MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 4, No. 6, pp. 545-552, 1991

Host-Pathogen Interactions XXXIX. A Soybean Pathogenesis-Related Protein with β-1,3-Glucanase Activity Releases Phytoalexin Elicitor-Active Heat-Stable Fragments from Fungal Walls

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Received 22 May 1991. Accepted 18 July 1991.

Evidence has been obtained that fungal cell wall oligosaccharide fragments (oligosaccharins) solubilized by a pathogenesis-related (PR) β-1,3-glucanase elicit phytoalexin accumulation in soybean. Soybean leaves were treated with salicylic acid, polyacrylic acid, or mercuric chloride, or they were infected with *Phytophthora megasperma* H20 (a fungal pathogen of Douglas fir) to which soybean responds with nonhost resistance. Only mercuric chloride and the fungus induced the leaves to synthesize PR proteins. Both β-1,3-glucanase (EC 3.2.1.39) and chitinase (EC 3.2.1.14) activities were induced by treatment with mercuric chloride and by infection with the fungus. During purification of elicitor-releasing activity to homogeneity those fractions containing elicitor-releasing activity also contained β-1,3-glucanase activity, providing evidence that β-1,3-glucanase is a principal elicitor-releasing enzyme of the extracts. Antiserum raised against a tobacco PR β-1,3-glucanase cross-reacted with the purified soybean β-1,3-glucanase that accounted for major elicitor-releasing activity in the basic fraction of the soybean leaf extracts. Soybean β-1,3-glucanase, induced by mercuric chloride treatment or pathogenic infection with *P. m.* f. sp. *glycinea* race 1 (incompatible to the soybean cultivar used), could not be detected by immunoblots of extracts of control plants, indicating that the β-1,3-glucanase is a PR protein. These results suggest that a β-1,3-glucanase, induced in soybean seedlings by pathogenic infection or by chemical stress, functions in defense by releasing a phytoalexin elicitor from the mycelial walls of a pathogenic fungus.

Additional keywords: defense response, glucan elicitor, hypersensitive response.

Infection of plants with viruses, fungi, or bacteria, or treatment with any of several chemicals that stress the plants frequently leads to the induction of "pathogenesis-related" (PR) proteins (Bol and Van Kan 1988; Carr and Klessig 1989; Van Loon 1985). Although PR proteins have been widely studied, and some of their catalytic functions identified (for reviews see Bol *et al.* 1990; Carr and Klessig 1989), their biological functions have been inferred but not substantiated by experimental evidence.

The identification of several PR proteins as β-1,3-glucanases and chitinases has first been shown in tobacco (Kauffmann *et al.* 1987; Legrand *et al.* 1987) and confirmed in several other dicots (Joosten and De Wit 1989; Kombrink *et al.* 1988) and in monocots (Nasser *et al.* 1988). The substrates for these enzymes, β-1,3-glucan and chitin, are known components of many fungal cell walls (Farkas 1979). Furthermore, both chitinase and β-1,3-glucanase, induced by ethylene treatment or pathogenic infection, have been shown to inhibit fungal growth, and the combination of the two enzymes has been even more effective at inhibiting fungal growth (Mauch *et al.* 1988; Schlumbaum *et al.* 1986).

It has been suggested that PR proteins might release elicitor-active oligosaccharides from fungal cell walls (Darvill and Albersheim 1984; Hahn *et al.* 1989). This hypothesis has been supported by the observation that a β-1,3-glucanase purified from extracts of healthy (untreated) soybean cotyledons released elicitor-active oligosaccharides from the isolated mycelial walls of *Phytophthora megasperma* Drechs. f. sp. *glycinea* T. Kuan & D. C. Erwin (Keen *et al.* 1983; Keen and Yoshikawa 1983). Recently, Yoshikawa and colleagues (Takeuchi *et al.* 1990, Yoshikawa *et al.* 1990) reported that a β-1,3-glucanase, induced in soybean cotyledons by ethylene treatment, generated elicitor-active fragments from *P. m.* f. sp. *glycinea* mycelial walls, and exogenous application of this enzyme to hypocotyls of soybean partially inhibited fungal growth with induction of glyceollin accumulation. This β-1,3-glucanase activity did not increase further after microbial attack or elicitor treatment. They proposed that this enzyme may be an important component in the mechanism of ethylene-induced resistance to the pathogen in soybeans. It is known that exogenously applied ethylene, pathogenic infection, and elicitors extracted from mycelial walls induce both β-1,3-glucanase and chitinase in a number of plants (Boller 1985). But, experiments with a specific inhibitor of ethylene biosynthesis indicated that, in pea pods, the induction of these enzymes by ethylene occurs by a different mechanism than induction by fungal infection or fungal elicitors (Mauch *et al.* 1984). Thus, the question remained as to whether the proteins induced by pathogenic infection

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are capable of generating phytoalexin elicitor-active fragments from mycelial walls.

The purpose of the present study was to determine whether a pathogen-induced PR protein generates phytoalexin elicitor-active fragments from the mycelial walls of a pathogen. The soybean-*P. m.* f. sp. *glycinea* host-pathogen pair was selected for these investigations because the system has been widely studied and because the best characterized elicitor of phytoalexins, a hepta-β-glucoside, has been isolated from a partial acid hydrolysate of the mycelial walls of this pathogen (Sharp *et al.* 1984a; Sharp *et al.* 1984b).

