Plant Physiol. (1981) 67, 633-639
0032-0889/81/67/0633/07/$00.50/0

Polygalacturonase from *Rhizopus stolonifer*, an Elicitor of Casbene Synthetase Activity in Castor Bean (*Ricinus communis* L.) Seedlings[1]

Received for publication July 21, 1980 and in revised form October 20, 1980

SUNG-CHUL LEE[2] AND CHARLES A. WEST
Division of Biochemistry, Department of Chemistry, University of California, Los Angeles, California 90024

ABSTRACT

Apparently homogeneous polygalacturonase-elicitor purified from the filtrates of *Rhizopus stolonifer* cultures stimulates germinating castor bean seedlings to produce greatly increased levels of casbene synthetase activity. The purification procedure involved gel-filtration chromatography on Sephadex G-25 and G-75 columns followed by cation-exchange chromatography on a Sephadex CM C-50 column. Homogeneity of the purified preparation was indicated by the results of cationic polyacrylamide disc gel electrophoresis and isoelectric focusing (pI = 8.0). The identity of the casbene elicitor activity and polygalacturonase were indicated by the coincidence of the two activities at all stages of purification, the coincidence of both activities with the single protein-staining band detected on a cationic polyacrylamide disc gel and an isoelectric focusing gel, and the identical behavior of both activities on an agarose gel affinity column. The purified polygalacturonase-elicitor is a glycoprotein with approximately 20% carbohydrate content and an estimated molecular weight of 32,000 by polyacrylamide disc gel electrophoresis.

The accumulation of low-molecular weight, antimicrobial compounds called phytoalexins in an area of localized necrosis during the hypersensitive response of plants to invasion by potentially pathogenic microorganisms is a subject of considerable current interest (6, 10). The antibiotic properties of phytyoalexins against a wide range of microorganisms (10), their high levels in necrotic areas in the presence of potential pathogens in comparison with low or nondetectable levels in healthy tissue (10), and the general correlation of accumulated phytoalexin levels with the success or failure of compatible and incompatible strains of pathogenic fungi in some instances (10, 31) have provided evidence for the involvement of phytoalexins in plant disease resistance. More than 64 different substances from 75 species representing 20 families have been proposed to serve as phytoalexins (10). It is too early to say if their production is a general feature of all plants because most of the reported phytoalexins are either isoflavonoid or sesquiterpenoid or biogenetically related compounds from the Leguminosae or the Solanaceae families (10).

The accumulation of phytoalexins in response to infection implies the presence of substances of microbial origin that are recognized by plants to trigger the response. Such substances have been called elicitors. In a few cases, elicitors of phytoalexins in plants have been purified from fungal culture filtrates or mycelial extracts to an extent which has permitted an evaluation of their chemical nature (3, 10).

Work in this laboratory showed that a cell-free system from castor bean seedlings catalyzes the conversion of GGPP[3] to several diterpenes (21). One of these diterpenes, casbene, was shown to have antibiotic properties against *Escherichia coli* and *Aspergillus niger* (23). Also, the rate of synthesis of casbene from mevalonate was seen to be up to 100 times greater in cell-free extracts of young seedlings which have been exposed to the fungi *A. niger* or *Rhizopus stolonifer* in comparison with uncontaminated controls (23). These properties prompted the suggestion that casbene might serve the castor bean plant as a phytoalexin. Elicitors of the casbene synthetase activity were detected in filtrates from *R. stolonifer* cultures by means of a bioassay which measures casbene synthetase activity in extracts of treated castor bean seedlings (26). A high-molecular weight elicitor fraction partially purified by gel-filtration chromatography from this source was shown to possess the properties expected of a glycoprotein which required both protein and carbohydrate for its activity (26).

This paper reports a procedure for the purification of the high-molecular weight elicitor from *R. stolonifer* to homogeneity and presents evidence that the elicitor is a polygalacturonase.

MATERIALS AND METHODS

Chemicals. Thin-layer silica gel plates were purchased from E. Merck Laboratories. Sephadex sieving gels (G-10, G-25, G-75, and G-100) and ion-exchange (DEAE A-25 and CM C-50) resins were from Pharmacia Fine Chemicals. Bio-Gel P-2 resin, Bio-Lyte, dye reagent concentrate for Bradford protein assay, Coomassie brilliant blue R250, and Alcian blue were bought from Bio-Rad. Dehydrated potato extract was Difco brand. Polyclar AT was purchased from General Aniline and Film Corp. Millipore filters of RA (1.2 μm), HA (0.45 μm), and GS (0.22 μm) were purchased from Millipore Corp. The following materials were purchased from Sigma: polygalacturonic acid (grade III), polygalacturonic acid (sodium salt, grade II), D-galacturonic acid, blue dextran, *N,N,N′,N′*-tetramethylethylenediamine, acrylamide, *N,N′*-methylenebisacrylamide, BSA, and ovalbumin (chicken, grade V).

Biological Materials. Castor bean seeds were obtained from

[1] This work was supported by National Science Foundation Grants 77-14027 and 79-23142.

[2] Supported as a Cell and Molecular Biology Trainee by National Institutes of Health Training Grant GM-07185 and as a predoctoral trainee by United States Public Health Service Training Grant GM-00463. This work was presented in partial fulfillment of the requirements for the PhD degree from the University of California, Los Angeles, 1980. Present address, Department of Chemistry, University of California-San Diego, La Jolla, CA 92093

[3] Abbreviation: GGPP, all *trans*-geranylgeranyl pyrophosphate.

plants growing in the botanical gardens at the University of California, Los Angeles. *R. stolonifer* was a subculture derived from the original isolate obtained by D. Sitton (23) from spontaneously contaminated castor bean seeds and identified by N. T. Keen, University of California, Riverside.

Preparation of [2-^{14}C]GGPP. [2-^{14}C]GGPP was prepared by pyrophosphorylation of 2-^{14}C-labeled all *trans*-geranylgeranoil (a generous gift of Dr. R. M. Coates at the University of Illinois, Urbana) by procedures modified from those described by Upper and West (28).

