodium azide was present, by the phenol-sulfuric acid method (12). Glucose was used as a standard. The glucan and oligosaccharide elicitors were quantitated by their carbohydrate content and are reported as nanograms of glucose equivalents. Protein and reducing sugars were measured as described (8). The percent hydrolysis of polysaccharide and oligosaccharide was determined from the ratio of reducing sugars to total carbohydrate as described (8). The contribution of the sugar residue at the reducing end of unhydrolyzed oligosaccharides to the total hexoses and reducing sugar content of the oligosaccharides was subtracted before the percentage of hydrolysis was calculated.

The activity of either β-glucosylase I or cell wall enzyme preparations was quantitated by the β-1,3-glucanase assay (8). Units of enzyme refer to units of β-1,3-glucanase. One unit of hydrolytic enzyme activity is the amount required to hydrolyze 1 μmol glycosidic bonds/min under the standard assay conditions (8).

Glycosyl and Glycosyl Linkage Composition. Glycosyl compositions were determined by the alditol acetate method as described (1). Glycosyl linkage compositions were determined by methylation analysis as described by Darvill *et al.* (10). Oligosaccharides were reduced with sodium borodeuteride prior to methylation. GS and MS of alditol acetates and of partially methylated alditol acetates were performed as described (8).

Gel Filtration Chromatography. Gel filtration on Bio-Gel P-2 was performed as described (8). Gel filtration on Sephadex G-100 was performed in a 1.7 × 30 cm glass column. The column was equilibrated and eluted with 50 mM Na-acetate (pH 5.2). The flow rate was 5 ml/h. The collected fractions were 1.25 ml. The void volume (25 ml) and the included volume (70 ml) were determined with Blue Dextran (Sigma) and glucose, respectively.

Enzymic Hydrolysis of Elicitors. All enzymic hydrolyses of

elicitors were carried out at 30 C in glass test tubes equipped with Teflon-lined screw caps. Reaction mixtures contained elicitor at a concentration of 1 mg/ml, enzyme, and sodium acetate (pH 5.2) (0.1 M for hydrolyses with β-glucosylase I, 0.8–1.0 M for hydrolyses with the mixture of cell wall enzymes). Control reactions, in which enzyme was absent, were run in parallel. All reaction and control mixtures were supplemented with sodium azide at a concentration of 0.02% to prevent the growth of microorganisms. Reactions were initiated by the addition of enzyme to otherwise complete reaction mixtures which had been prewarmed at 30 C for 10 min. Reactions were terminated by heating the tightly capped reaction tubes in a boiling water bath for 15 min.

RESULTS

Hydrolysis of the Glucan Elicitor by Soybean Cell Wall Enzymes and by β-Glucosylase I. A high molecular weight fraction of the glucan elicitor was used in these experiments to assess unambiguously the effect of the enzyme hydrolysis on the molecular size of the elicitor. The high molecular weight glucan elicitor was prepared by gel filtration chromatography and is comprised of glucans with mol wt greater than 100,000 (Fig. 1a). This elicitor preparation will be referred to as the "glucan elicitor" for the remainder of these studies. The glucan elicitor is a very potent stimulator of glyceollin accumulation in the cotyledon assay. As little as 5 ng glucan elicitor/cotyledon results in a 50% saturation (Fig. 2a, $A/A_{max} = 0.5$). The glucan elicitor has a carbohydrate composition which is entirely glucosyl residues, and a glucosyl linkage composition shown in Table I.

The glucan elicitor was treated with a mixture of soybean cell wall enzymes or with purified β-glucosylase I, and the percent hydrolysis monitored over time. To compare the two treatments, the concentrations of enzyme units were identical, as were the concentrations of elicitor. Both enzyme preparations hydrolyze the glucan elicitor (Fig. 3). The mixture of cell wall enzymes is more active than β-glucosylase I in hydrolyzing the elicitor. A calculation of units of hydrolytic activity for the 1st h of incubation shows that the mixture of cell wall enzymes is approximately half as active with the glucan elicitor as substrate as with laminarin as substrate. Laminarin is the substrate used to assay the β-1,3-glucanase activities of these enzymes. β-glucosylase I is about one-third as active with the glucan elicitor as with laminarin.

Hydrolysis of the glucan elicitor by either enzyme preparation continued for up to 24 h; however, the rate of hydrolysis had slowed considerably. Addition of fresh enzyme did not significantly increase either the rate or degree of hydrolysis (see arrows, Fig. 3). In 36 h, the mixture of cell wall enzymes hydrolyzed 91% of the glycosidic bonds of the glucan elicitor. In 32 h, β-glucosylase I hydrolyzed 58% of the glucan elicitor.

The values for percent hydrolysis of the glucan elicitor were confirmed by Bio-Gel P-2 chromatography of portions of the hydrolysis mixtures (not shown). Approximately 10% of the cell wall enzyme-hydrolyzed elicitor eluted as polysaccharide in the void volume of the P-2 column. The remaining 90% eluted as monosaccharide. The polysaccharide component was the only material which showed elicitor activity. This polysaccharide is the residual glucan from hydrolysis by the mixture of cell wall enzymes.

