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Purification and Properties of an Elicitor of Castor Bean Phytoalexin from Culture Filtrates of the Fungus *Rhizopus stolonifer*[1]

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ABSTRACT

Evidence has been obtained for the presence in filtrates of 3-day-old cultures of the fungus *Rhizopus stolonifer* grown on potato-dextrose medium of both high molecular weight and low molecular weight elicitors of the production of the phytoalexin casbene in cell-free extracts of castor bean (*Ricinus communis* L.) seedlings. The high molecular weight elicitor activity was purified by means of gel filtration chromatography. Both protein and carbohydrate are associated with the most purified fraction containing elicitor activity. The elicitor is inactivated by treatments at 60 C or higher temperatures for 15 minutes. The molecular weight of the purified elicitor was estimated from gel filtration chromatography in 10 mM Na-phosphate (pH 7) to be 30,000 ± 5,000. Treatments of the purified elicitor fraction with either sodium periodate or the nonspecific protease preparation, pronase, substantially reduced its activity as an elicitor of casbene production. On the basis of these properties it is concluded that the elicitor is most likely a protein and may be a glycoprotein. It is estimated that 2×10^{-8} M elicitor gives about a 14-fold increase in casbene synthetase activity in extracts of treated split seedlings in comparison with controls. This corresponds to about 50% of the maximal activity obtainable in this assay system developed to measure elicitor activity.

The phytoalexin concept (6, 19) was developed to explain some features of the general resistance of plants to invasion by potentially pathogenic fungi. The generic term phytoalexin has been used in this context to refer to substances whose production in the plant is greatly stimulated by exposure of the plant to a potential pathogen and which possess antifungal activity against a broad range of fungi. Substances with these general properties which are thought to serve as phytoalexins have been identified in a wide variety of higher plants (11). The substances proposed as phytoalexins to date are low mol wt compounds, principally from among the members of the isoflavanoid and isoprenoid families of plant metabolites. Active research on the identification of new phytoalexins and the role of phytoalexins in disease resistance continues in many laboratories. Several reviews in recent years have summarized developments in this field (11, 14, 27).

The macrocyclic diterpene hydrocarbon, casbene, is synthesized from [^{14}C]mevalonic acid along with several other cyclic diterpene hydrocarbons in cell-free extracts of young castor bean (*Ricinus communis* L.) seedlings (20, 21). Subsequent to this discovery it was shown by Sitton and West (24) that the initial rates of casbene synthesis are much higher in extracts from seedlings which have been exposed to any one of several fungal pathogens of plants than in extracts prepared from comparable seedlings maintained in sterile conditions. Among the fungi which stimulated casbene biosynthesis were *Rhizopus stolonifer* and *Aspergillus niger*. It was also demonstrated that casbene in concentrations as low as 10 μg/ml inhibits the radial growth of *A. niger* on agar and possesses other antibiotic properties as well. For these reasons it was proposed that casbene is serving as a phytoalexin in the castor bean seedlings. This is the first instance of a diterpene suggested as a phytoalexin and also the first case of a hydrocarbon proposed for this role.

The molecular basis for regulation of phytoalexin production in the plant in response to exposure to fungi is not well understood. A number of reports have indicated the presence in filtrates from fungal cultures of substances which are capable of stimulating phytoalexin production when applied to a responsive plant. Such substances have been called "inducers" or "elicitors." The latter term, introduced by Keen *et al.* (12), will be utilized in this paper since it has no connotations about the mode of action. In a few instances, elicitors have been purified from fungal culture filtrates or mycelial extracts to an extent which as permitted an evaluation of their chemical nature (1-5, 7, 18). This paper reports the purification from culture filtrates of *R. stolonifer* of a material capable of eliciting casbene biosynthesis in castor bean seedlings. The properties of this elicitor differ in some respects from those of other reported elicitors.

EXPERIMENTAL PROCEDURE

Cultures of *Rhizopus stolonifer*. The *R. stolonifer* strain employed was originally isolated by D. Sitton (24) from castor bean seedlings as a spontaneous contaminant. It was maintained on potato-dextrose agar (Difco) plates. Liquid cultures were started from asexual spore suspensions. A few sporangiospores collected from a plate were transferred to the center of a fresh, sterile potato-dextrose agar plate which was then incubated in the dark at 30 C until a ring of spores encircling the outer perimeter of the plate was evident (approximately 3 days). Sporangia were removed with tweezers, suspended in water, and filtered through glass wool to remove mycelial fragments.

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The concentration of spores was adjusted to about 3×10^6/ml by dilution. One ml of this suspension was added to 250 ml of sterile potato-dextrose medium (20 g dextrose and 4 g dehydrated potato extract [Difco]/l H_2O) contained in a 2.8-l Fernbach flask. The cultures were swirled initially to aerate the medium and then incubated in the dark at 30 C without shaking for 72 hr.

Preparation of Culture Filtrate from 3-Day-Old Cultures. After 72 hr of growth the liquid cultures were filtered successively through Whatman No. 1 qualitative filter paper, 1.2-μm (RA) and 0.45-μm (HA) Millipore filters and finally through a sterile 0.22-μm (GS) Millipore filter. These sterile filtrates, which could be stored at 4 C for several months without loss of activity, served as the source of elicitor.

ASSAY FOR CASBENE ELICITOR ACTIVITY

The assay described below measures the casbene elicitor activity of test solutions when administered to young germinated castor bean seeds. Split, germinated seeds are exposed to the test solution for a period of time and then the casbene synthetase activity is assayed in a cell-free extract prepared from those seeds. For more quantitative results the assays of a test solution were performed in duplicate. A positive control solution containing *R. stolonifer* spores and a negative control solution of either 10 mM Na-phosphate (pH 7) or water were administered to test plants along with a group of solutions to be tested for elicitor activity.