MATERIALS AND METHODS

Culture of organisms. Soybean seeds (*Glycine max* (L.) Merr. 'Williams 82') were seeded in 9-cm trays and grown in a growth chamber for a 14-hr photoperiod at 5,400 lux and 60% relative humidity. The temperatures during the light and dark periods were 26 and 20° C, respectively. *Phytopthora megasperma* H20 (a pathogen of Douglas fir), against which soybean shows nonhost resistance, and *P. m.* f. sp. *glycinea* race 1 (incompatible with the soybean cultivar used) were obtained from D. Kuhn (Florida International University, Miami) and were maintained at room temperature in the dark on V8 juice agar slants or plates. The fungus was transferred to fresh medium every 6–8 wk.

Chemical treatments and fungal infection. Chemical treatments of 10-day-old soybean seedlings were performed by spraying their primary leaves with 1 mM salicylic acid solution, 0.2% polyacrylic acid solution (2,000 average mol wt, Aldrich Chemical Co., Milwaukee, WI), or 0.2% mercuric chloride solution.

The primary leaves of 10-day-old soybean seedlings were inoculated with the fungus by rubbing the leaves with a mycelial suspension of *P. megasperma* H20 using Celite 545 (0.3% w/v) as an abrasive. The mycelial suspension of *P. megasperma* H20 was prepared by collecting the mycelia of 2- to 3-wk-old fungi grown on V8 agar plates by rubbing the plates in distilled water (6 ml/plate). Control plants were either sprayed (control for chemical treatment) or rubbed (control for fungal infection) with distilled water.

Infection of soybean leaves with *P. m.* f. sp. *glycinea* race 1 was performed on detached leaves from 9-day-old soybeans by floating the leaves for 24 hr under a 14-hr photoperiod (26° C) and a 10-hr dark period (20° C) on a zoospore suspension (2.5×10^4 zoospores per milliliter) of *P. m.* f. sp. *glycinea* race 1. The zoospore suspension was prepared by the procedure of Eye *et al.* (1978). A control experiment was carried out by floating the leaves on water instead of the zoospore suspension.

Extraction of PR proteins. Chemically treated leaves were harvested 4 days after treatment; fungus-inoculated leaves were collected 10 days after inoculation. Five grams of soybean leaves was ground in liquid nitrogen, and the resulting leaf powder was stored at −80° C. PR proteins were extracted by grinding this powder in a glass tissue grinder with 13 ml of phosphate/citrate buffer (pH 2.8) containing 84 mM citric acid, 32 mM Na_2HPO_4, 14 mM 2-mercaptoethanol, and 6 mM ascorbic acid (Jamet and Fritig 1986). This low pH extraction procedure has been shown to extract PR proteins selectively (Van Loon 1985). After centrifugation of the homogenate at 15,000 *g* for 20 min, the supernatant was desalted on a Sephadex G-25 column (2.5 × 32 cm) equilibrated with 50 mM Tris buffer (pH 8.0) containing 1 mM EDTA and 3 mM 2-mercaptoethanol. The protein fraction was concentrated by ultrafiltration using YM-10 membrane (Amicon Corp., Beverly, MA) and concentrated further by Centricon-10 (Amicon).

Soybean cotyledon assay for elicitor activity. Mycelial walls of *P. m.* f. sp. *glycinea* race 1 were prepared following the procedure of Hahn *et al.* (in press). *P. m.* f. sp. *glycinea* mycelial walls were suspended in 10 mM sodium acetate (pH 5.2) by homogenizing with a Waring blender followed by sonication to disperse cell wall aggregates. The suspension of cell walls was washed six times with the same buffer in a sintered glass funnel (pore size 10–15 μm) and adjusted to a final concentration of 10 mg/ml. A quantity (usually 250 μl) of the mycelial wall suspension was gently shaken at 37° C for 1 hr with the protein solution being assayed for elicitor-releasing activity. The reaction mixture was then filtered through a cellulose acetate membrane (0.2 μm, Nalge Co., Rochester, NY) and inactivated by boiling for 10 min. The amount of carbohydrate solubilized from mycelial cell walls was determined by the anthrone method with glucose as a standard, according to the procedure described by Hahn *et al.* (in press).

Aliquots of the solubilized mycelial cell wall carbohydrate solution were diluted with water and analyzed for phytoalexin elicitor activity in the soybean cotyledon assay as described by Sharp *et al.* (1984a), except that 9-day-old soybean cotyledons were used, and the samples were diluted with water instead of buffer. To reduce day-to-day variation in the cotyledon elicitor assay, the elicitor activities were expressed as A/A_{max} in which A is the absorbance at 286 nm of the wound droplet solution treated with the sample being assayed, and A_{max} is the absorbance at 286 nm of the wound droplet solution treated with 1 μg/ml of the *P. m.* f. sp. *glycinea* void glucan solution. The *P. m.* f. sp. *glycinea* void glucan elicitor, a mixture of glucan fragments generated from *P. m.* f. sp. *glycinea* cell walls by partial acid hydrolysis and that eluted in the void volume of a low-resolution P-2 gel-permeation column described by Sharp *et al.* (1984a), was kindly provided by M. Hahn of the Complex Carbohydrate Research Center, The University of Georgia.

Purification of elicitor-releasing protein. Soluble proteins, extracted from mercuric chloride-treated soybean leaves by the procedure of Kauffmann *et al.* (1987), were applied to a CM-Sepharose fast flow cation-exchange column (2.5 × 8 cm, Sigma, St. Louis, MO) that had been equilibrated with 20 mM sodium acetate, pH 5.2 (buffer A). Proteins were eluted with a 1-L, linear gradient of 0–0.5 M NaCl in buffer A. Those fractions that were active in releasing elicitor from the mycelial walls were pooled, dialyzed against buffer A, and then applied to a Mono S fast protein liquid chromatography (Pharmacia, Piscataway, NJ) cation-exchange column (0.5 × 5 cm) that had been equilibrated with buffer A. Adsorbed proteins were eluted with a 30-ml linear gradient of 0–250 mM NaCl in the same buffer. Fractions containing the major peak of elicitor-releasing activity were pooled and adjusted to 1.7

M ammonium sulfate, and then loaded onto a Pharmacia Phenyl Superose hydrophobic interaction column (1 × 10 cm) that had been equilibrated with buffer A, containing 1.7 M ammonium sulfate. Desorption of the proteins was achieved by a linear salt gradient decreasing from 1.7 to 0 M ammonium sulfate in the same buffer. The active fractions were pooled and further purified on a Pharmacia Superose 12 gel-permeation column (1 × 30 cm) that had been equilibrated with 50 mM sodium acetate, pH 5.2.