Approximately 45% of the β-glucosylase I-hydrolyzed elicitor eluted in the void volume of the P-2 column, and the remaining 55% eluted as monosaccharide. As with the cell wall enzyme-hydrolyzed elicitor, only the material eluting in the void volume showed any elicitor activity.

Effect of Enzyme Hydrolysis on the Molecular Size of the Glucan Elicitor. The molecular size distribution of enzyme-hydrolyzed elicitor was determined by Sephadex G-100 chromatography of the hydrolysis mixtures (*i.e.* the 32 h β-glucosylase I hydrolysis mixture and the 36 h cell wall enzymes hydrolysis mixture). The

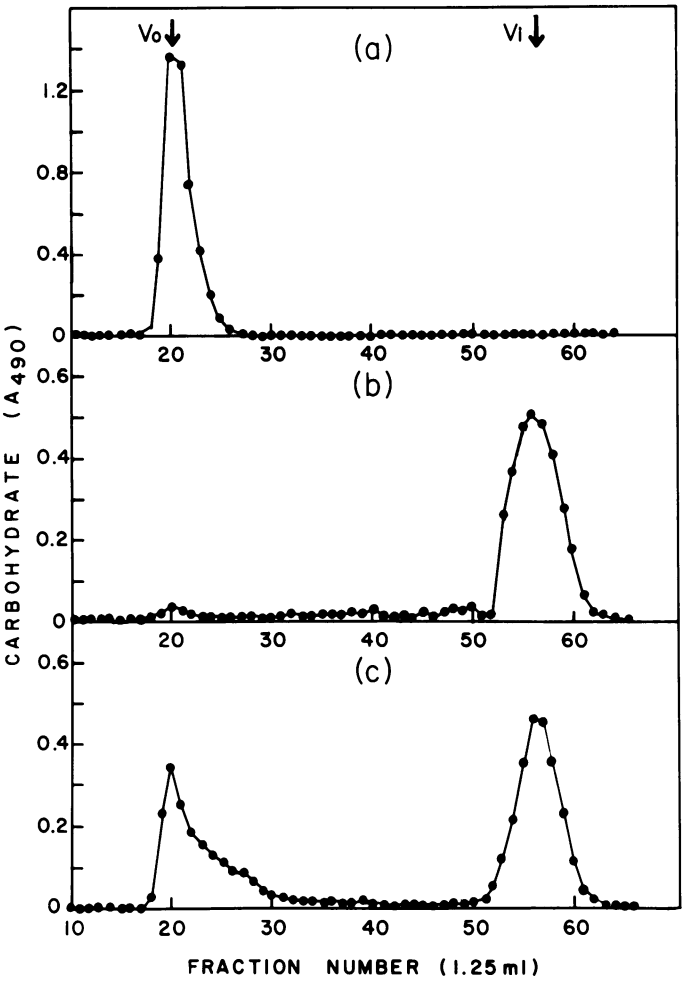

FIG. 1. The molecular size distribution of the Pms glucan elicitor (high molecular weight fraction) before and after enzyme hydrolysis. Samples of the glucan elicitor (0.8 mg) and of enzyme-hydrolyzed glucan elicitor (0.8 mg) were chromatographed on a 1.7 × 30 cm Sephadex G-100 column as described under "Materials and Methods." Fractions of the column effluent were assayed for carbohydrate by the phenol-sulfuric acid method. *a*, Glucan elicitor. *b*, Glucan elicitor hydrolyzed by a mixture of soybean cell wall enzymes during a 36-h incubation. *c*, Glucan elicitor hydrolyzed by purified β-glucosylase I during a 32-h incubation. Polysaccharides with mol wt greater than 100,000 elute in the void volume of this column (based on the fractionation range for dextrans) (18).

untreated glucan elicitor elutes in the void volume of the G-100 column as polysaccharide with mol wt greater than 100,000 (Fig. 1a). In contrast, most of the cell wall enzyme-hydrolyzed elicitor (83%) elutes as low molecular weight hydrolysis products in the included volume of the G-100 column (Fig. 1b). Only 4% of the cell wall enzyme-hydrolyzed elicitor elutes in the void volume (mol wt greater than 100,000) and the remaining 13% elutes in the partially included volumes of the G-100 column as a mixture of polysaccharide with mol wt ranging from 1,000 to 100,000 (18).

Most of the β-glucosylase I-hydrolyzed elicitor (55%) elutes in the totally included volume of the G-100 column (Fig. 1c) (mol wt less than 1,000). Only about 22% of the β-glucosylase I-hydrolyzed elicitor voids the G-100 column. The remaining 23% of the hydrolyzed elicitor elutes in the partially included volume of the column as polysaccharides with mol wt from 1,000 to 100,000. Thus, hydrolysis by the mixture of cell wall enzymes or by β-glucosylase I by itself results in a marked decrease in the molecular size of the glucan elicitor.