Germination of Castor Bean Seeds. Approximately 200 seeds (Hale variety obtained from the Baker Castor Oil Co.) were surface-sterilized by stirring in a 300-ml suspension of 3.3% $Ca(OCl)_2$ in H_2O for 8 hr. The seeds were freed of their coats and treated for 1 min with agitation with 300 ml of a 0.1% solution of Clorox in water and washed five times with 200 ml of sterile distilled H_2O. Ten seeds/dish were placed in sterilized glass 9-cm Petri dishes containing three layers of filter paper covered with a single layer of cheesecloth and 15 ml of distilled H_2O. The seeds were incubated at 30 C in the dark for 56 hr to germinate.

Treatment of Split Seedlings with Solutions to be Tested for Elicitor Activity. Germinated, healthy seedlings at a comparable stage of development after 56 hr were selected. The seeds were split under aseptic conditions with a scalpel in the plane of the cotyledons to form two hemispherical portions, one of which contained the radicle. Both halves were transferred with the split surface down onto three layers of filter paper and a layer of cheesecloth plus 10 ml of the solution to be tested contained in a sterile Petri dish. A total of 10 seedlings (20 halves) were used/treatment. The dish was then incubated in the dark at 30 C for 9 hr.

Preparation of Cell-free Extracts. Cell-free extracts were prepared according to previously published procedures (20, 24). The washed seedling halves fron one dish were mixed with one-third of their fresh wt of Polyclar AT (GAF) and 0.05 M tris-bicarbonate (pH 6.8) in the ratio of 2.5 ml of buffer/g fresh wt of seedlings. This suspension was ground at top speed in a ViRtis '23' homogenizer for 30 to 45 sec while being cooled in an ice bath. The homogenate was squeezed through four layers of cheesecloth and the resulting suspension was centrifuged at 37,000*g* for 15 min. The supernatant fraction, minus the floating lipid layer, was centrifuged at 150,000*g* for 1 hr. The supernatant fraction from the second centrifugation (S_{150}) was utilized in the assay for casbene synthetase activity.

Assay for Casbene Synthetase Activity. The assay mixture contained in a total volume of 1.5 ml: 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM K-phosphate (pH 6.8), 4 mM ATP, 9.2 μM RS-[2-^{14}C]mevalonic acid (N,N'-dibenzylethylenediamine salt from New England Nuclear; 8.05 mCi/mmol) and 1 ml of the S_{105} enzyme preparation. The tubes were incubated at 30 C for 30 min in a water bath without shaking. It was established with kinetic measurements while developing the assay that 30-min incubations gave a reasonable estimate of the initial rates of casbene synthesis even in extracts of fully stimulated seedlings. The reaction was stopped by the addition of 1 ml of acetone and the resulting suspension was centrifuged. The pellet was extracted once with 1 ml of acetone and twice with 1 ml of benzene. These extracts from the pellet were mixed with the supernatant fraction, and the upper benzene layer was removed. The lower water-phase was extracted twice with 1 ml of benzene. The combined benzene extracts were frozen and concentrated to about 0.1 ml under N_2. The concentrated extract was subjected to TLC on a 0.25-mm silica gel plate (Brinkmann) which had been impregnated in the upper region (from 3 cm above the origin to the front) with $AgNO_3$ from a solution of 4% (w/v) $AgNO_3$ in acetonitrile-ethanol (9:1)(v/v). The plate was developed with petroleum ether-benzene (7:3)(v/v) in a tank with an unsaturated atmosphere. Casbene was immobilized as a sharp peak at the $AgNO_3$ origin in this system (20). This region was scraped into 10 ml of scintillation fluid (0.4% Omnifluor [New England Nuclear]) in 95:5 (v/v) toluene-dioxane and the associated radioactivity was measured (84% efficiency) by means of liquid scintillation spectrometry. A unit of casbene synthetase activity is defined as the production of one cpm of radioactivity associated with casbene under these circumstances; 1,785 units correspond to the production of 1 pmol of casbene/min under these conditions.

One unit of elicitor is defined as that amount which produces 1 unit of casbene synthetase activity/mg of S_{150} protein (cpm/mg protein) in the above assay.

ANALYTICAL PROCEDURES

Protein was determined by the method of Lowry *et al.* (16) or by the Biuret method (15) with BSA as the reference protein for the preparation of calibration curves. Total amino acids were measured by hydrolysis of the sample in 6 N HCl in sealed ampules at 110 C for 21 hr followed by ninhydrin assay of the hydrolyzed sample (25). Total carbohydrate was measured by the anthrone procedure (26) on samples containing 20 to 200 μg of carbohydrate. Results are expressed as μg of glucose equivalents.

