Plant Physiol. (1981) 67, 640–645
0032-0889/81/67/0640/06/$00.50/0

Properties of *Rhizopus stolonifer* Polygalacturonase, 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

Some properties of the polygalacturonase-elicitor from the filtrates of *Rhizopus stolonifer* cultures have been examined in an attempt to understand its mode of action as an elicitor of casbene synthetase activity in castor bean seedlings. Both the polygalacturonase activity and the elicitor activity are heat-labile with similar heat-sensitivity profiles. Also, the catalytic activity of the enzyme is lost on treatment with sodium periodate, as had been shown previously for the elicitor activity. The pH optimum of the enzyme activity with polygalacturonic acid as the substrate is 4.9. Exposures of germinating castor bean seedlings to the elicitor for short-term periods of 1 to 10 minutes followed by washing and incubation in sterile, distilled water are partially effective in elicitation in comparison with the continuous exposure of the seedlings over 11 hours to the same amount of the elicitor. The initial rate of reaction catalyzed by the enzyme is about 3 times faster with polygalacturonic acid as a substrate than with partially (50%) methylated polygalacturonic acid (pectin). The K_m value of the enzyme for polygalacturonic acid is about 4.2 millimolar in terms of monomeric units and about 0.07 millimolar in terms of polymer concentration. Examination of the types of products formed by the action of the enzyme suggests that it is an endo-hydrolase. The amino acid composition of this enzyme is similar to those of other extracellular fungal proteins reported. The carbohydrate moiety of the glycoprotein polygalacturonase-elicitor is composed of 92% mannose and 8% glucosamine by gas chromatography-mass spectrometry analysis. The linkage group analysis of the carbohydrate moiety showed that mannosyl residues which are 1,2-linked comprise about 70% of the total glycosyl residues and demonstrated the presence of some 1,3,6- and 1,2,6-linked branching mannosyl residues.

Elicitors of plant-produced antifungal metabolites derived from various microorganisms are mostly either polysaccharides (3, 10, 19) or glycoproteins (6, 12, 23). Although there is not much experimental evidence on the mode of action of these elicitors, comparisons with other biological phenomena involving cell recognition mechanisms prompted Albersheim and Anderson-Prouty (1) to suggest that elicitors may be recognized by plant cell surface receptors in a process involving carbohydrate-protein interactions. There is little direct evidence available to date to evaluate this proposal.

Previous studies in this laboratory by Stekoll and West (23) showed that a partially purified elicitor from culture filtrates of *Rhizopus stolonifer*, which acts to elicit casbene synthetase activity in castor bean seedlings, possesses the characteristics expected of a glycoprotein requiring both protein and carbohydrate for its activity. The dependency of the elicitor activity on the native protein structure, and an evolutionary argument that the elicitor must have a role of the advantage of the fungus in addition to its seemingly deleterious role for the fungus of being recognized as a signal to initiate casbene biosynthesis, led us to search for an enzymic activity that might be associated with the elicitor. In the accompanying paper (15), the identification of the elicitor from *R. stolonifer* with a polygalacturonase is described. The apparently homogeneous polygalacturonase-elicitor catalyzes the hydrolysis of polygalacturonic acid, a substrate prepared from a major constituent of plant outer cell-wall components (18). It is also notable that the polygalacturonase-elicitor is a glycoprotein with about 20% carbohydrate content and, thus, contains a glycosyl moiety itself.

The finding of the enzymic activity associated with the elicitor, the first such instance reported, raises the question of how the glycoprotein polygalacturonase from *R. stolonifer* acts as an elicitor of casbene synthetase activity in castor beans. The characteristics of the polygalacturonase-elicitor described in this report were determined to gain some understanding of its mode of action as an elicitor.

MATERIALS AND METHODS

Chemicals. Pectin (grade I, from citrus fruit), methylated on about 50% of its carboxyl groups, was purchased from Sigma.

Source of Polygalacturonase-Elicitor. Polygalacturonase-elicitor preparations from filtrates of *R. stolonifer* cultures were obtained by the purification procedures described in the preceding paper (15).

Elicitor Activity and Polygalacturonase Activity Assays. Measurements of elicitor and polygalacturonase activities were made as described in the preceding paper (15). One unit of elicitor activity is defined as the amount of elicitor that will elicit a casbene synthetase activity of 1 pmol casbene/min in 100 μl S_{37} enzyme extract under the assay conditions described in the preceding paper (15). One unit of polygalacturonase activity is defined as that amount of enzyme which will yield 1 μmol reducing ends/min at 30 C under the assay conditions described in the accompanying paper (15).