Assay for Elicitor Activity. Procedures for assays of the elicitor activity were originally developed by Stekoll and West (26). The assay measures the casbene elicitor activity of test solutions when administered to young germinated castor bean seedlings. Split, germinated seeds were exposed to the test solution for a period of time and then the casbene synthetase activity was assayed in a cell-free extract prepared from these seeds. As a control, a solution of either sterile buffer or sterile distilled H_2O was used. In this work, some modifications were made in the methods for surface sterilization and germination of castor bean seeds, the preparation of enzyme extract, and the *in vitro* assay of casbene synthetase activity.

Castor Bean Germination. About 200 seeds were freed from their coats mechanically and the peeled seeds which were not damaged in the process were selected and surface-sterilized in 0.1% (w/v) NaOCl (5.7 ml commercial bleach in 300 ml sterile distilled H_2O) for 2 min, followed by nine rinses with a total of 3.5 liters sterile distilled H_2O. The seeds then were placed in a sterile germination dish which was prepared by autoclaving a 19-cm diameter crystallizing dish covered with aluminum foil and containing three layers of Whatman No. 1 filter paper covered with eight layers of cheesecloth plus 80 ml water. The seeds then were placed in the dark at 25 to 27 C to germinate.

Incubation of Split Seeds with Test Solutions. After 55 h germination, the castor bean seedlings were examined for growth and fungal contamination. Eighty seedlings with well-developed radicles and no sign of necrosis were selected, and each seedling was split in half through a plane parallel to the plane of the cotyledons, thus producing two hemispherical seedling-halves, one of which contained the radicle. Ten pairs of split halves were placed with their split sides down in a sterile Petri dish (9 cm diameter) which contained three layers of Whatman No. 1 filter paper covered with one layer of cheesecloth and 10.0 ml test solution to be assayed for elicitor activity. The Petri dishes then were incubated an additional 10 to 12 h in the dark at 25 to 27 C. After a total of 65 to 67 h germination, the seedlings were assayed for casbene synthetase.

Preparation of Enzyme Extract. Pools of the split halves of 10 germinating castor bean seedlings which had been exposed to a particular test solution were washed with distilled H_2O and then dried and weighed. Polyclar AT in an amount equal to one-third the weight of the seedlings was added. Usually 10 pairs of split halves weighed about 8 to 9 g. Cold homogenization buffer [50 mM Tris-HCl, 50 mM $NaHCO_3$ (pH 6.8), and freshly added 10 mM 2-mercaptoethanol) at the ratio of 2.5 ml/g fresh weight of seedlings was added to each batch of 10 half-seedling pairs. The resulting suspension was homogenized, while cooled on an ice bath, for 1 min in a VirTis "23" homogenizer at the top speed. The homogenate then was squeezed through cheesecloth and the resulting filtrate was centrifuged at 37,000*g* for 15 min at 4 C. Small portions of the supernatant (S_{37}) were carefully removed without the floating lipid layer for the incubations with [2-^{14}C]GGPP in the casbene synthetase activity assays.

Assay of Casbene Synthestase Activity. The incubations for assays of casbene synthetase activity were initiated by addition of [2-^{14}C]GGPP (6.6 μM final concentration; 17,800 dpm of 2.4 Ci/mol) to a mixture of various amounts of S_{37} enzyme extract, 100 mM Tris-HCl (pH 9.0), and 10 mM $MgCl_2$ in a final volume of 0.5 ml. The contents were mixed and the tubes were incubated for 30 min in a 30 C water bath. At the end of the incubation time, 2 ml 25% (v/v) ethanol in petroleum ether (30 to 60 C) was added, followed by vigorous mixing on a Vortex mixer. The organic layer was removed after centrifugation in a clinical centrifuge and the remaining aqueous layer was extracted three more times with petroleum ether (30 to 60 C). Residual water was removed from the combined organic layers by freezing. The remaining organic layer was concentrated to a small volume in a stream of N_2.

Precoated silica gel thin-layer plates (2 × 20 × 0.025 cm) were impregnated in the top 15 cm with 4% (w/v) $AgNO_3$ in acetonitrile: ethanol (9:1) (v/v). The concentrated organic extracts were applied at the origin which was 2 cm from the bottom of the plate and 3 cm below the $AgNO_3$ origin. The plates were developed with benzene:petroleum ether (3:7) (v/v) in a saturated tank. Casbene is immobilized as a sharp band at the $AgNO_3$ origin in this thin-layer system. The silica gel section of 1.0 cm width centered on the $AgNO_3$ origin was scraped into 10 ml scintillation fluid [0.4% Omnifluor in toluene:dioxane (95:5, v/v)] and the associated radioactivity was measured by means of liquid scintillation spectrometry. The counting rate was corrected for counting efficiency and background counting rate.

Definition of Elicitor Activity Units. Elicitor activities were expressed in terms of elicited casbene synthetase activity. One unit elicitor activity is defined as the amount that will elicit the production of 1 pmol casbene/min in 100 μl S_{37} enzyme extract under the assay conditions described above. The assays were reasonably linear up to 20 units elicitor activity.

Assay for Polygalacturonase Activity. Polygalacturonase activity was assayed from the rate of production of new reducing termini resulting from the hydrolysis of the substrate polygalacturonic acid at 30 C. Portions (0.9 ml) of 0.5% (w/v) polygalacturonic acid (grade III) in 100 mM acetate (pH 4.8), were added to various volumes of test solutions up to 0.1 ml. At the end of various periods of incubation time, catalysis was stopped by adding 1 ml alkaline copper reagent working solution and the amounts of reducing ends were measured by the procedures of Nelson and Somogyi (17, 24). The controls were prepared by adding the copper reagent working solution prior to substrate at the beginning of the incubation. Amounts of reducing groups released during hydrolysis were estimated from a standard curve prepared with D-galacturonic acid as the reference standard. It has been reported that oligomeric galacturonic acids up to pentamers give the same color yield on a molar basis as galacturonic acid in this assay (18).

One unit polygalacturonase activity is defined as that amount of enzyme which will yield 1 μmol reducing ends/min at 30 C under the above assay conditions. The assays gave a linear response up to about 2 unit-min with respect to incubation periods and amounts of enzyme.