Further Hydrolysis of the Glucan Remaining after the β-Glu-

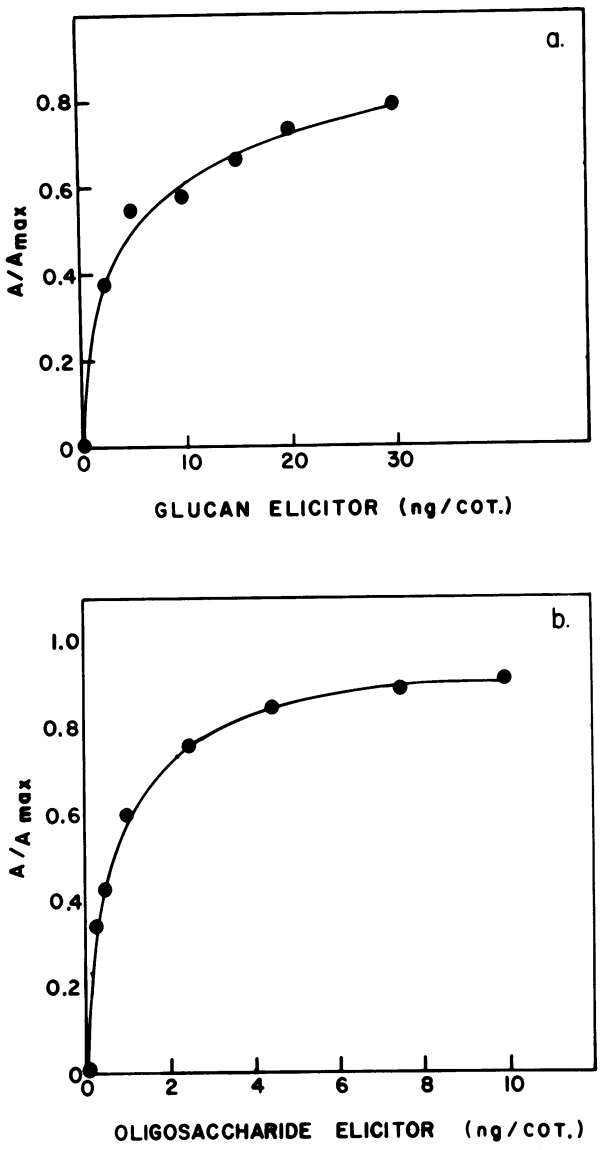

FIG. 2. Response of soybean cotyledons to the glucan and oligosaccharide elicitors. Varying amounts of the glucan or oligosaccharide elicitor were applied to cotyledons as described under "Materials and Methods." The response of the cotyledons to the elicitor samples, A, is equal to the A_{285} of the inoculation droplets measured under the conditions described under "Materials and Methods." The maximum response of cotyledons, A_{max}, was determined by applying 1 μg glucan or oligosaccharide elicitor per cotyledon. The fraction of maximum response, A/A_{max}, is plotted *versus* the amount of glucan elicitor (*a*) or oligosaccharide elicitor (*b*) applied. Each datum is an average value obtained from six determinations for the glucan elicitor or two determinations for the oligosaccharide elicitor.

cosylase I Treatment. The glucan remaining after the β-glucosylase I hydrolysis was subjected to further treatment by β-glucosylase I. β-Glucosylase I (0.16 U) was incubated with 2.5 mg of the residual glucan from the β-glucosylase hydrolysis (void material from the P-2 column; see above and Fig. 1c) under conditions described under "Materials and Methods." The residual glucan was hydrolyzed 26% during 14 h of hydrolysis. Addition of fresh β-glucosylase I (0.16 U) and continued incubation for another 22 h resulted in a total of 40% hydrolysis. This was confirmed by Bio-Gel P-2 chromatography of the hydrolysis mixture. Approximately 55% of the carbohydrate eluted as polysaccharide in the void volume of the column, and the remaining 45% eluted as monosaccharide.

These results indicated that the monosaccharide hydrolysis products interfere with the action of β-glucosylase I on the glucans. Thus, the residual glucan from the second β-glucosylase I hydrol-

Table I. *Glucosyl Linkage Compositions*[a] *of Elicitors and of Enzyme-Hydrolyzed Elicitors*

Elicitor Preparation	Glucosyl Residue[b]				
	t-Glc	3-Glc	4-Glc	6-Glc	3,6-Glc
Oligosaccharide elicitor	25	30	7	15	19
Glucan elicitor	20	43	2	4	26
Residual glucan remaining after hydrolysis by cell wall enzymes	14	48	2	8	19
Residual glucan remaining after limit hydrolysis by β-glucosylase I	8	45	2	28	10

[a] Glucosyl linkage compositions were determined by the methylation procedure as described under "Materials and Methods."

[b] The glucosyl residues: 2,3-, 3,4-, 2,3,6-, and 3,4,6-linked were also detected in minor amounts in all of the elicitor preparations. These residues comprise the difference between the sum of the mole percentages of the residues reported and 100%. None of these residues ever accounts for more than 4 mol %.

