Induction of Proteinase Inhibitors in Tobacco Cell Suspension Culture by Elicitors of Phytophthora parasitica var. nicotianae

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ABSTRACT

An elicitor preparation obtained from Phytophthora parasitica var. nicotianae, a pathogen of tobacco, induced an accumulation of proteinase inhibitors and a stimulation of ethylene synthesis in a tobacco (Nicotiana tabacum) cell suspension culture. About 30 micrograms per milliliter of elicitor were necessary for maximal induction of proteinase inhibitor accumulation, and the response was detectable after 12 hours of incubation with elicitor. Accumulation of proteinase inhibitors required de novo protein synthesis, since cycloheximide completely inhibited its elicitation, and actinomycin D inhibited it partially. One of the inhibitors was purified by a procedure that included heating, (NH₄)₂SO₄ precipitation, ion-exchange chromatography, and affinity chromatography. The purified inhibitor was shown to be a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular weight of about 10,500. It inhibited trypsin but not chymotrypsin.

Proteinase inhibitors (PIs) are proteins or polypeptides which occur in many different plants. The most widely studied group of plant PIs is that which inhibits animal serine proteases such as trypsin or chymotrypsin. They seem to accomplish multiple functions such as storage proteins, control mechanisms for endogenous proteinases, or protection against proteolytic attack by insects or microbial pathogens (19, 20). In 1972, Green and Ryan (6) showed that tomato plants accumulate chymotrypsin inhibitor I in their aerial parts after damage by insects or mechanical wounding and suggested a role in plant defense against leaf-eating insects by decreasing the digestibility of the plant. The reaction was mediated by a plant wound signal called proteinase inhibitor-inducing factor (PIIF), which was released at the wound site and transported throughout the plant. Later, PIIF was isolated and characterized as a pectic polysaccharide originating from the plant cell wall (1). PI accumulation in tomato plants was found to be induced not only by PIIF, but also by citrus pectic fragments, endopolygalacturonase and chitosan, which are potent elicitors of phytoalexin synthesis (25); on the other hand, PIIF could also induce phytoalexin synthesis. Accumulation of PIs has also been found in tomato plants after infection with Phytophthora infestans, showing differences in compatible and incompatible race-cultivar interactions (18). Thus, induction of PI seems to be an integral part of the plant defense system against pathogens.

In the present work, we investigated the induction of PI by elicitors isolated from Phytophthora parasitica var. nicotianae, the causal agent of the black shank disease of tobacco plants. Helgeson and Haberlach (7) demonstrated that the tobacco Phytophthora system can be studied at the whole plant level as well as in tissue cultures. A tobacco cell suspension culture was retained to set up a simplified system for the study of plant defense reactions. One of the several PIs was purified to electrophoretic homogeneity.

MATERIALS AND METHODS

Tobacco Cell Culture

Cells of Nicotiana tabacum L. cv Wisconsin 38 were grown in a liquid Murashige-Skoog medium modified by Jouanneau (9, 14) under constant light (10 Wm⁻²) at 24°C in Erlenmeyer flasks. The cells reached the stationary growth phase after 8 d and were subcultured every 14 d.

Elicitor Preparation

Elicitors were isolated from Phytophthora parasitica Tuck var. nicotianae as described previously (17). The ethanol-soluble fraction was used after dialysis (Spectrapor M6, cutoff mol wt 1000) against deionized water and lyophilization. Concentrations of elicitor were always based on dry weight.

Elicitor Treatment

Seven-day-old tobacco cell cultures (in late logarithmic growth phase) were used for incubations and prepared under sterile conditions as follows: cells were allowed to settle by gravity, the medium was gently removed by suction, and the cells were resuspended in twice their volume of fresh medium. This procedure was carried out three times.
Measurement of Ethylene Accumulation

One mL of tobacco cell suspension prepared as above was incubated with or without elicitor in sealed vials. After the incubation time (6 h unless stated otherwise), 1 mL of the gas phase was withdrawn from the vials with a gas-tight syringe through the rubber cap and injected into a gas chromatograph column for ethylene measurement. For time course measurements, 1 mL of air was re-injected into the vials after each withdrawal, and dilution was corrected by calculation.

PI Induction

Generally, 25 mL of the cell suspension prepared as above were incubated in 100 mL Erlenmeyer flasks under normal culture conditions after addition of elicitor and/or other substances, respectively. After 12 to 48 h, the cells were harvested by filtration on a Whatman G2 sintered glass filter. Their fresh weight was determined immediately and they were frozen at −40°C.