SDS-PAGE. SDS-PAGE was performed by the method of Laemmli (1970) with a 12.5% resolving gel and a 5% stacking gel. Gel fixation and Coomassie Brilliant Blue staining were carried out according to the procedure of Neuhoff *et al.* (1988). Some gels were silver-stained following a modification of the procedure of Blum *et al.* (1987). The modified steps were as follows. The gels were fixed in 12% trichloroacetic acid solution (w/v) and washed with 5% methanol (v/v). Development was terminated with 5% acetic acid (v/v), and the gels were washed with 5% methanol (v/v) instead of 50% methanol (v/v).

Immunoblotting. The basic procedure of Towbin *et al.* (1979) was used for protein transfer. The transfer of proteins from SDS-PAGE gels to nitrocellulose membranes (pore size 0.45 μm, Schleicher & Schuell, Keene, NH) was performed for 40 min with a voltage gradient of 8.5 V/cm. Immunodetection was carried out, with some modifications, according to the procedure of the Bio-Rad immunoblot assay kit instruction manual (Bio-Rad, Richmond, CA). After electrophoretic transfer of the proteins, the blots were soaked for 30 min in phosphate-buffered saline (PBS; 137 mM NaCl, 1.47 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 3 mM NaN_3, pH 7.8) containing 5% bovine serum albumin (w/v). The blots were washed with PBS-T (PBS + 0.05% Tween 20) and then incubated overnight in PBS-T containing rabbit antiserum raised against tobacco PR-O, an acidic β-1,3-glucanase (rabbit anti-tobacco-PR-O serum, 1/2,000 dilution) (Kauffmann *et al.* 1987). The immunoreacted blots were washed with PBS-T and incubated for 4 hr in PBS-T containing alkaline phosphatase conjugated goat-anti-rabbit IgG (1/1,000 dilution, Sigma). After washing with PBS-T, the blots were immersed in buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) containing 0.3 mg/ml nitro blue tetrazolium chloride (Aldrich) and 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate (p-toluidine salt form, Sigma). The reaction was terminated by washing with water.

β-1,3-glucanase assay. Activity of β-1,3-glucanase was assayed by measuring the rate of reducing sugar production using laminarin (Sigma) as the substrate. Assays were carried out following the procedure of Kauffmann *et al.* (1987), except that the absorbance was measured at 640 nm. One unit was defined as the amount of enzyme that produced 1 μmol of glucose equivalents per minute.

Chitinase assay. Chitinase activities were determined using chitin from crab shells (Sigma) as the substrate by the procedure of Legrand *et al.* (1987), except that 20 μl of 25% (v/v) β-glucuronidase (type H-2: crude solution from snail, Sigma) was used to hydrolyze the chitin oligomers. One unit was defined as the amount of enzyme that released soluble chitin oligosaccharides containing 1 μmol of *N*-acetylglucosamine per minute.

RESULTS

Induction of PR proteins in soybean leaves. Infection of soybean leaves with *P. megasperma* H20 caused small necrotic spots that resembled a hypersensitive resistance response to form. Polyacrylic acid and salicylic acid, which are known inducers of PR proteins in tobacco (Van Loon 1983), and mercuric chloride, which is a known inducer of PR proteins in bean (Abu-Jawdah 1982; De Tapia *et al.* 1986) and maize (Nasser *et al.* 1988), were individually applied to soybean leaves to determine whether they induced PR proteins. Polyacrylic acid and salicylic acid did not cause any detectable necrotic spots on the leaves, but mercuric chloride caused necrotic symptoms that became visible 12 hr after treatment.

Proteins were extracted in a phosphate/citrate buffer (pH 2.8) from control, chemically treated leaves, and from fungal-inoculated leaves. The SDS-PAGE protein patterns in crude extracts are shown in Figure 1. Salicylic acid (Fig. 1, lane 2) and polyacrylic acid (Fig. 1, lane 3) did not induce any apparent low pH-soluble proteins that were not detectable in the water-treated control (Fig. 1, lane 1). However, mercuric chloride (Fig. 1, lane 4) induced at least seven new proteins, and fungal infection (Fig. 1, lane 6) induced at least nine new proteins. The protein bands induced by fungal infection are numbered according to their apparent mol wt on the SDS-PAGE gel. Eight proteins (Fig. 1, lane 6, numbers 1–8) were considered to be PR proteins because they displayed several of the characteristic properties ascribed to PR proteins, that is, they