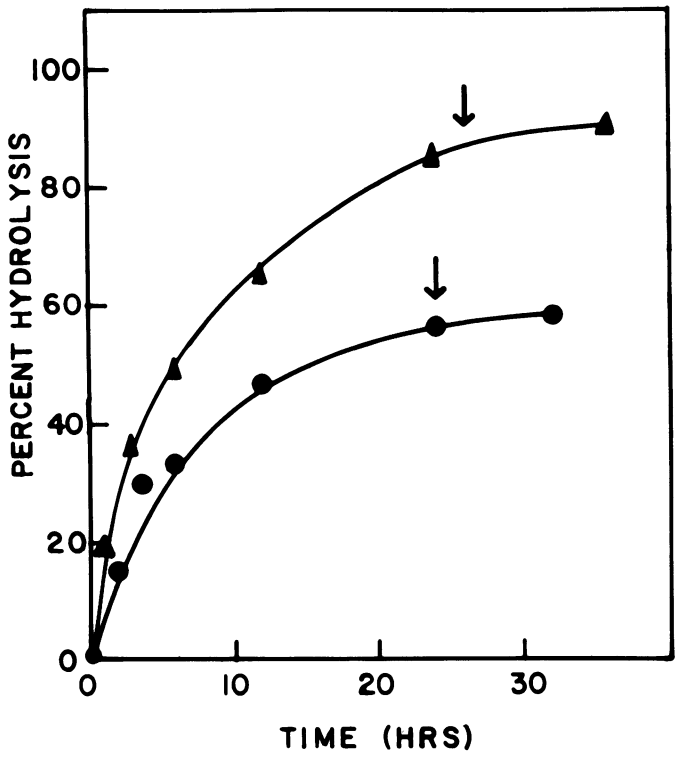

FIG. 3. Hydrolysis of the glucan elicitor by a mixture of soybean cell wall enzymes and by β-glucosylase I. The glucan elicitor (10 mg) was treated with 0.41 U of either a mixture of soybean cell wall enzymes or purified β-glucosylase I under the conditions described under "Materials and Methods." Aliquots of the reaction mixtures were removed at various times and were assayed for the percent hydrolysis of glucosidic linkages. The percent hydrolysis by the cell wall enzymes (▲——▲) or by β-glucosylase I (●——●) is plotted *versus* the time of incubation. An additional 0.14 U fresh enzyme was added to the respective reaction mixtures at the times indicated by the arrows. No hydrolysis occurred in control mixtures from which enzymes were deleted.

ysis was subjected to further β-glucosylase I treatment. Incubation of 0.08 U β-glucosylase I with 0.7 mg of the glucans remaining after the second hydrolysis (P-2 void; see above) for 12 h resulted in only 9% hydrolysis. The glucans remaining after this final hydrolysis (91%) were recovered by P-2 chromatography. This material is referred to as the residual glucan of limit hydrolysis by β-glucosylase I.

The percentage of the original glucan elicitor that was eventually hydrolyzed by β-glucosylase I was calculated to be 77% from the percentage of polysaccharide remaining after the three successive hydrolyses (as determined from the P-2 chromatograms).

Glycosyl Linkage Compositions of the Residual Glucans from the Enzyme Hydrolyses. The effect of enzyme hydrolysis on the structure of the glucan elicitor was determined from glucosyl linkage compositions (Table I) of the untreated elicitor and the residual glucans.

The untreated elicitor contains a high proportion of 3-linked glucosyl residues (43%), 3,6-linked glucosyl residues (26%), and terminal glucosyl residues (20%). This composition indicates that the glucan elicitor is a highly branched 3-linked glucan, one branch occurring for every three nonterminal residues. The residual glucan remaining after hydrolysis by the mixture of cell wall enzymes (see above) has a glucosyl linkage composition which is similar to that of the untreated glucan elicitor. However, the residual glucan has a lower degree of branching than the untreated elicitor. This is evidenced by the lower relative amounts of the terminal and 3,6-linked glucosyl residues which indicate that the residual glucan from the cell wall enzyme hydrolysis has one branch point for every four nonterminal residues.

The residual glucan remaining after limit β-glucosylase I hydrolysis has a glucosyl linkage composition which is substantially different from that of the untreated glucan elicitor (Table I). The residual glucan has a much higher content of 6-linked glucosyl residues than the untreated elicitor, and lower amounts of terminal glucosyl residues and 3,6-linked glucosyl residues. Therefore, the glucan remaining after β-glucosylase I hydrolysis is primarily a 3-linked and 6-linked glucan with only one branch point occurring for every eight nonterminal glucosyl residues.

The Effect of Enzyme Hydrolysis on the Activity of the Glucan Elicitor. The effect of enzyme hydrolysis on the activity of the glucan elicitor was determined with the aid of the cotyledon assay using the untreated glucan elicitor as a standard. The untreated glucan elicitor has a specific elicitor activity of 0.2 U/ng of carbohydrate (Fig. 2a, Table II).