PI Extraction

Extraction of PI was carried out according to Walker-Simmons and Ryan (23). The frozen tobacco cells were ground thoroughly in a mortar with a pestle under liquid N2. The fine powder was suspended in 0.5 M Tris-HCl, pH 8.5, containing 0.5% (w/v) polyethylene glycol and 3% (w/v) PVP (3 mL of buffer per g fresh weight of cells) and allowed to thaw under occasional shaking for about 1 h in the cold-room. The slurry was filtered through four layers of cheesecloth, and the liquid was cleared by centrifugation (1 h, 144,000g at 4°C). The supernatant was subsequently heated at 65°C for 10 min and the precipitate removed by centrifugation (5 min, 10,000g). The extract obtained by this procedure was either used for determination of PI activity in an enzymic assay or subjected to further purification.

Assay for PI Activity

For determination of PI activity, we modified an assay described by Walker-Simmons and Ryan (23). Each extract was assayed at different concentrations against 1 µg of trypsin (Trypsin TPCK Cooper Biomedical). Aliquots of the extracts (0–150 µL, each volume in duplicate) were brought to a volume of 0.5 mL with 46 mM Tris-HCl, pH 8.1, 11.5 mM CaCl2 in test tubes containing small magnetic bars and placed in a waterbath at 30°C where they were constantly stirred. One-hundred µL of a trypsin solution (10 µg/mL in 1 mM HCl) were added to each test tube except the blanks. After 3 min, 0.5 mL of 46 mM Tris-HCl, pH 8.1, 11.5 mM CaCl2, and 2 mL of a solution of 1% (w/v) Azocoll (Calbiochem, La Jolla, CA) in 46 mM Tris-HCl, pH 8.1, were added, and incubation was continued for further 12 min with constant stirring. The reaction was stopped by filtering the samples through paper filters, and the absorbance of the clear solution was measured in a spectrophotometer at 520 nm. The PI activity was expressed as µg trypsin inhibited by 1 mg of protein in the extract according to Walker-Simmons and Ryan (23).

PI specificity was determined according to Hummel (8) by measuring the ability to inhibit the enzymic activity displayed by chymotrypsin (Sigma) and trypsin on synthetic peptide esters. The chymotrypsin assay was composed of 0 to 150 µL of a PI solution preincubated with 100 µL of chymotrypsin (10 µg/mL in 1 mM HCl) in a total volume of 1.6 mL 80 mM Tris-HCl, pH 7.8, 0.1 M CaCl2 for 3 min at 25°C. After addition of 1.4 mL BTEE (1.07 mM in 50% methanol w/w), the rate of hydrolysis was determined from the increase in absorbance at 256 nm during 3 to 4 min. The trypsin assay was composed of 0 to 150 µL of PI solution preincubated with 100 µL of trypsin (10 µg/mL in 1 mM HCl) in a total volume of 1.1 mL 46 mM Tris-HCl, pH 8.1, 11.5 mM CaCl2 for 3 min at 25°C. After addition of 1.4 mL TAME (2 mM in the same buffer), the rate of hydrolysis was determined from the increase in absorbance at 247 nm during 3 min. Proteinase activity was calculated by measuring the slope of the linear portion of the curves. For controls the enzymes were added to their respective substrates without inhibitors and without prior incubation. The remaining proteinase activity was expressed as percent of the control. The inhibitory activity was calculated as in the Azocoll assay according to Walker-Simmons and Ryan (23).

PI Purification

The purification was carried out according to a procedure described by Carlberg et al. (3). About 60 g fresh weight of elicited cells were subjected to extraction, and the heat-cleared extracts (65°C, 10 min) were cooled and proteins precipitated with (NH4)2SO4 at 80% saturation during 3 h at 4°C. After centrifugation (20 min, 10,000g at 4°C), the pellet was resuspended in 10 mM Tris-HCl, pH 8.5, and dialyzed extensively against this buffer. The precipitate that formed during dialysis was removed by centrifugation (20 min, 50,000g at 4°C) and the supernatant applied to a DEAE-trisacryl M column (IBF; 2.6 x 7 cm) equilibrated with 10 mM Tris-HCl, pH 8.1. The column was washed with this buffer and subsequently eluted with a gradient of 0 to 1 M NaCl in this buffer. The protein peaks which eluted between 0.1 M and 0.2 M NaCl and contained a high PI activity were dialyzed against 0.1 M Tris-HCl, pH 8.5, 0.5 M NaCl, and applied to a trypsin-Sepharose column equilibrated with this buffer. For the affinity column, 4.5 mg trypsin were coupled to 1 mL of CNBr-activated Sepharose (Pharmacia) according to the manufacturer’s instructions. After the column was washed with the equilibration buffer, the absorbed protein was eluted with 0.5 M NaCl, pH 2.0, dialyzed against deionized water, and lyophilized.