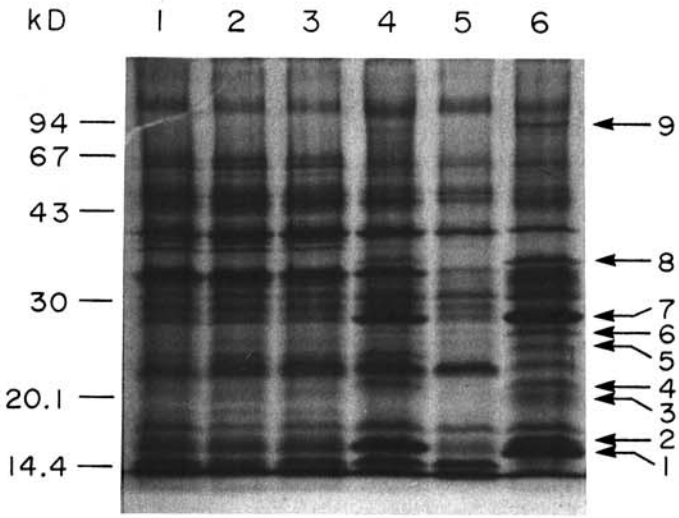

Fig. 1. SDS-PAGE of proteins extracted from soybean leaves sprayed with water (control) or treated with a chemical or *Phytophthora megasperma* H20. The proteins, extracted at pH 2.8 from 100 mg of leaves, were separated on a 12.5% polyacrylamide gel that was then stained with Coomassie Blue G-250. Leaves were sprayed with water (lane 1), with 1 mM salicylic acid (lane 2), with 0.2% polyacrylic acid (lane 3), and with 0.2% mercuric chloride (lane 4). The soybean leaves were collected 4 days after chemical treatment. The control for fungal-infected leaves (lane 5) and the fungal-infected leaves (lane 6) were harvested 10 days after treatment with water or the mycelial suspension. Protein bands induced by fungal infection are numbered according to their apparent mol wt. The mol wt standards were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400).

were soluble in low pH buffer, were low mol wt proteins, and were induced by fungal infection. Six of the seven proteins induced by mercuric chloride were also induced by fungal infection (Fig. 1, lane 6, numbers 1, 4, 6–9). Thus, six of the mercuric chloride-induced proteins (all except number 9) were also considered to be PR proteins. All of the induced proteins except number 9 (mol wt of 96,000) had a mol wt less than 40,000.

Several PR proteins from tobacco (Kauffmann *et al.* 1987; Legrand *et al.* 1987), potato (Kombrink *et al.* 1988), cucumber (Métraux *et al.* 1988), bean (Awade *et al.* 1989), maize (Nasser *et al.* 1988), and tomato (Joosten and De Wit 1989) have been shown to have chitinase or β-1,3-glucanase activity. Therefore, we analyzed the protein extracts of chemically treated and fungal-infected leaves for either or both of these enzyme activities (Table 1). Treatment of leaves with salicylic or polyacrylic acid did not induce significant increases, compared to the control extracts, in the activities of either β-1,3-glucanase or chitinase. However, the β-1,3-glucanase activity of the leaf extracts was increased eightfold 4 days after mercuric chloride treatment and twofold 10 days after fungal infection; chitinase activity was increased 67-fold and 13-fold, respectively. Thus, mercuric chloride and fungal infection both induce PR proteins in soybean.

Induction of phytoalexin elicitor-releasing activity and β-1,3-glucanase activity following mercuric chloride treatment. The time courses for the induction of elicitor-releasing and of β-1,3-glucanase activities were investigated in mercuric chloride-treated leaves (Fig. 2). Leaves sprayed with water were used as a control. One gram of leaves was extracted with 0.5 M sodium acetate (pH 5.2) containing 15 mM 2-mercaptoethanol. (Note: This buffer extracted more phytoalexin elicitor-releasing and β-1,3-glucanase activities than phosphate/citrate buffer, pH 2.8 [data not shown].) The β-1,3-glucanase activity increased during the first day following mercuric chloride treatment and reached about a 10-fold increase 4 days after treatment. There was no increase in β-1,3-glucanase activity in the control plants by 5 days after spraying with water.

The amount of carbohydrate solubilized from *P. m.* f. sp. *glycinea* cell walls correlated with the β-1,3-glucanase activity in the extracts (Fig. 2). Phytoalexin elicitor-releasing activity increased rapidly in mercuric chloride-treated plants and showed a similar increase relative to β-1,3-glucanase activity, suggesting a relationship between the elicitor-releasing and β-1,3-glucanase activities. This result indicated that phytoalexin elicitor-releasing activity was inducible by mercuric chloride treatment. Phytoalexin elicitor-releasing activity was also induced by pathogenic infection (data not shown), suggesting that PR proteins are responsible for elicitor-releasing activity. Therefore, we decided to monitor the β-1,3-glucanase activity while purifying elicitor-releasing activity from mercuric chloride-treated soybean leaves.

Purification of an elicitor-releasing protein. The proteins in the extracts of the mercuric chloride-treated leaves were applied to a CM-Sepharose fast flow column at pH 5.2. The adsorbed proteins were eluted with a linear gradient of 0–0.5 M NaCl, and the fractions with both phytoalexin elicitor-releasing and β-1,3-glucanase activities were pooled as shown in Figure 3. The pooled proteins were further

Table 1. Induction of β-1,3-glucanase and chitinase activities in soybean by various treatments[a]

Treatment	β-1,3-glucanase[b] (munit/g of leaves)	Chitinase[c] (munit/g of leaves)
Control (4 days after treatment)	17	0.6
Salicylic acid	20	1.2
Polyacrylic acid	28	1.8
Mercuric chloride	140	40
Control (10 days after treatment)	55	2.6
Fungal infection	108	34

[a] Chemically treated leaves were harvested 4 days after treatment; fungus-inoculated leaves were collected 10 days after inoculation. Proteins were extracted with phosphate/citrate buffer (pH 2.8).

[b] The relative β-1,3-glucanase activity was determined with laminarin as the substrate by measuring the amount of reducing sugars produced. One milli unit (munit) was defined as the amount of enzyme that produced 1 nmol of glucose equivalents per minute.