RESULTS

Evidence for the Presence of Elicitor Activity in Crude Culture Filtrate. Preliminary tests demonstrated that the application of crude culture filtrate from *R. stolonifer* (*Rs*)[3] to castor bean seedlings severely inhibited their growth. No elicitor activity was seen. It seemed likely that the factor in the filtrate which caused growth inhibition could also inhibit the ability of the seedlings to respond to elicitors which might be present in the filtrate. Dilutions of the culture filtrate were tested in the hope of finding a concentration which would minimize the effect of the growth inhibitor but permit elicitor activity to be expressed. A culture filtrate was prepared from 3-day-old cultures of *Rs* grown in potato-dextrose medium as described under "Experimental Procedure." Dilutions of this filtrate with sterile H_2O were tested for elicitor activity with the split seed assay. The results are summarized in Table I. Treatment of the seedlings with *Rs* spores suspended in half-strength potato-dextrose medium (a positive control) led to elicitation of casbene synthetase activity in a manner similar to that seen by Sitton and West (24) using a somewhat different assay system. Seedlings treated with water alone (a negative control) showed very little casbene synthetase activity. Significant elicitor activity was seen in the test of the 1:1 dilution of the culture filtrate. Not only were the seedling extracts stimulated to incorporate mevalonate

[3] Abbreviation: *Rs: Rhizopus stolonifer.*

TABLE I Elicitor activity in crude Rs culture filtrates

Treatment	Casbene synthetase[1] units	Appearance of Seedlings	
		Radicle	Necrosis at interface with test solution
H_2O control	197	normal	-
Spores[2]	34,200	normal	+
Filtrate (undiluted)[3]	272	arrested growth	-
Filtrate (diluted 1:1)	20,400	normal	+
Filtrate (diluted 1:9)	511	normal	-
Filtrate (diluted 1:99)	232	normal	-

[1] The split seed assay was employed as described in Experimental Procedure except that 12 hr exposure of the seedlings to test solutions was employed.

[2] 3×10^6 spores in half-strength potato-dextrose medium.

[3] The culture filtrate was prepared as described in Experimental Procedure and diluted with distilled water as indicated.

into casbene, but the seedlings also showed the characteristic necrosis at the interface with the test solution that is seen on exposure to live fungus. The higher dilutions of culture filtrate produced little or no effect on the seedlings. Treatment of seedlings with half-strength potato-dextrose medium gave no more response in the elicitor assay than treatment with distilled H_2O (not shown in Table I). These experiments demonstrated that *Rs* culture filtrates do contain elicitor activity, the presence of which is dependent on growth of the fungus in the medium. However, the presence of both elicitor activity and the growth inhibitor made it difficult to deal with the elicitor in a quantitative way in the crude culture filtrate.

Some general characteristics of the elicitor activity in the crude culture filtrate were determined. It was stable at 4 C indefinitely and at 25 C for several hr, but approximately half of the activity was lost if the filtrate were heated for 15 min at 60 C or autoclaved for 30 min at 123 C. The distribution of the elicitor activity after passage of the crude filtrate through a Sephadex G-25 column is illustrated in Figure 1. The largest portion was excluded from the column, and a small amount was present in the included fractions in what appeared to be two peaks. However, the pool of included fractions 49 through 59, which showed no elicitor activity when assayed directly, was active when dilutions of the pool were tested. It seems that the seedling growth inhibitor is included in the column along with a fraction with elicitor activity. These results indicate that there are at least two classes of elicitor molecules present in the crude culture filtrate, a high mol wt fraction and a low mol wt fraction. Further investigations were limited to the high mol wt fraction because of the complication of the growth inhibitor associated with the low mol wt fraction.

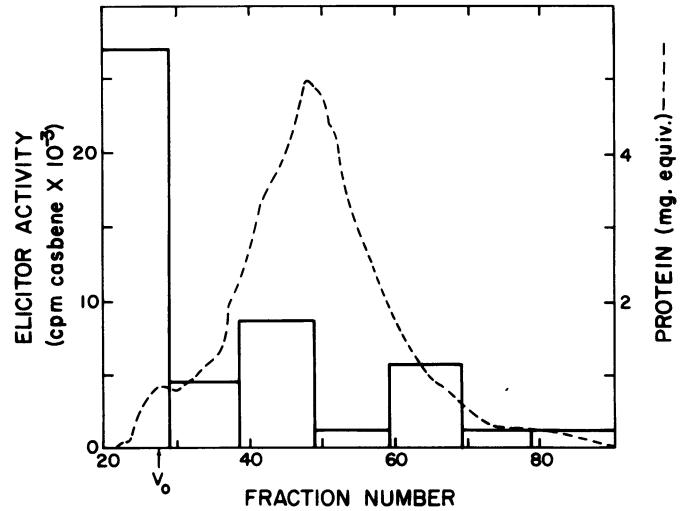

FIG. 1. Distribution of elicitor activity in fractions from a Sephadex G-25 gel filtration column. Two hundred ml of *Rs* culture filtrate were concentrated to 10 ml and applied to a column (3.6 × 31 cm) of Sephadex G-25 equilibrated with H_2O at 4 C. The column was eluted with water. Fractions of 5 ml were collected. Each fraction was assayed for Lowry-reactive material (- - -) which is expressed as mg of protein equivalents. Pools of 10 fractions were assayed for elicitor activity as described under "Experimental Procedure" (plotted as a histogram).

PURIFICATION OF ELICITOR ACTIVITY VOIDED FROM SEPHADEX G-25 GEL FILTRATION COLUMN

The split seed assay was examined with the elicitor fraction voided from the Sephadex G-25 column (G-25 void volume fraction) to determine whether it would be useful for following the course of purification of elicitor. The assay gave a linear increase in casbene synthetase activity with amount of elicitor fraction applied up to about 0.10 mg of protein in the elicitor fraction/ml of test solution. The assay also gave reasonable reproducibility when replicate samples were tested. The optimal time of exposure of seedlings to the G-25 void volume elicitor was evaluated (Fig. 2). A readily detectable increase in casbene synthetase activity could be measured after 4 hr of exposure, and the maximal stimulation was seen after 8 to 10 hr of exposure. A 9-hr exposure was utilized routinely in subsequent assays during the purification.