Preparation of *R. stolonifer* Culture Filtrate. Spores of *R. stolonifer* were collected with flame-sterilized tweezers from cultures grown on a potato-dextrose agar plate (4 g potato extract, 20 g D-glucose, and 20 g agar in 1 liter) and resuspended in a few ml distilled H_2O at 3 × 10^6 spores/ml. About 3 × 10^6 spores were used to inoculate 250 ml sterile growth medium in a 2.8-liter Fernbach flask. Alternatively, in some cases, 1.8 × 10^6 spores in 0.6 ml were used to inoculate 150 ml sterile medium in a Roux bottle.

The earlier studies were performed with *R. stolonifer* grown in potato-dextrose medium (20 g D-glucose and 4 g potato extract in 1 liter water). Later, two better-defined synthetic media containing glucose and salts were utilized to grow *R. stolonifer*. The Czapek medium (11) contained, per liter, 4.0 g NH_4NO_3, 0.5 g KCl, 1.4 g KH_2PO_4, 0.23 g $MgSO_4$, 0.01 g $FeSO_4$, plus various amounts of D-glucose and polygalacturonic acid. A glucose-asparagine me-

dium modified from a sucrose-asparagine medium (9) consisted, per liter, of 2.0 g L-asparagine, 1.4 g KH_2PO_4, 1 mg $FeSO_4 \cdot 7H_2O$, 0.49 g $MgSO_4$, 1 mg thiamine-HCl, 4.4 mg $ZnSO_4 \cdot 7H_2O$, 0.079 mg $CuSO_4 \cdot 5H_2O$, 0.053 mg $Na_2MoO_4 \cdot 2H_2O$, 0.086 mg $MnCl_2 \cdot 4H_2O$, plus various amounts of D-glucose and polygalacturonic acid. After investigation of both of these media, the glucose-asparagine medium was chosen to grow *R. stolonifer* for the large-scale purification of polygalacturonase.

The fungus was grown at 30 C either 3 days in potato-dextrose medium or for various periods in the cases of the other media. At the end of the incubation period, the spent medium was filtered in successive stages through Whatman No. 1 filter paper, Millipore filters of RA (1.2 μm) and HA (0.45 μm), and finally through a sterile GS (0.22 μm) filter. The resulting filtrate was quickly frozen on Dry Ice-acetone mixture and lyophilized to dryness.

Analytical Procedures. Protein was determined by the method of Bradford (4) or by the method of Lowry *et al.* (14) with ovalbumin as a standard. In column chromatography, *A* at 280 nm were routinely measured with a UV monitor coupled to a fraction collector to obtain an estimate of protein concentrations. Total carbohydrate content was measured by the anthrone assay (25). Standard curves in the anthrone assay constructed with D-glucose and D-mannose as reference standards were found to differ significantly from each other. One hundred μg D-glucose resulted in an A_{620} of 0.34, whereas the same amount of D-mannose gave an A_{620} of 0.18.

Cationic polyacrylamide disc-gel electrophoresis was performed by the procedure of Gabriel (8). The procedures of Segrest and Jackson (22) were used to stain gels for protein with Coomassie blue dye. Isoelectric focusing on thin-layer polyacrylamide gels containing ampholyte carriers were performed by the procedures of Williamson (30) with some changes. The staining for protein was done with Coomassie blue dye by the procedures of Righetti and Drysdale (20). The procedures of staining for carbohydrate were those of Wardi and Michos (29) with Alcian blue dye. For measurement of the pH gradient, a 2-cm wide strip was cut from the gel in the direction of the pH gradient and 0.25-cm transverse sections were cut from the strip. Each section was placed in 2 ml boiled, CO_2-free distilled H_2O. After 2 h extraction of carrier ampholytes into the CO_2-free water, the pH values of the extracts from each section were measured with a Corning pH meter.

RESULTS

Initial Indication of Polygalacturonase Activity. Two factors suggested that the elicitor of casbene from *R. stolonifer* might be an extracellular enzyme secreted by the fungus during its growth. First, the characteristics of heat inactivation of elicitor activity determined earlier by Stekoll and West (26) strongly suggested that the elicitor was dependent on a native protein structure for its activity. The other indication stems from the argument on evolutionary grounds that the elicitor must have a role to the advantage of the fungus in addition to its seemingly deleterious role for the fungus of being recognized by the castor bean plant as a signal to initiate casbene biosynthesis. Therefore, a search was made for enzyme activities which might be associated with the elicitor. Assays for a number of enzyme activities which seemed to be reasonable candidates for extracellular enzymes produced by *R. stolonifer* cultures were conducted with elicitor fractions that had been partially purified according to the procedures of Stekoll and West (26). The following enzyme activities were tested for and were not detected: α- and β-amylases, α- and β-xylosidases, α- and β-glucosidases, α-mannosidase, β-galactosidase, acid and alkaline phosphatases, invertase, and cellulase. However, polygalacturonase activity was readily detected in the partially purified elicitor preparation.

The initial discovery of the polygalacturonase activity associated with the elicitor from *R. stolonifer* was made as follows. Filtrates of six 250-ml *R. stolonifer* cultures were separated into high- and low-molecular weight elicitor fractions on a Sephadex G-25 column (4.1 × 50 cm) with distilled H_2O as the mobile phase (26). The high-molecular weight elicitor in the void volume was concentrated by lyophilization and then subjected to gel-filtration chromatography on a G-75 column (2.6 × 60 cm) with distilled H_2O as the mobile phase by the previously described procedures (26). The void volume fractions which contained all the elicitor activity applied, were pooled, lyophilized, and redissolved in 10 ml 30 mM potassium succinate (pH 5.5). A portion (0.1 ml) of the solution was assayed for polygalacturonase with 0.9 ml 0.5% (w/v) polygalacturonic acid in 100 mM sodium acetate (pH 4.7). A control of boiled enzyme was prepared as well. After 200 min incubation at 30 C, the number of reducing ends generated was measured by the Nelson-Somogyi procedure. It was found that a 0.1-ml portion of the solution produced 1.0 μmol new reducing ends in 200 min under these assay conditions.