Viscometry. The viscosities of polygalacturonic acid substrate

[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 Psychiatry, University of California-San Diego, La Jolla, CA 92093.

solution after various periods of incubation with polygalacturonase were measured with an Ostwald viscometer. Polygalacturonic acid (0.5% (w/v)) in 100 mM sodium acetate (pH 4.8), was incubated with polygalacturonase. At various periods of time, a portion (4.1 ml) of the incubation mixture was removed and the reaction was quenched by the addition of 0.1 ml 5 N NaOH. The resulting solution then was added to an Ostwald viscometer which had been immersed in a 30 C water bath. The time periods for the outflow of sample solutions through the capillary of the viscometer were measured three times for each sample and the average value was determined. Specific viscosities, *i.e.* fractional decrements in viscosity produced by changes in the sizes of polygalacturonic acids, were calculated from the following relationship:

$$N_{sp} = N_{rel} - 1 = \frac{t}{t_0} \times \frac{\rho}{\rho_0} - 1$$

where N_{sp} and N_{rel} refer to specific and relative viscosities, respectively, t and t_0 are the time periods of outflow of the sample with or without polygalacturonic acid, respectively, and ρ and ρ_0 are the densities of the sample with or without polygalacturonic acid, respectively. ρ and ρ_0 were assumed to be same in the calculation of specific viscosities.

Paper Chromatography. The products formed by the action of polygalacturonase on polygalacturonic acid were analyzed by descending paper chromatography according to the procedures of Young and Cordin (26).

Carbohydrate Analyses. The compositional and linkage analyses of the polygalacturonase-elicitor were performed by Dr. Alan Darvill in the laboratory of Dr. Peter Albersheim, University of Colorado, Boulder, by GC-MS analysis of alditol acetate derivatives of the sugar residues resulting from the trifluoroacetic acid hydrolysis of the enzyme with or without prior methylation (18).

Amino Acid Analysis. Acid hydrolysates of the polygalacturonase-elicitor, prepared by hydrolysis with 6 N HCl *in vacuo* at 110 C for 24 h, were analyzed for amino acid composition on Beckman UR-30 (56 × 0.9 cm) and PA-35 (5.5 × 0.9 cm) columns with a Beckman model 120 C amino acid analyzer. The eluting amino acids were coupled to *o*-phthalaldehyde and the fluorescence of the resulting complexes were measured at 550 nm. The contents of each amino acid and ammonia were quantitated by comparing their fluorescence intensities with those of 10-nmol samples of the corresponding standards subjected to the same procedure.

RESULTS

Heat Lability of Polygalacturonase and Elicitor Activities. The effects of pretreatment for 15 min polygalacturonase-elicitor at various temperatures on the polygalacturonase and elicitor activities are shown in Figure 1. Polygalacturonase activity was found to be heat-labile, as expected. Only 30% of the original activity remained after heating for 15 min at 50 C and less than 4% remained at 60 C. The elicitor activity was also heat-labile in accordance with the previous report by Stekoll and West (23). Except at 40 C, both polygalacturonase and elicitor activities showed nearly identical labilities to heating at all temperatures tested.

Periodate Treatment of Polygalacturonase. Previous studies in this laboratory by Stekoll and West (23) showed that the partially purified elicitor from *R. stolonifer* was completely inactivated by treatment with 70 mM sodium periodate for 9 h. Therefore, the effect of periodate oxidation of polygalacturonase activity was determined so that it could be compared to the effect of the same treatment on elicitor activity. The periodate treatment caused a progressive reduction of the polygalacturonase activity over 7 h until none could be detected, whereas there was only a slight decrease of enzyme activity in the control solution over the same period (Fig. 2).

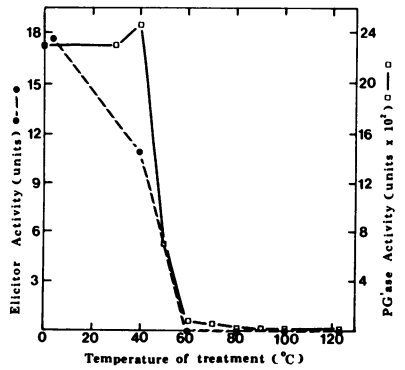

FIG. 1. Heat labilities of polygalacturonase (PG'ase) and elicitor activities. Twenty μl of a pure enzyme solution in 10 mM Na-phosphate (pH 7.0), which contained 0.23 unit of polygalacturonase activity, were treated for 15 min at temperatures ranging from 0 to 123 C (autoclaving). After the treatments, the tubes were quickly cooled on ice and the polygalacturonase activity of each sample then was determined as described in the preceding paper (15). The heat lability of elicitor activity was determined as follows. The void volume fractions from a Sephadex G-75 chromatography column were pooled and filtered through a sterile GS (0.22 μm) Millipore membrane to remove any possible contaminating microorganisms. Then 50-μl aliquots were treated for 15 min at temperatures from 0 C to 123 C (autoclaving). The sample tubes were quickly cooled on ice and diluted to 10 ml with sterile, distilled H_2O. The elicitor activities of these solutions were determined as described in the preceding paper (15).