[c] The relative chitinase activity was determined colorimetrically (Legrand *et al.* 1987) with chitin from crab shells as the substrate. One milli unit (munit) was defined as the amount of enzyme that released soluble chitin oligosaccharides containing 1 nmol of *N*-acetylglucosamine per minute.

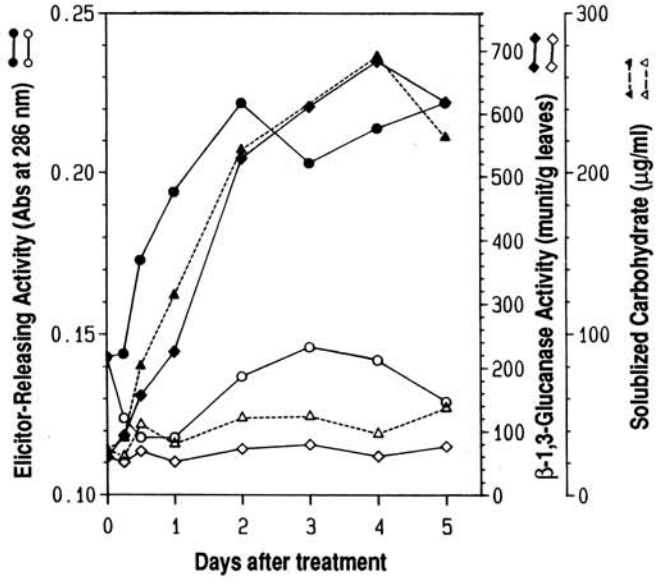

Fig. 2. Induction of β-1,3-glucanase and phytoalexin elicitor-releasing activities after mercuric chloride treatment of soybean leaves. Proteins from 1 g of leaves were extracted by grinding in a mortar in 5 ml of 0.5 M sodium acetate, pH 5.2, containing 15 mM 2-mercaptoethanol. After centrifugation, the protein extracts were dialyzed against 50 mM sodium acetate (pH 5.2) and concentrated with Centricon-10. The β-1,3-glucanase activity was measured using laminarin as the substrate. Elicitor-releasing activity and total carbohydrates solubilized from *Phytophthora megasperma* f. sp. *glycinea* cell walls were analyzed in aliquots of the leaf extracts by incubating the aliquot (equivalent to the extraction of 3 mg of leaves) with 250 ml of *P. m.* f. sp. *glycinea* cell wall suspension (10 mg/ml) in 10 mM sodium acetate (pH 5.2) at 37° C for 1 hr. The reaction was terminated by passing the mixture through a membrane filter (0.2 μm) and then boiling for 10 min. A portion (20 μl) of this heat-inactivated solution was diluted (100 times) and analyzed for its ability to elicit phytoalexins in the soybean cotyledon assay. The remainder of the heat-inactivated solution was analyzed for soluble carbohydrates by the anthrone assay. Closed symbols represent results obtained with the extracts of mercuric chloride-treated leaves; open symbols represent results obtained with the extracts of water-treated leaves.

purified by fast protein liquid chromatography using a Mono S cation-exchange column, then a Phenyl Superose hydrophobic interaction column, and, finally, a Superose 12 gel-permeation column (see Materials and Methods). In each column, the peaks of elicitor-releasing activity correlated exactly with the peaks of β-1,3-glucanase activity (for example, see Fig. 4). The amount of carbohydrate solubilized from *P. m.* f. sp. *glycinea* mycelial walls also correlated with both elicitor-releasing and β-1,3-glucanase activities (data not shown), suggesting that phytoalexin elicitors released by β-1,3-glucanase were carbohydrates. At least three β-1,3-glucanase activities were found. Two activities were found in the eluted fractions of the Mono S fast protein liquid chromatography column (Fig. 4). The third was found in the nonadsorbed fraction of the CM-Sepharose column (data not shown). The fractions containing the most active β-1,3-glucanase/elicitor-releasing material eluted from the Mono S column were pooled and further purified as described in Materials and Methods. The purified β-1,3-glucanase (Fig. 5A, lane 2), which also had elicitor-releasing activity, yielded a single band (apparent mol wt of 32,000) when analyzed by SDS-PAGE and stained with silver. A major band of the pooled basic proteins (Fig. 5A, lane 1) on the CM-Sepharose column was observed at the position corresponding to the purified β-1,3-glucanase, suggesting that the purified β-1,3-glucanase was a major component of the basic proteins in the extracts from the mercuric chloride-treated leaves.

Elicitor-releasing activity of a PR protein. Rabbit anti-tobacco-PR-O serum cross-reacted with the purified β-1,3-glucanase (Fig. 5B, lane G), indicating homology between the basic β-1,3-glucanse of soybean and the acidic β-1,3-glucanase (called PR-O) of tobacco.

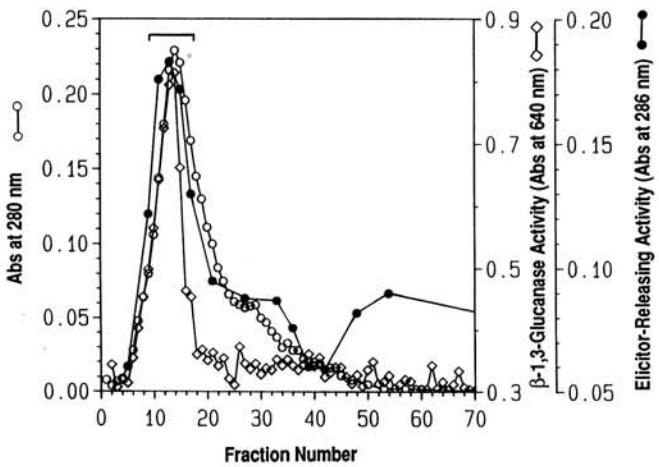

Fig. 3. CM-Sepharose fast flow chromatography of pooled proteins desalted by a Sephadex G-25 column. Proteins were extracted from mercuric chloride-treated leaves with 0.5 M sodium acetate (pH 5.2) containing 15 mM 2-mercaptoethanol and then desalted on a Sephadex G-25 column equilibrated with 20 mM sodium acetate, pH 5.2 (buffer A). The desalted proteins were applied to a CM-Sepharose fast flow column that had been equilibrated with buffer A. The adsorbed proteins were eluted with a 1-L linear gradient of 0–0.5 M NaCl in buffer A. The bar indicates those fractions that were pooled. Elicitor-releasing activity was analyzed as described in Figure 2 (30 μl of each fraction was incubated with 250 μl of *Phytophthora megasperma* f. sp. *glycinea* cell wall suspension).