Purification of Polygalacturonase and Elicitor from Culture Filtrates of *R. stolonifer* Grown in Potato-dextrose Medium. Polygalacturonase-elicitor from the filtrates of *R. stolonifer* cultures grown in potato-dextrose medium was purified by gel-filtration chromatography on the Sephadex G-25 and G-75 columns as described above. The void volume fractions of the G-75 column chromatography were pooled, concentrated by lyophilization, and subjected to further gel filtration chromatography on a Sephadex G-100 column (5.0 × 50 cm) with 10 mM Na-phosphate (pH 7.0) as the mobile phase. Both elicitor and polygalacturonase activities eluted with a K_{av} value of 0.43. The fractions containing both activities were pooled and chromatographed on an anion-exchange Sephadex DEAE A-25 column (1.8 × 30 cm) with 10 mM Na-phosphate (pH 7.0). Both polygalacturonase and elicitor activities were found to elute without binding to the resin. The fractions which contained the activities were combined for chromatography on a cation-exchange Sephadex CM C-50 column (1.1 × 23 cm) in 10 mM Na-phosphate (pH 7.0). All the polygalacturonase activity and the elicitor activity were bound to the column at pH 7.0 and co-eluted in the same fractions with identical elution profiles during the application of a salt gradient of 0 to 0.5 M KCl in 10 mM Na-phosphate (pH 7.0).

The fractions which contained polygalacturonase-elicitor from the cation-exchange CM C-50 chromatography were combined and desalted on a Bio-Gel P-2 column (1.8 × 30 cm). Twenty units of the salt-free polygalacturonase were chromatographed on an agarose Bio Gel A-0.5m column (1.1 × 25 cm) with 50 mM sodium acetate (pH 5.2) as the eluting buffer. Both the elicitor and polygalacturonase activities eluted from this column in essentially identical fashion (Fig. 1). Furthermore, both activities were retarded even beyond the completely included volume of the column. This is thought to be due to an affinity between the polygalacturonase enzyme and the agarose resin, a co-polymer of galactose and 3,6-anhydrogalactose (7). The *A* at 280 nm of the column fractions were measured as an indication of their protein concentrations. The A_{280} profile generally coincided with the two activities, although this could not be assessed accurately because of the low *A* involved.

The homogeneity of the polygalacturonase-elicitor preparation from the CM C-50 chromatography was also indicated by an analysis of a portion of the preparation by cationic polyacrylamide disc-gel electrophoresis. The positions of elicitor and polygalacturonase activities of extracts from gel slices were exactly coincident with each other and with the single protein staining band of a paired gel (Fig. 2). The molecular weight of the polygalacturonase-elicitor was estimated to be approximately 32,000 on the basis of a comparison of the relative electrophoretic mobility of polygalacturonase-elicitor with those of the other globular protein standards.

Large-scale Purification of *R. stolonifer* Polygalacturonase-

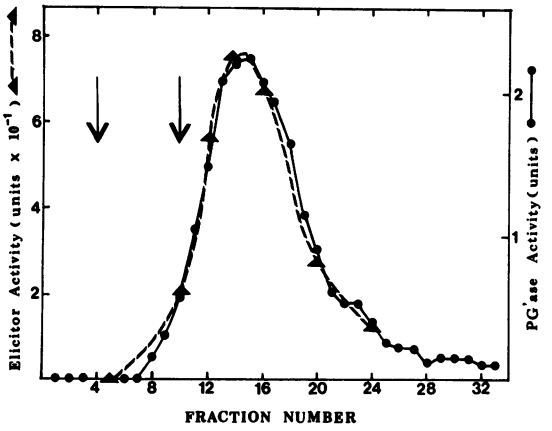

FIG. 1. Agarose affinity column chromatography. A polygalacturonase (PG'ase)-elicitor preparation from CM C-50 chromatography was desalted on a Bio-Gel P-2 column (1.8 × 30 cm). Twenty units salt-free polygalacturonase in 1 ml of 50 mM Na-acetate (pH 5.2) was chromatographed on an agarose Bio-Gel A-0.5m column (1.1 × 25 cm) with the same buffer at a flow rate of 40 ml/h. Thirty-three 3-ml fractions were collected. The void volume and the completely included volume of the column, determined with blue dextran and D-glucose in a separate run under identical conditions, are indicated by the two arrows in the figure. The polygalacturonase activity of each fraction and the elicitor activity of selected fractions (5, 10, 12, 14, 16, 20, 24) were determined as described under "Materials and Methods."

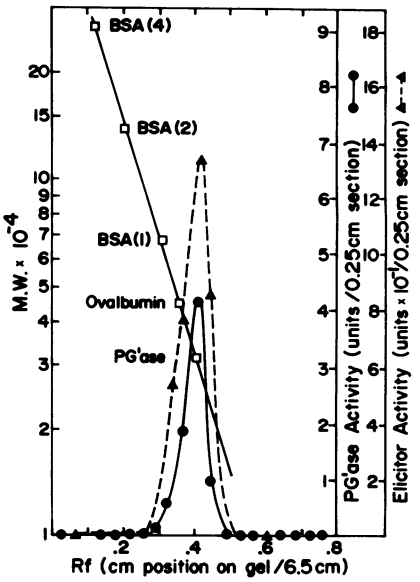

FIG. 2. Polygalacturonase disc-gel electrophoresis of polygalacturonase-elicitor. The following samples were subjected to tube gel electrophoresis: two portions of a desalted polygalacturonase-elicitor preparation from CM C-50 chromatography (each with 10 units polygalacturonase in 50 μl water), BSA (50 μg in 50 μl water), and ovalbumin 50 μg in 50 μl water). The gels consisted of a stacking gel (0.6 × 2.0 cm) of 2.5% (w/v) acrylamide and 0.625% (w/v) *N,N'*-methylenebisacrylamide, and a resolving gel (0.6 × 6.5 cm) of 7.5% (w/v) acrylamide and 0.2% (w/v) *N,N'*-methylenebisacrylamide. The electrophoresis was carried out with a running buffer of pH 4.5 at 1 to 2 mamp/tube at 4 C. After electrophoresis, one of the two sample gels was cut into 0.25-cm sections and each section was extracted with 1 ml 1 M NaCl overnight. The other sample gel and the reference gels were stained with Coomassie blue dye as described (22). BSA(1), BSA(2), and BSA(4) are monomeric, dimeric, and tetrameric forms of BSA, respectively, which are present in commercially available preparations.