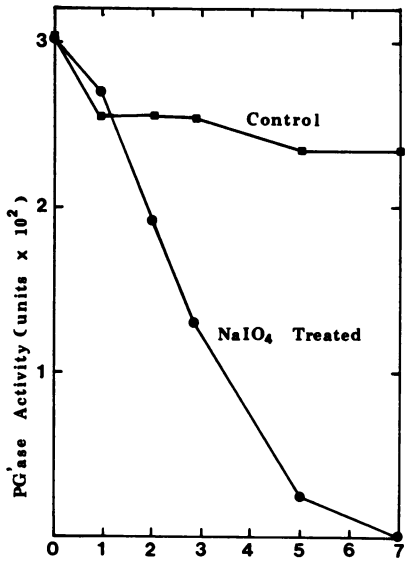

FIG. 2. The effect of sodium periodate on polygalacturonase (PG'ase) activity. Twenty μl 0.7 M sodium periodate was added to 200 μl of a pure enzyme solution in 10 mM Na-phosphate (pH 7.0) containing 0.56 unit polygalacturonase activity. The mixture was incubated at 30 C in the dark. After the lengths of time indicated in the figure, 22-μl aliquots were taken and quenched with 58 μl 4% ethylene glycol. Twenty μl water was added to each sample solution before it was assayed for polygalacturonase activity. The controls were prepared by exchanging the order of additions of water and 0.7 M sodium periodate solution in the above procedure.

pH Optimum. The optimum pH for the catalytic reaction was determined from the results shown in Figure 3. The enzyme is most active at pH 4.9 and completely inactive at pH values above 6.5 or below 3.2

Short-term Exposure of Seedlings to Elicitor. One approach to understanding the characteristics of the elicitation process was to vary the time periods of exposure of seedlings to elicitor. Previous

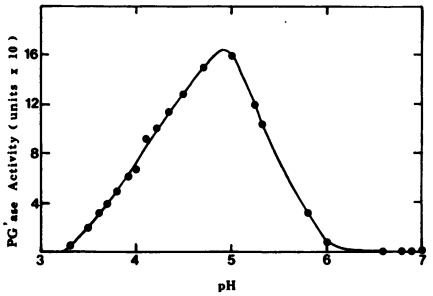

FIG. 3. pH optimum of polygalacturonase (PG'ase) activity. Fifty mg polygalacturonic acid was dissolved in 10 ml of each of the following buffer solutions: citrate (100 mM)-phosphate (0.2 M buffer) solutions ranging from pH 3.4 to 6.8, phosphate (0.2 M) buffer solutions ranging from pH 7.2 to 8.0, Tris-HCl (0.2 M) buffer solutions ranging from pH 8.2 to 9.0, and bicarbonate-carbonate (0.2 M) buffer solutions ranging from pH 9.2 to 10.0. The measured pH value of each resulting solution is indicated in the figure. One hundred μl of a partially purified G-75 void volume pool of polygalacturonase (15) was added to 1 ml buffered substrate solution and the resulting mixture was assayed for enzyme activity as described previously (15) with a 10-min incubation. No enzyme activity was observed between pH 7 and 10.

studies by Stekoll and West (23) showed that exposure times to elicitor of about 4 h were required to see a measurable response. Longer times of exposure beyond 4 h gave proportionally higher responses until a maximum was reached at 8 to 10 h. It was not known whether these initial 4 h were required for direct recognition of elicitors by the castor bean seedlings or for secondary processes of expression, such as protein synthesis, after a brief primary determinative recognition process. To examine this question, seedlings were exposed for short periods of time to elicitor before they were washed with large amounts of water to remove elicitors from the medium. After this, the seedlings were incubated for a total period of 11 h before cell-free extracts were prepared for measurements of the casbene synthetase activity. It was hoped that this experiment would indicate whether a brief exposure to elicitor followed by 11 h incubation is as effective as a continuous exposure to elicitor for 11 h.