The elicitor activity in the G-25 void volume fraction was stable to 15 min of treatment at temperatures up to 40 C, but was totally inactivated by exposures for 15 min to temperatures of 60 C or higher. This suggested that the elicitor activity might be associated with a heat-labile protein. Attempts to develop ion exchange chromatographic procedures for the purification of the elicitor were unsuccessful. However, gel filtration molecular sieving columns produced more useful results. Elicitor activity was eluted at the void volume from Sephadex G-25, G-50, and G-75 columns which were equilibrated with distilled H_2O. The active material also eluted at the void volume from Sephadex G-25 and G-50 columns which were equilibrated with 10 mM Na-phosphate (pH 7) but it eluted after the void volume from columns of Sephadex G-75 or G-100 in the presence of this dilute buffer. Advantage was taken of this behavior in the development of the following purification scheme.

a. Sephadex G-25. Twenty-two and five-tenths liters of *Rs* culture filtrate were concentrated by lyophilization to about 100 ml of a viscous dark brown solution. A portion of this was applied to a column (6.4 × 19 cm) (600 ml bed volume) of Sephadex G-25 equilibrated with H_2O at 4 C. The column was

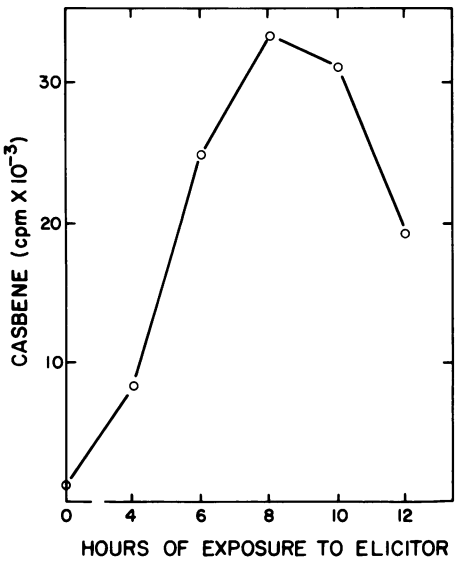

FIG. 2. Time course of elicitation of casbene synthetase activity by G-25 void volume elicitor. Ten ml of G-25 void volume elicitor were employed per treatment for the exposure period indicated in the split seed assay as described under "Experimental Procedure." Total time of growth of all seedlings (including time of exposure to elicitor) was 64 hr before they were assayed for casbene synthetase activity.

eluted with H_2O and fractions were collected and assayed for elicitor activity. The void volume fractions containing active material were concentrated to 8 to 9 ml by lyophilization. A total of 15 columns were run to process this amount of culture filtrate.

b. Sephadex G-75. The concentrated void volume fraction from each Sephadex G-25 column was applied to a column (2.6 × 59.5 cm) (530 ml bed volume) of Sephadex G-75 equilibrated with water at 4 C. Once again the elicitor activity was found associated with the void volume fractions while most of the anthrone-reactive carbohydrate material was distributed in the included fractions. The void volume fractions from three such Sephadex G-75 columns were pooled and lyophilized to dryness.

c. Sephadex G-100. The lyophilized residues from three G-75 columns were dissolved in 2.5 ml of 10 mM Na-phosphate (pH 7) and applied to a column (5 × 144 cm) (900 ml bed volume) of Sephadex G-100 which was equilibrated with the same buffer. The column was eluted with 10 mM Na-phosphate (pH 7) and 10-ml fractions were collected. Three peaks of 280 nm absorbance were detected in the fractions. One of these peaks corresponded to the void volume and the other two were centered on V_e/V_o = 2.1 and 3.5. The elicitor activity was coincident with the peak at $V_e{}^1P_o$. The fractions containing elicitor activity were pooled and lyophilized to dryness.

d. Sephadex G-100, Recycle. The pooled, lyophilized elicitor-containing fractions from five Sephadex G-100 columns run as described in step c were redissolved in 40 ml of 10 mM Na-phosphate (pH 7) and recycled on the same G-100 column eluted with the same buffer. The profiles of A_{280}, carbohydrate as measured by the anthrone assay, and elicitor activity in the fractions from this column are shown in Figure 3. The peak of elicitor activity was coincident with one of the four peaks of A_{280}(II). There was also anthrone-positive carbohydrate in the fractions containing elicitor, but the profiles of carbohydrate and elicitor activity did not coincide.

Table II summarizes the characteristics of elicitor fractions at various stages of this purification. The total yield of elicitor activity is high, presumably because of the presence of contaminants in the G-25 void volume fractions which interfere with estimates of the elicitor activity. Thus, the fold-purifications based on elicitor-specific activities in the G-25 void volume

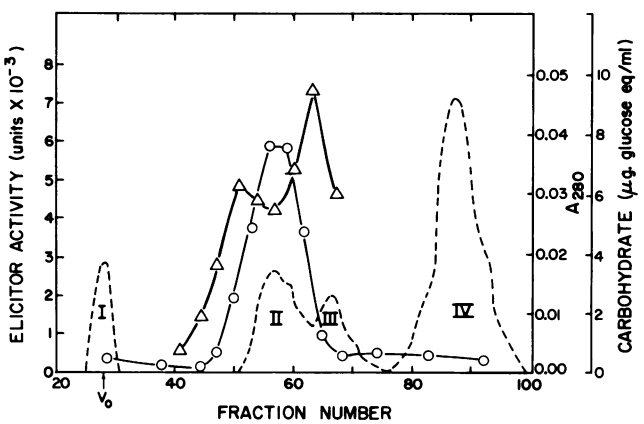

FIG. 3. Profiles of elicitor activity, A_{280}, and carbohydrate in fractions from the Sephadex G-100 gel filtration column in final elicitor purification step. Forty ml of pooled fractions containing elicitor from the initial G-100 columns (see text) were applied to a column (5 × 144 cm) (900 ml bed volume) of Sephadex G-100 and eluted with 10 mM Na-phosphate (pH 7) at 4 C. Fractions of 10 ml were collected. Each fraction was assayed for carbohydrate by the anthrone test (△——△) and A_{280} (- - -). Pooled fractions were tested for elicitor activity (○— —○) by split seed assay as described under "Experimental Procedure." V_0: void volume of column.