Evidence was obtained that the purified β-1,3-glucanase was induced both by mercuric chloride treatment and fungal infection. The purified β-1, 3-glucanase and the mixture of proteins extracted at low pH (pH 2.8) from control and mercuric chloride-treated plants were immunoblotted with rabbit anti-tobacco-PR-O serum (Fig. 5B, lanes M and C). A band corresponding to the position of the purified β-1,3-glucanase was observable in crude extracts from mercuric chloride-treated plants (Fig. 5B, lane M) but not in extracts from control plants (Fig. 5B, lane C), indicating that the β-1,3-glucanase that was purified had been, in fact, induced by mercuric chloride treatment.

Detached leaves of soybean were infected with a zoospore suspension of *P. m.* f. sp. *glycinea* race 1 as described in Materials and Methods to show that the purified β-1,3-glucanase was also induced by fungal infection. Proteins extracted at low pH (pH 2.8) from detached leaves that were floated either on the zoospore suspension or on water were immunoblotted with rabbit anti-tobacco-PR-O serum. A band corresponding to the position of the purified β-1,3-glucanase was observed in the extract from detached leaves that were infected with zoospores (Fig. 5B, lane I) but not in the crude extract from detached leaves that were floated on water (Fig. 5B, lane CI). Thus, the purified β-1,3-glucanase was induced by fungal infection as well as by mercuric chloride treatment, establishing that the purified β-1,3-glucanase was a PR protein. Two additional

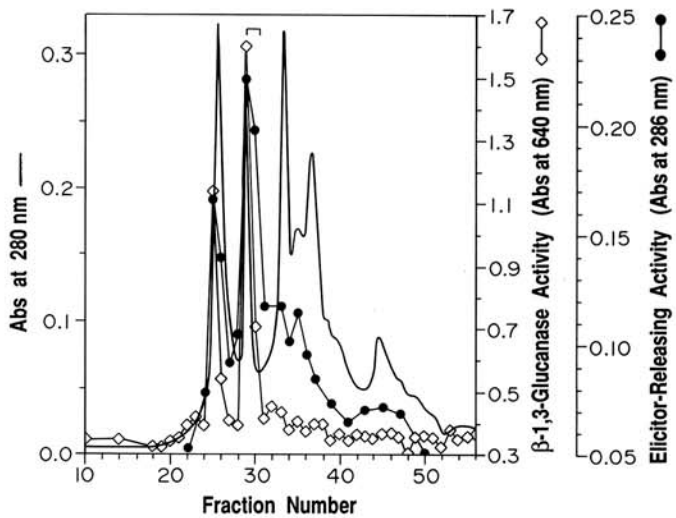

Fig. 4. Mono S cation-exchange fast protein liquid chromatography of the pooled fractions of CM-Sepharose fast flow chromatography. The pooled fractions of CM-Sepharose fast flow chromatography (Figure 3) were dialyzed against buffer A. An aliquot (approximately 4.5 mg of total protein) of the dialyzed, pooled fractions was loaded onto a Mono S column that had been equilibrated with buffer A. The adsorbed proteins were eluted with a 30-ml linear gradient of 0–250 mM NaCl in buffer A. The bar indicates the fractions (nos. 29 and 30) that were pooled and subsequently subjected to further chromatographic steps as described in Materials and Methods. Elicitor-releasing activity and total carbohydrates solubilized from *Phytophthora megasperma* f. sp. *glycinea* cell walls were analyzed as described in Figure 2 (carbohydrate measurements not shown). The amount of carbohydrate determined from each fraction correlated with both elicitor-releasing and β-1,3-glucanase activities. Ten microliters of each fraction was incubated with 200 μl of *P. m.* f. sp. *glycinea* mycelial wall suspension. The solubilized material was heat-inactivated, diluted 200 times, and assayed for elicitor activity.

cross-reacted bands were detected in lane I and lane M of Figure 5B. These bands also contained protein induced by fungal infection and mercuric chloride treatment, respectively, and probably also were β-1,3-glucanases. They may have been isozymes of the purified β-1,3-glucanase.

The elicitor activity released by the purified β-1,3-glucanase is shown in Figure 6. The amount of carbohydrate solubilized from the *P. m.* f. sp. *glycinea* mycelial walls correlated with the amount of elicitor activity solubilized from the *P. m.* f. sp. *glycinea* mycelial walls. Purified β-1,3-glucanase (7.4 μl; 10^{-3} units) released about 30 μg of glucose equivalent of soluble, anthrone-positive carbohydrates from *P. m.* f. sp. *glycinea* mycelial walls. Heat-inactivated β-1,3-glucanase did not solubilize elicitor activity and did not solubilize anthrone-positive carbohydrates (Fig. 6).