Gel Filtration

Molecular weights were estimated by gel filtration on a column of Sephacryl S-200 (Pharmacia; 60 x 2.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. Calibration standards (Pharmacia) were BSA (mol wt 67,000), chymotrypsin (mol wt 25,000), and ribonuclease A (mol wt 13,700).
SDS-PAGE

SDS-PAGE was conducted according to the method of Laemmli (11) with a 5% stacking gel and a 15% resolving gel, and the gel was stained with silver according to Oakley et al. (15).

Protein Determination

Protein contents were determined by the method of Lowry et al. (13) after precipitation with cold TCA. In samples with low protein contents (DEAE-Trisacryl fractions and further purification steps), the protein content was determined by the Bio-Rad protein assay according to Bradford (2). Bovine serum albumin was used as a standard in both assays.

RESULTS

Time Course of the Effects of Phytophthora Elicitor on Tobacco Cells.

Seven-day-old tobacco cells were used for incubations because it was previously found that the responsiveness of the culture to elicitor treatment (as estimated by ethylene production) was maximal after 6 and 7 d. The cells were washed with fresh medium before incubation in order to eliminate eventual endogenous elicitors as well as cell debris and to standardize nutritional conditions during the incubation period.

Tobacco cells which were incubated with elicitor (30 μg/mL) for more than 10 h showed a slight browning compared to the light green control cells. Additionally, the fresh weight of elicited cells was always lower than that of control cells (60–80% of control), and the protein content of their extracts was also lower (33–56% of control).

Control or elicitor-treated cells were assayed for ethylene production and PI activity after various incubation times. Both parameters remained low in control cells throughout the experiment (Fig. 1). The stimulation of ethylene production was detected 1 to 2 h after addition of elicitor and lasted for several hours. This is one of the earliest events occurring upon elicitation, as already reported elsewhere (4, 16, 21, 22). Elicitor treatment leads also to an increase in PI activity, which was detectable after 12 to 15 h and reached a maximum level after 30 h (Fig. 1). The PI activity was stable at 65°C for 10 min, which eliminated some proteins and resulted in a partial (about twofold) purification of PI activity.

Dose Response of PI Accumulation and Ethylene Production

The increase in ethylene production and PI activity was measured after incubation with elicitor at various concentrations for 6 h and 38 h, respectively. Saturation curves were obtained for both responses (Fig. 2). In the case of ethylene, saturation was achieved at about 60 μg elicitor/mL, whereas maximal stimulation of PI activity occurred at 30 μg/mL. Elicitor treatment induced approximately a sixfold increase of the two parameters over the values found in control cells.

In the following experiments we centered on the induction of PI. Therefore, the tobacco cells were always treated with elicitor at a concentration of 30 μg/mL and harvested after 38 h of incubation.

Requirement for Protein Synthesis

The sensitivity of the induction of PI activity to actinomycin D and cycloheximide was investigated. Incubation with 10−3 M cycloheximide almost completely suppressed induction of PI activity by elicitor treatment (Table I). In the case of actinomycin D, the incubations were carried out in the dark because of the light sensitivity of this antibiotic. As shown in Table I, incubation in the dark did not modify
Table I. Effect of Protein Synthesis Inhibitors on PI Production

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>PI activity (%) of control</th>
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<tbody>
<tr>
<td>Light</td>
<td>Cycloheximide 10^-5 M</td>
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<tr>
<td>Light</td>
<td>Elicitor 30 µg/mL</td>
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<tr>
<td>Light</td>
<td>Cycloheximide 10^-5 M +</td>
</tr>
<tr>
<td></td>
<td>elicitor 30 µg/mL</td>
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<tr>
<td>Darkness</td>
<td>Actinomycin D 10^-4 M</td>
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<tr>
<td>Darkness</td>
<td>Elicitor 30 µg/mL</td>
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<tr>
<td>Darkness</td>
<td>Actinomycin D 10^-4 M +</td>
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<td>elicitor 30 µg/mL</td>
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* Control values were 2.8 µg trypsin inhibited/mg for light and 4.2 µg trypsin inhibited/mg for dark conditions.

The PI activity from elicitor-treated cells was assayed against trypsin and chymotrypsin with the respective substrates, TAME and BTEE, because reaction of chymotrypsin with Azocoll was poor under our standard incubation conditions. No pronounced specificity was found in the heat-cleared extracts, although trypsin was a slightly better substrate (Fig. 3). The specific inhibitory activity was 20.2 µg trypsin inhibited per mg protein and 11.3 µg chymotrypsin inhibited per mg protein.