Seedlings were exposed to elicitors at room temperature for time periods ranging from 1 to 10 min before they were washed several times with large amounts of sterile, distilled H_2O and incubated for a total of 11 h. A positive control in which seedlings received 11 h continuous exposure to elicitor and a negative control in which the seedlings were treated with sterile distilled H_2O for a short time before they were washed and incubated were included. The short-term exposures of 1 to 10 min were 20 to 60% effective, compared to the positive control of 11-h exposure. This apparent effectiveness of short-term exposure was not due to the elicitation caused by the processes of washing and transferring of the seedlings inasmuch as the response of seedlings in the negative control was the same as that of seedlings which were treated with sterile, distilled H_2O continuously for 11 h. In several experiments with various amounts of the elicitor and various time periods of exposure, the responses of seedlings to the same amounts of elicitor were not proportional to the time periods of exposure to the elicitor. Also, there were some quantitative variations even in the responses of replicates. However, in all of the experiments performed, there were significant responses to short-term exposures. We realize that the apparently effective short-term exposures may have been due to the ineffectiveness of the washing procedure in removing nonspecifically bound elicitors. However, the finding is also consistent with some other possibilities which will be presented under "Discussion."

Substrate Specificity of Polygalacturonase. Polygalacturonic acid is found naturally in the plant cell wall as its partially methylated derivative (pectin). Therefore, the catalytic activity of polygalacturonase with pectin as the substrate was examined. Although the enzyme catalyzed the release of reducing substances from pectin, the initial reaction rate was about one-third of that with polygalacturonic acid as the substrate at equivalent concentrations of 0.25% (w/v) in 100 mM sodium acetate (pH 5.0) (Fig. 4).

K_m Value for Polygalacturonic Acid. The K_m value of the polygalacturonase for polygalacturonic acid was determined from the initial reaction rates with substrate concentrations ranging from 0.01% (w/v) to 0.50% (w/v). Higher substrate concentrations could not be used because of the limited solubility of polygalacturonic acid in 100 mM sodium acetate (pH 5.0). The pH of the substrate solutions was changed from 5.0 by the addition of the polygalacturonic acid; they ranged more or less linearly from 5.9 (0.01% solution) to 4.6 (0.50% solution). Therefore, the reaction rates, expressed in units of μmol reducing ends produced/min, were corrected for the effect of pH changes on enzyme activity from the pH *versus* enzyme activity relationship shown in Figure 3. The K_m value for the polygalacturonic acid was found to be 4.2 mM in terms of monomeric units of the polymer from an Eadie-Hofstee plot of the results. There were rather large deviations from the Michaelis-Menten pattern at the higher concentrations of substrate; the least squares analysis of the data indicated a linear regression coefficient of −0.871. The reason for the deviations is not known with certainty. One possibility is that the substrate or products or both are inhibitors of the enzyme. Another possibility is that the corrections for the effect of pH changes of substrate solutions on enzyme activity were inadequate. An average degree of polymerization of 60 was estimated for the polygalacturonic acid sample used as substrate from a measurement of the number of reducing ends in a weighed sample on the assumption that the reducing ends in the polymer give the same color yield as the free monomer in this assay. This assumption is based on the observations of Rexova-Benkova (21) that the galacturonic acid monomer and oligomers of galacturonic acid as long as pentamers give the same molar color yield in the Nelson-Somogyi assay. Therefore, the K_m for the polygalacturonic acid in terms of polymer concentration can be estimated to be about 70 μM.

Mode of Catalytic Action. Polygalacturonases are divided into hydrolases and lyases (also referred to as transeliminases), depending on their mode of catalytic action. The hydrolases catalyze the hydrolytic cleavage of the glycosidic bonds between galacturonic acid residues in the polygalacturonic acid substrate, whereas

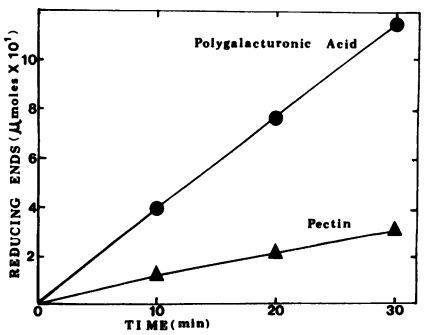

FIG. 4. Polygalacturonase activities with polygalacturonic acid and pectin (partially methylated polygalacturonic acid) as substrates. Fifty-μl aliquots of pure enzyme in 50 mM sodium acetate (pH 5.2) (each containing 0.038 unit of polygalacturonase activity) were added to 1 ml substrate solution, 0.25% (w/v) polygalacturonic acid or 0.25% (w/v) or partially (about 50%) methylated polygalacturonic acid in 100 mM sodium acetate (pH 5.0). The resulting mixtures were incubated for 10, 20, and 30 min at 30 C. Zero time controls were prepared by adding Nelson-Somogyi copper reagent working solution to the buffered substrate prior to the addition of enzyme.