The effect of hydrolysis on the glucan elicitor by the mixture of soybean cell wall enzymes was determined by assays of the 36 h hydrolysis mixture (Fig. 2a) and of the isolated residual glucan. The hydrolysis mixture had a specific activity equal to 15% of the untreated glucan elicitor (Table II). This indicates that in a single treatment, the mixture of cell wall enzymes destroys 85% of the activity of the glucan elicitor while hydrolyzing 91% of its glycosidic bonds. This result is confirmed by the activity of the residual glucan. The residual glucan had a specific activity equal to 1.5 times the specific activity of the untreated elicitor. Since the residual glucan represents 10% of the carbohydrate of the untreated elicitor, the residual glucan possesses 15% of the activity of the untreated elicitor. This conclusion is strengthened by the observation that neither the 36 h hydrolysis mixture nor the residual glucan had any inhibitory effect on the activity of the glucan elicitor. This was shown by assaying a known amount of glucan elicitor, with and without added aliquots of the enzyme-hydrolyzed elicitor preparations.

The effect of β-glucosylase I hydrolysis on the activity of the glucan elicitor was determined from the activity of the 32 h hydrolysis mixture (Fig. 2a). The 32 h hydrolysis mixture had a specific elicitor activity equal to 0.50 that of the untreated elicitor (Table II). This hydrolysis mixture had no inhibitory effect on the activity of the glucan elicitor. Therefore, in one treatment, β-glucosylase I destroys 50% of the activity of the glucan elicitor during hydrolysis of 58% of its glycosidic bonds.

Table II. *Effect of Enzyme Hydrolysis on the Activity of Elicitors*

Elicitor Preparation	Specific Elicitor Activity[a]	Carbohydrate	Activity[b]
	U/ng	*% untreated elicitor*	
1. Glucan elicitor untreated	0.2	100	100
Hydrolyzed by cell wall enzymes			
36-h hydrolysis mixture[c]	0.03 ± 0.01	100	15
Residual glucan after hydrolysis[d]	0.3 ± 0.1	10	15
Hydrolyzed by β-glucosylase I			
32-h hydrolysis mixture[c]	0.1± 0.03	100	50
Residual glucan after limit hydrolysis[d]	0.05 ± 0.01	23	6
2. Oligosaccharide elicitor untreated	1.7	100	100
Hydrolyzed by cell wall enzymes			
24-h hydrolysis mixture[c]	<0.01	100	<0.6
Hydrolysis by β-glucosylase I			
24-h hydrolysis mixture[c]	<0.01	100	<0.06

[a] Elicitor activities were determined using the cotyledon assay described under "Materials and Methods." One U of elicitor activity is defined as the amount of elicitor per cotyledon required to give an A/A_{max} of 0.5 in the cotyledon assay corrected to the standard curves shown in Figure 2. Specific activity is presented as U elicitor/ng carbohydrate.

[b] The percentage of the activity of the untreated elicitor which each enzyme hydrolyzed elicitor represents was computed from the specific activity of the elicitor preparations and from the percentage of carbohydrate of untreated elicitor which each enzyme hydrolyzed elicitor represents.

[c] The unfractionated hydrolysis mixtures contain glucose in addition to elicitor active oligo- or polysaccharides.

[d] Elicitor-active glucan remaining after enzyme hydrolysis isolated by P-2 chromatography.

It was shown by sequential treatment of the glucan elicitor that β-glucosylase I is able to hydrolyze up to 77% of its glucosidic bonds (see above). The effect of this exhaustive hydrolysis on the activity of the glucan elicitor was determined from the activity of the residual glucan remaining after limit β-glucosylase I hydrolysis. The residual glucan from the limit digest of the glucan elicitor by the β-glucosylase I possesses a specific activity equal to 25% of the specific activity of the untreated elicitor (Table II). The residual glucan had no inhibitory effect on the activity of the glucan elicitor. Since the residual glucan represents 23% of the carbohydrate of the original glucan elicitor, and is the only reaction product which possesses elicitor activity, the residual glucan contains an elicitor activity equal to 6% of the activity of the original glucan elicitor. Therefore, β-glucosylase I destroys 94% of the elicitor activity of the glucan elicitor (Table II) under conditions in which 77% of the glucosidic linkages are hydrolyzed.

Enzyme Hydrolysis of Elicitor-Active Oligosaccharides. Elicitor-active oligosaccharides were obtained from Pms mycelial walls as described under "Materials and Methods." A relatively size-homogeneous oligosaccharide elicitor (prepared by Bio-Gel P-2 chromatography) was used for these studies. This elicitor has an apparent mol wt of about 2,200 (as determined by Bio-Gel P-2 chromatography) and is comprised entirely of glucosyl residues. The oligosaccharide elicitor has a glucosyl linkage composition similar to that of the glucan elicitor (Table I) with the exception that the oligosaccharide elicitor has relatively high amounts of 4-

linked and 6-linked residues. The oligosaccharide elicitor is extremely potent in the cotyledon assay, an A/A_{max} of 0.5 results from as little as 0.5 ng/cotyledon.

The oligosaccharide elicitor was treated with either the mixture of cell wall enzymes or with purified β-glucosylase I, and the degree of hydrolysis was monitored. Both enzyme preparations rapidly hydrolyze the elicitor at nearly equivalent rates (Fig. 4). Calculation of units of hydrolytic activity during the 1st h of treatment indicates that these enzymes are about 60% as active in hydrolyzing the oligosaccharide elicitor as they are in hydrolyzing laminarin.