Elicitor from Cultures Grown in Glucose-asparagine Medium. Milligram quantities of *R. stolonifer* polygalacturonase were required for studies of the characteristics of the enzyme and its mode of action as an elicitor of the phytoalexin casbene. To improve the efficiency of the purification, various changes were made. First, conditions were sought to increase the amount of polygalacturonase in the source from which a purification scheme starts.

The yields of polygalacturonase from cultures of *R. stolonifer* grown in several media and for varying lengths of time are given in Table I. It seems that *R. stolonifer* produces polygalacturonase constitutively. Among the media tested, the glucose-asparagine medium with 15 g glucose/l supported the production of the highest level of polygalacturonase in the filtrates. The reason for the difference in polygalacturonase activities in filtrates from cultures of the glucose-asparagine medium with 15 g glucose/l after about 97 h growth in three different cultures is not known. Over 90% of the polygalacturonase in *R. stolonifer* cultures was present in the spent medium.

Details of Purification. *R. stolonifer* was grown for 4 days in five lots, each in 3 liters glucose-asparagine medium with 15 g D-glucose/l. The spent medium was sterile-filtered, lyophilized, and chromatographed on a Sephadex G-25 column (4.1 × 50 cm) with distilled H_2O as the mobile phase. All the polygalacturonase eluted in the void volume. A total of 10 such columns were run. The void volume fractions from two such G-25 columns were combined and lyophilized to dryness. The residual material was redissolved in 16 ml 10 mM Na-phosphate (pH 7.0) and the solution was applied to a Sephadex G-75 column (5.0 × 50 cm) which was eluted with the same buffer. The enzyme eluted with a K_{av} value of 0.35 (Fig. 3). The fractions containing polygalacturonase activity were pooled.

One or two such pooled samples from G-75 chromatography were loaded on a CM C-50 column (1.1 × 23 cm) and washed with 200 ml 10 mM Na-phosphate (pH 7.8). Then 400 ml of a linear gradient of 0 to 0.1 M KCl in 10 mM Na-phosphate (pH 7.8) was applied. A typical run of the CM C-50 column in the large-scale purification is shown in Figure 4. The polygalacturonase-containing fractions from CM C-50 chromatography were combined, concentrated by lyophilization, and desalted on a Sephadex G-10 column (2.6 × 40 cm).

Electrophoretic Analysis. The homogeneity of the polygalacturonase-elicitor from the large-scale purification was shown by cationic polyacrylamide disc-gel electrophoresis and by isoelectric focusing. The number of protein bands of preparations at various stages of purification as revealed by Coomassie blue staining of the gel decreased progressively from six in the G-25 gel to one in the CM C-50 gel (Fig. 5). The glycoprotein staining procedure with Alcian blue showed that only two of the six protein bands in the G-25 gel were glycoproteins and one of the two glycoproteins was purified to homogeneity by this purification scheme (Fig. 5). The extracts from the slices of a paired gel were assayed for the enzyme activity. There was a single peak of polygalacturonase activity with an R_F value of 0.40, coincident with the Coomassie blue and the Alcian blue staining bands. This result is consistent with the previous run with the preparation from the potato-dextrose medium (Fig. 2).

The homogeneity of the preparation was also indicated by the results of isoelectric focusing to provide more rigorous evidence by means of a technique with a different basis of separation. Figure 6 shows that only one Coomassie blue staining band at an isoelectric pH of 8.0 was detected; the position of this band coincided with the peaks of polygalacturonase and elicitor activities in the extracts of sections from another strip. The tailing of the activity peak was probably due to overloading of the gel. The carbohydrate staining method did not reveal any band, perhaps due to an interference with staining by the ampholytes.

Table I. *Polygalacturonase Activities in Cultures of R. stolonifer Grown under Various Conditions*

Growth Medium[a]	Growth Period	Source[b]	Polygalacturonase Activity
	h		*units/l*
Potato-dextrose	48	Filtrate	140
	60	Filtrate	156
	72	Filtrate	136
	84	Filtrate	128
	96	Filtrate	116
	120	Filtrate	100
Czapek			
+ Glucose (20 g/l)	145	Filtrate	5
+ Glucose (20 g/l) + PGA (2 g/l)	145	Filtrate	4
+ PGA (2 g/l)	145	Filtrate	3
GA + glucose (15 g/l)	72	Filtrate	464
	97	Filtrate	520
	132	Filtrate	412
GA + glucose (15 g/l) + PGA (1 g/l)	72	Filtrate	444
	97	Filtrate	352
	132	Filtrate	380
GA + glucose (1 g/l) + PGA (1 g/l)	72	Filtrate	92
GA + PGA (1 g/l)	72	Filtrate	4
GA + glucose (15 g/l)	98	Filtrate	1,220
GA + glucose (25 g/l)	119	Filtrate	708
GA + glucose (35 g/l)	218	Filtrate	307
GA + glucose (15 g/l)	96	Filtrate	1,130
	96	Mycelial wash	41
	96	Mycelial extract	44

[a] The compositions of potato-dextrose, Czapek, and glucose-asparagine (GA) media are presented under "Materials and Methods." PGA, polygalacturonic acid (grade II, sodium salt).

[b] Filtrates were prepared as described under "Materials and Methods." Mycelia from which as much filtrate as possible was removed was placed in 500 ml of 50 mM Na-phosphate (pH 7.0) and shaken at 30 C for 1 h. The filtrate from this procedure is referred to as the mycelial wash. The washed mycelia then were frozen and ground with glass beads in a VirTis homogenizer for 5 min with cooling. The resulting homogenate was centrifuged at 37,000*g* for 15 min; the supernatant fraction from this treatment is referred to as the mycelial extract.