the lyases catalyze the cleavage of the glycosidic bonds through an elimination mechanism. Consequently, the lyases can be distinguished from the hydrolases by their characteristic catalytic products which contain 4,5-unsaturated galacturonosyl nonreducing termini with an *A* maximum at 235 nm (22). A pure polygalacturonase-elicitor preparation was assayed with polygalacturonic acid as the substrate at various pH values in the presence or absence of 0.5 mM Ca^{2+}, which was reported to activate some lyases (22). No lyase activity as evidenced by an increased *A* at 235 nm was detected under any of the assay conditions tested. The inference from these results is that the polygalacturonase from *R. stolonifer* is a hydrolase.

Whether the enzyme possessed an "endo" or "exo" mode of action was examined by two different methods. In the first, the viscosity of an enzyme-incubated substrate solution was measured as a function of time of incubation with the enzyme. Figure 5 shows that the specific viscosity of the solution was reduced to 10% of that of the zero time control during a period of time in which only 6.6% of the glycosidic bonds in the polygalacturonic acid substrate were being hydrolyzed. Even though a change in specific viscosity is not linearly related to the size of polygalacturonic acids, such a sharp decrease in specific viscosity with a relatively slow increase in the number of reducing ends is a strong indication that the polygalacturonase from the culture filtrates of *R. stolonifer* has en endo catalytic mode.

In the second approach, the products from an exhaustive hydrolysis of polygalacturonic acid with the enzyme were analyzed by paper chromatography. The products from the exhaustive incubation showed a large spot with an R_F value of 0.56 and no material at the origin of the paper chromatogram, whereas monomeric D-galacturonic acid and polygalacturonic acid have R_F values of 0.73 and 0.00, respectively, in this chromatographic system. From these results, it was clear that the polygalacturonase in question was an endo enzyme inasmuch as there was no evidence for the production of monomeric D-galacturonic acid as would have been expected to an exo enzyme. Reference samples of oligogalacturonic acids were not available, so it can only be surmised that the large spot at R_F 0.56 represented a mixture of oligogalacturonic acids.

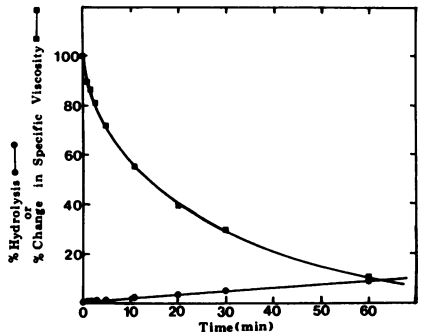

FIG. 5. Per cent hydrolysis and per cent changes in specific viscosity as a function of incubation time. One hundred μl of a partially purified G-75 void volume pool of polygalacturonase (0.29 unit), prepared as described in the preceding paper (15), were added to each of nine test tubes containing 5 ml 0.5% (w/v) polygalacturonic acid in 100 mM sodium acetate (pH 4.8). The resulting mixtures were incubated at 30 C and, after the periods of time indicated in the figure, 1 ml mixture was withdrawn and the reaction was quenched quickly by the addition of the working copper reagent. Each solution then was assayed for reducing ends by the Nelson-Somogyi test as described in the accompanying paper (15). The zero time control for the measurement of readding the copper reagent before the enzyme solution. The rest of the enzyme-incubated substrate solution (4.1 ml) was also quenched quickly by adding 100 μl 5 N NaOH, which raised the pH of the solution from 4.5 to 12.4. The viscosity of the resulting solution (4.2 ml) then was measured with an Ostwald viscometer and the changes in specific viscosity were calculated as described under "Materials and Methods."

Carbohydrate Analysis. The composition and the types of linkages of the carbohydrate moiety of the polygalacturonase-elicitor from the filtrates of *R. stolonifer* cultures grown in the glucose-asparagine medium (15) were analyzed (Table I). The carbohydrate moiety of the polygalacturonase-elicitor is composed of 92% D-mannose and 8% glucosamine, the latter presumably originating from *N*-acetylglucosamine residues in the glycoprotein. The linkage group analysis showed that mannosyl residues which are 1,2-linked predominate, with small but significant levels of 1,4-linked mannose residues. Due to the characteristically low yield of amino sugars in the linkage group analysis, the percentage of glucosamine in the linkage group analysis was much smaller than the 8% determined from the compositional analysis. It is also notable that other potentially important glycosyl components, such as 3-glucosamine and 3-mannose, were looked for and were not detected.