Both enzyme preparations had hydrolyzed nearly all of the oligosaccharide elicitor after 12 h of incubation. To ensure complete hydrolysis, fresh enzyme was added to each reaction mixture (see arrow, Fig. 4), and incubation was continued for an additional 12 h. During the 24-h treatment, the mixture of cell-wall enzymes hydrolyzed 99% of the oligosaccharide elicitor. Purified β-glucosylase I hydrolyzed 96% of the oligosaccharide elicitor.

These results were confirmed by Bio-Gel P-2 chromatography of the 24-h hydrolysis mixtures. Whereas the untreated oligosaccharide elicitor elutes in a peak just into the partially included volume of the column (Fig. 5a), essentially all of the enzyme-hydrolyzed elicitor elutes as monosaccharide (Fig. 5, b and c).

Cotyledon assays of the enzyme-hydrolyzed oligosaccharide elicitor showed that both the mixture of cell wall enzymes and β-glucosylase I destroy greater than 99% of the activity of oligosaccharide elicitors (Table II). Thus, both the mixture of cell wall enzymes and β-glucosylase I by itself are very effective in hydrolyzing nearly all of the glycosidic bonds of the oligosaccharide elicitor and destroying essentially all of its activity.

DISCUSSION

We have demonstrated that a mixture of enzymes extracted from the walls of suspension-cultured soybean cells is able in a single treatment to hydrolyze 91% of the glucosidic linkages of the glucan elicitor isolated from the mycelial walls of the fungal pathogen, Pms (Fig. 2). This hydrolysis destroys 85% of the activity of the elicitor (Table II). β-Glucosylase I, one of the enzymes present in the walls of suspension-cultured soybean cells, can eventually hydrolyze 77% of the glucosidic bonds and, in the process, destroy 94% of the activity of the glucan elicitor (Fig. 2, Table II).

It appears that β-glucosylase I is responsible for much, but not all, of the ability of the mixture of soybean wall enzymes to degrade the glucan elicitor. This is apparent from the higher rate and extent of hydrolysis of the glucan elicitor achieved by the mixture of soybean wall enzymes than that achieved by β-glucosylase I alone. β-Glucosylase I hydrolyzes about 58% of the glucosidic linkages of the glucan elicitor under conditions in which the mixture of cell wall enzymes hydrolyzes about 91% of the elicitor. Thus, the hydrolysis of the glucan elicitor by the mixture

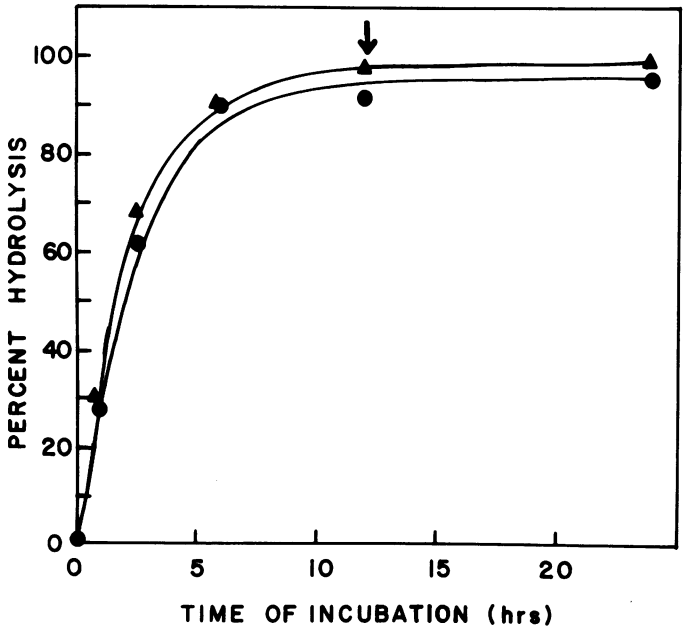

FIG. 4. Enzymic hydrolysis of the oligosaccharide elicitor. The oligosaccharide elicitor (3 mg) was treated with 0.16 U of either the mixture of cell wall enzymes or purified β-glucosylase I under the conditions described under "Materials and Methods." The percent hydrolysis by the cell wall enzymes (▲——▲) or by β-glucosylase I (●——●) is plotted *versus* the time of incubation. An additional 0.08 U of the mixture of cell wall enzymes or of β-glucosylase I was added to the respective reaction mixtures at the time indicated by the arrow. No hydrolysis occurred in control reactions from which enzyme was deleted.