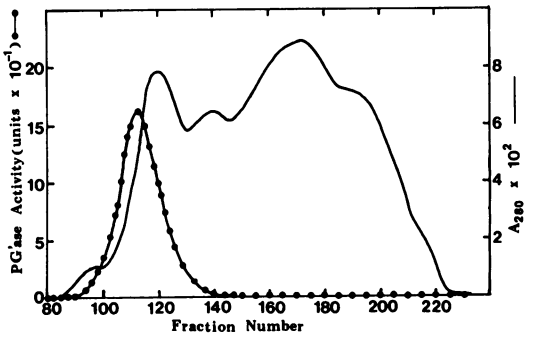

FIG. 3. Sephadex G-75 gel filtration chromatography in the large-scale purification. A typical column in the large-scale purification is shown. About 3,100 units polygalacturonase from two pools of G-25 void volume fractions were combined and lyophilized to dryness. The residual material was redissolved in 16 ml 10 mM Na-phosphate (pH 7.0), and the solution was added to a Sephadex G-75 column (5 × 50 cm). The column was eluted with 10 mM Na-phosphate (pH 7.0) at a flow rate of 25 ml/h. A_{280} was measured with a UV monitor coupled to the fraction collector. Fractions from 94 to 130 were pooled.

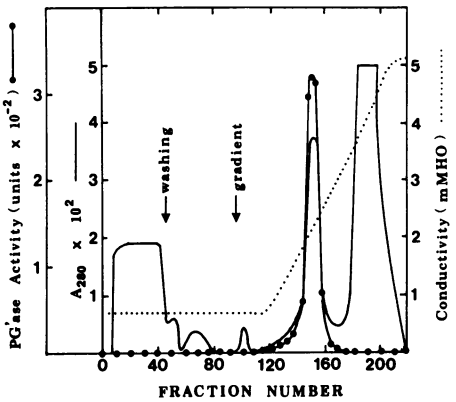

FIG. 4. Sephadex CM C-50 chromatography in the large-scale purification. A typical column in the large-scale purification is shown. About 2,900 units polygalacturonase in 180 ml 10 mM Na-phosphate (pH 7.0) from G-75 chromatography was added to the CM C-50 column (1.1 × 23 cm). The column was washed with 200 ml 10 mM Na-phosphate (pH 7.8). Then, 400 ml of a linear gradient of 0 to 0.1 M KCl in 10 mM Na-phosphate (pH 7.8) was applied. A flow rate of 20 ml/h was maintained and 210 4-ml fractions were collected. The points where washing and the gradient were started are indicated by the arrows. Conductivity was measured on a London Co. conductivity meter. Fractions from 132 to 157 were pooled.

Fold Purification. One of the five lots from the large-scale purification was analyzed for the amounts of elicitor and polygalacturonase activities, and the protein and carbohydrate contents, at various stages of the purification (Table II). Polygalacturonase activity was purified over-all 350-fold with respect to carbohydrate and 10-fold with respect to proteins as measured by the Bradford dye-binding assay. The fold purification of elicitor activity was similar to that of polygalacturonase activity. Elicitor activity was purified 560-fold with respect to carbohydrate and 16-fold with respect to protein, as measured by the Bradford dye-binding assay. The Lowry assay (14) for protein gave high values with the crude culture filtrates and at intermediate stages of purification in comparison with the dye-binding assay. This was apparently a result of the presence of nonprotein contaminants which react with the Lowry reagent. However, the protein values indicated for the pure enzyme by both procedures were comparable.

The apparently homogeneous polygalacturonase-elicitor preparation (650 units polygalacturonase and 8,230 units elicitor) contained 0.32 mg total carbohydrate measured by the anthrone assay with D-mannose as a standard and 1.4 mg protein based on the Bradford dye-binding assay with ovalbumin as a standard. If these reference standards in the assays are appropriate, the carbohydrate comprises about 19% of the polygalacturonase-elicitor on a weight basis.

DISCUSSION

The identity with polygalacturonase of the elicitor of casbene synthetase activity in castor bean seedlings present in the high-molecular weight fraction from *R. stolonifer* culture filtrates is clearly established by the results presented in this paper. The major points of evidence in support of this conclusion are as follows.

(*a*) Both activities co-purify through a series of gel filtration and ion-exchange chromatography steps which yield an apparently homogeneous polygalacturonase preparation. The two activities remained essentially coincident in the elution profiles from these columns. There was no evidence for the resolution of either

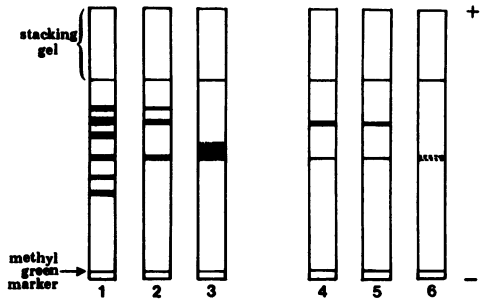

FIG. 5. Polyacrylamide disc-gel electrophoresis of polygalacturonase-elicitor preparations from the large-scale purification. Six tubes consisting of a stacking gel (0.6 × 2.5 cm) of 2.5% (w/v) acrylamide and 0.625% (w/v) *N,N'*-methylenebisacrylamide and a resolving gel (0.6 × 7.0 cm) of 7.5% (w/v) acrylamide and 0.2% (w/v) *N,N'*-methylenebisacrylamide were prepared by the procedures of Gabriel (8). The following portions of polygalacturonase-elicitor preparations from various stages of the large-scale purification, each in 50 μl water with 25 μl of 0.2% (w/v) methyl green dye in 40% (v/v) glycerol, were analyzed by the procedures of Gabriel (8). For protein staining with Coomassie blue dye, 75 μg G-25 preparation, 70 μg G-75 preparation, and 45 μg CM C-50, G-10 preparation were analyzed on gels 1, 2, and 3, respectively. For carbohydrate staining with Alcian blue dye, the same amounts of G-25, G-75, and CM C-50, G-10 preparations were analyzed on gels 4, 5, and 6, respectively.