Amino Acid Analysis. Acid hydrolysates of 250 μg of the homogeneous polygalacturonase-elicitor purified from the filtrates of *R. stolonifer* cultures grown in the glucose-asparagine medium (15) were analyzed for amino acid composition (Table II). The cysteine and cystine content could not be determined by the procedure used because of their poor fluorescence yields. Proline could not be detected by this method either, inasmuch as it lacks a primary amine. Any tryptophan present would be destroyed in the process of acid hydrolysis and, therefore, cannot be determined. In addition to the usual amino acids found, a small peak which may represent glucosamine according to Benson and Patterson (5) was detected between the peaks of phenylalanine and histidine. However, this peak was not identified with certainty and, thus, was not quantitated.

DISCUSSION

Homogeneous polygalacturonase from the culture filtrate of *R. stolonifer* was shown to function as an elicitor of casbene synthetase in germinating castor beans as described in the accompanying paper (15). Some of the structural and catalytic properties of this enzyme have been examined to understand better its mode of

Table I. *Carbohydrate Compositional and Linkage-group Analyses of Polygalacturonase-elicitor from Filtrates of R. stolonifer Cultures*

Apparently homogeneous polygalacturonase-elicitor was obtained from the filtrates of *R. stolonifer* cultures grown in the glucose-asparagine medium by the procedures described in the accompanying paper (15). The figures presented are the mol per cent of each sugar residue found in the carbohydrate moiety of the glycoprotein polygalacturonase-elicitor. The glycosidic linkages to each sugar derivative are indicated by numeric prefixes: 2,6-mannose indicates that other glycosyl residues are glycosidically linked to carbons 2 and 6 of these mannose residues. T-Mannose refers to terminal mannose residues.

Sugar	Total Glycosyl Residues	
	Compositional analysis	Linkage group analysis
	mol %	
Mannose	92	
Glucosamine	8	
T-Mannose		18.6
2-Mannose		70.7
4-Mannose		4.5
2,6-Mannose		0.5
3,6-Mannose		5.6
4-Glucosamine		<1.0[a]

[a] Less than 1% of 4-linked glucosamine was detected due to the characteristically low yields of amino sugars in the linkage group analysis.

Table II. *Amino Acid Composition of Polygalacturonase-elicitor*

The numbers presented indicate the mol of each amino acid and ammonia in 1 mol polygalacturonase-elicitor (mol wt, 32,000) from the filtrates of *R. stolonifer* cultures. Asx, mixture of aspartate and asparagine, both detected as aspartate in this analysis; Glx, mixture of glutamate and glutamine.

Amino Acid	Residue/Polygalacturonase-elicitor
	mol/mol
Ala	23
Val	34
Leu	22
Ile	22
Phe	9
Gly	35
Ser	30
Thr	29
Tyr	4
Asx	59
Glx	15
Lys	14
Arg	3
His	5
NH_3	54

action as an elicitor. A large number of pectic enzymes from plants, fungi, and bacteria have been described (7–9, 16, 17, 22). These have been classified into subgroups according to their substrate preferences and modes of action. The *R. stolonifer* polygalacturonase-elicitor behaves as an endopolygalacturonase with a hydrolytic mode of action. Although it catalyzes the hydrolysis of polygalacturonic acid most efficiently at an optimum pH of 4.9, it also promotes the hydrolysis of partially methylated pectin at a significant rate under the same conditions. Thus, it could be expected to degrade pectic substances in the plant cell wall.

R. stolonifer polygalacturonase is a glycoprotein with glycosyl moieties containing mannose and glucosamine (presumably as *N*-acetylglucosamine in the native protein). A partial analysis of the amino acid composition indicates a close similarity to other fungal extracellular proteins. The acid hydrolysate is rich in acidic amino acids and lacks methionine. Even if all the ammonia released were generated from glutamine and asparagine residues, the balance between acidic and basic residues would indicate an isoelectric pH somewhat lower than the observed value of 8.0 (15). The basis for this discrepancy is not known. The glycosyl portion contains mannose and glucosamine in the approximate ratio of 92:8. Linkage group analysis revealed terminal 1-linked mannosyl residues along with a preponderance of 1,2-linked-, much smaller amounts of 1,4-linked-, and both 1,3,6- and 1,2,6-linked branch-point mannosyl units. No 1,3-linked mannosyl residues were detected. Also, a low yield of 1,4-linked glucosamine residues were detected by the linkage group analysis. It is proposed that the glycosyl units are most likely partially branched mannans linked through chitobiose units to asparagine of the polypeptide as is typical of other fungal glycoproteins (13, 27). The finding of relatively large amounts of terminal units (18.6%) in comparison with branch-point units (6.1%) indicates the presence of a large number of short, unbranched chains. Ziegler and Albersheim (27) suggested this explanation to account for similar analytical results in the case of invertases isolated from *Phytophthora megasperma* var. *sojae* culture filtrates.