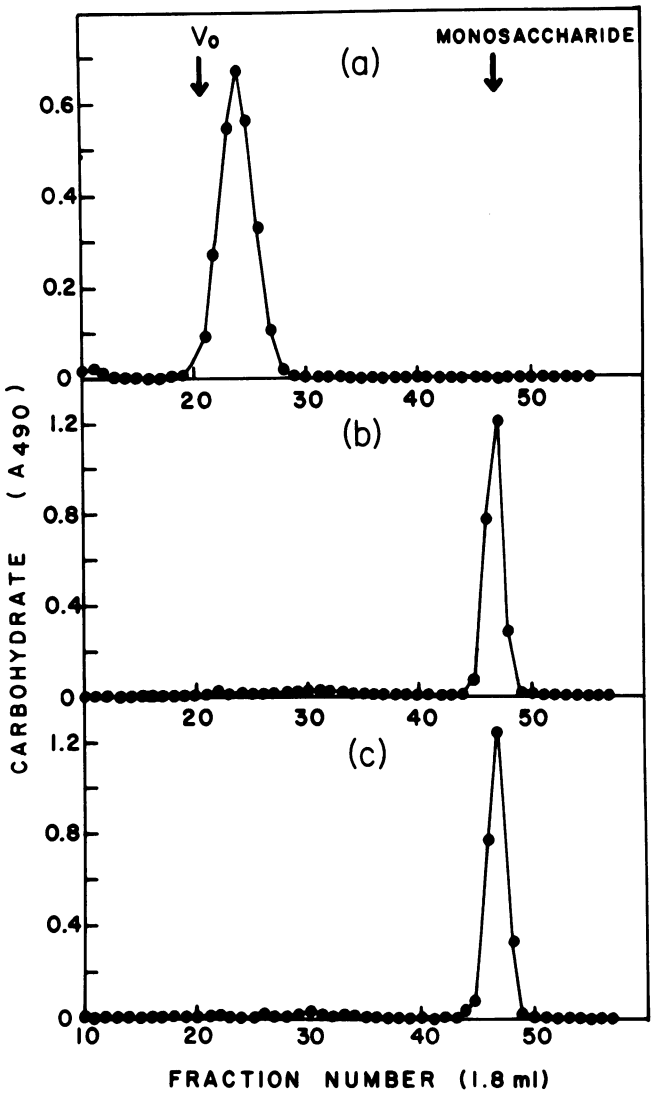

FIG. 5. The molecular size distribution of the oligosaccharide elicitor before and after enzyme hydrolysis. Samples (0.5 mg) of the oligosaccharide elicitor and of enzyme-hydrolyzed oligosaccharide elicitor were chromatographed on a 1.7 × 50 cm P-2 column as described under "Materials and Methods." Fractions of the column effluent were assayed for carbohydrate by the phenol-sulfuric acid method. *a*, Oligosaccharide elicitor. *b*, Oligosaccharide elicitor hydrolyzed by a mixture of soybean cell enzymes during a 24-h incubation (Fig. 4). *c*, Oligosaccharide elicitor hydrolyzed by β-glucosylase I alone during a 24-h incubation (Fig. 4).

of cell wall enzymes is a concerted effort by β-glucosylase I and either at least one additional enzyme or some sort of an activation factor. This is not surprising since the enzyme mixture extracted from the walls of suspension-cultured soybean cells contains several β-1,3-glucanase and β-glucosidase activities in addition to those activities of β-glucosylase I (8).

The present studies provide some additional information concerning the structural requirements for polysaccharide substrates of β-glucosylase I. It was shown in the preceding paper (8) that β-glucosylase I will hydrolyze linear β-glucans comprised of 3-linked glucosyl residues or of a mixture of 3-linked and 4-linked glucosyl residues. However, the question of whether β-glucosylase I can hydrolyze branched β-glucans was left unanswered. In addition, it was not clear whether β-glucosylase I can hydrolyze the glucosidic bonds to carbon 6 of glucosyl residues in polysaccharides, *i.e.* β-glucosylase I exhibited a low rate of hydrolysis of no more than 10% of pustulan, a 6-linked β-glucan, and this hydrolysis might have been of a contaminant polysaccharide in the pustulan preparation. Both of these questions are addressed by an analysis of the β-glucosylase I-catalyzed hydrolysis of the glucan elicitor.

The glucan elicitor is a highly branched β-glucan, as evidenced by its high percentage of 3,6-linked glucosyl residues (26%) and high percentage of terminal glucosyl residues (20%) (Table I). β-Glucosylase I converts 77% of the glucan elicitor into monosaccharide (see above). In addition, the glucan which remains after this exhaustive hydrolysis is even less branched than the original glucan elicitor (Table I). Therefore, β-glucosylase I is effective in hydrolyzing branched glucans.

The glucan elicitor also contains a considerable amount of glucosidic bonds to C-6 of other glucosyl residues, as demonstrated by its 6-linked glucosyl residues (4 mol %) and the 3,6-linked glucosyl residues (26 mol %) (Table I). An accounting of the total number of glucosidic bonds to C-6 in both the glucan elicitor and the residual glucan of β-glucosylase I hydrolysis (Table I) indicates that β-glucosylase I hydrolyzed 71% of the glucosidic bonds to C-6 which were present in the glucan elicitor.