TABLE II Yields of Elicitor Activity, Protein and Carbohydrate during Purification

This represents the result of purification of elicitor from 22.5 liters of Rs culture filtrate by the scheme described in the text.

Purification Step	Total Elicitor Activity	Total Carbohydrate	Protein		Elicitor specific activity	
			Total[1]	TCA Precipitable[2]		
	Units x 10^{-6}	mg	mg		Units/mg carbohydrate	Units/mg protein[3]
G-25	1.99	30,500	1,520	23.3	6.52×10^1	8.54×10^4
G-75	15.6	681	53.3	8.1	2.29×10^4	1.94×10^6
G-100	8.72	12.2	13.5	-[4]	7.15×10^5	—
G-100 (recycled)	4.5	2.42	4.94	4.42	1.86×10^6	1.02×10^6

[1] Total Lowry-reactive material expressed as mg protein equivalents.
[2] Trichloroacetic acid-precipitable Lowry-reactive material expressed as mg protein.
[3] Units/mg of TCA-precipitable Lowry-reactive material.
[4] Not assayed.

fraction are not very meaningful. However, the purification scheme removed large amounts of carbohydrate and total Lowry-reactive material from the elicitor preparation. A much smaller proportion of the trichloroacetic acid-precipitable protein was removed. The nature of the nontrichloroacetic acid-precipitable, Lowry-reactive material in these fractions is not known. The most purified elicitor fraction from 22.5 liters of culture filtrate contained 2.42 mg of glucose equivalents of carbohydrate and 4.94 mg of protein associated with 4.5×10^6 units of elicitor activity. An assay for protein based on the ninhydrin colorimetric assay of amino acids released on acid hydrolysis indicated the presence of 4.42 mg of protein amino acids in the purified elicitor.

The results shown in Figure 3 suggest that some, and perhaps all, of the carbohydrate is present in the purified elicitor as a contaminant. Analytical polyacrylamide gels were run with samples of 42 μg of purified elicitor essentially as described by Davis (9) with half-strength tris-glycine (pH 8.3) as the running buffer. These showed the presence of one major band and two minor bands when stained with Coomassie blue R. No elicitor activity could be detected in direct assays of slices from paired, unstained gels. Also, no staining bands were detected when identical gels were stained with the periodate-Schiff reagent for glycoproteins (17, 23). The sample applied to the gel contained about 14 μg of total carbohydrate. A sample of 100 μg of ovalbumin, which contains 3.5 μg of carbohydrate, does give a positive stain with the periodate-Schiff reagent under these conditions. Either one or both of the following may have contributed to the failure to obtain a reaction for glycoprotein with the purified elicitor: (*a*) the purified elicitor contained a large amount of contaminating carbohydrate which did not react; or (*b*) the elicitor does not give a substantial reaction with this reagent. We concluded from these observations that the purified elicitor is relatively pure with respect to protein, but still contains an unknown amount of contaminating carbohydrate. There is no evidence for more than one type of elicitor in the high mol wt fraction.

OTHER PROPERTIES OF THE PURIFIED ELICITOR FRACTION

The UV spectrum of the purified elicitor in H_2O showed only one peak of absorbance superimposed on a background absorbance which generally increased at wavelengths below 300 nm. Subtraction of the background absorbance showed an *A* maximum at 279 nm typical of a polypeptide. The mol wt of the purified elicitor was estimated to be 30,000 ± 5,000 daltons from its K_{av} on a Sephadex G-100 column (V_e/V_o = 1.95 – 2.02) equilibrated and run in dilute buffer (Fig. 3). The column was calibrated with BSA (68,000), ovalbumin (45,000), α-chymotrypsinogen A (25,700), and Cyt *c* (12,400).

The elicitor is stable at 4 C indefinitely when dissolved either in sterile distilled H_2O or 10 mM Na-phosphate (pH 7). It is also stable to freeze-drying. Figure 4 illustrates the elicitor activity remaining after 15-min exposures to temperatures ranging from 4 to 123 C. No significant activity remained at temperatures of 60 C or higher.

Dilutions of the most purified elicitor were made with 10 mM Na-phosphate (pH 7) and assayed for activity with the split seed assay. The seedlings show an approximately linear increase in casbene synthetase with increasing concentration of applied elicitor up to 1 μg of protein/ml (Fig. 5). Elicitor solutions containing as little as 0.15 μg of protein/ml give a significant response in the seedlings of about four times the control level. If a mol wt of 30,000 is assumed for the elicitor and all of the protein in the purified fraction is assumed to be elicitor, then the ED_{50} (effective dose for 50% of the maximal response in this assay) is about 20 nM.