The demonstration that a fungal endopolygalacturonase acts as an elicitor of an enzyme activity presumably involved in the production of an antifungal antibiotic in the plant raises the interesting question of how this enzyme functions as an elicitor. Two sets of results are consistent with the idea that the elicitor activity is in some way dependent on the catalytic activity of the enzyme. First, both elicitor and polygalacturonase activities show nearly identical sensitivities to thermal denaturation at all temperatures tested (Fig. 1). We do not believe that the difference at 40 C in the data summarized in Figure 1 is significant inasmuch as a reduction in elicitor activity by the heat treatment at 40 C was not seen in other experiments. Second, it is now clear that both the elicitor activity and the polygalacturonase activity are destroyed by periodate treatment. The susceptibility of elicitor activity are destroyed by periodate treatment. The susceptibility of elicitor activity to oxidation by periodate was described previously (23). At that time, this finding was interpreted as an indication of the dependency of the elicitor activity on the glycosyl moiety of the glycoprotein. However, in this work, it has been shown that the *R. stolonifer* polygalacturonase activity is also completely lost after periodate treatment under comparable conditions. Therefore, the results of periodate oxidation could equally well be interpreted in favor of a requirement for catalytic activity in elicitor function.

The basis for inactivation of polygalacturonase with periodate is not known. A direct attack of periodate on the vicinal hydroxyl and unsubtituted primary amine functions of an amino-terminal serine or threonine of the polypeptide is conceivable. However, the coincidence of the occurrence of one of these residues at the amino-terminus and of the indispensability of this amino-terminal residue for catalytic activity seems unlikely. It seems more likely, instead, that the periodate is modifying the glycosyl portion of the glycoprotein in a way which affects the stability of the enzyme.

The partial effectiveness of the short term exposures of castor bean seedlings to the polygalacturonase-elicitor in the eventual elicitation of casbene synthetase activity is consistent with a sequence of elicitation processes proposed by Keen and Bruegger (11). Their model was based on studies by Yoshikawa *et al.* (25) of the effectiveness of metabolic inhibitors added at various stages in the process on the accumulation of the phytoalexin, glyceollin, in soybeans. In that model, a short determinative period for initial recognition and DNA transcription is followed by a relatively long expressive phase for protein synthesis and finally glyceollin synthesis.

There is insufficient evidence to date to permit any conclusions as to how *R. stolonifer* polygalacturonase is acting as an elicitor in castor bean seedlings. One possibility is that the enzyme acts to degrade the cell wall so that a structural feature of the enzyme itself, such as a glycosyl moiety, can be recognized by an appropriate receptor in the plant. A second possibility is that the plant recognizes through an appropriate receptor a degraded portion of its own cell wall produced by the action of polygalacturonase. Obviously other possibilities also exist. Work is in progress at present to evaluate these possibilities.

This is the only reported instance of a fungal enzyme serving as an elicitor of a higher plant enzyme system that catalyzes the synthesis of a stress metabolite. Although the castor bean-*R. stolonifer* system utilized here does not constitute a well-defined host-parasite interaction, it is nonetheless possible that the features of this system have relevance for cases where true phytopathogens interact with higher plants to elicit phytoalexin production. Polygalacturonases and other types of pectic enzymes are produced by many types of plant pathogens (22), and these enzymes have been implicated in phytopathogenesis (2, 4, 7, 14, 20, 24). It is known that pectic enzymes can degrade the pectic substances in plant cell walls since maceration is often observed when plant tissues are treated with purified pectic enzymes. But it is not clear that the removal of a cell wall barrier is the only factor of importance in the action of these enzymes in phytopathogenesis. The possibility that polygalacturonases and other pectic enzymes may act more

generally as elicitors in phytoalexin-producing systems should be considered.

Acknowledgments—We sincerely thank Robert J. Bruce and Gloria Turner for their excellent technical assistance. Our sincere appreciation is also extended to Alan Darvill and Peter Albersheim of the Department of Chemistry, University of Colorado for the carbohydrate composition and linkage-group analyses.