Purification of PI

In order to better characterize the PI activity of elicitor-treated tobacco cells, we followed a purification procedure described by Carlberg et al. (3). In the first step, heating of the extracts at 65°C for 10 min eliminated about half of the protein and led to only a 15% loss of total activity. Yields in protein and activity were approximately the same after the second step, precipitation with (NH₄)₂SO₄ at 80% saturation. During the third step, chromatography on DEAE-Trisacryl M, we separated PI activity into one fraction which was not retained on the column and another one, which eluted broadly between 0.1 and 0.2 M NaCl (Fig. 4). Each fraction contained about half of the total activity eluting from the column, but about 40% of the activity loaded onto the column was lost in this step.

We continued the purification procedure with the PI activity retained on DEAE-Trisacryl and eluted between 0.1 and 0.2 M NaCl. The corresponding fractions were applied to a trypsin-Sepharose column equilibrated with 0.1 M Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl. Elution with 0.5 M NaCl at pH 2.0 resulted in a sharp protein peak which contained 24% of the total activity of the sample applied to...
the affinity column; harsher elution conditions such as 7 M urea at pH 3.0 or 0.05% Triton X-100 in 0.5 M NaCl at pH 2.0 did not improve this value.

Characterization of the Purified PI

The PI obtained from trypsin-Sepharose was apparently homogeneous as can be seen on SDS-PAGE (Fig. 5), which showed only a single band migrating below the 14,400 mol wt marker. Its mol wt, as estimated by gel filtration on a Sephacryl S-200 column, was about 10,500. When assayed for its specificity with the esters TAME and BTEE, it proved to be specific for trypsin; chymotrypsin was not inhibited (data not shown).

The specific activity, determined with Azocoll, was 1.8 μg trypsin inhibited by 1 μg of protein (means of four preparation), which suggests a 1:1 molecular interaction of the enzyme and the inhibitor, according to their respective mol wt (24,000 for trypsin and 10,500 for the purified inhibitor).

**DISCUSSION**

In a previous work, Walker-Simmons and Ryan (24) reported on the induction of proteinase inhibitor I in a tomato suspension culture by fungal and plant cell wall derived fragments. In this paper, we demonstrate that tobacco cells develop a high PI activity when incubated in the presence of glycopeptide elicitors of *P. parasitica* var. *nicotianae*, a true pathogen of tobacco plants. As an early reaction, a pronounced elicitation of ethylene is also observed, as already reported in other systems (4, 16, 22).

From elicitor-treated tobacco cells, we have purified to homogeneity an inhibitor which has the same characteristics reported for other PIs (heat stability and low mol wt) and which is highly specific for trypsin. As such, it resembles the PI isolated from the culture medium of embryogenic carrot cells (3); however, we could not detect a significant PI activity in the medium of elicited 7-d-old tobacco cells under normal incubation conditions. The possibility that some elicitor-induced PI could be bound to the cell wall was not investigated.

Induction of PI by *Phytophthora* elicitor was not affected by transferring the tobacco cells from light to darkness. Tobacco plants, callus and cell suspension cultures were previously reported to accumulate PI I under the effect of environmental factors such as light and aging (10, 24, 26). In our work we have characterized a PI which is induced by a different stimulus (treatment with elicitors) and seems to differ from the PI I in that it has a lower mol wt and is not oligomeric. The PI activity induced in tobacco cells by elicitor treatment is obviously composed of several inhibitors. Upon ion exchange chromatography we separated the activity into two main fractions. These two fractions showed about the same nonspecificity against trypsin and chymotrypsin as the crude extract (data not shown). However, chromatography of one of the fractions on a trypsin-Sepharose column resulted in a pure PI which was highly specific for trypsin but represented only 24% of the activity loaded onto the column. An important fraction of protein and of activity was not eluted from the trypsin-Sepharose column and corresponded to the nonspecific PI in our extract before this purification step. Therefore, we assume that at least three PIs occur in elicited tobacco cells, which differ in specificity and isoelectric points. It remains to be shown if they are all equally regulated and induced by elicitor treatment. One of the most extensively studied systems of PI induction is that of tomato plants which are wounded or treated with PHI. In this system, Ryan and co-workers (12) have isolated and characterized the genes for PI I and II and demonstrated that the transcription of the PI I gene was induced by wounding. Besides proteinase inhibitors, several elicitor-induced defense reactions are now well characterized at the molecular level. In contrast, almost nothing is known concerning the cellular events leading from elicitor perception to gene activation. The elicitor preparation of *Phytophthora* used in this work elicits, in addition to PI, an early production of ethylene, a high lipoxygenase activity (5), and a slight browning of the cells. The same responses are observed in tobacco plants infected with *P. parasitica* var. *nicotianae* (data not shown). Thus, tobacco cell suspension culture is a model system to study the effects of elicitors on infection-related events and their regulation.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**