The purified elicitor was subjected to a periodate oxidation in order to test whether the associated carbohydrate is essential for its activity. The experimental procedure is described in the legend to Figure 6 and the results are portrayed in the Figure. Essentially all of the activity is lost after periodate oxidation. This indicates that some portion of the carbohydrate present is essential for elicitor activity. Alternatively, N-terminal serine or threonine residues in a protein component could be oxidized by periodate. This might also account for the loss of elicitor activity, although this seems a less likely explanation to us.

In an analogous experiment to test for the essentiality of the protein component for activity, an aliquot of purified elicitor was treated with the nonspecific protease preparation, pronase (Sigma Chemical). The experimental details and the results are indicated in Figure 7. Pronase treatment under these conditions

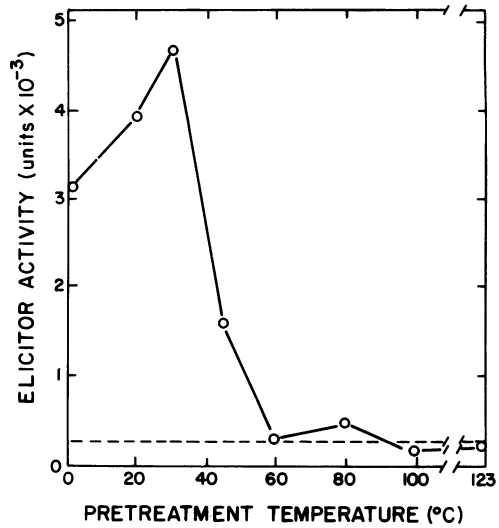

FIG. 4. Effect of pretreatment of various temperatures on elicitor activity. Aliquots of recycled elicitor fraction from the G-100 column (0.2 ml) were diluted with 9.8 ml of 10 mM K-phosphate (pH 7) and exposed to the indicated temperature for 15 min. The solutions were then cooled and assayed for elicitor activity by split seed assay as described under "Experimental Procedure." The control with phosphate buffer in the assay gave a value of 250 cpm in casbene/mg of S_{150} protein.

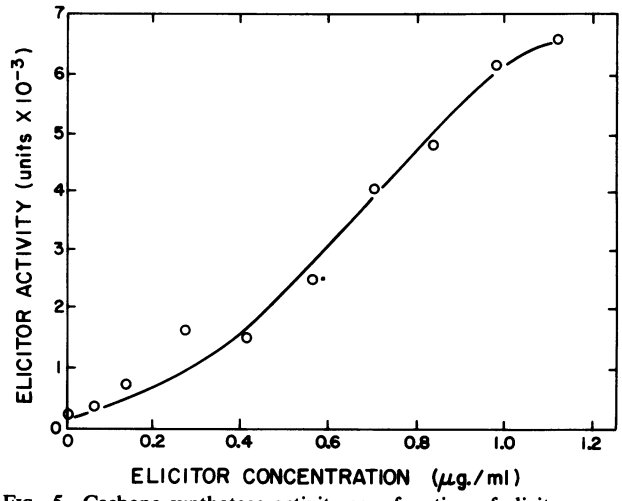

FIG. 5. Casbene synthetase activity as a function of elicitor concentration. Dilutions of the purified *Rs* elicitor (G-100) were made with 10 mM Na-phosphate (pH 7) to give the indicated concentrations. Each level was assayed for elicitor activity with the split seed assay as described under "Experimental Procedure." Maximum response possible in this assay is between 6,000 and 7,000 units of elicitor activity. Values for amount of elicitor are final concentrations applied to seedlings.

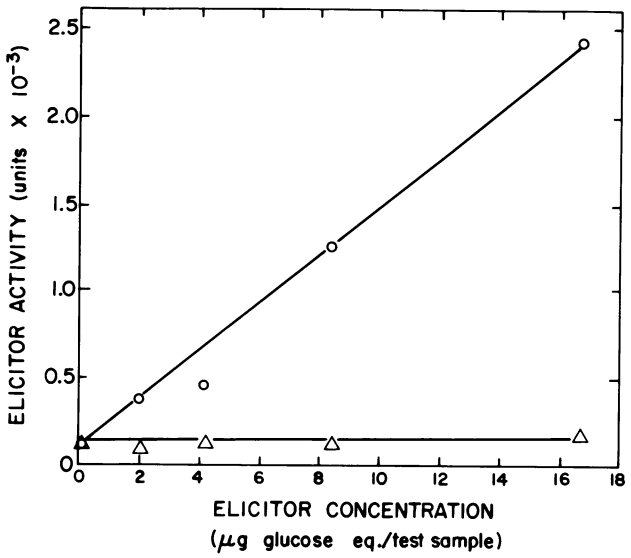

FIG. 6. Effect of pretreatment of purified elicitor with periodate on elicitor activity. Purified elicitor (G-100) (14 μg total weight) was treated with 0.2 ml of 0.07 M $NaIO_4$ at room temperature for 9 hr. Ethylene glycol (2.0 μl) was added to stop the reaction by removing unreacted periodate. Dilutions of the sample were made with 0.01 M $NaPO_4$ (pH 7) and each diluted sample was assayed for elicitor activity by split seed assay as described under "Experimental Procedure" (△——△). Elicitor concentrations are given in terms of amounts of elicitor fraction-associated carbohydrate present in diluted solutions used to treat split seedlings. A control was run as above except for the presence of ethylene glycol before the initial addition of the $NaIO_4$ solution (○——○).