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 interactions. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol 57: 766–774
4. BARMORE CR, GE BROWN 1979 Role of pectolytic enzymes and galacturonic acid in citrus fruit decay caused by *Penicillium digitatum*. Phytopathology 69: 675–678
5. BENSON JV, JA PATTERSON 1971 Chromatographic advances in amino acids and peptide analysis using spherical resins and their applications in biochemistry and medicine. *In* A Niederwieser, G Patake, eds, New Techniques in Amino Acid, Peptide, and Protein Analysis. Ann Arbor Science Publisher, Ann Arbor, pp 1–73
6. BRUEGGER BB, NT KEEN 1979 Specific elicitors of glyceollin accumulation in the *Pseudomonas glycineae*-soybean host-parasite system. Physiol Plant Pathol 15: 43–52
7. CERVONE F, A SCALA, F SCALA 1978 Polygalacturonase from *Rhizoctonia fragariae*: further characterization of two isoenzymes and their action toward strawberry tissue. Physiol Plant Pathol 12: 19–26
8. COOKE RD, CEM FERBER, L KANAGASBATHY 1976 Purification and characterization of polygalacturonases from a commercial *Aspergillus niger* preparation. Biochim Biophys Acta 452: 440–451
9. ENGLISH PD, A MAGLOTHIN, K KEEGSTRA, P ALBERSHEIM 1972 A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. Plant Physiol 49: 293–297
10. HAHN MG, P ALBERSHEIM 1978 Host-pathogen interactions. XIV. Isolation and partial characterization of an elicitor from yeast extract. Plant Physiol 62: 107–111
11. KEEN NT, B BRUEGGER 1977 Phytoalexins and chemicals that elicit their production in plants. *In* PA Hedin, ed, Host Plant Resistance to Pests, ACS Symposium Series, Vol 62. American Chemical Society, Washington DC pp 1–26
12. KEEN NT, M LEGRAND 1980 Surface glycoproteins: evidence that they may function as the race specific phytoalexin elicitors of *Phytophthora megasperma* f. sp. *glycinea*. Physiol Plant Pathol 17: 175–192
13. KORNFELD, R, S KORNFELD 1976 Comparative aspects of glycoprotein structure. Annu Rev Biochem 45: 217–237
14. LANGCAKE P, RB DRYSDALE 1975 The role of pectic enzyme production in the resistance of tomato to *Fusarium oxysporum* f. *lycopersici*. Physiol Plant Pathol 6: 247–258
15. LEE S-C, CA WEST 1981 Polygalacturonase from *Rhizopus stolonifer* is an elicitor of casbene synthetase activity in castor bean (*Ricinus communis* L.) seedlings. Plant Physiol 67: 633–639
16. LISKER N, J KATAN, Y HENIS 1975 Sequential production of polygalacturonase, cellulase, and pectin lyase by *Rhizoctonia solani*. Can J Microbiol 21: 1298–1304
17. LIU YK, BS LUH 1978 Purification and characterization of endopolygalacturonase from *Rhizopus arrhizus*. J Food Sci 43: 721–726
18. MCNEIL M, AG DARVILL, P ALBERSHEIM 1981 The structural polymers of the primary cell walls of dicots. *In* E Tolbert, ed, The Biochemistry of Plants. Academic Press, New York pp. 91–162
19. METLITSKII LW, OL OZERETSKOVSKAVA, LA YURGANOVA, ON SAVELEVA, LI CHALOVA, YT DYAKOV 1976 Induction of phytoalexins in potato tubers by metabolites of the fungus *Phytophthora infestans* (Mont) de Bary. Dokl Akad Nauk USSR. 226: 1217–1220
20. MUSSELL H, LL STRAND 1977 Pectic enzymes: involvement in pathogenesis 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
21. REXOVA-BENKOVA L 1973 The size of the substrate-binding site of an *Aspergillus niger* extracellular endopolygalacturonase. Eur J Biochem 39: 109–115
22. REXOVA-BENKOVA L 1976 Pectic enzymes. Adv Carbohydr Chem Biochem 33: 323–385
23. 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
24. WOOD RKS 1967 The degradation of cell walls of higher plants by parasites. Cell-wall degrading enzymes in different plant diseases. *In* Physiological Plant Pathology, Chaps 5 and 6. Blackwell Scientific Publications, Oxford pp 122–187
25. YOSHIKAWA M, K YAMAUCHI, H MASAGO 1978 *De novo* messenger RNA and protein synthesis are required for phytoalexin-mediated disease resistance in soybean hypocotyls. Plant Physiol 61: 314–317
26. YOUNG RJ, ME CORDIN 1964 Paper chromatography of galacturonic acids to determine polygalacturonase activity. Biochim Biophys Acta 83: 124–126
27. ZIEGLER E, P ALBERSHEIM 1977 Host-pathogen interactions. XII. Extracellular invertases secreted by three races of a plant pathogen are glycoproxeins which possess different carbohydrate structures. Plant Physiol 59: 1104–1110