The fact that β-glucosylase I is able to hydrolyze the glucosidic bonds to C-6 of residues in the glucan elicitor, but has little if any ability to hydrolyze pustulan might be explained as follows. Most of the linkages to C-6 in the glucan elicitor are to a branched 3,6-linked glucosyl residue, while the linkages to C-6 in pustulan are to an unbranched 6-linked glucosyl residue. This suggests that β-glucosylase I is able to hydrolyze glucosidic bonds to C-6 of polysaccharides only if the C-6 is part of a branched glucosyl residue. Most of the linkages to C-6 in the glucan which remain after exhaustive β-glucosylase I hydrolysis of the glucan elicitor are to unbranched 6-linked glucosyl residues. This residual glucan is resistant to further β-glucosylase I hydrolysis.

It should be pointed out that the inability of β-glucosylase I to hydrolyze the glucosidic linkages to C-6 of unbranched glucosyl residues is true only for polysaccharide substrates. β-Glucosylase I rapidly hydrolyzes the β-6-linked diglucoside, gentiobiose (8), as well as the oligosaccharide elicitor (Fig. 4). The oligosaccharide elicitor contains 15% 6-linked glucosyl residues (Table I).

The studies presented here demonstrate that soybean cell wall enzymes are capable *in vitro* of degrading the glucan and oligosaccharide elicitors from Pms. Although there is no direct evidence that the soybean wall enzymes hydrolyze the elicitor *in situ*, it is reasonable to believe that this does occur. The elicitor, as a foreign molecule, must approach its site of action in the soybean cell from outside the cell. Previous studies have shown that glycosidases bound to the walls of intact plant cells will hydrolyze exogenously added substrates (14, 17).

If the soybean wall enzymes do hydrolyze the glucan elicitor *in situ*, what is the physiological significance of such a hydrolysis? The presence of enzymes in soybean tissues which can hydrolyze the elicitor might appear to be disadvantageous to soybeans since the elicitor-stimulated phytoalexin accumulation is thought to be a defense response. However, it is possible to envision advantages which accrue to plants which possess enzymes capable of hydrolyzing elicitors. Two functions for the hydrolysis of the glucan elicitor by plant enzymes might be to facilitate passage of the elicitor through the cell wall by reducing the size of the elicitor and to localize the effect of the elicitor by eventually destroying its activity.

The primary site of action of the elicitor is probably the plasma membrane of the plant cell. Some evidence that plants have receptors for glucan elicitors in their plasma membranes has been provided by the work of Peters *et al.* (16). They observed that a glucan elicitor, isolated from the mycelial walls of *Phytophthora infestans*, agglutinates potato protoplasts. They also demonstrated that this agglutination was inhibited by the polysaccharide, laminarin. It has been observed in our laboratory that laminarin inhibits the elicitor-stimulated accumulation of glyceollin in soybeans (unpublished results of L. Pillus and P. Albersheim). This evidence is suggestive that the biological receptor for the elicitor is located in the plasma membrane, but the evidence is far from conclusive. Nevertheless, it is reasonable to hypothesize that the receptors for the elicitor are in the plasma membrane.

The biological receptors for many biologically active macromolecular ligands, including peptide hormones (9) and toxic proteins (6, 15), are located in the plasma membrane. If the primary site of action of the elicitor in soybean cells is in the plasma membrane, the elicitor must pass through the cell wall before it can stimulate the cell. For this to occur, elicitors must be small enough to pass through the pores of the wall. However, the Pms elicitor is a large polysaccharide. Indeed, glucans with mol wt in excess of 100,000 are active elicitors (Figs. 1 and 2). This is particularly interesting because recent studies by Carpita *et al.* (7) demonstrate that glucans with mol wt in excess of 6,500 do not readily pass through primary walls of plant cells. This suggests that enzymes, such as β-glucosylase I, could be advantageous to plants because of their ability to reduce the site of glucan elicitors (Fig. 1) and, thereby, expedite passage of elicitors through the cell wall. This could, in turn, permit a more rapid response of soybeans to an invading pathogen.

A second beneficial function for the ability of the plant to hydrolyze the elicitor might be to localize the effect of the elicitor by eventually destroying its activity. The elicitor-stimulated accumulation of phytoalexins is useful because phytoalexins are toxic to the invading pathogen. However, phytoalexins have also been shown to be toxic to plants (5, 13). Therefore, the presence of enzymes, such as β-glucosylase I, which could eventually destroy the elicitor (Table II), could protect the plant from unnecessary elicitation of phytoalexins by preventing migration of the elicitor to different tissues of the plant not challenged by the pathogen. This would be of obvious advantage to the plant because it would minimize the deleterious effects of the elicitor-stimulated accumulation of phytoalexin.

The suggestion that the cell wall enzymes of soybean serve these two beneficial functions is highly speculative. However, it provides a framework for future studies of the roles of the glucan-degrading enzymes present in plant cell walls. Careful studies need to be carried out to determine whether these enzymes play a role in plant disease resistance. If, however, these enzymes do function in the manner which we have suggested, the studies which have been presented in this and the accompanying papers will provide a model system for research into how plant cells mediate the transport of large molecules through their cell walls, and how plants regulate molecules which are both beneficial and toxic to the plant.

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