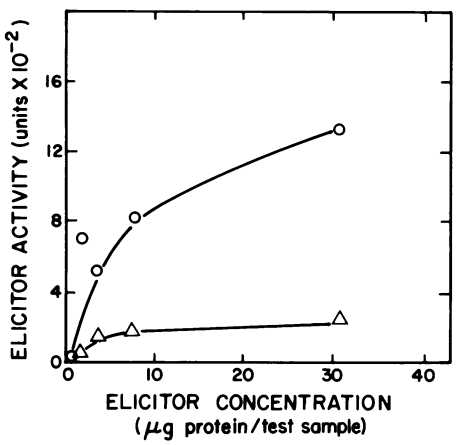

FIG. 7. Effect of pretreatment of purified elicitor with pronase on elicitor activity. Four-ml aliquots of purified elicitor (72 μg of protein) were lyophilized to dryness and redissolved in 0.4 ml of 1.0 mg/ml pronase dissolved in 10 mM Na-phosphate (pH 7). The tubes were sealed with Parafilm and incubated at 30 C in a water bath for 10 hr. Dilutions were made with 10 mM Na-phosphate (pH 7) and assayed for elicitor activity by split seed assay as described under "Experimental Procedure" (△——△). Elicitor concentrations are given in terms of amounts of elicitor fraction-associated protein present in the diluted solutions to treat split seedlings. Controls were run as above except that no pronase was added (○——○). Preliminary experiments indicated that pronase alone at these levels did not affect the development of treated seedlings.

reduced the elicitor activity to about 20 to 30% of the control value. Preliminary experiments showed that treatment of seedlings with pronase alone had no observable effect on their development.

DISCUSSION

The results of these investigations support the idea that *R. stolonifer* produces a relatively high mol wt elicitor of casbene production which is released into the surrounding medium. It seems reasonable to assume that this elicitor plays a part in the biochemical reaction of castor bean tissues to the presence of this fungus. It is estimated from the apparent size of the elicitor and the quantitative aspects of its activity in the assay system that the castor bean seedlings will respond to 5 nM or less of the elicitor. The seedling response to the elicitor resembles closely its response to exposure to live fungus. Dark gray-green necrotic regions appear on the surface of the seedlings when contact is made either with the elicitor or the fungus. Correlated with the necrosis is the stimulation of the production of the phytoalexin, casbene, in cell-free extracts of seedlings exposed to either agent. This is similar to the observations of Anderson-Prouty and Albersheim (1) who correlated browning of bean tissues exposed to either the fungus *Colletotrichum lindemuthianum* or elicitors derived from it with the accumulation of the phytoalexin, phaseollin.

The nature of the low mol wt elicitor which was included in the Sephadex G-25 column has not been investigated. Therefore, its relationship to the high mol wt elicitor remains unknown. Kim and Uritani (13) have described some properties of a water-soluble, dialyzable and heat-stable low mol wt fraction obtained from extracts of mycelia and conidia of two strains of *Ceratocystis fimbriata* which was capable of eliciting terpene phytoalexins of the ipomeamarone group in sweet potato root tissue. This same fraction also caused cellular injury and ethylene production in a manner similar to that seen in the hypersensitive response of the plant tissue to the fungus. The active molecules in this fraction appeared to be neutral substances, but they have not been further characterized. These observations show that low mol wt as well as high mol wt substances with elicitor activity can be obtained from fungal pathogens.

No elicitor activity was detected in 3-day-old filtrates of *R. stolonifer* cultures which had been grown in a fully defined glucose-minerals medium even though the fungus had grown as well on a fresh wt basis and produced seedling growth inhibitors in the medium. Dilutions of these culture filtrates also failed to reveal elicitor activity. The presence of a different balance of the seedling growth inhibitors and elicitor might be responsible for this failure to detect elicitor activity. Alternatively, the fungus may require exposure or access to some substance(s) in the potato extract utilized in the standard growth medium before it develops the capacity to produce phytoalexin elicitors. Frank and Paxton (10) have noted the need for exposure to soybean extract if *Phytophthora megasperma* var. *sojae* is to produce "inducers" for the synthesis of a fluorescent metabolite by soybean seedlings.

The experiments reported here were all conducted with elicitor obtained from culture filtrates. Some efforts were made with little success to extract elicitor activity from *R. stolonifer* mycelia by various procedures. All of the mycelial extracts contained growth inhibitors which interfered with the elicitor assay. One type of extract obtained by the passage of frozen mycelia through a Sagers' press (22) had a low level of elicitor activity; however, this was not pursued further. It is possible that the failure to detect substantial activity in the mycelia was a consequence of either inadequate extraction techniques or problems with the presence of interfering materials in the assay solutions or both of these factors.

The properties of the *Rs* elicitor indicate that the active molecule may be a glycoprotein. A number of different analytical criteria indicate that the most purified fraction contained both carbohydrate and protein. The heat inactivation profile of the elicitor activity is similar to that expected for denaturation of a protein; and treatment of the most purified elicitor with a