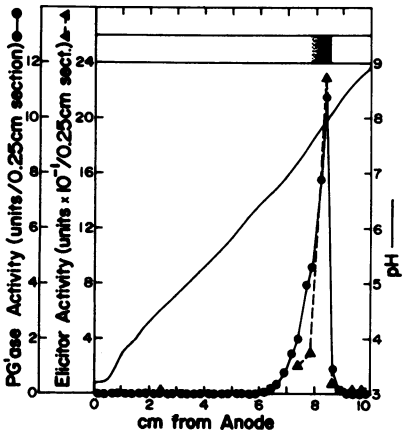

FIG. 6. Isoelectric focusing of the purified polygalacturonase (PGase)-elicitor from the large-scale purification. A thin-layer polyacrylamide gel (0.21 × 8.5 × 11.5 cm) containing 2% (w/v) carrier ampholytes was prepared by polymerizing the following mixture with UV light: 0.1 ml 5% (v/v) *N,N,N',N'*-tetramethylethylenediamine, 4.5 ml 33% (w/v) acrylamide and 0.9% (w/v) *N,N'*-methylenebisacrylamide, 1.0 ml 40% (w/v) Biolyte (pH 3 to 10), 2.0 ml 2% (w/v) riboflavin, plus 12.4 ml distilled H_2O. A portion of the CM C-50 preparation, containing 30 units polygalacturonase and 65 μg protein in 150 μl water, was applied on a piece of Whatman GF/A glass filter paper (1.2 × 5.0 cm) which was placed on the center of the gel. The gel was focused with a voltage difference of 500 v for 24 h. After focusing, the gel was cut into four strips which were used for the pH gradient measurement, Coomassie blue staining, Alcian blue staining, and the polygalacturonase-elicitor extraction of 0.25-cm transverse sections.

activity from the other at any stage. The apparently homogeneous polygalacturonase-elicitor showed similar, but not identical, overall -fold purifications of both the elicitor and polygalacturonase activities with respect to protein and carbohydrate (Table II). The basis for the differences seen is not known with certainty, but it is probably due to the inherent difficulties in accurately measuring the amounts of elicitor activity. We believe that the ratio of elicitor activity to polygalacturonase activity is constant within experimental error at all stages of purification after the preparation of the Sephadex G-25 void volume fraction.

(*b*) The preparations of enzyme after cation-exchange chromatography showed a single band staining for protein which was coincident with both polygalacturonase and elicitor activity on both cationic polyacrylamide electrophoresis disc gels and isoelectric-focusing gels.

(*c*) Both polygalacturonase and elicitor activities were eluted from an agarose gel column in essentially coincident peaks after the totally included volume (Fig. 1). This behavior indicates that agarose, which is a galactose-anhydrogalactose copolymer, has an affinity for the polygalacturonase-elicitor; this behavior has been reported previously for polygalacturonase from a different source (7). Chromatography on agarose was not used routinely for purification since the binding of the enzyme to the gel was weak and was sensitively dependent on the salt concentration in the sample as well as the pH and molarity of the eluting buffer, as reported by Thibault and Mercier (27).

It would be of interest to know how effective the polygalacturonase-elicitor is in stimulating casbene synthesis at sites of fungal contamination of seedlings *in vivo*. But at present, we do not have satisfactory methods for quantitating either casbene accumulation or the levels of polygalacturonase at sites of fungal contamination of seedlings *in vivo*. It is possible to correlate the levels of casbene synthetase activity elicited with the amounts of polygalacturonase-elicitor supplied from the results of the *in vitro* assay system employed. The specific activity of the pure enzyme is seen to be about 153 units elicitor activity/nmol enzyme from the results in Table II and the assumption that the mol wt of the enzyme is 32,000. The dose-response curve for the elicitor assay system indicates that 0.1 nmol in 10 ml (10 nM) polygalacturonase-elicitor leads to the production of casbene synthetase capable of synthesizing about 5 nmol casbene/min · 10 seedlings, or about 0.5 nmol/min · seedling, under optimal incubation conditions. Previous studies by Sitton and West (23) showed that 27 μM casbene in agar was sufficient to inhibit the radial growth of *A. niger* inoculated onto the plate. On the basis of crude assumptions, it can be calculated that the levels of casbene synthetase elicited by 0.1 nmol polygalacturonase-elicitor in the elicitor assay system would be sufficient to accumulate 27 μM casbene in the seedlings in about 50 min if the enzyme is operating under optimal conditions. It might also be noted that approximately 0.10 nmol polygalacturonase accumulates in 1.0 ml culture filtrate after 4 days growth of *R. stolonifer* in the glucose-asparagine medium under the conditions described. Obviously, the results of these calculations tell us little about the amounts of casbene which may accumulate in the plant tissues under more usual physiological circumstances of elicitation. But they do give some feeling for the effectiveness of polygalacturonase as a casbene synthetase elicitor.

Although the castor bean-*R. stolonifer* interaction is not an authentic host-parasite system and casbene has not been clearly demonstrated to serve the castor bean plant as a phytoalexin, it is our belief that an understanding of the molecular basis for elicitation in this system will be instructive and should be considered in connection with the elicitation of phytoalexins in more established host-parasite systems. This is the first reported instance of the association of enzyme activity with an elicitor. Most of the elicitors of biotic origin reported to date are either polysaccharides (3, 10) or glycoproteins (12). Even though there is not much experimental evidence on the question of how these elicitors cause accumulations of phytoalexins, comparisons with other biological systems involving cell-cell recognition mechanisms prompted Albersheim and Anderson-Prouty (1) to suggest that carbohydrate-protein interactions at plant cell surfaces may be involved in triggering the response. In this connection, two properties of the *R. stolonifer* polygalacturonase-elicitor should be mentioned. First, it is a glycoprotein with about 19% carbohydrate and thus contains a glycosyl moiety or moieties. And second, it catalyzes the hy-

Table II. *Fold Purification and Yields of Elicitor Activity, Polygalacturonase Activity, Carbohydrate, and Protein at Various Stages of the Large-scale Purification*

Polygalacturonase-elicitor was purified according to the scheme described in the text from 3 liters *R. stolonifer* grown in the glucose-asparagine medium for 4 days. Carbohydrate was determined by the anthrone procedure with D-mannose as the reference standard and protein was determined by the Bradford procedure with ovalbumin as the reference standard. Fold purifications with respect to the culture filtrate are indicated in parentheses. PGase, polygalacturonase.

Purification Step	Total Elicitor Activity	Total PGase Activity	Total Carbohydrate	Total Protein	Specific Activity	
					Elicitor	PGase
	units		*mg*		*units/mg protein*	
Culture Filtrate	2.69×10^5	3,430	590	72	3.74×10^3 (1.0)	47.6 (1.0)
G-25	2.04×10^5	2,510	150	55	3.71×10^3 (1.0)	45.6 (0.96)
G-75	3.13×10^5	3,120	38	34	9.21×10^3 (2.5)	91.8 (1.9)
CM C-50 and G-10	8.23×10^4	650	0.32	1.4	5.88×10^4 (15.7)	464.0 (9.7)

drolysis of polygalacturonic acid, a substrate prepared from a major constituent of the plant outer cell-wall components (15).