DISCUSSION

This report describes the ability of a PR protein to generate phytoalexin elicitor-active oligosaccharides from the mycelial walls of a fungal pathogen. We have shown that PR proteins are induced in soybean leaves by fungal infection and by treatment with mercuric chloride. Proteins were extracted from a leaf homogenate in a low pH buffer, and the PR proteins were separated by one-dimensional SDS-PAGE. Eight PR proteins were identified with mol wts of less than 40,000, which agrees with the size of the PR proteins that had been previously shown to be present in the intercellular fluid of soybean leaves infected with a tobacco necrosis virus (Roggero and Pennazio 1989 and 1990).

We have also shown that β-1,3-glucanase and chitinase activities were increased after treatment with mercuric chloride or fungal infection, indicating that some of the induced PR proteins are β-1,3-glucanases and chitinases, as in many other plants (Carr and Klessig 1989). We went on to demonstrate that one of the basic PR proteins is indeed a β-1,3-glucanase.

The possibility that some of the proteins induced by fungal infection are of pathogen rather than plant origin has not been excluded. But fungal infection caused small, hypersensitive, resistance-like necrotic spots to appear on the leaves, indicating that fungal growth was restricted. Thus, the amount of fungal protein in the leaf extracts was probably too little to be detected by SDS-PAGE stained with Coomassie Brilliant Blue, suggesting that the newly appearing proteins are of plant origin. In any case, five of the eight PR proteins induced by fungal infection were also induced by treatment with mercuric chloride. Thus, at least those five proteins, which include the purified β-1,3-glucanase, are encoded and synthesized by the plant rather than by the pathogen.

It has been reported that exogenously applied ethylene increases defense responses of several plants against pathogens (Boller 1982). Recently, Yoshikawa and colleagues (Takeuchi *et al.* 1990) purified from ethylene-treated soybean cotyledons a major basic β-1,3-glucanase that could

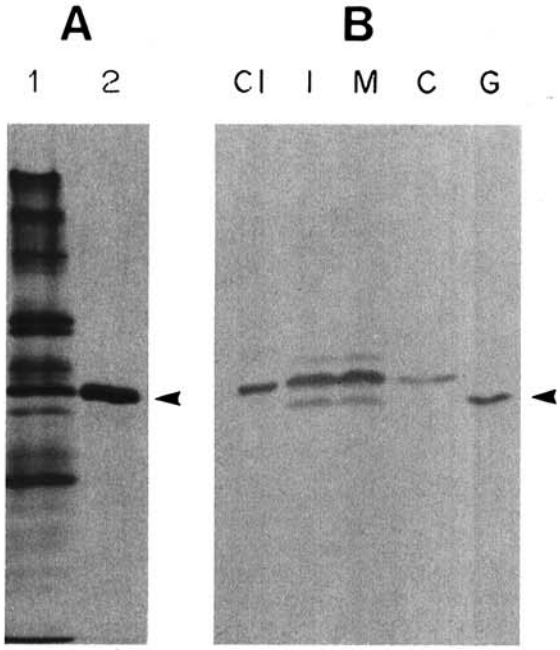

Fig. 5. A, silver-stained SDS-PAGE of the basic pooled proteins of CM-Sepharose column (lane 1) and purified β-1,3-glucanase (lane 2). **B,** proteins extracted at pH 2.8 from an equivalent amount of leaves (10 mg) were loaded in each lane. Extracts of leaves that were floated for one day on water (control for fungal-infected leaves) are in lane CI and extracts of leaves floated on a zoospore suspension of *Phytophthora megasperma* f. sp. *glycinea* race 1 are in lane 1. Extracts of leaves of whole plants harvested 4 days after treatment with mercuric chloride are in lane M; extracts of leaves treated with water (control for chemically treated leaves) are in lane C. Purified β-1,3-glucanase is in lane G. The extracts were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane and immunodetected with rabbit anti-tobacco-PR-O serum. The position of the purified β-1,3-glucanase is indicated.

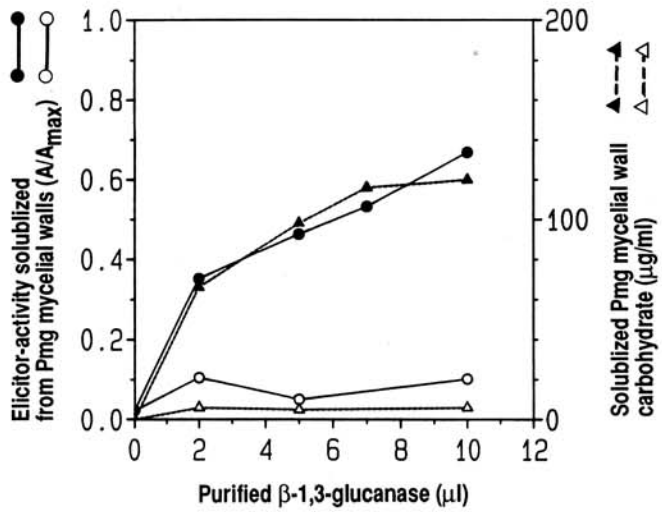

Fig. 6. Elicitor-releasing activity of purified β-1,3-glucanase. The samples to be analyzed for elicitor- and carbohydrate-releasing activities were prepared as described in Figure 2. Heat-inactivated elicitor solutions were diluted 1,000 times with water and analyzed by the soybean cotyledon phytoalexin elicitor assay. The closed and open symbols show the results obtained when 250 μl of the *Phytophthora megasperma* f. sp. *glycinea* cell wall suspension was incubated with various amounts of the purified β-1,3-glucanase or with heat-inactivated purified β-1,3-glucanase, respectively. The purified β-1,3-glucanase contained one milli unit (munit) of activity in 7.4 μl.

release phytoalexin elicitor-active fragments from *P. m.* f. sp. *glycinea* mycelial walls. This enzyme activity was induced two- to threefold by ethylene treatment. This purified β-1,3-glucanase was the same enzyme that had been previously purified from extracts from untreated soybean cotyledons (Keen and Yoshikawa 1983). Therefore, the β-1,3-glucanase was a constitutive component of healthy soybean cotyledons. This enzyme activity was not induced by microbial attack or elicitor treatment (Takeuchi *et al.* 1990; Yoshikawa *et al.* 1990).