TABLE III Characteristics of Purified Elicitors

Source of elicitor	Phytoalexin assay system	Chemical nature of elicitor	Molecular weight estimate	Heat stability	References
Phytophthora megasperma var sojae (Pms elicitor) Culture filtrate and cell wall	Glyceollin in Glycine max	Branched β-glucan with predominantly 3- and 3,6-linked glucosyl residues	Heterogeneous-macromolecules	Stable at 120 C	Ayers, et al. (2,3,4)
Colletotrichum lindemuthianum Culture filtrate and cell wall	Phaseollin in Phaseolus vulgaris	Glucan with predominantly 3- and 4-linked glucosyl residues	$1 - 5 \times 10^6$	Stable at 120 C	Anderson-Prouty and Albersheim (1)
Phytophthora infestans Cell wall and mycelia	Rishitin in Solanum tuberosum	Glucan	—	Stable at 120 C	Melitskii, et al. (18) Chalova, et al. (5)
Monolinia fructicola (monilicolin A) mycelia	Phaseollin in Phaseolus vulgaris	Polypeptide	~ 8,000	Partially inactivated at 99 C	Cruickshank and Perrin (7)
Rhizopus stolonifer (Rs elicitor) Culture filtrate	Casbene in Ricinus communis	Glycoprotein (?)	$30 \pm 5 \times 10^3$	Inactivated at 60 C	This paper

nonspecific protease preparation (pronase) greatly reduced the elicitor activity. The latter observation must be regarded with some reservation since the commercial preparation of pronase used was not further purified and conceivably might have contained some other types of hydrolases. The major piece of evidence supporting the involvement of carbohydrate in the active molecules is the fact that periodate treatment completely inactivates the elicitor. These observations are most easily explained if the elicitor is a glycoprotein in which both moieties are essential for activity. However, further purification of *Rs* elicitor and chemical characterization will be required to confirm this proposal.

Several observations made in the course of these investigations can be explained by assuming (*a*) that the elicitor molecules form aggregates with a lower specific activity when the elicitor is present in distilled H_2O or in the presence of large amounts of contaminating carbohydrate material; and (*b*) that the presence of low concentrations of salts in the elicitor solutions favors disaggregation and a relatively higher specific elicitor activity. This would explain why the elicitor is voided from a Sephadex G-75 column equilibrated and eluted with H_2O, but is included when the column is equilibrated and eluted with dilute phosphate buffer. It also would explain why the total elicitor activity obtained from the Sephadex G-75 column in the large scale purification is much higher than that in the G-25 void volume fractions applied to the column since much of the contaminating carbohydrate is removed from the elicitor during this step. Although it has not been tested systematically, it appears that the elicitor-containing fractions from the G-75 and G-100 columns are less active when tested in the split seedling assay in distilled H_2O solution rather than the usual dilute phosphate buffer.

Table III summarizes the characteristics of high mol wt elicitors reported in the literature which have been purified sufficiently to permit at least tentative conclusions about the chemical nature of the elicitor molecules. The most definitive work has been done by Albersheim and his associates (2–4) with the elicitors derived from the cell walls and culture filtrates of several races of *P. megasperma* var. sojae, a pathogen of soybeans. Several fractions with elicitor activity were purified from the cell wall. Structural and biological studies with these fractions led to the important conclusion that the activity is associated with a highly branched portion of a β-1,3-glucan with 3,6-linked branch points. The filtrate elicitor is closely related to the wall elicitor and probably derived from it during growth. Protein is associated with some of the active wall fractions, but is believed not to be essential for their activity. Several lines of evidence support the conclusion that the elicitor activity is due to a discrete structural arrangement within the β-1,3-glucan. An elicitor has also been obtained in Albersheim's laboratory (1) from the cell walls and culture filtrates of *C. lindemuthianum*, a pathogen of beans. The activity again is associated with a structural glucan of the cell wall, although this glucan contains predominantly 3-linked and 4-linked glucosyl residues. Recent reports from a group of Russian investigators (5, 18) indicate that a similar cell wall glucan elicitor can be obtained from the potato pathogen *Phytophthora infestans*. All of these elicitors are quite heat-stable and survive prolonged heating at neutral pH and autoclave temperatures. Each acts as the elicitor of a different phytoalexin. Thus, components associated with cell wall glucans constitute an important class of phytoalexin elicitors.

The *Rs* elicitor of casbene production differs strikingly from the above group in its heat lability and seeming requirement for a native protein structure as well as a carbohydrate component for its activity. Elicitors from some other sources also have a dependence on a polypeptide or protein component for activity. Monilicolin A, which was purified by Cruickshank and Perrin (7) from the mycelia of *Monolinia fructicola*, is a polypeptide. Unlike the *Rs* elicitor, there was no evidence for the presence of carbohydrate in the active material, and the activity was relatively stable to heat. A recent report by Daniels and Hadwiger (8) summarizes their work with fractions from the culture filtrates of two strains of *Fusarium solani* which act as elicitors of pisatin production in pea plants. The fact that activity was partially lost after either digestion with pronase or heat treatment led them to suggest that at least part of the activity might be associated with protein. The purification was not sufficient to permit very definitive statements about the chemical nature of the elicitor molecules present. It would be premature on the basis of such limited information to suggest that there is a class of elicitors dependent on protein for activity which differs in a fundamental way from the glucan wall elicitors. Because of the diversity seen in the characteristics of the few elicitors studied to date, one should be cautious in proposing a general model to explain the basis for fungal stimulation of phytoalexin production.

Clearly, many questions about the functions of elicitors have been left unanswered by the work done to date. For example, not very much is known about the specificity of elicitor action.

Monilicolin A, which was purified on the basis of its ability to stimulate phaseollin production in beans, did not function to stimulate the production of either pisatin in peas or viciatin in the broad bean (7). In the case of the other elicitors, the response of a range of test plants has not been reported. Ayers *et al.* (2, 4) have shown that the elicitors derived from three different races of *P. megasperma* var. *sojae* which differ in their abilities to infect soybean cultivars have the same activity in eliciting glyceollin in all of these cultivars. They conclude that these elicitors have a role in general resistance but cannot account for race-specific resistance. These and other observations have led to the suggestion that fungal factors not yet identified must be of importance in stimulating race-specific resistance in plants.

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