Polygalacturonase is one of a family of pectic enzymes that occurs primarily in plants, bacteria, and fungi (19). Even though the involvement of these enzymes in phytopathogenesis has been known for some time (2, 16, 19) and their indispensability has been suggested (2), their exact role is not known. A frequent suggestion is that they make possible the entry of pathogens into plants by degrading the cell-wall pectin as evidenced by the maceration of plant tissues with these enzymes (2, 5). The results of the study presented here indicate that polygalacturonase may have a more direct role in initiating the accumulation of a stress metabolite in castor beans. The accompanying paper (13) describes some of the characteristics of the polygalacturonase-elicitor which were determined as an initial step in attempting to understand its mode of action as an elicitor.

Acknowledgment—Sincere appreciation is expressed to Robert J. Bruce for his capable technical assistance.

LITERATURE CITED

1. ALBERSHEIM P, AJ ANDERSON-PROUTY 1975 Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. Annu Rev Plant Physiol 26: 31–52
2. ALBERSHEIM P, TM JONES, PD ENGLISH 1969 Biochemistry of the cell wall in relation to infective processes. Annu Rev Phytopathol 7: 171–194
3. AYERS AR, B VALENT, J EBEL, P ALBERSHEIM 1976 Host-pathogen interaction. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol 57: 766–774
4. BRADFORD M 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254
5. COOPER RM, B RANKIN, RKS WOOD 1978 Cell wall-degrading enzymes of vascular wilt fungi. II. Properties and modes of action of polysaccharidases of *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Plant Pathol 13: 101–134
6. CRUICKSHANK IAM 1963 Phytoalexins. Annu Rev Phytopathol 1: 351–374
7. ENGLISH PD, A MAGLOTHIN, K KEEGSTRA, P ALBERSHEIM 1972 A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. Plant Physiol 49: 293–297
8. GABRIEL O 1971 Analytical disc gel-electrophoresis. Methods Enzymol 22: 565–578
9. KEEN NT 1974 Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187: 74–75
10. KEEN NT, B BRUEGGER 1977 Phytoalexins and chemicals that elicit their production in plants. *In* P Hedin, ed, Host Plant Resistance to Pests, ACS Symposium Series, Vol 62. American Chemical Society, Washington, DC, pp 1–26
11. KEEN NT, JC HORTON 1966 Induction and repression of endopolygalacturonase synthesis by *Pyrenochaeta terrestris*. Can J Microbiol 12: 443–453
12. KEEN NT, M LEGRAND 1980 Surface glycoproteins: evidence that they may function as the specific elicitors of *Phytophthora megasperma* f. sp. *glycineae*. Physiol Plant Pathol 17: 175–192
13. LEE S-C, CA WEST 1980 Properties of *Rhizopus stolonifer*, an elicitor of casbene synthetase activity in castor bean (*Ricinus communis* L.) seedlings. Plant Physiol 67: 640–645
14. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin-phenol reagent. J Biol Chem 193: 265–275
15. MCNEIL M, AG DARVILL, P ALBERSHEIM 1981 The structural polymers of the primary cell walls of dicots. *In* NE Tolbert, ed, The Biochemistry of Plants, Vol 1. Academic Press, New York In press
16. MUSSELL H, LL STRAND 1977 Pectic enzymes: involvement in pathogensis and possible relevance to tolerance and specificity. *In* B Solheim, J Raa, eds, Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions. Universitetsforlaget, Oslo, pp 31–70
17. NELSON N 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem 153: 375–380
18. REXOVA-BENKOVA L 1973 The size of the substrate-binding site of an *Aspergillus niger* extracellular endopolygalacturonase. Eur J Biochem 39: 109–115
19. REXOVA-BENKOVA L 1976 Pectic enzymes. Adv Carbohydr Chem Biochem 33: 323–385
20. RIGHETTI PG, JW DRYSDALE 1974 Isoelectric focusing in gels. J Chromatogr 98: 271–321
21. ROBINSON DR, CA WEST 1970 Biosynthesis of cyclic diterpenes in extracts of *Ricinus communis*. II. Conversion of geranylgeranyl pyrophosphate into diterpene hydrocarbons and partial purification of cyclization enzymes. Biochemistry 9: 80–89
22. SEGREST JP, RL JACKSON 1972 Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Methods Enzymol 28: 54–63
23. SITTON D, CA WEST 1975 Casbene, an antifungal diterpene produced in cell-free extracts of *Ricinus communis* seedlings. Phytochemistry 14: 1921–1925
24. SOMOGYI M 1952 Notes on sugar determination. J Biol Chem 195: 19–23
25. SPIRO RG 1966 Analysis of sugars found in glycoproteins. Methods Enzymol 8: 3–26
26. STEKOLL MS, CA WEST 1978 Purification and properties of an elicitor of castor bean phytoalexin from culture filtrates of the fungus *Rhizopus stolonifer*. Plant Physiol 61: 38–45
27. THIBAULT JF, C MERCIER 1977 *Aspergillus niger* endopolygalacturonase. I. Studies on purification by agarose gel chromatography. J Solid-Phase Biochem 2: 295–304
28. UPPER CD, CA WEST 1967 Biosynthesis of gibberellins. II. Enzymatic cyclization of geranylgeranyl pyrophosphate to kaurene. J Biol Chem 242: 3285–3292
29. WARDI AH, GA MICHOS 1972 Alcian blue staining of glycoproteins in acrylamide disc electrophoresis. Anal Biochem 49: 607–609
30. WILLIAMSON AR 1973 Isoelectric focusing of immunoglobulins. *In* DM Weis, ed, Handbook of Experimental Immunology, Ed 2 Vol 1, Chap 8. Blackwell Scientific Publications, Oxford, pp 1–23
31. YOSHIKAWA M 1978 Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls with *Phytophthora megasperma* var. *sojae*. Physiol Plant Pathol 12: 73–82