We have reported the induction of elicitor-releasing activity by both mercuric chloride treatment (Fig. 2) and by fungal infection (data not shown). Our purification procedures were made towards elicitor-releasing activity as well as β-1,3-glucanase activity. Our rationale was to look for any protein induced on fungal infection that could release elicitor(s) from fungal cell walls. We found that a major elicitor-releasing protein, isolated from the basic fraction of soybean extracts, was, in fact, a β-1,3-glucanase. This enzyme was not detectable by immunoblotting extracts from 14-day-old control soybean plants (Fig. 5B, lane C). Two other isozymes have been shown to occur in the extracts of chemical-treated soybean leaves (see Fig. 4 and results). At least one, the second basic β-1,3-glucanase, was capable of releasing elicitor-active oligosaccharides from fungal cell walls (data not shown). We do not know whether the isozymes act in a synergistic or additive fashion to produce more active elicitor(s) from fungal cell walls.

Phytoalexins are thought to be important in the mechanisms by which soybeans resist fungal infection (Keen 1990; Keen *et al.* 1982; Yoshikawa *et al.* 1978). We have now shown that a β-1,3-glucanase PR protein, induced either by fungal infection or mercuric chloride treatment, releases phytoalexin elicitor-active fragments from mycelial walls. During purification of elicitor-releasing activity, the elicitor-releasing activity co-migrated in four types of chromatography with the β-1,3-glucanase activity. We did not find elicitor-releasing activity that did not co-chromatograph with β-1,3-glucanase activity. These observations provide evidence that β-1,3-glucanase is the quantitatively predominant and perhaps the only enzyme activity in soybean leaf extracts that is capable of releasing elicitor-active fragments from mycelial walls. This can be taken as evidence that β-1,3-glucanase induced by pathogenic infection plays an important role in the defense of soybeans against infection.

It has been shown that chemical-treated soybean leaf extracts contain several isoforms of β-1,3-glucanase activity (see Fig. 4 and results). Therefore an important consideration, relevant to the mechanism of action of these β-1,3-glucanases, is their localization. It is well documented in tobacco that hypersensitive reacting leaves, on infection with tobacco mosaic virus, contain several isozymes of β-1,3-glucanase (Kauffmann *et al.* 1987): some are vacuolar, the basic enzymes, some are apoplastic, the acidic isoforms. Thus, it may be possible that the different soybean β-1,3-glucanases also have different locations. Determining the location of these enzymes may be crucial in understanding the mechanisms of defense induced by pathogen infection. Despite numerous reports concerning localization of β-1,3-glucanases, it is still debated which isoforms are acting first. One possibility would be that the vacuolar isozymes are quickly released, to be in contact with the invading fungus, as part of the rapid host cell death, which is one of the main characteristics of the hypersensitive response, and the apoplastic isoforms would act later as a signal amplifier. Another point of view suggests that any function the vacuolar glucanase isoforms play in plant defense is restricted to an intracellularly coordinated defense process, because these particular isozymes have not been found to be secreted to the extracellular spaces of the plant following pathogen infection (Van den Bulcke *et al.* 1989). The vacuole-localized β-1,3-glucanases have also been thought to be a last line of defense, released when the attacked host cells lyse; the apoplastic isoforms, on the other hand, would be involved in the recognition process, releasing defense-activating signaling molecules from the walls of the invading fungus (Mauch and Staehelin 1989).

The purified β-1,3-glucanase hydrolyzes glycosidic linkages of fungal cell walls, solubilizing oligo- and/or polysaccharides. The phytoalexin elicitors released from *P. m.* f. sp. *glycinea* mycelial walls by the purified β-1,3-glucanase were heat stable. The ability of elicitors solubilized from *P. m.* f. sp. *glycinea* mycelial walls to induce phytoalexin accumulation was proportional to the amount of carbohydrate solubilized from those walls, providing additional evidence that the chemical nature of the phytoalexin elicitors released by β-1,3-glucanase was carbohydrate. This conclusion agrees with the finding that elicitor-active molecules solubilized by autoclaving *P. m.* f. sp. *glycinea* mycelial walls were β-glucans consisting primarily of 3-, 6-, and 3,6-linked glucosyl residues in the β-configuration (Ayers *et al.* 1976a; Ayers *et al.* 1976b). The smallest elicitor-active β-glucan fragment obtained by partial acid hydrolysis of the *P. m.* f. sp. *glycinea* mycelial walls was isolated and shown to be a specific hepta-β-glucoside (Sharp *et al.* 1984a; Sharp *et al.* 1984b). We will attempt to isolate and characterize the phytoalexin elicitor-active *P. m.* f. sp. *glycinea* mycelial wall fragments generated by the purified β-1,3-glucanase that has been shown to be a pathogenesis-related protein.

ACKNOWLEDGMENTS

We thank M. Hahn for his generous gifts of fungal void glucan, assistance, and helpful discussions. We are also grateful to D. Kuhn (Florida International University, Miami) for donating fungal strains, and to Carol L. Gubbins Hahn for drawing Figure 4. Serge Kauffmann was supported by an EMBO fellowship. This work was supported by a grant from the U.S. Department of Energy (DE-FG09-85ER13425) and supported, in part, by the USDA/DOE/NSF Plant Science Centers Program, this center being funded by Department of Energy grant DE-FG-09-87ER13810.

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